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DIFFERENT FECAL SHEDDING PATTERNS OF TWO COMMON STRAINS OF HEPATITIS E VIRUS AT THREE JAPANESE SWINE FARMS

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Abstract. Zoonotic infections caused by eating the meat of deer, wild boar, and pig have been suggested in Japan, a country that is not epidemic for hepatitis E caused by hepatitis E virus (HEV). This virus is widely spread in domestic pigs in both epidemic and non-epidemic countries. We studied fecal HEV shedding patterns on three Japanese farms that had two common genotype III HEV strains. Two of the three farms had high shedding peaks (75% and 100%) in pigs 1–3 months of age, suggesting that these animals had the highest risk of spreading HEV through feces. Another farm had a low shedding rate in animals six months of age and a low prevalence of the IgG antibody to HEV. Fecal IgA antibody to HEV was found in sucking pigs < 13 days of age on farms that had high and low shedding patterns. A small fraction of pigs (3 of 43 [7%]) at the finishing stage (5–6 months of age) still shed HEV on the three farms.

INTRODUCTION

Human acute hepatitis E caused by hepatitis E virus (HEV) is a major cause of viral hepatitis in many disease-epidemic countries. Outbreaks of acute hepatitis E are usually associated with fecally contaminated drinking water.[1,2] This virus was recently classified into the *Hepevirus* genus of the family *Hepeviridae* and divided into four genotypes: I, II, III, and IV. Genotypes I and II are detected in outbreaks in disease-epidemic areas, and genotypes I, III, and IV are detected in sporadic cases of hepatitis E in areas that are not epidemic for this disease. In the latter areas, genotype I is generally isolated from patients who have traveled to HEV-epidemic areas weeks before their symptoms appeared, and genotypes III or IV are often isolated from patients who have not traveled to areas epidemic for HEV. In addition, frequent asymptomatic infections have been detected in countries not epidemic for HEV because antibodies against HEV have been identified in a significant proportion of blood donors in these areas.[3–9]

Antibodies against HEV have been detected in several animal species, and HEV or HEV-like genomes have been detected in domestic pigs,[10] wild boars,[11–14] wild deer,[15] mongooses,[16] and chickens.[17,18] Hepatitis E viruses isolated from chickens, known as avian HEVs, are distantly related to human HEV, and HEVs isolated from non-human mammalian species are mostly of genotype III or IV and are closely related to or sometimes indistinguishable from human HEVs.[14,19] A swine HEV strain of genotype III was shown to infect and cause hepatitis in non-human primates.[20,21] Thus, zoonotic transmission has been suspected. Direct transmission from animals to humans was shown by two clinical cases in which patients who had eaten uncooked or undercooked meats were infected by genotype III HEV whose sequences were identical to those from residual deer meat[15] or residual wild boar meat.[11] Several other cases found in Japan also suggested infection by genotype III or IV viruses by eating contaminated meat of wild boars and domestic pigs.[22–24]

A high prevalence of antibodies to HEV has been reported in domestic pigs in many countries that are epidemic for this virus and in non-epidemic countries.[5,10,25–31] Transmission among pigs is suspected through a fecal-oral route analogous to that in human cases. However, fecal shedding of HEV is less characterized than viremia in natural infection, and experimental infection by the oral administration of feces was only recently accomplished with a low frequency (one of three infected pigs).[32] However, infection was readily achieved by intravenous administration or co-housing of infected pigs.[32,33] Experimental intravenous infection of pigs with HEV induced fecal shedding of HEV 1–2 weeks after infection that lasted for 3–5 weeks, and viremia was associated with fecal shedding.[33] However, HEV has been shown to replicate not only in the liver but also in other tissues, including the small intestine and colon.[34] Thus, fecal HEV is probably derived from both the liver and intestinal tract. To understand pig-to-pig and farm-to-farm transmissions of HEV in the field, fecal shedding needs to be further characterized. This paper reports the different patterns of fecal HEV shedding at three farms in Japan and genetic variation in fecal HEVs.

MATERIALS AND METHODS

Animals. The three swine farms (A, B, and C) studied in Japan were independently run. Farm C was located 17 km from farms A and B, which were 7 km apart. The total number of pigs including piglets, sows, and boars was approximately 200 on farm A, 500 on farm B, and 800 on farm C. At farms A and C, pigs of the same age or size from different litters were housed together after the farrowing phase. At farm B, only pigs of the same litter were housed together throughout the growth/finishing stages.

Serologic analysis. Antibody to HEV was measured by an enzyme-linked immunsorbent assay (ELISA) as previously described.[3] The antigen used in the ELISA was HEV-like particles composed of a truncated open reading frame 2 (ORF2) protein of genotype I HEV expressed by a recombinant baculovirus in insect cells.[35] Sera were frozen at −20°C until tested by the ELISA. Serum samples were tested at a dilution of 1:200 with antigen. The secondary antibody was peroxidase-labeled goat anti-swine IgG (heavy plus light chain) (Kirkegaard and Perry Laboratories, Gaithersburg, MD). At least one negative control sample and one positive

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control sample were run for each ELISA plate. At the end of assay, the negative control (NC) optical density (OD) value was subtracted from each sample OD value and from the positive control (PC) OD value. The result was reported as a sample-to-positive ratio (S/P) (S/P = (S – NC)/(PC – NC)). The cutoff value of the S/P ratio was 0.55, which was determined based on the distributions of the S/P ratios of 91 samples from pigs 1–10 months of age from a farm considered free of HEV infection.[36] The S/P ratios of the individual samples ranged from –0.15 to 0.51, and the mean S/P ratio was 0.005 with an S.D. of 0.091. The cutoff value (0.55) was calculated from the mean + 6 SD (0.005 + 6 × 0.091).

Fecal IgA antibody to HEV was measured by the same ELISA used for serum IgG antibody. Fecal samples were clarified supernatants from 10% fecal homogenates that were tested at a dilution of 1:20. The secondary antibody used was horseradish peroxidase-labeled goat anti-swine IgA (Serotec, Ltd., Oxford, United Kingdom). The cutoff value of the S/P ratio was 0.078 based on 50 serum samples from pigs 1–10 months of age used for the determination of the IgG cutoff value. The IgA S/P ratios ranged from –0.006 to 0.055, and the mean S/P ratio was 0.012 with an SD of 0.011. The cutoff value (0.078) was determined using the same formula as that for IgG antibody. Since fecal samples from HEV-negative farm were not available, we tentatively used the cutoff value of serum IgA for the fecal samples.

The IgA S/P ratio of the fecal samples from the three farms ranged from –0.21 to 7.73, and when the serum cutoff value was used, 260 of the 280 tested fecal samples were negative for IgA antibody. The mean S/P ratio of the IgA-negative group was –0.00078 with an SD of 0.023, which was significantly lower (P = 0.00026, by Student's *t* test) than that of serum samples from the farm considered HEV negative. A nonspecific IgA reaction was less in fecal samples than in serum samples in the ELISA.

Extraction of RNA and reverse transcription–polymerase chain reaction (RT-PCR). Feces were sent to our laboratory by a commercial transportation system, kept in cold storage for approximately one day, and stored at –80°C until subsequent experiments. The frozen feces were thawed to room temperature, suspended in an appropriate volume of saline, crushed well, and mixed by vortexing. Ten percent suspensions of fecal samples were centrifuged at 1,500 × *g* for 15 minutes, and supernatants were further centrifuged at 20,000 × *g* for 15 minutes to obtain the final clarified sample. RNA was extracted with Isogen-LS (Nippon Gene Co., Ltd., Toyama, Japan) from 250 μL of clarified 10% suspensions of fecal samples. cDNA was synthesized from the total RNA fraction isolated from 2.5 mg of feces using Superscript II reverse transcriptase and primers of random hexamers according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The HEV genome was amplified from cDNA by PCR with ExTaq DNA polymerase (Takara Co., Ltd., Tokyo, Japan). Primers (previously reported or slightly modified from the original primers) for two regions of the HEV genome were used to amplify HEV strains of all genotypes. The PCR primers for the ORF1 region[37] were HE5-1 (sense): 5′-TCGATGCCATGGAGGCCCA-3′ and HE5-4m (antisense, m = modified from the original primer): 5′-CATVGCCTCBGCAACATCRG-3′ for the first PCR, and HE5-2 (sense): 5′-GCCYTKGCGAATGCTGTGG-3′ and HE5-3m (anti-sense) 5′-TCAAARCAGTARGTSCGGTC-3′ for the second PCR. They generated 542-basepair and 365-basepair PCR products, respectively. The PCR primers for the ORF2 region[38] were 3156N (sense): 5′-AATTATGCYCAGTAYCGBGTKG-3′ and 3157N (antisense): 5′-CCCTTRTCYTGCTGMGCRTTCTC-3′ for the first PCR, and 3158N (sense): 5′-GTWATGCTYTGCATWCATGGCTC-3′ and 3159N (anti-sense): 5′-AGCCGACGAAATCAATTCTGTC-3′ for the second PCR. They generated 731-basepair and 348-basepair PCR products, respectively.

The first-round PCR was performed in a 20-μL volume that included an amount of cDNA equivalent to 0.125 mg of feces. The reaction conditions of the first-round PCR consisted of an initial denaturation step at 95°C for 9 minutes, followed by 40 cycles of denaturation (94°C for 1 minute), annealing (54°C for 1 minute), and extension (72°C for 1 minute), and a final extension at 72°C for 7 minutes. The second-round PCR used 1 μL of the first-round PCR product in a 20-μL volume under the same reaction conditions as the first-round PCR. The PCR products were examined by electrophoresis on a 2% agarose gel.

Cloning, sequence determination, and genetic analysis. The PCR products were excised from the agarose gel, purified using the Geneclean II kit (Bio 101, Inc., La Jolla, CA) and inserted into the cloning vector pCR2.1 using a TOPO TA cloning kit (Invitrogen, Inc., USA) for subsequent transformation of the competent *Escherichia coli* DH5a cells with a chemical method. Plasmids were purified using a commercial kit (Wizard Plus SV Minipreps DNA purification System; Promega, Madison, WI). Inserts of plasmids were sequenced using both standard M13 forward and reverse sequencing primers (obtained from our laboratory or Hokkaido System Science Co., Ltd., Sapporo, Japan). Sequence alignment was done using the computer program Genetyx (Genetyx Co., Ltd., Tokyo, Japan). Phylogenetic analyses were performed with ClusterW[39] and TreeviewX version 0.4.[40] The 26 nucleotide sequences of the fecal HEVs were available from DDVJ database (accession no. AB270965-AB270990).

RESULTS

Frequencies of HEV shedding in feces. We examined 386 pigs from three swine farms (farms A, B, and C) 7–17 km apart. Feces were collected from randomly selected pigs one week to six months of age, and each sample was tested with the ORF1 and ORF2 PCR primers. The frequency of HEV-positive pigs differed among the three farms (Table 1). Farms

TABLE 1
Frequency of pigs shedding hepatitis E virus (HEV) in feces

Age (months)	Frequency of HEV-positive pigs* (no. of HEV+ pigs/no. of pig tested)(%)		
	Farm A	Farm B	Farm C
0	0/22 (0)	0/33 (0)	0/16 (0)
1	2/10 (20)	4/44 (9)	16/16 (100)
2	15/20 (75)	1/16 (6)	20/20 (100)
3	10/17 (59)	0/26 (0)	11/29 (38)
4	1/7 (14)	1/30 (3)	0/7 (0)
5	0/7 (0)	1/23 (4)	1/7 (14)
6	1/7 (14)	2/23 (9)	0/6 (0)
Total	29/90 (32)	9/195 (5)	48/101 (48)

* HEV-positive pigs represent those from which HEV RNA in feces was detected by either the first and second polymerase chain reactions for the open reading frame 1 (ORF1) or ORF2 genes.

A and C were similar in the HEV shedding pattern but were different from that observed at farm B. At farms A and C, HEV shedding was undetectable until one month of age, detectable at high frequencies between one and three months of age (20–75% at farm A and 38–100% at farm C), and detectable at lower frequencies in older pigs (0–14% at both farms) (Table 1).

In 1–3-month-old pigs at farms A and C, HEV genomes were detected by the first PCR at higher frequencies compared with pigs of other ages (Figure 1). This was characterized by a high frequency of HEV-positive animals (Table 1) and a higher amount of HEV RNA in feces. In contrast, farm B showed no peak in the frequency of fecal HEV shedding: HEV was undetectable in piglets less than one month of age, but was detected at low frequencies (0–9%) throughout the observation period (Table 1). Of 195 samples tested, HEV genomes were detected in 9 HEV-positive samples only by the second PCR (Figure 1). This suggests a low level of HEV RNA in these samples.

Because HEV shows extensive genetic variation, it was not known whether the PCR systems we used were valid for the fecal field samples. The PCR systems for the ORF1 and ORF2 regions seemed comparably efficient at the level of the second-round PCR because the frequencies of the ORF1- and ORF2-positive pigs in the second-round PCR were comparable for farms A and B, and 61% (52 of 85) of HEV-positive samples were detected by both the ORF1 and ORF2 PCR systems in the second-round reaction. However, in the first-round PCR systems, the detection efficiency appeared slightly higher with the ORF2 PCR than with the ORF1 PCR (Figure 1).

Nucleotide sequences of isolated HEV genomes. Eight to ten PCR products (731 basepairs or 348 basepairs) of the ORF2 region isolated from each farm were sequenced. All the sequenced genomes belonged to two genetic clusters in genotype III, clusters III-A and III-B (Figure 2). Farm A had five III-A viruses and three III-B viruses, farm B had five III-A viruses and three III-B viruses, and farm C had one III-A virus and nine III-B viruses (Table 2). The intracluster nucleotide identities were 99.0–100% in III-A and 98.7–100% in III-B, and the intercluster nucleotide identities were 89.3–91.0% between the III-A and III-B clusters. The intra-farm and inter-farm nucleotide identities were not significantly different in both the III-A and III-B clusters. These results indicated that the three farms shared two HEV strains, in contrast to a previous report in which each of the investigated farms with multiple HEV isolates had identical virus strains, and none of the farms shared these HEV strains.[29]

The nucleotide sequences (300 nucleotides) of the III-A and III-B viruses isolated from the farms were compared with other HEV isolates in the database by BLAST analysis. The 10 sequences most closely related to III-A sequences were Japanese HEVs isolated from human patients or pigs with 93–95% identities (Figure 2). The III-B sequences were also related to the Japanese HEVs from human patients or pigs with 94–95% nucleotide identities.

Prevalence of antibody to HEV. Sera from pigs at the three farms were tested for the prevalence of IgG antibody to HEV by an ELISA. Seroprevalences were 82% (14 of 17) in 3–5-month-old pigs from farm A, 5% (3 of 65) (2–6 months old) from farm B, and 100% (25 of 25) (2–3 months old) from farm C (Figure 3). Farm B had a low seroprevalence compared

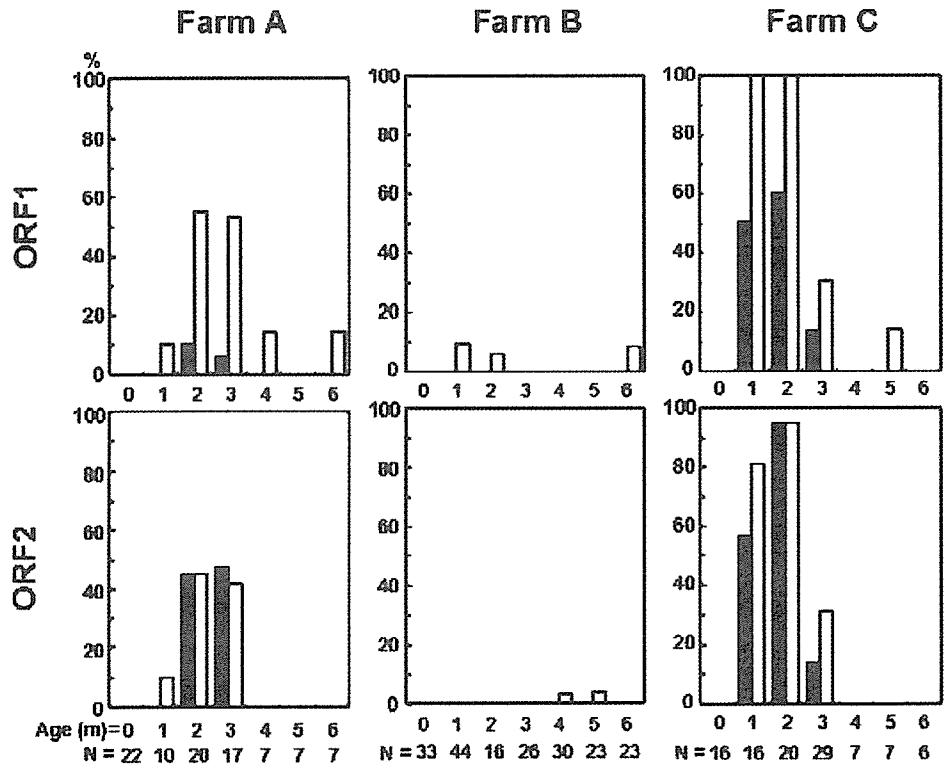

FIGURE 1. Frequencies of pigs shedding hepatitis E virus (HEV) in feces on three farms in Japan. The open reading frame 2 (ORF1) and ORF2 genes of HEV were amplified from each fecal sample by reverse transcription–polymerase chain reaction (RT-PCR). Solid and open bars represent frequencies of HEV-positive pigs detected by the first PCR and the second PCR, respectively.

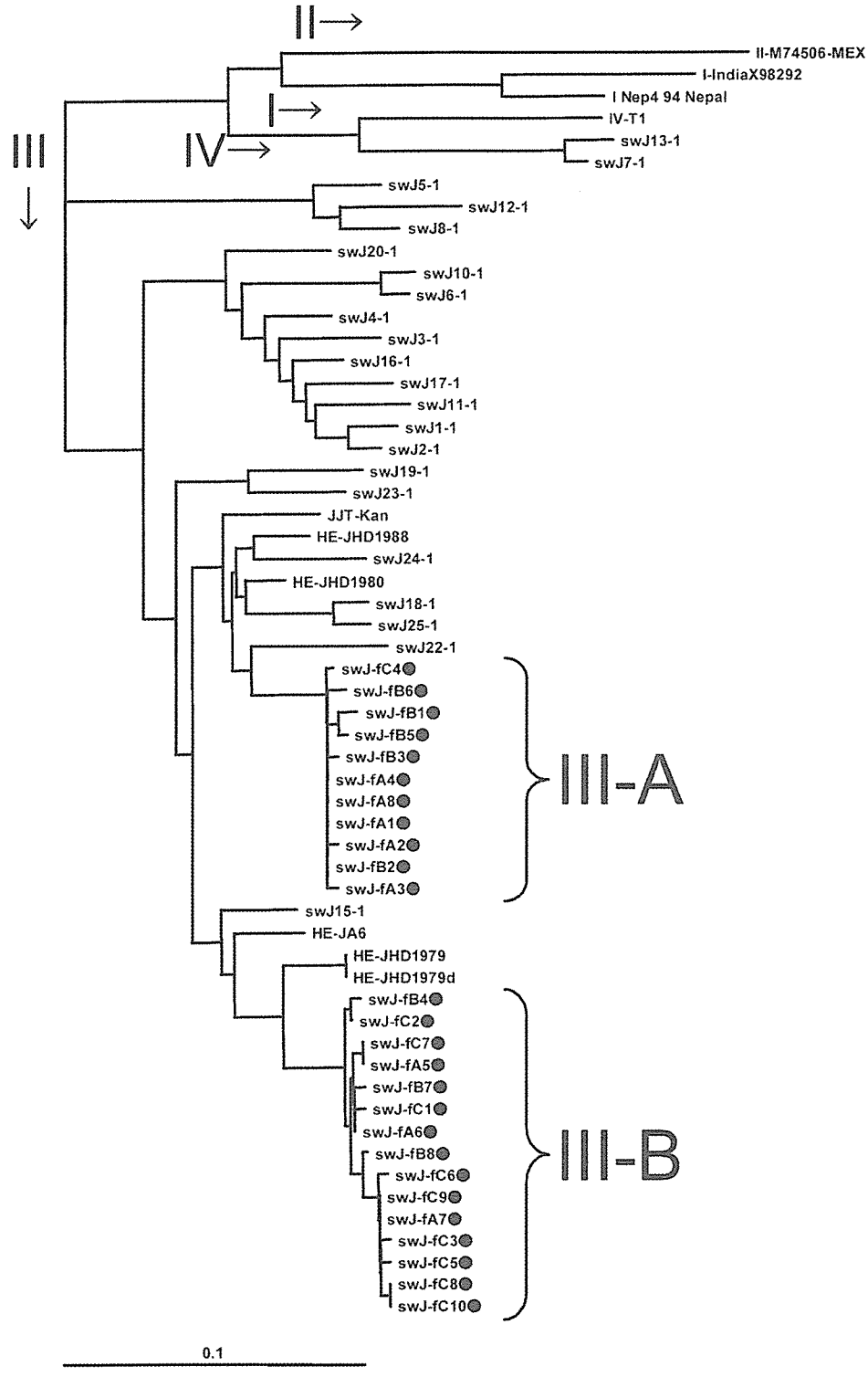

FIGURE 2. Phylogenetic tree constructed by the neighbor-joining method for hepatitis E virus (HEV) open reading frame 2 (300 bases). The 26 sequences isolated from farms A, B and C in this study (Table 2) belonged to either cluster III-A or III-B. The tree included our 26 isolates, typical sequences of genotypes I, II, and IV, genotype III sequences isolated from pigs at 19 Japanese farms (Takahashi, 2003 #58), and six human HEV sequences isolated in Japan (JJT-Kan, HE-JHD1988, HE-JHD1980, HE-JA6, HE-JHD1979, and HE-JHD1979d), which had the highest nucleotide identities (94–95%) with clusters III-A or III-B. Scale bar at the lower left shows percent relatedness.

with those of many previously tested Japanese commercial farms,[29] but antibody titers (S/P ratio) of two of three seropositive pigs from farm B were equivalent to the highest titers from farms A and C (Figure 3).

Fecal IgA antibody to HEV was measured. IgA antibody was detected only from suckling pigs less than 13 days old at the three farms (Figure 4). At farm A, all 8 two-day-old pigs were positive for antibody to HEV, and 82 older pigs, includ-

TABLE 2
HEV ORF2 sequences isolated from swine feces from three farms*

Farm	Pig	Age (months)	HEV sequence name	Gene cluster
A	1	2	swJ-fA1	III-A
	2	2	swJ-fA2	III-A
	3	2	swJ-fA3	III-A
	4	2	swJ-fA4	III-A
	5	3	swJ-fA5	III-B
	6	3	swJ-fA6	III-B
	7	3	swJ-fA7	III-B
	8	3	swJ-fA8	III-A
B	1	1	swJ-fB1	III-A
	2	1	swJ-fB2	III-A
	3	1	swJ-fB3	III-A
	4	1	swJ-fB4	III-B
	5	1	swJ-fB5	III-A
	6	2	swJ-fB6	III-A
	7	6	swJ-fB7	III-B
	8	6	swJ-fB8	III-B
C	1	1	swJ-fC1	III-B
	2	1	swJ-fC2	III-B
	3	2	swJ-fC3	III-B
	4	2	swJ-fC4	III-A
	5	2	swJ-fC5	III-B
	6	2	swJ-fC6	III-B
	7	2	swJ-fC7	III-B
	8	2	swJ-fC8	III-B
	9	3	swJ-fC9	III-B
	10	3	swJ-fC10	III-B

* HEV = hepatitis E virus; ORF2 = open reading frame 2.

ing 4 four-day-old pigs and 7 14-day-old pigs, were negative. At farm B, which showed a low seroprevalence (Figure 3), fecal IgA antibody was detected in 2 (40%) of 5 four-day-old pigs, 4 (80%) of 5 six-day-old pigs, 2 (40%) of 5 11-day-old pigs, and 4 (57%) of 7 13-day-old pigs, in which each age group contained the same littermates. Seventy-nine other pigs more than 13 days of age were negative for antibody to HEV. At farm C, all 90 serum samples from pigs more than 18 days of age were negative for antibody to HEV. The IgA antibody to HEV detected in feces of these young pigs appeared to have maternal antibody because newborn pigs have virtually no serum antibody if they are not infected with pathogens,[41] and vertical transmission of HEV from sow to fetus was not evident in an experimental infection of pregnant pigs.[42] Newborn pigs absorb maternal antibody from sow colostrums 24–36 hours after birth.[41] Titers of fecal IgA and IgG antibodies to HEV were significantly correlated (Spearman's correlation coefficient rank test, $r_s = 0.784 > 0.483$, $P < 0.01$) in 28 fecal samples from newborn pigs at farms A and B with various titers of IgG or IgA antibodies to HE, which suggests that both IgA and IgG were maternally transmitted.

DISCUSSION

We investigated fecal shedding patterns of HEV at three swine farms and analyzed the genetic variation in the shed HEVs. Although HEV transmission by the fecal-oral route is suspected in pigs, it is still unclear how the transmission occurs among pigs and within or between swine farms. Domestic pigs from Japanese farms were heavily infected by diverse HEV strains of genotypes III or IV.[19,24,29] An analysis of 25 Japanese farms showed that multiple HEV genomes were isolated from 19 farms and that each farm had one strain of HEV with a minor variation, which was clearly distinguishable from those of other farms.[29] In contrast to this report, our study indicated that the three farms harbored the same two HEV strains. Thus, multiple HEV strains can coexist on a farm, and HEV pre-existing in a farm may not interfere with additional infection by new HEV strains from other farms. However, it is not known why many farms exclusively harbor one strain of HEV.[29] The three farms we studied were 7–17 km apart, but there was no close contact between these farms for at least the past 20 years. However, we have not identified the factors responsible for inter-farm transmission of HEV strains, such as a common animal source, personnel relationships, and shared water supply, food, or workers.

The three farms shared two virus strains but differed in virus shedding rate and seroprevalence (Table 1 and Figure 1). Therefore, different shedding patterns may not be caused by characteristics of the two viruses but may be caused by other non-viral factors, such as sanitary conditions, hosts, facilities, or type of farming. Farm B, the farm with the lowest rate of HEV shedding, differed from the other two farms in that it housed only pigs of the same litter, had a lower density of pigs, and was a cleaner facility. These characteristics might reduce circulation of virus within a farm.

On farms A and C, the farms with higher rates of HEV shedding, fecal shedding peaked between one and three months of age with respect to the amounts of HEV RNA (Figure 1) and the frequencies of the HEV-positive pigs (Table 1). These results correspond to those of other reports in which viremic stages occurred in pigs approximately 3–4 months of age on many farms in Japan[29,43] and the prevalence of antibody to HEV increased in pigs 2–3 months of age in Japan[29] and in other countries.[5,9,28,44] Therefore, 1–4-month-

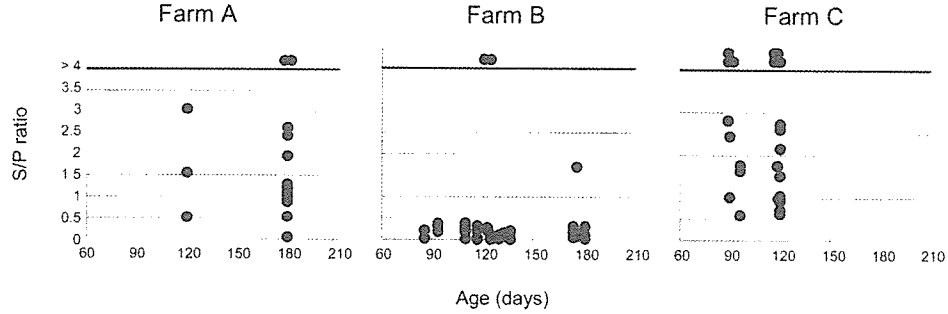

FIGURE 3. IgG antibody titers to hepatitis E virus in pigs from three farms in Japan. The enzyme-linked immunosorbent assay cutoff value was a sample-to-positive (S/P) ratio of 0.55. The percentages of pigs with an S/P ratio above the cutoff value were 82% (14 of 17) from farm A, 5% (3 of 65) from farm B, and 100% (25 of 25) from farm C.

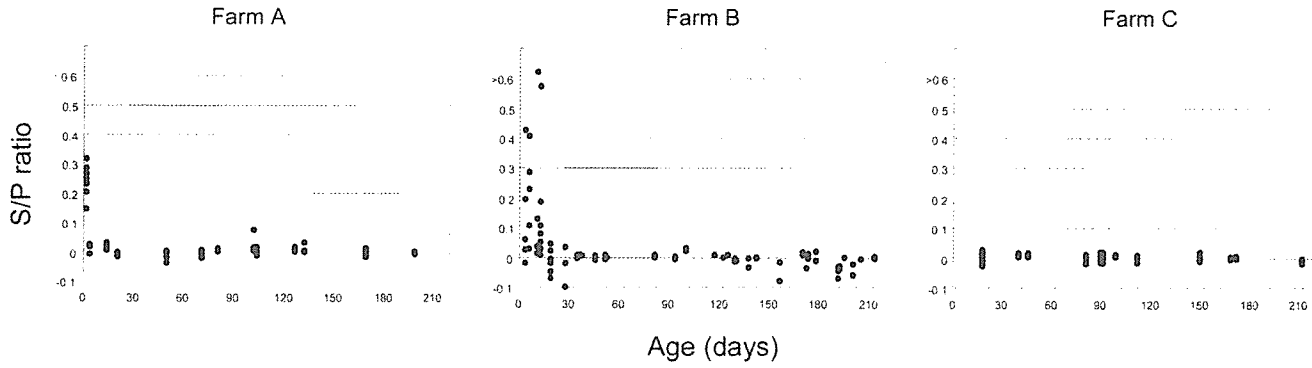

FIGURE 4. IgA antibody titers to hepatitis E virus in swine feces from three farms in Japan. The enzyme-linked immunosorbent assay cutoff value was a sample-to-positive (S/P) ratio of 0.078. A total of 90 samples were tested from farm A, 101 from farm B, and 90 from farm C. Samples having an S/P ratio greater than the cutoff value included 8 of 8 from two-day-old pigs on farm A, and 2 of 5 from four-day-old pigs, 4 of 5 from six--day-old pigs, 2 of 5 from 11-day-old pigs, and 4 of 7 from 13-day-old pigs from farm B. All animals with an S/P ratio below the cutoff value were > 4 days of age on farm A and > 19 days of age on farm B. All samples from farm C were from pigs > 18 days of age and had S/P ratios below the cutoff value.

old pigs seem to be the group at greatest risk for HEV shedding, which is responsible for the intra-farm and inter-farm spread of the virus. Furthermore, at approximately six months of age when most pigs are slaughtered in Japan, fecal HEV genomes were still detected in a small fraction of the pigs (3 of 43 [7%]) (Table 1). This observation is also consistent with a previous study in which HEV genomes were detected in 1.9% of 363 packages of raw pig liver marketed in Japanese grocery stores,[24] although there was a report that HEV genomes were not detected in sera of 250 pigs at six months of age.[29] Thus, pigs of slaughtering age are also a lower but potential risk group.

None of the pigs from farm B shed a high amount of HEV RNA, but a low amount of HEV RNA was detected in pigs of various ages in different pens (Tables 1 and 2 and Figure 1); 3 (5%) of 65 pigs had IgG antibody to HEV IgG (Figure 3), and fecal IgA antibody to HEV was detected in newborns (Figure 4). These observations suggest that HEV was spreading on this farm. Restricted spread on a farm and low multiplication of HEV in infected pigs may be responsible for the low prevalence or low antibody titer. Studies of farms such as farm B may provide information on methods to eradicate HEV from swine farms or to eliminate pigs producing large amounts of HEV and reduce risks of pig-to-pig virus transmission through feces or pig-to-human virus transmission through meats.

An interesting observation in our study was that fecal IgA antibody to HEV was found only in suckling pigs less than 13 days old. This antibody was detected on farms A and B, those with high and low levels of virus shedding, but no fecal samples of such young pigs were available from farm C. Although there seems to be no correlation between the fecal shedding rate of HEV and fecal antibody to HEV, maternal antibody to HEV may provide resistance to young pigs against HEV. Young pigs 0–2 months of age appear to be more resistant to infection by HEV than older pigs. For example, the three farms studied, like many swine farms, used a rearing practice in which piglets stayed with their mothers until approximately one month of age. No fecal HEV was detected in pigs less than one month of age (Table 1). In addition, pigs 0–2 months of age did not show viremia or production of serum antibody to HEV.[5,9,28,29,43,44] It is not known whether resistance is innate or acquired after birth, but maternal antibody may be one of the determinants.

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Epidemiological study of hepatitis E virus infection of dogs and cats in Japan

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HEPATITIS E virus (HEV) is the causative agent of human hepatitis E in many developing and some industrialised countries (Aggarwal and Krawczynski 2000, Meng 2000a, Schlauder and Mushahwar 2001, Huang and others 2002, Mizuo and others 2002, Takahashi and others 2003). It is widely known that in humans HEV is primarily transmitted by the faecal-oral route through contaminated water, and occasionally causes large epidemics in endemic areas (Arankalle and others 1994, Aggarwal and Krawczynski 2000). However, in non-endemic areas, the reservoir for sporadic human cases with no history of travel to HEV-endemic areas has remained unknown for many years (Harrison 1999, Schlauder and Mushahwar 2001, Mizuo and others 2002, Takahasi and others 2002). In 1997, the first animal HEV strain, swine HEV, was discovered in a pig in the USA (Meng and others 1997). Subsequent epidemiological studies indicated that, for example, in Japan, most farmed pigs had been exposed to swine HEV (Takahashi and others 2003). There is a growing consensus that HEV is a potential zoonotic agent and that pigs can act as a reservoir for humans.

HEV has also been detected in chickens (Haqshenas and others 2001) and wild rodents (Kabrane-Lazizi and others 1999, Favorov and others 2000, Arankalle and others 2001, He and others 2002, Hirano and others 2003a), and anti-HEV antibodies have been found in cattle, water buffaloes, sheep, goats, non-human primates, dogs and cats (Tsarev and others 1993, Meng 2000b, Arankalle and others 2001, Hirano and others 2003b, Usui and others 2004). In an unusual case, an incident was reported in which a cat was suspected to be a reservoir for human infection (Kuno and others 2003). This short communication describes the examination of blood samples and rectal swabs from dogs and cats for evidence of HEV infection.

An ELISA using the purified, empty virus-like particles (VLPs) of HEV (Li and others 1997) was performed with 424 canine and 202 feline serum samples. Samples were collected from animals at animal hospitals over a five-year period, from 2000 to 2004, in over 30 prefectures covering northern Hokkaido and south-western Okinawa, Japan, and stored at –20°C. The ELISA method used was the same as that described by Li and others (2000). The sera were examined simultaneously using VLP-coated and VLP-uncoated (blank) plates, and the antibody titre was expressed as an optical density at 492 nm (OD_{492}) by subtracting the OD_{492} value of the blank plate from that of the VLP-coated plate.

OD_{492} values of greater than 0·1 were obtained from 10 canine and eight feline serum samples (Table 1). These samples were subsequently absorbed with the same VLPs used as the antigen in the ELISA to confirm the specificity of the reaction. The serum sample (100 µl at a dilution of 1:200) was mixed with 1 µg of VLPs, and the mixture was incubated at 37°C for one hour, and then re-examined. A reduction of the OD_{492} value of the sample by greater than 50 per cent after the absorption was considered to indicate an anti-HEV antibody-positive result, as described by Arankalle and others (2001). On this basis all the canine serum samples were considered to be anti-HEV antibody-negative, and four feline serum samples (1·98 per cent) were considered to be anti-HEV antibody-positive (Table 2).

A reverse transcriptase-PCR (RT-PCR) assay, described for the detection of swine HEV (Huang and others 2002), was used to test 100 canine and 66 feline rectal swabs from animal hospitals located in Tokyo and its environs obtained over a six-year period from 1999 to 2004. In addition, the 18 sera showing an OD_{492} value of greater than 0·1 (Table 1) were also examined by RT-PCR. When a questionable PCR product was obtained, it was analysed by sequencing. No specific PCR products were amplified from any of the canine or feline samples examined.

Only a small number of cases of HEV infection in dogs and cats have been reported (Tien and others 1997, Arankalle and others 2001, Usui and others 2004). In the present study, the samples were collected nationwide in Japan and anti-HEV antibody was found in a very small percentage of the cats and in none of the dogs tested by the ELISA. These results were in contrast to a previous report. Among 135 cats visiting an animal hospital in a provincial capital in Japan, 44 (33 per cent) possessed anti-HEV antibody but no HEV RNA was recovered from the 135 sera (Usui and others 2004). No convincing explanation for the discrepancy in the seroprevalence rates between the previous and the present reports was possible. It should be noted that there is a possibility that the recombinant antigen itself could have non-specific, cross-reacting epitopes among antibodies in animal sera. According to a report describing HEV infection in dogs from India (Arankalle and others 2001) and Vietnam (Tien and others 1997), 22·7 per cent to 27 per cent of the dogs were anti-HEV antibody-positive. It may be true that dogs and cats in such HEV-endemic areas have been exposed to HEV more frequently than animals in non-endemic areas.

In non-endemic areas, there may be some common infectious source affecting both human beings and their companion animals, as they share mostly the same living environment. One probable reservoir, especially for companion animals, is rodents, and it has recently been reported in Japan that wild rats have been found to be infected with HEV (Hirano and others 2003a). Cats are rodent hunters, which is inherent to their nature, but this is not so much the case in dogs. This causal relationship between disease prevalence and a behavioural characteristic has already been seen in other viral diseases in cats, such as Borna disease virus (Berg and

TABLE 1: Results of an ELISA for antibody to hepatitis E virus in dog and cat sera from Japan

Animal	Number of samples tested	Optical density (mean) at 492 nm ≤0	0·001-0·099	0·1-0·499	≥0·5
Dog	424	254	160 (0·039)	10 (0·113)	0
Cat	202	83	111 (0·044)	7 (0·178)	1 (1·189)

TABLE 2: Details of cats suspected or confirmed as hepatitis E virus (HEV) antibody-positive by ELISA

Cat	Place of residence	Age (years)	Sex	Clinical signs	Reduction rate of ELISA optical density at 492 nm after absorption (%)	HEV antibody*
1	Niigata	Unknown	MN	None	60	+
2	Tokyo	11	MN	Kidney dysfunction	49·6	Suspected
3	Tokyo	6	F	None	58·6	+
4	Kanagawa	7	M	Stomatitis	32·3	Questionable
5	Aichi	15	FN	None	82·1	+
6	Okinawa	2-3†	M	None	96·2	+

* Greater than 50 per cent reduction was regarded as HEV antibody-positive
† Estimated
MN Male neutered, F Female, M Male, FN Female neutered, + Positive

others 1998) and cowpox virus infections (Nowotny and others 1994).

In conclusion, natural HEV infections in both dogs and cats in Japan appear to be rare and these animal species may be accidental hosts for human beings.

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Influence of food intake on the clinical response to cyclosporin A in canine atopic dermatitis

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CYCLOSPORIN A (CsA) (Atopica, Atoplus soft capsules; Novartis Animal Health) was introduced recently as an oral microemulsion formulation for the treatment of dogs with atopic dermatitis. Several randomised, controlled and open clinical studies have proven its clinical efficacy (Olivry and others 2002, Steffan and others 2003, 2004). Based on pharmacokinetic data, administration of CsA is recommended two hours before or after feeding, as its bioavailability decreases and the variability of individual blood concentrations increases when the drug is given with food (Steffan and others 2004). Dosing fasted dogs is therefore recommended to optimise the drug's bioavailability. However, the clinical response of dogs after dosing with or without food has never been evaluated. This short communication describes a study to determine whether the administration of CsA with or without food could influence the clinical response in dogs with atopic dermatitis.

Twenty-five dogs with non-seasonal atopic dermatitis, diagnosed by Prélaud's criteria (Prélaud and others 1998) and by exclusion of differential diagnoses, were included in a multicentre, randomised study involving seven investigators

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Comparison of clinical features of acute hepatitis caused by hepatitis E virus (HEV) genotypes 3 and 4 in Sapporo, Japan

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Abstract

In Japan, indigenous acute hepatitis E is not a rare disease, and is mainly caused by hepatitis E virus (HEV) genotypes 3 and 4. Whether there is a difference in clinical features between the two genotypes remains unclear. This study compares the clinical features of patients infected with the two. From January, 1994, to December, 2003, 9 infected with HEV genotype 3 and 27 patients with genotype 4 were enrolled. Patients with genotype 4 had significantly higher peak alanine aminotransferase levels (median 3430 IU/L, interquartile range 1747–4763 versus 1052 IU/L, 845–2707; $p=0.01$). The lowest prothrombin time was lower in the genotype 4 group (61%, 42–77 versus 84%, 70–96; $p=0.05$). In our series, patients with genotype 4 had longer median duration of hospital stay (26.5 days, 18–31 versus 18 days, 12–23.5; $p=0.06$). The patients with genotype 4 infection tended to have more severe clinical manifestations than those with genotype 3 infection.

Keywords: Hepatitis E virus; Acute hepatitis E; Clinical features; Genotype; Severe acute hepatitis; Fulminant hepatitis

1. Introduction

Hepatitis E virus (HEV) is a major etiological agent that causes sporadic as well as epidemic hepatitis in tropical and subtropical areas. The majority of HEV infections in these endemic areas are caused by genotype 1 [1]. In the last decade, patients with sporadic hepatitis E have been reported in North America and Europe [2–5]. Based on RNA sequence analyses, HEV isolates in non-endemic areas have been found to be genotypes 3 and 4 [6,7].

In Japan, ever since an indigenous HEV strain of genotype 3 (JRA1) was isolated from a patient with acute hepatitis with no relevant traveling history [8], many cases infected with HEV have been reported [9–12]. The reports have shown that viruses of the two genotypes (3 and 4) have co-circulated in Japan, that some cases progressed to fulminant hepatitis (FH) [13,14], that some cases had ingested raw or undercooked wild deer, boar or pig livers [15–18], and that in Honshu Island most patients were infected with genotype 3, whereas there were more infection with genotype 4 than 3 in Hokkaido [10].

However, the differences in the demography, mode of transmission, and clinical features of patients infected with HEV genotypes 3 and 4 have not been sufficiently studied. Also unknown is the incidence of FH and severe acute hepatitis (SAH) according to the genotypes. Recently, Mizuo et al. [19] maintained that HEV genotypes and the presence of underlying disease(s) influence the severity of hepatitis E. To further clarify the difference in disease according to the genotype, we compared patients infected with HEV genotypes 3 and 4 in Sapporo, Hokkaido, Japan.

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2. Patients and methods

2.1. Patients

Between January, 1994, and December, 2003, we diagnosed 315 cases of acute hepatitis at two community teaching hospitals in Sapporo, which is a metropolitan city of 1.8 million people located in the island of Hokkaido in northern Japan. Acute hepatitis was arbitrarily defined as acute illness with symptoms and signs compatible with acute hepatitis and with alanine aminotransferase (ALT) >120 IU/L, and asymptomatic hepatitis with ALT >1000 IU/L. All patients had serum samples drawn for serological diagnosis of acute viral hepatitis when first seen, which were all within 2 weeks of the onset of illness. Serum samples had been stored frozen (at or below −20 °C) and not thawed until this analysis. The samples were screened for immunoglobulin M (IgM) antibody to hepatitis A virus (IgM HAV), hepatitis B virus surface antigen (HBsAg), IgM antibody to hepatitis B core antigen (anti-HBc IgM), PCR for HBV DNA and antibody to hepatitis C virus (anti-HCV Ab). Subsequently, the serum was also tested for IgM anti-Epstein-Barr virus antibody (IgM-EBV) and IgM anti-cytomegalovirus antibody (IgM-CMV). Diagnosis of drug-induced hepatitis was based on the history and clinical course; lymphocyte stimulation index for drugs was used as an adjunct. Acute hepatitis of unknown etiology was a diagnosis of exclusion.

Acute hepatitis E was defined by the presence of HEV RNA in acute-phase sera. We enrolled 36 patients with HEV, excluding one case because of coinfection with two genotypes.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board at each institution. Informed consent was obtained from each patient.

2.2. Methods

2.2.1. Detection of HEV RNA and determination of their strains

HEV sequences were analyzed according to the methods reported previously [8,20]. The nucleic acids extracted from the sera were applied to the first cDNA synthesis and then the c DNA subjected to a nested polymerase chain reaction (PCR). The 326-nt region amplified from ORF1 of the HEV genome was sequenced to compare with the prototype sequences of the four major genotypes of HEV.

2.2.2. Epidemiological survey and laboratory examinations

Between the two groups, we compared demographics, symptoms and signs, risk factors for contracting hepatitis, clinical course and outcome. In this study, an underlying disease was defined as an active illness which had already been under medical care before the onset of hepatitis. Overconsumption of alcohol was defined as daily ethanol ingestion over 50 g for 5 years or longer. We performed an extensive interview on 32 out of 37 patients (86%) and their family members to supplement the information from the medical records. As the incubation period of acute hepatitis E is approximately 2–10 weeks, we inquired into the presence or absence of epidemiological risk factors for hepatitis such as traveling abroad, exposure to bodily fluid, and ingestion of raw and/or undercooked liver and/or intestines within 10 weeks before the onset of illness. To minimize recall bias and/or interview bias, we performed the following: we visited each patient's home twice in 3 months with an open-ended questionnaire, and assisted them to recall their daily ingestion and/or alcohol intake during the 2 months preceding the onset of illness, comparing it with their current eating and drinking habit. Fulminant hepatitis was diagnosed according to the Inuyama Symposium Criteria in Japan as follows: acute hepatitis in which patients develop hepatic encephalopathy grade II or higher within 2 months after the onset of hepatitis symptoms, showing a plasma prothrombin time (PT) ≤ 40% [21]. Severe acute hepatitis was diagnosed according to the criteria as acute hepatitis with PT ≤ 40% in the absence of encephalopathy.

2.2.3. Statistical analyses

We used medians with interquartile range for numerical variables because the samples did not show a normal distribution. For comparisons of medians and proportions, Mann–Whitney *U* and chi-square (without Yates correction) tests were used, respectively. Significant predictors in the univariate analysis were then included in a forward, stepwise multiple logistic regression model. A p-value of <0.05 was considered to indicate statistical significance; all tests were two-tailed. Data were analyzed using a statistical software StatView for Macintosh (Version 5.0 StatView, SAS Institute Inc. Japan).

3. Results

3.1. Patients infected with HEV: characteristics, laboratory data and outcome

Of the 119 patients with acute hepatitis of unknown etiology, 37 were diagnosed with definite acute hepatitis E. There were 29 males, and 8 females (all non-pregnant), and the median age was 45 years (range 19–69). One patient (case 4) infected with genotype 4 had traveled to the Republic of Indonesia, which is an endemic area. No other case had history of a travel to endemic areas. Characteristics, laboratory data and outcome of all patients are shown in Table 1. Nineteen patients (51%) had underlying disease(s) and their median age was 52 years (34–69). There were 15 patients (41%) with alcohol overconsumption. Six cases had PT ≤ 40%, and two of them developed hepatic coma grade II or higher. Two of them died: one (case 22) died from a bleeding tendency after liver transplantation from a living donor,

Table 1
Characteristics, laboratory data and outcome of 37 patients with acute hepatitis E

Case number	Sex/age	Genotype	Underlying disease(s)[a]	Alcohol overconsumption[b]	Laboratory data				Outcome		
					AST (IU/L)	ALT (IU/L)	T.Bil. (mg/dL)	PT (%)	Severity[c]	Hospital stay (days)	Outcome
1	F/37	4	–	+	3790	6690	10.7	53	Self-limited	29	Survived
2	M/44	4	–	+	5232	6419	9.0	41	Self-limited	31	Survived
3	M/34	4	Fatty liver, HBV carrier	+	2660	4040	17.4	34	SAH	54	Survived
4	M/41	4	–	–	1554	3738	16.1	48	Self-limited	31	Survived
5	M/47	4	–	–	3760	6330	7.1	53	Self-limited	36	Survived
6	M/41	3	–	–	3370	4490	5.9	62	Self-limited	25	Survived
7	M/41	3	–	–	2300	2940	3.3	84	Self-limited	26	Survived
8	M/59	4	HT	–	4770	4930	6.4	55	Self-limited	25	Survived
9	M/45	3	DM, HT	–	1535	1590	1.4	99	Self-limited	11	Survived
10	M/40	4	Essential thrombocytopenia	+	962	1318	11.1	74	Self-limited	25	Survived
11	M/57	4	DM, HT, HU, IHD	–	5200	3790	31.9	22	SAH	48	Survived
12	F/58	4	Bechet disease	–	1112	1058	10.6	90	Self-limited	18	Survived
13	M/19	3	–	–	38	151	0.8	100	Self-limited	Outpatient	Survived
14	M/36	4	–	–	2000	2676	1.6	78	Self-limited	16	Survived
15	M/66	3	DM, IHD	+	54	1052	5.5	100	Self-limited	16	Survived
16	F/51	3	HBV carrier, hypothyroidism	–	484	854	2.2	94	Self-limited	8	Survived
17	F/20	4	–	–	5490	4040	2.8	49	Self-limited	7	Survived
18	M/64	4	–	–	1574	1641	19.9	68	Self-limited	31	Survived
19	M/54	3	HT, BA	–	1275	2630	7.4	95	Self-limited	19	Survived
20	F/69	3	DM, HT	–	900	667	2.5	75	Self-limited	17	Survived
21	M/60	4	–	+	12100	17900	4.3	42	Self-limited	28	Survived
22	F/34	3	–	–	2170	1810	12.7	20	FH	91	Died
23	M/51	4	IHD	–	814	1493	14.2	61	Self-limited	33	Survived
24	M/54	4	–	–	3762	4263	10.0	44	Self-limited	31	Survived
25	M/45	3+4	HU	+	266	440	3.6	100	Self-limited	20	Survived
26	M/51	4	HT, GU	–	7617	3111	12.2	25	FH	22	Survived
27	F/54	4	–	+	247	1000	0.8	73	Self-limited	Outpatient	Survived
28	M/35	4	–	–	2890	3430	24.8	62	Self-limited	28	Survived
29	M/41	4	IHD, hypothyroidism, ML	+	5770	5860	4.2	63	Self-limited	28	Survived
30	M/69	4	Fatty liver, BA, HL, HU	–	1670	2065	16.9	37	SAH	20	Died
31	M/38	4	DM, HU	+	260	1120	0.6	92	Self-limited	12	Survived
32	M/52	4	Amyotrophic lateral sclerosis	–	1015	2295	0.8	97	Self-limited	7	Survived
33	M/60	4	DM	+	1254	3485	7.6	93	Self-limited	14	Survived
34	M/45	4	–	–	5600	7620	7.0	34	SAH	20	Survived
35	M/43	4	–	+	157	1269	5.3	100	Self-limited	33	Survived
36	F/45	4	–	+	2057	3034	5.4	100	Self-limited	Outpatient	Survived
37	M/51	4	DM	+	2660	2422	4.8	72	Self-limited	Outpatient	Survived

[a] Underlying disease defined as disease which had been under treatment before onset of hepatitis (see text).

[b] Alcohol overconsumption defined as daily ethanol ingestion over 50 g for 5 years or longer (see text). HT, essential hypertension; DM, diabetes mellitus; HU, hyperuricemia; IHD, ischemic heart disease BA, bronchial asthma; GU, gastric ulcer; ML, malignant lymphoma; HL, hyperlipidemia; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T.Bil., total bilirubin; PT, prothrombin time (in percent). AST, ALT, T.Bil. are peak levels, PT are lowest levels.

[c] Severity was classified as: (1) "self-limited" in which acute hepatitis had a benign course and lowest PT levels were not below 40%, (2) "SAH" in which PT was less than or equal to 40% without encephalopathy and (3) "FH" in which patients developed encephalopathy within 8 weeks after onset.

Table 2
Comparison of background characteristics, according to genotype

Patient characteristics	Genotype 3 ($N=9$)	Genotype 4 ($N=27$)	p-Value
Male (no., %)	6(67)	22(81)	0.36
Age median (years) (interquartile range)	45 (39–57)	47 (40 56)	0.86
Occupation (no. of patients, %)			
Handling with animals[a]	0	0	
Handling with raw food	1(11)	7(26)	0.35
Underlying disease	5(56)	13 (48)	0.70
History of (no., %)			
Traveling to endemic area	0	1(4)	0.56
Blood transfusion intake	0	0	
Wild animals[b]	0	0	
Raw pig liver or intestine	2(22)	9(33)	0.53
Raw fish or shellfish	7(78)	20 (74)	0.67
Alcohol overconsumption	1(11)	12 (44)	0.07

[a] SAH/FH was defined as with lowest PT ≤ 40%.
[b] Wild animals means as boar, deer and bear.

and the other (case 30) died of ischemic heart disease while recuperating from hepatitis.

3.2. Comparison between patients infected with HEV genotypes 3 and 4

3.2.1. Comparison of base-line characteristics between the two groups

The comparison of characteristics between HEV genotypes 3 and 4 is shown in Table 2. One patient (case 24) who had coinfection with HEV genotypes 3 and 4 was excluded from the analysis. There was no significant difference in age, the male-female ratio, occupations, or presence of underlying disease(s) between the two genotypes. Overconsumption of alcohol was seen in a higher proportion of patients infected with genotype 4 than with genotype 3 (12/27 versus 1/9; $p=0.07$). There was no patient with history of ingesting raw wild deer, boar, bear or rabbits. No patients in the two groups had history of blood transfusion, ingesting raw seafood, raw pig liver, and/or undercooked pig intestines before the onset of illness.

3.2.2. Comparison of clinical features between the two groups

The clinical features are shown in Table 3. Patients infected with genotype 4 had significantly higher peak serum levels of ALT (median: 3 430 IU/L, interquartile range: 1747–4763 versus 1052 IU/L, 845–2707; $p=0.01$), and had a trend toward having a higher peak serum aspartate aminotransferase (AST) levels (2660 IU/L, 1206–5092 versus 1275 IU/L, 451–2202; $p=0.07$) and total bilirubin (T.Bil.) levels (7.1 mg/dL, 4.4–13.7 versus 3.3 mg/dL, 2.0–6.3; $p=0.09$) than those with genotype 3. The median of the lowest PT tended to be lower in the genotype 4 group (61%, 42.5–77 versus 84%, 70–96; $p=0.06$). The proportion of patients with PT ≤ 60% was significantly higher in

Table 3
Comparison of clinical manifestations, laboratory data and outcome, according to genotype

Clinical features	Genotype 3 ($N=9$)	Genotype 4 ($N=27$)	p-Value
Symptoms and signs (no. of patients, %)			
Fever	5(56)	12 (44)	0.44
Malaise	7(78)	21 (78)	0.53
Jaundice	5(56)	19 (70)	0.33
Hepato-splenomegaly	3(33)	6(22)	0.84
Laboratory data median			
Peak AST (IU/L) (interquartile range)	1275 (451 2202)	2660 (1206 5092)	0.07
Peak ALT (IU/L) (interquartile range)	1052 (845–2707)	3430 (1747–4763)	0.01
Peak T.Bil. (mg/dL) (interquartile range)	3.3	7.1	0.09
Lowest PT (%) (interquartile range)	84 (70–96)	61 (42–77)	0.05
PT < 60% (no., %)	1(11)	13 (48)	0.03
SAH or FH[a] (no., %)	1(11)	5(19)	0.61
Duration for admission, median (days) (interquartile range)	18 (12 23.5)	26.5 (18–31)	0.06

[a] SAH/FH was defined as with lowest PT < 40%.

Table 4
Comparison of characteristics and laboratory data, according to lowest prothrombin time ≦40%

Patient characteristics/laboratory data	PT > 40% (N = 31)	PT ≤ 40% (N = 6)	p-Value
Male (no., %)	24 (77)	5(83)	0.72
Age (median) (interquartile range)	45 (41–54)	48 (34 57)	0.93
Genotype 3/4	8/23	1/5	0.61
History of			
Intake raw/undercooked pig liver/intestine (no., %)	8(26)	3(50)	0.41
Alcohol overconsumption	10 (32)	3(50)	0.44
Underlying disease(s)	14(41)	4(67)	0.37
Laboratory data median			
Peak AST (IU/L) (interquartile range)	1554	3930	0.05
Peak ALT (IU/L) (interquartile range)	2630	3450	0.32
T.Bil. on admission day or on initial visit (mg/dL) (interquartile range)	4.6	10.6	0.01

the genotype 4 group (13/27 versus 1/9; $p = 0.03$). Patients infected with genotype 4 showed a trend toward longer median duration of hospital stay (26.5 days, 18–31 versus 18 days, 12–23.5; $p = 0.06$), excluding four outpatients and two inpatients who died.

3.3. Analyses of factors related to the patients with lowest PT ≤ 40%

We used PT ≤ 40% (in patients with SAH/FH) as the basis of epidemiological factors to evaluate the influences of age, gender, genotype and history of exposure (Table 4). None of these demographic factors was significantly associated with PT being equal to or less than 40%. Infection with genotype 4 showed a trend toward significant association with the lowest PT ≤ 60% (odds ratio 13, $p = 0.1$, 95% CI 0.4–38).

4. Discussions

4.1. Summary of the patients with hepatitis E in Sapporo

Our report is rather unique in that detailed personal interviews were performed to explore patients' exposures that may not have been recalled when the patient was originally seen for hepatitis E. We maintained an attitude of strict neutrality during the interview to minimize recall bias and/or interview bias. The characteristics of patients with hepatitis E in our study are summarized as follows: many patients with hepatitis E were 40–60 years of age, as old as the non-endemic cases reported thus far in East Asia [7,22–24]; 28 patients (78%) were male, which gives a male-female ratio close to that reviewed by Okamoto et al. [32]; 27 patients (75%) were infected with genotype 4 in Sapporo, which gives a genotype 4 to 3 ratio close to that previously reported in Hokkaido [9,12]; all but one patient seem to have been infected with HEV in Japan; no patient had history of ingesting wild animal meat; only 11 patients (34%) had history of ingesting raw and/or undercooked intestines, which is different finding from the earlier reports [19,9]. The authors suspect that there may be other routes of infection than ingestion of wild animal meat [25].

4.2. Differences in clinical course between patients infected with HEV genotypes 3 and 4

Most cases of acute hepatitis E in Japan are shown to be caused by HEV genotypes 3 or 4 [9,10]. Whether viral genotypes have any influence on susceptibility and/or severity of illness remains unclear.

In our series, patients infected with genotype 4 consumed alcohol on a regular basis than those infected with genotype 3, whereas there was no significant difference in the other background characteristics between the two groups. Alcohol overconsumption, however, were not associated with severity of illness. Whether or not overconsumption of alcohol is associated with susceptibility to acute hepatitis E of a specific genotype awaits further research.

Patients infected with genotype 4 had more severe clinical manifestations (including laboratory data such as AST, ALT, T.Bil. levels, PT and duration of hospital stay) than those with genotype 3. The proportion of patients with PT ≤ 40% (SAH/FH), however, was not significantly different in the two groups, which may be due to the small number of cases. In order to avoid underestimating the severity of genotype 4, we used two different cut off levels of PT in the analysis; the cut off value of PT 40% is used in the definition of SAH/FH, and the cut off of PT 60% was close to the median PT of 63% in our study. The fact that having a PT ≤ 60% showed a trend toward association genotype 4 infection suggest the possibility that hepatitis E infection with genotype 4 tend to be more severe compared with infection with genotype 3.

Recently, Takahashi et al. [26] reported on four cases of severe hepatitis caused by a subtype of HEV genotype 4. In their report, the authors maintain that a certain cluster may possibly be associated with more severe clinical manifestations in HEV infections.

Mizuo et al. [19] reported that HEV genotype 4 and underlying disease(s) were possibly associated with more severe

disease of acute hepatitis. Although our result is similar to that by Mizuo et al. in that genotype 4 may be associated with severe disease, it differs from theirs in that the underlying disease(s) were not significantly associated with severity. The difference might be partly accounted for by different patient's backgrounds. The number of cases in both studies is too small to draw a conclusion on this issue. More research is being done on HEV genotypes and subtypes as a possible viral factor for SAH/FH.

4.3. Limitations of this study

The limitations of this study are as follows. (1) The number of cases was small and this might be the reason why statistical significance was not reached in the proportion of patients who fulfilled the criteria of SAH/FH (PT $\leq$ 40%) (a possible β error). (2) The study was done retrospectively, and thus limited information was available on the patient demographics and history of exposure.

5. Conclusions

In our cases of acute hepatitis E in Sapporo, patients with genotype 4 infection tended to have more severe disease. Age, gender, history of ingestion of raw and/or undercooked pig liver and/or intestines, underlying disease(s) were not significantly associated with HEV genotypes or with clinical course.

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Original Article

Prevalence of hepatitis E virus among wild boar in the Ehime area of western Japan

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Aims: Transmission of hepatitis E virus (HEV) from wild boar to humans has been reported, particularly from Japan. We attempted to clarify this issue.

Methods: We assessed the IgG class antibodies against HEV (anti-HEV) in serum samples taken from 406 boar living in the Ehime area of western Japan from 2001 to 2004, of which 392 were captured in the wild (wild-caught boar) and 14 had been kept in a breeding farm (bred boar).

Results: Anti-HEV positive rate in the bred boar (10/14, 71.4%) was significantly higher than in the wild-caught boar (100/392, 25.5%) ($P < 0.001$). Of the 392 wild-caught boar, 12 (3.1%) were positive for HEV-RNA, 10 of which were then subjected to phylogenetic analyses by sequencing an 821-nt fragment within ORF1. All the 10 isolates segregated to genotype 3, and eight of them were mutually related to form a cluster. All the eight HEV isolates in this cluster were from the wild-caught boar living in one and the same habitat within the studied area, while the other two independent isolates were from different regions.

Conclusion: HEV infection is endemic in wild boar in the Ehime area, and we should regard the wild boar as an important reservoir of HEV.

Key words: genotype, hepatitis E virus, wild boar, zoonosis

INTRODUCTION

HEPATITIS E VIRUS (HEV), a nonenveloped virus with a single-stranded RNA of approximately 7200 bases,[1] is prevalent mostly in tropical and subtropical countries, such as those located in south Asia, north and central Africa, and Mexico.[2] Hepatitis E in other areas had been thought to be an 'imported' infection until recently many reports described a patient(s) infected with HEV domestically in the USA, European nations or in Japan.[3–13] While transmission via blood transfusion undoubtedly occurs,[14–17] a significantly more important transmission route seems to be "zoonotic": from swine,[18–24] wild boar[25–28] and wild deer[29,30] to humans. In wild boar, Sonoda *et al.*[31] and Nishizawa *et al.*[32] found HEV-RNA in 3% (1/35) and 2.3% (2/87), respectively. However, the numbers of boar tested in those studies were limited, and thus the prevalence of HEV infection among wild boar is yet to be fully understood. In the present study, we assessed the prevalence of HEV in boar in western Japan on a much larger scale in order to clarify this issue.

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METHODS

Subjects

WE TESTED 406 boar (*Sus scrofa*) in the Ehime area of western Japan, which is located in the northwestern part of Shikoku Island. Of those, 392 were captured in the wild (wild-caught boar) from 2001 to 2004, while 14 had been kept in a breeding farm (bred boar) in the investigated area. Sixty-two of the wild-caught boar were estimated to be 0–1 years old, 65 were 1–2 years old, 130 were 2–3 years old, 41 were 3 or more years old, and 94 were age unknown: 155 were males, 150 were females and 87 were sex unknown. As for bred boar, 13 were aged 1–2 years and one was aged 2–3 years: five were males and nine were females. Serum samples were obtained from each and stored at −70°C until tested.

Detection of anti-HEV

IgG class antibodies against HEV in the wild boar's sera were determined using an in-house enzyme-linked immunosorbent assay (ELISA), with some modifications from the previously reported method.[33] Briefly, we used a recombinant ORF2 protein of HEV (kindly provided by Dr Li Tian-Cheng) as capture antigen, and horseradish peroxidase-labeled antipig IgG (MP Biomedicals Inc., OH) as tracer antibodies.

Detection and sequencing of HEV genome

Detection and nucleotide sequencing of the HEV-RNA in the wild boar's sera were performed by the methods described previously.[5,34] Phylogenetic analyses were done on the partial HEV-RNA sequence of 821 nucleotides (nt) in length within ORF1, which corresponds to a region coding for RNA-dependent RNA polymerase.[35]

Statistics

Statistic analyses were done by χ^2-test. A *P*-value of less than 0.05 was considered as significant.

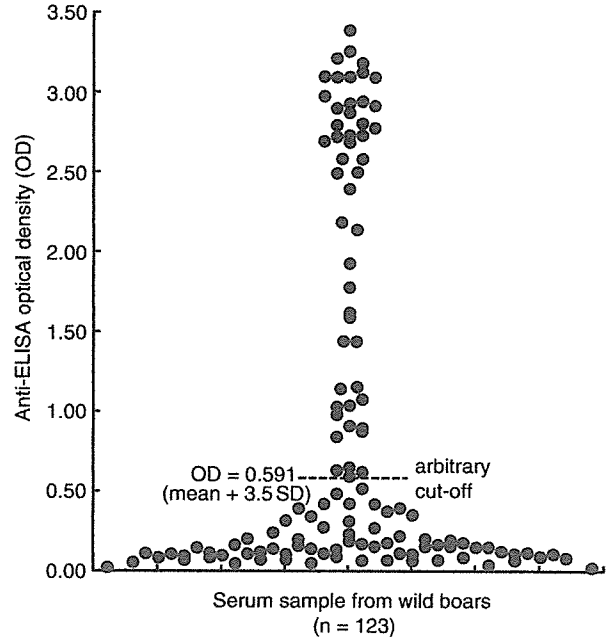

Figure 1 Optical density (OD) of anti-HEV determined by enzyme-linked immunosorbent assay (ELISA).

RESULTS

SERA FROM 123 wild boar caught in 2004 were assayed for anti-HEV, and optical density (OD) values were analyzed to determine the cut-off value (Fig. 1). An OD value of 0.591 calculated using 3.5 standard deviations above the mean value was employed as the cut-off value.

Anti-HEV was positive in 25.5% of the wild-caught boar, with no significant difference between males and females (Table 1). The positive rate in wild-caught boar less than 1 year old was 6.4% (4/62), while it was approximately 30% in those older than 1 year. The anti-HEV prevalence differed also by place and by year of capture, as shown in Table 2, in which the investigated area (Ehime prefecture, which is approximately 5700 km^2) was divided into three parts: east, central and south-west.

HEV-RNA was found in 12 (3.1%) of 392 wild boar, and nine of the 12 were also positive for anti-HEV

Table 1 Anti-HEV in the wild-caught boar in relation to estimated age and sex

	Number	Positive for anti-HEV	Anti-HEV (OD) Range (central)
Sex			
Male	155	40 (25.8%)	0.046–3.396 (0.280)
Female	150	46 (30.7%)	0.029–3.220 (0.318)
Unknown	87	14 (16.1%)	0.090–3.159 (0.245)
Age in years			
0–1	62	4 (6.4%)	0.075–3.190 (0.201)
1–2	65	21 (32.3%)	0.029–3.264 (0.274)
2–3	130	44 (33.8%)	0.046–3.181 (0.311)
3+	41	14 (34.1%)	0.160–3.396 (0.382)
Unknown	94	17 (18.1%)	0.029–3.220 (0.257)
Total	392	100 (25.5%)	0.029–3.396 (0.275)

OD, optical density.

Table 2 Anti-HEV in the wild-caught boar in relation to year and area of capture

Year of capture	Area of capture				Total
	East	Central	South-west	Unknown	
2001	1/38 (2.6%)	10/30 (33.3%)	3/27 (11.1%)	1/10 (10.0%)	15/105 (14.3%)
2002	2/15 (13.3%)	3/50 (6.0%)	1/27 (3.7%)	1/4 (25.0%)	7/96 (7.3%)
2003	4/21 (19.0%)	24/44 (54.5%)	0/3 (0%)	0/0 (0%)	28/68 (41.2%)
2004	5/42 (11.9%)	38/64 (59.4%)	7/15 (46.7%)	0/2 (0%)	50/123 (40.7%)
Total	12/116 (10.3%)	75/188 (39.9%)	11/72 (15.3%)	2/16 (12.5%)	100/392 (25.5%)

(Table 3). An 821-nt sequence was determined in 10 of the 12 HEV-RNA-positive samples (the other two samples were unable to sequence the 821-nt, but a shorter sequence, 69-nt, was available from them) for phylogenetic analyses. As shown in Figure 2a, all the wild boar-derived 821-nt sequences analyzed in this study segregated to genotype 3 (those for which only 69-nt was available were also of genotype 3), but were clearly distinct from any of the previously reported sequences. Interestingly, eight isolates from the wild boar in this study conformed to a compact cluster. This was conceivable because all of the eight isolates were derived from boar living in the same habitat within the studied area. The other two isolates, genetically distant from the eight isolates, were from boar captured at different sites (Fig. 2b).

The positive rates of anti-HEV showed a significant difference between wild-caught boar and bred boar in total (25.5% vs. 71.4%, $P < 0.001$), as well as in an age-matched comparison: anti-HEV was positive in 18.2% (14/44) of the wild-caught, but in 69.2% (9/13) of the bred boar ($P < 0.05$) when only those aged 1–2 years were compared. Despite quite a high rate of anti-HEV, none of the bred boar was positive for HEV-RNA, similarly to the reported case of bred swine in which viremia was recognized only within 6 months of birth.[36]

DISCUSSION

OUR PRESENT RESULTS showed that approximately 25% (100/392) of boar caught in the wild were positive for anti-HEV, with HEV-RNA in 3% (12/392).

Table 3 The boar positive for HEV-RNA

Age (years)	Sex	Place	Year	Anti-HEV (OD)	Genotype	HEV isolate name
0–1	F	South-west	2003	(-)	3	JBOAR221-Ehi03R
1–2	F	Central	2001	3.010	3	‡
1–2	F	Central	2003	2.938	3	JBOAR247-Ehi03R
1–2	M	Central	2003	2.366	3	JBOAR243-Ehi03R
1–2	F	Central	2004	1.451	3	JBOAR288-Ehi04R
2–3	F	Central	2003	3.000	3	JBOAR261-Ehi03R
2–3	F	Central	2003	2.723	3	JBAOR263-Ehi03R
2–3	M	East	2004	(-)	3	JBOAR351-Ehi04R
†	M	South-west	2002	1.557	3	JBOAR024-Ehi02R
†	†	East	2002	2.175	3	‡
†	F	Central	2004	3.220	3	JBOAR300-Ehi04R
†	†	South-west	2004	(-)	3	JBOAR398-Ehi04R

†Unknown; ‡Only a 69-nt sequence was available.
OD, optical density.

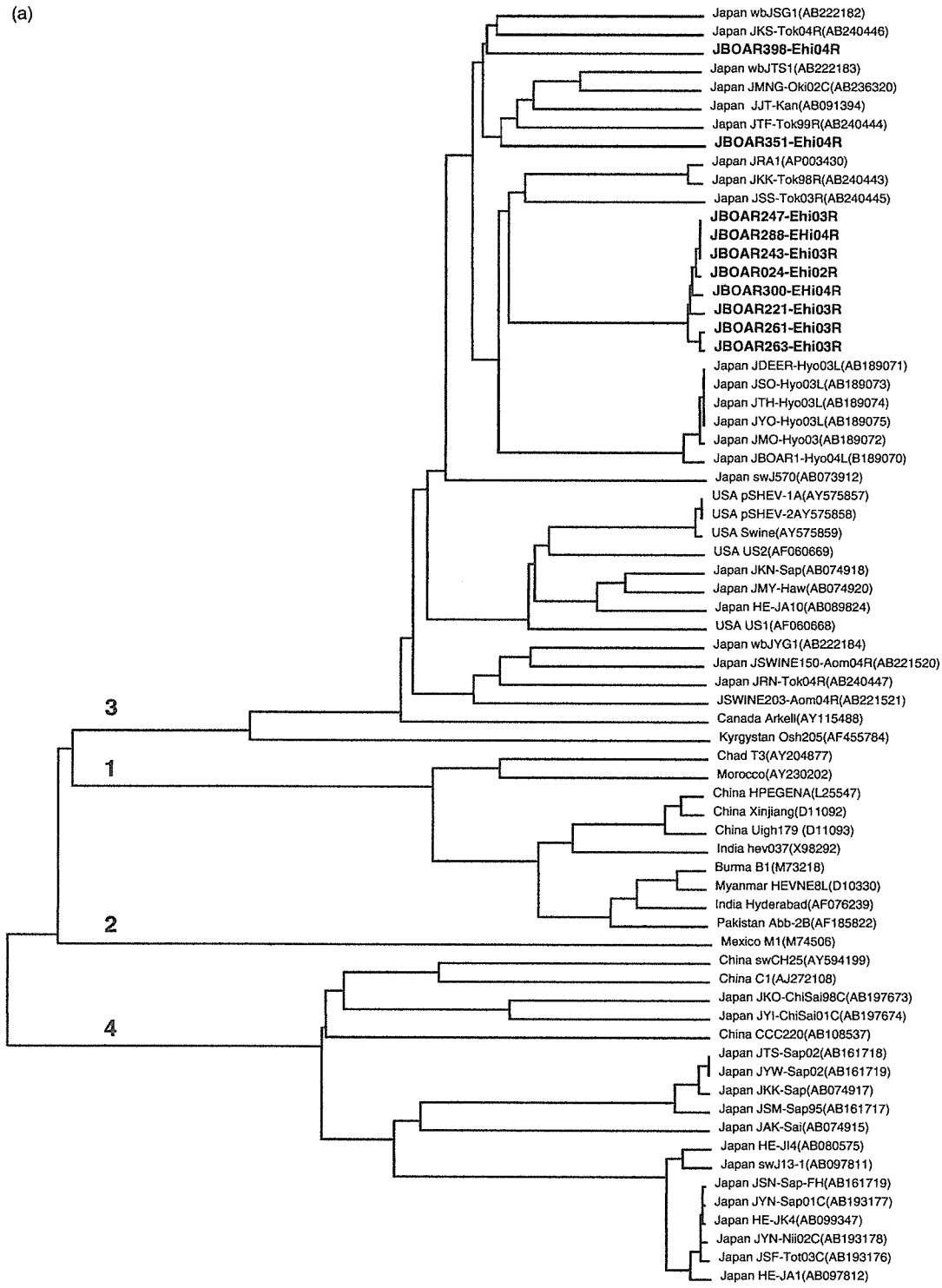

Figure 2 (a) Phylogenetic tree based on the partial nucleotide sequence of ORF1 (821-nt). DDBJ/EMBL/GenBank accession numbers are shown in parentheses.

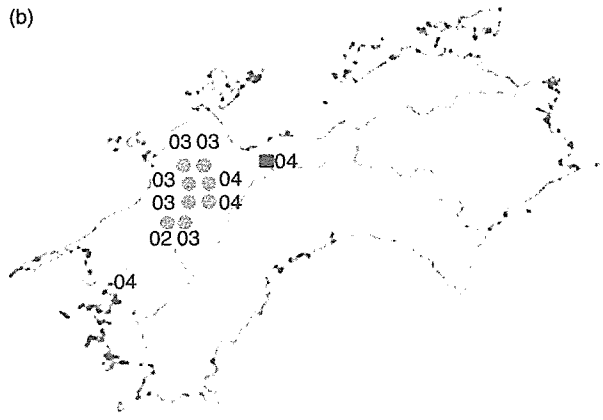

Figure 2 *Continued.* (b) Information on the place and year of capture for the 10 wild boar from which the 821-nt sequence was available. Circles, squares, and stars correspond to the isolates similarly highlighted in Figure 2(a). Numbers shown in the figure represent the year in which the boar was caught (02, 03, and 04 represent the years 2002, 2003, and 2004, respectively).

In the present study, three of 12 HEV-RNA-positive boar were negative for anti-HEV, which may suggest that these three were infected with HEV very recently. The rate of viremic boar is striking, because HEV infection is basically transient, not persistent, which would allow viremia be detected much easier. Our findings indicate that HEV is endemic in wild boar, at least in the Ehime area of Japan. We have a patient with acute hepatitis E in this area who had a history of cooking and eating the meat of wild boar (unpublished data), therefore wild boar should be regarded as an important infectious source.

Wild boar are distributed throughout Europe, Africa, Asia and some areas of North America, and their habitat includes broad-leaved woodland and grassland areas. They eat both plants and small animals, such as roots, fruits, nuts, insects and small mammals. They often live near muddy puddles where they routinely wallow so as to remove parasites from their body surface. Female wild boar remain with their offspring and form a family group, whereas older males live alone. During the mating season in autumn, several family groups often live together and form a herd. Although the transmission routes of HEV among wild boar remain unknown, it is possible that their habit of wallowing in mud and their tendency to form herds may have some association with fecal–oral transmission.

As for the bred boar, in which quite a high rate of anti-HEV was found in this study, their situation is more comparable to farm pigs than to the boar in the wild. They are kept together in a limited space with a limited freedom, this means the horizontal spread of infective agents occurs more easily than in the wild. In such circumstances HEV infection occurs very early on: it has been reported that pigs kept in farms get infected with HEV within a few months of birth.[36] The same thing may have happened in the bred boar population studied here. In the same way that most farm pigs lost HEV-RNA before 6 months of age, our bred boar may have lost it by the time they were subjected to this study (all were aged more than 1 year).

HEV is classified into four major genotypes[37] with different distribution by geographic regions: genotype 1 in Asia and Africa, genotype 2 in Mexico and Nigeria, genotype 3 in the USA, European countries, Argentina, Korea and Japan, and genotype 4 in east Asian countries.[38–45] All of the 12 HEV-RNA-positive wild boar in the present study had HEV of genotype 3. In other studies in Japan, genotypes 3 and 4 HEV have been isolated from both humans and boar.[25,30,31,34] Being the same genotype as those previously reported from Japan notwithstanding, the HEV isolates we report here are phylogenetically unique, as shown in Figure 2a. In addition, the finding that there were three different strains (one cluster consisting of eight isolates, and two solitary strains) corresponding to three different habitats might reflect the fact that wild boar tend to establish their own territory and hardly leave that area.

In conclusion, the results of the present study of wild boar captured in the Ehime area suggest that the wild boar in this area are an important reservoir of HEV, and should be regarded as an infectious source for humans.

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