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Research Article

Phylogenitc analysis and immunogenicity comparison of porcine genotype G9 rotavirus in China from 2020–2023

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ABSTRACT

As an emerging genotype, the G9 genotype rotaviruses (RVs) are widespread among humans and pigs, and have been reported in many countries and regions in the recent years. Moreover, porcine G9 strains could cross the interspecies barrier to infect human. To investigate the epidemic trends of porcine G9 strains as well as the cross-immunoreactivity among different isolates, an epidemiological investigation about porcine G9 genotype RVs (PoRVs) was performed during the period 2020–2023 in multiple provinces of China. A total of nine representative strains were identified. The phylogenetic analysis based on viral *VP7* gene showed that these strains mainly clustered with lineages III and VI, which revealed the predominant G9 PoRVs in China. Moreover, a new lineage, lineage VII, was identified, and strains of this lineage were found to be circulating in Guangdong and Taiwan. Except lineages I and IV, some isolates from other lineages could co-circulate in pigs and humans. Three G9 strains, namely 923H, 923E, and 923X, which belonged to the largest sub-lineage III, were isolated. Then, the significant cross-reactivity was observed among strains of the same or different lineages. This study is the first to systematically investigate the genetic and immunogenetic characteristics of porcine G9 genotype rotavirus in China, as well as the potential cross-species transmission between pigs and humans, providing a valuable direction for the effective prevention of porcine rotavirus.

INTRODUCTION

Rotavirus (RV) displays a broad host range, including mammals and birds. RV consists of 11 segments of double-stranded RNA wrapped in a triple layered proteins, and produces six structural (VP1–VP4, VP6 and VP7) and five or six nonstructural proteins (NSP1–NSP6) in host cells. Based on the serotype of VP6 protein, the virus can be divided into 11 serum groups (A–D, F–K), according to the International Committee on Taxonomy of Viruses (ICTV) web site (http://ictv.global). Of these, group A rotavirus (RVA) infection is the most widespread, which causes viral diarrhea in the young children (under 5 years of age) and animals (Kim et al., 2013; Parashar et al., 2003). Especially in low-income countries and war zones, the lack of effective treatment after infection can cause death in children (Parashar et al., 2003). Even in developed countries, RVA infection can also cause a lot of expenses for the treatment of rotavirus infection (Troeger et al., 2018). With industrial breeding increasing, single or mixed co-infection with porcine epidemic diarrhea virus often cause serious diarrhea in the newborn and weaning piglets (Liu et al., 2024), which would result in a huge economic loss.

Rotavirus belongs to the *Reoviridae*, with a segmented, double-stranded RNA genome. In addition to gene mutations, rotavirus displays a high frequency of genetic reassortment, which promotes rotavirus genetic exchange. While rotavirus can be detected in animals, birds and humans, species-specific RVs have a restricted host range. Some uncommon genotype strains can cross the interspecies barrier and increase viral diversity. Co-infection between/among different genotype strains or between human and animal strains contributes to the exchange of viral genome segments. Analysis of genetic sequences of human and animal rotaviruses reveals a close relationship (Cook et al., 2004). Gene segments of circulating rotaviruses in the human population have been found in domestic animals, and these reassortant strains may become more and more epidemic in the human population through zoonotic transmission (Rojas et al., 2019). It is necessary to pay attention to the

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cross-infection of human and animal rotaviruses. Moreover, more cases indicate that humans can be infected by swine and bovine rotaviruses. Therefore, farmed animals, especially pigs, are considered as the reservoir hosts of rotavirus (Li et al., 2022).

As an emerging genotype, the G9 genotype RVs are widespread among humans and pigs, and have been reported in many countries and regions. In 1974, G9 rotavirus was first isolated in pigs in Britain, then efficiently spread throughout the world. The detection rate for this genotype is extremely low in China (Yang et al., 2007), and it was not reported until 2008 (Shi et al., 2012). Following that, the G9 genotype RV gradually became epidemic. According to recent epidemiological data, the proportion of G9 rotavirus is as high as 56.55% from more than twenty thousand porcine samples of diarrhea in 23 provinces in China in 2022 (Qiao et al., 2024).

In addition to infecting pigs, animal G9 strains have transferred to humans. Following detection in pigs, G9 rotavirus was first isolated from humans in the United States in 1983, but it was not found in the following ten years (Hoshino et al., 2005). In 1994, G9 re-emerged in China and many other places, and then only sporadically appeared (Yang et al., 2007). Until 2012, G9 became the most prevalent genotype and its detection rate reached 77% in 2019–2021 (Dong et al., 2023). Now it has replaced G1, G3 and other main genotypes in human RV infection in China and Thailand (Khamrin et al., 2006; Dong et al., 2016; Dong et al., 2023; Liu et al., 2022).

Although *VP7* gene is not associated with the host tropism of the virus, it is an important gene that induces neutralizing antibodies. Viral VP7 protein is a N-liked glycoprotein and contains two antigenic epitopes: 7-1 (7-1a and 7-1b) and 7-2 (Reslan et al., 2021). These epitopes determine virus genotype and immunogenicity. VP7 appears to be immunodominant in hyperimmunised animals. Vaccination with the WC3 vaccine elicited neutralizing antibody response almost solely to VP7. Likewise, immune responses to epitopes on VP7 showed significant correlations to protection against RV infection.

Due to the importance of G9 RV in humans and pigs, development of an efficient vaccine is necessary to inhibit virus infection. Although a few reports exist about the porcine G9 genotype rotavirus isolation and pathogenicity, it is still less known about the characteristics of *VP7* gene of epidemic strains and its immunogenicity between different strains. In this study, we analyze the *VP7* gene of pig G9 RVs from piglets diarrhea samples collected from 2020 to 2023 in China and assess the cross-interspecies transmission risk of these strains. Three strains were isolated from the mainly epidemic branch and their cross-immunoreactivity was analyzed and compared, which would provide a basis for the development of porcine G9 RV vaccine.

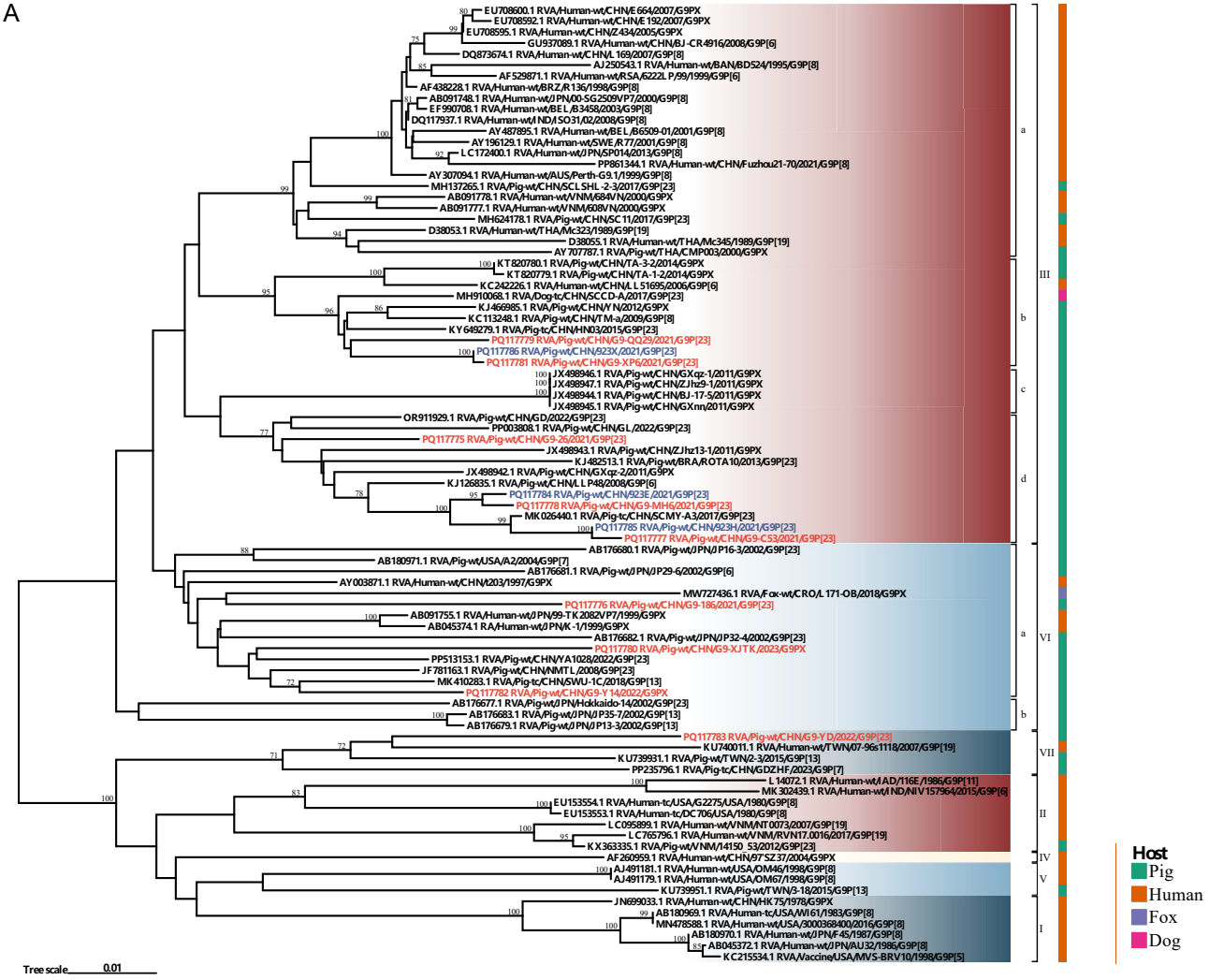

Fig. 1. Phylogenetic analysis based on the *VP7* sequence and epidemic characterization. **A** The *VP7* sequences identified in this study (color label) and reported in previous studies were analyzed. Representative and key sequences identified in the different period as well as sequences with higher similarity to these were included. Tree reliability was evaluated using the bootstrap method with 1000 replications. Only bootstrap values >70% at branch points are shown. Scale bar at the bottom indicates nucleotide substitutions per site. **B** The detection rate of representative strains and their distribution in China. **C** Transmission diagram of G9 RVs among human and animals. The arrow represents the cross-species transmission.

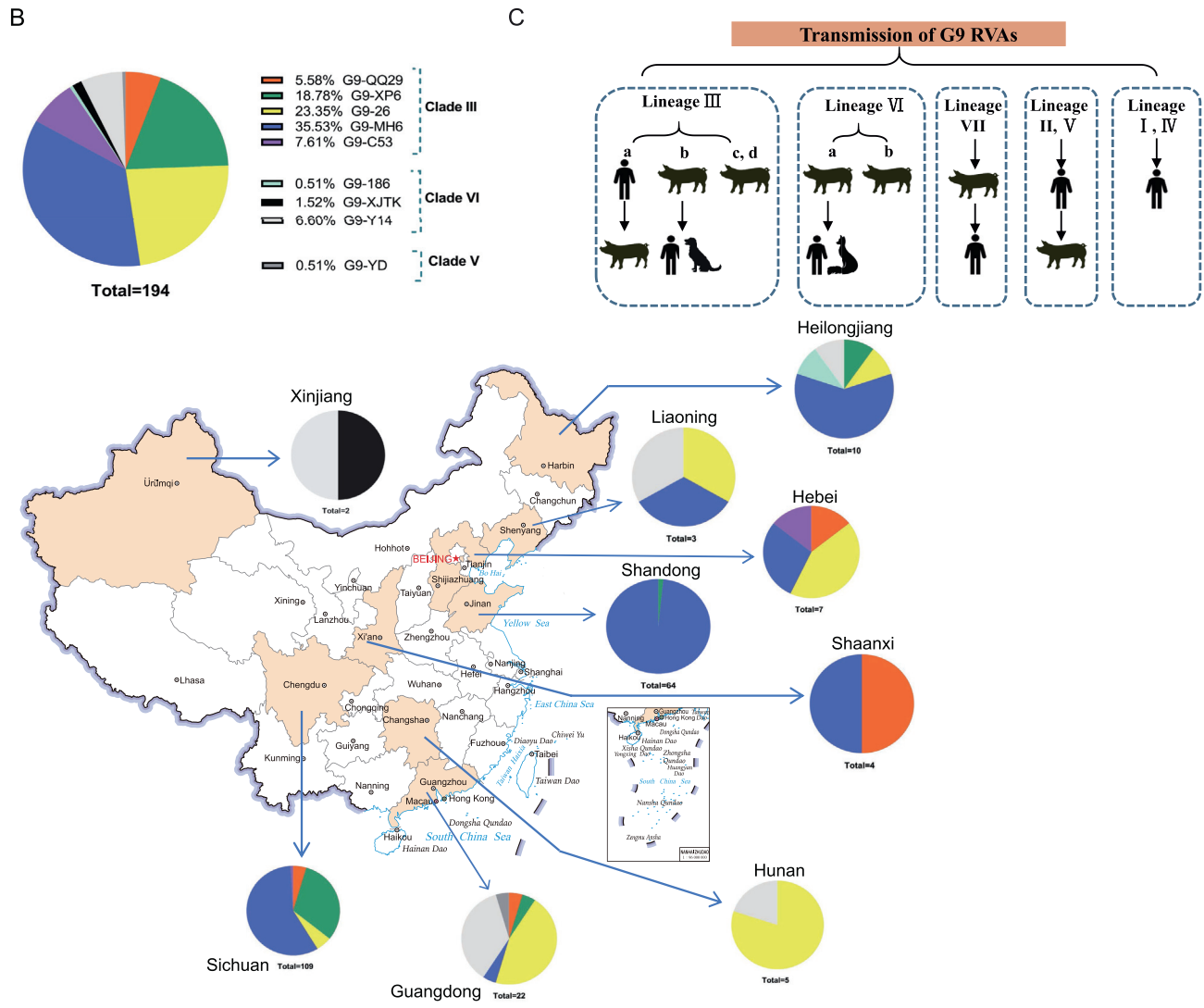

Fig. 1. (*continued*).

RESULTS

Phylogenetic analysis of *VP7* gene reveals that lineage III strains are the most prevalent in pigs in China

Fecal samples were collected from diarrheal piglets on breeding farms in Heilongjiang, Gansu, Inner Mongolia, Hunan, Fujian, Guangdong, Shandong, Sichuan and Xinjiang provinces from 2020 to 2023. In the 307 RVA positive samples, 194 (63.19%) samples were positive for G9 genotype virus. Sequences of the G9 VP7-encoding gene were obtained from nine represented strains, then phylogenetic tree was constructed. As shown in Fig. 1A, G9 RVA infection occurs mainly in the human and pigs, and only two isolates were identified in a dog and a fox (Colic et al., 2021), respectively. According to previous reports, G9 VP7 was divided into six lineages. Lineages I and IV strains were only found in humans and most of them were detected at an early stage (1980s). Most of strains from lineages II and V were detected in humans, and only one isolate from each lineage was found in porcine. Lineages I, II, IV and V strains were rarely detected in the last ten years.

Strain YD identified in Guangdong shares the highest identities with strain 2-3 and 07-96s detected in Taiwan and strain GDZHF detected in Guangdong, respectively. The four stains forms an independent branch. Compared with strains from other six lineages previously determined, strain YD shares highest identities (91.43%) with human strain OM67 reported in the 1998 from lineage V. So the YD strain with other three strains were defined as lineage VII and this clade consisted of porcine and human origin isolates (Fig. 1A).

Lineages III and VI, two biggest branches, predominantly consisted of both human and pig strains, suggesting that isolates in those branches might have cross-interspecies transmission ability between humans and pigs. Strains in lineage III are the most prevalent in humans and pigs. Sub-lineage IIIa mainly contains human strains, while sub-lineage IIIb, IIIc, IIId mainly contain pig strains (Fig. 1A).

Then, the detection ratio of each represent strain in all the isolates identified in this study was analyzed (Fig. 2B). Our investigation showed that lineage III strains of G9 RVA are the most prevalent in pigs in China during 2020–2023, accounting for a significant majority of G9 PoRV infections. Strains QQ29, XP6, 26, MH6 and C53 were classified in the lineage III and those five representative strains as well as their similar strains accounted for 89.92% of the total, which revealed the predominance of G9 PoRVs in China. Another three representative strains, 186, XJTK and YD, accounted for 9.59% of the total, which were detected only in a few pig farms. According to the sampling map, the represent strain G9-MH6 was prevalent in the Heilongjiang, Liaoning, Hebei, Shandong, Shaanxi and Sichuan provinces; strain G9-26 was prevalent in the Hunan and Guangdong provinces. The strains G9-XJTK and Y14 were prevalent in the Xinjiang, which was different from other provinces.

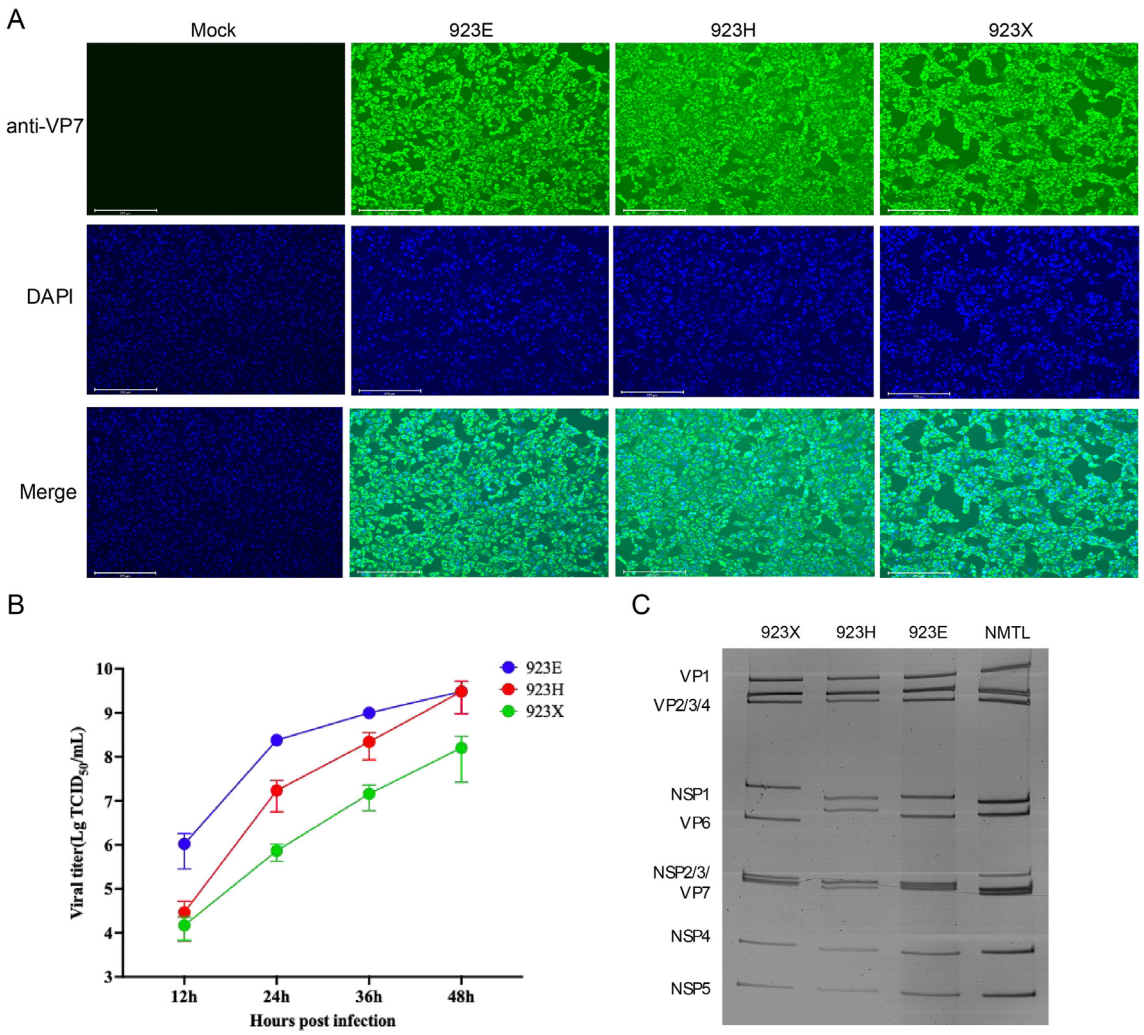

Fig. 2. Virus isolation and identification. **A** After five serial passages, each isolate pre-treated with 10 μg/mL of trypsin was inoculated into MA104 cell monolayers for 24 h. The cells were fixed and immunofluorescence assay was conducted to identify the isolates using an anti-VP7 monoclonal antibody. Scale bar represents 275 μm. **B** The MA104 cells were inoculated with each virus with an MOI of 1, and the suspension was collected for the analysis of growth curve. **C** RNA profile analysis of the three isolates using PAGE.

By analyzing the host range of each lineage, human isolates were present in sub-lineage IIIb and VIa, characterized by porcine strains, which suggests that human infections were of zoonotic origin (Fig. 1C). In the sub-lineage IIIa, human strains are prevalent but three porcine isolates were detected, which suggests that porcine infections were of reverse-zoonotic origin (Fig. 1C). The zooanthroponotic and reverse-zoonotic potential of G9 RVs would facilitate continuous spread.

Isolation and identification of strains 923E, 923H, 923X from the lineage III

Three strains 923E, 923H, 923X were isolated from the prevalent lineage III. During passaging on the MA104 cells, significant cytopathic effect (CPE) was observed on passage 9, 10, 9 for strains 923E, 923H, 923X, respectively (data not shown). The three isolates were further identified by immunofluorescence assay using a monoclonal antibody against RVA VP7 protein (Fig. 2A). The results confirmed that these three isolates are RVA stains. Then, viral growth curve was analyzed. The MA104 cells was infected with each strain with an MOI of 0.1, and the suspension was harvested at the indicated time points. As shown in Fig. 2B, strain 923E displays a fastest growth ability followed by strain 923H and strain 923X. RNA PAGE-testing of the isolates further revealed that all isolates showed a typical RNA pattern of mammalian RVA strains, respectively, and the bands of each isolate displayed a migration pattern of 4:2:3:2 (Fig. 2C). While the 11 segmented dsRNAs of each isolate showed a similar migration pattern compared with strain NMTL, a G9 strain isolated from a piglet in the 2008, the NSP1 and VP6 bands of strain 923X had a little difference in the migration speed.

Phylogenetic analysis of other ten genes reveals that the three strains are typical porcine RVs with a Wa-like backbone

Using the genotyping system and online BLAST analysis, the gene constellation and the most similar sequences of five structural genes (*VP1*, *VP2*, *VP3*, *VP4*, *VP6*) and five non-structure genes (*NSP1*, *NSP2*, *NSP3*, *NSP4*, *NSP5*) were identified. The genotyping results showed that the three strains display a constellation of I5-R1-C1-M1-A8-N1-T1-E1-H1 (Table 1). BLAST analysis of strain 923E showed that *NSP3* and *VP3* share the most identities with human strain and panda strain, and other gene segments are from porcine strains. For the strain 923H, *NSP1*, *NSP3* and *VP6* share the most identities with human strains, *NSP5* shows a highest similarity with a Yak strain, *VP3* share the most identities with panda strain, and other gene segments are from porcine strains. For the strain 923X, *NSP4* and *NSP5* share the most identities with human strains, *NSP3* shows a highest similarity with a rabbit

Table 1
Genotype constellation.

Strain		923E		923H		923X	
Gene	Genotype	Closest strain	Identity%	Closest strain	Identity%	Closest strain	Identity%
NSP1	A8	RVA/Pig-wt/HRV/S243-VS/2019/G4G5G11P[6]P[13]	92.45	RVA/Human-tc/VNM/NT0042/2007/G4P[6]	97.77	RVA/Pig-tc/CHN/NMTL/2008/G9P[23]	94.75
NSP2	N1	RVA/Pig-wt/CHN/KY-2022/2022/G9P[23]	98.11	RVA/Pig-wt/CHN/KY-2022/2022/G9P[23]	97.64	RVA/Pig-wt/CHN/CN1P7/2021/G1P[7]	97.17
NSP3	T1	RVA/Human-wt/CHN/R1954/2013/G4P[6]	98.51	RVA/Human-wt/CHN/LL4260/2001/G5P[6]	97.76	RVA/Rabbit-tc/CHN/Z3171/2020/G3P[22]	97.4
NSP4	E1	RVA/Pig-wt/MOZ/MZ-MPT-200/2016/G9P[13]	98.26	RVA/Pig-wt/MOZ/MZ-MPT-200/2016/G9P[13]	98.8	RVA/Human-wt/CHN/R479/2004/G4P[6]	97.73
NSP5	H1	RVA/Pig-wt/CHN/CN127/2021/G12P[7]	98.64	RVA/Yak-tc/CHN/HY-1/2018/G6P[11]	98.8	RVA/Human-wt/IND/RCM-G60/2004/G9P[19]	98.64
VP1	R1	RVA/Pig-tc/CH/TM-a-P20/2018/G9P[23]	97	RVA/Pig-wt/BGD/H14020027/2014/G4P[49]	95.77	RVA/Pig-wt/CHN/SC11/2017/G9P[23]	98.55
VP2	C1	RVA/Pig-wt/CHN/SD-1/2021/G9P[23]	96.75	RVA/Pig-tc/CHN/SCJY-5/2017/G9P[23]	99.18	RVA/Pig-wt/GHA/14/2016/G5P[7]	94.99
VP3	M1	RVA/Panda-tc/CHN/CH-1/2008/G1P[7]	95.87	RVA/Panda-tc/CHN/CH-1/2008/G1P[7]	96.49	RVA/Pig-tc/CHN/NMTL/2008/G9P[23]	94.4
VP4	P[23]	RVA/Pig-wt/CHN/SCYB-C3/2019/G5P[23]	97.59	RVA/Pig-tc/CHN/SCJY-11/2017/G9P[23]	98.03	RVA/Pig-tc/CHN/NMTL/2008/G9P[23]	97.55
VP6	I5	RVA/Pig-wt/VNM/14150_53/2012/G11P[13]	95.91	RVA/Human-wt/CHN/LI3354/2000/G5P[6]	96.53	RVA/Pig-wt/CHN/TA-4-1/2014/G3PX	96.68
VP7	G9	RVA/Pig-wt/CHN/LLP48/2008/G9P[6]	97.17	RVA/Pig-tc/CHN/SCJY-11/2017/G9P[23]	99.18	RVA/Pig-wt/CHN/TM-a/2009/G9P[23]	96.7

strain, and other genes are from porcine strains. So the three isolate were assortment strains among porcine and human as well as other animal strains.

Phylogenetic analysis of the structural and non-structure genes (Figs. 3 and 4) reveals that those three strains are typical porcine RVs with a Wa-like backbone. Except *VP4* gene, other nine gene segments share a higher similarities and belong to the same clade. Of note, *VP6* (Fig. 3E) and *NSP1* (Fig. 4A) gene of the three isolate belong to genotypes I5 and A8, respectively, both of which reveal that the three isolates were typical porcine RVs. All the *VP4* gene of the three strains belongs to P[23] genotype (Fig. 3D). This genotype was further divided into six clades, and strain 923H and 923X clustered with the clade I and strain 923E with the clade V (Fig. 3D).

As shown in Fig. 3A, B, C, E and Fig. 4B, C, D, E, phylogenetic trees of other ten genes reveal that the three strains belong to type I clade and display feature of human Wa strain. These results showed that the three strains are reassorted RVs among porcine, human and other animals, and have a Wa-like backbone.

Cross-immunoreactivity test suggests the cross-protection among different strains

To further compare the breadth of cross-immunoreactivity induced by different lineage strains from lineage III and VI, the serum of each strain was prepared. The same immune dose (10^8 $TCID_{50}$/mL) was inoculated into 40-day old pigs. The serum was isolated after two doses of immunizations with a three-week interval and viral neutralization tests were performed.

As shown in Table 2, the neutralizing antibody (NA) titres of the homologous strain were 1:6400 (923H), 1:12800 (923E), 1:12800 (923X), respectively. Next, the cross-protective immunities against heterologous strains were conducted. The serum of anti-strain 923H could neutralize strain 923E and 923X, and the NA titres were 1:6400. The serum of anti-strain 923E could neutralize strain 923H and 923X, and the NA titres were 1:6400. The sera of anti-strain 923X could neutralize strain 923H and 923E, and the NA titres were 1:3200. To compare the cross-protective breadth of different lineage strain serum, the strain NMTL from lineage VIa was used. As shown in Table 2, the NA titres of the homologous strain and heterologous strains were 1:4096 (NMTL), 1:2048 (923H), 1:2048 (923E), 1:1024 (923X), respectively. To depict the antigenicity distances, the antigenicity map was constructed (Fig. 5). 923H serum was found to be located in the relative center of the antigenic map, and its breadth covered other strains. That suggests that this strain may be a potential broad-spectrum vaccine strain against G9 virus infection. The variance of NA titers among other strains serum was less than 8-fold, suggesting the existing of cross-protection among different lineage strains.

Antigen analysis of VP7 and VP4

Both VP7 and VP4 play an important role in inducing neutralizing antibody. Based on the identification of neutralizing epitopes in VP7, it contains two antigenic epitopes: 7-1 (7-1a and 7-1b) and 7-2 (Reslan et al., 2021). A total of five sites display the variance among the three isolates (Table 3). The cross-neutralizing activity of strain 923X was less

Table 2
Cross-neutralization activity assay.

	Serum				
		anti-923H	anti-923E	anti-923X	anti-NMTL
Isolates	923H (III-d)	6400	6400	3200	2048
	923E (III-d)	6400	12800	3200	2048
	923X (III-b)	6400	6400	12800	1024
	NMTL (VI-a)	3200	3200	1600	4096

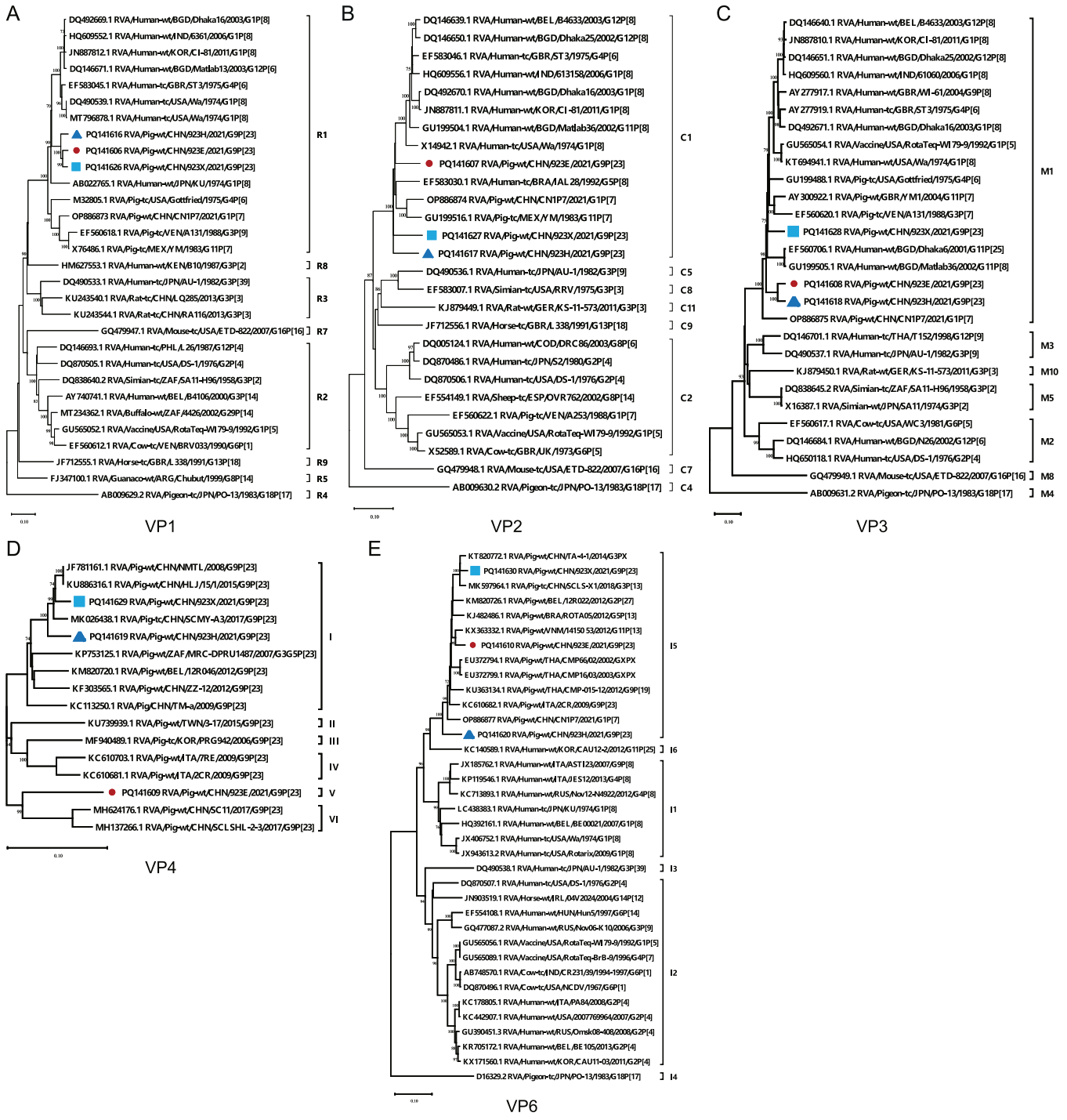

Fig. 3. Phylogenetic analysis of structural proteins. The phylogenetic tree was constructed based on the target gene ORFs (VP1, VP2, VP3, VP4, VP6) identified in this study (color labeled) and those reported in previous studies. Tree reliability was evaluated using the bootstrap method with 1000 replications. Only bootstrap values >70% at branch points are shown. Scale bar at the bottom indicates nucleotide substitutions per site.

than that of other isolates, and these unique sites may account for this. After screening, it was found that aa 87 and aa 96 were consistent with the prediction.

VP4 is divided into the VP8 and VP5, which play vital roles in virus entry process. VP8* consists of four epitopes (8-1 to 8-4), and VP5* consists of five (5-1 to 5-5) epitopes (Aoki et al., 2009; Dormitzer et al., 2002; Dormitzer et al., 2004). A total of six sites display the variance among the three isolates (Table 4). Of these, only position 88 of strain 923X has a unique amino acid residue, which may contribute to its cross-neutralizing activity being less than that of other isolates.

Through our analyzing, it appears that a total of three distinct sites within VP7 (aa 87, aa 96) and VP4 (aa 88) may be responsible for determining the cross-neutralizing activity.

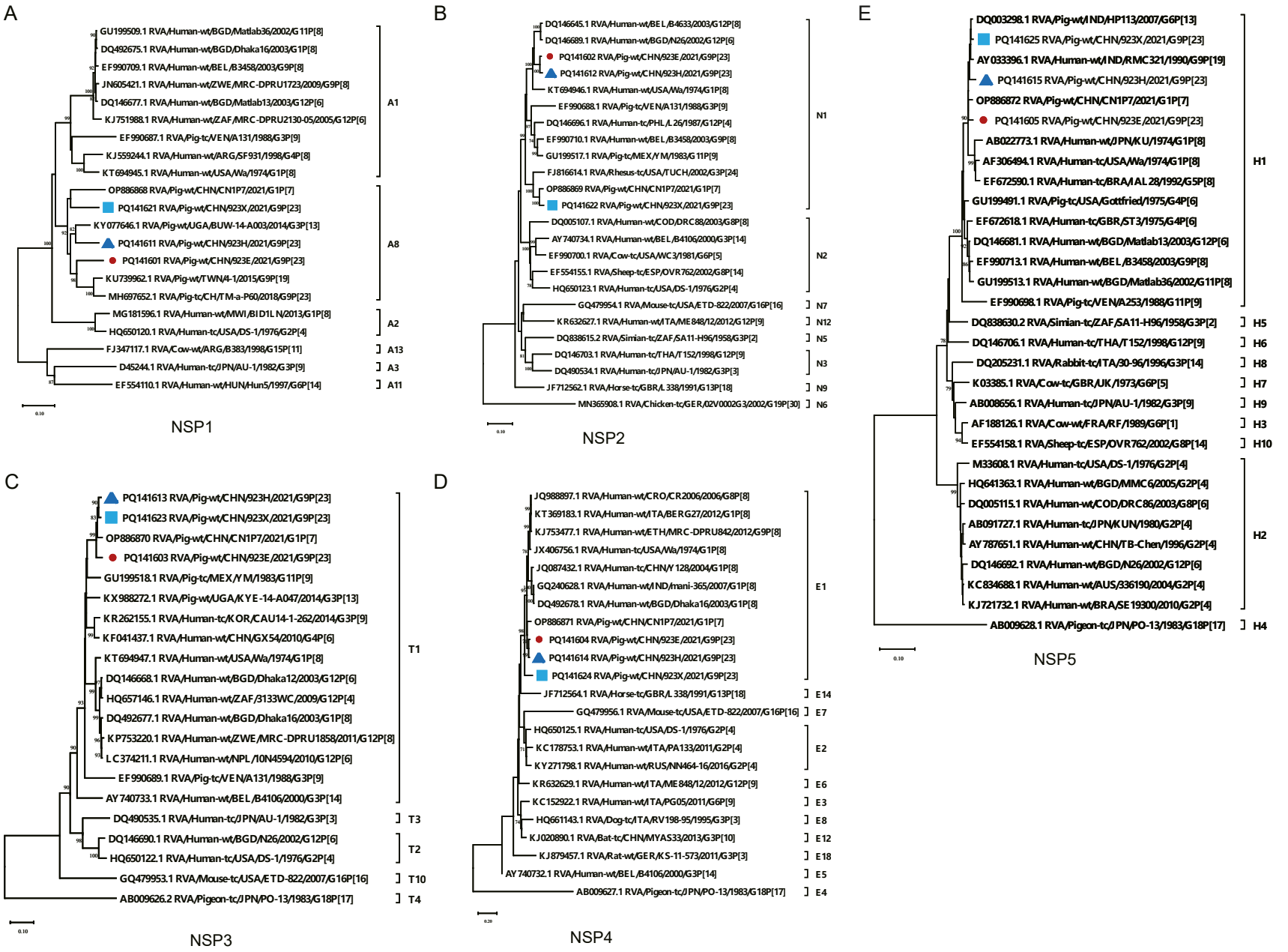

Fig. 4. Phylogenetic analysis of non-structural proteins. The phylogenetic tree was constructed based on the target gene ORFs (NSP1, NSP2, NSP3, NSP4, NSP5) identified in this study (color labeled) and those reported in previous studies. Tree reliability was evaluated using the bootstrap method with 1000 replications. Only bootstrap values >70% at branch points are shown. Scale bar at the bottom indicates nucleotide substitutions per site.

DISCUSSION

Human G9P[8] strain WI61 was first identified in the United states in 1983 (Clark et al., 1987), and the G9 genotype strains subsequently spread globally to become the fifth dominant genotype over the past two decades (Matthijnssens et al., 2010). In China, the human G9 genotype strains were first detected in 2002 (Fang et al., 2002). The prevalence rate (0.9%) of G9 strains was low and the incidence (72.6%) of G1 strains was high at that time (Fang et al., 2002). Now, the human G9 genotype has become the dominant genotype in China (Liu et al., 2022). The first porcine G9 strain was identified in China in 2008 (Shi et al., 2012), and it has also became the dominant genotype in Chinese farms now. Due to the importance of G9 RV in humans and pigs, its epidemiology, cross-interspecies transmission risk and cross-immunoreactivity of different porcine strains were assessed in this study.

The zoonotic feature of RVAs has been reported, showed that different genotype strains detected in humans exhibit highly genetic identity with porcine origin strains. Anupam Mukherjee et al., detected a human porcine-like strain (mcs/13-07) from a 3-year-old child in Eastern India (Mukherjee et al., 2009). Its VP8* was closely related to porcine P[6] strains (P[6] sublineage 1D) and its VP7 was clustered with G9 lineage-III strains. Among the other nine segments, only VP6 and NSP4 exhibited genetic relatedness to Wa-like human subgroup II strains, and VP1-3, NSP1-3 and NSP5 were closely related to porcine strains. Mark Zeller et al., identified another human porcine-like G9P[6] strain BE2001 from a one month old boy (Zeller et al., 2012). Except the P[6] genotype strains often detected in the cross-interspecies infections, the P[19] genotype strains also play an important role in promoting the zoonotic transmission. Arpaporn Yodmeeklin et al., first reported that a non-reassorted porcine rotavirus G9P[19] was detected from a 3-year-old girl with diarrhea (Yodmeeklin et al., 2017). In this study, lineages IIIb and VI were mainly consist of the porcine G9 strains, but some human isolates were also identified, which reveals that both lineages strains may have the zoonotic potential.

For the VP6 and NSP1, genotypes I5 and A8 are commonly found in porcine RVA strains. The three strains display a same genotype constellation: G9-P[23]-I5-R1-C1-M1-A8-N1-T1-E1-H1. However, the BLAST results showed that the three strains are reassorted RVs among the porcine, humans and other animals and have a Wa-like backbone. Due to the close contact between human and porcine, interspecies transmission may contribute the emerging of more and more porcine-human reassorted RVA strains.

Both VP7 and VP4 are the outer layer proteins of virus particle and play an important role in inducing neutralizing antibody. VP7, a calcium-binding glycoprotein, mainly contains six main antigenic domain and forms trimers on the surface of the virion (Lopez and Arias, 2004). Only two unique areas, 7-1 and 7-2 seem to be targeted by neutralizing antibodies (Aoki et al., 2009). Epitope 7-1 located at the three corners of each trimer comprises residues from two adjacent VP7 subunits (7-1a and 7-1b) (Aoki et al., 2009). Binding to the epitope 7-1 by neutralizing antibody can prevent virus uncoating (Aoki et al., 2009). Epitope 7-2 is in the center of each VP7 subunit that is a flexible region. It is less uncovered about the neutralization mechanism by antibodies (Aoki et al., 2009). In the mature virion, VP4 is cleaved into VP5* and VP8* by cellular trypsin. VP8* has four antigenic domain, 8-1, 8-2, 8-3, 8-4. Most neutralizing antibodies induced by the four epitopes block virus attachment. In this study, strains 923H exhibits high cross-neutralizing abilities than other strains. Sequence comparison reveals that only position 130 of VP7 and position 196 of VP8* were different from other strains. We speculate that epitope 7-1a of VP7 and epitope 8-1 affect the cross-protection abilities of different strains.

CONCLUSION

RVAs belong to zoonotic viruses and have the risk of cross-infection between human and animal rotaviruses. As an emerging genotype, the G9 genotype is the most common in humans and pigs. Currently, the combination of G9 and P[23] is considered to be epidemiologically significant. Moreover, pig G9 strains can cross the interspecies barrier to infect humans. This study systematically explores the genetic and immunogenetic characteristics of pig G9 genotype rotavirus in China and finds that strain 923H could serve as a candidate G9 vaccine strain.

MATERIALS AND METHODS

Viruses and cells

Porcine rotavirus strain NMTL belongs to G9P[23] genotype was isolated from stool specimens from a Chinese piglet with acute diarrhea in the 2008 (Shi et al., 2012). Another three G9P[23] strains 923E, 923H, 923X were isolated from stool specimens in the 2021. MA104 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM)

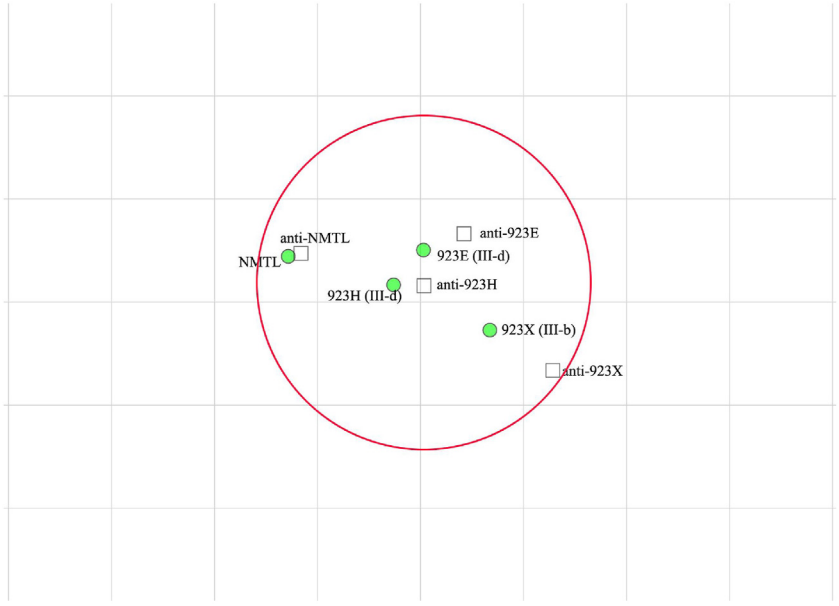

Fig. 5. Protective breadth of serum. The scope of protective efficacy was created with NA titer data. Each square represents a 2-fold difference. Viruses and serum samples are represented by circles and rectangles, respectively. The red circle on the map marks the region where the NA titers fall below the threshold of 8-fold.

Table 3
Alignment of the amino acid residues in VP7 antigenic epitopes.

	7-1a														7-1b						7-2									
	87	91	94	96	97	98	99	100	104	123	125	129	130	291	201	211	212	213	238	242	143	145	146	147	148	190	217	221	264
923E	T	T	G	A	E	W	K	N	Q	D	A	I	D	K	Q	N	A	A	D	N	K	D	S	T	L	S	E	G	G
923H	T	T	G	A	E	W	K	D	Q	D	A	I	N	K	Q	N	A	A	D	N	K	D	S	T	L	S	E	G	G
923X	A	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	N	G
NMTL	T	T	G	T	E	W	K	N	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	N	G

Table 4
Alignment of the amino acid residues in VP4 antigenic epitopes.

	8-1											8-2		8-3									8-4		
VP8	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
923E	D	T	V	G	Y	T	T	N	Y	D	T	E	N	T	S	Q	S	Q	E	N	A	T	S	N	A
923H	D	T	V	G	Y	T	T	N	Y	D	A	E	N	T	S	Q	S	Q	E	N	V	T	S	N	A
923X	D	T	V	G	Y	T	T	N	Y	D	T	E	N	T	S	Q	S	Q	E	N	V	T	S	S	A
NMTL	D	T	V	G	Y	T	T	N	Y	D	T	E	N	T	S	Q	S	Q	E	N	V	T	S	N	A

	5-1								5-2	5-3	5-4	5-5
VP5	384	386	388	393	394	398	440	441	434	459	429	306
923E	N	D	R	A	W	M	A	R	G	N	S	T
923H	N	D	R	A	W	T	A	R	E	K	S	T
923X	N	D	R	A	W	T	A	R	E	K	S	T
NMTL	N	N	R	A	W	T	A	R	E	K	S	T

with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin, and maintained at 37 °C with 5% CO_2.

Sampling

Fecal samples were collected from diarrheal piglets under 30 days old on breeding farms in Heilongjiang, Gansu, Inner Mongolia, Hunan, Fujian, Guangdong, Shandong, Sichuan and Xinjiang provinces. Samples were collected during the winter and spring of each year from 2020 to 2023, as this period is a higher incidence of diarrhea on the farms.

Viral RNA extraction and identification

Each swab sample was added with 500 μL of PBS, then centrifuged at 12,000 ×*g* for 5 min. The supernatant (200 μL) was harvested for extraction of viral RNA using the Viral RNA extraction kit (Axygen, China) following the manufacturer's instructions. One-step method PCR kit (Solomon biotech, China) was used for amplifying the ORF of *VP7* gene. The primers were listed: forward 5′-GGCTTTAAAAGAGA-GAATTTC-3′; Reverse 5′'-GGTCACATCATACARTTCTAACC-3′. The amplified target band in agarose gel electrophoresis was cut for sequencing.

Sequence analysis and phylogenetic trees construction

The *VP7* gene segments were compared by Basic Local Alignment Search Tool (BLAST), and the typical gene sequences from NCBI were selected to construct genetic tree. Genotype constellation analysis was performed on the Bacterial and Viral Bioinformatics Resource Center (https://www.bv-brc.org/).

Based on the previous study, phylogenetic trees of each gene were constructed using MEGA11 software, and the maximum likelihood method was used. New clades would be defined based on the similarities among the target gene and genes from Genbank.

Virus isolation

The swab supernatants were filtered with 0.45 μm, then the filtrate was treated with 10 μg/mL of trypsin without EDTA at 37 °C for 30 min. The inoculum was inoculated into monolayer MA104 cells at 37 °C for 1 h, then discarded and replaced with DMEM medium containing 0.5 μg/mL of trypsin for 4 days. Through serial passage, the cytopathic effect (CPE) could be observed.

Virus identification

For the immunofluorescence assay (IFA), a mouse monoclonal antibody (mAb) (Wu et al., 2024) against the VP7 protein prepared in our laboratory was used. Briefly, MA104 cell monolayers were washed twice with PBS and infected for 24 h. The cells were fixed with prechilled methanol for 20 min at 4 °C. After washing with PBS, cell plate was incubated for 1 h at 37 °C with the VP7 mAb (1:1000 dilution in 0.1% of bovine serum albumin). After washing, the plate was incubated at 37 °C for 1 h with goat anti-mouse IgG labeled with FITC (1:500 dilution, Abcam), and fluorescence was detected under an ultraviolet microscope.

For the polyacrylamide gel electrophoresis (PAGE), it had been described in our previous study (Wu et al., 2024). Briefly, virus dsRNA was extracted, then were used for analysis of dsRNA shifting pattern using PAGE. A total of 20–30 μg of viral RNA was loaded into 10% gels, and TBE Running buffer was used for electrophoresis. After electrophoresis at 4 °C for 10 h, silver staining was performed to make dsRNA visible using a Fast Silver Stain Kit (Beyotime, China), and the gels were scanned on an ImageScanner III (GE HealthCare).

Mono-factor serum preparation

To prepare the vaccine used in this study, ISA 15A adjuvant was added into the inactivated virus of passage 10 (P10) by the formaldehyde. The pigs with 40 days of age were immunized intramuscularly with the prepared vaccine with an interval of three weeks. Whole blood was collected after three immunizations, centrifuged at 2000 ×*g*, 10 min, the serum was aliquoted and stored at −20 °C.

Neutralizing antibody (NA) test

The inactivated serum at 56 °C for 30 min was serially diluted 2-fold with DMEM, and the virus with 10^3 $TCID_{50}$/100 μL pretreated with 10 μg/mL of trypsin was added into the diluted serum. After being incubated at 37 °C for 1 h, the mixture was added into MA104 cells seeded in 96-well plates for 1 h. After washing three times, maintenance medium containing 2 μg/mL of trypsin was added, then the cells were cultured for 3 days at 37 °C with 5% CO_2. The CPE was

observed, and the highest serum dilution inhibiting half of the CPE was considered as the neutralization titre.

Antigenic cartography construction

The antigenicity distances between viruses were evaluated at https://www.antigenic-cartography.org/, as previously described (Zhang et al., 2024). The NA titers suffered from mathematical transformation to generate the antigenic distances table. This transformation was performed with the equation Dij = bj-log$_2$ (Hij), where Hij represents the titer corresponding to antigen i against serum j, b is the log of the peak titer of serum j, and Dij is the target distance between virus i and serum j. To optimize the representation, the error function represented as (Dij − dij) was minimized, and dij indicates the Euclidean distance between two points on the map.

DATA AVAILABILITY

All data relevant to the study are included in the article or uploaded as Supplementary Material. All the sequences have been uploaded in the GenBank with the accession number PQ117775–PQ117786, and in Science Data Bank (https://scidb.cn) under project: doi.org/10.57760/sciencedb.20588.

ETHICS STATEMENT

The animal experimental protocols in this study were approved by the Harbin Veterinary Research Institutional Animal Care Committee (Approval number: 230913-05-GR).

AUTHOR CONTRIBUTIONS

Conceptualization, Li Feng, and Jin Tian; methodology, Yudi Pan, Qian Miao; validation, Hongyan Shi; investigation, Jin Tian, Yudi Pan, and Zixin Li; writing-original draft, Jin Tian, Longjun Guo; writing-review & editing, Jin Tian, Yudi Pan.

CONFLICT OF INTEREST

We declare that we have no conflicts of interest with respect to the research authorship and/or publication of this article.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2025.02.003.

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