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Research paper

Characterization of a moderately pathogenic pseudorabies virus variant isolated in China, 2014

Mingming Zhou[a,1], Xiangju Wu[a,1], Dandan Jiang[a,1], Chao Sui[a,1], Lei Chen[a,c], Xiaoyan Cong[a], Xu Xin[b], Guisheng Wang[d], Yujie Li[d], Fulin Tian[d], Zhi Chen[a], Hongliang Zhang[b], Jing Qi[a,c,**], Zhao Wang[e,*], Jiaqiang Wu[a,*], Hu Shan[b,*], Yijun Du[a,b,c,**]

[a] Shandong Key Laboratory of Animal Disease Control and Breeding, Institute of Animal Science and Veterinary Medicine, Shandong Academy of Agricultural Sciences, Sangyuan Road No. 8, Jinan 250100, China
[b] College of Veterinary Medicine, Qingdao Agricultural University, Qingdao 266109, China
[c] College of life science, Shandong Normal University, Jinan 250014, China
[d] Shandong Provincial Center for Animal Disease Control and Prevention, Jinan 250022, China
[e] China Institute of Veterinary Drug Control, 8 Nandajie, Zhongguancun, Haidian, Beijing, 100081, China

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ABSTRACT

In this study, we reported a moderately pathogenic pseudorabies virus (PRV) variant isolated from one Bartha-K61-vaccinated pig farm in Weifang, Shandong Province, China, 2014. The sick piglets in the farm were characterized by anorexia, weight loss and neurologic symptoms but did not die. Sequence alignment of the gE gene indicated that it belonged to a new mutated PRV strain and about 15% amino acid sites had mutations, deficiencies and insertions compared to the other PRV strains. The gD gene had two amino acid insertions and ten amino acid mutations in comparison with the Bartha-K61 vaccine strain. The TK and gM genes were the same as one highly pathogenic PRV TJ strain. Evidence from virus isolation, laboratory challenge, serological detection and histopathologic examination confirmed that the etiological agent of the disease is PRV SD1404, which is a moderately pathogenic strain and causes piglets to be sick but not to die. PRV SD1404 strain is different from other reports and should be paid more attention to avoid economic losses.

1. Introduction

PRV, the agent of pseudorabies, is a member of the *Alpha herpesvirinae* subfamily within the family *Herpesviridae*. The virus contains a double-stranded DNA genome with strong genetic stability molecule (~143 kb) long and contains at least 72 genes (Nauwynck et al., 2007; Wang et al., 2015). The virus has a broad host range (Pomeranz et al., 2005), occurs worldwide and causes great economic losses in swine industry, owing to respiratory distress, nervous system disorders, genital disorders and consequent high mortality rate, according to the age of the host and the virulence of the virus strain (Pomeranz et al., 2005; Marcaccini et al., 2008; Ye et al., 2015; Tong et al., 2015; Tang et al., 2017).

PRV vaccines have been widely used to effectively control pseudorabies > 30 years, and cases of the disease were rarely reported in pig farms (de Leeuw and van Oirschot, 1985; Yuan et al., 1987; Mettenleiter, 1995; Zhou et al., 2017). Among the vaccines, the Bartha-K61 strain currently used has played a key role in the control and eradication of pseudorabies. The first PRV outbreak from China was reported in 1950s. In 1970s, the Bartha-K61 vaccine was imported from Hungary to China (Yuan et al., 1987). Between the 1990s and late 2011, most pigs in China were vaccinated with the Bartha-K61 vaccine and pseudorabies was well controlled (Tong and Chen, 1999; An et al., 2013). Since late 2011, however, PRV outbreaks emerged in a large number of Bartha-K61-vaccinated swine herds in > 6 provinces (including autonomous cities and regions) of China (An et al., 2013; Yu et al., 2014; Wang et al., 2014; Luo et al., 2014). The infected pigs had high fever (> 40.5 °C), anorexia, respiratory distress, conjunctival

* Corresponding authors.
** Corresponding authors at: Shandong Key Laboratory of Animal Disease Control and Breeding, Institute of Animal Science and Veterinary Medicine, Shandong Academy of Agricultural Sciences, Sangyuan Road No. 8, Jinan 250100, China.
E-mail addresses: qj-happy2008@163.com (J. Qi), zhaowang2007@outlook.com (Z. Wang), wujiaqiang2000@sina.com (J. Wu), shanhu67@163.com (H. Shan), duyijun0916@163.com (Y. Du).
[1] These authors contributed equally to this article.

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serous and mucinous secretion, posterior paralysis, shiver and opisthotonos. 2–3 days old piglets died very quickly and most pig deaths were recorded within 3 days after the clinical signs appeared. The disease caused by highly pathogenic PRV was characterized by high-mortality among newborn piglets (Luo et al., 2014). Pathologic examination showed that the most striking gross lesions were consolidated in the kidney, lymph node, tonsil and brain.

In this study, we collected the brain tissue samples of sick piglets in two Bartha-K61-vaccinated farms in Weifang, Shandong Province, China. The sick piglets in one farm were characterized by anorexia, weight loss and neurologic symptoms but did not die. Although the mortality was 0%, the morbidity of pigs in the farm was 60%. The PRV gE gene could be amplified by PCR from the brain tissue samples of sick piglets. Then the PRV strain was isolated, plaque-purified and designated as PRV SD1404. The full-length of gE, gD, TK and gM genes and the pathogenicity of the strain were further characterized.

2. Methods

2.1. Cells, virus and plasmid

Porcine kidney (PK-15) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 2 mM L-glutamine, 100 U penicillin/ml and 100 μg streptomycin/ml in a humidified incubator with 5% CO_2 at 37 °C. PRV Bartha-K61 vaccine strain was obtained from Nanjing Tianbang Bio-industry Co., LTD. Plasmid pCDNA3.1-gE containing PRV gE gene was kindly provided by Dr. Ping Jiang (Nanjing Agricultural University, China).

2.2. Viral genome extraction and PCR

DNA was extracted from the homogenized brain tissue samples of sick piglets as described previously (Sui et al., 2010). The sense primer used for PCR amplification of partial PRV gE gene was 5'-TCTGGCTC TGCGTGCTGTG-3′, and the reverse primer was 5'-GGGTCCATTCGTC ACTTCCG-3′. The amplification was performed in a 50 μl reaction mixture containing 1.5 mM $MgCl_2$, 1 × PCR buffer, 0.2 mM of each dNTP, 20 pmol of each primer, 1.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 2 μl of DNA. The reaction was run in a thermocycler (DNA Engine, PTC-0200; Bio-Rad Laboratories, Hercules, CA, USA) with the following program: denaturation at 94 °C for 5 min; followed by 35 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 2 min; and a final extension at 72 °C for 7 min. The partial PRV gE gene was amplified. The PCR products were subjected to electrophoresis on a 1% agarose gel and stained with ethidium bromide for visualization using an ultraviolet transilluminator (Gel Doc™ XR + with image Lab™ Software, Bio-Rad Laboratories).

2.3. Virus isolation and purification

Homogenates of the brain tissue samples of sick piglets were centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatants were collected, filtered by 0.22 μm filter, and used to inoculate PK-15 cells. The cells were incubated at 37 °C and examined daily for cytopathic effect (CPE). The supernatants were harvested and used to infect fresh PK-15 cells again when CPE was observed. The second passage PRV was plaque-purified three times. In detail, viruses were serially 10-fold diluted in DMEM to infect PK-15 monolayers in six-well plates. After adsorption for 1 h, the monolayers were washed twice with PBS and overlaid with 2 ml DMEM containing 1% low melting-point agarose and 2% FBS, and then incubated at 37 °C. Then single plaque was taken out using a snapped tip and used to infect new PK-15 monolayers. After plaque-purified for three times, the virus was designated as PRV SD1404. PRV Bartha-K61 strain was used as a control for plaque-purification.

2.4. One-step growth curves of PRV SD1404 or Bartha-K61 strain

PK-15 cells were grown in 24-well plates to 80% confluency and infected with PRV SD1404 or Bartha-K61 strain at the same multiplicity of infection (MOI) of 1.0. Virus was allowed to adsorb for 1 h at 37 °C. The inoculum was removed, and the cells were washed twice and then replaced with fresh medium. At 12 h, 24 h, 36 h, 48 h and 60 h post-infection (hpi), the supernatants were collected and titrated by a microtitration infectivity assay and recorded as $TCID_{50}$/ml. All assays were repeated at least three times, with each experiment performed in triplicate.

2.5. PCR amplification of full-length gE, gD, TK and gM genes of PRV SD1404 and phylogenetic analyses

Viral genome DNA of PRV SD1404 was extracted using the DNA Tissue Kit (Omega, Irving, TX, USA) according to the manufacturer's instructions. The full-length gE, gD, TK and gM genes of PRV SD1404 were amplified from viral DNA using primer pairs as following: gE-Fwd (sense primer): 5'-TCGCACACACCGGGGTTGAG-3′, gE-Rev (reverse primer): 5'-GGTGGGCATGTCGGAATG-3′; gD-Fwd (sense primer): 5'-ATGCTGCTCGCAGCGCTATT-3′, gD-Rev (reverse primer): 5'-TACTGC GGAGGCTACG-3′; TK-Fwd (sense primer): 5'-ATGCGCATCCTCCGGAT CTACCT-3′, TK-Rev (reverse primer): 5'-TCACACCCCCATCTCCGACGT GAA-3′; gM-Fwd (sense primer): 5'-TTATTCAAAGCCGAGGTTC-3′, gM-Rev (reverse primer): 5'-ATGTCCGGGCCGCGCAAC-3′. The amplification was performed in a 50 μl reaction mixture containing 20 pmol of each primer, 1 × TransTaq® HiFi buffer (TransGen, Beijing, China), 1 × GC enhancer, 0.2 mM of each dNTP, 2.5 U of TransTaq® HiFi DNA Polymerase (TransGen, Beijing, China), 1 μl of each DMSO and 2 μl of DNA. The reaction was run in a thermocycler with the following program: denaturation at 94 °C for 5 min; followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min; and a final extension at 72 °C for 10 min. Then the PCR products were cloned into the pEASY-Blunt vector and sequenced. We analyzed sequence data as described (An et al., 2007) and compared the gE, gD, TK and gM genes with the sequences available in the GenBank database (Table 1). Lasergene sequence analysis software MegAlign (DNASTAR, Madison, WI, USA) was used to perform multiple sequence alignments and phylogenetic analyses. In detail, sequences were added in MegAlign and aligned using clustal W method. Phylogenetic tree was viewed using phenogram mode as described (Fauquet et al., 2008).

2.6. Animal experiments

The animal experiments were approved by Shandong Provincial Science and Technology department in China and conducted accordingly. Experiments conformed to the local (Regulations for the administration of affairs concerning experimental animals) and international (Dolan K. 2007 Second Edition of Laboratory Animal Law. Blackwell, UK) guidelines on the ethical use of animals.

2.7. PRV SD1404 or Bartha-K61 strain infection of chicken embryos

Seventy 10-day-old specific-pathogen-free (SPF) chicken embryos were randomly divided into fourteen groups containing five chicken embryos per group. Groups 1–7 were individually inoculated with different doses (10^1–10^7 $TCID_{50}$) of PRV SD1404 strain in 200 μl PBS. Groups 8–14 were inoculated with PRV Bartha-K61 strain in the same pattern. The embryos were sealed with paraffin wax, incubated at 37 °C and examined daily for up to 7 days. The dead embryos or surviving ones were recorded and LD_{50} (50% lethal dose) of PRV SD1404 or Bartha-K61 strain was calculated.

Table 1
PRV isolates used to compare the gE, gD, TK and gM genes with PRV SD1404 strain.

PRV isolates	Country	Year	GenBank accession No.
00 V72	Belgium	2000	FJ605137
20,130,620	China	2013	KF311112
Bartha	USA	2011	JF797217
Becker	United States	2011	JF797219
CL15	Argentina	1988	JF460026
Ea	China	1999	AF171937
Fa	China	2002	AF403049
FZ	China	2007	EF622042
GDSH	China	2007	EF552427
Guangdong	China	2001	AF403050
HBBD	China	2013	KC415026
HBCX2013	China	2013	KF360830
HBHD	China	2013	KC415027
HBHS	China	2013	KC415028
HBLF	China	2013	KC415029
HeN1	China	2012	KP098534
HNBA2012	China	2014	KF010499
HNHB2012	China	2014	KF010500
HNJZ	China	2008	EU561349
JS-2012	China	2012	KP257591
Kaplan	Hungary	Unknown	JF797218
LA	China	2002	AY173124
Min-A	China	2002	AY170318
Namyangju	South Korea	2011	GQ325660
NC	USA	2010	NC_006151
Nia-1	Ireland	1962	FJ605136
NiA3	Spain	2008	EU502923
NS374	Belgium	1971	FJ605135
NYXY	China	2013	KF360835
PRV-SH	China	1999	AF207700
QBA	China	2013	KF017333
QXX	China	2013	KF017332
QXY	China	2013	KF017335
QYY	China	2013	KF017334
SA215	China	2006	DQ367438
SC	China	1986	KT809429
TJ	China	2014	KJ789182
Yangsan	South Korea	2003	AY217094
ZJNB2012	China	2014	KF010504

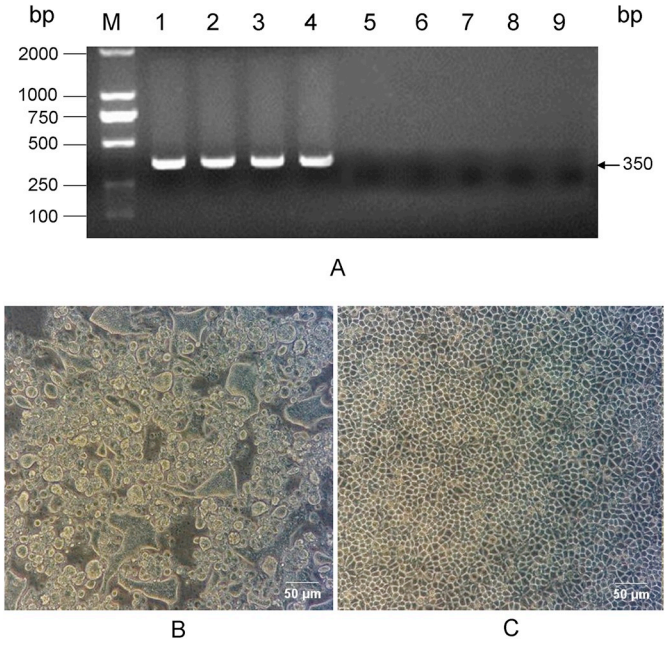

Fig. 1. (A) PCR amplification of partial PRV gE gene from brain tissue samples. Samples from 3 sick piglets in the farm in Weifang were verified PRV gE gene positive (Lanes 2, 3 and 4), other samples of piglets in the other farm were negative (Lanes 5, 6, 7 and 8). Lane 1 and 9 was a positive and negative control, respectively. M: DL2000 Marker. (B) Obvious CPE produced in PK-15 cells infected with PRV SD1404 strain. (C) No CPE in the control PK-15 cells.

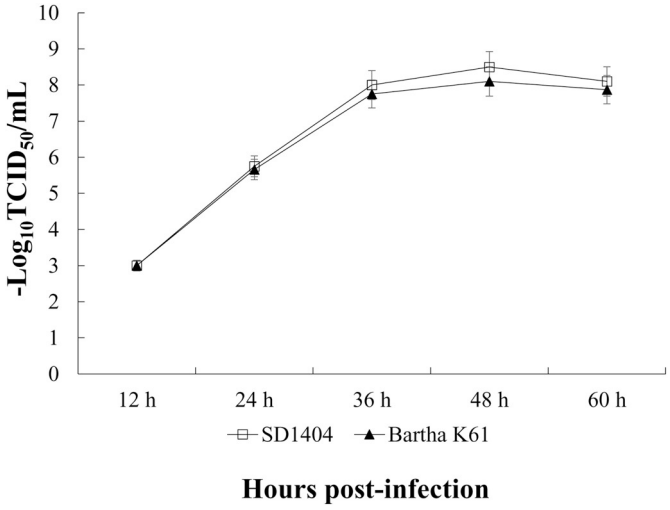

Fig. 2. One-step growth curve of PRV SD1404 or Bartha-K61 strain in PK-15 cells. Error bars indicate the standard deviations of three experiments. Data are the means ± the standard deviations.

2.8. Challenge of rabbits with PRV SD1404

Twelve 4-month old healthy rabbits were randomly divided into four groups each with three and housed in separate cages. Groups 1–3 were injected intramuscularly with $1 \times 10^{7.5}$ $TCID_{50}$, $1 \times 10^{6.5}$ $TCID_{50}$ and $1 \times 10^{5.5}$ $TCID_{50}$ of PRV SD1404 strain in 500 μl PBS, respectively. Group 4 was injected with 500 μl PBS. Clinical signs were checked daily and all rabbits were humanely euthanized on day 5 post-challenge. The brain tissue samples were collected from each rabbit to detect PRV using the gE-specific PCR as mentioned above.

2.9. Challenge of piglets with PRV SD1404

Twenty 5-week old Yorkshire piglets were obtained from a local farm. All piglets were tested to be free from PRV, porcine reproductive and respiratory syndrome virus, porcine circovirus 2, porcine parvovirus and Actinobacillus pleuropneumoniae infections and proven to be seronegative for pseudorabies by gE and gB ELISA kit (HerdChek PRV; IDEXX Laboratories, Westbrook, ME, USA). The piglets were then randomly divided into four groups with five piglets each group, numbered, and housed in separate rooms. Groups 1–3 were injected intramuscularly with $1 \times 10^{7.5}$ $TCID_{50}$, $5 \times 10^{7.5}$ $TCID_{50}$ and $2 \times 10^{8.5}$ $TCID_{50}$ (concentrated virus) of PRV SD1404 strain in 1 ml PBS, respectively. Group 4 was injected with 1 ml PBS. Rectal temperatures and clinical signs were checked and recorded daily during the study. The sera were collected from each piglet on day 2, 4, 6, 8, 10, 12 and 14 post-challenge to detect antibodies to PRV gE using IDEXX gE ELISA kit. All piglets were humanely euthanized on day 14 post-challenge and the body weights were measured. The tissue samples of brain, heart, lung,

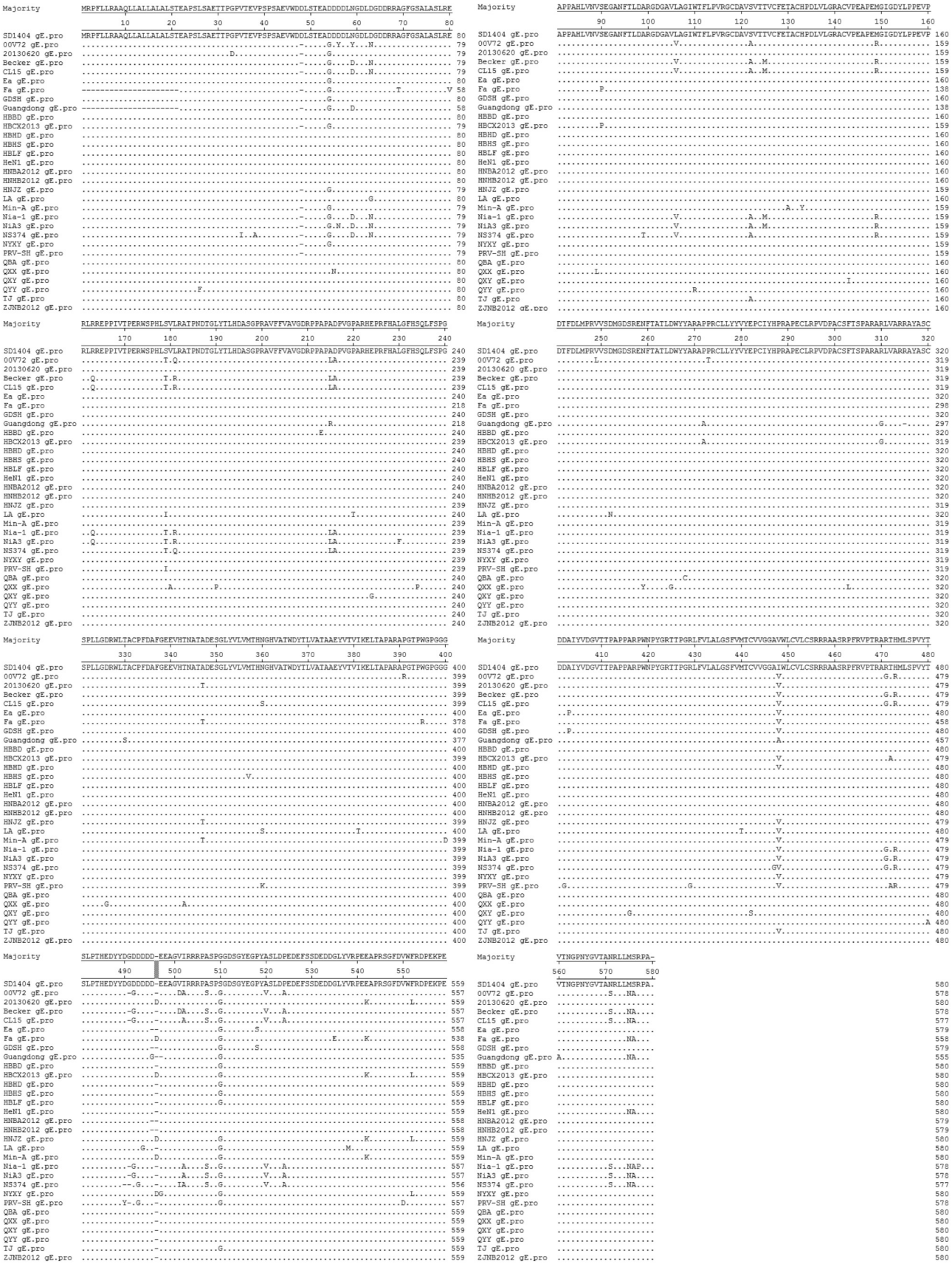

Fig. 3. Comparison of PRV SD1404 gE amino acid sequence with other PRV strains available in the GenBank database.

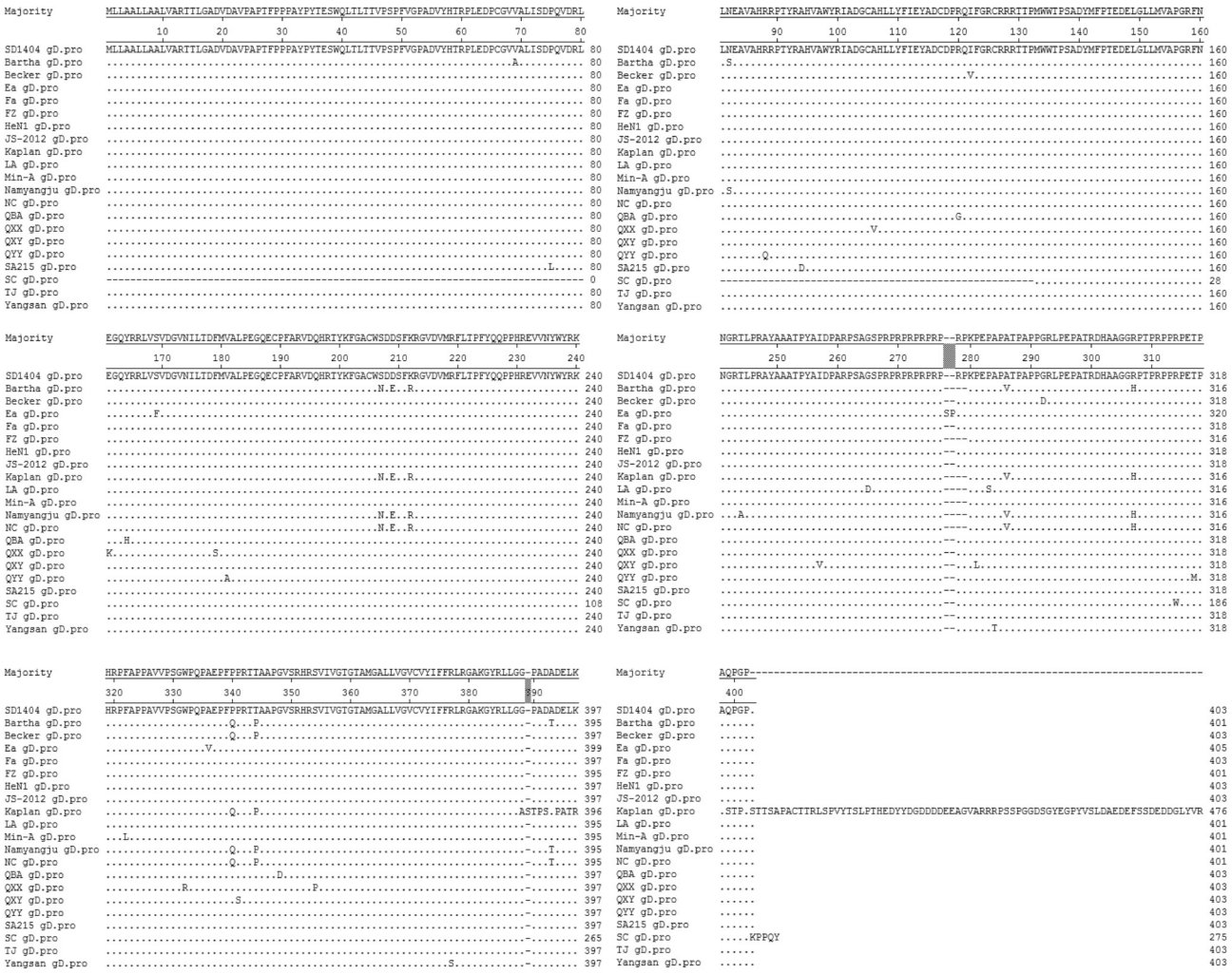

Fig. 4. Comparison of PRV SD1404 gD amino acid sequence with other PRV strains available in the GenBank database.

liver, spleen, kidney, tonsil and lymph node were collected for histopathologic analysis. The brain tissue samples were also used to detect PRV with gE-specific PCR as mentioned above.

3. Results

3.1. PCR amplification of partial PRV gE gene from brain tissue samples

The sick piglets in one farm in Weifang (Shandong Province, China) presented with multiple clinical signs, including high fever (≥40.5 °C), depression, anorexia, weight loss and neurologic symptoms but did not die. Viral genome DNA was extracted from the brain tissue samples, and the partial PRV gE gene was amplified by PCR. As shown in Fig. 1A, samples from 3 sick piglets which came from the farm were verified to be PRV gE gene positive (Lanes 2, 3 and 4), suggesting that the sick piglets in the farm were infected with PRV. Samples from 4 sick piglets which came from the other farm were verified to be PRV gE gene negative (Lanes 5, 6, 7 and 8), suggesting that the sick piglets in the farm were not infected with PRV. Plasmid pCDNA3.1-gE (Lane 1) was used as a positive control and tissue sample from a healthy piglet (Lane 9) was used as a negative control.

3.2. PRV isolation and purification

The supernatants of homogenates of the brain tissue samples were used to inoculate PK-15 cells, and obvious CPE was observed in the second passage. After plaque-purification for three times, PRV SD1404 was designated and produced obvious CPE in PK-15 cells (Fig. 1B). However, no CPE was observed in the control PK-15 cells (Fig. 1C). There was no obvious difference in morphology of plaques formed between PRV SD1404 and Bartha-K61 strain (data not shown). Viral genome DNA was extracted from PRV SD1404 and the PRV gE gene was still positive (data not shown). The sixth passage of the purified PRV SD1404 was used further for challenge experiments.

3.3. One-step growth curves of PRV SD1404 or Bartha-K61 strain

One-step growth curves of PRV SD1404 and Bartha-K61 strains were conducted in PK-15 cells. As seen in Fig. 2, the growth kinetics of PRV SD1404 was similar to that of Bartha-K61. The virus titer of PRV SD1404 at 48 h was significantly higher than that of Bartha-K61 ($P < 0.05$), although their growth rates were similar.

3.4. Phylogenetic analysis

The gE, gD, TK and gM genes were amplified from viral genome DNA of PRV SD1404 by PCR, cloned into the pEASY-Blunt vector and sequenced, respectively. The results showed that the full-length gE, gD, TK and gM gene was 1740 bp, 1209 bp, 963 bp and 1182 bp, respectively. The complete gE, gD, TK and gM gene sequences of PRV SD1404 strain were deposited in GenBank under the accession numbers KP315914, KP315913, MG581434 and MG581433. We compared the

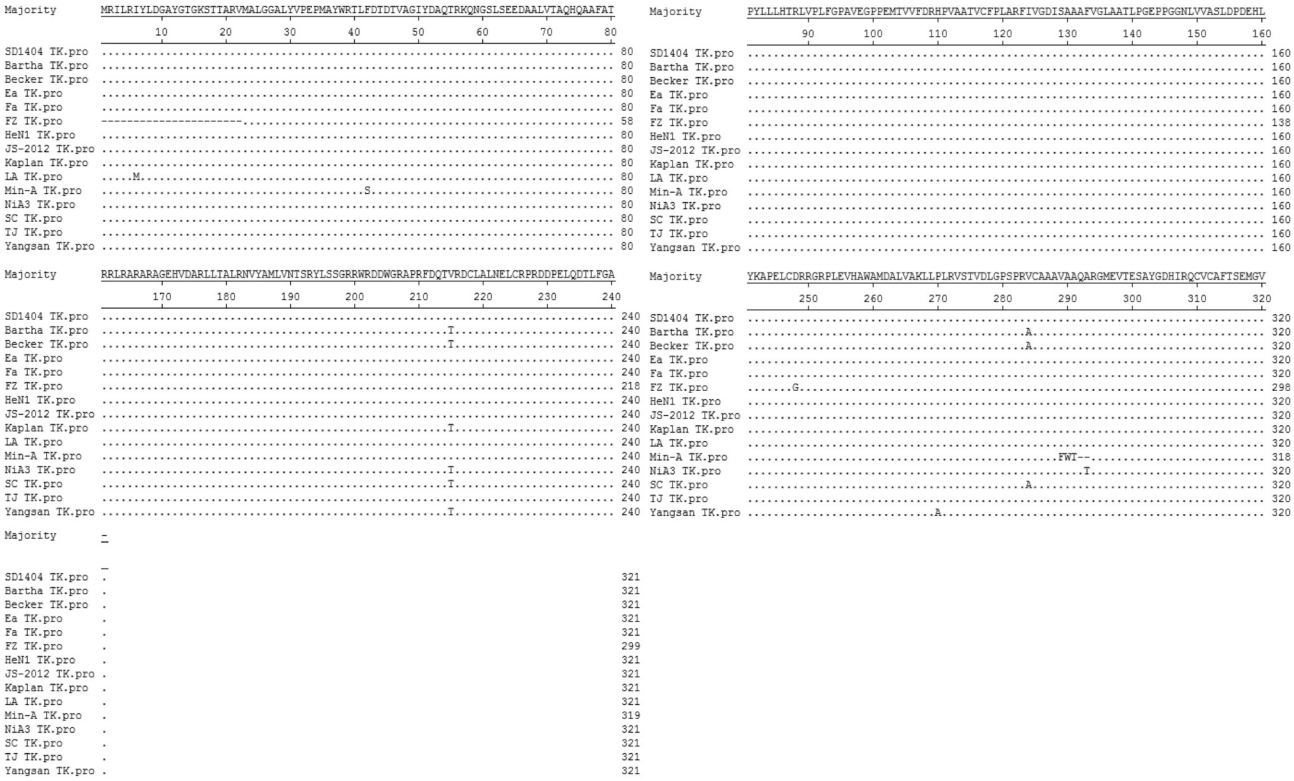

Fig. 5. Comparison of PRV SD1404 TK amino acid sequence with other PRV strains available in the GenBank database.

gE, gD, TK and gM gene of PRV SD1404 with other PRV strains available in the GenBank database (Table 1). Sequences alignment of gE indicated that about 15% amino acid sites of PRV SD1404 gE had mutations, deficiencies and insertions as compared to the other strains of PRVs (Fig. 3). The gD gene sequence alignment showed that there were two amino acid site insertions at positions 278–279 and ten amino acid site mutations at positions 69, 82, 207, 209, 212, 286, 307, 340, 344 and 393 in comparison with the sequence of Bartha-K61 vaccine strain (Fig. 4). The TK and gM genes were the same as one highly pathogenic PRV TJ strain (Luo et al., 2014) (Figs. 5 and 6). Compared to HeN1 strain, there were only two mutations at positions 575–576 in gE gene while gD, TK, and gM were all the same (Figs. 3–6).

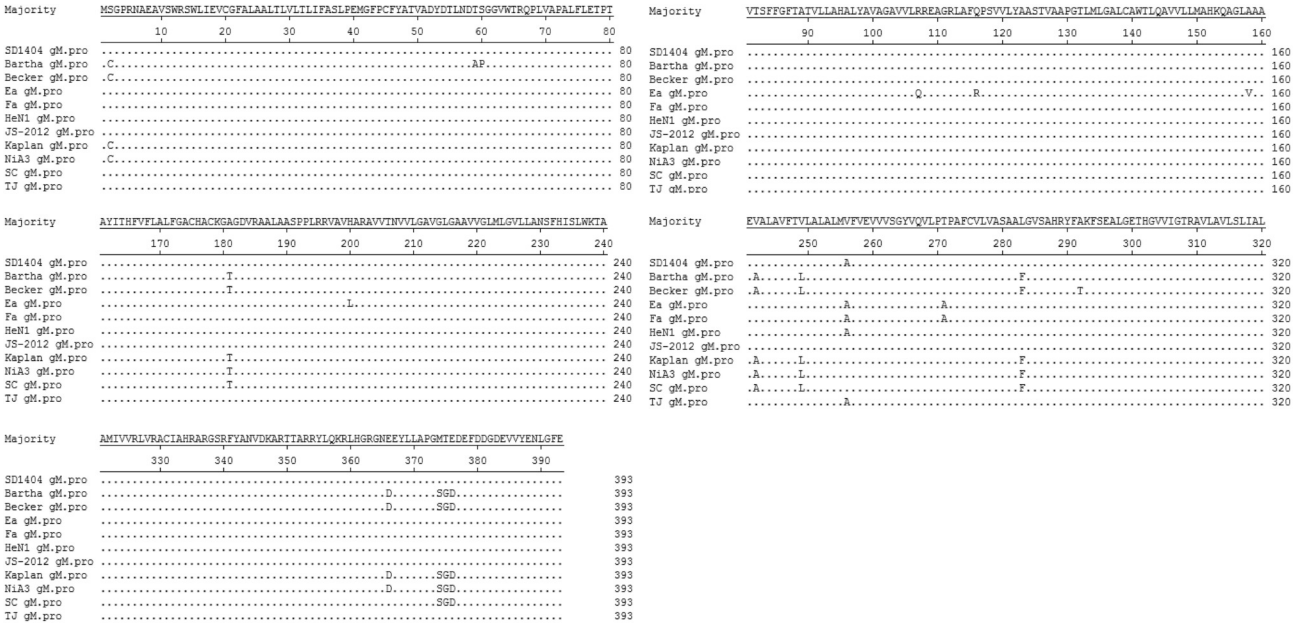

Fig. 6. Comparison of PRV SD1404 gM amino acid sequence with other PRV strains available in the GenBank database.

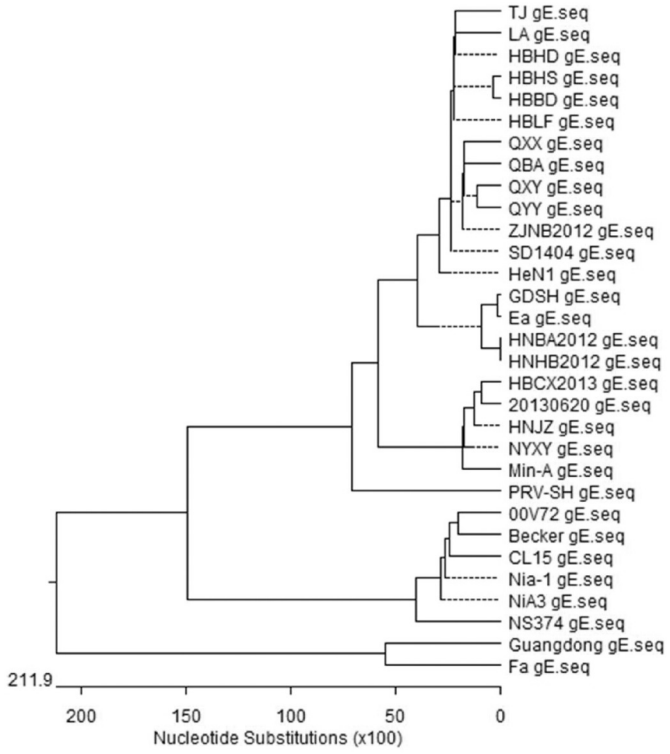

Fig. 7. Phylogenetic analysis of PRV isolates based on the gE gene sequence.

Table 2
Mortality of chicken embryos infected with PRV SD1404 or Bartha-K61strain.

PRV strains	Group	Numbers of chicken embryos	Doses of PRV ($TCID_{50}$)	Mortality
SD1404	1	5	10^7	5/5
	2	5	10^6	5/5
	3	5	10^5	5/5
	4	5	10^4	3/5
	5	5	10^3	1/5
	6	5	10^2	0/5
	7	5	10^1	0/5
Bartha-K61	8	5	10^7	5/5
	9	5	10^6	5/5
	10	5	10^5	3/5
	11	5	10^4	0/5
	12	5	10^3	0/5
	13	5	10^2	0/5
	14	5	10^1	0/5

We further analyzed the relationship of PRV SD1404 strain with other PRV isolates using a phylogenetic tree based on the gE gene. Phylogenetic analysis of PRV gE gene showed that PRV SD1404 strain was clustered to an independent branch together with some recent PRV isolates in China, such as ZJNB2012 and HeN1 (An et al., 2013) strains (Fig. 7).

3.5. PRV SD1404 or Bartha-K61 strain infection of chicken embryos

The dead or surviving embryos of PRV SD1404 or Bartha-K61 strain were recorded and the mortality in each group was calculated (Table 2). The LD_{50} of PRV SD1404 strain in chicken embryos was $1 \times 10^{3.3}$ $TCID_{50}$ while that of Bartha-K61 strain was $1 \times 10^{4.2}$ $TCID_{50}$ in 200 µl PBS.

3.6. Challenge of rabbits with PRV SD1404

Two days after challenge, itching and nibble at injected position was observed in all rabbits of groups 1–3. Subsequently, nerve symptom appeared. Four days after challenge, all rabbits of groups 1–3 died. However, the rabbits of group 4 were normal and healthy during the experiment and humanely euthanized on day 5 post-challenge. The results of PCR detection of brain tissue samples collected from each rabbit showed that all rabbits of groups 1–3 were PRV SD1404 strain positive while all rabbits of group 4 were negative (Table 3).

Table 3
PRV SD1404 detection in brain tissue samples collected from each rabbit with gE-specific PCR.

Groups	Rabbit No.	Inoculum	PCR [a]
1	1–1	$1 \times 10^{7.5}$ $TCID_{50}$ PRV	+
	1–2	$1 \times 10^{7.5}$ $TCID_{50}$ PRV	+
	1–3	$1 \times 10^{7.5}$ $TCID_{50}$ PRV	+
2	2–1	$1 \times 10^{6.5}$ $TCID_{50}$ PRV	+
	2–2	$1 \times 10^{6.5}$ $TCID_{50}$ PRV	+
	2–3	$1 \times 10^{6.5}$ $TCID_{50}$ PRV	+
3	3–1	$1 \times 10^{5.5}$ $TCID_{50}$ PRV	+
	3–2	$1 \times 10^{5.5}$ $TCID_{50}$ PRV	+
	3–3	$1 \times 10^{5.5}$ $TCID_{50}$ PRV	+
4	4–1	PBS	−
	4–2	PBS	−
	4–3	PBS	−

[a] , + represents positive result; − represents negative result.

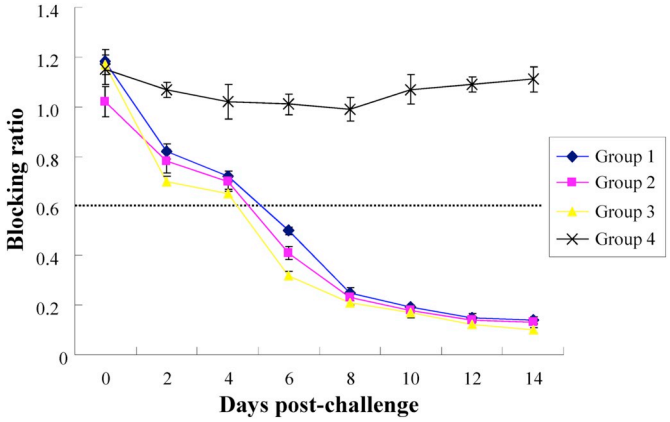

Fig. 8. Detection of PRV gE antibodies in challenged piglets using IDEXX gE ELISA kit. PRV gE antibodies were tested and reported as blocking ratios. A ratio < 0.6 was considered positive.

3.7. Challenge of piglets with PRV SD1404

As shown in Fig. 8, PRV gE antibodies were positive from day 6 post-challenge in all PRV SD1404 inoculated piglets of groups 1–3. At 2–6 days after challenge, fever was recorded in PRV SD1404 inoculated piglets of groups 1–3 with rectal temperature about 41.0 °C. At 3 days after challenge, shivering, depression, emaciated, anorexia and muscle tremors in trail legs were observed (Fig. 9A). At 7 days after inoculation, temperatures and appetites returned to normal, but piglets were obviously weak and emaciated. Symptoms of group 3 were most severe and average body weight was lowest (Table 4). Pathologic examination showed brain hemorrhage, bleeding in myocardium, cell infiltration in lung, local necrosis in liver, spleen, kidney and tonsil, and edema in lymph node in the challenged piglets (Figs. 9B–I). Histopathologic examinations showed that multiple lesion sites were observed in brain cortex, myocardial fibers, alveolar ducts and terminal bronchiolar cavities, liver cells, splenic cord, renal corpuscle cells, tonsil tissue and lymphoid nodules (Fig. 10). The PRV gE antibodies were negative and the temperature, clinical signs, pathologic and histopathologic

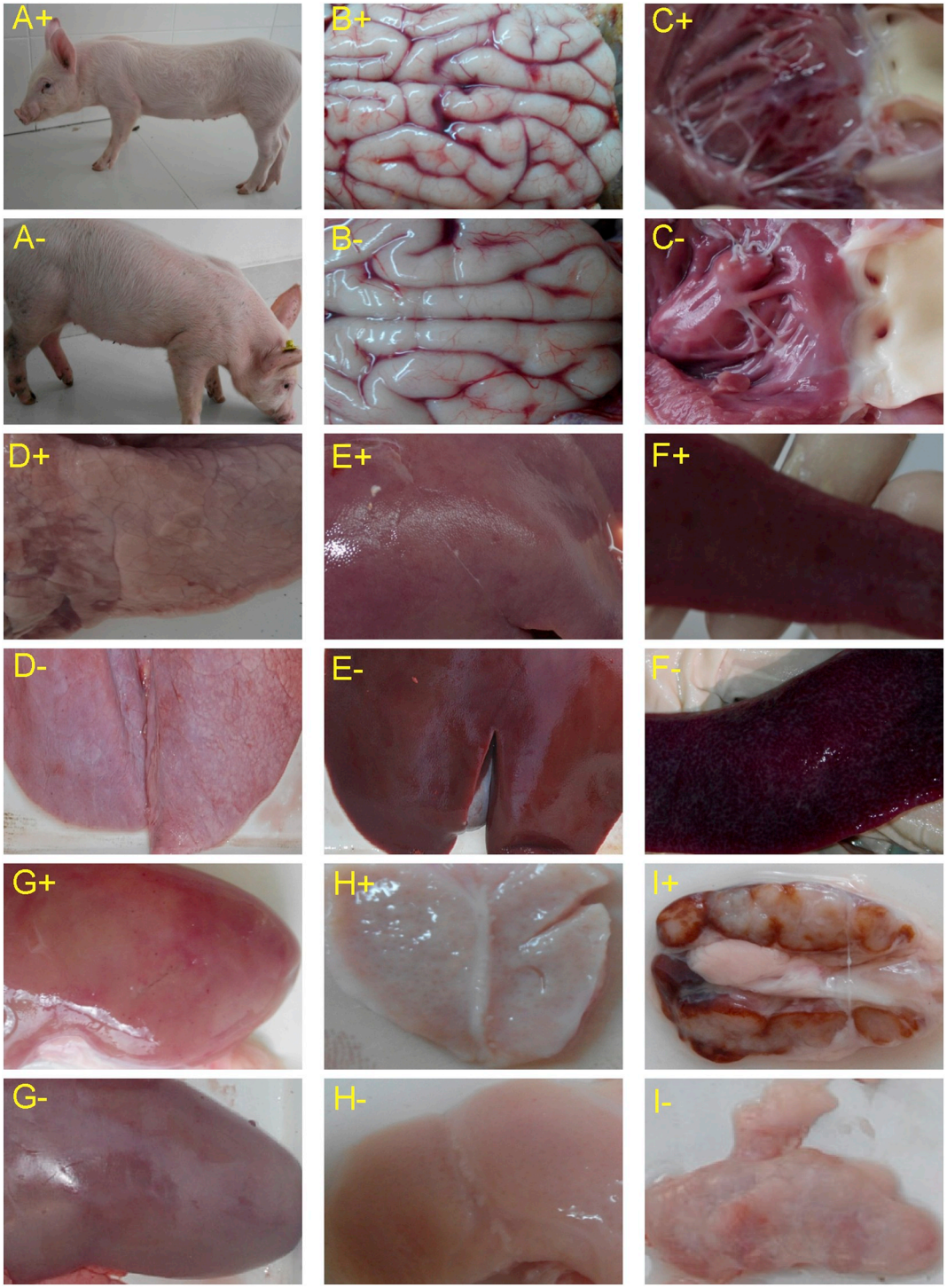

Fig. 9. Piglets and necropsy specimens in the challenge experiment. (A+) Piglet infected with PRV SD1404. (A-) Piglet free of PRV infection. (B+) Encephalic hemorrhage of brain of piglet challenged with $2 \times 10^{8.5}$ $TCID_{50}$ of PRV SD1404 strain. + means organs of piglet challenged with $2 \times 10^{8.5}$ $TCID_{50}$ of PRV SD1404 strain, the same with follows. (B-) Normal brain of control piglet. - means organs of control piglet, the same with follows. (C+) Bleeding in myocardium. (D+) Lung necrosis. (E+) Liver edema. (F+) Spleen infarct. (G+) Kidney with bleeding spots. (H+) Tonsil necrosis. (I+) Edema and hemorrhagic in lymph node.

examinations were normal in all piglets of group 4. In addition, all PRV SD1404 inoculated piglets of groups 1–3 were gE gene positive by PCR, while all piglets of group 4 were negative (Table 4).

4. Discussion

Pigs infected with virulent PRV showed high fever, depression, diarrhea, shivering, anorexia, respiratory distress, cough and a higher risk for death in newborn piglets (An et al., 2012; Yu et al. 2012; Wang et al., 2014). The outbreak of PRV occurred in Bartha-K61-vaccinated pig farms in China has caused great economic losses to the swine industry (Tong et al., 2015; Ye et al., 2015; Tang et al., 2017). The clinical signs in new born piglets are sudden, spanning about 5 h from onset to death (Wang et al., 2014). The mortality of infected neonatal piglets is up to 50% (An et al., 2012; Yu et al. 2012). It has been demonstrated that the Bartha-K61 vaccine is effective against the lethal challenge

Table 4
Symptoms, body weights and PRV SD1404 gE gene detection in brain tissue sample of each piglet inoculated with PRV SD1404 or PBS.

Groups	Piglet No.	Inoculum	Symptom scores [a]	Weights (kg)	gE PCR [b]
1	1–1	$1 \times 10^{7.5}$ $TCID_{50}$ PRV	+	11.3	+
	1–2	$1 \times 10^{7.5}$ $TCID_{50}$ PRV	+	11.0	+
	1–3	$1 \times 10^{7.5}$ $TCID_{50}$ PRV	+	12.0	+
	1–4	$1 \times 10^{7.5}$ $TCID_{50}$ PRV	+	11.8	+
	1–5	$1 \times 10^{7.5}$ $TCID_{50}$ PRV	+	11.0	+
2	2–1	$5 \times 10^{7.5}$ $TCID_{50}$ PRV	+	11.2	+
	2–2	$5 \times 10^{7.5}$ $TCID_{50}$ PRV	+	11.7	+
	2–3	$5 \times 10^{7.5}$ $TCID_{50}$ PRV	+	11.9	+
	2–4	$5 \times 10^{7.5}$ $TCID_{50}$ PRV	+	11.1	+
	2–5	$5 \times 10^{7.5}$ $TCID_{50}$ PRV	+	11.0	+
3	3–1	$2 \times 10^{8.5}$ $TCID_{50}$ PRV	+ +	9.7	+
	3–2	$2 \times 10^{8.5}$ $TCID_{50}$ PRV	+ +	8.5	+
	3–3	$2 \times 10^{8.5}$ $TCID_{50}$ PRV	+ +	9.3	+
	3–4	$2 \times 10^{8.5}$ $TCID_{50}$ PRV	+ +	9.1	+
	3–5	$2 \times 10^{8.5}$ $TCID_{50}$ PRV	+ +	9.5	+
4	4–1	PBS	−	12.2	−
	4–2	PBS	−	12.5	−
	4–3	PBS	−	12.6	−
	4–4	PBS	−	12.1	−
	4–5	PBS	−	12.2	−

[a] , + + represents severe symptoms; + represents mild symptoms; − represents no obvious symptoms.
[b] , + represents positive result; − represents negative result.

with the classical PRV SC strain, but does not provide full protection against the emerging PRV variant strain (An et al., 2012), indicating that the new outbreak PRV has different immunogenicity as compared to Bartha-K61. In this study, we reported one case of PRV-infected piglets in one Bartha-K61-vaccinated farm in Weifang, Shandong Province, China. The sick piglets presented with multiple clinical signs, including high fever (≥40.5 °C), depression, anorexia, weight loss and neurologic symptoms. What surprised us was that the piglets did not die. The etiological agent of PRV SD1404 caused the piglets to be sick, emaciated and grow very slowly, so economic losses to the farm was great. It is necessary and important to characterize the genetic variations and pathogenicity of the PRV SD1404 strain.

In order to find out the pathogen of piglets in the farm, PRV SD1404 strain was isolated and then gE, gD, TK and gM genes of PRV SD1404 were sequenced. It was shown that there were about 15% amino acid sites with mutations, deficiencies and insertions of PRV SD1404 gE gene (Fig. 3). Results of phylogenetic analysis of PRV gE gene showed that PRV SD1404 strain was clustered to an independent branch together with some recent PRV isolates in China (Fig. 7). It is well known that gD is one of critical factors that mediate cell attachment of PRV (Luo et al., 2014). Compared to Bartha-K61 vaccine strain, the gD gene of PRV SD1404 strain had two amino acid site insertions at positions 278–279 and ten amino acid site mutations (Fig. 4). Whether the insertions or mutations in the gD gene of PRV SD1404 strain are responsible for the escape from the immune response induced by Bartha-K61 vaccination needs to be further studied.

In order to further confirm and evaluate the pathogenicity of PRV SD1404 strain, we conducted inoculation experiments in chicken embryos and rabbits. PRV SD1404 strain could kill the chicken embryos and the LD_{50} was $1 \times 10^{3.3}$ $TCID_{50}$ in 200 μl PBS.

Rabbits are extremely sensitive to PRV infection and typical symptoms observed in PRV-infected rabbits are itching, convulsions, nibble at injected position and difficult breathing (Pomeranz et al., 2005). So rabbits are commonly used as laboratory animals to test the safety and inactivation of PRV vaccine. Rabbit challenge experiment was chosen to further confirm that the isolated virus was PRV in our study and typical symptoms were observed. 100% of the infected rabbits died, which are typically used for observing the efficacy of virus challenge in quality standards for various virus strains (Maeda et al., 2002).

To further demonstrate whether the isolated PRV SD1404 strain is responsible for the sickness of piglets in the farm, challenge experiment of piglets with PRV SD1404 was conducted. The challenged piglets presented with multiple clinical signs, including high fever, depression, emaciated, anorexia and muscle tremors in trail legs (Fig. 9A). What surprised us was that the virus didn't cause piglets to die. We conducted the challenge experiment for 3 times, all results showed that PRV SD1404 strain could not result in the death of piglets. Pathologic and histopathologic examinations further confirmed that the strain was moderately pathogenic (Figs. 9B–I and 10). The natural transmission of PRV occurs mostly via direct nose-to-nose contact between pigs. It was found that the mortality was 3/5 and 5/5 when 6-week-old healthy pigs were challenged intramuscularly or intranasally with $1 \times 10^{6.0}$ $TCID_{50}$ of the highly pathogenic PRV TJ strain, and the mortality was 3/5 when 5-week-old healthy pigs were inoculated intramuscularly with $1 \times 10^{5.0}$ $TCID_{50}$ of PRV TJ strain (Luo et al., 2014). However, PRV SD1404 strain could not cause 5-week old piglets to die even if we used a higher dose of $2 \times 10^{8.5}$ $TCID_{50}$ via intramuscular challenge in our study. Recent study demonstrated that the thymidine kinase (TK) and glycoprotein M (gM) were related to the virulence of PRV (Tang et al., 2017). However, the TK and gM genes of PRV SD1404 strain were the same as the highly pathogenic PRV TJ strain (Luo et al., 2014) (Figs. 5 and 6). Based on gE gene, there were three amino acid site mutations of PRV SD1404 strain at positions 122, 448 and 510 in comparison with TJ strain (Fig. 3). In addition, there were only two mutations at positions 575–576 in gE gene between PRV SD1404 and HeN1 strain, while gD, TK, and gM were all the same (Figs. 3–6). Whether these mutations of gE or other genes determine the pathogenicity of PRV SD1404 strain needs to be further studied.

In summary, we reported a moderately pathogenic strain named PRV SD1404, which was the etiological agent of piglet disease in the farm in Weifang, Shandong Province, China. Piglets infected with PRV SD1404 strain showed anorexia, weight loss and neurologic symptoms. PRV SD1404 strain is not lethal for piglets but can cause economic losses to pig farms, which should be paid more attention.

Competing financial interests

The authors declare that they have no competing interests.

Author contributions

M.Z., X.W., D.J. and C.S. designed and performed the experiments; L.C., X.C., X.X. and G.W. prepared the reagents and samples; Y.L., F.T., Z.C. and H.Z. were involved in data discussion; J.Q., Z.W., J.W., H.S. and Y.D. initiated the study, designed the experiments and wrote the manuscript. All authors reviewed the manuscript.

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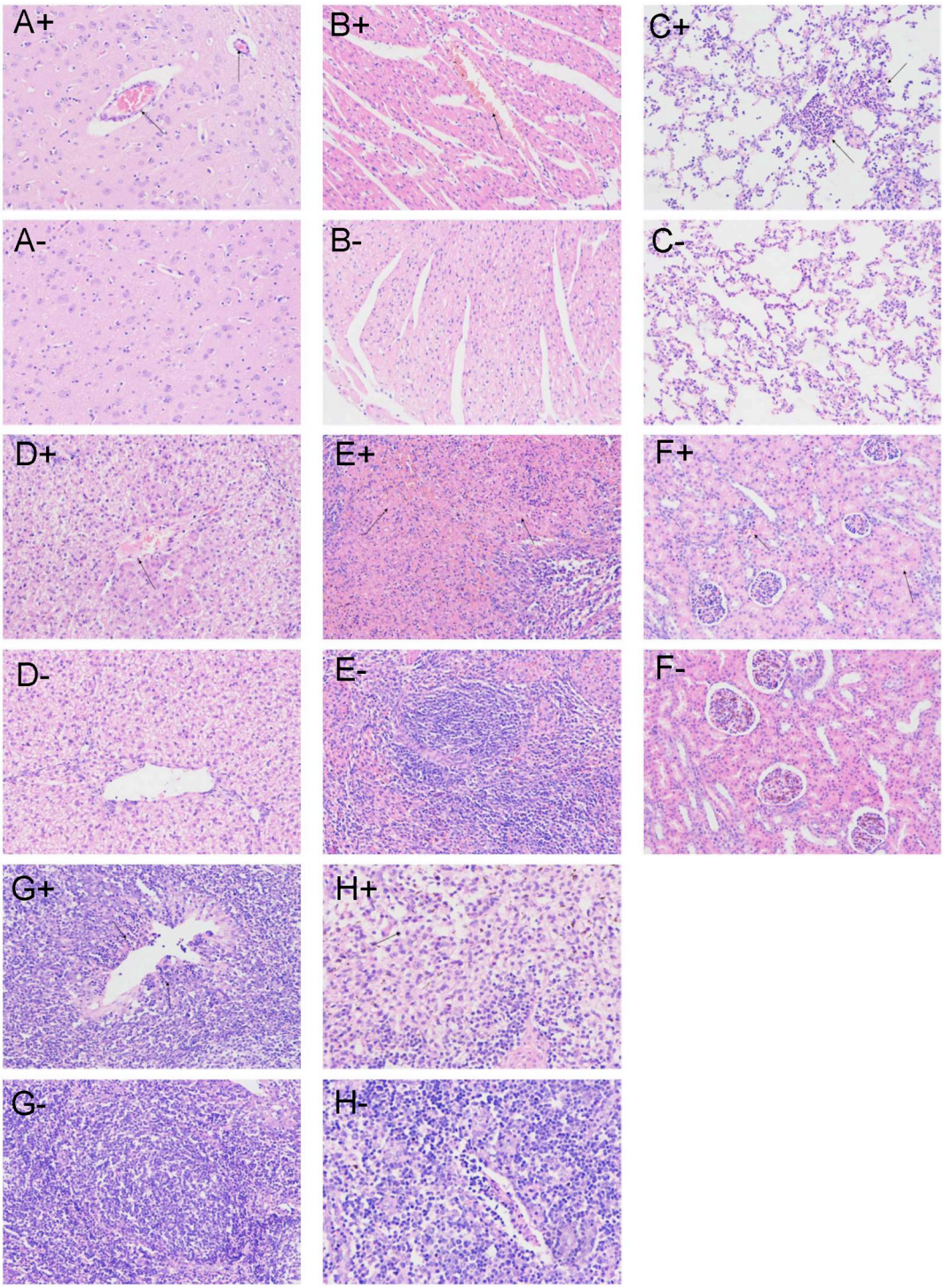

Fig. 10. Histopathologic examinations of the tissue samples of brain, heart, lung, liver, spleen, kidney, tonsil and lymph node. Original magnification × 200. (A+) Lymphocyte infiltration around the small blood vessels in the brain cortex of piglet challenged with $2 \times 10^{8.5}$ $TCID_{50}$ of PRV SD1404 strain. + means organs of piglet challenged with $2 \times 10^{8.5}$ $TCID_{50}$ of PRV SD1404 strain, the same with follows. (A-) Histopathologic examination of normal brain of control piglet. - means histopathologic examinations of organs of control piglet, the same with follows. (B+) Breakage and disintegration of myocardial fibers. (C+) Alveolar ducts and terminal bronchiolar cavities filled with cellular and serous exudates. (D+) Swelling and degeneration of liver cells. (E+) Splenic cord with unclear structure and reduced lymphocytes. (F+) Swelling and disintegration of renal corpuscle cells. (G+) Tonsil tissue necrosis, epithelial cells filled with eosinophilic intranuclear inclusions. (H+) Reduced lymphoid nodules with irregular structures.

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