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Possibility of Gene Rearrangement between Porcine Rotavirus H and Porcine Rotavirus C in Nonstructural Protein 3

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Abstract

Rotavirus species H (RVH) has been detected in pigs, humans and bats. Moreover, porcine RVHs have been recently identified in several swine-producing countries. Despite their zoonotic impact, genome information of RVHs is still limited. This study aimed to establish a tentative complete genome-based genotyping system for RVHs, by appending genomic sequences from 12 porcine RVHs identified in Japan between 2013 and 2015 to those from human and other porcine RVHs reported in previous studies. Phylogenetic analysis of 11 RNA segments indicated that porcine RVHs could be classified into multiple genotypes. Consequently, the genotype classification for RVHs revealed the existence of genotypes 10G, 6P, 6I, 3R, 4C, 7M, 6A, 2N, 4T, 6E, 3H for the genes *VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4* and *NSP5,* respectively. Surprisingly, two distinctive types in *NSP1* and *NSP3* genes were identified from among the twelve porcine RVHs. Our data suggest a potentially novel gene rearrangement event between porcine RVH and rotavirus species C in the *NSP3* gene. These findings would provide a new insight in understanding for evolution of RV.

Keywords: Porcine rotavirus H; Porcine rotavirus C; Full genome; Classification; Genotype; Non-structural protein 3; Gene rearrangement

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1. Introduction

Rotaviruses (RVs), member of the family *Reoviridae*, are major causative agents of gastroenteritis in humans and animals worldwide [1]. RVs are currently classified into nine species, rotavirus species A to I (RVA– RVI), based on sequence diversity of the inner capsid protein, VP6 [2, 3]. The genome comprises 11 segments of double-stranded RNA (dsRNA), which encode six structural proteins (VP1–VP4, VP6, and VP7), and five non-structural proteins (NSP1–NSP5). The structural proteins form infectious triple-layered particles surrounding the dsRNA. NSPs are primarily involved in dsRNA replication and transcription, cellular pathogenesis, and maturation of virions [4].

RVH was first isolated from fecal samples of pigs less than 30 days of age from Japan during 1991–1995 as a causative agent of diarrhea; this porcine RVH strain was designated as SKA-1 [5]. Subsequently, human RVHs have were detected in cases of epidemic and sporadic diarrhea in adults in China and Bangladesh, in 1997 and 2002, respectively [6-8]. During 2007 to 2012, porcine RVH infections have been reported in many countries, including the United States, Brazil, South Africa, and Vietnam [9-12]. Currently, RVH has been also identified in bats in Cameroon [13]. However, in Japan, the prevalence and epidemiology of porcine RVHs are unclear since the discovery of the SKA-1.

Recently, we identified twelve porcine RVH strains from fecal samples of pigs of various ages, in six farms in Nagasaki prefecture, Japan, between 2013 and 2015, most of which co-infected with other porcine rotavirus species, porcine rotaviruses C (RVCs). In this study, we attempted to establish a provisional full genome-based classification system for RVHs by appending further complete genomic sequences from the twelve Japanese porcine RVHs to classical porcine RVH, multiple porcine RVHs from other countries, and human RVHs available in GenBank, and by calculating the cut-off values for genotype classification on each RNA segment based on the definition recommended

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by the Rotavirus Classification Working Group. Simultaneously, we performed detailed analysis for unique genetic characteristics found among the *NSP3* gene from the twelve porcine RVHs. The data presented in this study would facilitate the molecular epidemiology and evolutionary study of RVHs.

2. Materials and Methods

2.1 Samples

Twelve porcine RVH strains was obtained from fecal samples of pigs from six farms in Nagasaki prefecture, Japan, from 2013 to 2015 (Table 1). Viral RNA was extracted from a 10% fecal suspension in minimum essential medium, using a QIAamp viral RNA mini kit (Qiagen, Venlo, Limburg, Netherlands), in accordance with the manufacturer's instructions. Genomic sequences of individual RNA segments were amplified via reverse transcription polymerase chain reaction (RT-PCR), using a set of primers (Table S1) designed in reference to terminal sequences in the 5'- and 3'-ends of all 11 segments from MRC-DPRU1575 (GenBank accession nos. KT962027–KT962037) [11]. RVC *NSP3* gene was also amplified via RT-PCR with specific primers reported in our previous study [14]. RT-PCR was carried out using a PrimeScript II High Fidelity One Step RT-PCR Kit (Takara Bio, Inc., Shiga, Japan) with the following cycling conditions: 45°C for 10 min and 94°C for 2 min; 35 cycles of 98°C for 10 s, 55°C for 15 s, and 68°C for 20 s; final extension step at 68 °C for 7 min.

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Table 1. Origin of twelve porcine rotavirus H strains detected in Nagasaki prefecture, Japan from 2013 to 2015.

Strain	Collection date	Collection farm	Collected age group	Co-infection with RVC	Type of RVH NSP1	Type of RVH NSP3
RVH/Pig-wt/JPN/NGS-3/2014/G5P6	2014.5	A	Sows	+	Short-type	Long-type
RVH/Pig-wt/JPN/NGS-5/2014/G7Px	2014.5	A	Piglet (< 30 days old)	-	Short-type	Long-type
RVH/Pig-wt/JPN/NGS-6/2014/G5Px	2014.5	B	Nursing (61–120 days old)	+	Short-type	Long-type
RVH/Pig-wt/JPN/NGS-7/2014/G5P6	2014.5	B	Nursing (61–120 days old)	+	Short-type	Short-type
RVH/Pig-wt/JPN/NGS-8/2015/G8Px	2015.3	C	Weaned (31–60 days old)	+	Long-type	Short-type
RVH/Pig-wt/JPN/NGS-9/2015/G9P5	2015.11	C	Fatting (>120 days old)	-	Long-type	Short-type
RVH/Pig-wt/JPN/NGS-10/2015/G9Px	2015.3	C	Weaned (31–60 days old)	+	Long-type	Short-type
RVH/Pig-wt/JPN/NGS-12/2013/G5Px	2013.8	D	Piglet (< 30 days old)	+	Long-type	Short-type
RVH/Pig-wt/JPN/NGS-14/2014/G10Px	2014.1	D	Piglet (< 30 days old)	+	Long-type	Short-type
RVH/Pig-wt/JPN/NGS-16/2014/G5Px	2014.8	E	Fatting (>120 days old)	-	Long-type	Short-type
RVH/Pig-wt/JPN/NGS-17/2013/G5Px	2013.9	F	Weaned (31–60 days old)	-	Long-type	Short-type
RVH/Pig-wt/JPN/NGS-18/2013/G9P5	2013.9	F	Sows	-	Long-type	Short-type

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2.2 Comparative sequence and phylogenetic analyses

PCR products were sequenced using a BigDye Terminator v3.1 Cycles Sequencing Kit on an automated ABI Prism 3130 Genetic Analyzer (Thermo Fisher Scientific, Carlsbad, CA, USA). Each gene sequences from the twelve porcine RVH strains determined herein were submitted to the DNA Data Bank of Japan (DDBJ), which are retrievable from GenBank (Table 2). The sequence data were aligned using the ClustalW method in the MEGA6 software [15]. Genetic distances were calculated using the Kimura two-parameter correction at the nucleotide level [16]. Phylogenetic analyses were conducted using the maximum-likelihood method with the general time reversible nucleotide substitution model and 1,000 bootstrap replicates. Genotype classification of individual RVH genes was conducted using cut-off values calculated per the definition recommended by the Rotavirus Classification Working Group [16, 17]. In addition, genotype classification of RVC *NSP3* gene was performed in accordance with our previous report [18].

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Table 2. Full-length (open reading frame) and GenBank accession number of individual genes from the twelve porcine rotavirus H strains.

Strain	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVH/Pig-wt/JPN/NGS-3/2014/G5P6	803 (768) LC348462	2412 (<1..2412) LC348479	1173 (<1..1164) LC348471	ND	2859 (<1..>2859) LC416239	2079 (<1..>2079) LC416240	1230 (1203) LC348482	980 (891) LC348491	1369 (1218) LC348500	720 (651) LC348507	654 (543) LC348513
RVH/Pig-wt/JPN/NGS-5/2014/G7Px	804 (768) LC348463	ND	1173 (<1..1164) LC348472	ND	2886 (<1..>2886) LC416241	ND	1230 (1203) LC348483	980 (891) LC348492	1369 (1218) LC348501	731 (651) LC348508	654 (543) LC348514
RVH/Pig-wt/JPN/NGS-6/2014/G5Px	804 (768) LC348464	ND	1173 (<1..1164) LC348473	ND	ND	ND	1230 (1203) LC348484	976 (891) LC348493	ND	731 (651) LC348509	654 (543) LC348515
RVH/Pig-wt/JPN/NGS-7/2014/G5P6	804 (768) LC348465	2406 (<1..>2406) LC348481	1173 (<1..1164) LC348474	ND	2853 (<1..>2853) LC416242	ND	1230 (1203) LC348485	980 (891) LC348494	1369 (1218) LC348502	731 (651) LC348510	654 (543) LC348516
RVH/Pig-wt/JPN/NGS-8/2015/G8Px	761 (<1..738) LC348466	ND	1173 (<1..1164) LC348475	ND	ND	ND	1233 (1206) LC348486	938 (891) LC348495	910 (801) LC348503	731 (651) LC348511	654 (543) LC348518
RVH/Pig-wt/JPN/NGS-9/2015/G9P5	804 (768) LC416254	2421 (<1..>2421) LC416252	1176 (<1..1167) LC416253	3432 (<1..>3432) LC416249	2871 (<1..>2871) LC416250	2097 (<1..2097) LC416251	1233 (1206) LC416244	975 (891) LC416245	901 (801) LC416246	720 (651) LC416247	642 (543) LC416248
RVH/Pig-wt/JPN/NGS-10/2015/G9Px	804 (768) LC348467	ND	1173 (<1..1164) LC348476	ND	ND	ND	1233 (1206) LC348487	977 (891) LC348496	910 (801) LC348504	ND	654 (543) LC348517
RVH/Pig-wt/JPN/NGS-12/2013/G5Px	804 (768) LC348468	ND	ND	ND	ND	ND	1233 (1206) LC248488	980 (891) LC348497	910 (801) LC348505	ND	ND
RVH/Pig-wt/JPN/NGS-14/2014/G10Px	804 (768) LC348469	ND	1173 (<1..1164) LC348477	ND	2778 (<1..>2778) LC416243	ND	1233 (1206) LC348489	980 (891) LC348498	910 (801) LC348506	731 (651) LC348512	654 (543) LC348519
RVH/Pig-wt/JPN/NGS-16/2014/G5Px	804 (768) LC348470	ND	1173 (<1..1164) LC348478	ND	ND	ND	1228 (1206) LC348490	980 (891) LC348499	ND	ND	ND

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RVH/Pig-wt/JPN/NGS-17/2013/G5Px	817 (768) LC416262	ND	1286 (1191) LC416263	ND	3001 (2949) LC416260	2197 (2160) LC416261	1315 (1206) LC416255	995 (891) LC416256	928 (801) LC416257	735 (651) LC416258	655 (543) LC416259
RVH/Pig-wt/JPN/NGS-18/2013/G9P5	817 (768) LC416274	2503 (2448) LC416272	1286 (1191) LC416273	3532 (3504) LC416269	3001 (2949) LC416270	2197 (2160) LC416271	1315 (1206) LC416264	995 (891) LC416265	928 (801) LC416266	735 (651) LC416267	655 (543) LC416268
RVH/Pig-wt/ZAF/ MRC-DPRU1575/ 2007/G4P3	817 (768) KT962032	2503 (2448) KT962030	1286 (1191) KT962031	3532 (3504) KT962027	3001 (2949) KT962028	2197 (2160) KT962029	1323 (1206) KT962033	1003 (891) KT962034	937 (801) KT962035	747 (651) KT962036	666 (543) KT962037

ND: not determined

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3. Results and discussion

3.1 Comparative sequence analysis

The amplification by RT-PCR using a set of primers designed in reference to complete genome of porcine RVH strain, MRC-DPRU1575 detected in South Africa succeeded to determine the nearly full-length nucleotide sequences of all genes from the twelve porcine RVH strains (Table 2). The *NSP1* open reading frame (ORF) nucleotide sequences from four porcine RVH strains (NGS-3, NGS-5, NGS-6, and NGS-7) were 1203 nt in length, which different from those (1206 nt) from the eight remaining strains, the same as the reference MRC-DPRU1575 strain (Table 1). The ORF nucleotide sequences of the *NSP3* products from seven porcine RVH strains (NGS-8, NGS-9, NGS-10, NGS-12, NGS-14, NGS-17 and NGS-18) were 801 nt in length, identical to that of the reference strain; however, the sequences of three RVH strains (NGS-3, NGS-5, and NGS-7) were 1218 nt in length. Beside the two genes, the products from the remaining genes were similar to those of the reference porcine RVH strain. These facts show that each terminal sequence in the 5'- and 3'- ends was also highly conserved among RVHs as well as other RVs, and all products were originated from porcine RVHs [19].

Aside from *NSP2* gene, the remaining genes displayed highly variable identity among the twelve porcine RVHs, higher than that observed among human RVHs. Furthermore, comparisons of ORFs of all genes between human and porcine RVHs, including the twelve porcine RVHs analyzed herein, revealed that porcine RVHs exhibit high diversity and are clearly distinct from human RVHs.

3.2 Phylogenetic analysis

All 11 genes of the twelve porcine RVH strains analyzed herein, the classical porcine RVH strain from Japan, numerous porcine RVH strains from the United States, Brazil, South Africa, and Vietnam, and 2–3 human RVH strains were differentiated (Fig.

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1 and Table 3). Cut-off values for differentiating among VP4 and VP7 genotypes were calculated from the frequency distribution of pairwise sequence identities and were set to 86% and 86% at the nucleotide level, respectively (Table S2). Based on these cut-off values, we observed six (P1–P6) and ten (G1–G10) genotypes for *VP4* and *VP7* genes, respectively (Fig. 1). The genotype classification for the *VP6* gene, based on a cut-off value of 87%, revealed the presence of six genotypes (I1–I6). Analysis of *VP1, VP2, VP3, NSP1*, *NSP2*, *NSP3*, *NSP4*, and *NSP5* genes, based on cut-off values of 85%, 87%, 86%, 84%, 67%, 87%, 83%, and 89% revealed the presence of three (R1–R3), four (C1–C4), seven (M1–M7), six (A1–A6), two (N1–N2), four (T1–T4), six (E1–E6), and three (H1–H3) genotypes, respectively (Table 3). Thus, for the ten remaining genes other than *NSP2* gene analyzed herein, porcine RVHs were classified into multiple genotypes, in contrast to human RVHs being grouped into one genotype. Moreover, our analysis indicates that porcine RVHs from Japan might belong to multiple genotypes; however, porcine RVHs from the United States, Brazil, South Africa, and Vietnam might be classified into 1–2 different genotypes in accordance with the country of collection.

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Table 3. Genotypes for individual genes of representative rotavirus H strains used in this study.

Strains	Genes	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
	Cut-off [%]	86	86	87	85	87	86	84	67	87	83	89
	Total number of genotypes*	10	6	6	3	4	7	6	2	4	6	3
RVH/Pig-wt/JPN/NGS-3/2014/G5P6		G5	P6	I5	ND	C3	M6	A4	N1	T3	E6	H3
RVH/Pig-wt/JPN/NGS-5/2014/G7Px		G7	ND	I5	ND	C1	ND	A4	N1	T3	E6	H3
RVH/Pig-wt/JPN/NGS-6/2014/G5Px		G5	ND	I5	ND	ND	ND	A4	N1	ND	E6	H3
RVH/Pig-wt/JPN/NGS-7/2014/G5P6		G5	P6	I5	ND	C3	ND	A4	N1	T3	E6	H3
RVH/Pig-wt/JPN/NGS-8/2015/G8Px		G8	ND	I5	ND	ND	ND	A6	N1	T1	E6	H1
RVH/Pig-wt/JPN/NGS-9/2015/G9P5		G9	P5	I1	R1	C4	M7	A6	N1	T1	E6	H1
RVH/Pig-wt/JPN/NGS-10/2015/G9Px		G9	ND	I5	ND	ND	ND	A6	N1	T1	ND	H1
RVH/Pig-wt/JPN/NGS-12/2013/G5Px		G5	ND	ND	ND	ND	ND	A5	N1	T1	ND	ND
RVH/Pig-wt/JPN/NGS-14/2014/G10Px		G10	ND	I1	ND	C1	ND	A5	N1	T1	E1	H1
RVH/Pig-wt/JPN/NGS-16/2014/G5Px		G5	ND	I6	ND	ND	ND	A6	N1	ND	ND	ND
RVH/Pig-wt/JPN/NGS-17/2013/G5Px		G5	ND	I1	ND	C4	M7	A6	N1	T4	E6	H1
RVH/Pig-wt/JPN/NGS-18/2013/G9P5		G9	P5	I1	R1	C4	M7	A6	N1	T4	E6	H1
RVH/Pig-wt/JPN/SKA-1/199x/G1P1		G1	P1	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVH/Pig-wt/USA/MN9.65/2008/G5P1		G5	P1	I1	R1	C1	M1	A5	N1	T1	E4	H1
RVH/Pig-wt/ZAF/MRC-DPRU1575/2007/G4P3		G4	P3	I3	R3	C3	M3	A6	N1	T1	E3	H1
RVH/Pig-wt/VNM/VNM12087-40/2012/GxPx		ND	ND	I5	ND	C3	M4	A4	ND	T3	E5	ND
RVH/Pig-wt/VNM/VNM12089-8/2012/G6P4		G6	P4	I4	R3	C3	M5	A3	N1	T3	E6	H1
RVH/Pig-wt/VNM/VNM14176-13/2012/G6P5		G6	P5	I5	R3	C3	M4	A4	N1	ND	E6	H3
RVH/Pig-wt/VNM/VNM14250-11/2012/G6P4		G6	P4	I4	R3	C3	M4	ND	N1	T3	E5	H1
RVH/Pig-wt/VNM/VNM14254-1/2012/G6P5		G6	P5	I5	R3	C3	M4	A4	N1	T3	E6	H3
RVH/Pig-wt/BRA/BR59/2012/G3P1		G3	P1	I3	ND	ND	ND	ND	ND	ND	E3	ND
RVH/Pig-wt/BRA/BR60/2012/G3P1		G3	P1	I3	ND	ND	ND	ND	ND	ND	E3	ND
RVH/Pig-wt/BRA/BR61/2012/G3P1		G3	P1	I3	ND	ND	ND	ND	ND	ND	E3	ND
RVH/Pig-wt/BRA/BR62/2012/G3P1		G3	P1	I3	ND	ND	ND	ND	ND	ND	E3	ND
RVH/Pig-wt/BRA/BR63/2012/G3P1		G3	P1	I3	ND	ND	ND	ND	ND	ND	E3	ND
RVH/Pig-wt/BRA/BR64/2012/G4P1		G4	P1	I3	ND	ND	ND	ND	ND	ND	E3	ND
RVH/Human-wt/CHN/J19/1997/G2P2		G2	P2	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVH/Human-wt/CHN/ADRV-N/1997/G2P2		ND	ND	I2	ND	ND	ND	A2	ND	T2	ND	ND
RVH/Human-wt/BAN/B219/2002/G2P2		G2	P2	I2	R2	C2	T2	A2	N2	T2	E2	H2

*Each genotype shown in Fig. 1. ND: not determined.

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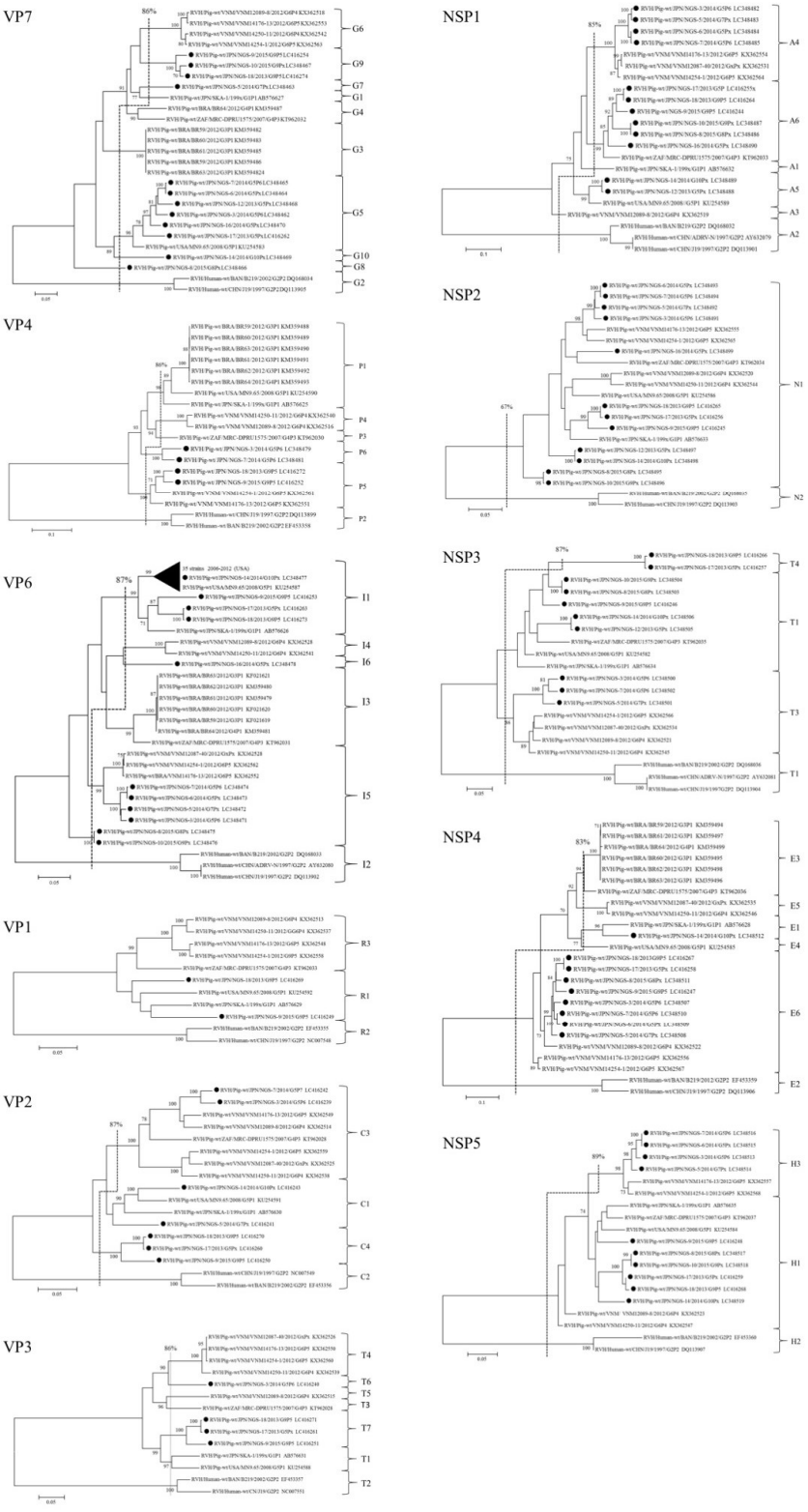

Figure 1. Phylogenetic tree constructed from the open reading frames of individual genes

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of rotavirus H strains.
Phylogenetic trees were constructed using the maximum-likelihood method with MEGA6 software. The number beside each node represents the percentage of bootstrap support (of 1,000 replicates) for the cluster. The dotted lines represent the division of genotypes based on cut-off values at the nucleotide level recommended by the Rotavirus Classification Working Group. Genotypes are specified on the right. The symbol (filled circle) above the strains indicates the porcine rotavirus H strains analyzed in this study. GenBank accession numbers are also shown below the strains.

3.3 Genetic analysis of the RVH NSP3 gene

Comparative sequence analysis of the twelve porcine RVHs and other porcine RVHs revealed the presence of two types of *NSP3* gene: long- and short-types. To investigate the origin of the extra nucleotide sequences in long-type RVH *NSP3*, a BLAST search was performed using the non-overlapping region (positions 807−1308) on multiple-sequence alignment of the nucleotide sequences between short-type and long-type RVH *NSP3* (Fig. 2). The extra sequences from long-type RVH *NSP3* showed the highest identity (79–85%) with the middle region (positions 550–970) of the *NSP3* gene (GenBank accession no. LC307034) from a porcine RVC (RVC/Pig-wt/JPN/134-9/2010/G6P5) of the T6 genotype [18]. In addition, the 421 nucleotide sequences (positions 840–1240) from long-type RVH *NSP3* displayed the highest identities (82.5–83.1%) with two porcine RVC strains (RVC/Pig-wt/JPN/87-G2/2008/G1P4 and RVC/Pig-wt/JPN/CJ10-1/2002/G6P5) on pairwise comparison between all porcine RVC strains belonging into the T6 genotype of the RVC *NSP3* (Fig. S1). Furthermore, phylogenetic analysis using partial RVC *NSP3* nucleotide sequences including extra sequences (positions 840-1240) from long-type RVH *NSP3* revealed that three RVH strains (RVH/Pig-wt/JPN/NGS-3/2014/G5P6, RVH/Pig-wt/JPN/NGS-5/2014/G7Px, and

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RVH/Pig-wt/JPN/NGS-7/2014/G5P6) grouped into long-type RVH *NSP3* were classified into the T6 genotype (Fig. 3). In contrast, phylogenetic analysis using entire *NSP3* nucleotide sequences indicated that the three RVH strains were clearly distinguished as outgroup in the RVC *NSP3* genotype classification. Therefore, our data suggest that the long-type RVH *NSP3* might be formed by insertion of *NSP3* nucleotide sequences from porcine RVC into the short-type RVH *NSP3* (Fig. 4).

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Figure 2. Multiple-sequence alignment of the full-length nucleotide sequences of the *NSP3* gene from the ten porcine rotavirus H strains.

Multiple-sequence alignment was performed using the ClustalW method. The boxes

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shown in blue represent a pair of primers used for amplification. The boxes shown in red represents start and stop codons of open reading frame in the *NSP3* gene from each strain. The underline shown in green represents nucleotide sequences (positions 807-1308) used for a Blast search. The box shown in orange represents nucleotide sequences (positions 820-940) exhibited the highest identity with *NSP3* nucleotide sequences from porcine rotavirus C (T6 genotype) by a Blast search. The symbols below sequence mean that the sequence is identical (*) and non-identical (.) among the ten porcine rotavirus H strains.

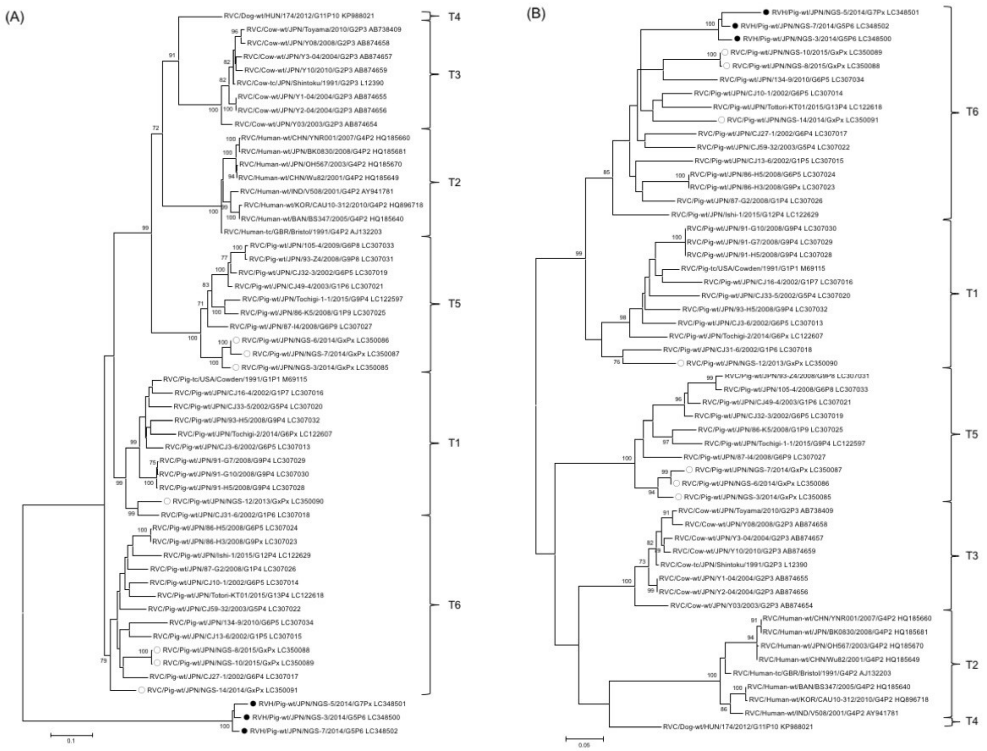

Figure 3. Phylogenetic tree constructed from entire (A) and partial (B) *NSP3* nucleotide sequences from rotavirus C strains including seven porcine rotavirus C and three porcine rotavirus H strains.

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In the analysis using partial *NSP3* nucleotide sequences, the 421 nucleotide sequences from all rotavirus C strains including seven rotavirus C and three rotavirus H strains were used. Phylogenetic tree was constructed using the maximum-likelihood method with MEGA6 software. The number beside each node represents the percentage of bootstrap support (of 1,000 replicates) for the cluster. Genotypes classifications are performed in accordance with our previous study. Genotypes are specified on the right. The symbols (filled circle and open circles) above the strains indicates the porcine rotavirus H and porcine rotavirus C analyzed in this study, respectively. GenBank accession numbers are also shown below the strains.

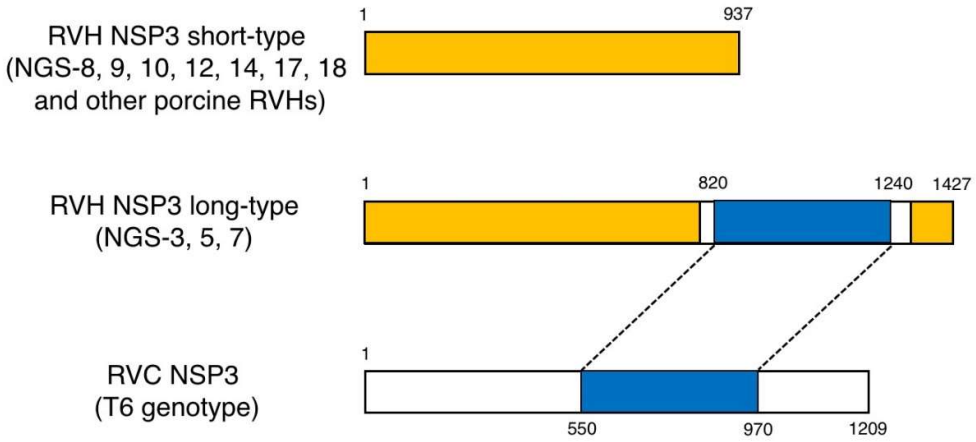

Figure 4. Schema of gene rearrangement between porcine RVH and porcine RVC in the *NSP3* gene.

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We also determined *NSP3* nucleotide sequences from the seven porcine RVCs co-infected with the twelve porcine RVHs in accordance with our previous study [14]. The seven RVC *NSP3* sequences were deposited into GenBank under accession nos. LC350085-LC350091. Phylogenetic analysis of the seven RVC *NSP3* revealed a division into three (T1, T5, and T6) genotypes (Fig. 3). In addition, comparative analysis of the extra sequences (positions 420–820) from the three RVH strains (NGS-3, NGS-5, and NGS-7) grouped into long-type RVH *NSP3* and the RVC *NSP3* sequences (positions 550–970) from the two same strains (NGS-3 and NGS-7) revealed low nucleotide sequence identities (67.5–70.5%). These findings indicate a potentially novel gene rearrangement event between porcine RVH and RVC in the *NSP3* gene, which would not have occurred in the swine herds assessed herein. Therefore, our results suggest the possibility that these mutants originate from either a hybrid between porcine RVHs and RVCs, which may have spontaneously occurred on another farm or an unknown RVH that has not been reported previously. The function of NSP3 in porcine RVH and RVC remains unknown; hence, future studies are required to perform functional analyses for NSP3.

4. Conclusions

Our data indicate that porcine RVHs of multiple genotypes have been present in Japan since the discovery of the first porcine RVH, SKA-1. In addition, our data point out that there are porcine RVHs with different genotypes in each swine-producing country other than Japan. Taken together, our results suggest that recently detected porcine RVHs in Japan would be transmitted via pig trade from other countries or may have independently evolved in Japan over the last 20 years. The twelve porcine RVHs analyzed herein displayed genetic variations in *NSP1* and *NSP3* genes, compared with other previously reported porcine RVHs. In particular, we report two different types in RVH *NSP3*, and that long-type RVH *NSP3* might have been derived from a gene rearrangement

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between porcine RVH and porcine RVC. Furthermore, we establish a tentative complete genome-based genotyping system for RVHs from pigs and humans. In future, a better complete genome-based genotyping system for RVHs, the same as RVAs, would be constructed, by adding complete genomic sequence data from new host species to these sequence data. The finding presented in this study would further proceed the current understanding of the evolution of RV.

Supplementary Materials: Figure S1: Multiple alignment between RVH *NSP3* nucleotide sequence (positions 820-1240) from three strains grouped into long-type and RVC *NSP3* nucleotide sequences (positions 550-970) from two strains grouped into T6 genotype; Table S1: A set of specific primers used for reverse transcription amplification of complete genomes from twelve porcine rotaviruses H strains; Table S2: Identities of individual genes at the nucleotide level among RVHs based on genotype classification as shown in Fig. 1

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