

A G3P[13] porcine group A rotavirus emerging in China is a reassortant and a natural recombinant in the VP4 gene

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Funding information

The National Key Research and Development Program of China, Grant/Award Number: 2015BAD12B02, 2016YFD0500103, 2017YFD0500104; National Natural Science Foundation of China, Grant/Award Number: 31572541, 31502092, 31602072

Summary

Group A rotaviruses (RVAs) are a major cause of serious intestinal disease in piglets. In this study, a novel pig strain was identified in a stool sample from China. The strain was designated RVA/Pig/China/LNCY/2016/G3P[13] and had a G3-P[13]-I5-R1-C1-M1-A8-N1-T1-E1-H1 genome. The viral protein 7 (VP7) and non-structural protein 4 (NSP4) genes of RVA/Pig/China/LNCY/2016/G3P[13] were closely related to cogent genes of human RVAs, suggesting that a reassortment between pig and human strains had occurred. Recombination analysis showed that RVA/Pig/China/LNCY/2016/G3P[13] is a natural recombinant strain between the P[23] and P[7] RVA strains, and crossover points for recombination were found at nucleotides (nt) 456 and 804 of the VP4 gene. Elucidating the biological characteristics of porcine rotavirus (PoRV) will be helpful for further analyses of the epidemic characteristics of this virus. The results of this study provide valuable information for RVA recombination and evolution and will facilitate future investigations into the molecular pathogenesis of RVAs.

KEYWORDS

porcine group A rotavirus, reassortant, recombinant, VP4

1 | INTRODUCTION

Rotaviruses (RVs) are a major cause of intestinal disease in animals, including weaned piglets and young children (Vlasova, Amimo, & Saif, 2017). RVs form a genus of the *Reoviridae* family and contain a genome consisting of 11 double-stranded RNA segments. The RV genome encodes six structural viral proteins (VPs; VP1-VP4, VP6 and VP7) and five or six non-structural proteins (NSP1-NSP5/6) (Ramig, 1997). The RV VP6 protein has been used to differentiate RV species by classifying them into 10 groups from group A rotavirus (RVA) to group J rotavirus (RVJ) (Banyai et al., 2017; Matthijnsens et al., 2012; Mihalov-Kovacs et al., 2015). Continuous RV classification is being performed by the Rega Instituut, KU Leuven (Belgium) (<https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/7th-RCWG-meeting>). The most common group infecting humans and

animals is RVA, which causes acute dehydrating diarrhoea (Vlasova et al., 2017).

To facilitate the classification of novel RVA strains, a Rotavirus Classification Working Group (RCWG) was established to develop a classification system for RVs (Matthijnsens, Ciarlet, Rahman et al., 2008b). Due to the high genetic diversity of RVA strains, the dual typing system (G and P genotypes) was extended to a whole-genome sequence classification system with all 11 gene segments (Matthijnsens, Ciarlet, Heiman et al., 2008a). The classification and nomenclature of RVA genes is G_x-P_[x]-I_x-R_x-C_x-M_x-A_x-N_x-T_x-E_x-H_x, where x indicates the corresponding number of genotypes (Matthijnsens et al., 2011). To date, 32 G, 47 P, 24 I, 18 R, 17 C, 17 M, 28 A, 18 N, 19 T, 24 E and 19 H genotypes have been identified among RVAs from humans and animals (Esona et al., 2017), as described at the 7th RCWG meeting (<https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/7th-RCWG-meeting>) at Rega Instituut, KU Leuven (Belgium). In pigs, 12 G genotypes (G1 to G6,

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G8 to G12 and G26) and 16 P genotypes (P[1] to P[8], P[13], P[19], P[23], P[26], P[27], P[28], P[32] and P[34]) of RVA have been identified, and G3, G4, G5, G9 and G11 usually occur in combination with P[5], P[6], P[7], P[13] and P[28], respectively (Vlasova et al., 2017).

Infections are confirmed in piglets by the association of RVAs with diarrhoea worldwide. An analysis of molecular characterization data for RVAs in Kenya showed that 18.5% of pigs were positive for RVA (Amimo, Otieno, Okoth, Onono, & Bett, 2017). RVA is an important pathogen for diarrhoea in Belgian suckling pigs (Amimo et al., 2016). Epidemiological surveillance of porcine rotavirus (PoRV) conducted in Ohio, USA, showed that 9.4% of pigs were positive for RVA (Amimo, Vlasova, & Saif, 2013). Genetic diversity analyses showed that different genotype combinations of PoRV strains were present in Thailand (Saikruang et al., 2013). Previous reports have shown that diarrhoea caused by PoRV can also be found in China (Shi et al., 2012).

To study the biological characteristics of PoRV, further investigation of PoRV diversity is necessary. In this study, the whole genome of an RVA strain belonging to the G3P[13] genotype was sequenced. The genome sequence identified an emerging reassortant G3P[13] PoRV strain from China with natural crossover points in the VP4 gene. The results will be helpful for further analysis of the epidemic characteristics of PoRV. The results provide valuable information on RVA recombination and evolution and facilitate future investigations into the molecular pathogenesis of RVA.

2 | MATERIALS AND METHODS

2.1 | Specimen collection

In 2016, an intestinal tissue sample from a pig with diarrhoea was collected from a pig farm in Liaoning Chaoyang (LNCY), China. The intestinal contents were collected and mixed with PBS at a 1:10 ratio. The suspension was centrifuged at 8000× *g* and 4°C for 10 min, and the supernatant was stored at −20°C.

2.2 | Viruses

The positive control viruses PoRV strain RVA/Pig/China/NMTL (Accession No. JF781163) (Shi et al., 2012), porcine epidemic diarrhoea virus (PEDV) strain CV777 (Accession No. AF353511) and transmissible gastroenteritis virus (TGEV) strain AHFH (Accession No. KX499468) (Zhang, Zhu, Shi et al., 2017) were maintained in our laboratory.

2.3 | Reverse transcription (RT)-PCR and sequencing

Total RNA was extracted from the supernatant of the intestinal contents using the QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. RT-PCR was performed using the PrimeScript™ One Step RT-PCR Kit Ver. 2 (TaKaRa, Dalian,

China) according to the manufacturer's instructions. Primers are designed as shown in Table S1.

2.4 | Sequence analysis

Sequences of the PoRV reference strains were obtained from GenBank (Tables S2 and S3). The nt sequences were analysed with DNASTAR software (DNASTAR Inc., Madison, WI, USA). Phylogenetic trees were constructed with MEGA5.2 software using the maximum likelihood method.

2.5 | 3D model of the VP4 protein

Using PyMOL software, the spatial distribution of the VP4 protein (70-210 aa) was analysed in a 3D model using the SWISS-MODEL server (Biasini et al., 2014).

2.6 | Recombination analysis

Sequence recombination was analysed using RDP4 software (Martin, Murrell, Golden, Khoosal, & Muhire, 2015). The RDP, GENECONV, Chimaera, MaxChi, Bootscan, SiScan and 3Seq methods were employed with respective default parameters.

3 | RESULTS

3.1 | Genome sequence of the PoRV RVA/Pig/China/LNCY/2016/G3P[13] strain

To determine the causative agent of diarrhoea in the sample from the LNCY pig farm in China, total RNA was extracted from the small intestine contents and subjected to RT-PCR. The samples were positive for PoRV but negative for TGEV and PEDV (Figure S1). The complete open reading frame (ORF) sequences for all 11 genome segments of strain **RVA/Pig/China/LNCY/2016/G3P[13]** were obtained using the primers as shown in Table S1. The accession numbers for the 11 segments deposited in GenBank are MF462321, MF462322, MF462323, MF462324, MF462325, MF462326, MF462316, MF462317, MF462318, MF462319 and MF462320 for VP1, VP2, VP3, VP4, VP6, VP7, NSP1, NSP2, NSP3, NSP4 and NSP5, respectively. The individual genotypes for VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 of **RVA/Pig/China/LNCY/2016/G3P[13]** were assigned as G3-P[13]-I5-R1-C1-M1-A8-N1-T1-E1-H1 using the online RotaC^{2.0} automated genotyping tool (Maes, Matthijnsens, Rahman, & Van Ranst, 2009). The VP7 and NSP4 genes of **RVA/Pig/China/LNCY/2016/G3P[13]** were closely related to cogent genes of human origin viruses (Table 1).

3.2 | Sequence analysis of the VP7 gene

The VP7 gene contains 981 nt encoding 326 amino acids (aa). The VP7 type was determined for the PoRV strains. A phylogenetic tree was

TABLE 1 Nucleotide and amino acid identities of genome segments of strains RVA/Pig/China/LNCY/2016/G3P[13] compared with the closest strains from GenBank database

Gene	Closely related strains	Nucleotide (%)	Amino acid (%)	Genotype	Accession No.
VP1	RVA/Pig-tc/USA/Gottfried/1983/G4 [P6]	95.2	99.2	R1	KR052746
VP2	RVA/Pig-tc/USA/OSU/1977/G5 [P7]	93.0	98.8	C1	GU199515
VP3	RVA/Pig-tc/MEX/YM/1983/G11 [P7]	87.5	95.0	M1	AY300922
VP4	RVA/Pig-wt/VNM/14150_54/VP4	95.7	97.3	P[13]	KX363348
VP6	RVA/Pig-tc/MEX/YM/1983/G11 [P7]	90.2	96.7	I5	X69487
VP7	RVA/ Human-tc/USA/P/1974/G3 [P8]	86.0	94.2	G3	EF672602
NSP1	RVA/Pig-tc/USA/Gottfried/1983/G4 [P6]	82.9	84.2	A8	U08431
NSP2	RVA/Pig-tc/MEX/YM/1983/G11 [P7]	95.0	96.9	N1	GU199517
NSP3	RVA/Pig-tc/MEX/YM/1983/G11 [P7]	88.7	96.5	T1	GU199518
NSP4	RVA/ Human-tc/USA/P/1974/G3 [P8]	94.7	96.0	E1	EF672603
NSP5	RVA/Pig-tc/MEX/YM/1983/G11 [P7]	96.5	98.0	H1	X69486

constructed using a whole VP7 gene sequence (981 nt) with selected G genotype sequences from GenBank. **RVA/Pig/China/LNCY/2016/G3P[13]** demonstrated the closest relationship with RVA/Human-tc/USA/P/1974/G3[P8] (EF672602) (Figure S2A). An analysis of PoRV G3 strains

identified as **RVA/Pig/China/LNCY/2016/G3P[13]** and the PoRV VP7 gene sequences available in GenBank showed that the nt identity rates varied from 79.3% to 88.0%. In a previous study, G3 genotypes were shown to be genetically diverse and clustered into two distinct lineages (G3a and G3b) (Mino et al., 2013). The **RVA/Pig/China/LNCY/2016/G3P[13]** strain found in this study belonged to G3a (Figure 1). Furthermore, a close relationship was found for RVAs (Maes et al., 2009) between **RVA/Pig/China/LNCY/2016/G3P[13]** and a number of RVA G3 strains using the RotaC^{2.0} automated genotyping tool. The RotaC results showed that the sequence was genotype G3 (glycolated) and that the query sequence was most similar to G3-RVA/Human-tc/USA/P/1974/G3[P8] (86.0%) (Figure S2B).

3.3 | Sequence analysis of the VP4 gene

The VP4 gene contains 2337 nt encoding 778 aa. The VP4 type was determined for the PoRV strains. A phylogenetic tree was constructed using the whole VP4 gene sequence (2337 nt) with selected P genotype sequences from GenBank. **RVA/Pig/China/LNCY/2016/G3P[13]** demonstrated the closest relationship with RVA/Pig-wt/VNM/14150_54/VP4 (KX363348) (Figure S3A). In a previous study, P[13] genotypes were shown to be genetically diverse and clustered into three distinct lineages (an India/Thailand lineage, a Japanese lineage and a new USA lineage) (Amimo et al., 2013). In this study, a phylogenetic tree was constructed using whole VP4 gene sequences (2337 nt) with selected P[13] genotype sequences from GenBank. Analysis of PoRV P[13] identified as **RVA/Pig/China/LNCY/2016/G3P[13]** and the PoRV VP4 gene sequences available in GenBank showed that the nt identity rates varied from 82.4% to 95.7%. We clustered the P[13] sequences into four lineages (lineage I, lineage II, lineage III and lineage IV) and found that **RVA/Pig/China/LNCY/2016/G3P[13]** belonged to lineage IV (Figure 2). Furthermore, a close relationship was found for group A rotaviruses (Maes et al., 2009) between **RVA/Pig/China/LNCY/2016/G3P[13]** and a number of RVA P[13] strains using the RotaC^{2.0} automated genotyping tool. The RotaC results showed that the sequence was genotype P[13] (protease-sensitive), and the query sequence was most similar to PoRV strain A46 (AY050274) with the highest similarity (83.5%); this strain was collected in 2001 in the USA (Figure S3B).

3.4 | Sequence analysis of VP6, VP1, VP2 and VP3

Using the RotaC^{2.0} automated genotyping tool for RVAs (Maes et al., 2009), the VP6, VP1, VP2 and VP3 sequences were compared between **RVA/Pig/China/LNCY/2016/G3P[13]** and a number of RVAs. The VP1 sequence was genotype R1 (RNA-dependent RNA polymerase); the query sequence was most similar to R1-RVA/Pig-tc/USA/Gottfried/1983/G4[P6] (95.2%) (Figure S4A). The VP2 sequence was genotype C1 (core shell protein); the query VP2 sequence was most similar to C1-RVA/Pig-tc/USA/OSU/1977/G5[P7] (93.0%) (Figure S4B). The VP3 sequence was genotype M1 (methyltransferase); the query VP3 sequence was most similar to M1-RVA/Pig-tc/MEX/YM/1983/G11[P7]

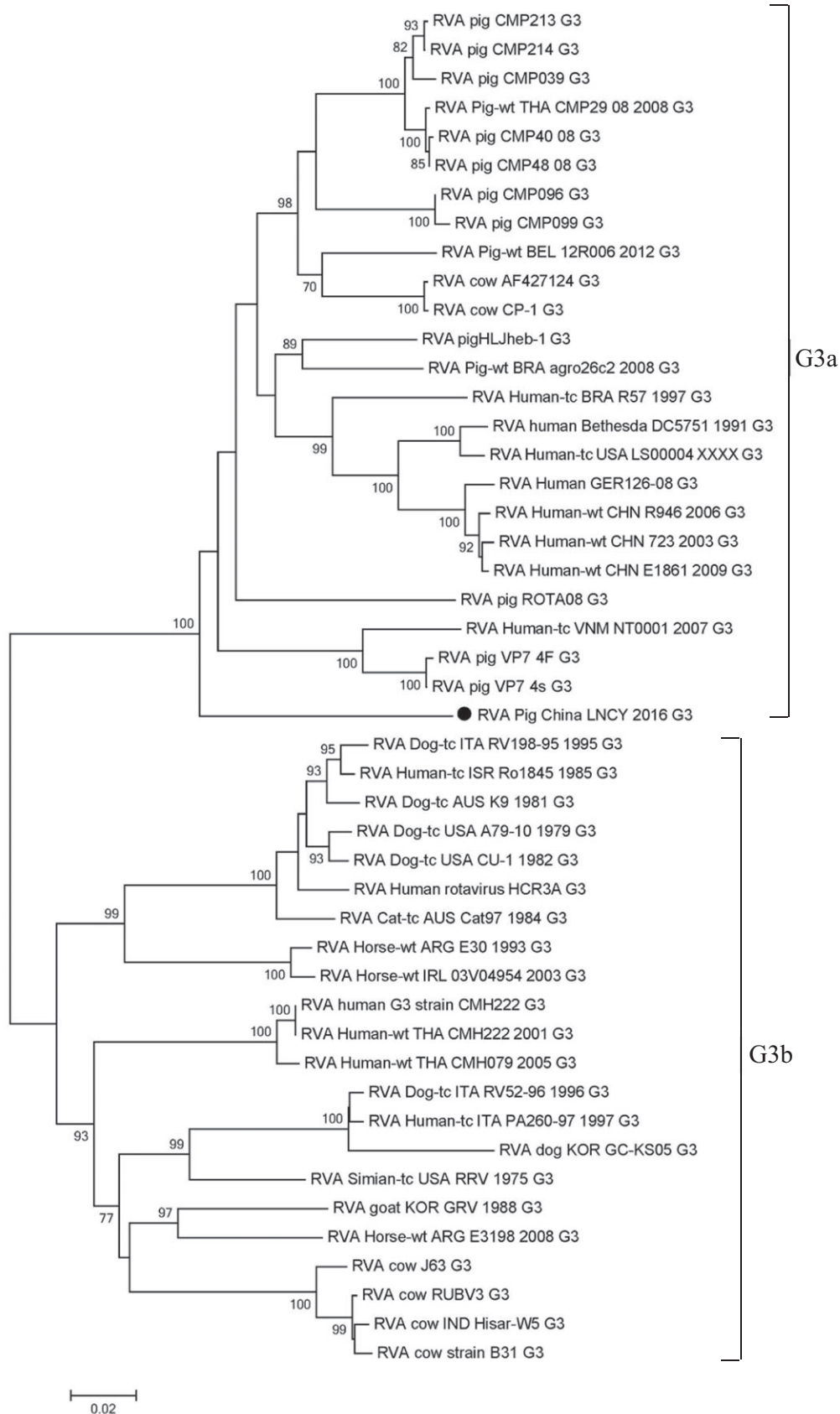


FIGURE 1 Phylogenetic analysis of RVA/Pig/China/LNCY/2016/G3P[13] based on the VP7 sequence in the G3 genotype. Phylogenetic trees were constructed using the Construct/Test Neighbour-Joining method with MEGA 5.2 software, and tree reliability was evaluated using the bootstrap method with 1,000 replications. Only bootstrap values >70% at branch points are shown

(a)

	7-1													7-2								
	87	91	94	96	97	98	99	100	201	211	213	238	291	147	148	190	217	221	223	242	264	
Consensus	T	T	G	T	A	E	W	K	T	T	T	L	K	S	T	Q	E	T	E	T	V	
LNCY/China/G3	.	.	N	N	.	S	A	.	S
A2/USA/G9	K
OSU/USA/G5	N	.	A	.	.	K	.	T	.	.	I	.	.	G	N	T	.	N
Gottfried/USA/G4	S	.	N	N	G	S	.	D	.	S	.	.
BJ-17-5/China/G9	A
DN3020/China/G5	N	.	A	T	.	.	I	.	.	G	N	T	.	N
DZ-2-G5/China/G5	N	.	A	.	.	K	.	T	.	.	I	.	.	G	N	T	.	N
GXnn-G9/China/G9	A
GXqz-1-G9/ChinaG9	A
GXqz-2-G9/ChinaG9	A
GZFF01/China/G4	.	.	S	N	G	S	.	E	.	S	.	.
GZFF02/China/G4	.	.	S	N	G	S	.	E	.	S	.	.
GZMJ01China/G4	.	.	S	N	A	.	.	.	G	S	.	E	.	S	.	.
GZMJ02/China/G4	.	.	.	N	A	.	.	.	G	S	.	E	.	S	.	.
HeN3/G3China/G3	I	.	N	N	.	S	A	A	T
HLJ-15-1/China/G4	.	.	.	N	A	G	S	.	E	.	S	.	.
HLJheb-1/G3	.	.	N	N	.	S	A	A	S
JL94/China/G5	N	.	A	.	.	K	.	T	.	.	I	.	.	G	N	T	.	N
JN-1-2/ChinaG9
JN-2China/G5	N	.	A	A	.	K	.	T	A	.	I	.	.	G	N	T	.	N
LLP48/ChinaG9	.	.	R	A
LY-2/China/G9	A
NMTL/China/G9	K	.	.
TA-1-2/China/G9	K	.	.
TA-3-2/China/G9	K	.	.
TA-4-2/China/G3	.	.	N	N	.	S	A	.	T
TM-a/China/G9	P	K	.	.
WF-1-2/China/G5	N	.	A	.	.	K	.	T	A	.	I	.	.	E	N	T	.	N
WF-5-2/China/G5	.	.	A	.	.	K	.	T	A	.	I	.	.	E	N	T	.	N
ZJhz13-4/China/G2	.	.	S	E	.	.	V	I	.	T	.	N	.	Q

(b)

	306	384	386	388	393	394	398	429	434	440	441	459
Consensus	T	N	D	R	A	S	T	S	E	A	R	K
LNCY/China/P[13]	.	S	S	A	N	F	S	R	.	T	.	.
OSU/USA/P[7]	A	S	S	A	H	Y	.	R	.	T	.	R
Gottfried/USA/P[6]	L	.	.	K	.	.	S	.	.	L	.	N
JL94-China/P[7]	A	S	S	A	H	Y	.	R	.	T	.	R
NMTL/China/P[23]
TM-a/China/P[23]	.	.	N
HLJ-15-1/China/P[23]
ZZ-12/China/P[23]

(c)

	8-1													8-2		8-3						8-4			
	100	146	148	150	188	190	192	193	194	195	196	180	183	113	114	115	116	125	131	132	133	135	87	88	89
Consensus	D	T	I	G	Y	T	T	N	Y	D	T	E	N	T	S	R	S	Q	E	N	V	T	S	N	A
LNCY/China/P[13]	.	S	Q	D	.	E	N	.	.	S	A	D	S	.	R	E	N	.	.	.	T	Q	T	D	K
A2/USA/P[7]	.	.	L	.	.	S	.	D	.	.	.	T	.	Q	A	T	N	.	.	.	T	Q	T	V	E
OSU/USA/P[7]	.	.	P	.	.	S	T	.	Q	T	T	D	.	.	.	T	Q	I	V	E
Gottfried/USA/P[6]	.	N	A	I	S	.	S	.	L	P	D	.	H	P	.	.	.	A	.	.	S	D	.	.	K
NMTL/China/P[23]	.	D	V
HLJ-15-1/China/P[23]	.	D	V
JL94-China/P[23]	.	.	P	.	.	S	T	.	Q	T	T	N	.	.	.	T	Q	T	V	E
TM-a/China/P[23]	.	D
ZZ-12/China/P[23]	E
WH-a/China/P[23]	.	D

FIGURE 3 Neutralizing epitopes on the VP7 and VP4 (VP5* and VP8*) proteins in Chinese strains. (a) Neutralizing epitopes on the VP7 protein. (b) Neutralizing epitopes on the VP5* protein. (c) Neutralizing epitopes on the VP8* protein. Dots represent the same residues

(Figure 3a). Six aa (94, 96, 98, 213, 147 and 190) in the 7-1 and 7-2 aa sequences differed between *RVA/Pig/China/LNCY/2016/G3P[13]* and the RCE strain A2-G9P[6]-USA. Nine aa (87, 94, 98, 100, 213, 147, 148, 190 and 221) in the 7-1 and 7-2 aa sequences differed between *RVA/Pig/China/LNCY/2016/G3P[13]* and the RCE strain OSU-G5P[7]-USA. Six aa (87, 98, 147, 148, 221 and 242) differed in the 7-1 and 7-2 aa sequences between *RVA/Pig/China/LNCY/2016/G3P[13]* and the RCE strain Gottfried G4P[6]-USA. Furthermore, in *RVA/Pig/China/LNCY/2016/G3P[13]*, the two aa positions located at 98S in 7-1 and 147A in 7-2 were not found in any of the RCE strains.

Trypsin cleavage of VP4 generates two fragments (VP5* and VP8*) (Dunn et al., 1995; Patton, Hua, & Mansell, 1993; Ruggeri & Greenberg, 1991). Only a partial sequence of the VP4 of porcine rotavirus strain A2 was found (AB180977, 783 nt). Therefore, we only compared the VP5* neutralizing epitope region of *RVA/Pig/China/LNCY/2016/G3P[13]* with all PoRV strains from China obtained from GenBank and the RCE strains (Gottfried G4P[6], OSU G5P[7]) available in the ProSystems RCE (Merck). The results show that two aa positions (434E and 441R) in the neutralizing epitope in VP5* were conserved (Figure 3b). Five aa (306, 393, 394, 398 and 459) in the VP5* neutralizing epitope differed between *RVA/Pig/China/LNCY/2016/G3P[13]* and the RCE strain OSU-G5P[7]-USA (Figure 3b). Nine aa (306, 384, 386, 388, 393, 394, 429, 440 and 459) in the VP5* neutralizing epitope differed between *RVA/Pig/China/LNCY/2016/G3P[13]* and the RCE strain Gottfried G4P[6]-USA (Figure 3b). Only two aa positions were conserved in the neutralizing epitope in VP8*: one aa (100D) in 8-1 and the other aa (131E and 132N) in 8-3 (Figure 3c).

3.7 | A six-nt insertion impacts the spatial structure of the VP4 protein

We identified an additional six nt (nt 559-561 and nt 568-570) in the VP8* gene of *RVA/Pig/China/LNCY/2016/G3P[13]* compared with the corresponding genes of other Chinese strains (Figure 4a). No other Chinese strains were previously reported to contain six additional nt in the VP8* protein (Figure 4a). However, the presence of six-nt gains at the same positions was found in strains from other countries, including Thailand, India, Japan, Australia, and the USA (Figure 4a). The additional six nt resulted in four aa changes (aa 184, 185, 186 and 190) and the loss of two aa from the VP8* protein (aa 188 and 189) (Figure 4a). Further analysis of the influence of these aa changes on the spatial structure of the VP8* protein was performed. The VP8* protein sequences (70-210 aa) of *RVA/Pig/China/LNCY/2016/G3P[13]* and *RVA/Pig/China/NMTL* were searched against the SWISS-MODEL template library. For the best homology modelling with VP8* protein sequences (70-210 aa) of *RVA/Pig/China/LNCY/2016/G3P[13]* while searching against templates, the solution structure of the rotavirus spike protein (SMTL id 2p3j.1) (Kraschnefski et al., 2009) was selected for model construction. Based on the results, the spatial structure of the VP8* protein of *RVA/Pig/China/LNCY/2016/G3P[13]* was different from that of *RVA/Pig/China/NMTL*. The *RVA/Pig/China/NMTL* strain forms a longer β -sheet in the aa region of 184TTGY187 than in the aa region of 184APQEF190 in *RVA/Pig/China/LNCY/2016/G3P[13]* (Figure 4b).

3.8 | Recombination of *RVA/Pig/China/LNCY/2016/G3P[13]*

To further analyse the association between VP4 from *RVA/Pig/China/LNCY/2016/G3P[13]* and the existing isolates, a genetic analysis was completed between *RVA/Pig/China/LNCY/2016/G3P[13]* and the existing RVA isolates using RDP4 software (Martin et al., 2015). The VP4 sequence of *RVA/Pig/China/LNCY/2016/G3P[13]* is shown in Figure 5a. Crossover points for a potential recombination zone were found at nt 456-804 of the VP4 gene (Figure 5b), but no gene recombination was detected in the other viral proteins. The major parental strain for the recombination located in the VP4 gene of *RVA/Pig/China/LNCY/2016/G3P[13]* was *RVA/Pig/PRG9121/2006/G9P[7]* (JF796737) (Kim et al., 2012), and the minor parental strain was *RVA/Pig-wt/BEL/12R046/2012/G9P[23]* (KM820720) (Theuns et al., 2015) (Figure 4b). These results indicate that P[13] of *RVA/Pig/China/LNCY/2016/G3P[13]* was the result of recombination of P[23] and P[7] RVA genes.

4 | DISCUSSION

Many types of viruses cause diarrhoea in animals, including TGEV (Zhang, Zhu, Zhu, Chen et al., 2017), swine enteric coronaviruses (SeCoV) (Belsham et al., 2016), PEDV (Lohse et al., 2016; Tian et al., 2014; Zhang, Tian et al., 2017), porcine deltacoronavirus (Lee et al., 2016; Mai et al., 2017; Saeng-Chuto et al., 2017) and rotaviruses (Kim et al., 2016). Rotaviruses cause acute dehydrating diarrhoea in various hosts, including children and young animals (Jiang, Liu, & Tan, 2017). Further information on rotavirus diversity is necessary to improve our understanding of the biological characteristics and evolution of rotaviruses. In 2016, intestinal tissue was collected from a piglet with LNCY-induced diarrhoea accompanied by vomiting at a pig farm in China. The entire *RVA/Pig/China/LNCY/2016/G3P[13]* genome was successfully sequenced, and evidence of a recombination event was identified.

Reassortment events between humans and animal strains have been found previously (Banyai et al., 2009; Doan et al., 2013; Esona et al., 2009, 2017; Ghosh et al., 2010; He et al., 2013, 2017; Jere, Mlera, O'Neill, Peenze, & van Dijk, 2012; Malik et al., 2016; Masuda et al., 2014; Matthijnsens et al., 2009; Mullick et al., 2013; Steyer, Sagadin, Kolenc, & Poljsak-Prijatelj, 2013). Of the 11 segments of the *RVA/Pig/China/LNCY/2016/G3P[13]* strain, nine gene segments (VP1, VP2, VP3, VP4, VP6, NSP1, NSP2, NSP3 and NPS5) possessed sequences nearly identical with those of the porcine strains, suggesting that *RVA/Pig/China/LNCY/2016/G3P[13]* was possibly of porcine origin. However, the VP7 and NSP4 genes of this porcine strain had human origins because the VP7 and NSP4 genes had the closest relationships with G3-RVA/Human-tc/USA/P/1974/G3P[8] and E1-RVA/Human-tc/USA/P/1974/G3P[8], respectively. These results indicated that *RVA/Pig/China/LNCY/2016/G3P[13]* might be a reassortant between porcine and human strains.

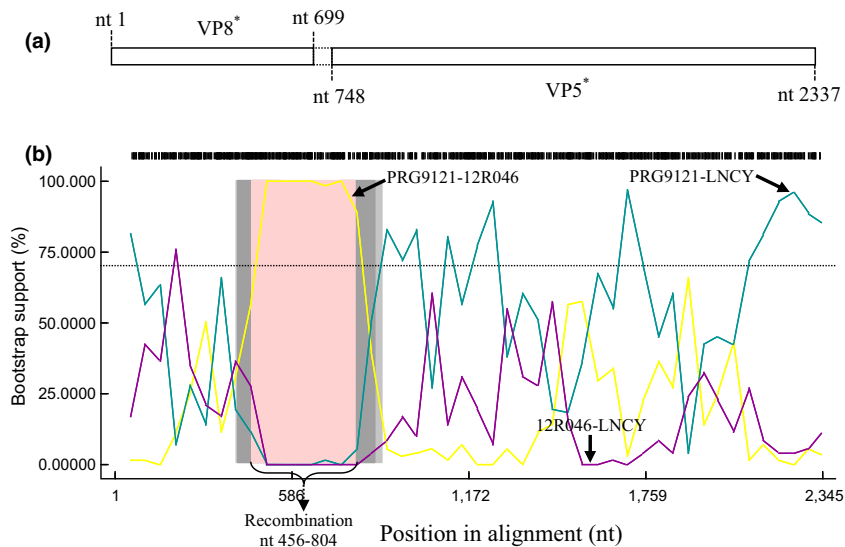


FIGURE 5 Recombination analysis of RVA/Pig/China/LNCY/2016/G3P[13] and other PoRV strains in the VP4 gene. (a) VP4 gene organization of PoRV. (b) Recombination analysis of the VP4 gene in RVA/Pig/China/LNCY/2016/G3P[13], RVA/Pig/PRG9121/2006/G9P[7] and RVA/Pig-wt/BEL/12R046/2012/G9P[23]. LNCY represents RVA/Pig/China/LNCY/2016/G3P[13] (MF462324), PRG9121 represents RVA/Pig/PRG9121/2006/G9P[7] (JF796737), and 12R046 represents RVA/Pig-wt/BEL/12R046/2012/G9P[23] (KM820720). Recombination crossover points are located at nt 456-804

present, relatively few P genotypes from China can be found in GenBank. More reports of the P genotype of PoRV are needed in China, which will provide information for the prevention of PoRV and the development of PoRV vaccines.

Recombination has been shown to be an important means of viral evolution (Simmonds, 2006). Recombination is considered a potential mechanism for antigenic diversity and a possible mechanism to escape from vaccine-imposed selective pressure but may not contribute to the long-term evolution of RVAs (Woods, 2015). Several reports have identified sequences with recombination in VP4 (Esona et al., 2017), VP6 (Jere, Mlera, Page, van Dijk, & O'Neill, 2011; Marthaler et al., 2014), VP7 (Martinez-Laso et al., 2009; Parra, Bok, Martinez, & Gomez, 2004; Phan et al., 2007a, 2007b; Suzuki, Gojobori, & Nakagomi, 1998), NSP1 (Esona et al., 2017), NSP2 (Jere et al., 2011), NSP3 and NSP4 (Donker, Boniface, & Kirkwood, 2011) (Table S4). Intramolecular recombination could contribute to the development of new RVs phenotypes (Ramig, 1997). To date, no reports have demonstrated a recombinant PoRV in China. In this study, evidence for a recombination crossover event in the VP4 gene (nt 456-804, aa 152-268) was provided in a Chinese isolate. This result may indicate that PoRV infection in farms in China is becoming increasingly complex. In the future, studies of the genetic evolution of new PoRVs in China will be necessary.

In the current study, we identified aa differences in the neutralizing antigen epitopes of VP7, VP5* and VP8* (VP4 is cleaved into VP5* and VP8*) between Chinese PoRVs and the porcine RCE strains. Some of the same epitopes were found for the VP7 protein in the PoRV strains, including *RVA/Pig/China/LNCY/2016/G3P[13]*, suggesting that the RCE strains would provide some protection against Chinese porcine genotypes. However, many neutralizing

antigen epitope differences were identified on the VP4 protein between the Chinese PoRV strains and the RCE strains, suggesting the reduced effectiveness of the RCE strains. Rotavirus antigens and serotypes are diverse, suggesting that obtaining extensive protective immunity requires immunization or infection with multiple antigens (Nair et al., 2017). More information is needed to understand the diversity of field strains and to develop an effective polyvalent vaccine as a possible means of PoRV control in China.

VP8* is a cleaved product of the VP4 spike protein that contains a shallow groove domain responsible for binding to SAs on cellular glycans (Desselberger, 2014; Lopez & Arias, 2004) or interacting with HBGA for virus particle attachment (Jiang et al., 2017; Sun et al., 2016). In this study, we reported a six-nt gain in the VP8* protein that resulted in a special structural change, which might affect attachment of the VP8* protein to host cells through interactions with SAs or HBGAs. This possibility will be a major focus of our future study.

Concurrent infection of a single host cell line by different RVs strains is a prerequisite for the occurrence of recombination events in nature (Esona et al., 2017). In developing countries, animal husbandry (i.e., cattle, sheep and pigs) is closely related to human populations, which provides potential possibilities for reassortment and recombination events. Monitoring newly emerging RVs in animals is important for the control of RVs infection in both humans and animals. The exact mechanism of recombination in rotaviruses is still unclear. Hypotheses have suggested that there may be a direct link between recombination and the formation of the secondary RNA structure, because these RNA structures induce stops, stalls or delays that favour strand invasion (Perez-Losada, Arenas, Galan, Palero, & Gonzalez-Candelas, 2015). The secondary structures in recombinant gene segments and in short stretches of AU-rich sequence might be associated with recombination events in RVs

(Esona et al., 2017). However, copy choice plays an important role in RNA virus recombination (Kirkegaard & Baltimore, 1986). Copy choice recombination might occur in RVs during plus- and minus-strand RVs RNA synthesis (Desselberger, 1996). Rotavirus recombination most likely occurs during the genome transcription stage in the viral life cycle (Kojima, Taniguchi, Urasawa, & Urasawa, 1996). **RVA/Pig/China/LNCY/2016/G3P[13]** is a recombinant virus between the P[7] and P[23] genotypes. It will be important to investigate the pathogenicity of this virus in pigs in future work.

In summary, in this study, an example of a natural recombination event in China between the P[7] and P[23] genotypes was observed, and crossover points (nt 456-804) for potential recombination were found in the VP4 gene. **RVA/Pig/China/LNCY/2016/G3P[13]** was a reassortant strain between pig and human strains. These results provide valuable information on RVs recombination and evolution and will facilitate future investigation of the molecular pathogenesis of porcine RVAs.

ACKNOWLEDGEMENTS

This study was supported by The National Key Research and Development Program of China (2015BAD12B02, 2016YFD0500103, 2017YFD0500104) and the National Natural Science Foundation of China (31572541, 31502092 and 31602072).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Jing Z, Zhang X, Shi H, et al. A G3P [13] porcine group A rotavirus emerging in China is a reassortant and a natural recombinant in the VP4 gene. *Transbound Emerg Dis.* 2018;65:e317–e328. <https://doi.org/10.1111/tbed.12756>