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Short communication

First identification of Sapoviruses in wild boar

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ABSTRACT

Sapoviruses (SaVs) are enteric viruses that have been detected in human and animals previously; however, SaVs have not been identified in wild boar yet. Using a metagenomics approach, we identified SaVs in fecal samples of free-living wild boars in Japan for the first time. Six of the 48 specimens identified belonged to one genogroup (G)III, one GV and four GVI SaV sequence reads. We successfully determined complete genome of GV and GVI SaV strains using the long reverse transcription PCR strategy and the 5' rapid amplification of cDNA end method. Phylogenetic tree analysis and pairwise distance calculation revealed that GV SaV detected from wild boar was related to recently assigned GV.5 strains from pig, while GVI SaV was assigned to a new genotype within GVI. Moreover, wild boar may act as a reservoir for transmission of SaVs to the pig population (and vice versa) because GIII, GV, and GVI SaVs were all detected in pigs previously.

Sapoviruses (SaVs) are enteric viruses that show a Star-of-David structure when viewed under an electron microscope and belong to the genus *Sapovirus* within the family *Caliciviridae* (Oka et al., 2015, 2017a). SaV strains that are pathogens of humans and pigs cause gastrointestinal disorders in their respective hosts (Oka et al., 2015). SaVs have been also found from asymptomatic animals, including mink (Guo et al., 2001), sea lion, (Li et al., 2011a), dog (Li et al., 2011b), chimpanzee (Mombo et al., 2014), rat (Firth et al., 2014), hyena, lion, fox (Olarte-Castillo et al., 2016), and bat (Kemenesi et al., 2016; Tse et al., 2012; Wu et al., 2016; Yinda et al., 2017). SaVs are non-enveloped viruses with a positive-sense, single-stranded RNA genome, approximately 7.1–7.7 kb in length. The 5' end of the genome is covalently linked to a small virus-encoded protein and the 3' end of the genome possesses a polyadenylated A [poly (A)] tail (Oka et al., 2016). The SaV genomes commonly have two open reading frames, (ORFs), ORF1 and ORF2, which encode non-structural proteins and the capsid protein VP1 and a minor structural protein VP2, respectively (Oka et al., 2015). At present, 19 genogroups (GI-GXIX) and at least 52 genotypes have been reported within the genus *Sapovirus* (Diez-Valcarce et al., 2019; Li et al., 2018; Oka et al., 2016; Yinda et al., 2017).

In Japan, population of free-living wild boar is increasing in numbers and distribution range (Ohdachi et al., 2009; Yamazaki et al., 2016). Wild boar is susceptible to pathogens from domestic pigs and may thus act as a viral disease reservoir for pathogens from domestic pigs (Meier and Ryser-Degiorgis, 2018; Meng et al., 2009); however, SaV has never been identified in wild boars. In the present study, we identified SaVs in the fecal samples of wild boars for the first time using a metagenomics approach. Furthermore, we determined complete genome sequences of two of the wild boar SaVs identified here and phylogenetically analyzed them with the sequences of other SaVs.

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Forty-eight fecal samples (rectal contents) of wild boars were collected during 2018-2019 from four prefectures (Toyama, Ishikawa, Mie, and Ibaraki) of the central region of the main land of Japan. Fecal samples were diluted at 1:9 (w/v) in sterile phosphate buffered saline and stored in a −80 °C freezer until use. Total RNA was extracted from the supernatants of the samples using TRIzol LS Reagent (Life Technologies, Carlsbad, CA, USA); this was followed by treatment with DNase I (Takara Bio, Shiga, Japan). Deep sequencing was performed using a MiSeq bench-top sequencer (Illumina, San Diego, CA, USA) and cDNA libraries were constructed from RNA using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). Sequence data were analyzed using CLC Genomics Workbench 7.5.5 (CLC bio, Aarhus, Denmark). SaV sequence reads were identified in six samples using the BLAST program of the National Center for Biotechnology Information website of the CLC Genomics Workbench. One sample identified from 2-3 month old (estimated age) wild boar (Ishikawa8) contained 5 GIII SaV sequence reads. The other sample identified from 6-7 month old (estimated age) wild boar (Ishikawa12) contained 406 GV SaV sequence reads. The other four samples identified from 4-5 months old (estimated age) wild boar (Toyama1-4) contained 54, 142, 99, and 120 GVI SaV sequence reads (Table 1, Supplementary Fig. 1).

For further analyses, we used the long reverse transcription (RT)-PCR strategy and the 5' rapid amplification of cDNA end (5'RACE) method. Ishikawa8 was found to contain only five sequence reads of SaV. Sequences obtained from deep sequencing of Toyama1, Toyama2, Toyama3, and Toyama4 were quite identical. Thus, we chose two samples, Ishikawa12 and Toyama2, which contained the greatest number of the sequence reads of GV and GVI SaVs [WB/Ishikawa12/2018 (Ishikawa12) and WB/Toyama2/2018 (Toyama2), respectively] for further study. cDNA synthesis using SuperScript IV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and single- or second- round long PCR using PrimeSTAR GXL DNA Polymerase (Takara Bio) or KOD-Plus DNA polymerase (Toyobo, Osaka, Japan), were performed as recently described (Oka et al., 2017b), using the primers listed in the Supplement Table. For the 5′-region targeting RT-PCR, we designed universal forward primers based on nucleotide sequences of representative GV strains (DDBJ/EMBL/Genbank accession numbers: AY646856, AB775659, AB521771, KX000383, and JN420370) from human, porcine, and sea lion and from two complete genome sequences of porcine GVI strains (AY974192 and KJ508818) that were available. The 5′ termini nucleotide sequence was confirmed by DNA linker ligated 5' RACE following semi-nested PCR as recently described (Oka et al., 2017b). The amplified regions and primer combinations are summarized in Supplementary Fig. 2. The complete genome lengths, excluding those of the poly(A) tails, of Ishikawa12 and Toyama2 were 7498 and 7201 nucleotides (nts), respectively. The nt sequences of Ishikawa12 and Toyama2 were deposited in DDBJ/EMBL/GenBank under accession numbers LC483440 and LC483441.

Complete sequences of Ishikawa12, Toyama2, and other SaVs were aligned using ClustalW in MEGA7 (Kumar et al., 2016), and phylogenetic analyses based on complete genome nt sequences and complete VP1 nt sequences were performed using the maximum likelihood method with a best fit model (the GTR + G model for the complete genome and the GTR + G+I for the complete VP1 sequences) in MEGA7. Tree topologies showed significant bootstrap support with 1000 replicates. Phylogenetic analysis of complete genome sequences revealed that Ishikawa12 and Toyama2 branched together with GV and GVI SaVs, respectively (Fig. 1A). In the VP1 tree, Ishikawa12 branched with US porcine GV.5 SaV WG194D-1 and formed a cluster with Japanese porcine GV.5 SaV HkKa2-1/2015 (Fig. 1B). Toyama2 generated a cluster with GVI SaVs but was distantly related to other GVI.1 and GVI.2 SaV strains. SaVs are classified into genogroups based on the complete VP1 amino acid (aa) sequences (Oka et al., 2016), while SaV genotyping employs complete VP1 nt sequences (Diez-Valcarce et al., 2018; Li et al., 2018; Oka et al., 2012, 2015). Therefore, pairwise distances were calculated using the complete VP1 nt sequences for genotyping using the MEGA7 software. The distance between Ishikawa12 and WG194D-1 was 0.247 and those between Toyama2 and other GVI viruses were 0.360-0.395, which were longer than the inter-cluster distances (0.031-0.270) of the established seven genotypes of GI viruses (Li et al., 2018) (Table 2). Therefore, Toyama2 may represent a new genotype, tentatively named GVI.3.

Primer-independent deep sequencing has allowed the discovery of many novel SaVs from humans and animals. Using this strategy, nearly full genome sequences of SaVs have been obtained previously, even though from total RNA directly extracted from fecal samples (Diez-Valcarce et al., 2019; Firth et al., 2014; Li et al., 2011a, b; Mombo et al., 2014; Shibata et al., 2015; Yinda et al., 2017). In the previous study, we obtained > 6000 nt SaV sequences from swine fecal samples that contained ≥690 (≥0.06%) SaV sequence reads [151 paired-reads, (SaV reads / total reads)] (Kuroda et al., 2017). Although in this study we could obtain only partial sequences and ≤406 SaV reads (≤0.0023%) from wild boar fecal samples by deep sequencing, we were able to determine the complete wild boar SaV genome sequences using the long RT-PCR and RACE method combined with deep sequencing as described previously (Oka et al., 2017b). This strategy could therefore be useful for samples with low sequence read counts which are hard to sequence by only deep sequencing.

Wild boars occasionally migrate close to pigs and human habitats which greatly increases the possibility of natural transmission of pathogens between domestic animals or humans and wild boars (Meier and Ryser-Degiorgis, 2018; Meng et al., 2009). Wild boars can be infected with zoonotic pathogens, such as hepatitis E viruses, which may represent a risk of virus transmission through contact with humans. (Meng et al., 2009). At present, there are no reports that describe detection of human SaV-like strains from wild animals and livestock (Oka et al., 2015). In this study, GIII, GV, and GVI SaVs were identified from wild boars; however, these viruses (except for GV.1 and GV.2) have never been identified from humans previously. In Japan, classical swine fever virus transmission between pigs and wild boars is a serious problem for the pig industry. GIII, GV, and GVI SaVs have been found in pigs in Japan (Kuroda et al., 2017); therefore, these viruses might be transmitted from Japanese pig population to wild boar or vice versa. Toyama2 GVI SaV was distantly related to known GVI strains, suggesting that Toyama2 may not be of a swine origin; however, this

Table 1
Information of sapovirus positive samples and sequence reads obtained from deep sequencing.

Sample name	Estimated age in month	Collected date	Sapovirus reads		Total reads	Genogroup
			Read count	% (SaV reads/total reads)	count	
Ishikawa 8	2-3	2018.07.23	5	0.0004	13,24,246	GIII
Ishikawa 12	6-7	2018.11.05	406	0.0765	5,30,902	GV
Toyama 1	4-5	2018.09.19	54	0.0044	12,23,296	GVI
Toyama 2	4-5	2018.09.19	142	0.0227	6,24,998	GVI
Toyama 3	4-5	2018.09.19	99	0.0169	5,87,106	GVI
Toyama 4	4-5	2018.09.19	120	0.0101	11,91,592	GVI

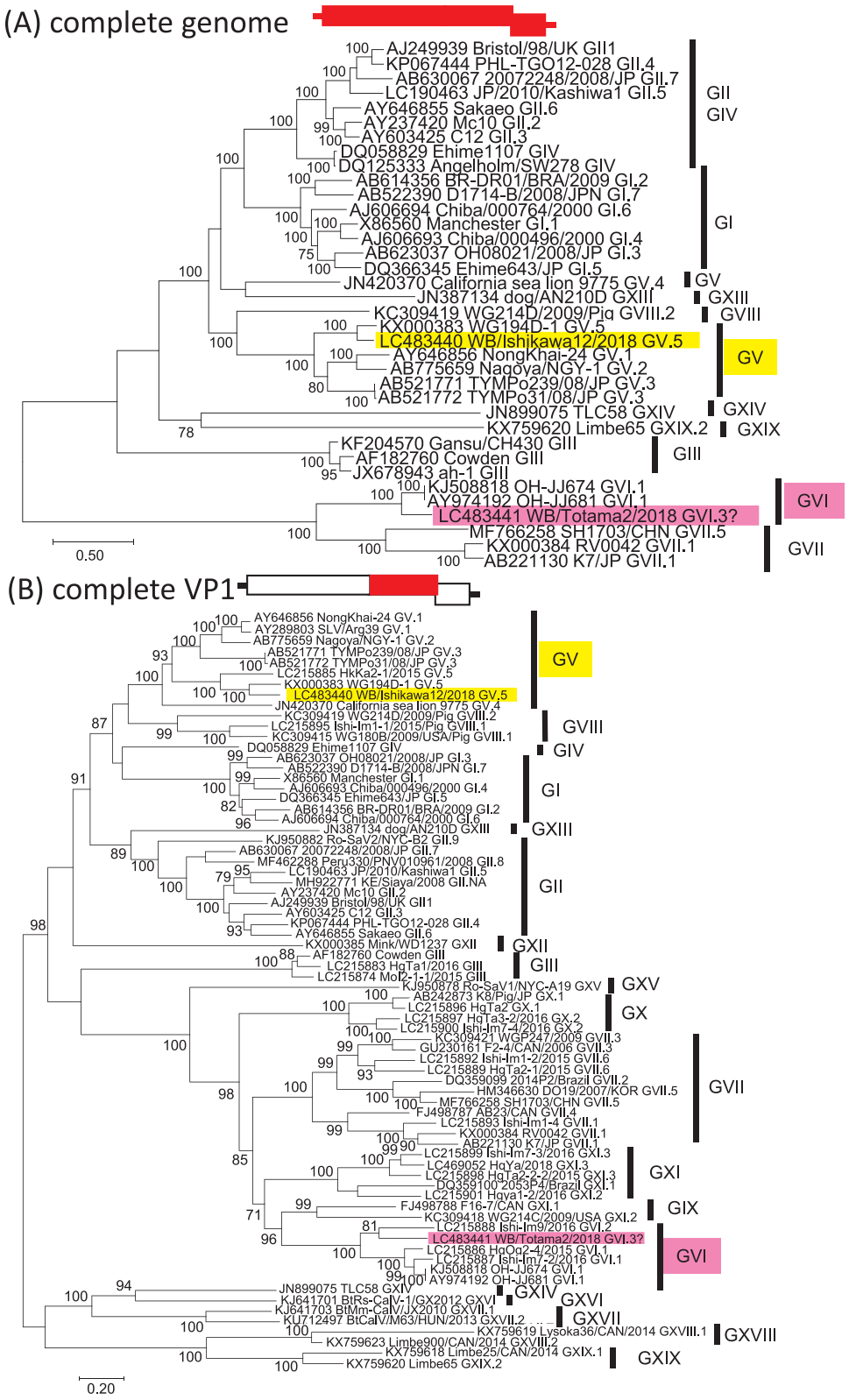

Fig. 1. Phylogenetic trees were constructed based on the nt sequences of the complete genome (A) and the complete VP1 region (B) of Japanese wild boar SaVs and SaVs from the DDBJ/EMBL/GenBank database. The phylogenetic tree was constructed using the maximum likelihood method of MEGA 7, and the bootstrap values (1000 replicates) above 70 are shown. The bar represents a corrected genetic distance. Ishikawa12 and Toyama2 SaVs are shown in yellow and pink color respectively.

Table 2
Pairwise distances of complete VP1 nucleotide sequences between strains of GV and GVI SaVs.

Genotype	Accession No.	Strains	GV.1		GV.2	GV.3		GV.4	GV.5			GVI.1				GVI.2
			NongKhai	SLV/Arg	NGY-1	TYMPo239	TYMPo31	sea_lion	HkKa2-1	WG194D-1	Ishikawa12	OH-JJ674	OH-JJ681	HgOg2-4	Ishi-lm7	Ishi-lm9
GV.1	AY646856	Human/NongKhai-24/Thailand														
	AY289803	Human/SLV/Arg39	0.079													
GV.2	AB775659	Human/Nagoya/NGY-1/2012/JPN	0.221	0.222												
GV.3	AB521771	Pig/TYMPo239/08/JP	0.373	0.373	0.375											
	AB521772	Pig/TYMPo31/08/JP	0.375	0.371	0.380	0.012										
GV.4	JN420370	California_sea_lion/9775	0.509	0.517	0.521	0.513	0.513									
GV.5	LC215885	Pig/HkKa2-1/2015	0.461	0.473	0.456	0.500	0.508	0.555								
	KX000383	Pig/WG194D-1	0.473	0.490	0.453	0.523	0.533	0.554	0.369							
	LC483440	WB/Ishikawa12/2018	0.490	0.473	0.448	0.516	0.518	0.541	0.368	0.247						
GVI.1	KJ508818	Pig/OH-JJ674/2000/US	0.988	1.004	0.953	0.996	1.001	0.918	0.999	1.017	0.995					
	AY974192	Pig/OH-JJ681/2000/US	0.988	1.004	0.953	0.996	1.001	0.918	0.999	1.017	0.995	0.000				
	LC215886	Pig/HgOg2-4/2015	0.994	0.993	0.957	0.975	0.980	0.901	1.050	1.010	1.004	0.169	0.169			
	LC215888	Ishi-lm7-2/2016	0.976	0.993	0.986	1.019	1.027	0.931	1.036	1.002	0.998	0.127	0.127	0.190		
GVI.2	LC215888	Ishi-lm9/2016	0.964	0.970	0.982	0.992	0.987	0.940	1.061	1.015	1.064	0.409	0.409	0.421	0.425	
GVI.3	LC483441	WB/Toyama2/2018	1.008	1.006	1.013	1.037	1.034	0.978	1.067	1.042	1.006	0.395	0.395	0.390	0.382	0.360

Pairwise distances between WB/Ishikawa12/2018 and GV SaVs, and WB/Toyama2/2018 and GVI SaVs are shown by yellow.

cannot be concluded with surety due to scarce data of porcine SaV in Japan. Therefore, further studies regarding the genogroup/genotype prevalence of wild boar SaVs and their pathogenicity towards wild boar and pigs are needed in the future.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197680.

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