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RESEARCH ARTICLE

Genome Characteristics and Evolution of Pseudorabies Virus Strains in Eastern China from 2017 to 2019

Xiaofeng Zhai[1] · Wen Zhao[1] · Kemang Li[1] · Cheng Zhang[1] · Congcong Wang[1] · Shuo Su[1] · Jiyong Zhou[2] · Jing Lei[1] · Gang Xing[2] · Haifeng Sun[1] · Zhiyu Shi[1] · Jinyan Gu[1]

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Abstract

Since late 2011, outbreaks of pseudorabies virus (PRV) have occurred in southern China causing major economic losses to the pig industry. We previously reported that variant PRV forms and recombination in China could be the source of continued epidemics. Here, we analyzed samples from intensive pig farms in eastern China between 2017 and 2019, and sequenced the main glycoproteins (gB, gC, gD, and gE) to study the evolution characteristics of PRV. Based on the *gC* gene, we found that PRV variants belong to clade 2 and detected a founder effect during by the PRV epidemic. In addition, we detected inter- and intra-clade recombination; in particular, inter-clade recombination in the *gB* genes of strains FJ-ZXF and FJ-W2, which were recombinant with clade 1 strains. We also found specific amino-acid changes and positively selected sites, possibly associated with functional changes. This analysis of the emergence of PRV in China illustrates the need for continuous monitoring and the development of vaccines against specific variants of PRV.

Keywords Pseudorabies virus (PRV) · Glycoproteins · Founder effect · Epidemic · Eastern China

Introduction

Pseudorabies virus (PRV) is a herpes virus belonging to the genus *Varicellovirus* (Murphy *et al.* 2000). Pigs are the primary hosts and reservoirs of PRV. PRV causes reproductive disturbances in pregnant sows and lethal disease in newborn piglets. This condition is referred to as Aujeszky's disease, which causes serious economic losses worldwide (He *et al.* 2019a). PRV is a double-stranded DNA virus that encodes more than 70 proteins, including the viral capsids, coats, and envelopes (Pomeranz *et al.* 2005). Among these proteins, glycoprotein B (gB), gD, and gC induce cellular and humoral immune responses (Ober *et al.* 1998, 2000; Ye *et al.* 2015), while gE is the major virulence determinant of pig PRV (Kimman *et al.* 1992; Wang *et al.* 2014). These four genes are commonly used to monitor the evolution of PRV (Sozzi *et al.* 2014; He *et al.* 2019a).

Many countries are currently declared free of PRV infection. In China, effective pseudorabies control was achieved by vaccination with the Bartha-K61 strain with 80% of pig farms adhering to vaccination between the 1990s and late 2011 (Tong and Chen 1999). However, in late 2011, PRV outbreaks occurred in several pig herds immunized with the Bartha-K61 strain. The outbreaks rapidly compromised most pig farms in northern China and resulted in huge economic losses to the swine industry (An *et al.* 2013). The causative agent was confirmed to be novel PRV strains that were genetically different from the classical PRV strains (He *et al.* 2019a). Due to enhanced pathogenicity and genetic differentiation, these strains were considered as newly emerged variants (Hu *et al.* 2015; Sun *et al.* 2016). Further investigation demonstrated that the Bartha-K61 vaccine does not provide full protection against the PRV variants (Hu *et al.* 2015). However, the

Xiaofeng Zhai and Wen Zhao have contributed equally to this work.

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✉ Shuo Su
shuosu@njau.edu.cn

[1] MOE Joint International Research Laboratory of Animal Health and Food Safety, Engineering Laboratory of Animal Immunity of Jiangsu Province, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210000, China

[2] Key Laboratory of Animal Virology of Ministry of Agriculture, Zhejiang University, Hangzhou 310058, China

Chinese vaccine reference strain Ea is genetically closely related to the newly emerged variant. It is suggested that the epidemic variant PRVs of China may have undergone mutations caused by host immune pressure over a long period of time and evolved into a new variant of PRV (Wang *et al.* 2017).

A major impediment to understanding the origin, evolution, and diversity of PRV in China is the lack of PRV sequences. Frequent recombination events have been detected in RNA and some DNA viruses, which explain the maintenance of a high evolutionary rate (Su *et al.* 2016, 2017; Li *et al.* 2018). Thus studying the evolution and recombination occurring in PRV could provide a new perspective on the potential direction of outbreak. To this end, the phylogenetic relationships between the new Chinese strains and the globally emerging and historic PRV strains were analyzed based on the main glycoproteins (gB, gD, gE, and gC) of 27 strains from eastern China obtained from 2017 to 2019.

Materials and Methods

Samples and Virus Isolation

A total of 587 samples were collected from the lungs, lymph nodes, kidney, spleen, and brain of pigs suspected to be infected with PRV from intensive pig farms in eastern China including the Anhui, Fujian, Shandong, and Jiangsu provinces (Fig. 1) between 2017 and 2019 (Table 1). Sample homogenates were prepared by freeze–thaw cycles followed by centrifugation at 5000 $\times g$ for 5 min. The supernatants were then harvested for DNA extraction. Positive samples confirmed by polymerase chain reaction (PCR) were used for virus isolation. For virus isolation, tissues were grinded, filtered using a 0.22-μm membrane, and inoculated into Vero cells. The cells were then incubated until obvious cytopathic effect (CPE) developed. Virus was further plaque purified.

DNA Extraction, PCR, and Sequencing

Virus DNA was extracted using the Virus Genomics DNA Isolation Kit (Tianlong Biotech, Suzhou, China) following the manufacturer's instructions. PRV was detected using a pair of specific PR-D-F/R primers (Supplementary Table S1) (Yue *et al.* 2009). PCR was performed in a 20-μL volume mixture comprising 10 μL of 2 × Taq Master mix (Vazyme Biotech, Shanghai, China), 7 μL of double distilled water (ddH_2O), 1 μL of template DNA, 1 μL each of 10 pmol/μL forward and reverse primers. Thermocycler conditions used for PCR were 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min before storage at 4 °C. The *gB*, *gC*, *gD*, and *gE* genes were also amplified (Supplementary Table S1) using the Phanta Max Super-Fidelity DNA polymerase (Vazyme Biotech, Shanghai, China) (Supplementary Table S1). Positive samples were sent to Tsingke (Nanjing, China) for DNA sequencing. Sequences were assembled using the BioEdit software (Hall 1999).

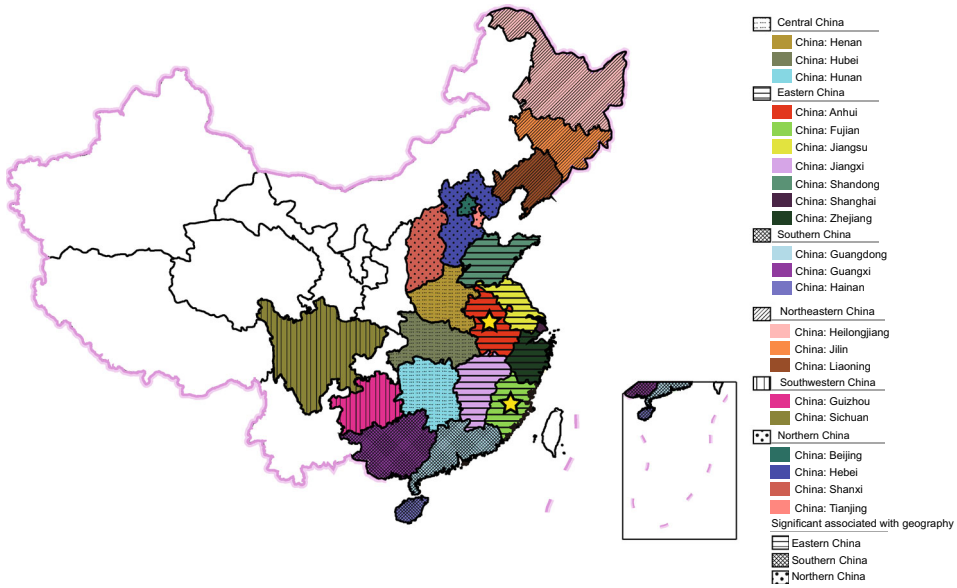

Fig. 1 Map indicating PRV-positive farms in China. Different colors indicate the different provinces in China. The PRV reference sequences used in this study were from these colored provinces. The provinces marked with yellow stars are the provinces with positive samples in this study. Provinces with shadow coverage indicate significant association between virus and geographic location.

Table 1 Sequence information of strains isolated in this study.

Strain name	Origin	Year	Month	No. GenBank			
				gC	gB	gD	gE
ANHUI-1	China: Anhui	2018	Janurary	MK922082	MK922098	MK610394	MK610413
ANHUI-2	China: Anhui	2018	Janurary	MK922083	MK922099	MK610395	MK610414
ANHUI-3	China: Anhui	2018	March	MK922084	MK922100	MK610396	–
ANHUI-4	China: Anhui	2018	April	MK922085	MK922101	MK610397	–
ANHUI-5	China: Anhui	2018	April	MK922086	MK922102	–	–
Anhui-CZ33	China: Anhui	2017	Janurary	MK934521	MK922119	–	MK934522
Anhui-BB11	China: Anhui	2017	Janurary	MK922079	MK922120	–	–
Anhui-ZJ1	China: Anhui	2018	October	–	MK922121	–	–
FJ-1620	China: Fujian	2018	April	–	MK922103	MK610398	MK610415
FJ-2125	China: Fujian	2018	April	–	MK922104	MK610399	MK610416
FJ-5	China: Fujian	2018	May	MK922087	MK922105	MK610400	MK610417
FJ-Z1	China: Fujian	2018	May	MK922088	MK922106	MK610401	MK610418
FJ-Y21	China: Fujian	2018	May	–	MK922107	MK610402	MK610419
FJ-N1	China: Fujian	2018	May	MK922089	MK922108	MK610403	MK610420
FJ-N2	China: Fujian	2018	May	MK922081	MK922109	MK610404	MK610421
FJ-N3	China: Fujian	2018	May	MK922090	MK922110	MK610405	MK610422
FJ-N4	China: Fujian	2018	May	MK922091	MK922111	MK610406	MK610423
FJ-W2	China: Fujian	2018	May	–	MK922112	MK610407	MK610424
FJ-ZXF	China: Fujian	2018	July	MK922080	MK922113	MK610408	MK610425
FJ-YXJSJ1	China: Fujian	2019	Janurary	MK922092	MK922116	MK610409	MK610428
FJ-YXJSJ2	China: Fujian	2019	Janurary	MK922093	MK922117	MK610410	MK610429
FJ-YY	China: Fujian	2019	Janurary	MK922094	MK922118	MK610411	MK610430
FJ-QYQ3	China: Fujian	2019	Janurary	MK922096	–	MK610412	–
FJ-QYQ4	China: Fujian	2019	Janurary	MK922097	–	–	–
FJ-QYQ2	China: Fujian	2019	Janurary	MK922095	–	–	–
FJ-SHS1	China: Fujian	2019	Janurary	–	MK922114	–	MK610426
FJ-GSG5	China: Fujian	2019	Janurary	–	MK922115	–	MK610427

Sequence Alignment and Phylogenetic Analysis

All PRV *gB*, *gC*, *gD*, and *gE* coding sequences from infected swine were collected from NCBI (https://www.ncbi.nlm.nih.gov/) (Supplementary Tables S2–S5), aligned using the MAFFT software (version 7.312) (Kazutaka *et al.* 2005) and manually adjusted using MEGA (version 7) (Kumar *et al.* 2016). The IQ tree software (version 1.6.5) was used to detect the best fit nucleotide substitution model according to the Bayesian information criterion (BIC) score (Lam-Tung *et al.* 2015). Maximum likelihood (ML) trees based on *gB*, *gC*, *gD*, and *gE* coding regions were reconstructed using RAxML (version 8.4.10) using the general time reversible (GTR) plus GAMMA distribution substitution model and 1000 bootstraps (Stamatakis 2014).

Geographical Correlation

To analyze the correlation between each PRV sequence and geographical location after Markov Chain Monte Carlo (MCMC) analysis (Drummond and Rambaut 2007; He *et al.* 2019a), PRV sequences were first classified according to their country of isolation, while isolates from China were classified as eastern, northern, central, southern, and western China. The correlation was determined using the Bayesian Tip-Significance testing software (BaTS) (Parker *et al.* 2008). The parsimony score (PS) and association index (AI) statistics were calculated based on the *gC* gene MCMC analysis. When the *P* values of AI and PS were < 0.05, the correlation between PRV and geographical distribution was considered significant.

Selection Analysis

The *gB, gC, gD,* and *gE* ML trees were uploaded to Datamonkey (http://www.datamonkey.org/) to estimate sites and branched under selection (Delport *et al.* 2010; He *et al.* 2019b). The algorithms single-likelihood ancestor counting (SLAC), fast unconstrained Bayesian approximation (FUBAR), fixed effects likelihood (FEL), and

mixed effects model of evolution (MEME) were used to identify selected sites. Positive selected branches were detected using an adaptive branch-site REL test for episodic diversification (aBSREL) algorithm (Kosakovsky Pond and Frost 2005; Murrell *et al.* 2012, 2013; Smith *et al.* 2015). A site was considered to be under positive selection if at least two algorithms were satisfied ($P < 0.1$ in SLAC, $P < 0.05$ in FEL and MEME, $P > 0.9$ in FUBAR).

Results

Characterization of PRV Strains Circulating in Eastern China

Samples are collected from four provinces in eastern China (Anhui, Fujian, Shandong, and Jiangsu), but PRV positive sample only detected in Anhui and Fujian Province (Table 1). Of 587 samples, 48 were positive for PRV (positive rate of 8.18%). Sequencing analysis of the main PRV glycoproteins *gB*, *gC*, *gD*, and *gE* revealed maximum nucleotide sequence divergences of 1.8%, 0.3%, 0.3%, and 0.6% within the isolates, and 2.6%, 5.6%, 2.0%, and 3.7% compared with the reference isolates from GenBank, respectively. Maximum amino acid sequence divergences were 4.0%, 0.7%, 0.3%, and 1.1% within the isolates for gB, gC, gD, and gE, and 4%, 9.5%, 3.5%, and 6.6% from other GenBank isolates, respectively. In addition, we observed some special amino acid site changes in the gC and gE proteins. For gC, more than half of the strains had a S to G change at position 41, and R162H and S345L changes were observed in four of the twenty strains. Compared with the Bartha vaccine strain, in addition to some specific amino acid mutations, there were some specific insertions from residues 59–65 on gC. A T386M change was observed in 8 gE strains, and 4 strains displayed a L575P change (Fig. 2). However, in case of gB, two strains (FJ-W2 and FJ-ZXF) were similar to the clade 1 strain NIA3.

PRV Phylogenetic Analysis

We reconstructed phylogenetic trees of PRV based on the *gB*, *gD*, *gE*, and *gC* genes using all PRV sequences available from infected pigs in GenBank and the sequences generated here (Figs. 3, 4). Strains sequenced in this study were mainly similar with strains distributed in Guangdong, Guangxi, Henan, Hainan, Shandong, Shanxi, Fujian and Hubei Province. Based on the ML trees, we concluded that four trees had similar structures and PRV could be divided into two main clades, clade 1 and clade 2. Most of the sequences generated here belonged to clade 2, similarly to variant PRV (He *et al.* 2019a). This indicates that variant PRV is epidemic in eastern China. However, we also found that the sequences of different genes clustered with different clades. For example, *gB* of strains FJ-W2 and FJ-ZXF located in clade 1 (containing the Bartha vaccine strain) and was genetically closer to strains from other countries, while *gD*, *gE* (Fig. 3B, 3C) and *gC* (Fig. 4) clustered in clade 2. This is indicative of inter-clade recombination events occurring in the PRV epidemic. In addition, sequences of different strains within clade 2 were grouped in different sub-clades for different genes, suggesting intra-clade recombination during PRV epidemic in eastern China.

Geographical Correlation of PRV Based on the *gC* Gene

Given that *gC* has the highest mutation rate and the largest number of reported sequences (Ye *et al.* 2015) among the glycoprotein-encoding genes, we studied the geographical correlation of PRV based on *gC*. We performed BaTS analysis and found that the *P* value of both AI and PS were < 0.01 (Table 2). Except for the *P* value of Malaysia, Belgium, Greece, United Kingdom, central China, and western China that equaled 1, the other 14 countries and regions showed a *P* value within 0.01. This is indicative of a strong correlation of PRV with geographical location, especially in some areas of China where the epidemic is more severe.

Selection and Amino Acid Function Analysis

Using the FUBAR and MEME methods, we detected positive selection at gB residues 43, 834 and 908, gE residue 348 according to all methods, and two gC residues (75 detected with FEL, SLAC and FUBAR; and 194 detected with SLAC and FUBAR). No positive selection was detected on gD (Supplementary Table S6). As expected, some selected residues have been associated with relevant functions. For example, gB site 43 is near the B-cell epitopes within residues 59 to 126 (Zaripov *et al.* 1999), site 75 on gC is associated with virus adsorption (Karger and Mettenleiter 1993), and site 194 is on the 1/3 N terminal of the gC protein, which is associated with the HS receptor-binding domain (Flynn and Ryan 1995, 1996). In addition, the amino acid sites in which we found changes in relation to the reference strains had also important functions. In particular, change S41G on gC results in a change in the glycosylation site from NSS to NGS.

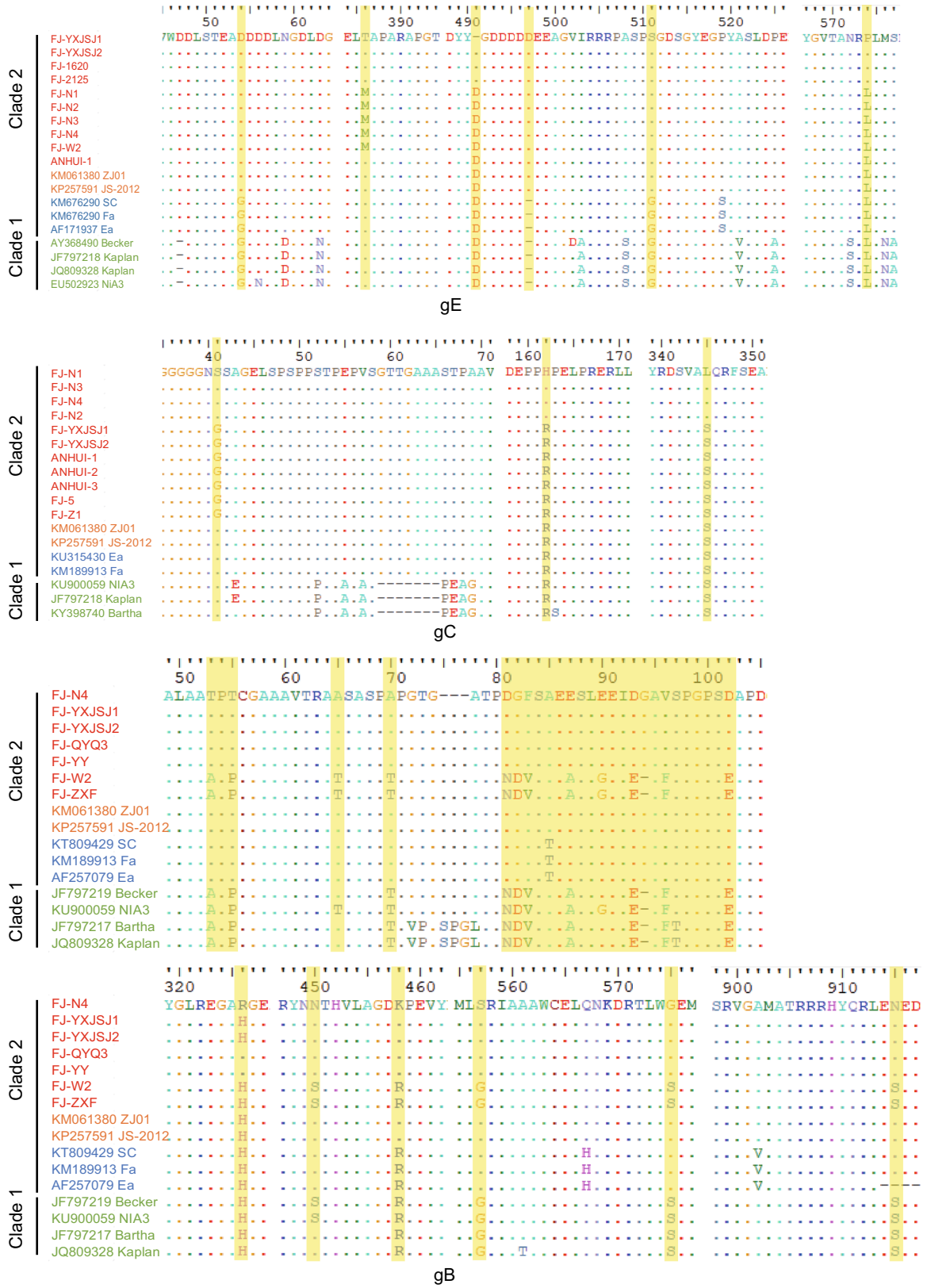

Fig. 2 Sequence alignment and amino acid differences between strains sequenced here and GenBank isolates. The strains sequenced here are indicated in red. Variant PRVs are indicated in orange, early clade 2 strains in blue, and clade 1 strains in green. The yellow bar indicates different site among these sequences.

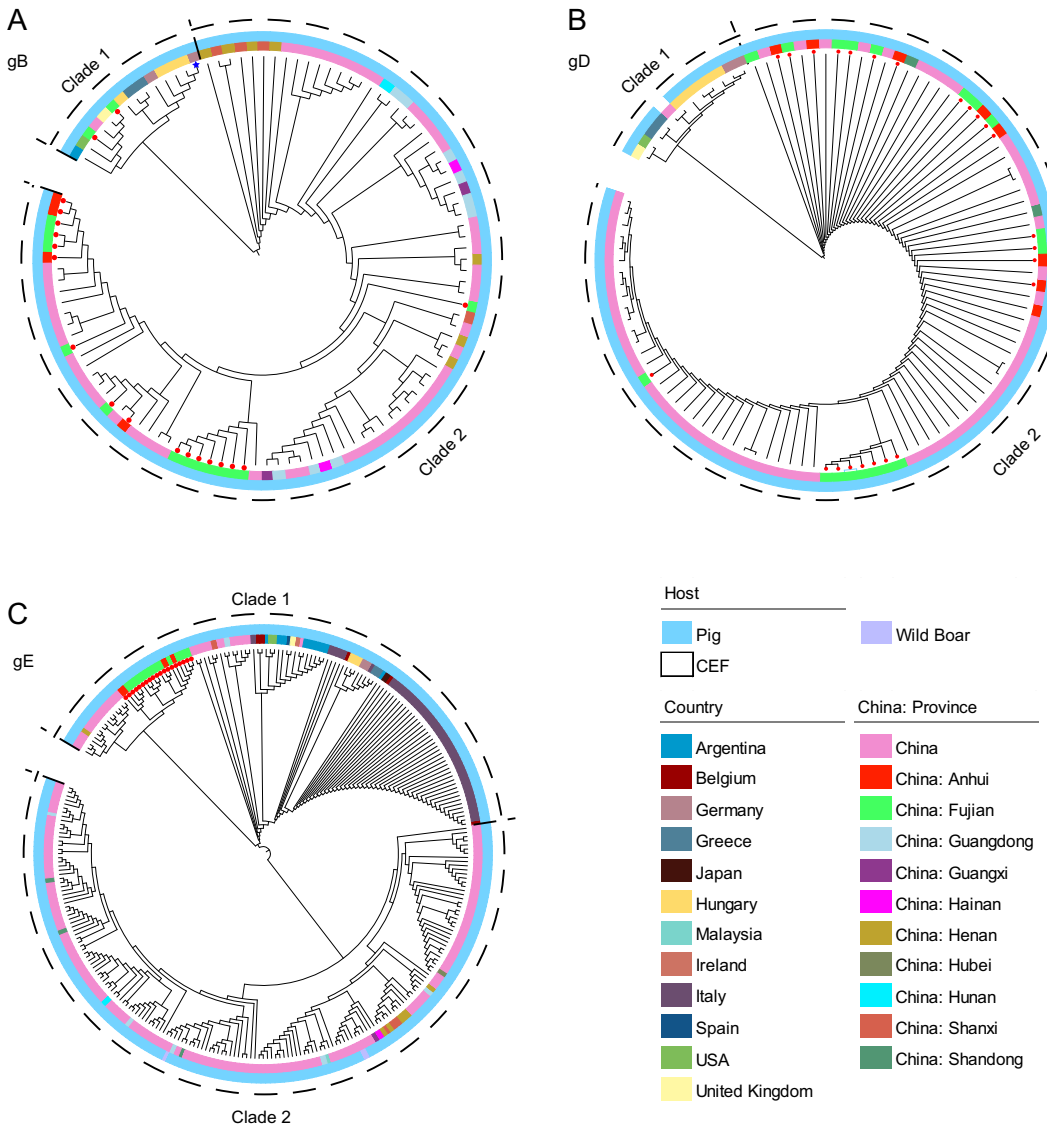

Fig. 3 Maximum likelihood trees reconstructed based on the *gB*, *gD*, and *gE* genes. Trees were reconstructed using RAxML (Version 8.4.10) with the general time reversible (GTR) plus GAMMA distribution substitution model, and 1000 bootstraps. The first layer of red dots indicates sequenced strains from this study. The second layer of colored circles indicates PRV host, and the third circle indicates country and regions of PRV. **A** *gB* gene. **B** *gD* gene. **C** *gE* gene. Red dots indicates PRV sequenced in this study.

Discussion

The Bartha-K61 vaccine was imported from Hungary to China and has been widely used since the late 1980s. Vaccination has been instrumental in controlling PRV for decades. Indeed, < 10% of serum samples were positive for PRV gE antibody between 2005 to 2010 (Tong and Chen 1999; Kong 2000). Since 2010, a PRV variant has emerged quickly in many pig farms that despite having vaccination programs in place, causing substantial losses to the pig industry in China (An *et al.* 2013; Wu *et al.* 2013; Luo *et al.* 2014). According to a recent report, 80.1% of investigated farms from 23 regions of China were PRV-positive (Yang 2015). Furthermore, gE-antibody positive rates increased to 58.2% in farms infected with variant PRV. We previously showed that from 2012 to 2017, the effective reproduction rate (R_e) during each year for the variant PRV was more than 1, indicating a high risk of a variant PRV epidemic and the need for continuous monitoring in China (He *et al.* 2019a).

From 2017 to 2019, some pig farms in the eastern China provinces of Fujian and Anhui suspected the presence of variant PRV, which we confirmed to be PRV by PCR. After virus isolation and sequencing of the main glycoprotein genes *gB, gD, gE*, and *gC*, we reconstructed the ML trees and classified these as variant PRVs (clade 2). Importantly, phylogenetic analysis of *gD*, *gE*, and *gC*

Fig. 4 Maximum likelihood trees reconstructed based on the *gC* gene. The tree was reconstructed using RAxML (Version 8.4.10) with the general time reversible (GTR) plus GAMMA distribution substitution model, and 1000 bootstraps. Colored lines indicate host and country of PRV. Red dots indicates PRV sequenced in this study.

revealed that all of the strains belong to clade 2. As for the *gB* gene, there were two strains (FJ-W2 and FJ-ZXF) similar to clade 1 strains, suggesting that some of these variant PRVs originated via recombination with clade 1. Although the recombination sites are different, similar recombination events have been reported in previous studies, suggesting recombination between wild strains and foreign epidemic strains (similar to the vaccine strains clade) in China (Ye *et al.* 2016; He *et al.* 2019a). Moreover, we also detected intra-clade recombination in clade 2. Recombination may result in changes of antigenicity, virulence, and thus immune failure (He *et al.* 2019a).

We found a strong association between PRV and geographical location, indicating a founder effect along the PRV epidemic. Interestingly, the phylogeographic associations we observed were similar to those observed in previous reports, indicating that the PRV strains in China may have evolved independently, leading to the emergence of a variant strain (Wang *et al.* 2017). A founder effect is the loss of genetic variation that occurs when a new population is established by a very small number of individuals from a larger population. As a result of the loss of genetic variation, the new population may be distinctively different, both genotypically and phenotypically, from the parent population from which it is derived (Templeton 1980; Provine 2004). Therefore, the immune failure and observed specific amino acid mutations in critical epitopes suggest that different regions of China may need to develop different vaccines, formulate corresponding local policies, and indicate compensation strategies to control the prevalence of PRV.

In addition, we also found some important amino acid differences and positively selected sites between the

Table 2 Geographical correlation analysis of PRV based on gC.

Statistic		Observed mean	Lower 95% CI	Upper 95% CI	Nulsl mean	Lower 95% CI	Upper 95% CI	Significance
AI		13.08	11.37	14.72	28.24	27.39	28.93	**0.00**
PS		102.28	98.00	107.00	201.63	198.27	204.85	**0.00**
MC	Eastern China	11.06	11.00	12.00	1.74	1.48	2.09	**0.01**
MC	USA	2.03	2.00	2.00	1.06	1.00	1.15	**0.01**
MC	Brazil	3.52	2.00	6.00	1.37	1.14	1.94	**0.01**
MC	Hungary	2.14	2.00	3.00	1.04	1.00	1.14	**0.01**
MC	China	6.65	3.00	10.00	2.44	2.21	3.02	**0.01**
MC	Malaysia	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MC	Spain	4.26	2.00	7.00	1.06	1.00	1.17	**0.01**
MC	Germany	3.06	2.00	5.00	1.17	1.03	1.40	**0.01**
MC	Slovakia	2.07	2.00	3.00	1.02	1.00	1.06	**0.02**
MC	Italy	7.55	4.00	13.00	2.13	1.85	2.71	**0.01**
MC	Argentina	2.90	1.00	5.00	1.09	1.00	1.31	**0.01**
MC	Austria	1.80	1.00	2.00	1.01	1.00	1.03	**0.01**
MC	Croatia	5.47	3.00	7.00	1.05	1.00	1.13	**0.01**
MC	Belgium	1.47	1.00	3.00	1.09	1.00	1.29	1.00
MC	Central China	1.45	1.00	2.00	1.20	1.06	1.39	1.00
MC	North China	1.52	1.00	2.00	1.00	1.00	1.01	**0.01**
MC	Western China	1.01	1.00	1.00	1.00	1.00	1.01	1.00
MC	Greece	1.37	1.00	2.00	1.00	1.00	1.01	1.00
MC	South China	3.03	3.00	4.00	1.56	1.32	2.02	**0.01**
MC	United Kingdom	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Bold number remind the difference in geographic associations significantly.

AI association index, *PS* parsimony score, *MC* monophyletic clade.

isolates sequenced here and reference sequences. Although some of the amino acid changes identified in this study were not positively selected, probably due to the limited number of sequences, some conclusions could be noted. In particular, S41G, R162H, and S345L changes on gC could alter receptor-binding and/or glycosylation that likely contributed to the emergence of variant PRV.

In general, we conclude that the outbreak of PRV in eastern China may be due to immune failure, caused by the emergence of variant PRV via recombination and specific amino acid mutations and insertions in gC protein epitope compared with the Bartha vaccine strain. In addition, a founder effect also promoted the epidemic spread of PRV. Therefore, the development of new vaccines and novel monitoring strategies are necessary.

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Author Contributions XZ, WZ and SS designed the experiments. XZ, KL, CZ, CW and WZ carried out the experiments. CZ and XZ analyzed the data. JZ, JL, GX, HS, ZS and JG reviewed drafts of the paper, XZ, WZ and SS wrote the paper. SS checked and finalized the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal and Human Rights Statement This article does not contain any studies with human participants or animals performed by any of the authors.

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