The Journal of Veterinary Medical Science

FULL PAPER

Laboratory Animal Science

# Risk assessment for hepatitis E virus infection from domestic pigs introduced into an experimental animal facility in a medical school

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**ABSTRACT.** Hepatitis E virus (HEV) is known to cause zoonotic infections from pigs, wild boars and deer. Domestic pigs have been used as an experimental animal model in medical research and training; however, the risks of HEV infection from pigs during animal experiments are largely unknown. Here, we retrospectively investigated the seroprevalence and detection rates of viral RNA in 73 domestic pigs (average 34.5 kg) introduced into an animal experimental facility in a medical school during 2012–2016. We detected anti-HEV immunoglobulin G antibodies in 24 of 73 plasma samples (32.9%), though none of the samples were positive for viral RNA. Plasma samples of 18 pigs were sequentially monitored and were classified into four patterns: sustained positive (5 pigs), sustained negative (5 pigs), conversion to positive (6 pigs) and conversion to negative (2 pigs). HEV genomes were detected in 2 of 4 liver samples from pigs that were transported from the same farm during 2016–2017. Two viral sequences of the overlapping open reading frame (ORF) 2/3 region (97 bp) were identical and phylogenetically fell into genotype 3. A 459-bp length of the ORF2 region of an amplified fragment from a pig transported in 2017 was clustered with the wbJYG1 isolate (subgenotype 3b) with 91.5% (420/459 bp) nucleotide identity. Based on our results, we suggest that domestic pigs introduced into animal facilities carry a potential risk of HEV infection to researchers, trainees and facility staff. Continuous surveillance and precautions are important to prevent HEV infection in animal facilities.

**KEY WORDS:** animal experimental facility, domestic pig, hepatitis E virus, zoonosis

*J. Vet. Med. Sci.*
81(8): 1191–1196, 2019
doi: 10.1292/jvms.19-0086

Received: 8 February 2019
Accepted: 26 June 2019
Advanced Epub: 8 July 2019

The use of pigs as an experimental animal model in medical research and training has expanded over the past several decades, because the anatomical and physiological properties of pigs are similar to those of humans [6]. Several types of pigs, i.e., domestic pigs, miniature pigs and micromini pigs, are available for medical research. Among them, domestic pigs are most commonly used due to their cost-effectiveness. However, use of domestic pigs carries a potential risk of exposing the researchers to zoonotic infectious diseases, since the animals are not kept under specific-pathogen-free (SPF) conditions. Miniature pigs and micromini pigs are easier to handle and more suitable for research, but the production of these pigs for animal experiments is limited and they are more expensive.

Hepatitis E is a notable zoonotic disease in swine industries [10]. Hepatitis E virus (HEV) is a causative agent which is transmitted from pigs and likely other animal species (e.g., wild boar and deer) that are known as viral reservoirs. HEV is a non-enveloped, positive-sense RNA virus with a genome of approximately 7.2 kb in length and is a member of the genus *Orthohepevirus* in the family *Hepeviridae* [18]. *Orthohepevirus* consists of four species, *Orthohepevirus A* to *D*. It is recognized that four genotypes (HEV-1 to -4) within species *Orthohepevirus A* infect humans and that the HEV-3 and HEV-4 cause zoonotic diseases. Recent studies revealed that HEV-7 and *Orthohepevirus C*, known as rat HEV, also infected to humans by liver

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transplantation [7, 20].

Anti-HEV immunoglobulin G (IgG) antibodies were detected in 58% of the serum samples from pigs aged 2 to 6 months at 25 commercial farms in Japan, and viral RNAs were also detected in 10% of the tested samples [21]. Interestingly, all the HEV RNA-positive samples were collected from 3- to 4-month-old pigs. In previous reports, swine HEV RNA has also been detected in various tissues during the viremia period, as well as for a remarkably long period of time in the feces [5, 25]. Therefore, pig handlers such as swine veterinarians and pig farmers who are in contact with pigs have high risk of infection, as evidenced by previous reports worldwide [2, 13, 26]. Accordingly, medical researchers and trainees using domestic pigs and staff rearing them in the animal experimental facilities might also be under a high risk of infection.

In the present study, we performed a serological survey retrospectively to detect HEV-specific IgG antibodies as well as a molecular detection assay in domestic pigs introduced into an experimental animal facility in Okayama University from a commercial pig farm in order to assess HEV infection risk in the facility.

## MATERIALS AND METHODS

### *Pigs*

Seventy-three domestic pigs at a farrow-to-finish farm (where pigs are bred and raised to their slaughter weight) located in Okayama prefecture, 3 domestic pigs guaranteed HEV-free for safe animal experiments (Zen-noh Premium Pigs: Zen-noh Livestock, Tokyo, Japan) and 3 SPF-miniature pigs aged 18 months (Göttingen Minipigs: Oriental Yeast, Tokyo, Japan) were introduced into the Department of Animal Resources, Advanced Science Research Center, Okayama University over the years 2012 to 2016 for animal experiments. All of these animal experiments were approved by the Animal Care and Use Committee of Okayama University. The numbers of domestic pigs introduced each year were as follows: 36 including 3 SPF-miniature pigs in 2012, 9 in 2013, 14 including 3 Zen-noh Premium Pigs in 2014, 6 in 2015 and 14 in 2016. The mean body weight of the 73 domestic pigs and 3 Zen-noh Premium Pigs at the time of the first animal experiment was 34.5 kg (25.0–73.0 kg), which corresponds to an age of approximately 80–90 days in domestic pigs. Furthermore, 4 domestic pigs whose body weight and estimated age were same as the above-mentioned, were transported from the same farm during 2016 to 2017 (2016: $n$=2; 2017: $n$=2). However, only liver samples were available for this study in these pigs, since this study was conducted by sample sharing from another medical research using domestic pigs.

### *Samples*

Plasma samples were collected at the first animal experiment after an acclimation period (more than 4–5 days) and stored at −80°C before use. Sequential blood samples were also collected in 18 of the 73 domestic pigs. Six plasma samples collected from 3 Zen-noh Premium Pigs and 3 SPF-miniature pigs were used as a negative control in this study. Liver samples were collected from 4 domestic pigs transported from the same farm (2016: $n$=2; 2017: $n$=2).

### *Enzyme-linked immunosorbent assay (ELISA)*

Anti-HEV IgG antibody was screened using a PrioCHECK HEV Ab porcine ELISA kit (Mikrogen, Neuried, Germany) according to the manufacturer's instructions. The optical density (OD) at 450 nm was measured using a Flex Station 3 (Molecular Devices, Sunnyvale, CA, U.S.A.). Samples with OD values above or at the cut-off, which was calculated in each 96-well plate according to the manufacturer's instructions, were considered positive.

### *Nested reverse transcription-polymerase chain reaction (RT-PCR)*

Total RNA was extracted from 140 $\mu l$ of swine plasma or 10% (w/v) liver homogenates with a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Two nested RT-PCR assays for genotypes 1–4 were performed as described elsewhere [4, 15]. RT-PCR (ORF2/3-137PCR) developed by Inoue *et al*. [4] was used for screening of plasma and liver samples; the sensitivity of this screening in our laboratory was 1 copy using pCAGGS-HEVcap(1-660) constructed from the genotype 4 strain JTF-Yamagu11 [28] as a template. Another RT-PCR (ORF2-457PCR) with a sensitivity of 1,000 copies was used for only liver samples.

### *Sequence analysis*

PCR-positive products were purified using a FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan) and then subjected to direct sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.), and a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.); all these procedures were carried out according to the manufacturer's instructions. The sequence data were aligned using Clustal W software, and then neighbor-joining phylogenetic trees were generated with 1,000 bootstrap replications using MEGA 6.0 software [23].

## RESULTS

Anti-HEV IgG antibodies were detected by ELISA in 24 of 73 specimens (32.9%) of domestic pigs introduced from the farm (Fig. 1A). Six samples derived from 3 Zen-noh Premium Pigs and 3 SPF-miniature pigs were all negative. The annual prevalences of anti-HEV IgG antibodies in domestic pigs introduced from the farm were 48.5% (16/33) in 2012, 44.4% (4/9) in 2013, 18.2%

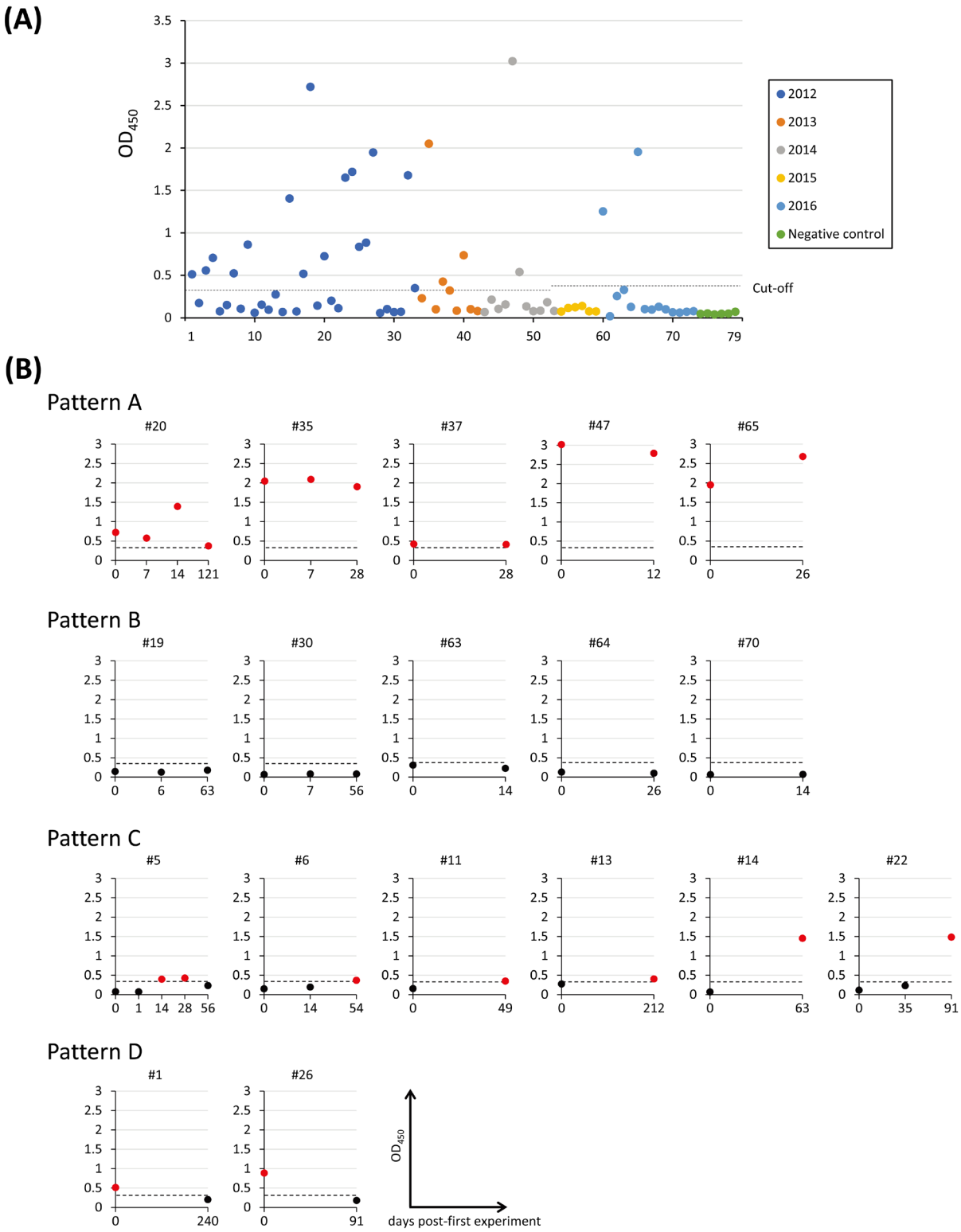


**Fig. 1.** Anti-hepatitis E virus (HEV)-specific immunoglobulin G (IgG) antibodies detected in the plasma samples collected from introduced pigs at the first animal experiment after the acclimation period (A) and 18 kinetics of anti-HEV IgG in the introduced domestic pigs (B). (A) The Y axis indicates the optical density (OD) values. The X axis indicates the sample identification numbers (#1 to 73: domestic pigs were numbered in ascending order based on the time of their introduction, and color-coded by introduction year). The negative control indicates the samples collected from 3 SPF-miniature pigs in 2012 (#74–76) and 3 Zen-noh Premium Pigs in 2014 (#77–79). (B) The sample identification numbers are shown at the top of the figure. The Y axis indicates the OD values. The X axis indicates the days post-first animal experiment (day 0: first ELISA detection as shown in Fig. 1A). Red and black dots indicate positive and negative values, respectively. Patterns A–D represents a sustained positive pattern, sustained negative pattern, seroconversion pattern, and conversion from positive to negative pattern, respectively.

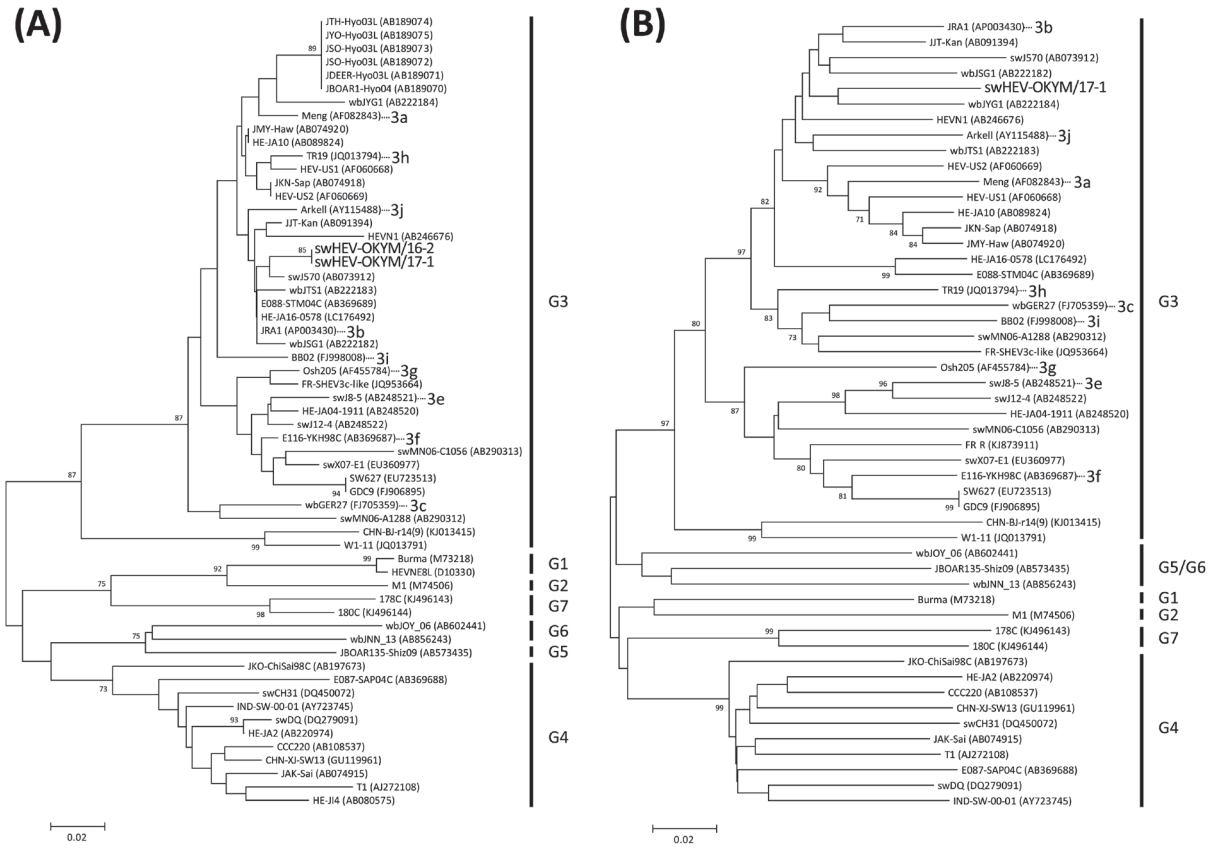


**Fig. 2.** Neighbor-joining phylogenetic trees based on the partial nucleotide sequences of the overlapping open reading frame (ORF) 2/3 (A) and ORF2 (B) regions of hepatitis E virus (HEV). The size of the partial sequences of ORF2/3 and ORF2 are 97 bp (nt 5326–5422) and 459 bp (nt 5959–6418), respectively (the nucleotide positions correspond to those in the HEV-3 strain Meng). The subtypes of HEV genotype 3 in the proposed reference sequences [19] are shown for each strain name. The dendrograms were constructed with a p-distance model using 1,000 replications. Numbers at the nodes indicate bootstrap supports >70%. The sequences determined in this study are shown in bold with a large font. GenBank accession numbers are indicated in parentheses. G1–G7 indicate genotypes 1–7 according to the current proposed classification [18].

(2/11) in 2014, 0% (0/6) in 2015 and 14.3% (2/14) in 2016.

The kinetics of the OD values in 18 sequential plasma samples of domestic pigs were classified into 4 patterns: pattern A represents sustained positives, pattern B represents sustained negatives, pattern C exhibits seroconversion, pattern D exhibits conversion from positive to negative. There were 5 pigs (#20, #35, #37, #47 and #65) showing pattern A (sustained positives), 5 pigs (#19, #30, #63, #64 and #70) showing pattern B (sustained negatives), 6 pigs (#5, #6, #11, #13, #14 and #22) showing pattern C (seroconversion) and 2 pigs (#1 and #26) showing pattern D (conversion to negative) (Fig. 1B).

Viral RNA was not detected in plasma samples collected at the first animal experiment by ORF2/3-137PCR, despite the fact that this RT-PCR was capable of detecting 1 copy of cDNA. Next, we tried to detect HEV in 4 liver samples collected in 2016 ($n$=2) and 2017 ($n$=2) by both ORF2/3-137PCR and ORF2-457PCR. Viral RNA was detected in a sample in 2016 (swHEV-OKYM/16-2) by only ORF2/3-137PCR and in a sample in 2017 (swHEV-OKYM/17-1) by both ORF2/3-137PCR and ORF2-457PCR. The sequences of the above RT-PCR products were deposited to DDBJ and assigned to accession numbers LC422839, LC422840 and LC422841. Phylogenetic analysis revealed that the amplified sequences fell into genotype 3 (Fig. 2). The two fragments (swHEV-OKYM/16-2 and swHEV-OKYM/17-1) revealed by ORF2/3-137PCR (Fig. 2a) were closely related with 97.9% nucleotide identity (95/97 bp) to the swJ570 isolate that was identified in swine serum in Tochigi Prefecture [17]. The sequences of swHEV-OKYM/16-2 and swHEV-OKYM/17-1 were identical, indicating that the same or similar genotype 3 strains were circulating in this farm in the years 2016 and 2017. The sequence of the ORF2-457PCR fragment in swHEV-OKYM/17-1 was clustered with the wbJYG1 isolate, which was identified from wild boar in Yamaguchi Prefecture in 2005 [16], with 91.5% (420/459 bp) nucleotide identity (Fig. 2b). On the other hand, swHEV-OKYM/17-1 showed 88.7% (407/459 bp) nucleotide identity to the swJ570 isolate in this sequence region.

## DISCUSSION

Domestic pigs are easily obtained and inexpensive, because they have been well established as a food source. Therefore,

domestic pigs are the most commonly used for animal experiments. For medical research in Japan, pigs of 2 to 3 months of age are widely used due to their anatomical size, and many pigs in this age range remain in the animal facilities for a month or two, resulting in a large number of 3- to 4-month-old pigs as well. Domestic pigs of these ages are sometimes infected with HEV, and become the source of human infection in the swine industries. In the present study, we tried to detect HEV-specific IgG antibodies and viral RNA using retrospective plasma samples stored at −80°C to assess HEV infection risk in the experimental animal facility, showing that 32.9% (24/73) of samples were sero-positive, although none of the plasma samples was positive for viral RNA. In the previous study, serum samples collected from 62 domestic pigs and 15 miniature pigs in 12 Japanese animal facilities were tested for anti-HEV antibodies in 2008 [27]. The results showed that 58.1% (36/62) of domestic pigs and 0% (0/15) of miniature pigs were positive for anti-HEV IgG antibody. Seroepidemiological findings including those from the present study revealed that the domestic pigs used for medical research in Japan were introduced from HEV infection-prevalent farms.

For the above reasons, it is highly desirable to find HEV-free farms to supply pigs for safety experiments. Indeed, Tanaka *et al*. [24] surveyed several commercial farms to find HEV-free farms to supply pigs for safety experiments at Jichi Medical School. However, it is practically difficult to supply HEV-free pigs continuously. Because farrow-to-finish farms generally supply slaughter houses with pigs approximately 6 months of age, followed by next farrowing, continued surveillance of HEV circulation may not always be accomplished on the farm and the HEV-free conditions are not necessarily kept constant. Therefore, when using domestic pigs, we should consider the viremia kinetics and virus shedding in feces. In a previous large-scale study designed to test 2,500 pigs at 25 swine farms in Japan from 2000 to 2002 [21], 58% of serum samples ($n$=1,448) were positive for anti-HEV IgG, of which 7% (37/500), 40% (301/750) and 87% (433/500) were from 2-, 3- and 4-month-old pigs, respectively. HEV RNA was detected from serum in 0% (0/180), 15% (113/750) and 13% (24/180) of 2-, 3- and 4-month-old pigs, respectively. Approximately 90% of pigs of 4–6 months of age are positive for anti-HEV IgG antibody [21]. Another previous study with long-term follow-up of virus shedding in naturally infected swine litters with and without maternal antibodies showed that viral RNA was detected in feces until 30–110 days of age in both litters [5], and the dates corresponded to the ages of the introduced pigs. During these periods, HEV was continuously shed for 63.5 days (range, 50–80 days) in the feces. In the serum, viral RNA was detected until 60–100 days of age in pigs in the maternal antibody positive-litter and for 40–100 days of age in pigs in the maternal antibody negative-litter. Viremia and seroconversion of serum antibodies occurred at 33.5 days (range, 10–60 days) and 32.3 days (range, 20–50 days) after the onset of HEV shedding in feces, respectively. In the present study, we could not identify the dates of viremia because none of the viral RNA was detected in plasma samples. However, 6 of 11 sero-negative samples (54.5%) in 18 sequential plasma samples showed seroconversion during the animal experiment period (Fig. 1B, pattern C), indicating that these pigs exhibited seroconversion before 32.3 days and shed the virus into the feces. The sero-positive days of pattern C pig plasma were 14, 54, 49, 212, 63 and 91 days after the animal experiment in pigs no. 5, 6, 11, 13, 14, and 22, respectively. Since the virus was continuously shed for 63.5 days into the feces [5], pigs may have been infected not only at the farm but also at the experimental animal facility. In our facility, introduced pigs were reared more than 4–5 days during the acclimation period and followed-up in individual cages after experiments on the same floor. HEV in pigs is mainly transmitted by a fecal-oral route [11]; therefore, non-infected pigs might be infected from HEV-contaminated feces and/or water during cage cleaning by facility staff. It is known that the prevalence of anti-HEV IgG among pig handlers is higher than that among non-pig handlers worldwide (e.g., China, Moldova, Taiwan and U.S.A.) [2, 3, 12, 13]. However, the staff at our facility wear the personal protective equipment (PPE) during cleaning, which is not the case for pig handlers at farms. Since viral RNA is detected not only in feces but also in various tissues during the viremia period [5, 25], in particular, the medical researchers and trainees should also pay attention to exposure to pig blood during experiments and manipulation using these pigs (e.g., needlestick). Indeed, there is a case report in which a surgeon was infected with HEV after exposure to 3-month-old pig blood during surgical training [1]. Several reports have documented the transmission of HEV through blood transfusions in human cases [9, 14]. The potential risk of HEV infection in experimental animal facilities has not been well established; in general, this risk has simply been inferred from the prevalence of HEV infection among pigs on farms. Our results indicate that researchers and trainees using domestic pigs as well as staff in the animal facilities are at increased potential risk of HEV infection; however, proper PPE use, proper performance of procedures on pigs, and adequate safety precautions based on our data will reduce the potential risk of HEV infection.

HEV-3 fragments were amplified in the liver samples from pigs introduced in 2016 and 2017. Two fragments (swHEV-OKYM/16-2 and swHEV-OKYM/17-1) were closely related subgenotype 3b strains that were indicated to be indigenous to Japan [8]. This subgenotype genome was also detected in wild boars in Okayama Prefecture in 2013 [22], indicating that these viruses were widely circulating between animals in Okayama Prefecture (i.e., pigs and wild boars). In general, domestic pigs are assigned to animal biosafety level 1, and therefore it is necessary to take precautions (e.g., biosafety and quarantine) in experimental animal facilities, because some domestic pigs in which HEV is not limited to subgenotype 3b might continue to be introduced.

In the present study, anti-HEV IgG antibodies were detected, but meanwhile no viral RNA was surprisingly detected in stored plasma samples collected from the approximately 80–90 days age of domestic pigs although viral RNA was frequently detected in these ages of pigs [5, 21]. In any case, the domestic pigs introducing into the facility carry a potential risk of HEV infection. To prevent infection during animal experiments using domestic pigs, continuous surveillance of the introduced domestic pigs is important and warranted. Because pigs infected with HEV shed viruses into feces for a long period of time [5], it might be also essential for detection of swine HEV RNA in the feces.

ACKNOWLEDGMENT. This work was supported by a JSPS KAKENHI Grant Number 25930022.

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