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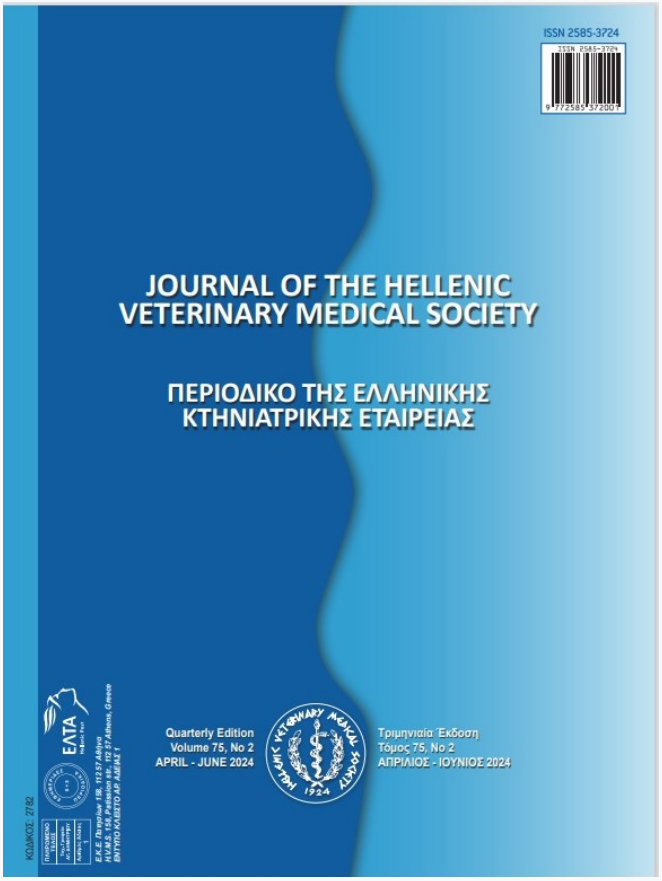

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Research article
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Isolation, identification and genetic variation analysis of pseudorabies virus mutant strain from Chinese pig farm

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ABSTRACT: Pseudorabies (PR) is an important infectious disease affecting pig farms worldwide. In this study, we reported a pseudorabies virus (PRV) isolated from a Bartha-K61-vaccinated pig farm in Linyi, Shandong Province, China. Evidence from virus isolation, electronic microscope observation laboratory animal infection, and histopathologic examination confirmed that the etiological agent of the disease is PRV SD-2017. Sequence alignment of the gE gene indicated that it belongs to a new mutated PRV strain. The gB sequence alignment showed that compared with the Bartha and Kaplan genotype 1 strains, SD-2017 strain had a deletion of three amino acids at positions 75-78 (SPG) and an insertion of one amino acid at position 94 (G). The gC gene had 7 amino acids (A, A, A, S, T, P and A) inserted at positions 69-75 in comparison with the Bartha-K61 vaccine strain. The median lethal dose (LD50) of PRV SD-2017 strain on rabbits was $3.16\times10^{2.0}$TCID50 by Karber method. Histopathologic examinations showed that multiple lesion sites were observed in brains, lungs, livers and kidneys. PRV SD-2017 is different from other reports and should be paid more attention to avoid economic losses.

Keywords: *Pseudorabies virus; Sequence alignment; Amino acid mutations; Mutant strain*

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INTRODUCTION

Pseudorabies virus (PRV), also known as Suid Herpesvirus 1 (SuHV1), belongs to the family *Herpesviridae*. It is the causative agent of pseudorabies (PR), also known as Aujeszky's disease (AD) (Mettenleiter, 2000). PRV can infect pigs (the only natural host), ruminants such as cattle and sheep, carnivores such as mink, and rodents, with a mortality rate close to 100% (Pensaert, 1989). The disease is an acute, severe, highly contagious infectious disease that causes fever, extreme itching (except in pigs) and encephalomyelitis. PRV is mainly transmitted through the exchange of saliva between animals, nasal secretions and particulate matter in the air (Wozniakowski and Samorek-Salamonowicz, 2015). PRV can be transmitted in multiple species and can infect humans, posing a public health safety risk (Wong *et al.*, 2019). Typical symptoms of PRV infection in humans include weakness, fever, sweating, difficulty swallowing, and neurological disorders. Available case studies reported that PRV infection can cause endophthalmitis and encephalitis in humans (Ai *et al.*, 2018; Yang *et al.*, 2019; Yang *et al.*, 2019).

PR had been popular since the early 1970s and spread almost globally after 1980(Freuling *et al.,* 2017; Müller *et al.,* 2011). At present, PR is still one of the important infectious diseases that need to be controlled in the pig breeding industry worldwide, especially in areas with intensive breeding (Müller *et al.,* 2011). Since the introduction and application of Hungary's Bartha-K61 strain vaccine, the PR epidemic in China had been controlled relatively stable. However, since the emergence of PRV variants in 2011, PR outbreaks had occurred in pig farms that had been immunized with the Bartha-K61 vaccine in many provinces and regions in China (Wu *et al.,* 2017; Yu *et al.,* 2014). Bartha-K61 vaccines showed reduced protection against the variant, although this view was controversial (An *et al.,* 2013; Gu *et al.,* 2015). PRV variants have spread in northern, eastern and southern China, which seriously threatens the healthy and sustainable development of the pig breeding industry (Sun *et al.,* 2018; Zhai *et al.,* 2019).

In this study, PRV detection and virus isolation were performed on specimens collected from a pig farm suspected of having a PR outbreak in Linyi, Shandong, China. The PRV virus was successfully isolated from PK-15 cells, and the isolated SD-2017 strain was proved to be a PRV variant by sequencing analysis. Through the genetic variation analysis of gE, gB and gC virulence genes, it was found that there were different degrees of variation in the main virulence genes of PRV SD-2017 strain. This study lays the foundation for the follow-up study of PRV virus characteristics and the development of new genetically engineered vaccines.

MATERIALS AND METHODS

Clinical samples

Six piglets at 2 weeks of age suspected of porcine pseudorabies infection in a pig farm in Linyi Shandong province, China, were sent for inspection, in Dec.2017. The main symptoms of piglets were fever, anorexia, depression, and then appear shivering, ataxia, hind limb paralysis, intermittent spasms, limb scratching and other neurological symptoms. Some piglets appeared vomiting, diarrhea symptoms. Samples (brain, lung, spleen, kidney, and inguinal lymph nodes) were collected after necropsy. Samples for viral isolation were stored at -80°C until use. 2~3 g of homogenized tissue samples were frozen and thawed three times and were centrifuged at 5000×g for 10 min at 4°C. The resulting supernatant was filtered by a needle type filter (0.22 μm) and stored at -80°C until use (An *et al.,* 2013).

Cell Lines and Virus

The Porcine kidney cell line (PK-15, ATCC CCL-33) was used to culture PRV. PK15 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, USA) at 37°C in 5% CO2 a humidified incubator. PRV Bartha-K61 vaccine strain and pMD19-T vector were preserved by the Key Laboratory of Preventive Veterinary Medicine of Shandong Province.

Viral genome extraction and PCR

DNA was extracted from the above treated clinical samples supernatant by extraction kit (TIANGEN, China). Referring to the PRV TJ strain in GenBank (accession No. KJ789182.1) and literature (Fan et al., 2016; Yu et al., 2016; Zhou et al., 2019), specific primers (Table 1) for amplifying the whole genes of PRV gE, gD, TK, gB, gC, gI and gM were designed by Primer Premier5.0 (Premier, Canada). Primers were synthesized by Shanghai Sangon Co., Ltd. The amplification was performed in a 50 μl reaction mixture containing 2.5U PrimeSTAR HS DNA Polymerase (TaKaRa, Beijing), 2×PrimeSTAR GC Buffer (Mg^{2+}plus), 2.5 mM of each dNTP, 10 μM of each

primer, and 200 ng of DNA. The reaction was run in a thermocycler with the following program: denaturation at 98 °C for 1 min; followed by 35 cycles of 98 °C for 10 s, gE,gB,TK and gC 60 °C for 5 s, gM 68 °C for 2min, gI 55 °C for 15 s, and 72 °C for 2 min; and a final extension at 72 °C for 5 min. The PCR products were subjected to electrophoresis on 1% agarose gel. The all target genes was recovered and ligated into the pMD19-T vector, transformed into DH5α competent cells, and positive clones were selected. The plasmids were extracted by High Pure Maxi Plasmid Kit (TIANGEN, China) from the positive clones and sent to Shanghai Sangon Co., Ltd for sequencing.

Virus isolation and purification

PK-15 cells were subcultured, and when the confluence reached about 70% in T25 cell flasks, 1.0 mL of the processed supernatant of clinical samples was inoculated with PK-15 cells for virus isolation and culture. 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 six times by virus plaque formation (Gu *et al.,* 2015). The virus was designated as PRV SD-2017. PRV Bartha-K61 strain was used as a control for plaque-purification, which was obtained from Shandong Huahong Biological Engineering Co., LTD (Huahong).

Observation of virus morphology

The purified PRV was seeded into PK-15 cells at 70% confluence in T25 cell flasks. Cultured at 5% CO2 and 37°C for 48~56h. The culture supernatant was harvested, and then the virus was purified use the ultracentrifuge (Beckman, L-80XP, Germany) by sucrose density gradient centrifugation (55%, 45%, 35%, 25%, 15% gradient sucrose solutions were added in sequence). The purified samples were sent to the Central Laboratory of Qingdao Agricultural University for transmission electron microscopy (Hitachi, HT7700, Japan) observation.

One-step growth kinetics of PRV

One-step growth kinetics was conducted as described previously (Smith and Enquist, 1999). PK-15 cells were grown in 96-well plates to 80% confluence and infected with PRV SD-2017 strain at the multiplicity of infection (MOI) of 1.0. At 4 h, 8 h, 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h postinfection (hpi), the supernatants were collected and titrated by a microtitration infectivity assay and recorded as TCID50/ml. The TCID50 of the virus was calculated by Reed-Muench method. All assays were performed in triplicate, and the resulting titers were averaged.

Phylogenetic analyses of PRV

We analyzed PRV SD-2017 sequence data and compared the gE, gB and gC genes with the sequences available in the GenBank database (Table 2). 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 constructed using the neighbor-joining method with the MEGA program (ver. 5.05) and bootstrap

Table 1 PRV primer sequence information

Primers	Primer sequences (5'-3')	Amplification size(bp)
PRV-gE-for	TCGCACACACCGGGGTTGAG	1734
PRV-gE-rev	GGTGGGCATGTCGGAATG	
PRV-gD-for	ATGCTGCTCGCAGCGCTATT	1220
PRV-gD-rev	TACTGCGGAGGCTACG	
PRV-TK-for	ATGCGCATCCTCCGGATCTACCT	963
PRV-TK-rev	TCACACCCCCATCTCCGACGTGAA	
PRV-gB-for	TGTACCTGACCTACGAGGCGTCATGC	2742
PRV-gB-rev	TATTTCCATCTGCGGGGAGGGGGCTA	
PRV-gC-for	GGATCCATGGCCTCGCTCGCGCGTGCGAT	1440
PRV-gC-rev	GAATTCTCACAGCGCGGACCGGCGGTAGT	
PRV-gI-for	ATGATGATGGTGGCGCG	1101
PRV-gI-rev	TTATTGTTCCTCTGCGATGGT	
PRV-gM-for	ACCATGTGCGATCGAAACGAA	1182
PRV-gM-rev	GGTCGCAACCGTATAGCATC	

Table 2 Background information in the NCBI database of reference PRV strains

PRV isolates	GenBank accession No.	Country	Year	Variant (Yes/No)	Species
Bartha	JF797217	Hungary	1960	No	Swine
SC	KT809429	China	1986	No	Swine
Ea	KU315430	China	1990	No	Swine
LA	KU552118	China	1997	No	Swine
Hercules	KT983810	Greece	2010	No	Swine
Kolchis	KT983811	Greece	2010	No	Swine
Kaplan	JF797218	Hungary	2011	No	Swine
BJ/YT	KC981239	China	2012	No	Dog
Fa	KM189913	China	2012	Yes	Swine
HN1201	KP722022	China	2012	Yes	Swine
HB1201	KU057086	China	2012	Yes	Swine
HeN1	KP098534	China	2012	Yes	Swine
TJ	KJ789182	China	2012	Yes	Swine
ZJ01	KM061380	China	2012	Yes	Swine
HNB	KM189914	China	2012	Yes	Swine
HNX	KM189912	China	2012	Yes	Swine
JS-2012	KP257591	China	2012	Yes	Swine
HLJ8	KT824771	China	2013	No	Swine
HLJ-2013	MK080279	China	2013	No	Swine
GD0304	MH582511	China	2015	Yes	Swine
MY-1	AP018925	Japan	2015	Yes	Swine
RC1	LC342744	Japan	2016	No	Racoon
HeNLH/2017	MT775883.1	China	2017	Yes	Swine
HuBXY/2018	MT468549.1	China	2018	Yes	Swine
SY7	MT150583.1	China	2018	Yes	Swine
JSY13	MT157263.1	China	2018	Yes	Swine
hSD-1/2019	MT468550.1	China	2019	Yes	Human
GD1802	MT949535.1	China	2020	Yes	Swine
SD18	MT949536.1	China	2020	Yes	Swine
HuB17	MT949537.1	China	2020	Yes	Swine

analyses were conducted using 1,000 replicates (Fan *et al.,* 1994). Phylogenetic tree was viewed using phenogram mode as described (Fauquet *et al.,* 2008).

Infection of rabbits with PRV

Three-month old healthy SPF rabbits (weight 2~2.5 kg) were randomly divided into six groups each with five. Groups 1-4 were injected intramuscularly with $1\times10^{2.0}$ TCID50, $1\times10^{3.0}$ TCID50, $1\times10^{4.0}$ TCID50 and $1\times10^{5.0}$ TCID50 of PRV SD-2017 strain in 500 μl DMEM, respectively. Group 5 was injected with 500 μl PBS. Clinical signs were checked by the state registered veterinarian daily. After the challenge, rabbits with serious clinical symptoms of depression, pruritus and anorexia at the same time were judged as the humanitarian endpoint. Rabbits were euthanized by intravenous injection of 100 mg/kg sodium pentobarbital and the death was counted as a statistical result. All surviving rabbits were humanely euthanized on day 7 post-challenge. The median lethal dose (LD50) was calculated and determined using the Karber method (Zhang *et al.,* 2021). The brain, lungs, livers and kidneys tissue samples were collected from each rabbit to detect PRV using the gE-specific PCR as mentioned above. The paraffin imbedding tissue samples were cut into 4 μm-thick sections, stained with HE, and pathological changes were observed under an optical microscope. All rabbit experiments were performed in the standardized animal room. In accordance with the protocols approved by the Animal Care and Ethics Committee, under the number 201907003, which adhere to the"Guidelines for ethical review of Laboratory Animal Welfare"(GB/T 35892-2018).

RESULTS

PCR amplification of PRV partial genes

We amplified the main PRV virulence genes gE, gD, TK, gB, gC, gI and gM respectively, and ligated

into the pMD19-T vector (Fig.1). The main genomic information of gE, gD, TK, gB, gC, gI and gM were published in GenBank (Acc. No. MW535259-MW535265).

PRV isolation and purification

The filtrate of tissue supernatant identified as positive for PRV gE gene by PCR was inoculated into PK-15 cells cultured in T25 cell flasks. At 36 hours post infection, obvious CPE was observed in SD-2017 infected PK-15 cells (Fig.2A), and similar CPE was seen in positive control cells seeded with PRV Bartha-K61 (Fig.2B), whereas no CPE was observed in control PK-15 cells (Fig.2C). The culture was successively passaged for 6 times, all of which could produce obvious CPE, and the cell cultures of each passage were all positive for PRV gE gene by PCR (data not shown).

Observation of virus morphology

After the PK-15 cell culture of the sixth-generation PRV SD-2017 strain was concentrated by ultracentrifugation, many obvious virus particles were found in transmission electron microscopy (Fig. 3). Virus particles were spherical, with a diameter of about 140-180 nm. There was a thick capsule with radial fibers on the surface of the capsule. And the particle core was dense, with the typical virion structure of herpes virus.

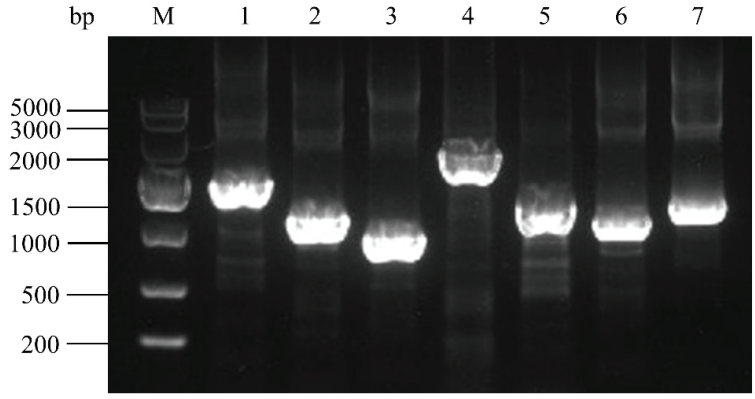

Fig.1. PCR amplification of PRV virulence genes.

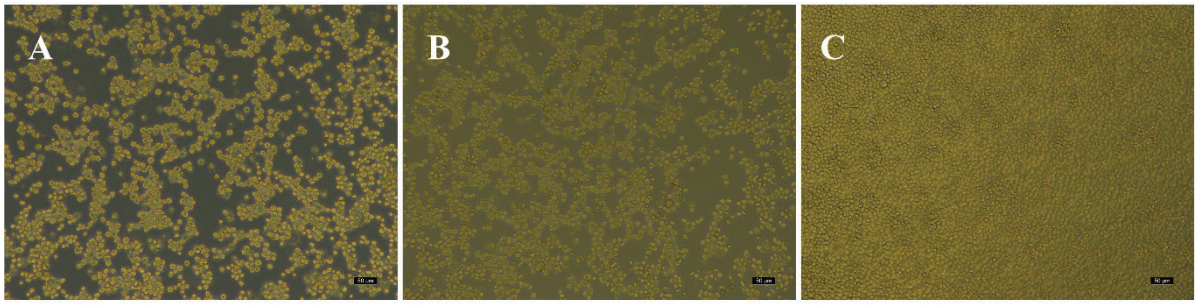

Fig.2. Obvious CPE produced in PK-15 cells infected with PRV strains (36h).

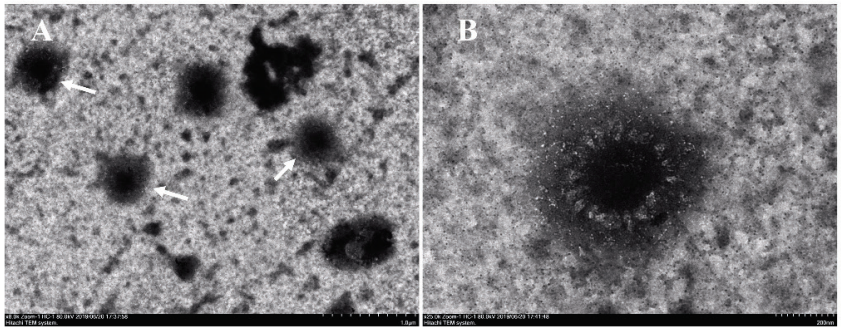

Fig.3. Electron microscopic observation of virus particle morphology (A.8000×, B.40000×).

One-step growth curves of PRV

The virus titer of PRV SD-2017 strain measured by Reed-Muench method at 48h had reached $10^{9.0}$TCID50/ml (Fig. 4). This indicated that PRV SD-2017 strain had good propagation characteristics in PK-15 cells.

Phylogenetic analyses of PRV

We uploaded the sequencing results of the main virulence genes gB, gE, gC, gD, TK, gI and gM of PRV SD-2017 strain to the GenBank under the accession numbers MW535259, MW535262, MW535260, MW535261, MW535265, MW535263 and MW535264. We compared the gE, gB, and gC gene of PRV SD-2017 with other PRV strains available in the GenBank database (Table 2). Compared with genotype I isolates, SD-2017 strain had insertion and substitution mutations at the gE amino acid site. Compared with the classic strains Kaplan, Hercules and Kolchis, SD-2017 strain had amino acid (D) inserted at positions 48 and 497, which was the typical of PRV popular variants. In addition, gE had amino acid site substitutions at positions 54 (G→D), 59 (D→N), 63 (N→D), 149 (R→M), 179 (T→S), 181 (Q→L), 503 (A→I), 503 (S→A), 521 (V→A) (Fig. 5).

The gB sequence alignment showed that compared with the Bartha and Kaplan genotype 1 strains, SD-2017 strain had a deletion of three amino acids at positions 75-78 (SPG) and an insertion of one amino acid at position 94 (G). In addition, gB gene had amino acid substitution at positions 53 (A→T), 55 (P→T), 70 (T→A), 72 (V→G), 73 (P→T), 81 (N→D), 82 (D→G), 83 (V→F), 87 (A→E), 93 (E→D), 96 (F→V), 102 (E→D), 454 (R→K), 553 (G→S), 571 (S→G) (Fig.6).

Compared with genotype I strains, SD-2017 strain and most Chinese isolates gC had 7 amino acids (A, A, A, S, T, P and A) inserted at positions 69-75. However, this variation was absent in GD1802 and HLJ-2013 isolates. In addition, compared with Bartha, Kaplan, Hercules, Kolchis and SC strains, SD-2017 strain and Chinese isolates after 2011 have amino acid substitutions at positions 16 (A→T), 25 (T→S), 52 (P→S), 61(A→E), 63(A→V), 82(A→V), 93(P→Q), 96(N→G), 108(A→S), 136(F→V), 148(Y→C), 200(G→E), 437(L→M), 443(V→I), 455(A→T), 463(S→T), 467(V →T) and 473(G→A) (Fig. 7).

We further analyzed the relationship of PRV SD-2017 strain with other PRV isolates using a phylogenetic tree based on the gE gene. Phylogenetic analysis of PRV gE gene showed that PRV SD-2017 strain was clustered to an independent branch together with recent PRV isolates in China, such as HeNLH/2017 and HLJ8 strains (Fig. 8).

Infection of rabbits with PRV

Two days after infection, itching and nibble at injected position was observed in all rabbits of groups 1-4. Subsequently, nerve symptom appeared. The rabbits were euthanized according to their symptoms. However, the rabbits of group 5 were normal and healthy during the experiment and humanely euthanized on day 7 post-challenge. The results of PCR detection showed that all rabbits of groups 1-4 were PRV SD-2017 strain positive while all rabbits of group 5 were negative. The median lethal dose (LD50) of PRV SD-2017 strain on rabbits was $3.16\times10^{2.0}$ TCID50 by Karber method. Histopathologic examinations showed that multiple lesion sites were observed in brains, lungs, livers and kidneys (Fig. 9).

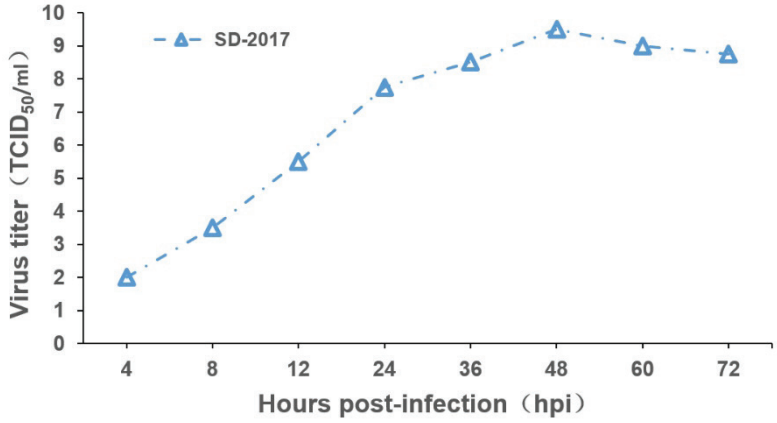

Fig.4. One-step growth curves of PRV SD2017 strain in PK-15 cells.

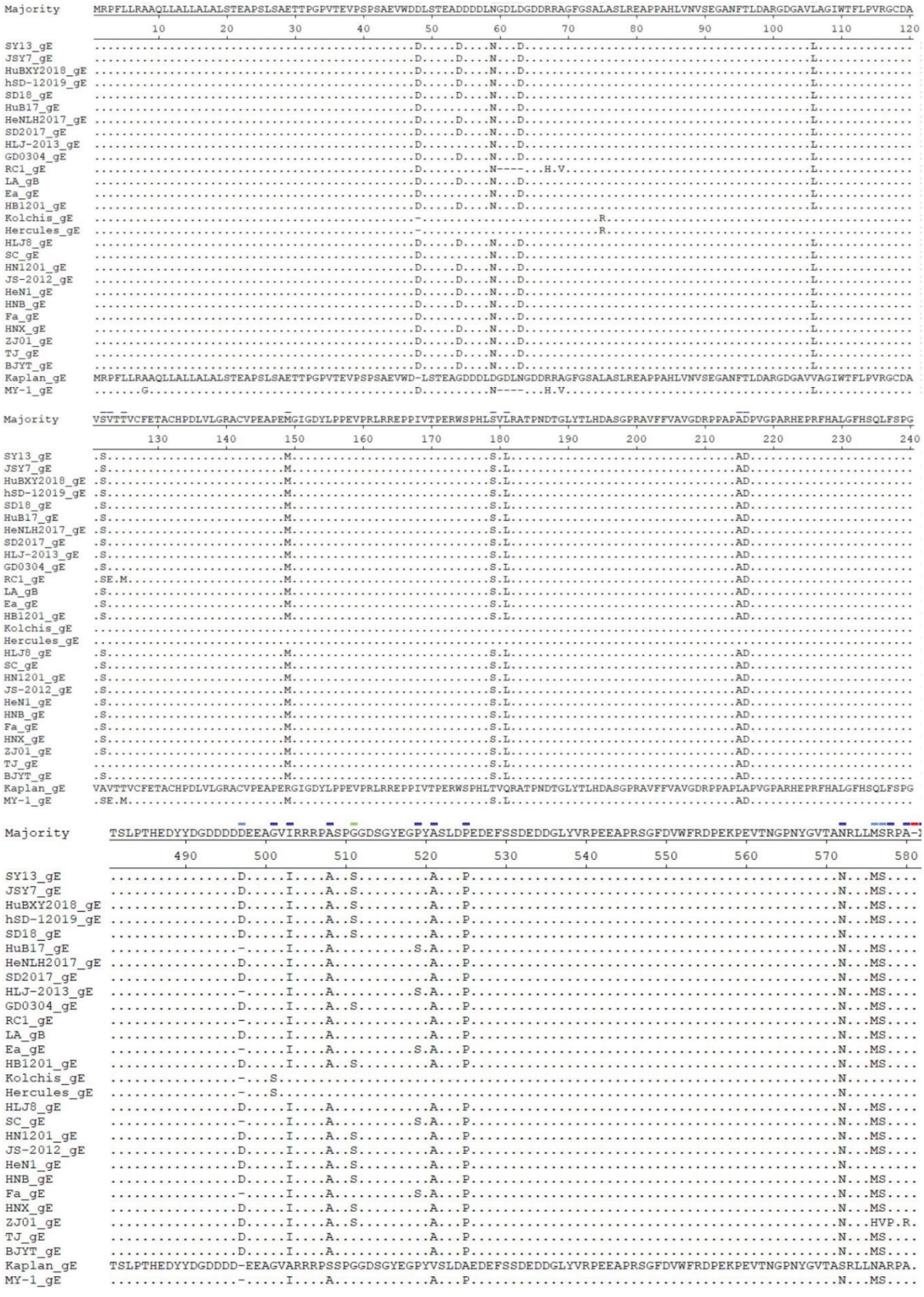

Fig.5. Comparison of PRV SD-2017 gE amino acid sequence with other PRV strains.

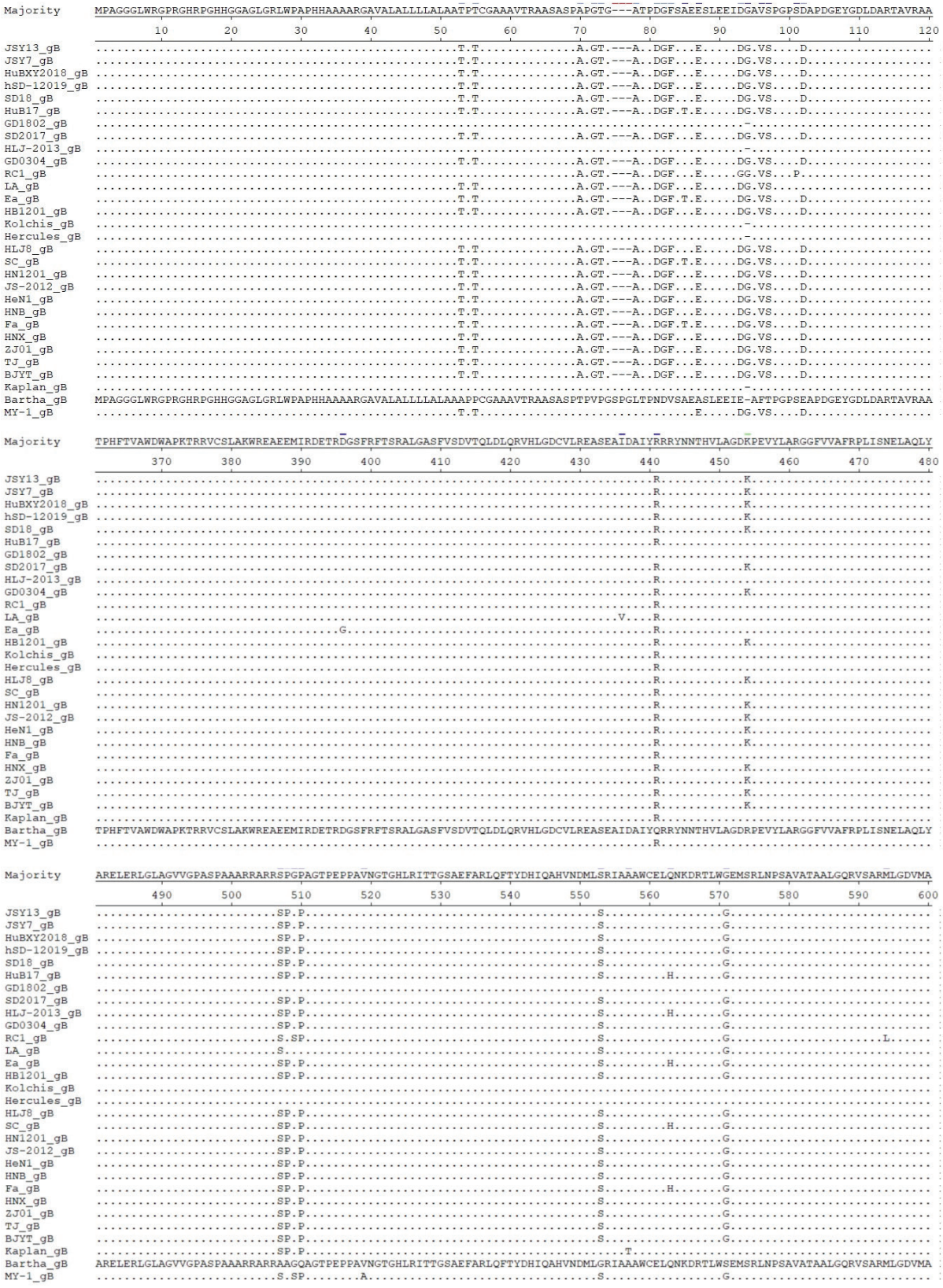

Fig.6. Comparison of PRV SD-2017 gB amino acid sequence with other PRV strains.

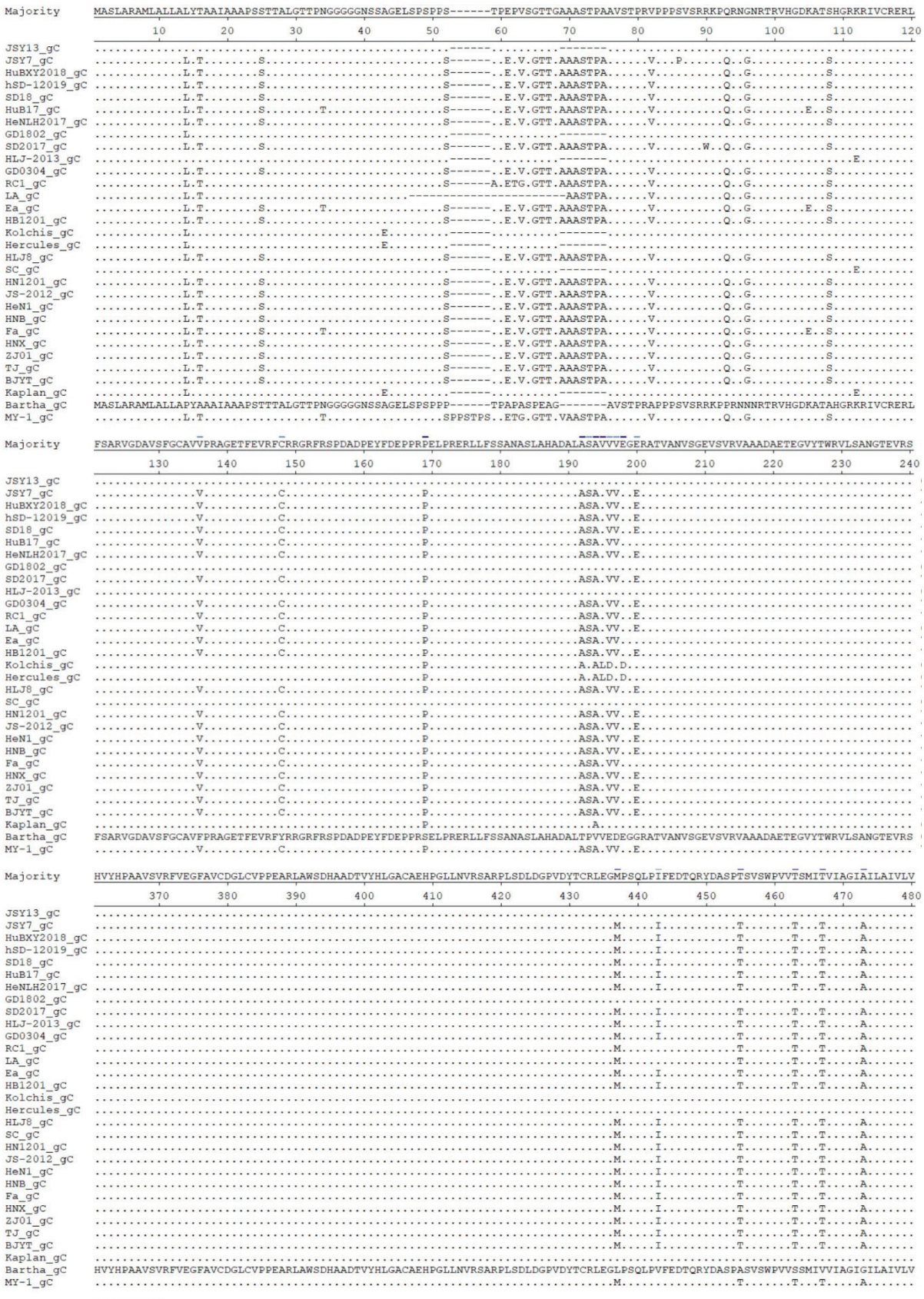

Fig.7. Comparison of PRV SD-2017 gC amino acid sequence with other PRV strains.

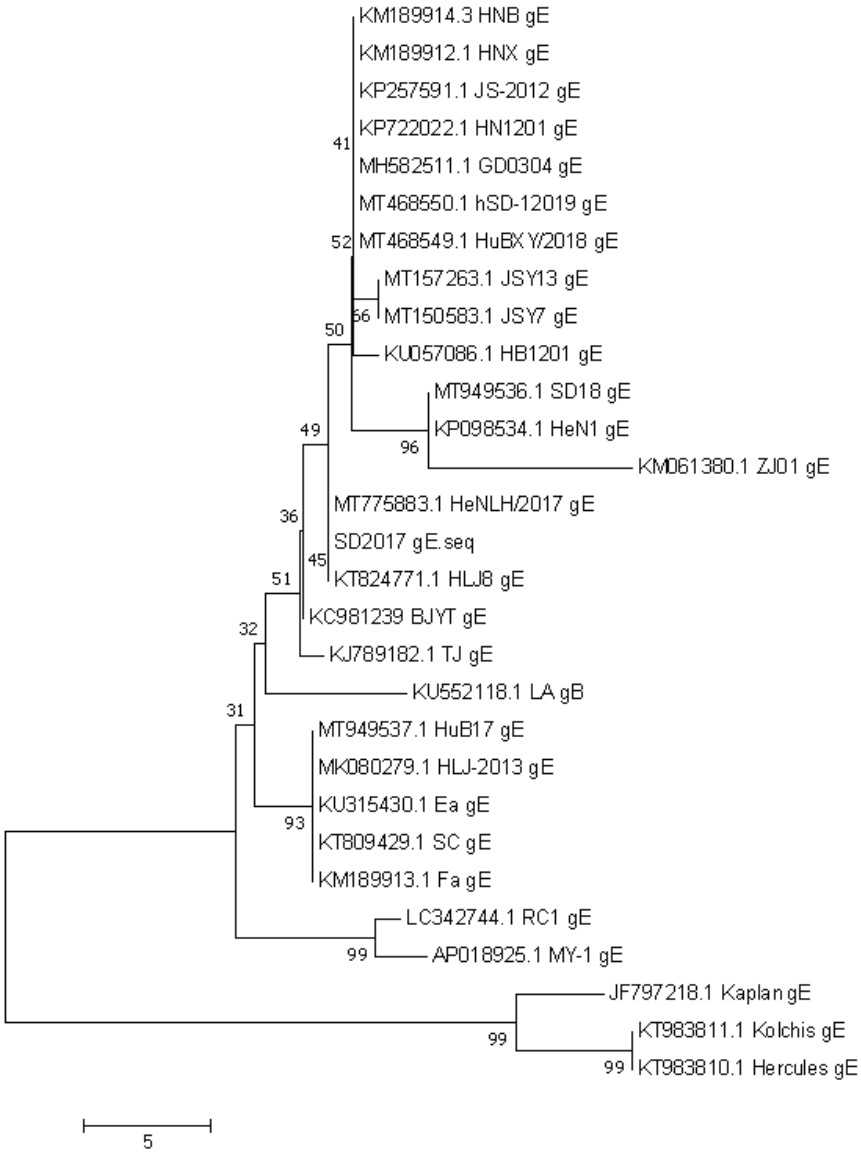

Fig.8. Phylogenetic analysis of PRV isolates based on the gE gene sequence.

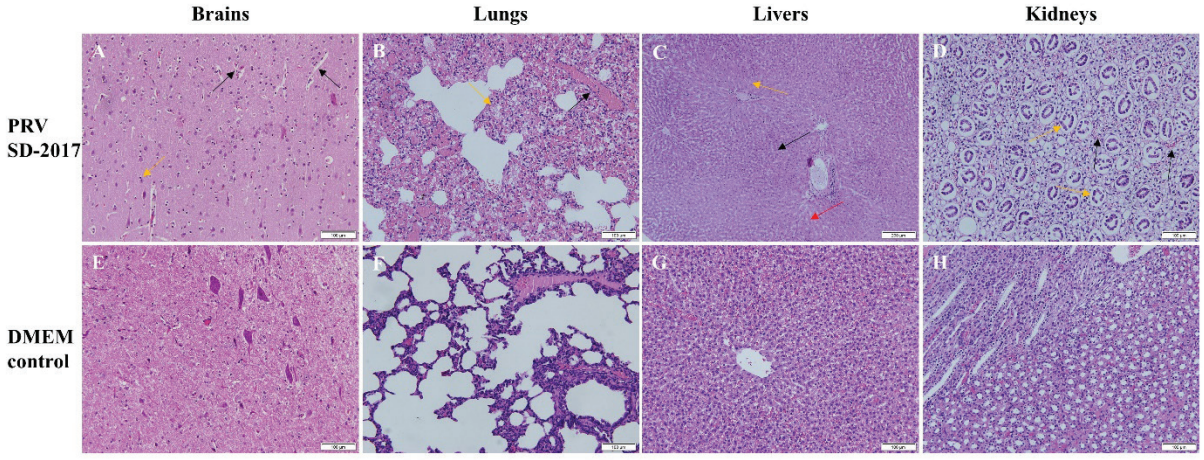

Fig.9. Histopathologic examinations of the tissue samples of brains, lungs, livers and kidneys.

DISCUSSION

Pseudorabies was one of the important infectious diseases that threatens the pig industry. Newborn piglets infected with virulent strains of PRV often exhibit high fever, depression, diarrhea, tremors, anorexia, respiratory distress, cough, and high mortality (An *et al.,* 2013; Wang *et al.,* 2014; Yu *et al.,* 2014). Affected by viral immune evasion and natural genetic evolution, PRV variant outbreaks had occurred in pig farms vaccinated with Bartha-K61 vaccine in China since 2011, causing serious damage to pig herds (Tang *et al.,* 2016; Ye *et al.,* 2016; Ye *et al., 2*015). Newborn piglets infected with PRV mutants develop sudden onset with significant clinical symptoms, which lasts only about 5 hours from onset to death, and the mortality rate was as high as 50% (An *et al.,* 2013; Wang *et al.,* 2014; Yu *et al.,* 2014). The Bartha-K61 vaccine was effective against lethal challenge with PRV SC classic strains, but did not provide comprehensive protection against PRV variant strains. It indicated that the PRV variant had antigenic changes and had different immunogenicity compared with the Bartha-K61 strain. Therefore, the generation of vaccines that antigenically more closely match emerging variant PRV strains may represent an added value to control these infections.

In this study, we successfully isolated a highly pathogenic PRV strain named SD-2017 from PRV-infected piglets in a Bartha-K61 vaccinated pig farm in Linyi, Shandong, China. Through virulence gene sequencing and genetic evolution analysis, the isolated SD-2017 strain was identified as a PRV epidemic variant. Homology comparison and genetic evolution analysis of the main virulence genes gE, gB and gC of PRV SD-2017 strain were carried out. The nucleotide homology of gE genes of 2017, HuB17, MY-1 and other mutant strains is between 99.4% and 100%, and there is an aspartic acid at positions 48 and 497 of the gE protein amino acid sequence (D), which is consistent with the mutation characteristics of the PRV variant strains prevalent in China. The gE protein is a major virulence protein of PRV (Wang *et al.,* 2015). It has been reported that changes in only a few gE amino acids can alter the virulence of PRV isolates (Mettenleiter *et al.,* 1994). The gB protein is the most conserved glycoprotein among herpesviruses and is essential for PRV to invade target cells and facilitate cell-to-cell spread (Mettenleiter 2003). Moreover, gB protein is the main immunogen of PRV and can stimulate the host to produce complement-dependent and complement-independent neutralizing antibodies (Okazaki, 2007). Compared with the Bartha-K61 strain, the SD-2017 strain and other mutant strains have three mutant forms of amino acid insertion, deletion and substitution through gB protein comparison analysis. These variants may lead to changes in the neutralizing epitope of the gB protein, thereby reducing the protective efficacy of the Bartha-K61 vaccine. According to the genetic evolution analysis of gC gene, SD-2017 strain belongs to the PRV genotype II strain circulating in China. It is on the same evolutionary branch as the Chinese PRV isolates that appeared after 2011. gC protein is another important neutralizing antigen involved in the adsorption process between PRV and target cells (Karger *et al.,* 1998). The amino acid sequence alignment of gC protein found that compared with Bartha-K61, SD-2017 strain and most domestic isolates had seven consecutive amino acid (AAAASTPA) insertions and multiple amino acid substitutions at positions 69-75. These changes may affect the gC glycoprotein structure of the variant strains, thereby affecting the adhesion of PRV to host cells.

Rabbits are extremely susceptible to PRV infection and the clinical symptoms are very typical, so they are often used to observe the efficacy of viral challenge in quality standards for various virus strains (Pomeranz *et al.,* 2005). PRV SD-2017 strain can kill rabbits with an LD50 of $3.16\times10^{2.0}$ TCID50 in 500 μl DMEM. Typical symptoms observed in PRV-infected rabbits are itching, convulsions, biting at the injection site, and difficulty breathing. In this study, we isolated and identified SD-2017 strain can be used for the development of new vaccines against PRV variants, as the vaccine parent strain, or the vaccine challenge strain. In the future research, we can try to develop new live attenuated vaccines and subunit vaccines using SD-2017 genome sequence information.

CONCLUSION

In conclusion, we reported a PRV variant named SD-2017, which was the causative agent of piglet disease on a farm in Linyi, Shandong Province, China. The genetic variation analysis of the main virulence genes showed that the SD-2017 strain had multiple positions of variation, and caused important harm to piglets in the farm, which needed to be paid enough attention. At the same time, the results of this study provided a new candidate strain for the construction of vaccines against PRV variant strains.

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CONFLICT OF INTEREST

None declared.

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