# Molecular epidemiology and biological characteristics of swine pseudorabies virus in Henan province of China during 2012 to 2019

Huihua Zheng[1], Yi-Lin Bai[1], Yu Zhang[1], Yu Zhao[1], Lan-Lan Zheng[1], Fang Liu[1], and Hongying Chen[1]

[1]Affiliation not available

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**Abstract**

Since late 2011, pseudorabies virus (PRV; Suid herpesvirus 1) infection was widely prevalent in vaccinated swine farms in China, and caused tremendous economic losses in the swine industry. To understand the epidemic and biological characteristics of the virus, a total of 1,174 tissue samples were collected from Bartha-K61-immunized swine farms in Henan province of China between 2012 and 2019, and PRV strains were isolated and the complete sequences of gE and gC genes were amplified by PCR. The detection rate of PRV was 15.25% (179/1174), which varied from 6.61% to 25.00% between 2012 and 2019. And 16 PRV isolates were obtained, and could cause clinical symptoms and death in mice. The phylogenetic trees based on the sequences of gE and gC genes showed that the 16 PRV strains in this study at these two phylogenetic trees all clustered to a relatively independent branch altogether with the Chinese variant PRV strains (after 2012), and sequence analysis of the isolates revealed that gE and gC both contained amino acid insertions, substitutions or deletions compared with European-American PRV strains and early Chinese PRV strains (before 2012). In addition, it was the first report that eight strains (8/16) in this study harbored a unique amino acid substitution at site 280 (F to L) of gC gene. In the protection assay, the emulsion containing inactivated PRV NY isolate could provide complete protection against variant NY, and the titer of neutralizing antibodies was 1:82. This study might enrich our understanding of the evolution of variant PRVs as well as pave the way for finding a model virus to develop a novel vaccine based on PRV variants.

## 1. Introduction

Pseudorabies (PR) disease, or Aujeszky's disease, caused by pseudorabies virus (PRV, a member of the family *Herpesviridae* , subfamily*Alphaherpescirinae* , and genus *Varicellocirus* ), is one of the most important viral diseases of pigs and results in gravely economic harm to swine industry in many countries (Pomeranz et al., 2005, Muller et al., 2011). Infected pigs display a range of symptoms, including the neurological disorders and high mortality in newborn piglets, severe respiratory illness in adult pigs and reproductive failure in sows (Gu et al., 2018). The first description of PR was made in America as early as 1813, and the first recorded case of China was in 1948, with following epidemic in pigs in the late 1980s (Ketusing et al., 2014). Due to the use of the gE-negative vaccine strain Bartha-K61 imported from Hungary, PR was well controlled from the 1990s to 2010 (Tong and Chen, 1999, An et al., 2013). Since late 2011, PR outbreaks have occurred in Bartha-K61-immunize pig herds on many Chinese farms and led to huge economic losses (An et al., 2013).

The genome of PRV contains 72 genes that encode 70 different proteins (Klupp et al., 2004). Among these proteins, glycoprotein B (gB) and glycoprotein C (gC) induce cellular and humoral immune responses (Ober et al., 1998, Ober et al., 2000, Wang et al., 2017). Furthermore, the gC is frequently used to analyze the evolutionary relationships of PRV, and the attachment of virus to cells is initiated by the binding of gC to heparan sulfate (HS) proteoglycans (Fonseca et al., 2012, Wang et al., 2015, Ye et al., 2015). The glycoprotein E (gE) is a major virulence determinant of PRV, but is not essential for virus replication (Kimman et al., 1992). In light of this fact, gE-deleted vaccines (Bartha-K61 vaccine) were developed, and the vaccines

together with a corresponding serological test to detect antibodies against gE protein have played a key role in a program for the elimination of PR.

Some researches indicated that the causative agent of currently circulating PRV was confirmed to be novel PRV strains which were genetically different from the early Chinese PRV strains, and the Bartha-K61 vaccine did not provide full protection against the PRV variants due to enhanced pathogenicity and genetic differentiation of PRV variants (He et al., 2019, Wang et al., 2019). Furthermore, PRV variants have been spread widely in China, and the PRV-positive rate was various in different years, regions and seasons (Gu et al., 2018, Sun et al., 2018, Zhai et al., 2019, Ma et al., 2020). Therefore, the present study aimed to investigate the molecular epidemiology and biological characteristics of PRV currently circulating in Henan province of China from 2012 to 2019.

## 2 Materials and methods

### 2.1 Cells and sample collection

Swine testicle (ST) cells were purchased from the China Institute of Veterinary Drug Control, Beijing, China, and passaged in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco).

A total of 1,174 tissue samples (including lungs, brains, kidneys, lymph nodes and spleens) were collected from Bartha-K61-immunized swine farms in the East (including in Kaifeng, Shangqiu and Zhoukou cities), West (including in Luoyang, Sanmenxia cities), South (including in Nanyang, Zhumadian, Xinyang cities), North (including in Anyang, Xinxiang, Jiaozuo, Puyang and Hebi cites) and Middle (including in Zhengzhou, Pingdingshan, Xuchang and Luohe cities) of Henan province, China, during 2012-2019 (Fig.1).

### 2.2 PCR detection

Viral DNA was extracted from 200 μL supernatants using DNA Miniprep Kit (Omega, Norcross, Georgia, USA) according to the manufacturer's instructions, and used for PRV detection by polymerase chain reaction (PCR). A pair of primers gEp-L/R (gEp-L: 5'-TGGGACACGTTCGACCTGATG-3', gEp-R: 5'-CCTT GATGACCGTGACGTACT-3') were designed to amplify partial gE gene. PCR was performed in a 25 μL volume mixture consisting of 12.5 μL Premix *Taq* (Takara, Dalian, China), 3 μL the extracted DNA template, 7.5 μL of dd$H_2O$, 1 μL DMSO and 0.5 μL each of primer (50 μM), and the cycling protocol was an initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min, with a final step of 72 °C for 10 min. PCR product was visualized by electrophoresis in a 1.5 % agarose gel containing ethidium bromide under ultraviolet light.

### 2.3 Virus isolation

For virus isolation, homogenate supernatants of positive tissue samples were filtered using a 0.22 μm filter (EMD Millipore, Billerica, MA, USA), and inoculated into ST cells. After cytopathic effect (CPE) appeared, cell cultures were collected and further used to 3 cycles of plaque purification, and confirmed by PCR with primers gEp-F/R. Subsequently, plaque fluid was inoculated into ST cells and cultured to $7^{th}$ generation. The 50% tissue culture infectious dose ($TCID_{50}$) were determined for PRV isolates, and calculated by Spearman-Karber method.

### 2.4 Experimental infection of mice

170 six-week-old healthy BALB/c female mice were randomly divided into seventeen groups of 10. Mice in PRV-injected groups were inoculated subcutaneously (s.c.) with 500 μL different isolates respectively. Mice in DMED-injected group were inoculated with 500 μL DMEM as control. After the inoculation, the mice were examined daily for clinical signs for 7 days.

### 2.5 Identification of PRV isolate

Physicochemical properties of NY isolate as a representative PRV strain were assayed as described previously (Binn et al., 1970). Briefly, the NY isolate was added into eight eppendorf tubes. Tubes 1-2 were treated

by water bath at 56 for 60 min and chloroform at 4 for 30 min, respectively. Tubes 3-4 were treated by adjusting the culture medium pH to 3.0 and 11.0 with 0.1 M HCl/NaOH solutions, and after incubation at 37 for 1 h, and the pH values were then adjusted back to 7.0. Tube 5 was digested with trypsin at 37 water bath for 1.5 h, and then added 4 mL of the inactivated fetal bovine serum to terminate the reaction. Tube 6 was incubated with formaldehyde at 37 for 2 d. Tube 7 was treated under ultraviolet ray for 30 min. Tube 8 was used as a negative control. Viruses of the eight tubes were inoculated on ST monolayers respectively and $TCID_{50}$ were determined. In addition, the $7^{th}$ passage of NY isolate was stained with uranyl acetate and examined using a Hitachi TEM transmission electron microscope (Hitachi, Japan).

### 2.6 Sequencing and phylogenetic analysis of gE and gC genes

The complete gE and gC genes of PRV were amplified from viral DNA extracted from PRV isolates using two specific primers gE-F/R and gC-F/R designed in our laboratory (Zhao et al., 2020). Then the genes were ligated with the vector pMD18-T (Takara), and separately introduced to*Escherichia coli* DH-5a cells (Takara). The positive recombinant plasmids carrying gE and gC genes were sent to Sangon Biotech Shanghai Co., Ltd for DNA sequencing and all sequencing reactions were performed in duplicate.

Phylogenetic trees were constructed based on the gE and gC gene sequences of PRV isolates and reference strains in GenBank using MEGA software, version 7.0 (www.megasoftware.net) by the neighbor-joining method with 1,000 bootstrap replicates (Kumar et al., 2016). Evolutionary distances were computed by the pairwise distance method with the maximum composite likelihood model. Sequences of PRV strains listed in Table 1 retrieved from NCBI were used as references.

### 2.8 Immunogenicity of PRV isolate

As the representative PRV isolate, NY isolate was inoculated into ST cells. The cell culture suspension was inactivated by incubating with moderate formalin (Sigma-Aldrich) at 37 for 24 h, and then emulsified with Freund's complete adjuvant and Freund's incomplete adjuvant, respectively. 60 six-week-old healthy BALB/c female mice were randomly divided into four groups of 15 mice each. Mice in Group 1 were injected with 0.5 mL of NY isolate containing Freund's complete adjuvant in $1^{th}$ week, followed by injecting with equal volume of emulsifier with Freund's incomplete adjuvant in $3^{th}$week, and finally with the inactivated virus in $5^{th}$week. Groups 2-4 were injected with 0.5 mL the Bartha-K61, Hubei 98 and DMEM in $1^{th}$, $3^{th}$ and $5^{th}$ respectively. At the $32^{th}$ day after the final injection, blood samples were collected from 5 mice of each group for serum neutralization assay, and the remaining mice of each group were challenged with PRV NY isolate. Serum neutralizing antibodies against PRV were detected as described previously (Zheng et al., 2020).

## 3 Results and discussion

A total of 1,174 samples, 179 samples were positive for the PRV gE detection, yielding an average positive rate of 15.25% (179/1174). The positive rates of PRV detection from 2012 to 2019 were 17.08% (41/240), 20.41% (30/147), 25.00% (45/180), 13.69% (23/168), 13.68% (13/95), 9.26% (10/108), 7.83% (9/115) and 6.61% (8/121), respectively, with the peak at 2014, showing that the positive rate gradually decreased after 2014, which might be related to the development and use of several new vaccines based on the epidemic strains and the Chinese government proposed the eradication program based on PR in 2012 (Wang et al., 2014, Hu et al., 2015). As for seasons, the overall PRV-positivity rates over the 2012-2019 study period were 14.02% (46/328) in the spring (March, April and May), 9.83% (17/173) in summer (June, July and August), 16.04% (60/374) in autumn (September, October and November) and 18.73% (56/299) in winter (December, January and February), respectively. The PRV-positive rate of summer was lower than that of the winter and autumn every year, and the positive rate was even 0% (0/21) in summer in 2019 (Fig.2A). The winter and autumn were the seasons with higher PRV-positive rate, suggesting that the morbidity of PR was relatively high in cold season, it might be interrelated with the epidemic characteristics of the disease. The result was consistent with the Sun's study that winter, spring and autumn were the seasons with the high positive rate in mainland China between 2012 and 2017 (Sun et al., 2018).

Henan province of China is divided into five parts including Eastern Henan, Western Henan, Southern Henan, Northern Henan and Middle Henan (Fig.1). Among regions, the PRV-positive rates for eight-year-total were 15.25% (43/282), 13.11% (24/183), 17.62% (40/227), 16.84% (50/297) and 12.09% (22/182) in Eastern Henan, Western Henan, Southern Henan, Northern Henan and Middle Henan respectively (Fig.2B). The highest PRV-positive rate of 40% was observed in Middle Henan in 2014. The positive rates of PRV detection during 2012 and 2019 were 15.38% (6/39), 28.13% (9/32), 17.54% (10/57), 16.98% (9/53), 20.00% (5/25), 12.50% (3/24), 13.33% (4/30) and 10.81% (4/37) in Southern Henan, respectively, which were higher than that of other regions, except for 2012 and 2014. Western Henan or Northern Henan was the region with the lowest PRV-positive rate among 2012 to 2019 (except for 2016), with the positive rates of 0% observed in 2018 for Northern Henan and 2019 for Western Henan. In addition, the detection rate of PRV in different regions between different years displayed diversity. For instance, the detection rates of PRV in Northern Henan during 2013 to 2016 were averaged approximately 15.00% (3/20), and 9.09% (3/33) in 2012, and 8.70% (2/23) in 2017, and 4.35% (1/23) in 2019, but zero in 2018; in Western Henan, the positivity rates in 2012 and 2014 were higher than 20.00%, but the positive rates in 2013, 2015, 2016, 2017 and 2018 were lower than 10.00%, even zero in 2019; in Middle Henan, the positivity rates in 2012, 2013, 2015, 2016 and 2017 were ranging from 11.11% (2/18) to 21.43% (6/28), and approximately 40.00% in 2014, but 8.00% (2/25) in 2018, but 9.52% (2/21) in 2019; in Eastern Henan, the positive rates in 2012, 2013 and 2014 were higher than 18.00%, and 10.00% (2/20) in 2018, but those of 2015, 2016, 2017 and 2019 were lower than 10.00%. These data demonstrated that PR remains in Henan province, China, which is coincidence with the reports of high prevalence of novel PR in China (An et al., 2013, Wu et al., 2013, Sun et al., 2018, Tian et al., 2020). The PRV-positive rate might be related to the different feeding and management methods, biological safety measures and geographical location.

Hence, PRV strains were isolated, and a distinct CPE was observed after three passages of virus on ST cells, which was characterized by cell rounding, pyknosis, and degeneration of the cell monolayer (Fig. 3A and Fig.3B). The gE gene in the infected cells was detected by PCR using the primers gEp-F/R, and the result of electrophoresis showed that the product was identical to the predicted DNA fragment size (429 bp, Fig.3C). Thus, these PRV isolates were formally named as NY, GY, LGX, MZ1, MZ2, ZM, ZK, JY, M5, YY, WY, BP, YZ, SMX, WZ and XC (Table 1). $TCID_{50}$ of these PRV isolates were NY $10^{9.0}$/0.1 ml, GY $10^{6.1}$/0.1 ml, LGX $10^{6.0}$/0.1 ml, MZ1 $10^{5.6}$/0.1 ml, MZ2 $10^{5.3}$/0.1 ml, ZM $10^{5.8}$/0.1 ml, ZK $10^{6.0}$/0.1 ml, JY $10^{7.4}$/0.1 ml, M5 $10^{6.0}$/0.1 ml, YY $10^{6.0}$/0.1 ml, WY $10^{6.0}$/0.1 ml, BP $10^{5.0}$/0.1 ml, YZ $10^{8.0}$/0.1 ml, SMX $10^{6.0}$/0.1 ml, WZ $10^{7}$/0.1 ml and XC $10^{5.375}$/0.1 ml, respectively. The 16 isolates were highly pathogenic in mice, causing skin inflammation, neural symptom and leading to death in all experimentally-infected mice at 48-72 h after challenge. In contrast, all mice survived in negative control groups, which were in agreement with previous study (Laval et al., 2018).

The identification results of representative PRV isolate NY were shown in Table 2. The NY isolate was sensitive to chloroform, trypsin, formaldehyde and ultraviolet ray, revealing that it belonged to the enveloped virus. The virus was not inactivated until the heating time was above an hour at , showing that NY isolate had good heat resistance. Otherwise, no detectable titers were observed when the culture medium pH was adjusted to 3.0 or 11.0. These results were consistent with the physicochemical properties of PRV. As shown in Fig. 3D, a circular viral particle of about 110˜150 nm was observed in the ST cells infected with NY isolate. Furthermore, virus particles exhibited envelope protein with a radially arranged spike. Thus, the morphological features were basically consistent with those of pseudorabies virus.

Sequencing analysis of the main PRV gE and gC genes for 16 PRV isolates revealed the maximal amino acid (nucleotide) sequence divergences of 0.3% (0.1%) and 1.2% (0.5%) within the 16 isolates, and 4.7% (2.2%) and 10.3% (5.2%) compared with those isolates from other countries, respectively. The maximal amino acid (nucleotide) sequence divergence of the two genes of the 16 isolates were 1.4% (0.9%) and 1.0% (0.4%) compared to those strains prevalent in China before 2012, and were 0.7% (0.2%) and 0.4% (0.1%) after 2012. The phylogenetic tree based on the sequences of gE gene revealed two distinct groups (Fig. 4A): one formed by the four European-American PRV strains (Clade 2), and the other (Clade 1) formed by three subgroups: one (Clade 1-1) formed by the 16 PRV strains in this study and the 11 Chinese variant PRV strains (after

2012), and the other (Clade 1-2 and Clade 1-3) formed by the 4 early Chinese PRV strains (before 2012), suggesting that gE gene of 16 isolates were genetically closer to the variants. In the deduced gE amino acid (aa) sequences of the 16 PRV isolates and 25 referent strains, compared to all strains of Clade 2, the complete gE sequences containing two aa insertions at position 48 (D) and 497 (D) were found in the strains of Clade 1-1, which were not observed in early Chinese strains Fa-2002-China and GX-NL-2007-China. Furthermore, all the 16 isolates of Clade 1-1 had eighteen aa interspersed substitutions (at positions 54, 59, 63, 106, 179, 181, 215, 216, 449, 472, 474, 504, 509, 512, 522, 526, 577 and 578) compared with the whole strains of Clade 2, whereas gE aa of the isolates YY and BP in this study were not changed at site 512 and 578 respectively. Remarkably, only four aa substitutions in 16 isolates at position 449 (V to I), 512 (G to S, except for YY), 577 (N to M) and 578 (A to S, except for BP) were different from the substitutions of 7 Chinese variant PRV strains. Compared with the Clade 1-2 and Clade 1-3, the 16 isolates had two aa substitutions at positions 449 (V to I) and 512 (G to S, except for YY). Compared with the variants, two isolates NY and BP existed one aa change at position 386 (T to M), and the WZ and ZK had two aa substitutions at positions 329 (W to R) and 532(S to G), respectively.

For the gC, phylogenetic tree revealed two distinct groups (Fig. 4B): one formed by the four European-American PRV strains (Clade 2), and the other (Clade 1) formed by three subgroups: one (Clade 1-1) formed by the 16 PRV isolates in this study and the 6 Chinese variant PRV strains, and the other (Clade 1-2) formed by the 6 early Chinese PRV strains and 2 Chinese variant PRV strains, indicating that 16 isolates had the relatively closely relationship with the variants. Compared to all strains of Clade 2, gC protein containing seven aa deletions at sites 63 – 69 (AAASTPA) was found in the strains of Clade 1-1, which was also observed in early Chinese PRV strains. Moreover, the strains of Clade 1-1 had 23 aa interspersed substitutions (at position 16, 43, 52, 55, 57, 59, 60, 61, 87, 90, 102, 130, 142, 188, 431, 437, 449, 457, 461, 467, 485, 486 and 487) compared with the strains of Clade 2. Interestingly, eight strains MZ1, NY, LGX, MZ1, XY, ZK, ZM and GY in this study harbored one aa substitution at sites 280 (F to L) compared to other European-American strains of Clade 2 and Chinese strains of Clade 1, which was the first reported that the characteristic aa substitution (at position 280) was existed in the Chinese variant PRV strains' gC. These might be the reasons that the virulence of PRV variants enhanced, and should be paid more attention.

All mice of four groups did not display adverse reactions after vaccination (data not shown). After challenge with PRV NY isolate, mice in group 1 survived without typical PR symptoms, and the mortality was 0% (0/10). While the mortalities of groups 2-4 were 50% (5/10), 30% (3/10) and 100% (10/10). As for neutralizing antibodies against PRV, mice in groups 1-3 induced neutralizing antibodies with titers of 1:82, 1:24 and 1:22, respectively. No neutralizing antibodies were detected in group 4 as control. The neutralization titer between PRV NY isolate and its own serum was the highest, followed by Bartha-K61 strain and Hubei 98 strain, which indicated that PRV NY strain might exist the cross antigenicity with both Bartha-K61 and Hubei 98 strain, with the different antigenicity. These results were consistent with the previous finding (An et al., 2013), further indicating that Bartha-K61 vaccine cannot provide the full-protection against the PRV variants. Therefore, the development of novel vaccine based on the variants was urgent.

Live vaccines are currently used to control pseudorabies in many swine farms in China, mainly based on strain Bartha-K61 which is an attenuated strain of PRV produced by extensive in vitro passages and contains a well-characterized deletion of the complete gE and partial gI genes encoding proteins that attenuate virulence, and Bartha-K61 has played a key role in the eradication of PR (Lomniczi et al., 1987). Thus, detection of gE-specific antibodies is used to differentiate vaccinated animals from those with wild-type virus infections. During 2005 to 2010, the positive PRV gE antibodies were detected in only 3%–5% of serum samples (Gu et al., 2015). Since 2012, severe PRV outbreaks have occurred on several pig farms and spread rapidly to most of China (An et al., 2013, Wu et al., 2013, Gu et al., 2015, Zhai et al., 2019). Although the PRV infection has recently decreased since Chinese government proposed the eradication program based on PR in 2011, it remains not completely eradicated (Wang et al., 2015, Sun et al., 2018, Zhai et al., 2019). Some researches demonstrated that the novel PR was caused by PRV variants (An et al., 2013, Hu et al., 2015, Ye et al., 2015, Sun et al., 2018, Zhai et al., 2019), and this study further confirmed PRV variants were prevalent in China. To control and prevent the PRV infection in pig farms, we should develop the effective labeled vaccines

and combine with other integrated control measures, such as serological and virological monitoring, and biosafety procedures. In addition, the Chinese government should call on all relevant practitioners (farmers, veterinarians, scientists, vaccine manufacturers, officials and communities) to join hands for fighting the disease, and learn from some countries in South America and North America which successfully eliminated PR for a long time. Facing the prevalence of PR, it is worthy of further study to evaluate whether the current immunization procedures and biosafety measures are reasonable and effective.

In conclusion, this study suggests that PR is not yet eradicated, and even exists in nearly whole Henan province, China, and the new PRV isolates identified by sequencing of the complete gE and gC genes may be PRV variants. Further, it can be speculated that these variations may be the cause of the re-emergence of PR in China. The pathogenicity of the genetic variations seen in complete gE and gC genes of these PRV isolates is currently under further investigation, and the complete genome sequencing of the isolates is also necessary.

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## CONFLICTS OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

## ETHICS STATEMENT

All experimental procedures were reviewed and approved by the Henan Agriculture University Animal Care and Use Committee (license number SCXK (Henan) 2013-0001).

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Fig.1 Samples collection from Henan province of China for PRV detection during 2012 to 2019.

Fig.2 Positivity rate of PRV detection in different seasons (A) and regions (B).

Fig. 3 The isolation and identification of the PRV. A.The normal ST cell control;B. The ST cells infected with NY strain; C. Amplified PCR product of 429 bp; D. Electronograph of PRV NY strain

Fig. 4 Phylogenetic tree constructed by alignment deduced nucleotide sequences of gE (A) and gC (B) genes of the PRV isolates and reference strains using the neighbor-joining method. Bootstrapping with 1,000 replicates was performed to determine the percentage reliability for each internal node. Black triangles indicate PRV field isolates in this study.

Note:express the PRV strains in this study; *express the Chinese re-emerging PRV strains (after 2012); express the early Chinese PRV strains (before 2012).

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