Short Communication

Prevalence and Phylogenetic Analysis of Hepatitis E Virus among Pigs in Japan

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SUMMARY: The number of reported cases of human hepatitis E virus (HEV) infection has increased since 2012. Pigs are considered an important source of viruses causing human HEV infection. It is possible that the prevalence of HEV among pigs at slaughter age (approximately 6 months old) has increased in the last decade. Therefore, we investigated the current prevalence of HEV among pigs in Japan. Although HEV RNA was detected in rectal content samples from pigs aged from one to 5 months, no HEV RNA was detected in any samples from 6-month-old pigs. The highest viral shedding prevalence (33%) was detected among 3-month-old pigs. This study shows that there has been no change in the prevalence of HEV among pigs at the slaughter age, in the prevalence of HEV by age group on pig farms, or in the phylogenetic classification of HEV isolates in the last decade. Therefore, factors downstream of the pork production stage may be contributing to the increased number of human HEV infection cases.

Hepatitis E virus (HEV) is the causative agent of hepatitis E and categorized into 4 major genotypes, G1–G4 (1). In Japan, 2 genotypes, G3 and G4, have been detected in human hepatitis E cases (2) and in pigs (3,4). Epidemiological studies suggest that pigs are an important source of viruses resulting in human HEV infection (5,6). In Japan, human hepatitis E is classified as a Category IV infectious disease according to the Law Concerning the Prevention of Infectious Diseases and Medical Care for Patients with Infections. When a patient receives a diagnosis of hepatitis E, even asymptomatic infection, his/her physician is obliged by law to notify the governor of the prefecture on physician practices. Although fewer than 100 cases were reported annually from 2005 through 2011, more than 100 cases have been reported annually since 2012 (7). The national health insurance system of Japan started covering the cost of an anti-HEV IgA detection assay in October 2011. A positive assay result triggers reporting an HEV infection case; this arrangement may in some part account for the increase in reported cases. There have been cases of human hepatitis E in Japan known to be caused by consumption of pork or pork products (5,6). This fact indicates that, although there have been no reports on detection of HEV in 6-month-old pigs in Japan, HEV may in fact remain in pigs at the slaughter age (approximately 6 months old) at a negligible viral titer, and the prevalence of HEV among pigs at the slaughter age may have increased in the last decade. Generally, HEV infects pigs at 2 to 4 months of age, and then HEV disappears from pigs at 6 months of age or later as a result of the biosynthesis of anti-HEV immunoglobulins (2,8). In 2010, a large

foot-and-mouth disease outbreak occurred in Miyazaki Prefecture on the Kyushu Island of Japan. During this epidemic, there were 292 infected farms, and ~290,000 animals were culled (9). This outbreak prompted pig farmers to enhance biosecurity measures on their farms, and this approach might have postponed HEV infection of pigs to a later growth stage. The delay may affect the period necessary for HEV to clear from the pig body, resulting in the presence of HEV in pigs at the slaughter age. Moreover, information on the current prevalence of HEV among pigs is useful not only for pig farmers but also for consumers to understand the risks associated with eating raw or undercooked pork. Therefore, we tried to estimate the current prevalence of HEV among pigs.

First, we examined the presence of HEV in pigs at the slaughter age on Kyushu Island between August and December 2013. To estimate the prevalence in a practical manner, we requested pig farmers in prefectures on Kyushu to participate in this study. A total of 21 pig farms (19 farrow-to-finish and 2 finishing farms) in 5 prefectures agreed to participate voluntarily in this survey on the condition of anonymity. Veterinarians visited each of these 21 farms once and sampled rectal contents in 5 healthy pigs of 5 months of age or older in one pig pen. A rectal content sample was placed in a sterilized container. The samples were sent to the Research Institute for Animal Science in Biochemistry and Toxicology by express delivery with refrigeration and were analyzed within 48 h of sampling. For the detection of HEV RNA, 0.25 g of each sample was mixed with 2.25 ml of buffered peptone water and was then centrifuged at $3,000 \times g$ for 15 min at 4 °C. The supernatant was tested for HEV RNA by nested reverse-transcription PCR, as described previously, with primers targeting the open reading frame 2 (ORF2) region (10). No HEV RNA was detected in any of the 105 samples, suggesting that the prevalence of HEV among pigs at the slaughter age on Kyushu is estimated to be low.

Second, we investigated the prevalence of HEV by age group on pig farms between November 2013 and

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February 2014. In response to our request, 24 pig farms (21 farrow-to-finish and 3 finishing enterprises) in 3 prefectures (A, B, and C) in the Kanto Region of Honshu Island agreed to participate voluntarily in the survey on the condition of anonymity. Veterinarians visited each of these 24 farms (indicated with lowercase letters) once, and sampled rectal contents on the same day in 20 healthy pigs from at least 2 age groups, each group originating from one pen. Among the age groups, there had to be at least one group composed of fattening pigs (i.e., ≥ 3 months old). HEV RNA was detected in 78 (16%) pigs from 17 (71%) farms (Table 1). HEV RNA was detected only in samples from the pigs of one to 5 months of age. HEV was not detected in any samples from the pigs of 6 months of age as in the first survey on Kyushu Island. The highest positivity rate (33%) was seen in the 3-month-old pig group. These results generally are consistent with those of the other studies conducted in Japan (2,8) in terms of the prevalence rate. The prevalence of HEV by age group on pig farms seems to have been unchanged in the last decade.

The 5-month-old pig groups on 2 farms (farm l in Prefecture B and farm p in Prefecture C) showed relatively high shedding rates of HEV. Nonetheless, in the 2-month-old pig group, in samples from these 2 farms, no HEV RNA was detected. These results indicate a delay of HEV infection on these farms and the presence of HEV in pigs at the slaughter age. The 2 farms were farrow-to-finish enterprises. To elucidate the cause of the delay epidemiologically, we collected information on biosecurity measures from all the 24 participating

farms. Most farms took some biosecurity measures. For example, all the farms required changed working clothes every day. Moreover, the rates of implementation of disinfection of vehicles before entry of the farms and footwear at each pig house entrance as well as the restriction of outsiders' access to the farms were 58%, 71%, and 88%, respectively. One of the 2 farms showing the delay of HEV implemented all of the above measures whereas the other implemented neither the disinfection of vehicles before entry of the farms nor the restriction of outsiders' access to the farms. Given the limited numbers of relevant farms, a clear interpretation was impossible regarding the relation between the delay of HEV infection and biosecurity measures. The major objective of most biosecurity measures is to prevent the invasion of farms by pathogens.

In Japan, it is common for farrow-to-finish and finishing farms to keep pigs of 2 to 4 months of age all year round in accordance with scheduled copulation or artificial insemination. Once HEV is introduced into a pig farm, the pig herds of 2 to 4 months of age on the farm get infected and then transmit HEV to other susceptible herds at those ages before the pigs start producing anti-HEV immunoglobulins. In short, once introduced into a farm, HEV is then present continuously there because of repeated transmission among susceptible herds, i.e., pigs without appropriate immunity. Recently, Salines et al. (11) reported that experimental porcine reproductive and respiratory syndrome virus (PRRSV) and HEV coinfection affect the HEV infection dynamics in 5-week-old pigs in the absence of the maternal antibody

Table 1. Prevalence of HEV RNA in the fecal contents from 480 pigs

Prefecture	Farm	Prevalence (%) of HEV RNA in pigs aged:						Total	Name of HEV RNA
		1 mo	2 mo	3 mo	4 mo	5 mo	6 mo		
A	a	0/1	1/4	0/5	0/1	0/5	0/4	1/20	P26
	b	0/3	0/2	1/4	2/5	0/5	0/1	3/20	P48, P51
	c	0/1	3/4	1/1	4/6	0/6	0/2	8/20	P61, P80
	d	-	4/5	2/5	-	1/5	0/5	7/20	P381, P382, P399
	e	-	1/3	2/5	0/2	0/4	0/6	3/20	P403
	f	-	-	-	3/10	0/7	0/3	3/20	P422
	g	0/6	0/4	0/3	0/1	0/6	-	0/20	
	h	0/4	0/6	-	0/2	0/6	0/2	0/20	
B	i	-	5/10	-	-	0/10	-	5/20	P82
	j	3/10	-	5/10	-	-	-	8/20	P103, P113
	k	-	2/10	-	3/10	-	-	5/20	P128, P129, P133
	l	-	0/10	-	-	4/10	-	4/20	P471, P474
	m	-	8/10	-	0/10	-	-	8/20	P142, P144
	n	-	0/10	-	-	-	0/10	0/20	
	o	-	0/10	-	-	0/10	-	0/20	
C	p	-	0/10	-	-	5/10	-	5/20	P213
	q	-	4/10	-	-	0/10	-	4/20	P163
	r	-	9/10	-	0/10	-	-	9/20	P221, P222
	s	-	1/10	-	0/10	-	-	1/20	P324
	t	-	2/10	-	-	0/10	-	2/20	P342
	u	-	0/10	-	2/10	-	-	2/20	P279
	v	-	0/10	-	-	0/10	-	0/20	
	w	-	0/10	-	0/10	-	-	0/20	
	x	-	0/10	-	0/10	-	-	0/20	
Total		3/25 (12)	40/178 (22)	11/33 (33)	14/97 (14)	10/114 (9)	0/33(0)	78/480 (16)	

-, not sampled; mo, month.

against these 2 endemic viruses. The simultaneous coinfection resulted in a prolonged latent period and HEV shedding period as compared to pigs infected with HEV only. Comprehensive studies addressing the immune status of pigs are needed to clarify the cause of this delay because PRRSV is one of the known immunosuppressive pathogens of swine.

The RNA of the PCR product of at least one HEV isolate from each of the HEV-positive farms was directly sequenced after amplification with primers HEV-F2 and HEV-R1. Evolutionary analyses were conducted by means of MEGA7 (<http://www.megasoftware.net>). A phylogenetic tree was constructed by the neighbor-joining methods (12) based on the sequences (338 nt long, when primer sequences at both ends are excluded). The sequences of 25 PCR products were determined completely, but those of 2 PCR products (P26 and P128) could not be obtained. All the 25 determined sequences belong to genotype G3 and were further categorized into 2 genotypes (branches): G3b (formerly known as G3jp) and G3a (formerly G3us). Among the 25 sequences, 21 (84%) were assigned to G3b, and 4 (16%) to G3a (Fig. 1). Extremely high (99.4–100%) sequence similarity between the 2 PCR products from the same farm was observed on 6 farms (farms b and c from Prefecture A and farms j, k, l, and m from Prefecture B). In addition,

7 PCR products from 4 farms (farms j, k, l, and m from Prefecture B) formed a cluster. These results suggest that HEVs circulate within a pig farm and occasionally spread to other farms in the same area with minor genetic mutations. The sequence similarity between the 2 PCR products (P221 and P222) from farm c of Prefecture C was 85.5%. P221 belongs to subtype G3b, whereas P222 belongs to subtype G3a. The sequences of P382 and P399 from farm d in Prefecture A were identical, but the sequence similarity between sequence P382 and the other PCR product from the same farm, P381, was 87.3%. These results suggested that some farms are contaminated with some distinct HEV strains via farm-to-farm transmission of HEV. The 2 subtypes (G3a and G3b) identified in this study were reported to be dominant HEV isolates among pigs in Japan a decade ago (13). These 2 subtypes have continued to dominate among pigs in Japan until now, with some minor genetic mutations.

This survey was conducted on Kyushu Island and in the Kanto region on Honshu Island, which are the main pork production areas in Japan: more than 60% of the pigs in the whole country. The 8 prefectures in these regions (where samples were collected for this study) rear approximately 40% of pigs in the whole country (14). Because no compulsory sampling was legally possible, the authors had to rely on voluntary offers of samples on the condition of anonymity. This arrangement may lead to insufficiently representative results with some bias. Nevertheless, it was not possible to obtain any samples for the survey by any other method.

There might be some seasonal and/or annual fluctuations in HEV prevalence among pigs. Although there have been no studies on the HEV fluctuations in Japan, a paper was recently published by Lu et al. (15) about the seasonal influence on the prevalence of HEV RNA among pigs in China: the only report on this topic. Lu et al. collected bile samples from pigs at 4 to 6 months of age in slaughterhouses and reported that the prevalence of HEV RNA shows a major peak between March and April and a smaller peak between September and October. On the other hand, they did not mention the age distribution of the slaughtered pigs and HEV detection or their relation, or whether 4-month-old pigs with high probability of having HEV are slaughtered in a specific season, and if so, whether there is any relation between that season and high prevalence of HEV. Given that pigs are slaughtered throughout the year for steady and constant supply of pork, further studies are necessary to confirm the seasonal or annual variability of HEV prevalence among pigs.

This study indicates that in the last decade, there has been little or no change in the prevalence of HEV among pigs at the slaughter age, in the HEV age distribution on pig farms, or in the phylogenetic classification of HEV isolates. The study also indicates that the prevalence of HEV is negligible among pigs at the slaughter age (6 months old) in both the Kyushu and Kanto regions. These findings imply that some factors in the pork supply chain downstream of the production stage may contribute more to the increase in the number of human HEV infection cases than can factors at the production stage. Recently, Kanayama et al. (16) suggested that the increase is most likely due to insurance coverage of the

anti-HEV IgA detection assay prompted by epidemiological assessment of nationally reported surveillance data regarding human HEV infection cases between 2007 and 2013. Takahashi et al. (17) conducted a nationwide survey of HEV infection in the general human population of Japan and reported that 5.3% (1,167/22,027) of serum samples collected between January 2002 and December 2007 tested positive for anti-HEV IgG. The positivity rate suggests that a large number of HEV infection cases remain unreported in Japan, and the anti-HEV IgA assay detects some of HEV infection cases that were previously undiagnosed, misdiagnosed, or misclassified. In fact, in 2015 and 2016, 212 and 354 human HEV infection cases were reported, respectively (18,19). Namely, the burden of human HEV infection in Japan has been considerably underestimated. Recently, Adlhoch et al. also reported that the increase in the number of reported HEV infection cases is observed in European countries and suggested that the increased awareness and improved diagnostic methods have contributed to this increase although some part of the increase might reflect a real rise (20). Because the anti-HEV IgA assay has become more popular in Japan, it is expected that the number of cases reported will continue to rise. To estimate the burden realistically, more scientific data along the food chain are needed.

On the 12th of June 2015, swine meat and organs, including liver, were banned by the Ministry of Health, Labour and Welfare of Japan from being offered for consumption in a raw form (without cooking) because of risks of HEV infection as well as food poisoning mediated by pathogenic microorganisms and parasites. Even under this ban, 4 human HEV infection cases were reported in 2016 to have been caused by the consumption of raw swine liver (16). It is therefore essential to educate restaurant owners and consumers about the risks of HEV infection associated with the consumption of raw or undercooked pork meat and organs on the basis of available scientific evidence.

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Conflict of interest None to declare.

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