described previously[8]. After centrifugation, supernatants were serially diluted, and added to plates containing HeLa cells. The cells were overlaid with DMEM containing 1% low melting agarose and incubated for 48 h. Then plaques were counted.

Echocardiography

Two days after EMCV infection, echocardiography was performed on mice anesthetized with 2.5% avertin (8 μl/g) using ultra-sonography (SONOS-5500, equipped with a 15-MHz linear transducer, Philips Medical Systems). Hearts were imaged in a two-dimensional parasternal short-axis view, and an M-mode echocardiogram of the midventricle was recorded at the level of the papillary muscles*. Heart rate, anterior and posterior wall thickness, and end-diastolic and end-systolic internal dimensions of the LV were obtained from the M-mode image.

Viruses

Mengo virus[1] was kindly provided by A. Palmenberg. Theiler's virus[2] have been described previously.

Preparation of viral RNA.

BHK cells and L cell plated on 10 X 15 cm dishes were infected with moi= 0.01 of wt VSV and EMCV, respectively. At 1h after infection, medium was removed and replaced with DMEM containing 10 % FCS and the cells were incubated for 2 days at 37 °C. Then the supernatants were collected and centrifuged at 3,000 rpm for 15 min to remove cells for avoiding cellar RNA contamination. Then the supernatants were harvested and centrifuged at 25,000 rpm for 90 min in an SW28 rotor at 4 °C. The viral pellet was suspended in TRIzol reagent (Invitrogen) and RNA was extracted. 5-10 μg/ml VSV RNA and 0.5-3 μg/ml EMCV RNA were obtained from single preparation.

Analysis of total protein synthesis.

Cultures of wild-type and MDA5$^{-/-}$ MEFs were infected with EMCV. At various time of labeled by incorporation of 50 μCi of [$^{35}$S]Met-Cys (GE Healthcare) for 1 h. Then the cells were lysed in a lysis buffer containing 1.0% Nonidet-P40, 150 mM NaCl, 20 mM Tris-Cl (pH7.5), 1 mM EDTA and protease inhibitor cocktail (Roche). Total cell extracts were separated by polyacrylamide gel electrophoresis, and the proteins were visualized by autoradiography.

1. Martin, L. R., Neal, Z. C., McBride, M. S. & Palmenberg, A. C. Mengovirus and encephalomyocarditis virus poly(C) tract lengths can affect virus growth in murine cell culture. J Virol 74, 3074-81 (2000).
2. Shin, T. & Koh, C. S. Immunohistochemical detection of osteopontin in the spinal cords of mice with Theiler's murine encephalomyelitis virus-induced demyelinating disease. Neurosci Lett 356, 72-4 (2004).

II. Supplemental Figure Legends

**Supplementary Fig. 1: Targeted disruption of the murine MDA5 gene.**

(a) Structure of the mouse *MDA5* gene, the targeting vector and the predicted disrupted gene. Closed boxes denote the coding exon. B; BamH I (b) Southern blot analysis of offspring from the heterozygote intercrosses. Genomic DNA was extracted from mouse tails, digested with BamHI, separated by electrophoresis and hybridized with the radiolabelled probe indicated in (a). Southern blot gave a single 9.4 kb band for wild-type (+/+), a 4.6 kb band for homozygous (-/-) and both bands for heterozygous (+/-) mice. (c) Nothern blot analysis of peritoneal exudates cells (PECs). Total RNA from wild-type (WT) and MDA5$^{-/-}$ PECs treated with 1000 U/ml IFN-β for 8 h was extracted and subjected to Northern blot analysis for the expression of *MDA5* mRNA. The same

membrane was rehybridized with a *β-actin* probe. (d) Western blot analysis of MDA5 expression. WT and MDA5$^{-/-}$ MEFs were treated with 1000 U/ml IFN-β for 8 h, and whole cell lysates were immunoblotted with antibody against MDA5. N.S. non specific.

**Supplementary Fig. 2. Involvement of MDA5 or RIG-I in the recognition of dsRNAs.**

(a) RIG-I$^{-/-}$ and MDA5$^{-/-}$, and their littermate WT mice were injected intravenously with 200 μg of poly I:C for the indicated periods and the production of IFN-β in the sera was measured by ELISA. The data are means ± S.D. of sera samples.

(b) GMCSF-DCs from RIG-I$^{-/-}$ and MDA5$^{-/-}$, TRIF$^{-/-}$ and their littermate control mice were incubated in the presence of 50, 250 μg/ml poly I:C for 24 h. The production of IFN-α in the culture supernatants was measured by ELISA.

(c) Generation of different lengths of dsRNAs corresponding to mouse lamin A/C. Different lengths of dsRNAs corresponding to mouse lamin A/C were synthesized as described in Methods section. 1 μg of dsRNAs were separated on 1% Agarose gel and visualized by staining with ethidium bromide. All dsRNAs synthesized appear with the estimated size.

**Supplementary Fig. 3: Contribution of RIG-I and MDA5 in the induction of genes encoding type I IFNs and IFN-inducible proteins in response to viral infection.**

(a) WT, RIG-I$^{-/-}$ or MDA5$^{-/-}$ MEFs were treated with 5 μg/ml poly I:C complexed with lipofectamine 2000 for the indicated periods. Total RNA was extracted and subjected to the Northern blot analysis for the expression of *IFN-β*, *IP10* and *β-actin* mRNA.

(b) WT, RIG-I$^{-/-}$ and MDA5$^{-/-}$ MEFs were treated with 5 μg/ml dsRNA corresponding to Lamin A/C (600 bps) complexed with lipofectamine 2000 for the indicated periods. Total RNA was extracted and subjected to the Northern blot analysis for the expression of

*IFN-β* and *IP10* mRNA. 28S and 18S ribosomal RNA bands on ethidium bromide-stained gel were used to control the RNA loading (lower panel).

(c) WT, RIG-I$^{-/-}$ and MDA5$^{-/-}$ MEFs were infected with moi=10 of SeV V(-) for the indicated periods. Total RNA was extracted and subjected to the Northern blot analysis for the expression of *IFN-β* and *IP10* mRNA. 28S and 18S ribosomal RNA bands on ethidium bromide-stained gel were used to control the RNA loading (lower panel).

(d) WT and MDA5$^{-/-}$ PECs were exposed to moi=10 of EMCV for the indicated periods. Total RNA was extracted and subjected to the Northern blot analysis for the expression of *IFN-β*, *IP10*, *IL-6* and *β-actin* mRNA.

**Supplementary Fig. 4: Role of MDA5 and RIG-I in the IFN-α responses against various viruses.**

(a) WT, RIG-I$^{-/-}$ and MDA5$^{-/-}$ MEFs were exposed to negative-sense ssRNA viruses, including NDV, VSV NCP, SeV Cm, SeV V- and influenza ΔNS1. IFN-α production in the culture supernatants was measured by ELISA.

(b and c) GMCSF-DCs from RIG-I$^{-/-}$ and MDA5$^{-/-}$ mice and their littermate WT mice were infected with indicated moi of EMCV for 24 h. The production of IFN-α (b) and IL-6 (c) in the culture supernatants was measured by ELISA.

(d) GMCSF-DCs from wild-type and MDA5$^{-/-}$ mice were infected with indicated moi of Theiler's virus or Mengovirus for 24 h. IFN-α production in the culture supernatants was measured by ELISA. The data are means ± S.D. of tripricates.

**Supplementary Fig. 5: EMCV-mediated protein synthesis shutoff was not altered between wild-type and MDA5$^{-/-}$ MEFs.**

Cultures of wild-type and MDA5$^{-/-}$ MEFs were infected with EMCV and labeled by incorporation of [$^{35}$S]Met-Cys for 1 h at various times after infection. Total cell extracts

were separated by polyacrylamide gel electrophoresis, and the proteins were visualized by autoradiography.

**Supplementary Fig. 6: Differential involvement of MDA5 and MyD88 in EMCV-mediated IFN production in cDCs and pDCs.**

DCs were induced from bone marrow cells obtained from MyD88$^{+/-}$, MyD88$^{-/-}$, MDA5$^{+/-}$ and MDA5$^{-/-}$ mice by cultivating in the presence of Flt3L. At day 7, B220$^{+}$CD11c$^{+}$ pDCs and B220$^{-}$CD11c$^{+}$ cDCs were purified by MACS, and infected with EMCV for 24h. IFN-α production in the culture supernatants was measured by ELISA. Error bars indicate ± S.D. of triplicates.

**Supplementary Fig. 7: The survival of MDA5$^{-/-}$, RIG-I$^{-/-}$ or IFNα/βR$^{-/-}$ mice in response to VSV infection.**

The survival of the mice (3-weeks old) infected with 4x10$^{6}$ pfu VSV intranasally was monitored for 9 days ($p<0.01$ by the generalized wilcoxon test between RIG-I$^{-/-}$ mice and their littermate controls).

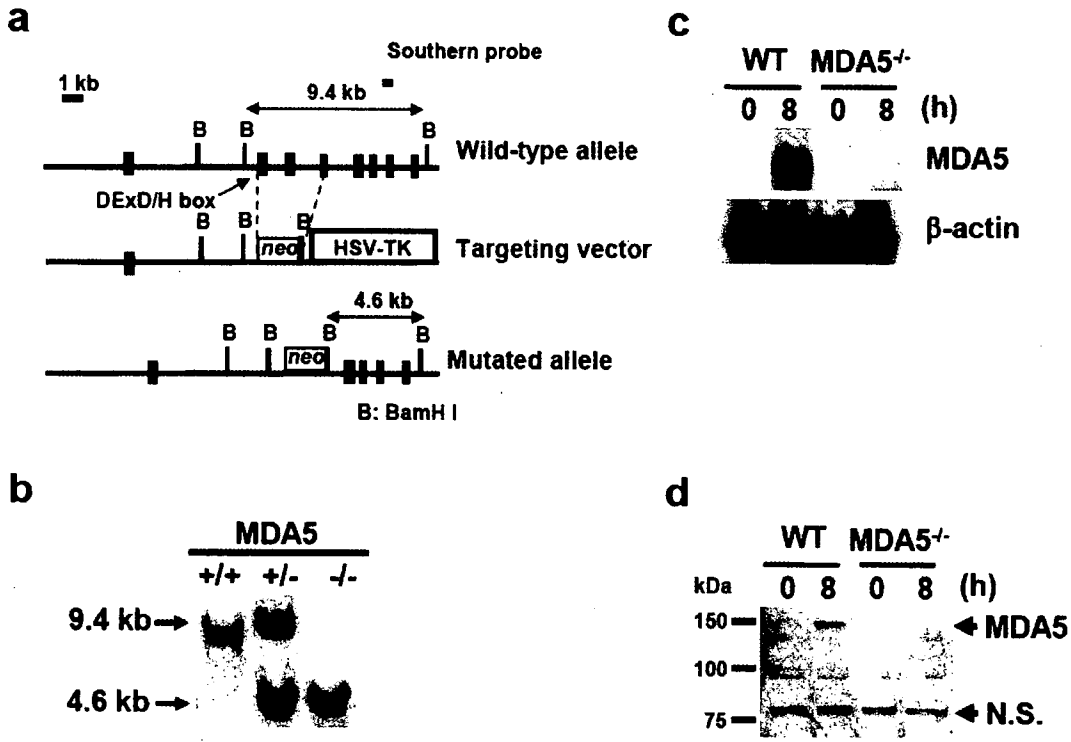
**Supplementary Fig. 8: Responses of MDA5$^{-/-}$ mice against EMCV infection**

(a) MDA5$^{+/-}$ and MDA5$^{-/-}$ mice (n=5) were intravenously inoculated with 1 X 10$^{7}$ pfu EMCV. Sera were taken at 4 h after injection, and IFN-β, Rantes and IL-6 production levels were determined by ELISA. *, $P<0.05$ versus controls by the student's t-test.

(b) Cardiac function of the mice 48 h after EMCV infection was assessed by echocardiography. Transthoracic M-mode echocardiographic tracings from MDA5$^{+/-}$ and MDA5$^{-/-}$ mice 48 h after EMCV infection.

**Supplementary Table I: Genotypes of mice derived from RIG-I$^{+/-}$ intercrosses or crosses between RIG-I$^{+/-}$ and RIG-I$^{-/-}$ mice.**

## Supplemental Figure 1.



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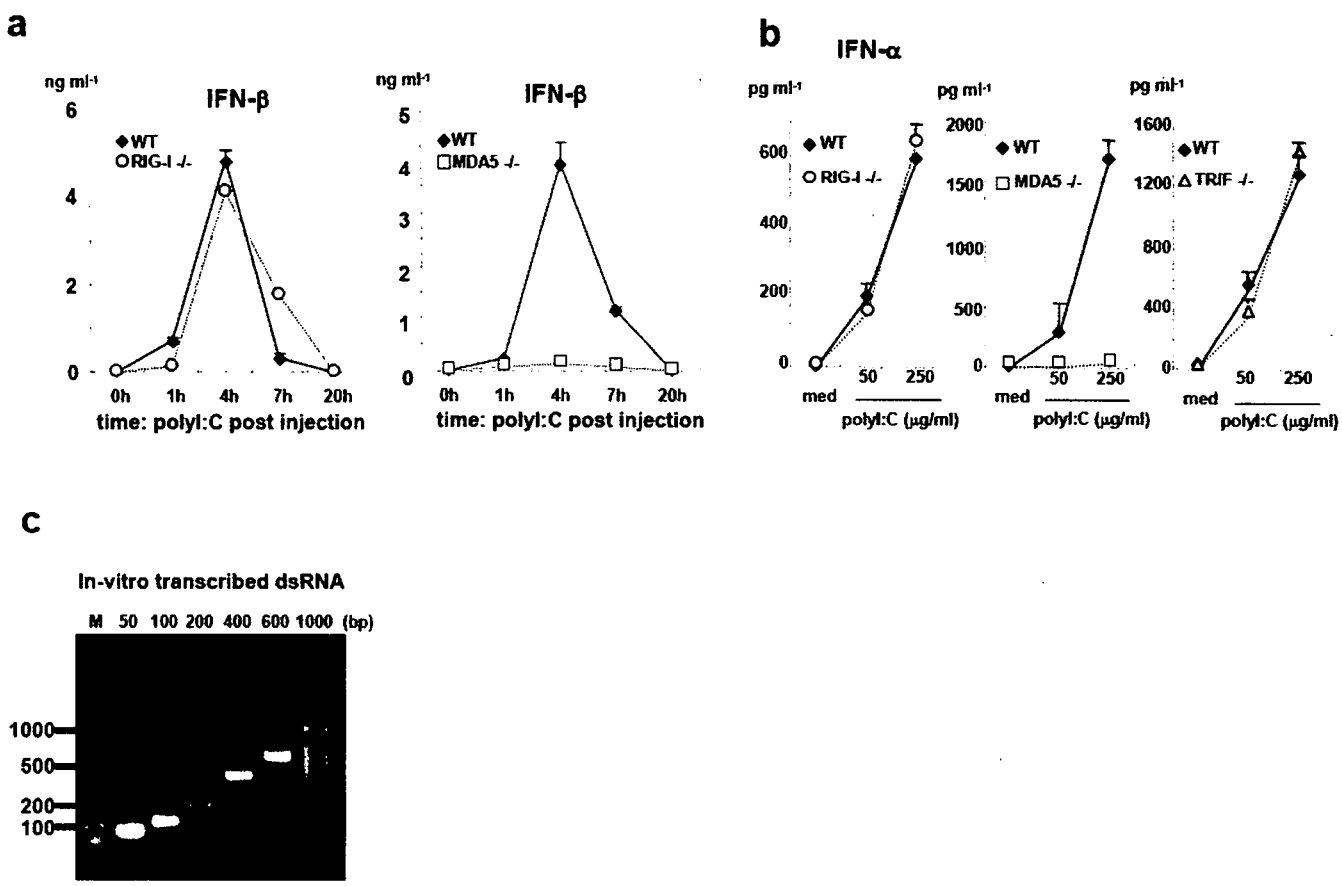

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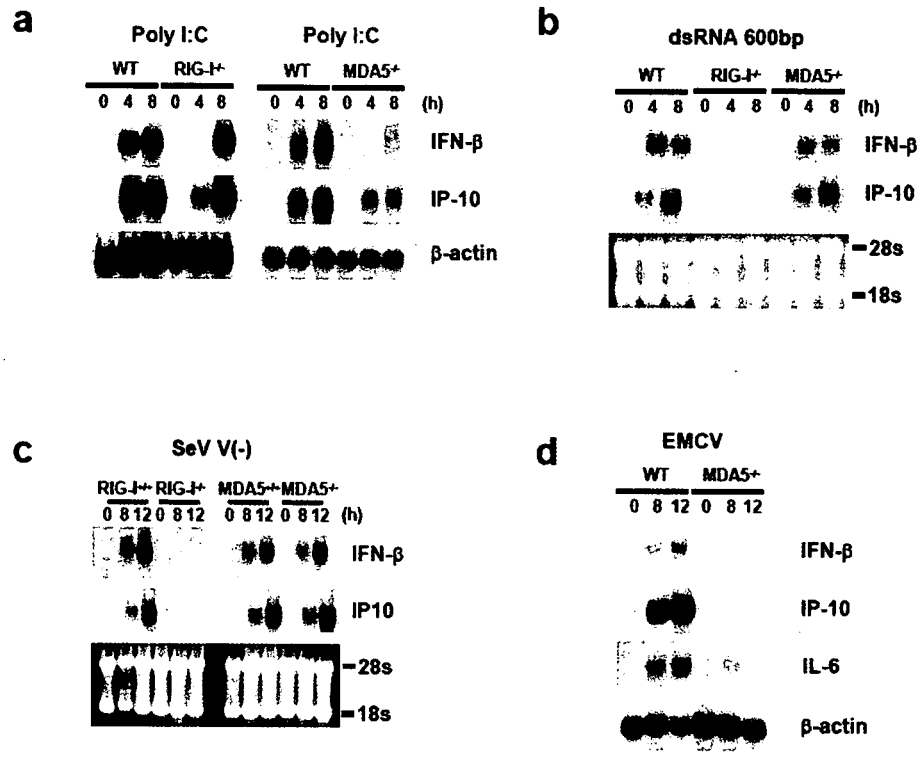

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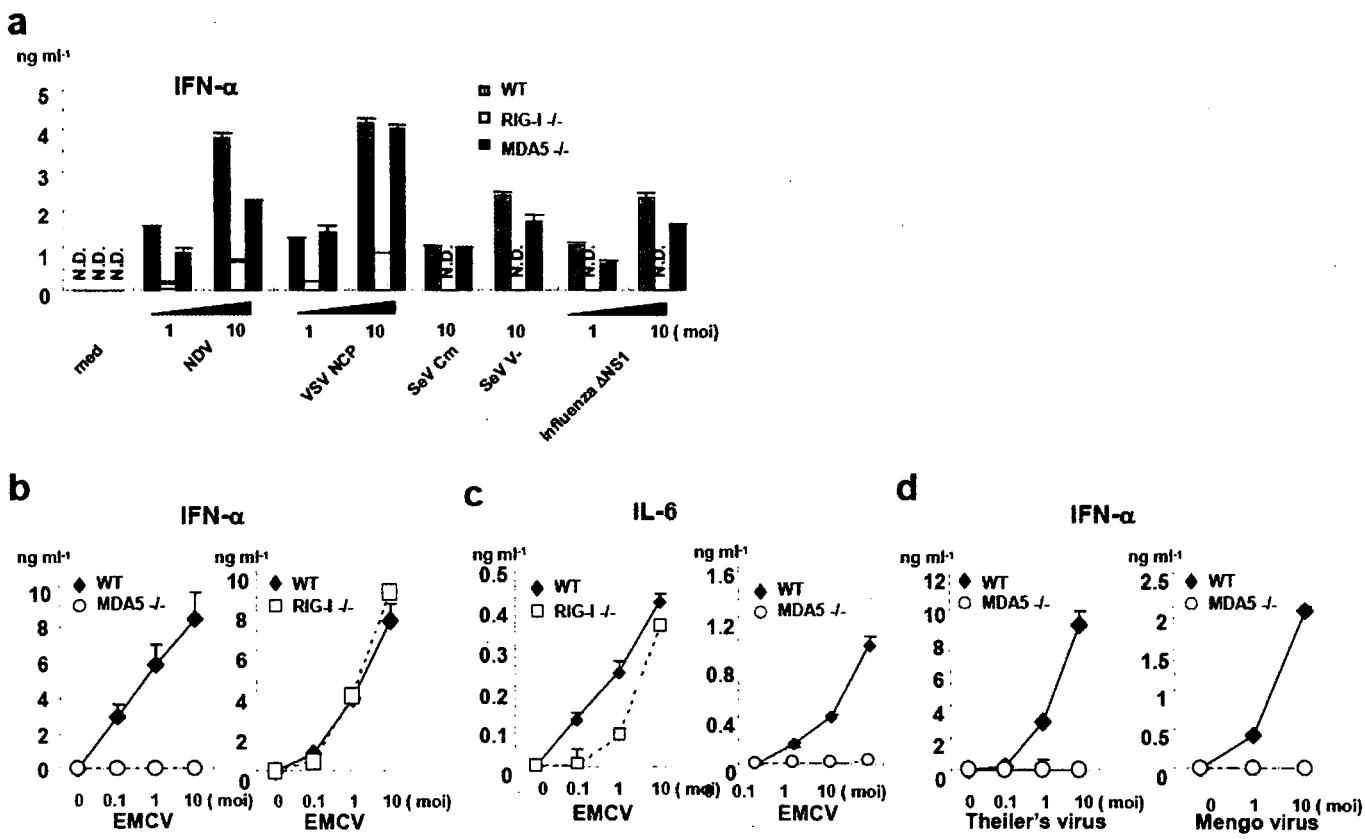

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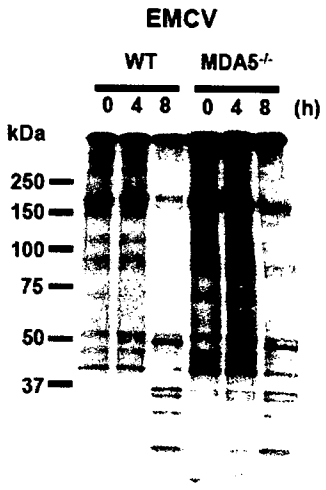

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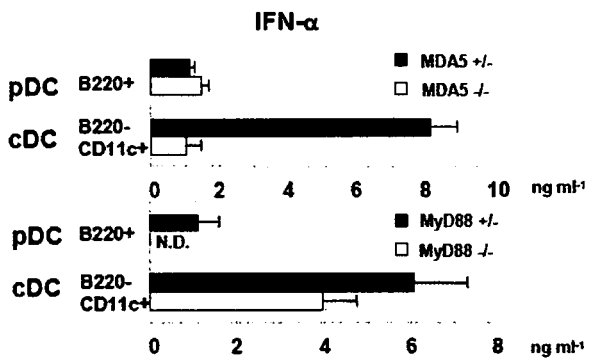
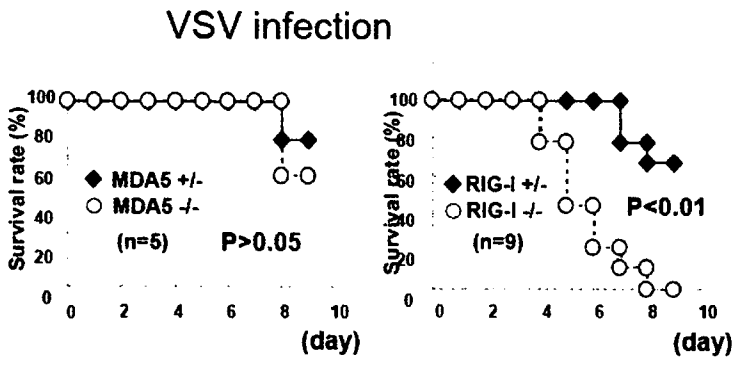


**Supplemental Figure 7.**



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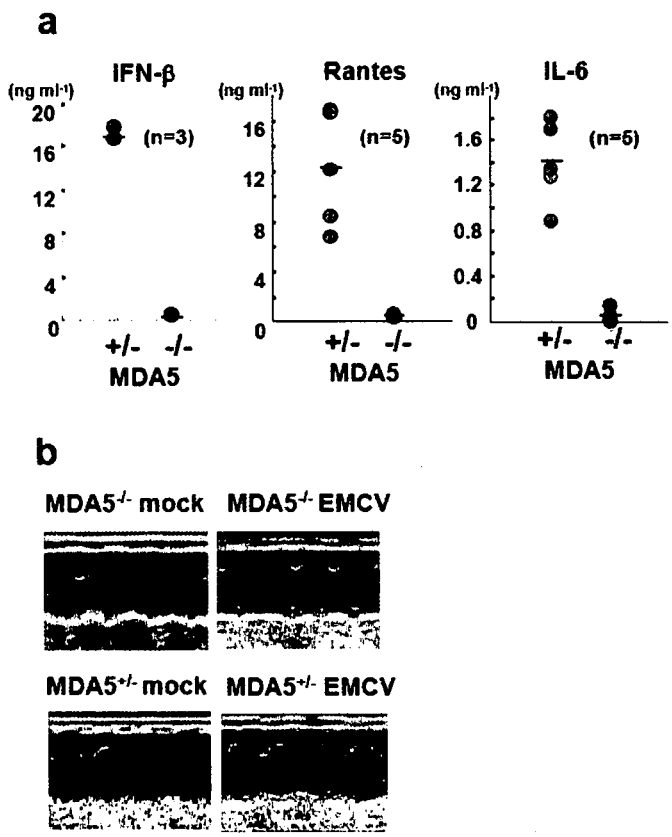

## Supplemental Table 1.

a

| | RIG-I$^{+/+}$ | RIG-I$^{+/-}$ | RIG-I$^{-/-}$ | Total (%) |
|---|---|---|---|---|
| Postnatal (6w~) | 16 (34) | 28 (60) | 3 (6) | 47 (100) |

b

| | RIG-I$^{+/-}$ | RIG-I$^{-/-}$ | Total (%) |
|---|---|---|---|
| Postnatal (6w~) | 108 (83) | 22 (17) | 130 (100) |

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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 (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.

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

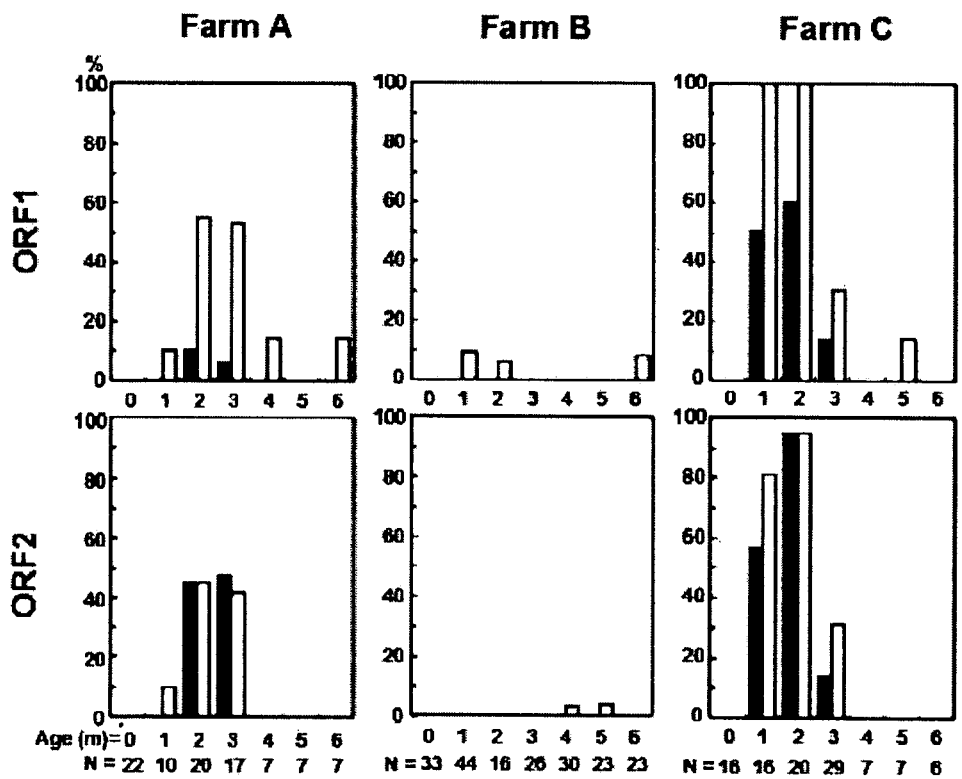


FIGURE 1. Frequencies of pigs shedding hepatitis E virus (HEV) in feces on three farms in Japan. The open reading frame 2 (ORF1) and ORF2 genes of HEV were amplified from each fecal sample by reverse transcription–polymerase chain reaction (RT-PCR). Solid and open bars represent frequencies of HEV-positive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

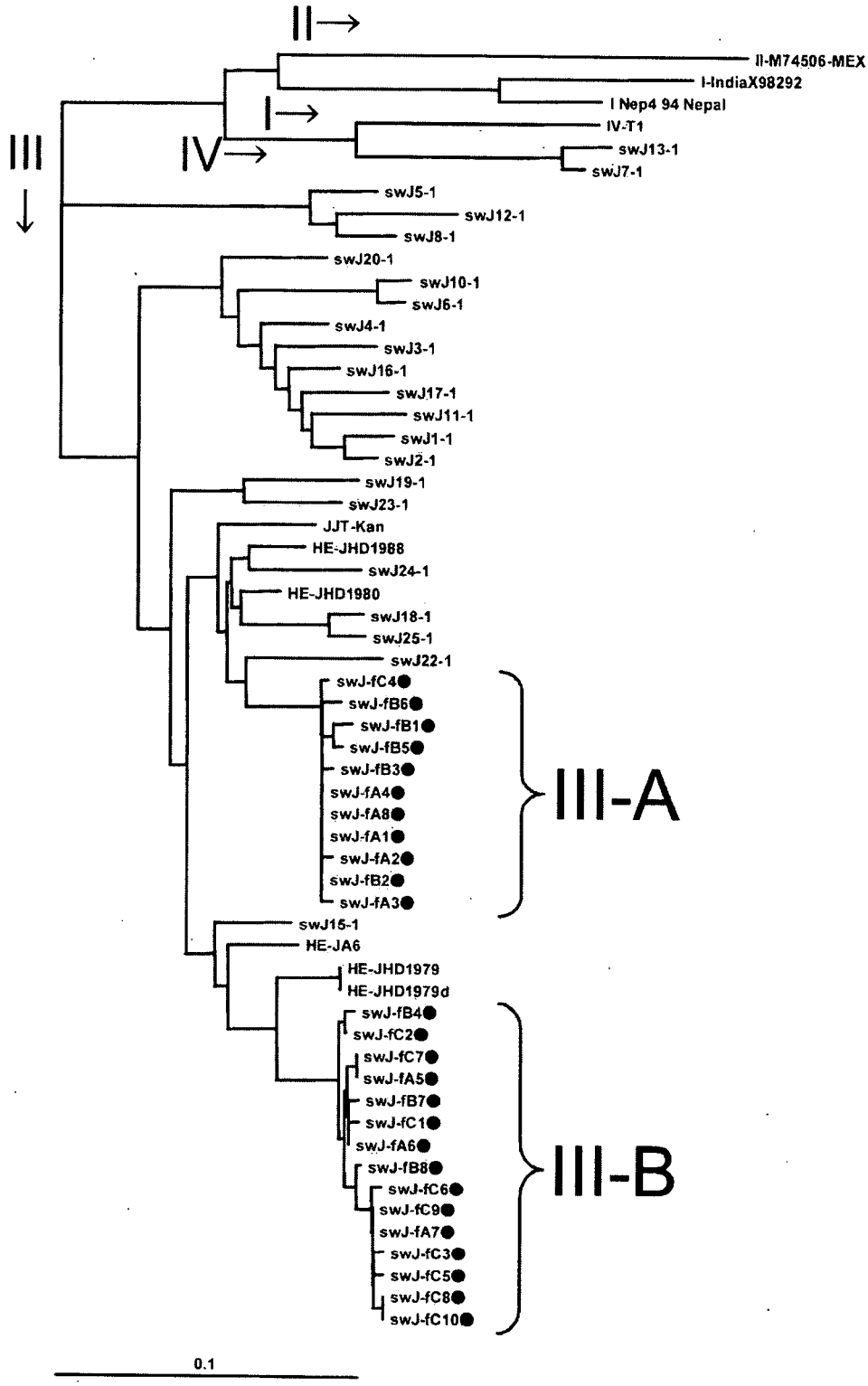


FIGURE 2. Phylogenetic tree constructed by the neighbor-joining method for hepatitis E virus (HEV) open reading frame 2 (300 bases). The 26 sequences isolated from farms A, B and C in this study (Table 2) belonged to either cluster III-A or III-B. The tree included our 26 isolates, typical sequences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

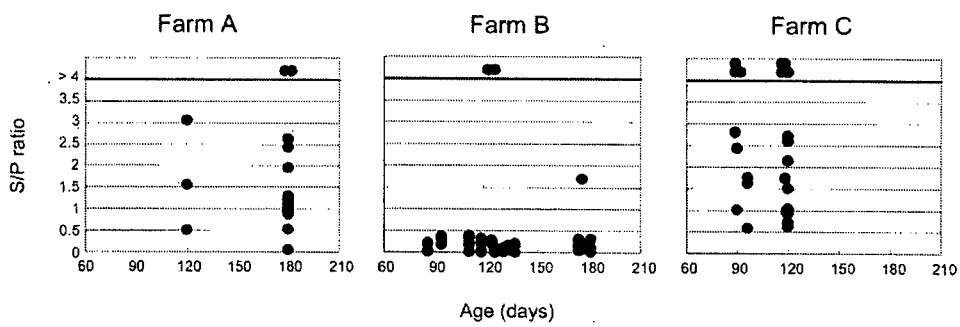


FIGURE 3. IgG antibody titers to hepatitis E virus in pigs from three farms in Japan. The enzyme-linked immunosorbent assay cutoff value was a sample-to-positive (S/P) ratio of 0.55. The percentages of pigs with an S/P ratio above the cutoff value were 82% (14 of 17) from farm A, 5% (3 of 65) from farm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

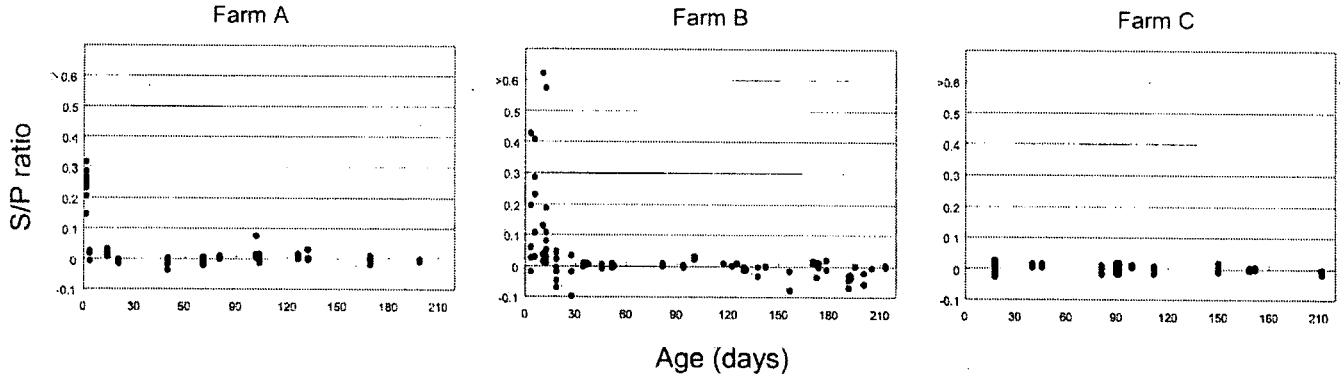


FIGURE 4. IgA antibody titers to hepatitis E virus in swine feces from three farms in Japan. The enzyme-linked immunosorbent assay cutoff value was a sample-to-positive (S/P) ratio of 0.078. A total of 90 samples were tested from farm A, 101 from farm B, and 90 from farm C. Samples having an S/P ratio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