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Growth characteristics and complete genomic sequence analysis of a novel pseudorabies virus in China

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Abstract Swine pseudorabies (PR) re-emerged in Bartha-vaccinated pig herds and caused death of millions of piglets in China since the later part of 2011. We isolated a novel pseudorabies virus (PRV), named HNX strain, from the brain of abortion fetuses to diagnose the disease. To reveal the genomic organization and characterize the HNX strain, the complete genomes of HNX and Fa strain, an isolate in the 1960s, were sequenced and analyzed. The genomic size of HNX and Fa strains were 142,294 and 141,930 nt, respectively, with corresponding G + C contents of 73.56 and 73.70 %. The two strains consistently possessed 70 open reading frames. In addition, comparative genomic analysis between HNX and Bartha strains was performed to understand the possible reason of immune failure. The major virulence-associated genes of HNX strain had slight changes, whereas glycoprotein B and glycoprotein C genes of HNX strain had 73 mutations; the homology at the whole genomic level between HNX and Bartha strains was 90.6 %. Genome-wide comparison between HNX and Fa strains indicated that the strains shared about 96.4 % of homology and clustered in a separate Chinese isolate group; the two strains are also distant from the isolates from other countries. Similarity plot and bootscanning analysis of complete genome sequences of nine PRV strains, including HNX and Fa, four newly Chinese strains, and three traditional reference strains, revealed that non-recombination events occurred in the HNX strain. The PRV HNX strain with genomic variations might contribute to the PR outbreak in China since the later part of 2011.

Keywords Pseudorabies virus · Phylogenetic analysis · Bartha strain · HNX strain · Genomic organization

Introduction

Pseudorabies virus (PRV), a member of family *Herpesviridae*, subfamily *Alphaherpesvirinae*, and genus *Varicellovirus*, is a double-stranded DNA virus with a genome of approximately 140 kb [1–3]. PRV causes the known Aujeszky's disease or pseudorabies (PR) in pigs [4–6]; PR is characterized by sow's reproductive failure and high morbidity in piglets that develop into a nervous system disease. Pseudorabies also causes severe economic losses to the pig industry in many countries [7]. To prevent and control PR, the attenuated Bartha-based vaccine has been widely used in China since the 1970s [8]. PR is well controlled in China through carrying out an eradication program [9, 10]. However, since the later part of 2011, PR broke out in more than 20 provinces in mainland China. Approximately 35 % abortion rate of pregnant sows and 24.5 % morbidity in piglets with neurologic and respiratory

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Nucleotide sequence accession numbers: The complete genomic nucleotide sequences of the PRV HNX and Fa strains were deposited in GenBank under accession numbers KM189912 and KM189913, respectively.

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problem in Bartha-vaccinated pig farms were observed [11]. The emergency of PR led to great economic losses to China's pig industry [8, 11–13]. Thus, clinical questions should be clarified; these questions include whether the pathogen is a new virus, what are its genomic features, and if any difference exists between the new and vaccine viruses at genomic level.

In the present study, we isolated the PRV HNX strain from affected farms. The whole genome of HNX was sequenced by Illumina paired-end sequencing for the molecular characterizations of HNX. The Fa strain isolated in China in the 1960s was also sequenced using the same strategy. Comparative genomic analyses between HNX, Bartha, Fa, and reference strains were performed to understand the causes of immune failure and evolution.

Materials and methods

Virus identification

The variant PRV HNX was isolated from the brain of abortion fetuses from Bartha-vaccinated pig farms in Henan province, China in our previous study [14]. Briefly, the homologized sample was freeze-thawed three times, centrifuged at 10000 rpm for 10 min, filtrated through 0.22 μm filter (Millipore, Milford, MA), and inoculated to the monolayer of PK-15 cells (ATCC CCL33). The cells were examined daily for cytopathic effect (CPE). Inoculated cells [0.1 multiplicity of infection, (MOI)] were fixed with cold absolute ethanol for 1 h, blocked with 1 % bovine serum albumin (Sigma, St. Louis, MO) for 30 min, and incubated with 200 times diluted mouse monoclonal antibody against gD of PRV (VMRD, Pullman, WA) for 1 h, followed by a 60 dilution of goat anti-mouse IgG-FITC-conjugated secondary antibody (SouthernBiotech, Birmingham, AL) for 1 h. Cell staining was examined under a fluorescence microscope. Virus titer was determined by measuring the 50 % tissue culture infective dose ($TCID_{50}$).

Growth kinetics

The growth kinetics of PRV HNX and Fa strains (both are the ninth passage) were determined by one-step growth curve as described previously [15]. PK-15 cells were inoculated with PRV HNX or Fa strain at 0.1 MOI in 24-well culture plates (Corning, USA). Cells and supernatants were harvested at 4, 8, 12, 16, 20, 24, 28, 32, 36, 42, and 48 h following the removal of viral inoculum. Virus titers in different time points were determined by measuring $TCID_{50}$ on PK-15 cells.

Genomic DNA extraction

Pseudorabies virus HNX and Fa strains (both are the ninth passage) were grown in the PK-15 cells and harvested at 24 h post-inoculation when typical CPE appeared. After three freeze-thaw cycles, the infected cells were centrifuged at 13,500 *g* for 10 min to discard the cell debris. The viruses in the supernatant were further purified using 20–60 % discontinuous sucrose density gradient ultracentrifugation at 141,000 *g* for 2 h. Subsequently, the purified virus particles in the second opalescent band (within 30–45 % sucrose density) were collected and diluted twofold with phosphate saline. Virus particles were examined under a transmission electron microscope (TEM) (Hitachi H-7650, Japan). The genomic DNA of HNX and Fa strains was extracted using DNA extraction kit (QIAGEN, USA) according to the manufacturer's instructions.

Complete genome sequencing

Five micrograms of viral genomic DNA was subjected to sequencing. The sequences of the complete genome of HNX and Fa strains were determined through next-generation sequencing (NGS) technology using Illumina paired-end sequencing in the Sangon Biotech (Shanghai) Co., Ltd. The total reads of each strain were compared with the Sus scrofa genome to remove the host sequences using Blast software (NCBI) and assembly was performed using Velvet 1.2.08 software to join the short reads into longer contigs. The final contigs and gaps of each PRV were determined using GapCloser software, and the sequences obtained via NGS technique were mapped to complete the genome of PRV strain (Accession No. NC_006151).

The spanned sequences were identified, and the remaining gaps were amplified with primers, which were designed on the basis of the assembly sequences and reference genome near gaps. The gaps were amplified with LA Taq polymerase (with GC buffer, TaKaRa), followed by gel-based purification, and subsequently cloned to pMD-18T vector for sequencing. The initial and terminal sequences of PRV were amplified using a pair of primers IT-F and IT-R and LA Taq polymerase with the GC Buffer I. The terminal repeat region was amplified using a pair of primers TR-F and TR-R and LA Taq polymerase with GC Buffer II (Table 1). The nucleotide sequencing was carried out using ABI3730 Genetic Analyzer automated sequencer (Applied Biosystems, USA). The glycoprotein B (gB), gC, gD, gE, TK, RR1, and RR2 gene sequences of PRV HNX and Fa strains were further confirmed by Sanger method (Table 1).

Homology and phylogenetic analyses

To determine the potential variations in virulence and immunogenicity of HNX strain, phylogenetic trees based

Table 1 Primers used for PCR amplification of the genome of PRV HNX and Fa strains

Target sequences	Primers (5′–3′)	Annealing temperature and GC buffer (°C)
Initial and terminal sequences	P1F: TGCGATATGCAGATGAGATCCGTG	60 (Buffer I)
	P1R: TGCCAATTCTCAGTGAGTGCCCAT	
Terminal repeat region sequences	P2F: TCTCGCTCTGGCACCCGATGACA	60 (Buffer II)
	P2R: TGTTCTTCCTCCACCCCCACCGT	
gB gene	P3F: TGTACCTGACCTACGAGGCGTCATGC	60 (Buffer II)
	P3R: TATTTCCATCTGCGGGGAGGGGGCTA	
gC gene	P4F: GGATCCATGGCCTCGCTCGCGCGTGCGAT	56 (Buffer II)
	P4R: GAATTCTCACAGCGCGGACCGGCGGTAGT	
gD gene	P5F: GGATCCATGCTGCTCGCAGCGCTATT	56 (Buffer II)
	P5R: GAATTCCTACGGACCGGGCTGCGCTT	
gE gene	P6F: TTGAGACCATGCGGCCCTTTCTGCT	60 (Buffer II)
	P6R: GACCGGTTCTCCCGGTATTTAAGCG	
TK gene	P7F: TAATGCGCATCCTCCGGATCTACCT	58 (Buffer II)
	P7R: TATCACACCCCCATCTCCGACGTGA	
RR1 gene	P8F: GAATTCATGGCCTCCGTCGTCGCGCCCGC	65 (Buffer I)
	P9R: AAGCTTTCACAGGTGGCAGCTCGTGCAGAC	
RR2 gene	P10F: GAATTCATGGAGTACTTTTACACGT	56 (Buffer I)
	P10R: AAGCTTCTACAGGTCGTTCACGAC	

on virulence-related and immunogenicity genes of Barth and Fa strains were constructed via the neighbor-joining method, with 1,000 bootstrap replicates, implemented by MEGA 5.05 software package. The selected immunogenicity and virulence-related genes of these PRVs were genes encoding gB, gC, gD, gE, gI, TK, RR, PK, AN, and dUTPase. The sequence alignment was performed with MegAlign (Version 5.01).

The comparative genomic analysis of HNX, Fa, four newly isolated PRVs, and three non-Chinese strains was performed. The reference strains included three non-Chinese PRV strains, including Bartha (JF797217), Kaplan (JF797218), and Becker (JF797219), and four recent Chinese PRV strains, including TJ (KJ789182), ZJ01 (KM061380), HeN1 (KP098534), and JS-2012 (KP257591). Similarity plots and bootscanning analysis via sliding window were performed as implemented in the SimPlot, v. 3.5.1 package [16].

Results

Virus characterization

Positive brain tissue homogenate of abortion fetuses was confirmed by PCR and inoculated onto PK-15 cells (Fig. 1a). A distinct CPE was observed from passage three after 24 h post-inoculation (Fig. 1b, c). Virus growth was identified by immunofluorescence assay (IFA) (Fig. 1d, e). Virus-like spherical particles with envelope (approximately 150 nm in diameter) were visualized by negative-staining TEM (Fig. 1f). During the first 10 serial passages, the infectious titers of HNX strain ranged within $10^{6.0}$–$10^{7.4}$ $TCID_{50}$/mL. The growth kinetics showed no significant difference in the replication kinetics of PRV HNX and Fa strains. However, the virus titer of HNX strain was similar to that of Fa strain during the first 20 h post-infection but lower at the remaining time points (Fig. 2).

Genomic organizations of PRV HNX and Fa strains

Over 28 million Illumina sequence reads were combined for PRV HNX and Fa strains, from which more than 20 million high-quality sequence reads were produced. The raw data were processed by Velvet and GapCloser software to produce high-quality viral sequence data used for de novo assembly process. Assembly quality was checked by BLAST-based alignment of each genome of PRV HNX and Fa strains versus the reference genome (NC_006151). More than 91 % genome sequences of HNX and Fa strains and 55 gaps (33 and 22 in HNX and Fa, respectively) were identified. These gaps, initial and terminal sequences, and terminal repeat region sequences of PRV HNX and Fa strains were further amplified by PCR for sequencing analysis. The gB, gC, gD, gE, TK, RR1, and RR2 gene sequences of PRV HNX and Fa strains were further validated (Table 1). The genome sizes of PRV HNX and Fa strains were 142,294 and 141,930 nt, respectively;

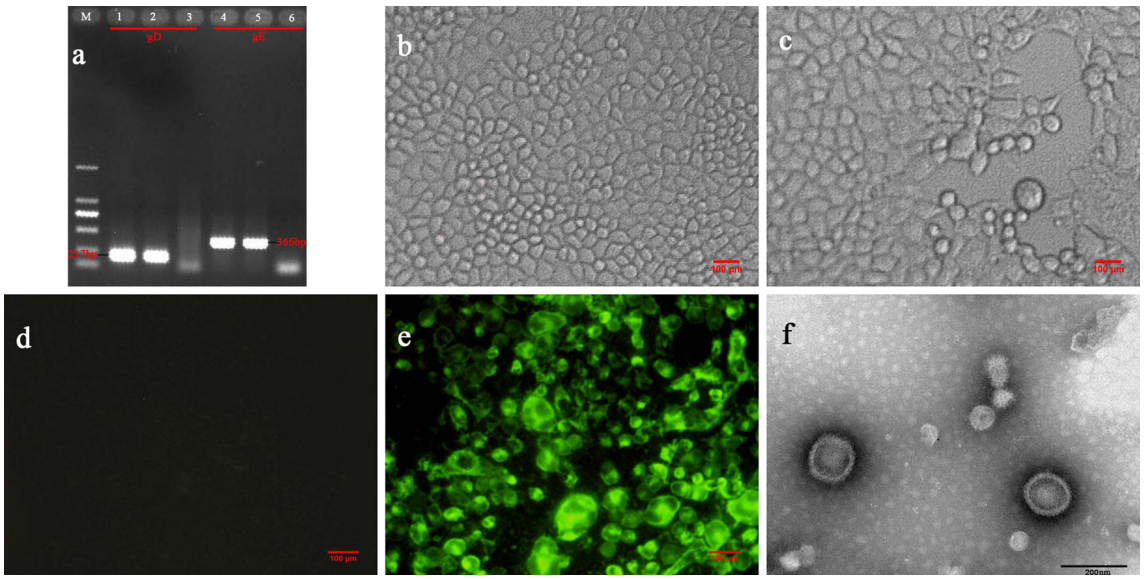


Fig. 1 Identification of pseudorabies virus (PRV) HNX strain. **a** PCR assay for PRV detection from the brain tissue of abortion fetuses. *Lanes 1 and 4*, amplicon using PRV gD and gE specific primers, respectively; *lanes 2 and 5*, positive control; *lanes 3 and 6*, negative control; lane M, DL2000 Marker. **b–e** Cytopathic effects (CPEs) and immunofluorescence assays (IFAs) of PRV HNX isolates in PK-15 cells. PK-15 cells (**b**) and CPEs (**c**) were recorded (*scale bars* 100 μm). Non-infected cells (**d**) and cells infected with PRV HNX strain [Multiplicity of infection (MOI) = 0.1] (**e**) were examined by IFA (*scale bars* 100 μm). **f** Transmission electron microscope (TEM) of PRV HNX strain with a scale of 200 nm

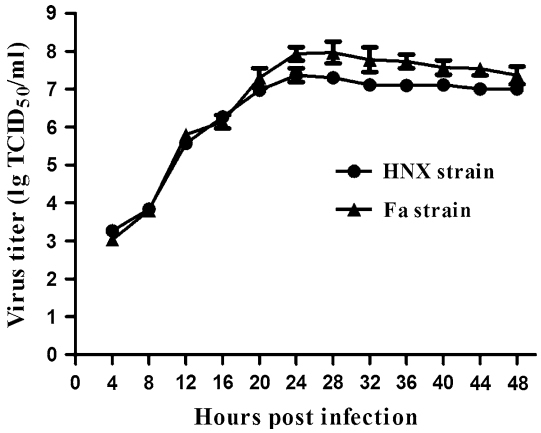


Fig. 2 One-step growth curves of the PRV HNX and Fa strains. PK-15 cells were infected at 0.1 MOI with the PRV HNX or Fa strain. The cells and supernatants were harvested at 4, 8, 12, 16, 20, 24, 28, 32, 36, 42, and 48 h, and virus titers were determined by measuring the $TCID_{50}$ on PK-15 cells

correspondingly, their G + C contents were 73.56 and 73.70 %. The genomic organizations of PRV HNX and Fa strains were similar to those of other alphaherpesviruses, which consist of two unique regions (UL and US), with the US region flanked by the internal and terminal repeat sequences (IR_S and TR_S, respectively) (Table 2). Overall, complete genome sequences were largely conserved between PRV HNX and Fa strains, whereas the main foci of divergence generally occurred in IR_S/TR_S and non-coding regions. Seventy open reading frames (ORF, included two copies of IE180, US1 genes, and major and minor forms of US3 gene) were identified. The gB, gC, gD, gE, TK, and RR1 gene sequences of PRV HNX and Fa strains determined by the Sanger method were remarkably consistent with those by NGS method.

Sequence comparisons

The complete genome sequences of the two PRV strains (HNX and Fa) examined in this study were compared to those of four Chinese strains isolated after 2012 (TJ, ZJ01, HeN1, and JS-2012) and three non-Chinese PRV strains (Bartha, Kaplan, and Becker). PRV HNX shared 90.1, 96.4, 97.2, 94.3, 96.9, and 95.9 % homology with Bartha, Fa, TJ, ZJ01, HeN1, and JS-2012 strains, respectively (Table 3). Notably, the major divergence was observed in non-coding regions at the whole-genome level.

When compared with the Bartha strain, HNX genome contained 74 singular insertions in 29 genes, 37 singular deletions in 19 genes, and 72 both singular insertions and deletions in 12 genes. In particular, 32, 41, and 12 mutations were observed in the gB, gC, and gD genes in the HNX genome, respectively (Fig. 3a; Table S1). The homology between HNX and Bartha strain in virulence and immune-associated genes was 97.3 and 94.5 % at

Table 2 Genome organization of PRV HNX and Fa strains

Strain	Genome sequence (nt)	G + C content (%)	UL region		IR region		US region		TR region	
			Location[a]	Length (nt)	Location	Length,(nt)	Location	Length,(nt)	Location	Length,(nt)
HNX	142,294	73.56	1–100,831	100,831	100,832–117,125	16,294	117,126–126,000	8875	126,001–142,294	16,294
Fa	141,930	73.70	1–101,024	101,024	101,025–117,053	16,059	117,054–125,901	8848	125,902–141,930	16,059

[a] Nucleotide sequence coordinates are given relative to the genome sequence of PRV HNX and Fa, respectively

nucleotide level, respectively, which resulted in 96.2 and 91.4 % identity at amino acid level.

A total of 29 genes in HNX genome were different from those of Fa strain (Fig. 3b; Table S2). The major variations occurred in the genes UL36, UL15, UL5, UL1, IE180, US1, and US8, which are responsible for capsid transport, DNA packaging, DNA replication, transcriptional regulator, and gene regulation. In addition, HNX had three insertions in IE180 and gE genes and 105 deletions in VP22, VP1/2, pUL15, gL, IE180, ICP22, and gD genes. A total of 17 variations also existed in the gB, gC, gD, and gE genes between HNX and Fa strains, However, no changes were found in gI, TK, RR1, RR2, PK, AN, and dUTPase (Fig. 3b; Table S2).

Phylogenetic analysis

Both singular genes and genome-wide phylogenetic analysis demonstrated that all tested PRV strains were clustered into group 1 and group 2 (G1 and G2) as in the previous study [17]. HNX, together with Fa, TJ, ZJ01, HeN1, and JS-2012, was clustered into G1; three non-Chinese PRV strains were clustered into G2. PRV HNX, TJ, and HeN1 strains were clustered within the same subgroup, which was a little distinct from Fa, JS-2012, and ZJ01 strains (Fig. 4). On the basis of gB, gC, gD, gE, gI, and PK genes, HNX, TJ, ZJ01, HeN1, JS-2012, and previous strains (Ea and Fa) were clustered into different subgroups; on the basis of the TK, AN, dUTPase, RR1, and RR2 genes, HNX and Fa strains were clustered into the same subgroup (Fig. 5).

In addition, similarity plots and bootscanning analysis of individual UL, IR/TR, and US region, as well as the complete genome sequences, showed that no recombination events occurred in the nine PRV strains tested (Fig. 6).

Discussions

The re-emergence of PR in both vaccinated and non-vaccinated herds in China raised many scientific concerns to scientists and farmers. These concerns include the degree of contribution of the pathogen to the outbreak and the cause of immune failure in herds. In the present study, we isolated and identified PRV HNX strain from a Bartha-vaccinated herd. The isolate was regarded a novel PRV with insertions and mutations through the sequencing analysis of gE, TK, gB, gC, and RR genes. To unveil the genomic characterization of HNX strain fully, the whole genome was sequenced using the NGS technology; comparative analysis with Bartha and Fa strain was performed.

More than 70 % G + C content was observed, and thus, numerous short sequence repeats and single-nucleotide

Table 3 Nucleotide identities of nine PRV strains with complete genome sequences available

Strain	Nucleotide identity (%)							
	Fa	TJ	ZJ01	HeN1	JS-2012	Bartha	Kaplan	Becker
HNX	96.4	97.2	94.3	96.9	95.9	90.1	92.1	91.2
Fa		96.2	94.9	95.6	95.2	90.7	92.6	91.7
TJ			94.5	95.7	95.9	90.0	92.0	91.3
ZJ01				94.3	94.0	90.3	92.7	92.3
HeN1					95.3	90.2	92.7	91.6
JS-2012						90.4	92.5	91.9
Bartha							95.3	93.7
Kaplan								96.4

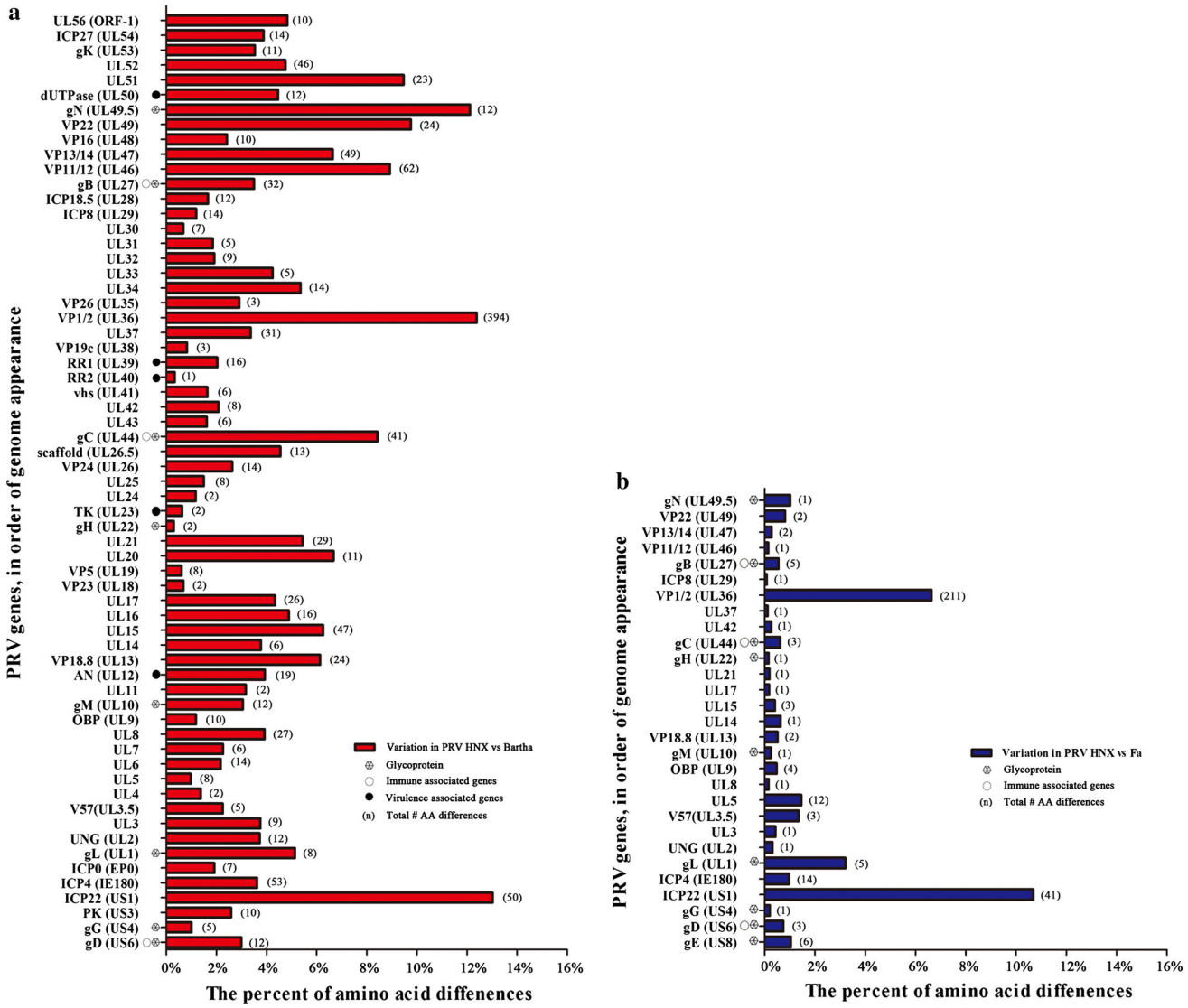


Fig. 3 Protein-coding variation in PRV HNX strain versus Bartha and Fa strains. The bars show the percent of amino acid differences in PRV HNX strain versus the Bartha (**a**) and Fa (**b**) strains. Protein names are listed on the left, and proteins without variation are not listed. The four proteins (US7, US8, US9, and US2) affected by the deletion in Bartha's US region are absent

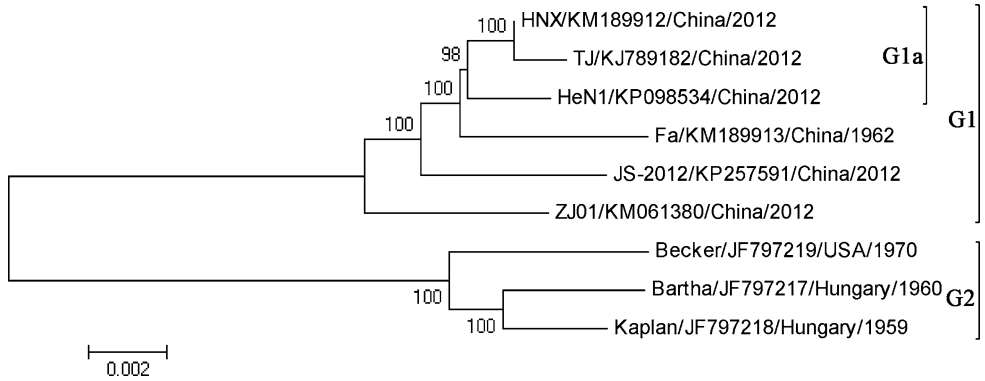
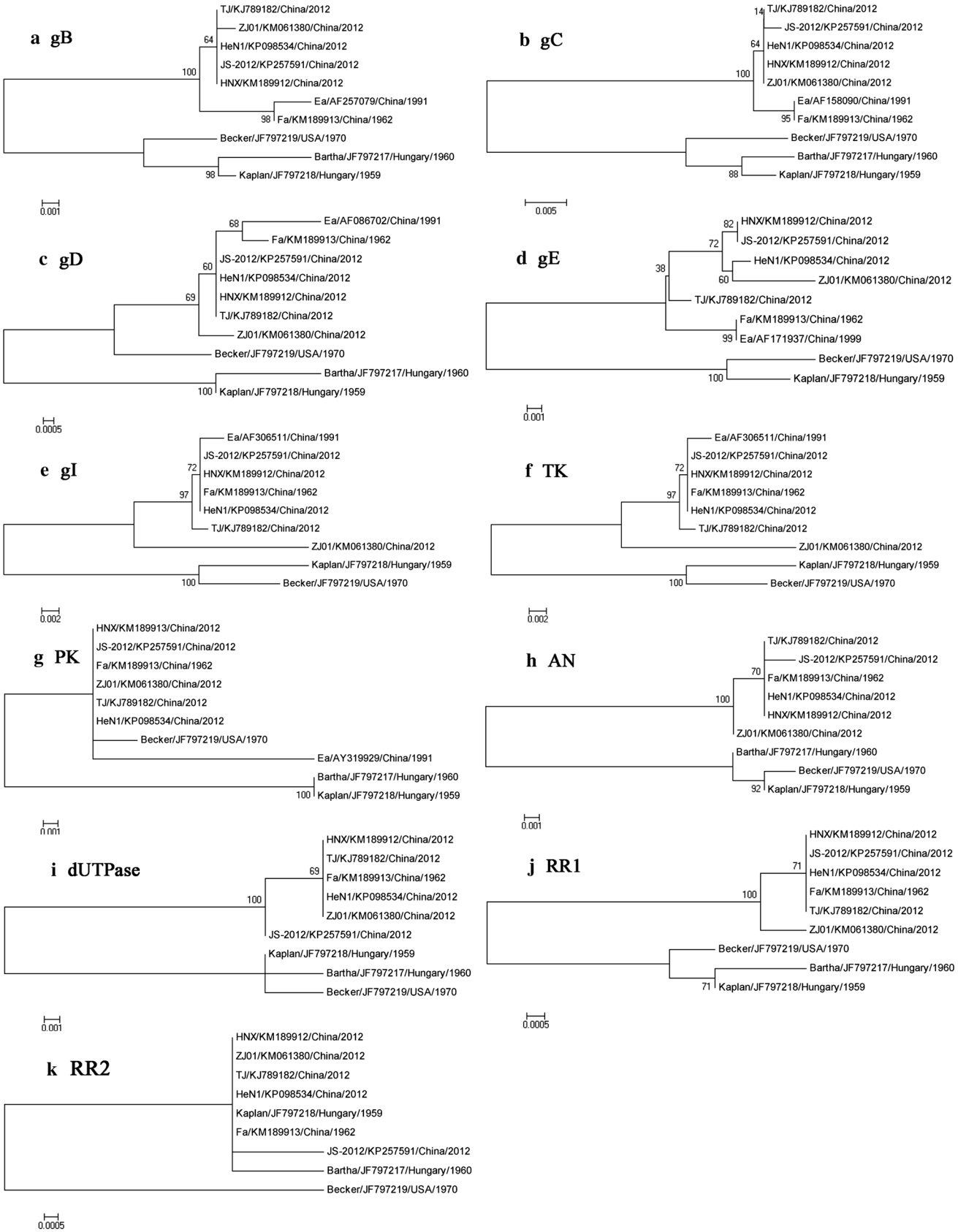


Fig. 4 Phylogenetic analysis based on the complete genome sequences of nine PRV strains. PRV from this study [HNX (KM189912) and Fa (KM189913)] and seven previously published PRV genome sequences [Bartha (JF797217), Kaplan (JF797218), Becker (JF797219), TJ (KJ789182), ZJ01 (KM061380), HeN1 (KP098534), and JS-2012 (KP257591)]. The tree was constructed with MEGA software package (v5.05) using the distance-based neighbor-joining method, with bootstrap values calculated for 1000 replicates

repeats existed in the PRV genome; moreover, 91 % genome sequences and 33 gaps in HNX genome were identified after the primary sequencing. For the amplification and sequencing of the remaining 9 % of genome, the fragments located at 100 nt downstream and upstream of gap junctions were selected as the target gene to design the primers. During PRV replication, the linear viral DNA genome is assumed to be in a circular form [7]; the initial and terminal fragments were amplified using the primer pair of P1F and P1R. Finally, the complete genomes of PRV HNX and Fa strains were sequenced. The genome sizes of HNX and Fa strains were 142,294 and 141,930 nt, respectively; nevertheless, both consisted of 70 ORFs, thereby demonstrating the diversity in genome sizes but the conservation in the ORF organization in PRV strains. The variations were present in 29 gene sequences, and 330 amino acids changes were found; among which, UL36, US1, IE180, and UL5 were the most variable. Recently, non-coding regions, including IR_S/TR_S, were found to induce beta-interferon promoter activity and regulate viral mRNAs in herpes simplex virus type 1 [18, 19]. We found that the main foci of divergence occurred in IR_S/TR_S and non-coding regions in genomes of all nine PRV strains, and their biological functions are worthy of further exploration.

Many factors, including host immunosuppression and vaccine antigen, contribute to immune failure. The variations in immunogenicity-related genes between the vaccine and field strains may be the partial reason. Glycoprotein B (gB), gD, and gC are involved in the immune-protection provided by PRV vaccine. The homology of gB and gD between HNX and Bartha strains was 96.50 and 97.01 %, respectively. In addition, comparative genomic analysis between HNX and Bartha strains revealed 1060 mutations, 80 deletions, and 221 insertions in the HNX genome. Also, the absence of gE in Bartha may lead to relatively weaker protection against PRV field strain, including HNX strain. Furthermore, only 92 % homology existing between HNX and Bartha strains at the genomic level may provide a clue for further research on other mechanism for weaker protection via the Bartha-based vaccine. In our neutralization test, lower neutralization activity against HNX (lower than 1:8) [14] than against Fa strain was observed when using the antiserum prepared through immunized pigs with Bartha strain (higher than 1:16); this result further supported the conclusion from the comparative genomic analysis. In our latest study, the PRV HNX–TK^-/gE^- strain-vaccinated mice or piglets were provided complete protection against challenge with PRV HNX or Ea strain [14]. Similar conclusions were also documented by other researchers [20–22]. These results indicated that the candidate TK^-/gE^- vaccine modified from the current prevalent PRV could be a better option to control the present PR in China.

Recombination between viruses (PRRSV) [23, 24], natural evolution through selection pressure (MDV, FMDV, AIV) [25–27], and predominance of quasispecies [28] are common mechanisms for the emergence of new viruses. The genetic evolution of Marek's disease virus, also a herpesvirus, increases the virus virulence, hence resulting in a very virulent strain. Interestingly, recombination between different PRV strains was previously reported, although the mechanisms are unclear [29, 30]. Fa and Ea strains were isolated in 1962 and 1994, respectively, and regarded as local reference strains. To elucidate the possibility, the comparative genomic analysis of HNX and Fa strains first revealed about 96.4 % homology. The phylogenetic analysis showed that the homologies between HNX and Ea, TJ, ZJ01, HeN1, and JS-2012 strains ranged within from 94.3 to 97.5 % and clustered into the same group and distinct from Bartha, Becker, and Kaplan strains.


a gB
TJ/KJ789182/China/2012
ZJ01/KM061380/China/2012
HeN1/KP098534/China/2012
JS-2012/KP257591/China/2012
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Ea/AF257079/China/1991
Fa/KM189913/China/1962
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TJ/KJ789182/China/2012
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JS-2012/KP257591/China/2012
Kaplan/JF797218/Hungary/1959
Bartha/JF797217/Hungary/1960
Becker/JF797219/USA/1970
69
100
0.001
j RR1
HNX/KM189912/China/2012
JS-2012/KP257591/China/2012
HeN1/KP098534/China/2012
Fa/KM189913/China/1962
TJ/KJ789182/China/2012
ZJ01/KM061380/China/2012
Becker/JF797219/USA/1970
Bartha/JF797217/Hungary/1960
Kaplan/JF797218/Hungary/1959
71
100
71
0.0005
k RR2
HNX/KM189912/China/2012
ZJ01/KM061380/China/2012
TJ/KJ789182/China/2012
HeN1/KP098534/China/2012
Kaplan/JF797218/Hungary/1959
Fa/KM189913/China/1962
JS-2012/KP257591/China/2012
Bartha/JF797217/Hungary/1960
Becker/JF797219/USA/1970
0.0005

◄**Fig. 5** Phylogenetic trees based on amino acid sequence of gB (**a**), gC (**b**), gD (**c**), gE (**d**), gI (**e**), TK (**f**), PK (**g**), AN (**h**), dUTPase (**i**), RR1 (**j**), and RR2 (**k**) among the HNX, Fa, Ea, TJ, ZJ01, HeN1, JS-2012, Bartha, Becker, and Kaplan strains

However, in this study, similarity plot and bootscanning analysis on the complete genomic sequences of all tested PRV strains indicated no recombination events in the HNX and other new isolates (TJ, ZJ01, HeN1, and JS-2012). This observation indicated that HNX was neither derived from the recombination between Bartha and field strains nor among field viruses. Therefore, the new isolate might result from natural evolution under pressure.

In conclusion, this is the first comparative genomic analysis of the novel PRV HNX strain and previous Fa strain, which is a previous PRV strain in China. The

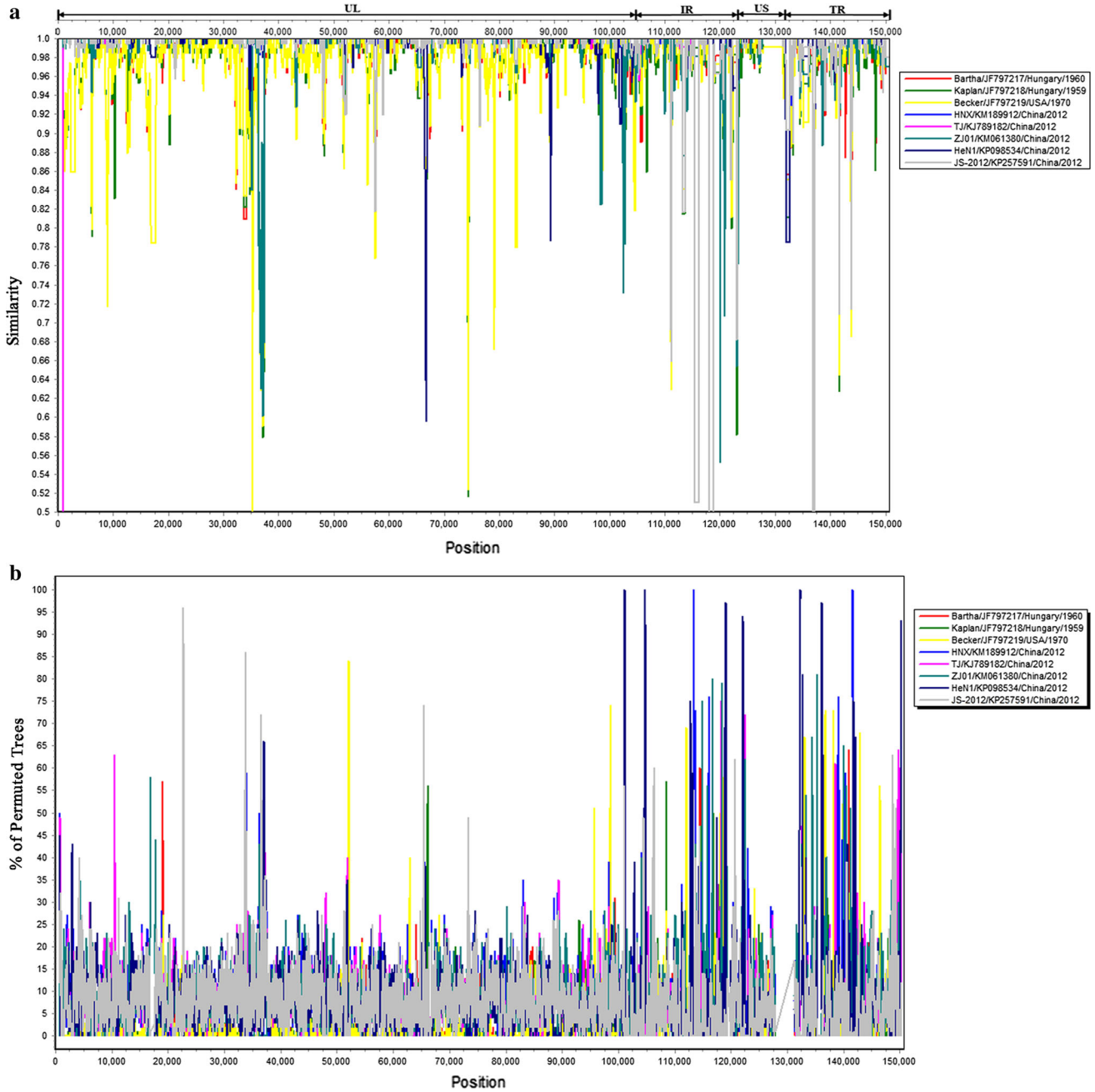

Fig. 6 Similarity plot (**a**) and bootscanning analysis (**b**) of the complete genome of Fa strain with other eight PRV strains. The complete genome of strain Fa/KM189913/China/1962 serves as a query sequence. Each point represents the similarity between the query sequence and a given heterologous sequence, with a 200 nt window moving in 20 nt steps. Positions containing gaps were excluded from the analysis

Chinese PRV strains were clustered into the same groups, which were different from those of non-Chinese PRV strains. The mutations in immune-associated genes may contribute to the immune escape of HNX from Bartha vaccination. The novel PRV strain might result from natural evolution, not from recombination events.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest in the present study and report.

Ethical approval The present study and the protocols used in this study were approved by the Committee on the Ethics of Animal Experiments at the College of Veterinary Medicine, Huazhong Agricultural University and the Guide for the Care and Use of Laboratory Animals Monitoring Committee of Hubei Province in China. The authors declare their compliance to publication ethics.

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