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Detection Frequency of Porcine Noroviruses in Healthy Pigs in Japan

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Abstract

Porcine noroviruses have been previously reported by some Japanese research groups. However, there is limited information regarding their epidemiology and pathogenicity in pigs. Here, we attempted to determine the frequency of norovirus infection in pigs in Japan. Fecal samples were collected from 190 healthy pigs in the Tokai region of Japan and checked for noroviruses by reverse transcription-PCR. Noroviruses were present in 11 of the 190 fecal samples (5.8%) and were genetically related to the porcine noroviruses previously detected in other regions of Japan. Our data suggest that porcine noroviruses are circulating among populations of healthy pigs without clinical signs in Japan.

Discipline: Animal Science
Additional key words: nested PCR, phylogenetic tree, swine

Introduction

Noroviruses (NoVs) in the family *Caliciviridae* are the major cause of acute gastroenteritis in humans worldwide. They have been detected also in many animal species (Bodnar et al. 2017, Di Martino et al. 2016, Ferragut et al. 2016 and Wolf et al. 2009). NoVs are classified into seven genogroups (GI-GVII) based on the sequence diversity of the capsid protein, VP1 (Vinjé 2015). Each genogroup is further subdivided into multiple genotypes (Zheng et al. 2006). Porcine NoVs (PoNoVs) belong to the GII genogroup, which is the major genogroup identified in human gastroenteritis cases (Zheng et al. 2006). Within the GII genogroup, the GII.11, GII.18, and GII.19 genotypes are porcine-specific (Vinjé 2015). PoNoVs were first reported in Shizuoka Prefecture in Japan in 1997 (Sugieda et al. 1998) where the GII.11 genotype was detected in healthy pigs. Subsequently, PoNoVs were detected in the United States (Wang et al. 2006), Belgium (Mauroy et al. 2008), China (Shen et al. 2012), and Brazil (Silva et al. 2015). In Japan, GII.11, GII.18, and/or GII.19 genotypes of PoNoVs VP1 genes have been detected in both apparently healthy (Sugieda et al. 1998, Nakamura et al. 2010 and Morimitsu et al. 2014) and diarrheic pigs (Yin et al. 2006). Thus, the association between genotypes and the healthy/diarrheic condition of pigs is unclear. A recombinant PoNoV was detected in a diarrheic pig in China, and this virus experimentally caused diarrhea to specific pathogen-free miniature pigs (Shen et al. 2012). Human noroviruses (HuNoVs) have also been detected in pigs (Sisay et al. 2016, Mattison et al. 2007 and Nakamura et al. 2010), indicating that pigs are sensitive to HuNoVs. These reports raise the possibility that, like in China, new recombinant PoNoVs that cause diarrhea in pigs may appear in Japan and indicate that continued surveillance of PoNoVs in pigs is required. However, in one of these studies (Nakamura et al. 2010), the genetic variation of each genotype of PoNoV detected was limited, suggesting that the likelihood of newly emerging recombinant PoNoVs may be low. No PoNoV nucleotide sequences isolated in Japan since 2009 have been published. Here, we investigated the current frequency of detection of PoNoVs in healthy pigs from several farms in Japan. The nucleotide sequences of the detected viruses were compared with previously reported sequences of limited diversity to further delineate the current genetic diversity of PoNoVs and to monitor the appearance of new recombinant PoNoVs in Japan.

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Materials and methods

Fecal samples were collected from the rectums of 190 apparently healthy pigs from eleven farms in the Tokai region of Japan at two slaughterhouses between May and July in 2017. Ten percent (w/v) fecal suspensions were prepared in sterile phosphate-buffered saline (1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM $KHPO_4$) and centrifuged (11,000 × *g*, 10 min, 4°C) to eliminate larger debris. Viral RNA was extracted from 140 µL of fecal suspensions using a Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The extracted RNA was stored at –80°C until use.

To detect PoNoVs, two rounds of polymerase chain reaction (PCR) assays were performed. One-step reverse transcription (RT)-PCR was performed using the primers G2SKF (5′- CNT GGG AGG GCG ATC GCA A -3′) and G2SKR (5′- CCR CCN GCA TRH CCR TTR TAC AT -3′) to amplify the partial (344 bp) VP1 genomic region of GII NoVs (Kojima et al. 2002). For the nested PCR assay, forward Nest-F (5′- GGT GTG AAT GAA GAT GGC GTC -3′) and reverse Nest-R (5′- TTC CGG GGG GAA ACA GTA AA -3′) primers were designed based on the PoNoVs nucleotide sequences available in the GenBank database to amplify an internal fragment (216 bp) of the one-step RT-PCR product. The nucleotide alignment used for designing the primers is shown in Figure 1. We used the GII.11, GII.18, and GII.19 genotypes to design the primers because they are porcine-specific (Vinjé 2015). Although some studies detected HuNoVs in pigs, they may not replicate in pigs (Nakamura et al., 2010). In the present study, primers were designed using PoNoVs as reference strains to detect NoVs circulating in pigs. The RT-PCR mixture was prepared to a final volume of 20 µL containing 2 µL of the extracted RNA using GoTaq 1-Step RT-qPCR System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The mixture was reverse transcribed at 37°C for 15 min, followed by denaturation at 95°C for 10 min and 30 cycles as follows: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and strand extension at 72°C for 30 s. After the cycling, the reaction mixtures were stored at 4°C. The nested PCR mixture was prepared to a final volume of 20 µL containing 2 µL of the RT-PCR product using GoTaq Green Master Mix (Promega, Madison, WI, USA) according to the manufacturer's instructions. The cycling conditions were as follows: denaturation at 95°C for 60 s, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and strand extension at 72°C for 30 s. After the cycling, the reaction mixtures were incubated for 7 min at 72°C and were then stored at 4°C. Amplified PCR products were sequenced for genotyping and phylogenetic analyses. The amplicons were purified with the QIAquick PCR Purification Kit (QIAGEN) and sequenced with the Nest-F or Nest-R primer in an ABI 310 Genetic Analyzer with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequences were analyzed using the Norovirus Typing Tool Version 2.0 (https://www.rivm.nl/mpf/typingtool/norovirus/) to determine the genotype of the NoVs. The obtained nucleotide sequences were aligned using ClustalW and phylogenetically analyzed with MEGA 7 (Kumar et al. 2016). A phylogenetic tree was constructed using the maximum-likelihood method, which provided statistical support via bootstrapping with 1,000 replications.

Results and discussion

The one-step RT-PCR analysis using the primer pair G2SKF/G2SKR detected only one NoV (pig ID 57) in 190 fecal samples. In contrast, the nested PCR analysis using the primers G2SKF/G2SKR in the first round and Nest-F/Nest-R in the second round detected 11 NoVs in 190 fecal samples (5.8%) (Table 1). The number of positive samples from each farm ranged from zero to two. It is unknown whether there are no PoNoVs in the farms without positive samples because of the small numbers of samples tested in the present study. To compare the detection rate of each farm, increased numbers of fecal samples might be required. Previous studies reported that the detection rate of viral genes of NoVs from healthy pigs was 15% in Toyama Prefecture (Nakamura et al. 2010) and 5% in Kochi Prefecture (Morimitsu et al. 2014); our results are thus similar to those of the latter study. Because we used nested PCR to detect PoNoVs, the detection rates of PoNoVs in this study might be lower than those in previous studies because of differences in the methods used. In the human population, HuNoVs were found predominantly in winter (Thongprachum et al. 2016), and PoNoVs were found to slightly increase in winter, although the change was not significant (Morimitsu et al. 2014). It might be partly due to the fact that our samples were collected in relatively warm months (May to July). The low detection rate might also be due to implementation of strict hygiene protocols at slaughterhouses and farms as a result of a huge outbreak of porcine epidemic diarrhea (PED) in Japan from 2013 to 2015 (Toyomaki et al. 2018).

The constructed phylogenetic tree is shown in Figure 2, and the nucleotide sequence identities of VP1 genes between PoNoVs detected in this study and PoNoVs detected in previous studies are shown in Table 2. In the phylogenetic tree, PoNoVs classified as GII.11 belonged

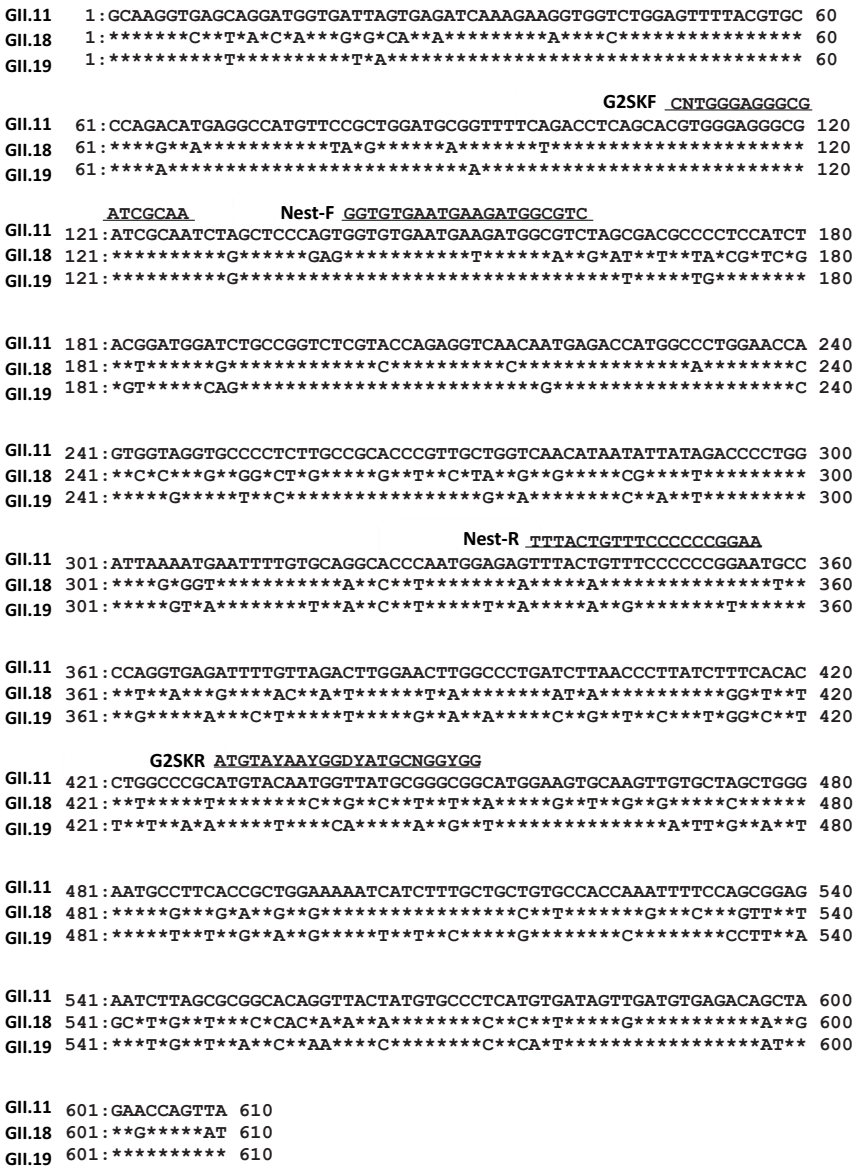

Fig. 1. Nucleotide sequence alignment of the partial VP1 regions of three genotypes of PoNoVs (GII.11, GII.18 and GII.19)

The primer sequences are underlined. Accession numbers in GenBank: GII.11 (AB074893), GII.18 (AY823304), and GII.19 (AY823306)

Table 1. Frequency of detection of noroviruses in healthy pigs in the Tokai region of Japan

Slaughterhouse ID	Farm	Numbers tested	Numbers positive	Detection rate (%)	Genotype
1	A	20	2	10	GII.11
	B	20	1	5	GII.11
	C	20	2	10	GII.11
	D	14	2	14	GII.11
	E	11	0	0	
	F	5	0	0	
2	G	20	2	10	GII.11
	H	20	1	5	GII.11
	I	20	1	5	GII.18
	J	20	0	0	
	K	20	0	0	
Total		190	11	5.8	

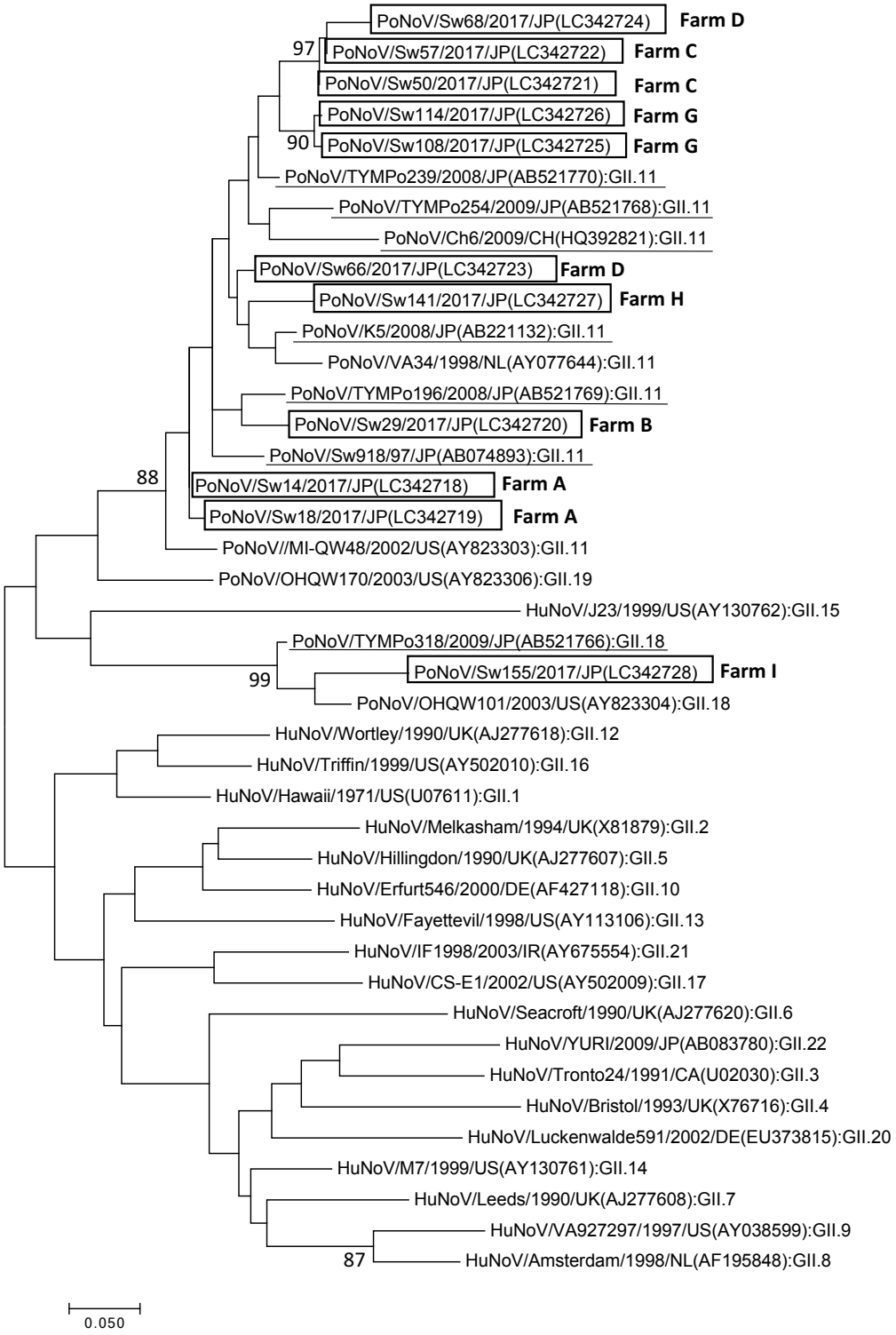

Fig. 2. Maximum likelihood phylogenetic tree of 174-nucleotide VP1 gene sequences of PoNoV strains identified in this study and other strains in GenBank

The GenBank accession numbers of representative sequences are provided in parentheses. Bootstrap values of more than 80% (1,000 replications) are shown on each node. The strains detected in this study are displayed in boxes and those previously detected in Japan are underlined.

Table 2. Percent nucleotide sequence identity between noroviruses detected in this study and reference strains based on a partial sequence of the capsid gene

	HQ392821	AB521770	AB521768	AB221132	AY077644	AB074893	AB521769	AY823303	AY823304
SwNoV/Sw14/2017/JP	88	94	90	93	94	94	94	95	68
SwNoV/Sw18/2017/JP	89	94	89	95	94	94	93	94	68
SwNoV/Sw29/2017/JP	90	92	87	93	92	93	95	90	67
SwNoV/Sw50/2017/JP	90	94	93	91	91	92	91	91	65
SwNoV/Sw57/2017/JP	91	94	92	92	91	92	91	89	68
SwNoV/Sw66/2017/JP	89	95	92	95	94	94	94	94	67
SwNoV/Sw68/2017/JP	88	91	90	89	88	90	89	88	63
SwNoV/Sw108/2017/JP	92	95	92	92	91	94	90	92	66
SwNoV/Sw114/2017/JP	91	95	91	92	91	94	90	92	66
SwNoV/Sw141/2017/JP	89	91	88	94	92	92	91	92	65
SwNoV/Sw155/2017/JP	77	76	75	74	84	80	78	79	92

to the same cluster as the reference strain of GII.11. Another PoNoV classified as GII.18 clustered together with the reference strain of GII.18. Previous studies detected both GII.11 (Sugieda et al. 1998, Nakamura et al. 2010) and GII.18 (Morimitsu et al. 2014, Nakamura et al. 2010) in Japan. Some PoNoVs detected in the present study are genetically close to the PoNoVs detected in Japan in 2008 or 2009, suggesting that the sequences of PoNoVs in Japan have not changed significantly in the last 8 y. In addition, PoNoVs originating from the same farms had a tendency to cluster together (Fig. 2), suggesting that PoNoVs circulated within farms. In one exceptional case, two genetically different strains were detected in one farm (farm D). The different strain might have been brought in with an external sow as previously reported (Morimitsu et al. 2014). However, the exact time and date of the introduction of the sow into farm D is unknown. We used previously reported primers on other PoNoV genes (Shen et al. 2012) but obtained no amplification. Thus, the virus in the fecal samples might lack part of the viral genome and might not be infectious. Another possibility is that the PoNoVs detected in this study might have diverged from previously reported PoNoVs. To test this idea, it might be necessary to analyze the full genome sequences by another method such as next-generation sequencing.

Taken together, our results revealed a very limited genetic variation of PoNoVs and a very limited viral transmission between the farms in the Tokai region of Japan. This suggests that genetically related PoNoVs might circulate in healthy pigs in Japan and that there is little zoonotic potential of PoNoVs in Japan.

However, the sequenced region in the recombinant strain in China (Shen et al. 2012) was also related to the Japanese strains (Table 2). We could not properly assess the possibility of recombinant PoNoVs by the nested PCR method with PoNoV-specific primers used in this study. Sequencing additional regions of the capsid region may be required to confirm the limited genetic variation of PoNoVs in Japan. Further sequencing of other PoNoV genes of the detected PoNoVs genome, combined with comparative analyses of geographically distinct PoNoV strains, will be required in order to fully understand how these viruses are circulating in Japan.

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