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RESEARCH ARTICLE

Genetic Properties of the New Pseudorabies Virus Isolates in China in 2012

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ABSTRACT

Since the beginning of 2012, a disease, that emerging with the clinical characteristics of neurologic symptoms and high mortalities in piglets, was observed in many farms in China. Clinical samples of brains and lungs, that collected from sick piglets, abortion and death fetuses in 8 different farms, were examined. The virulent and immunogenicity related genes of pseudorabies viruses (PRV) were detected from these samples and five PRV strains were isolated successfully. The amino acid sequence analysis of the gE, gB, gC, TK RR1 and RR2 genes showed that, these strains could form an independent cluster together despite of some amino acid changes, with the homology being 99.4~100%, 99.3~100%, 99.9~100%, 98.4~100%, 98.9~99.7%, 98.7~100%, respectively. If compared with the relative genes of PRV Bartha and Ea strain, these isolates have some unique amino acid insertions, deletions and changes which do not exist among Chinese strains isolated previously. These findings indicated that these newly isolates did not derive from the current vaccine strains. Oppositely, they might evolve from field strains and the process was still obscure. These results might partially explain the prophylactic failure and emergency of pseudorabies in some areas of China.

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INTRODUCTION

Pseudorabies virus (PRV), causative agent of Aujeszky's disease, is a member of the *Alphaherpes-virinae* subfamily within the family *Herpesviridae*. It contains a double-stranded DNA genome of around 143kb which encodes 72 genes (Dong *et al.*, 2014; Sozzi *et al.*, 2014). The virus encodes several glycoproteins and enzymes, of which glycoprotein E (gE), gB, gC, thymidine kinase (TK) and Ribonucleotide reductase (RR) have an important role in the whole invading process. gE, composing the PRV envelope, is one of the six main glycoproteins (Mettenleiter, 2000; Pontes *et al.*, 2016). Although it is not required for virus propagation, gE would affect the virulence, virus egression (Fuchs *et al.*, 1990) and the transportation process of virus to the central nervous system (Mettenleiter, 2000; Ch'Ng and Enquist, 2006). gB is essential for virus growth and take part in membrane fusion during virus initial attachment and infection (Enquist *et al.*, 2002; Serena *et al.*, 2016). gC is involved in the initiation of attachment by the virus to the host cell through binding to heparin sulfate (HS) proteoglycans (Karger and Mettenleiter, 1996). TK is a member of the enzymes that functions in the viral replication and spreading process within central nervous system (CNS) (Hu *et al.*, 2015). And it is also an important virulence factor in PRV infection. RR is composed of two subunits (RR1, large subunit and RR2, small subunit). RR negative mutants are avirulent for the swine, and can provide partial protective against virulent challenge (De Wind *et al.*, 1993).

The pigs are known as the main hosts and natural reservoir of this virus (Tischer and Osterrieder, 2010; Yu *et al.*, 2014). The clinical symptoms of PRV infection are characterized by the death of newly born piglets, nervous system disorder, respiratory disorder among the finishing pigs, and reproductive failure in the sows. Due to the widely application of the attenuated gene-deleted live or whole virus-based inactivated vaccines, the swine psuedorabies has been well controlled in many countries including China and the disease eradication program is on the way (Maresch *et al.*, 2012; Zhao *et al.*, 2012). However, in the end of 2011, the re-emergency of pseudorabies-like disease was observed firstly in north of

China and then spread to other areas. This disease occurred in the vaccinated herds and the sero-conversion of gE antibody, label of field virus infection, could be detected with or without significant clinical symptoms. Molecular epidemiology investigation should be conducted to understand the genetic variation of the re-emergent strains. To understand the etiology, we amplified six virulence-related and immunogenic genes from the clinical samples, subjected to sequence analysis. The alignments with traditional PRV strains suggested that the presence of the PRV variant strains may be responsible for the recently emergent pseudorabies in some areas of China.

MATERIALS AND METHODS

Samples collection: A total of 16 brain and lung samples from 8 dead fetuses, suckling piglets and weaned pigs were obtained from 8 farms among three provinces of China from March to June in 2012. In these farms, sows, boars and weaned pigs were vaccinated with attenuated live gE-negative vaccine. All pigs from these farms showed signs of high fever, neurological disorder and posterior paralysis at the time of sample collection. A strain surnamed HBYD-China-2008 isolated in 2008 was also contained in this study. These samples were collected, homogenized, and suspended in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) at ratios of 1:2 (W/V). The suspensions were freeze-thawed for three times and kept at -70°C freezer for further viral isolation and DNA extraction.

Virus isolation and identification: For detection of PRV by PCR, the gD gene was chosen as the target gene (Chen *et al.*, 1997). The filtered supernatant of the homogenized positive brain tissues were inoculated to the monolayer of PK-15 cells and incubated at 37°C incubator. Cells were observed daily for CPE. When typical CPE was observed, cells were acquired and stored under -20°C. RNA and DNA extracted from the cell cultures were detected by reverse transcription PCR or PCR and found to be PRV positive while negative for porcine reproductive and respiratory syndrome virus and classical swine fever virus. The isolates at dose of 1×10^2 $TCID_{50}$ were employed to infect Balb/C mice and the typical clinical signs were observed.

Genomic DNA extraction and polymerase chain reaction (PCR): Total DNA was extracted from 200μl sample suspension or equal volume of viral culture supernatant using a commercially available TIANamp Genomic DNA Kit (TIANGEN Biotech, China). Seven pairs of oligonucleotide primers were designed to amplify gB, gC, gE, TK, RR1 and RR2 genes from the samples, respectively. The amplicons of the expected size were purified, cloned into the pMD18-T vector (TaKaRa Biotechnology, Dalian, China) for sequencing of both directions on ABI 3730xl DNA Analyzer (Applied Biosystems, CA, USA).

Nucleotide sequence analysis: Nucleotide sequences were edited, and analyzed through the MegAlign program, a component of the Lasergene sequence analysis software package (DNASTAR Inc., Madison, WI, USA). Phylogenetic trees were acquired by Molecular Evolutionary Genetics Analysis (MEGA) software version 5.02, based on the neighbor-joining method. The accession numbers of genes of other PRV reference strains used in the research were contained in table 1.

RESULTS

Confirmation and isolation of PRV in the samples: The conservative gD genes (217bp) were amplified from the 16 samples (Fig.1A). Positive brain tissue samples were filtered to get rid of the possible contamination and then incubated with the cells. The cytopathogenic effect (CPE) could be observed after 3 to 5 passages (Fig.1C), indicating the stable growth of the isolates in PK-15 cell lines. Five PRV strains were isolated from the brains of 1 dead fetus, 2 suckling piglets and 2 weaned pigs, respectively. The viruses were designated as HNXX-China-2012, HNZK-China-2012, HNBA-China-2012, HNQZ-China-2012, HNQX-China-2012. These PRV isolates could induce strong itchy and nervous system disorder in the infected mice (Fig. 1E), suggesting the PRV-related typical signs in mice model.

Sequence homology and genetic characterization: Partial or complete gene sequences of these 8 strains were analyzed and aligned with the reference strains (Table 1). These strains were collected in 2012 from PRV-affected pig farms in the swine-breeding provinces (Henan, Shandong, Hebei) and the isolate HBYD-2008-China isolated in 2008 was also included in this study. The target genes of gE (1284bp), gB (2745bp), gC (1461bp), TK (936bp), RR1 (2367bp) and RR2 (912bp) were all amplified successfully.

Phylogenetic analysis based on gE: The 1284bp fragment encoding the 428-amino-acid extracellular domain of gE of these PRV strains were compared with the sequences of reference strains available at GenBank (Table. 1). All the new field strains shared highly similarity with each other (99.7~100% at nucleotide level and 99.3~100% at amino acid level). When new PRV gE sequences were compared with previous domestic and reference viruses, homology values decreased (97.8~99.8% nucleotide similarity and 98.1~99.5% amino acid sequence similarity). If compared with PRV reference Becker strain, these new isolates contained an amino acid insertion of Asp (D) at 48 and 12 amino acid changes were also observed in these strains (54G-D, 59D-N, 63N-D, 66V-L, 122A-S, 125M-T, 149R-M, 163Q-R, 179T-S, 181R-L, 215L-A, 216A-D) (Fig. 2A).

Phylogenetic tree of the gE gene exhibited that all field isolates could be divided into two groups (Fig. 3A). All the Chinese strains, together with a Korea strain, Yangsan strain, and a Malaysia strain, P-PrV, fell in Group 1. Five Europe strains and one America strain were classified in Group 2.

Phylogenetic analysis based on gB: Sequence comparison of gB proved that PRV strains in 2012 had 99.9~100% amino acid sequence similarity with each other. When the gB amino acid sequences of the newly

strains were compared with the previous domestic viruses, 98.9~99.5% amino acid similarity was found; If compared with the foreign isolates, 96.6~97.7% identities were found. Although relatively high level of identity with previous Chinese strains was indicated, there were still some differences in amino acid sequence between new isolates and previous ones. The variations were as follows: 85T-A; 454R-K; 563H-Q; 740T-A; 898V-A. Among these variations, R-K change at 454 can only be observed among the new isolates (Fig. 2B). Phylogenetic comparison of the gB protein showed that all field viruses could also form two branches (Fig. 3B). Group 1 consisted of all Chinese strains. Group 2 comprised Becker, Kaplan, Bartha, Namyangju.

Phylogenetic analysis based on gC: All gC genes of the new Chinese PRV strains were 1461 nt in length and encoded 487 amino acids. However, all foreign strains have a 7-aa continuous deletion (63AAASTPA69) (Fig. 2C). Three USA strains (Indiana_S, Becker, NIA3) contain an amino acid deletion at 192 position while our new strains do not. The gC protein of these new strains had 98.4~100% identity with each other and 89.8~100% similarity when compared with previous Chinese and other reference strains.

Phylogenetic analysis of the gC gene revealed that all strains could be classified into two different groups (Fig. 3C). Group 1 contained all Chinese strains. Group 2 comprised Bartha (Hungary), Yamagata S-81 (Japan), Indiana S (USA), NIA3 (USA).

Phylogenetic analysis of TK gene: PRV TK gene was 963 nt in length, encoding a protein of 321 amino acids. The TK region is rather conserved among all the PRV isolates in this study, with the amino acid identity ranging from 98.8 to 100% (except Min-A). When the TK protein of Min-A was compared with other isolates, amino acid identity was between 97.8 and 98.7%. All the strains except Min-A belong to Group 1 while Min-A formed a unique cluster because of 2-aa deletion at 292-293 (Fig. 3D).

Phylogenetic analysis based on RR1: The RR1 gene of PRV contained 2 367 nucleotides, deciphering a protein of 789 amino acids. All the Chinese strains shared 98.9~100% amino acid sequence identity with each other, and 97.6~98.5% similarity with the reference strains. Phylogenetic analysis of RR1 protein showed that all the viruses could be divided into two parts (Fig. 3E). All the Chinese strains could be classified in Group 1. Comparing with strains in Group 2, there was a deletion in position 11-12 (SS) among the isolates in Group 1 (Fig. 2D).

Phylogenetic analysis based on RR2: The gene RR2 of PRV had 912 nucleotides in length, encoding a protein of 303 amino acids. There was no deletion or insertion among these strains. The RR2 region is rather conserved among all the PRV isolates with the aa identity being 98.3~100% (except Becker strain). When the RR2 aa sequence of Becker was compared with other isolates, amino acid identities of 97.4~98% was observed. All the strains except Becker were divided into Group 1. Becker formed a unique Group (Fig. 3F).

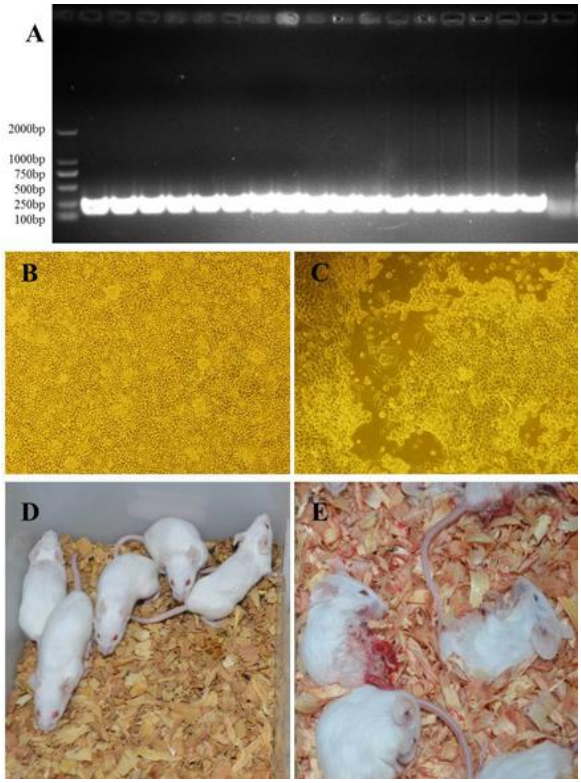

Fig. 1: (A) Normal control PK-15 cells. (B) Pseudorabies virus infected PK-15 cells. (A) and (B) original magnification ×100. (C) Uninfected control mice. (D) Mice infected with pseudorabies virus. (E) Amplification of PRV-gD fragment from the collected samples

Table 1: Sequences obtained from GeneBank used in this work.

Strain/gene	Accession No, Country	Strain/gene	Accession No., Country
(A)gE		Becker	M12778, USA
HB/BD	KC415026, China	NIA3	D49437, USA
HB/LF	KC415029, China	Bartha	EU719641, Hungary
HB/HS	KC415028, China	(D) TK	
Ea	AF171937, China	Becker	JF797219, USA
Min-A	AY170318, China	Kaplan	JF797218, Hungary
LA	AY173124, China	Bartha	JF797217, Hungary
HNJZ	EU561349, China	SA	AB440241, China
Yangsan	AY249861, Korea	ZMD2012	KF381398, China
P-PrV	FJ176390, Malaysia	SDCX2011	KF381397, China
Nia-1	FJ605136, Ireland	PYQF2013	KF381396, China
89V87	FJ605134, Belgium	LYYY2011	KF381395, China
00V72	FJ605132, Belgium	HBCX2013	KF381394, China
NS374	FJ605135, Belgium	Min-A	AY171242, China
Becker	AY368490, USA	(E) RR1	
NiA3	EU502923, Spain	BJ/YT	KC981239, China
(B) gB		Becker	JF797219, USA
Namyangju	GQ325658, Korea	Kaplan	JF797218, Hungary
Kaplan	JQ809328, Hungary	Bartha	JF797217, Hungary
Becker	JQ809328, USA	(F) RR2	
Batha	JF797217, Hungary	BJ/YT	KC981239, China
(C) gC		Becker	JF797219, USA
Fa	AF403051, China	Kaplan	JF797218, Hungary
Yamagata S-81	D49435, Japan	Bartha	JF797217, Hungary
Indiana S	D49436, Japan		

DISCUSSION

In the present study, five new PRV strains were isolated from the brains of abortion, dead fetuses and suckling piglets, and six important genes (TK, gE, gB, gC, RR1, RR2) were amplified, sequenced and comparison was processed with other PRV isolates. Comprehensive analysis of these genes indicated that new isolates shared high sequence identity with each other, and usually they,

together with the earlier Chinese strains, can be clustered into the same group (Fig. 3). Vaccine based on Bartha-K61 has been widely utilized in China for more than three decades, and is widely used as an efficient weapon against PRV. However, since late of 2011, cases of pseudorabies could be observed in some farms. And gradually the disease threatened the pig farms across the whole nation. The pigs immunized with PRV Bartha-K61 vaccine still showed the typical clinical symptoms, this indicated the low efficacy of the vaccine used.

Due to its high ratio of G+C bases, the complete gE gene is difficult to be amplified by PCR, For this reason the fragments encoding 428-amino-acid extracellular domain of gE was chosen for amplification in our study (Tirabassi *et al.*, 1997). Sequence analysis showed that the new isolates shared conserved multiple amino acid changes at the same part and were divided into the same group (Fig. 3A). These changes included one amino acid insertion and 12 amino acids variation. The insertion was also confirmed by other researchers (An *et al.*, 2013; Yu *et al.*, 2014). Virulence and spread mediated by gE are thought to be highly correlated and the gE gene has been considered as a target for understanding genetic variation of PRV viruses (Fonseca *et al.*, 2010; Pontes *et al.*, 2015). Our findings indicated that the gE genes of new PRV strains were highly homologous and these strains were phylogenetically distant from previous PRV isolates. This may partly explain the low immunological protection induced by the current Bartha-K61-based vaccine in China.

Amino acid sequence analysis of gB showed a 3-aa deletion (75SPG78) and one-aa insertion (94G) among the new strains while comparing with the reference isolates (Fig. 2B). Sequence comparison also indicated that the viruses contain a unique amino acid change (R454K) when comparing with previous Chinese isolates and other reference strains. Whether this change will affect the binding ability of gB needs further confirmation.

The absence of gC will decrease its virulence in pigs (Rue and Ryan, 2008). Thus, the gC gene is implicated as an important factor in the pathogenesis of PRV. Sequence homology analysis indicated two discontinuous insertions (63AAASTPA69, 192E) in the new PRV strains. But these insertions could also be detected in previous Chinese PRV strains.

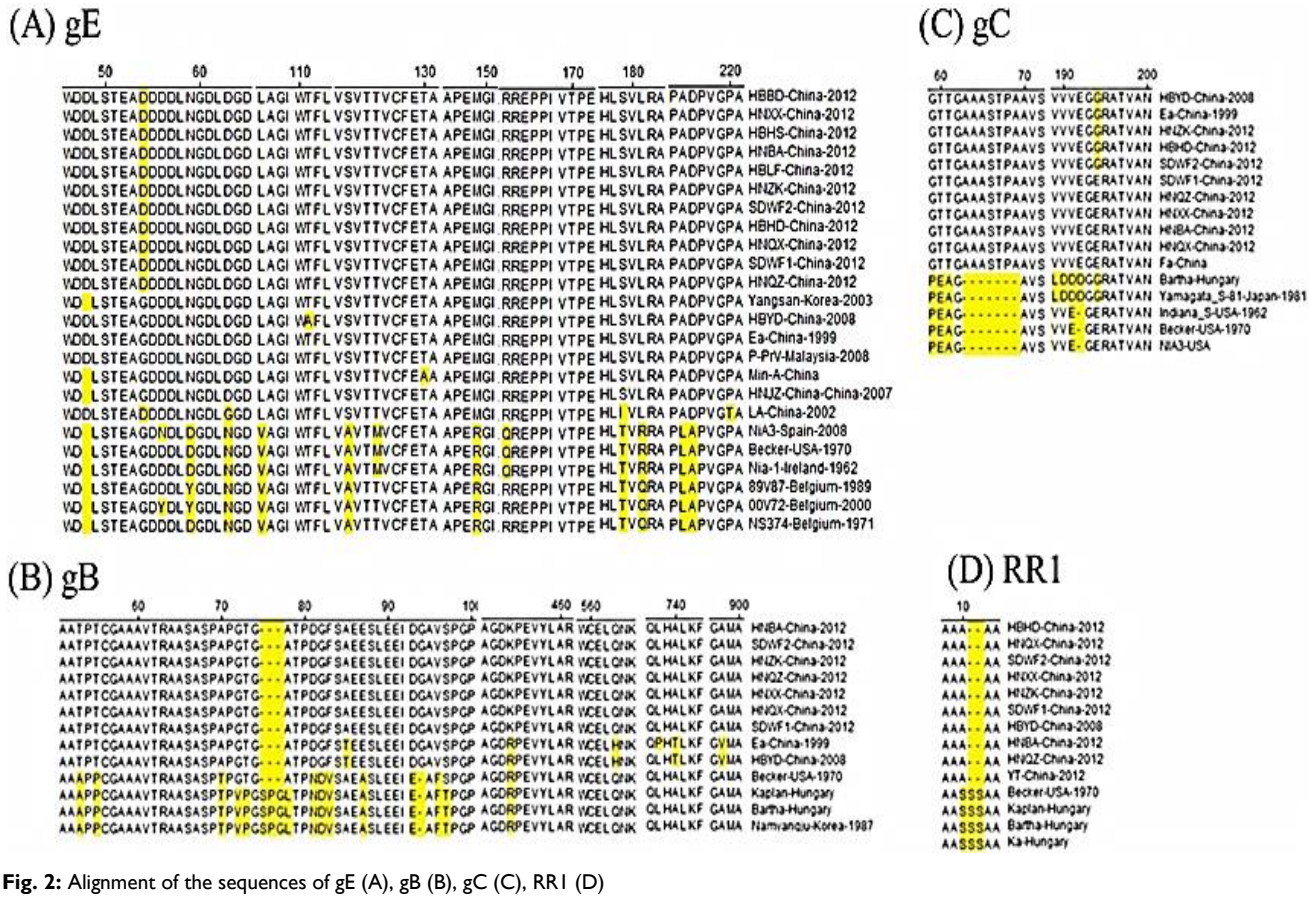

Fig. 2: Alignment of the sequences of gE (A), gB (B), gC (C), RR1 (D)

Table 2: Viral isolates used in this work

Strains	Date of isolation	Province of origin	Material	Accession number gE	Accession number gB	Accession number gC	Accession number TK	Accession number RR1	Accession number RR2
HNQX-China-2012	2012 March	Henan	Brain	KJ526425	KJ526437	KJ526445	KJ526449	KJ526460	KJ526469
HNQZ-China-2012	2012 March	Henan	Brain	KJ526430	KJ526435	KJ526442	KJ526450	KJ526461	KJ526470
HNXX-China-2012	2012 March	Henan	Brain	KJ526423	KJ526436	KJ526441	KJ526452	KJ526465	KJ526474
HNBA-China-2012	2012 March	Henan	Brain	KJ526424	KJ526434	KJ526443	KJ526453	KJ526458	KJ526467
HNZK-China-2012	2012 March	Henan	Brain	KJ526426	KJ526433	KJ526444	KJ526451	KJ526462	KJ526471
SDWF1-China-2012	2012 May	Shandong	Brain	KJ526427	KJ526438	KJ526446	KJ526455	KJ526463	KJ526472
SDWF2-China-2012	2012 July	Shandong	Brain	KJ526428	KJ526439	KJ526448	KJ526456	KJ526464	KJ526473
HBHD-China-2012	2012 June	Hebei	Lung	KJ526429	-	KJ526447	KJ526454	KJ526459	KJ526468
HBYD-China-2008	2008	Hubei	-	KJ526431	KJ526432	KJ526440	KJ526457	KJ526466	KJ526475

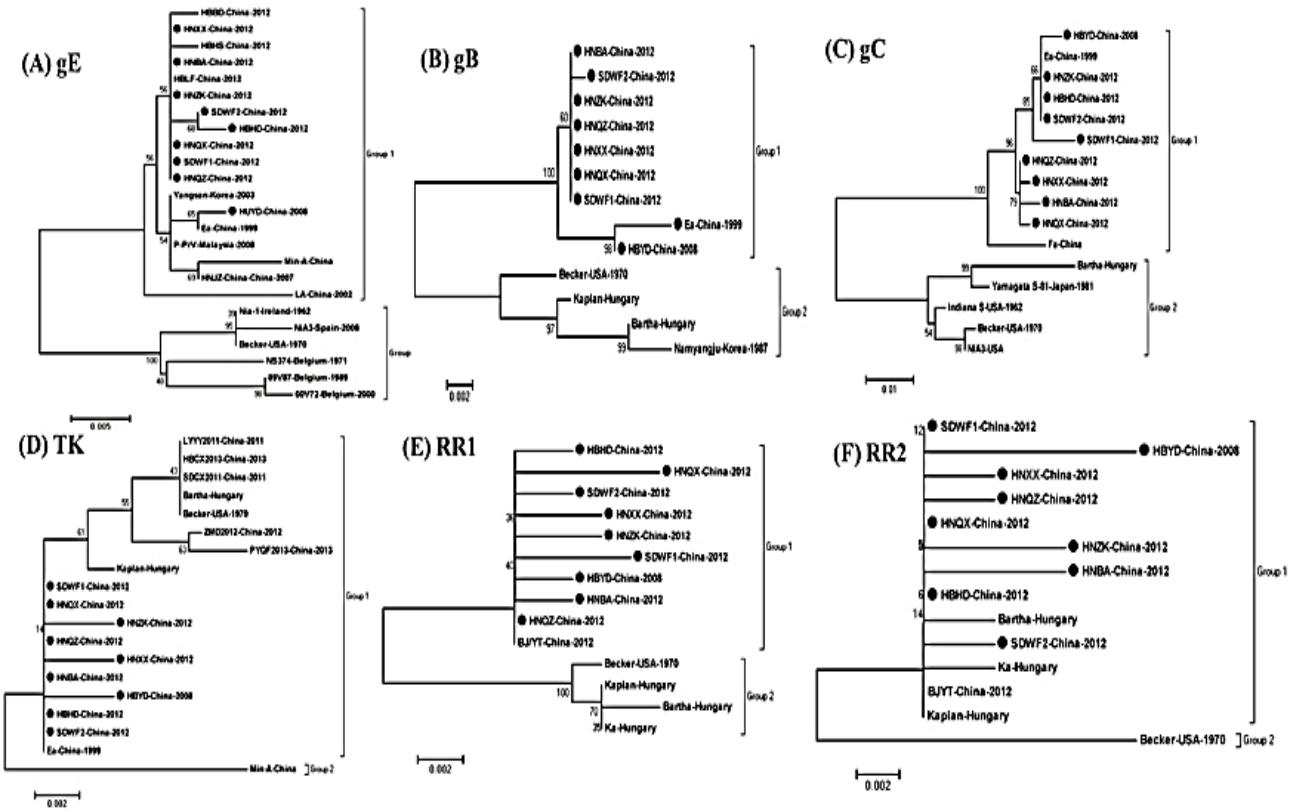

Fig. 3: Phylogenetic analysis of the current Chinese PRV strains and reference strains according to the amino acid sequences of gE (A), gB (B), gC (C), TK (D), RR1 (E), RR2 (F). Phylogenetic trees were processed by MEGA. ●, current Chinese isolates. GenBank accession numbers of all viruses used in this research are listed in Table 1 and Table 2.

Sequence analysis revealed an aa deletion in TK protein of Min-A. All the other strains formed one group. Comparison with reference strains showed that TK is highly conserved besides some point mutations. Alignment with reference strains suggested that RR is also highly conserved. A two-aa (11SS12) deletion of RR1 could be observed among all the Chinese strains, while there were not any insertions or deletions could be found in RR2.

Taken together, the phylenogentic analyses of the six important genes showed that the new PRV strains may contribute to the re-emergence of PR in China and are phylogenetically distant from previously PRV isolates. The reason for the immune failure may be partly due to the combination of variations in these important genes, in particular, the immunogenic-related gE, gB, gC genes. It is speculated that these new isolates had evolved from the previous Chinese PRV isolates. Because the gE, gB, gC, RR1 genes of new strains showed higher identities with earlier Chinese isolates rather than the reference ones. The influence of the amino acid insertion, mutation and deletion caused on the pathogenicity and immunogenicity of the new PRV strains needs to be further characterized.

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Author's contribution: HQG and WB designed the experiment. HH and YT helped with the manuscript. HH, YT, FJ, CS, LC, DF and GN executed the experiment and analyzed the data.

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