a humidified atmosphere. Plates were washed and 100 μl of substrate *o*-phenylenediamine (Tokyo Chemical Industry Co., Ltd., Japan) was applied to each well. Thirty minutes later, reactions were stopped by adding 100 μl of 2 N H_2SO_4 and the optimal density (OD) was measured at 492 nm in an ELISA plate reader (Corona, Japan). The inhibition rate was measured using the following formula:

Inhibition rate (%)=$(N-S)/(N-P)\times 100$ where N= mean OD_{492} value of negative controls, P=mean OD_{492} value of positive controls and S=mean OD_{492} value of specimens. Specimens with non-specific responses detected in the same manner using the non HAV-binding plates were excluded from this study.

Titration of anti-HAV positive specimens. Titers of anti-HAV were determined in all positive specimens and are expressed as mIU/ml using the parallel line assay (19) with a national human anti-HAV reference calibrated with a WHO standardized preparation. All positive sera were separated into five age categories as follows: 0–49 years (n=32), 50–59 years (n=103), 60–69 years (n=71), 70–79 years (n=60) and 80–92 years (n=31). The geometric mean titer (GMT) of anti-HAV in each age group was determined.

Data analysis. All data were statistically assessed by the χ^2-test or one-factor ANOVA using Statcel software (version 1; OMS, Japan, 1998). $P<0.05$ was accepted as the minimal level of significance.

Results

Prevalence of Anti-HAV

Figure 2 shows that all specimens plotted by inhibition rate were divided into two groups. Specimens with inhibition rates above 80% were regarded as anti-HAV positive. The cutoff inhibition rate of 80% was equivalent to 352.3 mIU/ml. Of the 2,340 serum specimens, 297 were anti-HAV positive (Table 1). Anti-HAV prevalence remained very low until the age of 45–49 years. Most individuals below or equal to 49 years of age were HAV susceptible. Seroprevalence gradually increased among persons above 50 years of age. The highest seroprevalence was 86.5% in the oldest age group (≥65 years). Seroprevalence between males (158/1,242) and females (139/1,188) did not significantly differ (P=0.59).

Geographic differences in anti-HAV seroprevalence in the Tohoku (north area), Kanto (central east area), Kansai (central west area) and Kyushu-Yamaguchi (south area) regions of Japan were analyzed over the age groups 0–9, 10–19, 20–29, 30–39, 40–49, 50–59 and over 60 years old (Fig. 3). Age-specific seroprevalence was very similar in populations under 59 years of age in each area. Statistical differences were evident in individuals over 60 years of age between Tohoku and Kansai (P=0.0066), and between Kansai and Kyushu-

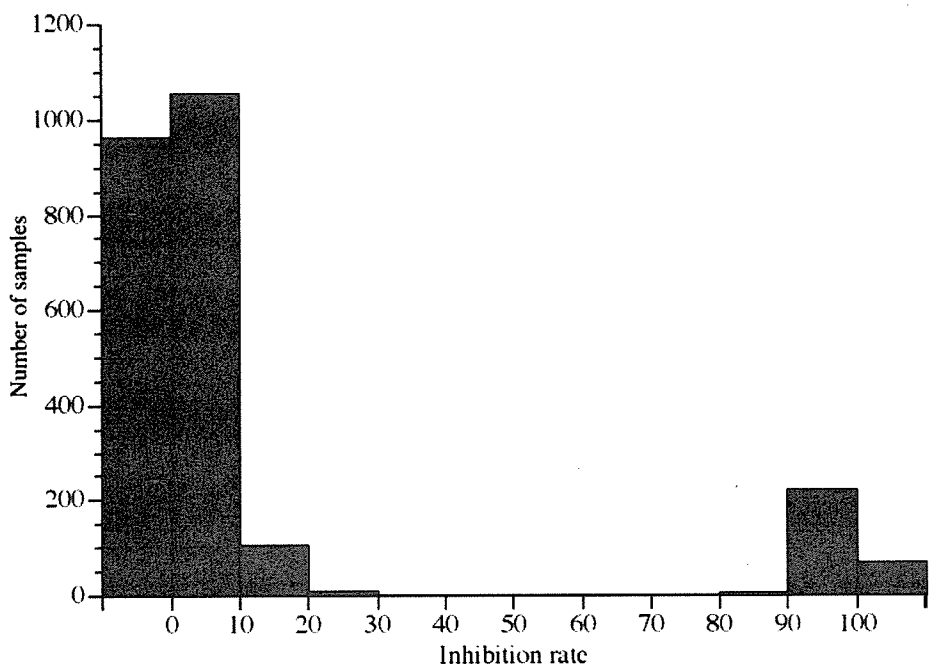

Fig. 2. Distribution of inhibition rates. Sera were divided into two groups by inhibition rate. Sera over 80% inhibition corresponding to 352.3 mIU/ml of anti-HAV antibody were identified as anti-HAV positive.

Yamaguchi (P=0.012).

Titration of Anti-HAV Positive Specimens

The GMT values of anti-HAV were 7,486 (95% confidence interval, 4,437–12,629), 7,397 (6,061–9,027), 6,210 (4,742–8,132), 7,944 (5,917–10,666), and 5,007 (3,351–7,481) mIU/ml for the age groups 0–49, 50–59, 60–69, 70–79 and 80–92 years old, respectively (no significant difference, P=0.34).

Discussion

In 2003, the overall anti-HAV prevalence in Japan was 12.2% (Table 1) and gender did not affect this value. The GMT of anti-HAV in five age groups remained high and did not change with age (P=0.34). This confirmed that individuals who acquired anti-HAV immunity would retain high titers of anti-HAV and lifelong immunity. Fifty percent of individuals over 50 years of age had anti-HAV whereas only 1.68% of people younger than 50 years of age had immunity. Because of the low incidence of hepatitis A, fewer younger people would acquire HAV-immunity, whereas anti-HAV positive individuals who mostly belong to older age groups have retained anti-HAV from past exposure. Seroprevalence also remained very low in individuals under the age of 40–44 years who were born around 1960. This finding implies that the incidence of hepatitis A in Japan has been rare since 1960. The seroprevalence of the oldest age group (over 65

Table 1. Age-specific prevalence of anti-HAV in Japan, 2003

Area	Tohoku								Kanto					
Prefecture	Akita		Yamagata		Miyagi		Fukushima		Niigata		Ibaraki		Nagano	
Age (years)	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive
0–4	13	0	NA		NA		16	0	NA		19	0	9	0
5–9	23	0	NA		NA		11	0	NA		21	0	23	0
10–14	8	0	NA		2	0	14	0	NA		15	0	NA	
15–19	17	0	NA		27	0	17	0	NA		7	0	4	0
20–24	4	0	1	0	14	0	2	0	NA		9	0	43	1
25–29	14	0	NA		11	0	23	0	1	0	14	0	12	0
30–34	9	0	NA		7	0	17	0	32	1	14	0	21	0
35–39	11	2	NA		1	0	8	0	29	3	11	0	14	1
40–44	15	0	NA		9	1	21	0	26	1	6	0	24	0
45–49	9	2	19	0	8	0	4	0	19	3	10	2	27	0
50–54	14	5	13	2	14	2	11	3	26	6	9	1	19	3
55–59	11	6	12	7	6	1	14	5	20	11	9	2	14	8
60–64	4	4	6	3	1	1	5	4	2	1	5	3	8	7
65–	23	23	19	19	NA		18	14	NA		NA		14	13
Total	175	42	70	31	100	5	181	26	155	26	149	8	232	33

Area	Kansai				Kyushu-Yamaguchi						Total		
Prefecture	Fukui		Kyoto		Yamaguchi		Fukuoka		Saga				
Age (years)	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	%
0–4	NA		25	0	34	2	16	0	24	0	156	2	1.3
5–9	NA		34	0	35	0	46	0	26	0	219	0	0
10–14	NA		24	0	71	0	35	0	18	0	187	0	0
15–19	NA		19	0	44	0	45	0	18	0	198	0	0
20–24	1	0	24	0	42	1	43	0	12	0	195	2	1
25–29	2	0	27	0	46	3	38	1	15	1	203	5	2.5
30–34	NA		19	0	41	0	37	0	14	0	211	1	0.5
35–39	NA		24	0	42	0	34	1	12	0	186	7	3.8
40–44	13	0	32	0	17	1	12	0	13	0	188	3	1.6
45–49	5	1	23	3	11	0	13	1	12	0	160	12	7.5
50–54	15	5	32	4	17	5	8	0	15	6	193	42	21.8
55–59	4	2	26	12	11	3	3	2	7	2	137	61	44.5
60–64	NA		9	5	2	2	1	1	6	3	49	34	69.4
65–	NA		26	19	26	23	NA		22	17	148	128	86.5
Total	40	8	344	43	439	40	331	6	214	29	2,430	297	12.2

NA: not available.

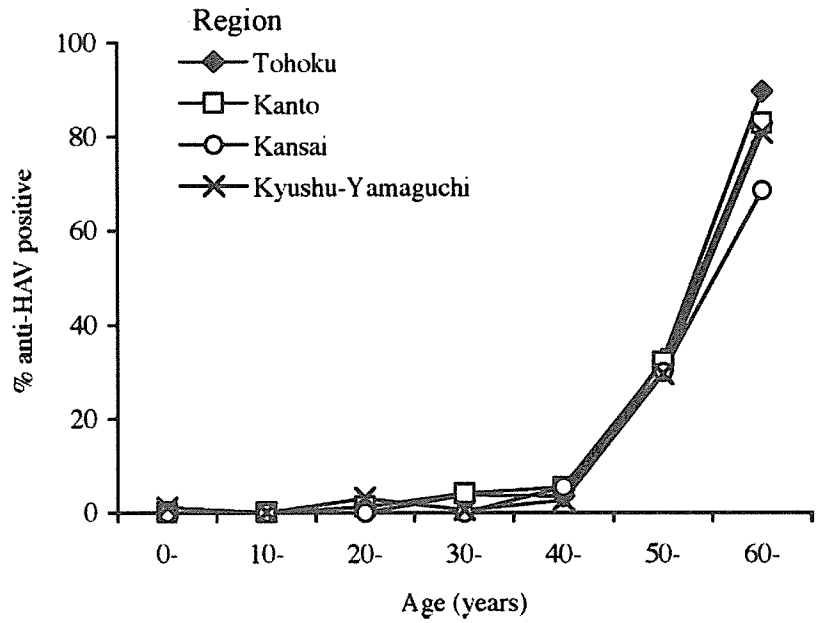

Fig. 3. Geographic distribution of anti-HAV antibody. Geographic distribution does not significantly differ among 4 areas of Japan until the population reaches 60 years of age. Seroprevalence in individuals over 60 years old significantly differs between Kansai and Tohoku (P=0.0066), and between Kansai and Kyushu-Yamaguchi (P=0.0121).

years old) is the highest at 86.5%. Individuals within this population, born before 1940, presumably acquired immunity during an era when hepatitis A was endemic in Japan. Between 1940 and 1960, risk of HAV-exposure was decreased by rapid infrastructural improvement, including hygiene conditions particularly after World War II.

Interestingly, regional differences in seroprevalence were evident only in individuals over 60 years of age (Fig. 3). We surmise that when HAV was endemic over 60 years ago, the frequency of hepatitis A endemicity varied across locations. However, regional features might have no impact on current hepatitis A status.

Figure 4 shows the shift in anti-HAV seroprevalence in Japan during the past three decades. The shapes of seroprevalence curves derived from studies reported in 1973, 1984, 1994 (12) and 2003 have remained similarly sigmoidal, and shift towards older groups in 10-year increments. Numbers of HAV-susceptible individuals have accumulated not only among younger generations, but also among older age groups. The seroprevalence of anti-HAV in individuals older than 50 years in 2003 was 50.3%, which is significantly lower than that of corresponding studies in 1994 (74.3%), 1984 (96.9%) and 1973 (96.9%). This has led to a shift in hepatitis A patients towards older age groups, in which clinical illness is more frequent and severe (Fig. 5) (8, 13).

Hepatitis A is one of the major vaccine preventable diseases. Hepatitis A vaccine is an inactivated preparation of cell-culture adapted HAV, and has been used in many parts of the world (20). Several hepatitis A mass vaccination programs have been started since 1997 in regions of intermediate endemicity. A universal vaccination program in the United States of America resulted in a 76% decline in the overall incidence of hepatitis A to 2.6 per 100,000 reported in 2003, compared with 10.7 per 100,000 during the baseline period of 1990–1997 (18). A study in Israel found that a toddler-only universal vaccination program effectively reduced the incidence of hepatitis A by 95% (4). In that study, the annual pre-vaccination incidence of 50.4 per 100,000 declined post-vaccination to 2.2–2.5 per 100,000. The authors of these reports suggested that this decline was not solely a result of the universal vaccination program but was also associated with improved environmental and hygiene conditions. However, both studies suggest that the implementation of a universal hepatitis A vaccination program for children provides a significant degree of immunity for individuals who live in intermediate epidemic regions. Determining the impact of hepatitis A on current health care resources and defining the benefit and cost of hepatitis A vaccination will greatly influence the decision as to whether to implement such programs in particular countries and population groups (1, 3). The recent incidence of hepatitis A of 0.34 per 100,000 in Japan is lower than the post-vac-

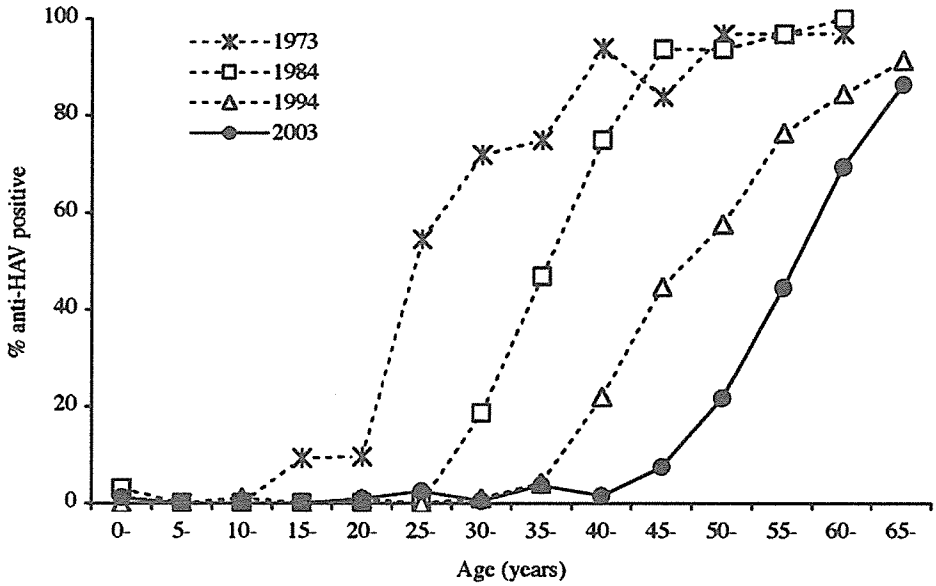

Fig. 4. Age-specific seroprevalence of anti-HAV from 1973 to 2003 in Japan. Data for 1973, 1984 and 1994 are cited from Ref. 12.

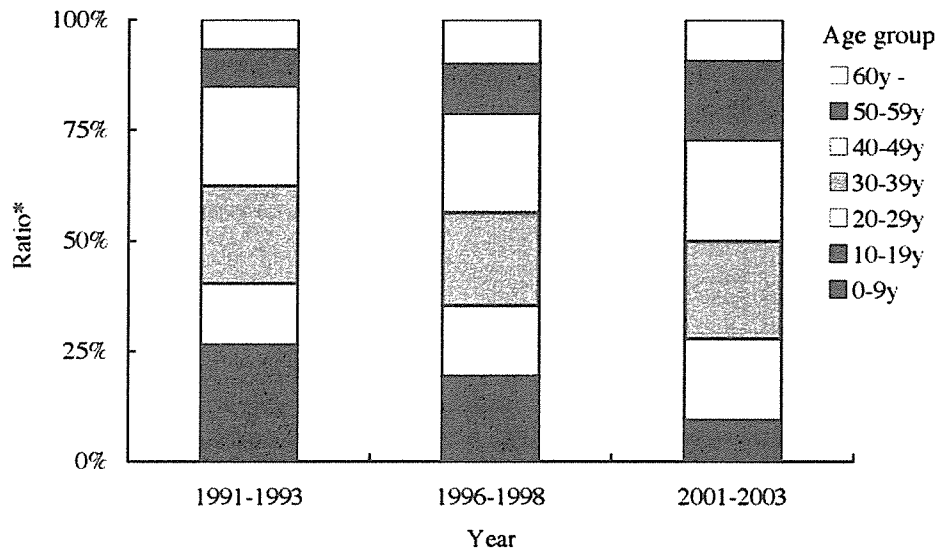

Fig. 5. Age-distribution of hepatitis A reported cases in Japan from 1991 to 2003. Data are cited from Ref. 15. Ratio of reported cases aged 50–59 increased during the period 1989 to 2003. Simultaneously, ratio of the 0–9 and 10–19 age groups decreased. * Ratio=Number of reported cases per age group in each study period/all reported cases in each study period (%). Study period was separated into 3-year intervals.

cination incidence in studies reported from the United States and Israel, namely 2.6 and 2.2–2.5 per 100,000, respectively. The current incidence of hepatitis A in Japan might be too low to warrant the introduction of a universal vaccination policy.

Hepatitis A infection might not be considered as a disease of great importance in Japan due to its low frequency and low mortality rate. However, our study suggests that susceptibility to hepatitis A among Japanese is increasing. As in other low HAV-endemic areas of the world, the major risk factors for hepatitis A in Japan have been travel to endemic areas (16), consumption of contaminated food including that imported from HAV endemic areas (6), patients medicated with clotting factors (9) and men who have sex with men (2). In addition, immunization of food handlers would seem

worthwhile, to prevent the possibility of food-borne, common-source outbreaks of hepatitis A. Furthermore, people with chronic liver disease are not more likely to be infected with hepatitis A virus, but they are more likely to develop fulminant hepatitis and die if infected (10).

In conclusion, the timely release of information regarding the current status of hepatitis A together with a vaccination program for high-risk groups, food handlers and persons with chronic liver disease in Japan would be suitable for the control of this virus at present.

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SEROLOGIC EVIDENCE FOR HEPATITIS E VIRUS INFECTION IN MONGOOSE

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Abstract. Although pig and wild boar are considered to be the reservoirs of hepatitis E virus (HEV) in Japan, the spread of HEV to other animals is unknown. Serum samples from 84 mongooses (Small Asian mongoose; *Herpestes javanicus*) collected in Okinawa, Japan were examined for antibodies to HEV by enzyme-linked immunosorbent assay and RNA was analyzed by reverse transcription–polymerase chain reaction. Seven (8.3%) of 84 mongooses were positive for IgG antibodies to HEV, and the antibody-positive rate increased with body weight and size, whereas HEV RNA was not detected in these samples. These results are consistent with the possibility that mongooses in Okinawa are occasionally infected with HEV; however, they are not considered the major zoonotic reservoir of HEV.

INTRODUCTION

Hepatitis E virus (HEV) is the most important cause of acute hepatitis in many developing countries in Asia, the Middle East and north Africa,[1] and was recently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae.*[2] Hepatitis E was first recognized when a large waterborne hepatitis outbreak occurred in India in 1955, where the antibody-positive rate of hepatitis A virus is extremely high in all age groups.[3] Because HEV is transmitted via an oral-fecal route, contaminated drinking water and food are the primary source of the infection. Although hepatitis E is self-limiting and neither chronic nor persistent infection is observed in the adult population in general, a high mortality rate of 15–20% is reported in pregnant women.[4]

Hepatitis E virus is a nonenveloped, single-stranded positive-sense RNA virus.[5] Phylogenetic analysis has identified at least four major genotypes of HEV.[6] Genotype 1 (G1) HEV was isolated from Asia and Africa,[1,4] genotype 2 (G2) from Mexico,[7] and genotypes 3 (G3) and 4 (G4) from the United states, European countries, China, Japan, and Vietnam.[8–13] These viruses are believed to compose a single serotype.[14]

Although most cases were imported into developed countries, recent studies have showed that hepatitis E occurred in patients who had never been abroad.[9,10] Genetically similar G3 and G4 HEVs isolated from pigs, deer, and wild boars,[8,15,16] and serum antibodies to HEV in a variety of animals including pigs, deer, wild boars, wild rats, dogs, cats, and cows[17,18] suggest that hepatitis E is a zoonosis. Recently, direct evidence of G3 HEV transmission from deer and wild boar meats to humans was clearly provided in Japan, suggesting that wild animals are the zoonotic reservoir of HEV in this country.[15,16] Transmission from visceral organs of pigs to humans has also been suspected.[19]

Okinawa is located southwest of Japan, where mongoose (Small Asian mongoose; *Herpestes javanicus*), an exotic animal, was introduced from India in 1910 for the control of a poisonous snake (habu) and rats. The number of mongooses and their living area increased quickly because there was no natural predator of this animal in Okinawa. This caused a disruption of the ecology. Since wild boars also live on this island, mongooses have an opportunity to be exposed to HEV from infected boars.

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To find HEV reservoirs other than pigs, deer, and wild boars, and to determine whether HEV spreads to other wild animals, we analyzed serum samples from 84 mongoose captured in Okinawa for antibodies to HEV and viral RNA. IgG antibodies to HEV were found in 8.3% of the mongooses, suggesting that although HEV infection may occur among mongooses, they are not the major zoonotic reservoir of HEV.

MATERIALS AND METHODS

Mongoose sera. Wild *H. javanicus* (54 males and 30 females) were captured between July 2004 and May 2005 in Okinawa. The mongooses were transferred to the laboratory and anesthetized. Their sex was identified, and their body weights and head and body length (body size) were measured. Blood was collected by cardiac puncture under anesthesia, and allowed to clot at room temperature for one hour. The serum fraction was collected by centrifugation and stored at −20°C until use.

Preparation of recombinant virus-like particles. A recombinant baculovirus, Ac5480/7126, that harbors the G1 HEV capsid protein gene with a 111-amino-acid deletion at the N-terminal was constructed as previously described.[20] Briefly, Tn5 cells (High Five™; Invitrogen, Carlsbad, CA) were infected with Ac5480/7126 at a multiplicity of infection of 10 and incubated at 26.5°C for 7 days. The intact cells and cell debris were removed from the culture medium, and the recombinant virus-like particles (VLPs) with a molecular mass of 53 kD were concentrated by centrifugation at 100,000 × *g* for 2 hours in an SW28 rotor (Beckman Instruments, Inc., Fullerton, CA). The VLPs were further purified by isopycnic binding in a CsCl gradient.[21] Recombinant baculoviruses that express N-terminal truncated capsid proteins of G3 and G4 HEV were similarly prepared, and the 53-kD VLPs were also prepared (Li T-C and others, unpublished data).

Detection of antibodies to HEV in mongoose. Flat-bottom 96-well polystyrene microplates (Immulon 2; Dynex Technologies, Inc., Chantilly, VA) were coated with the purified VLPs (1 μg/mL, 100 μL/well). The plates were incubated overnight at 4°C. Unbound VLPs were removed, and the wells were washed twice with 10 mM phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) and blocked at 37°C for 1 hour with 200 μL of 5% skim milk (Difco Laboratories, Detroit, MI) in PBS-T. After the plates were washed four times with PBS-T, mongoose serum (100 μL/well) was added in duplicate at a dilution of 1:200 in PBS-T containing 1%

skim milk. The plates were incubated at 37°C for 1 hour and washed four times as described above. The wells were incubated with 100 μL of peroxidase-conjugated goat anti-cat IgG (heavy plus light chain) (1:4,000 dilution) (Kirkegaard and Perry Laboratories, KPL, Guildford, United Kingdom) or anti-cat IgM (1:1,000 dilution) (Kirkegaard and Perry Laboratories) in PBS-T containing 1% skim milk. The plates were incubated at 37°C for 1 hour and washed four times with PBS-T. One hundred microliters of substrate (*o*-phenylenediamine; Sigma Chemical Co., St. Louis, MO) and H_2O_2 was added to each well. The plates were incubated in the dark at room temperature for 30 minutes and 50 μL of 4 N H_2SO_4 was added to each well. After the plates were incubated at room temperature for 10 minutes, the absorbance at 492 nm was measured.

Western blot assay. Approximately 1 μg of VLPs was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in 50 mM Tris–HCl, pH 7.4, and 150 mM NaCl and incubated with mongoose serum (1:500 dilution), followed by horseradish peroxidase (HRP)–conjugated goat anti-cat IgG (heavy plus light chain) (1:1,000 dilution). The membrane was treated with electrogenerated chemiluminescence detection reagent (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instruction and exposed to FP-3000B45 film (Fuji, Tokyo, Japan).

Detection of HEV RNA by reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was extracted with RNAzol-LS reagent (Tel-Test Inc., Friendswood, TX) using 200 μL of the mongoose serum and resuspended in 20 μL of DNase-, RNase-, and proteinase-free water. Reverse transcription was performed at 42°C for 50 minutes and 70°C for 15 minutes with 1 μL of oligo (dT) primer, 1 μL of superscript II reverse transcriptase (Gibco-Bethesda Research Laboratories, Gaithersburg, MD), 0.5 μL of 0.1 M dithiothreitol, 4 μL of 5× reverse transcription buffer, and 1 μL of 10 mM deoxynucleoside triphosphates. Two microliters of the resulting cDNA were amplified in a 50-μL nested PCR with ExTaq DNA polymerase (Takara Shuzo Co. Ltd., Kyoto, Japan) with an external sense primer HEV-F1 (5′-GGBGTBGCNGAGGAGGAGGC-3′, nucleotide residues 5903-5922 of the G1 Myanmar strain D10330) and an antisense primer HEV-R2 (5′-TGYTGGTTRTCRTARTCCTG-3′, nucleotide residues 6486-6467 of the G1 Myanmar strain, GenBank accession no. D10330) using the GeneAmp PCR System 9700 (Perkin Elmer Biosystems, Foster City, CA). Each cycle consisted of the denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds, followed by a final extension at 72°C for 7 minutes. The nested PCR was conducted with the internal sense primer HEV-F2 (5′-TAYCGHAAYCAAGGHTGGCG-3′, nucleotide residues 5939-5958) and the internal antisense primer HEV-R1 (5′-CGACGAAATYAATTCTGTCG-3′, nucleotide residues 6316-6297) using the same conditions.[16]

RESULTS

Detection of mongoose IgG and IgM with anti-cat IgG and IgM. Because no peroxidase-conjugated antibody to mongoose IgG or IgM is commercially available, we explored the cross-reactivity of mongoose IgG and IgM with those of other animals. Since the mongoose is in the family *Herpestiade* order *Carnivora*, the reactivity between mongoose IgG or IgM and anti-cat IgG or IgM was evaluated.

Two-fold dilutions of pooled mongoose sera were used to coat the microplate. After blocking, peroxidase-conjugated goat anti-cat IgG or IgM was added to determine whether anti-cat antibodies are capable of binding to the mongoose antibodies. Peroxidase-conjugated goat anti-rabbit IgG or IgM was added to the control wells. As shown in Figure 1, the mongoose serum reacted with both HRP-goat anti-cat IgG and IgM. The control well did not show any reactivity with these antibodies. These results indicated that the anti-cat antibodies cross-reacted with mongoose IgG and IgM, and that the HRP-goat anti-cat IgG and IgM are useful as the second antibody in detecting mongoose IgG and IgM by enzyme-linked immunosorbent assay (ELISA).

Detection of IgG and IgM antibodies to HEV in mongoose sera. The mongoose serum samples were tested for IgG and IgM antibodies to HEV at a dilution of 1:200 by ELISA. The distribution of optical density (OD) values is shown in Figure 2. The OD values for IgM antibodies to HEV ranged from 0.09 to 0.321, and one serum sample with a titer of 200 had an OD value greater than 0.20. The OD values for IgG antibodies to HEV ranged from 0.011 to 3.751, and 7 sera whose titers ranged from 200 to 12,800 had OD values greater than 0.20 (Table 1).

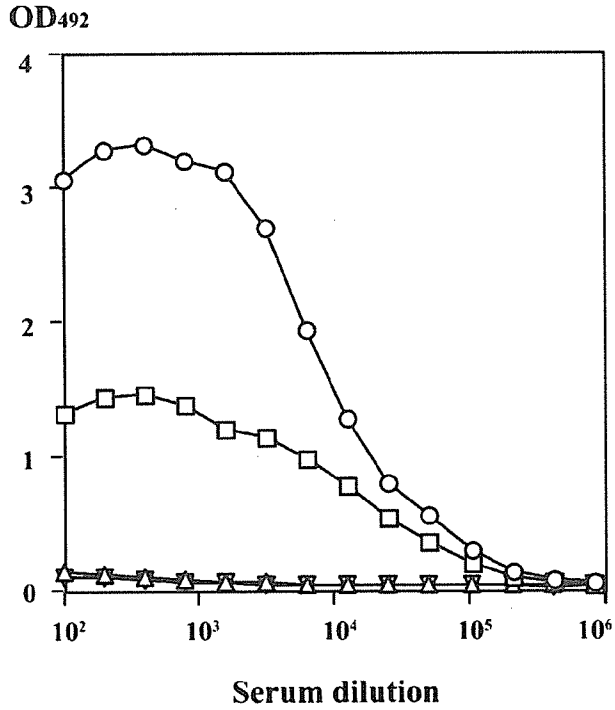

FIGURE 1. Detection of mongoose IgG and IgM with anti-cat IgG and IgM by enzyme-linked immunosorbent assay. Two-fold dilutions of pooled sera from 10 mongooses were used to coat a 96-well microplate. The reactivity of peroxidase-conjugated goat anti-cat IgG (○) and IgM (□) or horseradish peroxidase–conjugated goat anti-rabbit IgG (△) and IgM (▽) was measured. OD_{492} = optical density at 492 nm.

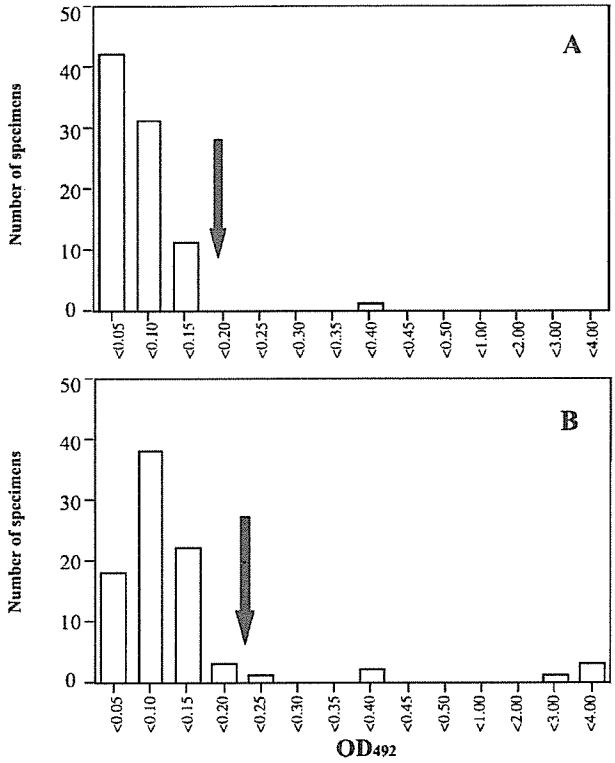

FIGURE 2. Distribution of mean optical density values at 492 nm (OD_{492}) of mongoose IgM (**A**) and IgG (**B**) antibodies. Serum samples from 84 mongooses were tested, and the values were plotted as a frequency distribution. The arrows indicate the cut-off values.

Specificity of IgG antibody in mongoose sera. To determine whether the IgG antibody detected in mongoose sera was specific for HEV, nine serum samples were selected and examined by Western blot assay. The G1, G3, and G4 VLPs were separated by SDS–PAGE and electrophoretically transferred onto a nitrocellulose membrane. A serum dilution of 1:500 was used for the assay. As shown in Figure 3, strong bands with a molecular mass of 53 kD corresponding to G1, G3, and G4 VLPs were detected with in serum samples 8, 39, 58, and 66. The OD values of these sera ranged from 2.529 to 3.751, as determined by ELISA. Weak bands were detected with in serum samples 52 and 65 whose OD values were 0.383 and 0.387, respectively. No bands were detected in serum samples 60, 41, and 10, which had low OD values, 0.204, 0.175, and 0.065, respectively, as determined by ELISA. These results indicated that the IgG antibodies to HEV detected in mongoose serum by ELISA were specific for HEV.

TABLE 1
OD values and antibody titers in mongoose sera*

Serum sample	OD values (IgG/IgM)	Antibody titers (IgG/IgM)
No. 8	3.751/0.321	12,800/200
No. 39	3.268/0.065	6,400/(< 200)
No. 52	0.383/0.047	400/(< 200)
No. 58	3.268/0.103	12,800/(< 200)
No. 60	0.204/0.049	200/(< 200)
No. 65	0.387/0.067	400/(< 200)
No. 66	2.529/0.098	3,200/(< 200)

* OD = optical density.

Prevalence of IgG and IgM antibodies to HEV in mongoose sera. The cutoff value of IgG for the ELISA was determined with 78 antibody-negative serum samples. The OD values for IgG antibodies to HEV in these serum samples ranged from 0.011 to 0.204 (mean ± SD value = 0.086 ± 0.037). The cutoff value (mean + 3SD) was 0.196 (Figure 2). When this value was used, the prevalence of IgG antibodies to HEV was 8.3% (7 of 84). The antibody-positive rate was 3.3% in females and 11.1% in males; however, the difference between sexes was not statistically significant ($P > 0.05$). The average body weight and body size were 565.3 grams (range = 182.5–1,037.9) and 553.8 mm (range = 402–654), respectively.

When the antibody-positive rate was analyzed according to body weight, the antibody-positive rate for IgG to HEV was 11.1% in animals with body weights of 500–599 grams, 12.5% in animals with body weights of 600–699 grams, 11.1% in animals with body weights of 700–799 grams, 20% in animals with body weights of 800–899 grams, and 33.3% in animals with body weights greater than 900 grams. IgG antibody to HEV was not detected in animals with body weights less than 500 grams. Thus, the antibody-positive rate increased with body weight.

The antibody-positive rate for IgG to HEV was 11.1% in animals with a body size of 550–599 mm and 21.2% in animals with a body size of 600–654 mm. IgG antibody to HEV was not detected in animals with body sizes less than 500 mm, which shows that antibody-positive rate also increased with the body size. The mean ± SD OD value of IgM antibody to HEV in these 78 sera was 0.062 ± 0.031. The cutoff value was 0.155 (0.062 + 3 × 0.031). When analyzed with this cutoff value, one mongoose serum sample (no. 8) was positive for IgM antibody; the prevalence rate was 1.2% (1 of 84).

Detection of HEV RNA by RT-PCR. All 84 mongoose serum samples tested by RT-PCR were negative for HEV RNA.

DISCUSSION

The mongoose is a small, cat-like carnivore that is a member of the family *Herpestidae*. It is between one and four feet in length, and inhabits in Asia, the Caribbean, and southern Europe, comprising more than 30 species. Although the small Asian mongoose was introduced into Okinawa for the control of habu snakes and rats, this animal eats not only habu snakes and rats, but also other small animals.

An ELISA with recombinant HEV VLPs was used to detect IgG and IgM antibodies to HEV in the mongoose. This assay was capable of detecting antibodies to HEV in human sera with high sensitivity and specificity.[22] Although only one serotype was recognized and four genotypes were identified in HEV, we used G1, G3, and G4 VLPs as antigens to compare the reactivity. No significant difference was found between the genotypes. The specificity of the ELISA was confirmed by Western blot assay. Among seven samples positive for IgG antibodies to HEV by ELISA, one sample (no. 60) was be negative by Western blot assay, a result that was probably due to a low antibody titer in the ELISA (OD value of the 1:200 serum dilution = 0.204 and antibody titer = 200) and the lower sensitivity of the Western blot assay.

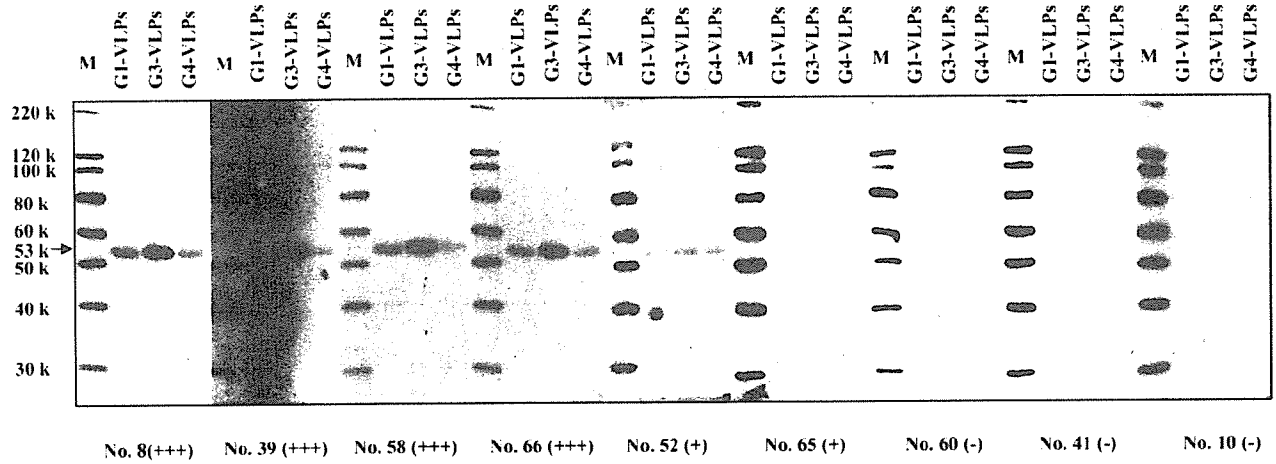

FIGURE 3. Specificity of the IgG antibody determined by Western blot assay. The G1, G3, and G4 virus-like particles (VLPs) were used as the antigens, and nine mongoose sera with different optical density values were evaluated. Strong bands (+++), weak bands (+), and no bands (−) by Western blot assay are indicated. Lane M = molecular weight marker.

In this study, only one mongoose was positive for IgM antibodies to HEV and the titer was low (200). We were not able to amplify any HEV sequence in these 84 mongoose serum samples, including the sample positive for IgM antibodies to HEV. Therefore, the genotype of the HEV-infected mongoose in Okinawa is unknown. In experimentally infected monkeys, the period of the viremia was very short, and HEV RNA was detected in serum only 1–2 weeks after seroconversion.[23] This could be one of the reasons why the HEV genome was not detected in serum.

The prevalence of antibodies to HEV was high in domestic pigs and wild boar; they are considered to be possible reservoirs of HEV in Japan. In contrast, the prevalence of IgG antibodies to HEV in mongooses (8.3%) was lower than that in pigs (58%), wild rats (44–94%), or wild boars (44%)[24,25] (Li T-C and others, unpublished data). This finding suggests that mongooses may not be the major reservoir of HEV in Okinawa, but may occasionally be infected with HEV. The current source and route of infection are not clear. Since many wild boars inhabit Okinawa and were eventually infected with HEV, the virus might spread to the surrounding environment by means of animals' stool. Mongooses also inhabit the region that wild boars inhabit, and might thus be exposed to HEV. Because mongooses catch insects, crabs, worms, lizards, and other small creatures for food, transmission of HEV may occur if these creatures are infected. The positive rate for IgG antibody to HEV increases with body weight and size, indicating that the exposure to HEV increases with age.

It has been reported that HEV is excreted in low concentrations by humans,[26] and that a small amount of HEV is excreted in experimentally infected monkeys. This may explain the lower efficiency of the transmission of HEV, and may result in the lower prevalence of antibodies to HEV in the mongoose.

In summary, mongooses in Okinawa were infected with HEV; however, the source of infection, the routes of transmission, and their genotype are still unknown. Further serologic and genetic investigations with larger number of specimens are needed.

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SHORT REPORT: DETECTION OF HEPATITIS E VIRUS RNA FROM THE BIVALVE YAMATO-SHIJIMI (*CORBICULA JAPONICA*) IN JAPAN

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Abstract. To evaluate whether bivalves are contaminated with hepatitis E virus (HEV), samples of a bivalve called Yamato-Shijimi (*Corbicula japonica*) were examined for HEV by reverse transcription–polymerase chain reaction. Genotype 3 HEV was detected from 2 of 32 packages of Yamato-Shijimi obtained from Japanese rivers, which indicated that HEV contaminates river water in Japan.

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus recently classified as the sole member of the genus *Hepevirus* in the family Hepeviridae.[1,2] This virus causes human hepatitis E and is transmitted primarily by the fecal-oral route through contaminated drinking water.[3,4] However, recent studies have demonstrated that various animal species have serum antibodies to HEV, and its viral genome has been detected in swine, wild deer, wild boar, and mongoose, which suggests that hepatitis E is a zoonotic disease.[5–10] Because HEV is excreted into feces,[11,12] there is a risk of HEV contamination in environmental water. In fact, HEV has been detected in sewage from industrialized countries, including Spain, the United States, and France.[13–15] However, HEV contamination of river water has not been examined. In the present study we detected the HEV genome from a bivalve called Yamato-Shijimi (*Corbicula japonica*), which suggested that river water in Japan is contaminated with HEV.

A total of 32 packages of Yamato-Shijimi were obtained at a fish market in December 2005 and March 2006. All samples were harvested at areas A, B, C, D, E, F, G, and H in western Japan. The package numbers and collection days are shown in Table 1. The samples were shucked, and the digestive diverticulum were removed by dissection and weighed. One gram of digestive diverticulum obtained from 10–15 Yamato-Shijimi was homogenized with an Omni-mixer (OCI Instruments, Waterbury, CT) in 10 mL of phosphate-buffered saline, pH 7.4, for two 30-second intervals at a maximum speed of 18,000 rpm. After centrifugation at 10,000 × *g* for 30 minutes at 4°C, the supernatant was centrifuged at 100,000 × *g* for 2 hours in an SW41 rotor (Beckman Instruments, Inc., Fullerton, CA). The pellet was resuspended in 140 μL of distilled water and stored at -80°C until use.

Total RNA was extracted with the QIAmp viral RNA mini kit (Qiagen, Hilden, Germany) and resuspended in 20 μL of DNase-, RNase-, and proteinase-free water. Reverse transcription (RT) was performed at 42°C for 50 minutes, followed by 70°C for 15 minutes in a 20-μL reaction mixture containing 1 μL of Superscript™ II RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA), 1 μL of oligo (dT) primer, 1 μL of RNaseOUT™ (Invitrogen), 2 μL of 0.1 M dithiothreitol, 4 μL of 5× RT buffer, 1 μL of 10 mM deoxynucleoside triphosphates, 5 μL of RNA, and 5 μL of distilled water. An RT–polymerase chain reaction was performed to amplify part of the open reading frame 2 (ORF2) as described previously.[8,10] Two microliters of the cDNA was used for the first PCR in a 50-μL reaction mixture with external forward primer HEV-F1 (5′-GGBGTBGCNGAGGAGGAGGC-3′) and external reverse primer HEV-R2 (5′-TGYTGGTTRT-CRTARTCCTG-3′), which corresponded to nucleotide residues 5903–5922 and 6486–6467, respectively, of the G1 Myanmar strain (D10330). Each cycle consisted of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds, followed by final extension at 72°C for 7 minutes. Two microliters of the first PCR product was used for a nested PCR with internal forward primer HEV-F2 (5′-TAYCGHAAYCAAGGHTG-GCG-3′; nucleotide residues 5939–5958) and internal reverse primer HEV-R1 (5′-CGACGAAATYAATTCTGTCG-3′, nucleotide residues 6316–6297) under the same conditions.

Two packages, B4 and B6, collected in area B on February 7, 2006, and March 1, 2006, were positive for HEV RNA by

TABLE 1
Detection of hepatitis E virus (HEV) in *Corbicula japonica*

Package no.	Collection day	HEV RNA
A1	12/08/05	–
A2	12/22/05	–
A3	1/14/06	–
A4	1/22/06	–
A5	1/22/06	–
A6	2/05/06	–
A7	2/17/06	–
A8	3/02/06	–
A9	3/14/06	–
A10	3/15/06	–
B1	12/10/05	–
B2	12/17/05	–
B3	1/24/06	–
B4	2/07/06	+
B5	2/19/06	–
B6	3/01/06	+
B7	3/17/06	–
B8	3/18/06	–
C1	1/10/06	–
D1	1/16/06	–
D2	1/20/06	–
D3	3/14/06	–
E1	1/21/06	–
E2	1/26/06	–
E3	2/25/06	–
E4	3/10/06	–
F1	3/13/06	–
F2	3/14/06	–
F3	3/18/06	–
F4	3/18/06	–
G	3/18/06	–
H	3/18/06	–

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RT-PCR. The PCR products were purified using the QIAquick PCR purification kit (Qiagen) and cloned into TA cloning vector pCR2.1 (Invitrogen). The nucleotide sequence of each of 10 clones was determined. Most of the sequences formed a single genotype 3 cluster. The exceptions (B4-13) formed a different cluster along with Sakai-9 detected from a wild boar in 2004 in Japan (Figure 1). We found large numbers of small different nucleotide sequences among the clones with 88.9–100% identity, even when they were derived from the same package, which indicated that multiple HEV strains were accumulated in the digestive diverticulum of Yamato-Shijimi.

To further analyze the HEV RNA detected in the Yamato-Shijimi, the entire ORF2 of B4 RNA was amplified as overlapping segments, and the nucleotide sequences were determined. The full-length ORF2 consisted of 1,980 basepairs and were phylogenetically classified into genotype 3. High amino acid identities (97.57–98.87%) were observed with HEV strains detected from hepatitis E patients, swine, wild boar, and wild deer in Japan. This is the first report on the detection of HEV from a bivalve.

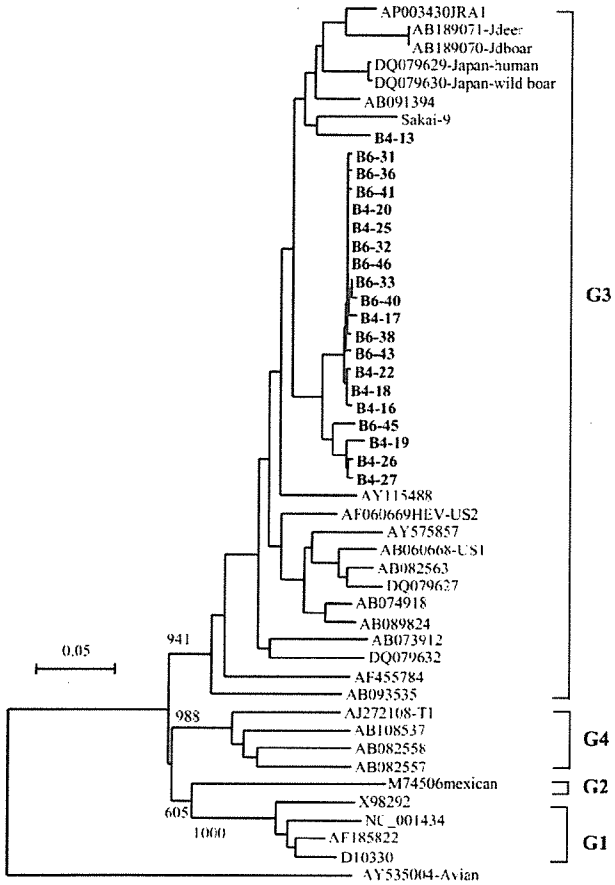

FIGURE 1. Phylogenetic trees of hepatitis E virus (HEV) constructed with avian HEV as an outgroup. A partial open reading frame 2 ORF2 (338 basepairs) of each of 10 clones of B4 (B4-13, B4-16, B4-17, B4-18, B4-19, B4-20, B4-22, B4-25, B4-26, and B4-27) and B6 (B6-31, B6-32, B6-33, B6-36, B6-38, B6-40, B6-41, B6-43, B6-45, and B6-46) were analyzed by the neighbor-joining method. The bootstrap values correspond to 1,000 replications. All nucleotide sequences determined in this study are shown in bold. Other HEV sequences were obtained from GenBank.

The HEV sequences were detected from Yamato-Shijimi (*Corbicula japonica*) harvested on February 7, 2006, and March 1, 2006, in western Japan. The Yamato-Shijimi, a brackish-water bivalve, grows in sandy mud in or near rivers, ponds, and lakes. During breathing and feeding, this bivalve filters a large amount of water. When the water is contaminated with HEV, the virus is ultimately concentrated in the digestive diverticula of the bivalves.

The source of HEV in this organism is not known. Since Japan was considered not to be endemic for this virus, and disposal of sewage is in this country is efficient, the risk of HEV contamination from human stool was believed to be low. There have been no outbreaks of hepatitis E in Japan from drinking water. However, HEV has been detected in wild deer, wild boar, and mongoose in Japan, and HEV shed in the feces of these animals may pollute environmental water. Wild deer and wild boar are controlled in Japan to eliminate their ability to damage agriculture and forestry; hunting is the main control strategy. Hunters usually wash killed animals in river water and this would increase the risk of HEV contamination in river water. These wild animals presumably play an important role in the contamination of environmental water.

In Japan, many outbreaks caused by bivalves contaminated with hepatitis A virus and noroviruses have been reported. Fortunately, Yamato-Shijimi is generally eaten as an ingredient in hot miso soup in Japan, and the heat, usually at 100°C for nearly 10 minutes, decreases the risk of HEV transmission from Yamato-Shijimi to humans. However, more efforts are needed to determine the infectivity and stability of HEV in the natural environment, including that in Yamato-Shijimi.

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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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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

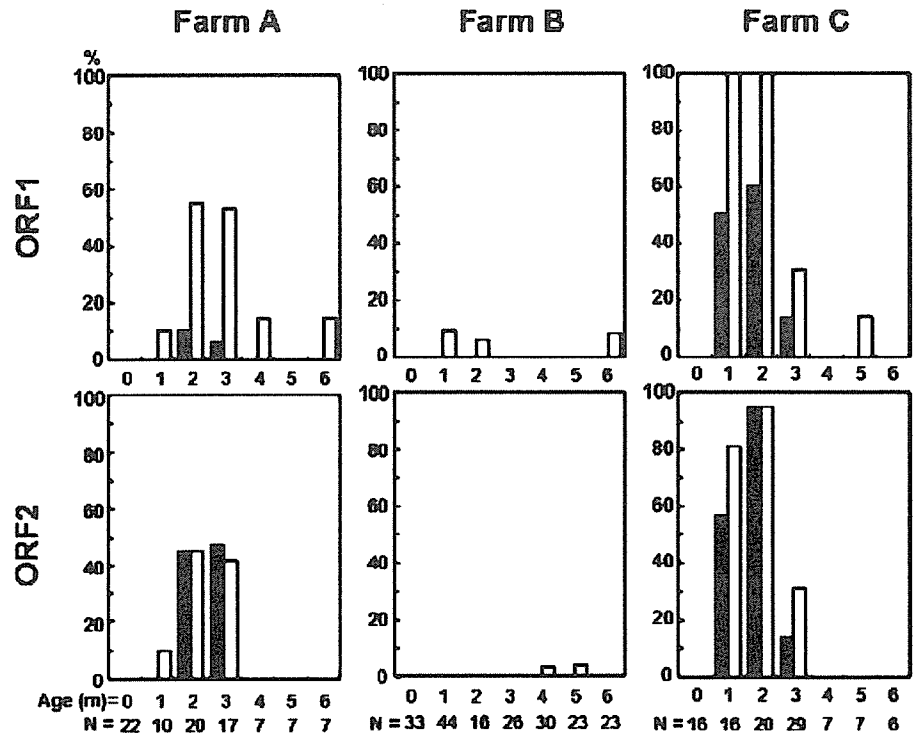

FIGURE 1. Frequencies of pigs shedding hepatitis E virus (HEV) in feces on three farms in Japan. The open reading frame 2 (ORF1) and ORF2 genes of HEV were amplified from each fecal sample by reverse transcription–polymerase chain reaction (RT-PCR). Solid and open bars represent frequencies of HEV-positive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

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 (anti-sense, 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 (anti-sense): 5′-CCCTTRTCYTGCTGMGCRTTCTC-3′ for the first PCR, and 3158N (sense): 5′-GTWATGCTYTGCATW-CATGGCTC-3′ and 3159N (anti-sense): 5′-AGCC-GACGAAATCAATTCTGTC-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.

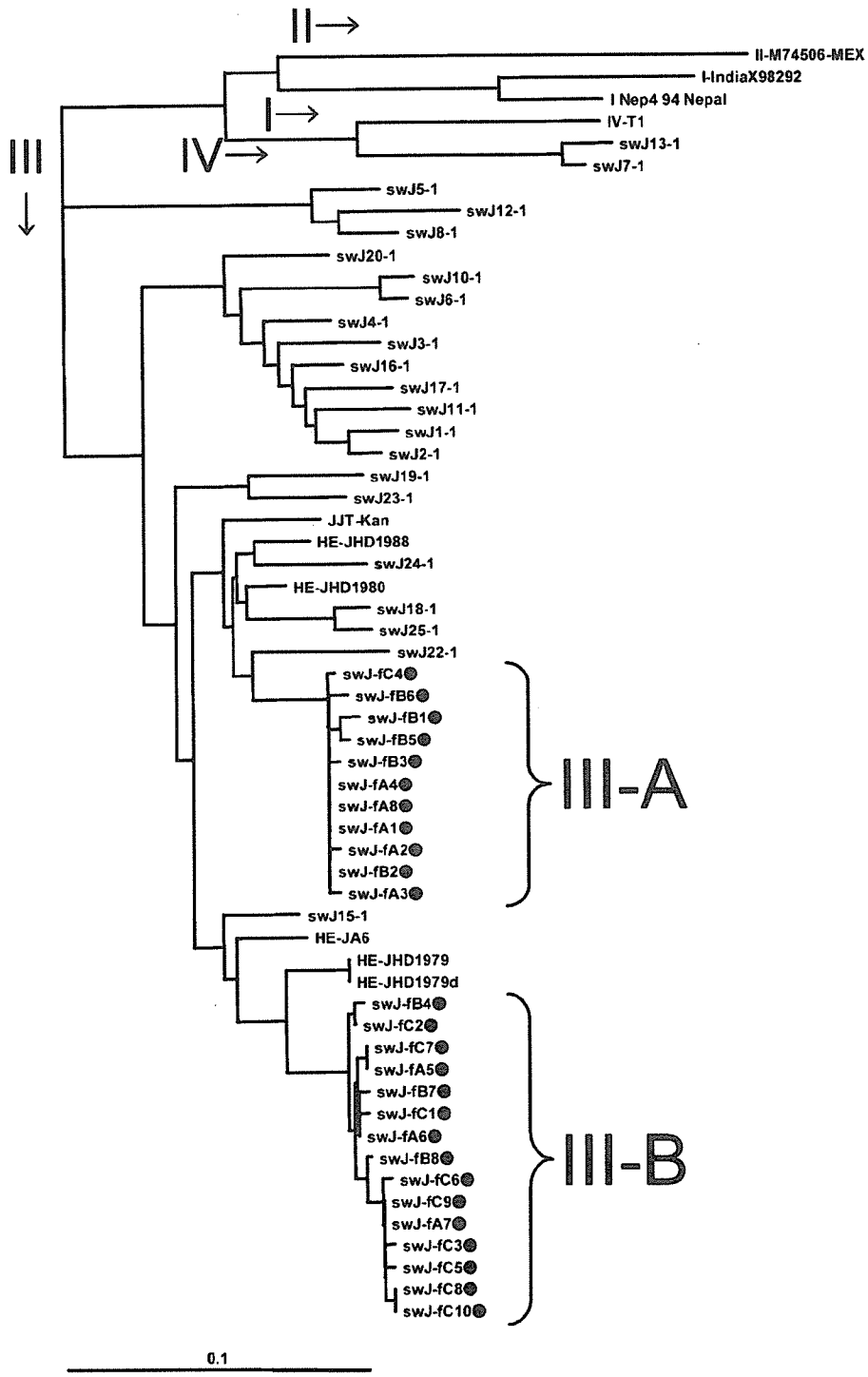

FIGURE 2. Phylogenetic tree constructed by the neighbor-joining method for hepatitis E virus (HEV) open reading frame 2 (300 bases). The 26 sequences isolated from farms A, B and C in this study (Table 2) belonged to either cluster III-A or III-B. The tree included our 26 isolates, typical sequences 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-

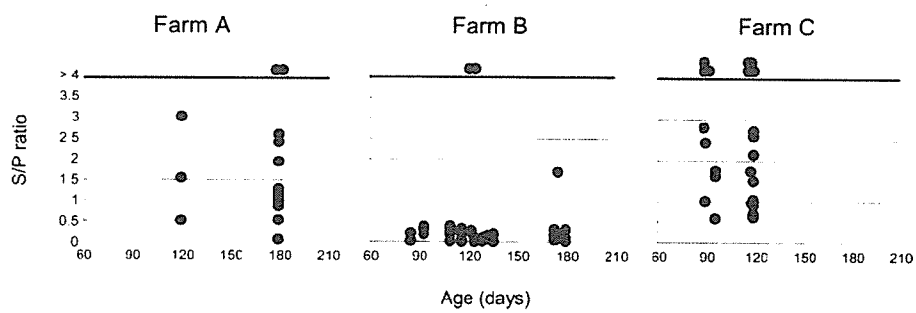

FIGURE 3. IgG antibody titers to hepatitis E virus in pigs from three farms in Japan. The enzyme-linked immunosorbent assay cutoff value was a sample-to-positive (S/P) ratio of 0.55. The percentages of pigs with an S/P ratio above the cutoff value were 82% (14 of 17) from farm A, 5% (3 of 65) from farm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

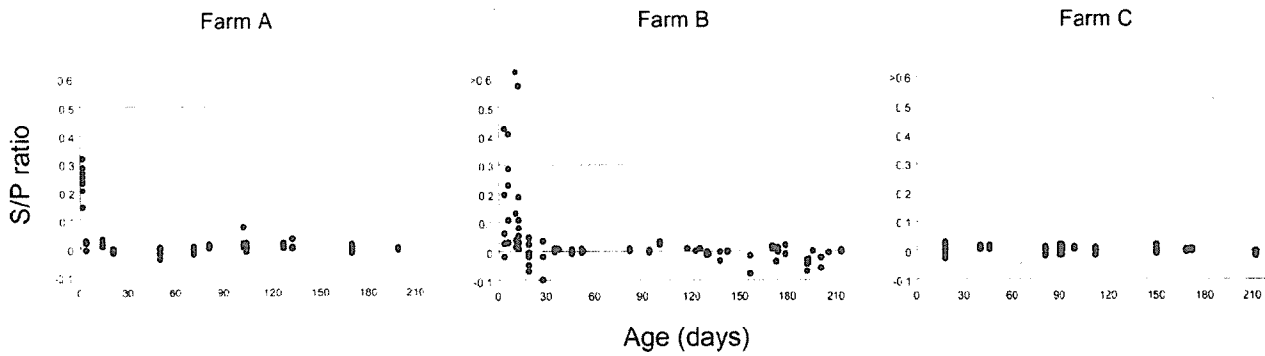

FIGURE 4. IgA antibody titers to hepatitis E virus in swine feces from three farms in Japan. The enzyme-linked immunosorbent assay cutoff value was a sample-to-positive (S/P) ratio of 0.078. A total of 90 samples were tested from farm A, 101 from farm B, and 90 from farm C. Samples having an S/P ratio 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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