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Molecular epidemiology of pseudorabies virus in Yunnan and the sequence analysis of its gD gene

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Abstract Outbreaks of pseudorabies (PRs) have occurred in Yunnan, China, which caused significant economic loss. To determine the prevalence and origin of PR in Yunnan, especially among vaccinated pigs, overall 791 samples of blood, tissue, semen, and sera were analyzed by serological methods, PCR, and sequence analysis of gD gene. Detection with viral gI antibody or PCR showed that the yearly positive rates of PR virus (PRV) in Yunnan from 2010 to 2014 were 48.15, 21.26, 2.17, 5.22, and 0.35%, respectively, with an average of 15.43%. In general, the incidence declined through the period of 2010–2014 probably due to the application of PRV eradication strategies. A phylogenetic tree was constructed based on the complete sequence of gD gene, with all strains clustered into two independent clades, i.e., Asian and European–American clades. The virus isolates from Henan, Tianjin, Heilongjiang, Sichuan, Shandong, Fujian, Xinjiang, Hubei, Guangdong, and Yunnan fell into Asian group, which harbored South Korea isolate. Four Yunnan virus isolates together with South Korean Namyangju fell into in the European–American clade. It showed that PR was pandemic as there was not a clear clue about the geographical origin of the PRV isolates in China since 2010.

Keywords Pseudorabies virus · gD gene · Phylogenetic tree · Yunnan

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Introduction

Pseudorabies (PR) disease was first found from an American cattle population in 1813 and listed as one of the top three epidemic diseases in pig industry. PR is caused by Pseudorabies virus (PRV), which belongs to suid herpesvirus type 1 (SuHV 1), a member of Alphaherpesvirinae subfamily within the Herpesviridae family. Pig is the natural host, reservoir, and source of PRV infection. PRV infection results in some obvious clinical manifestations such as fever, local intense pruritus, and encephalomyelitis in farm animals including cattle, sheep, pig, dog, some avian species, and cat, with exception of higher primates [1–5]. Hunting dogs and wild boars [6, 7] are also important potential carriers, which aggravated the PRV control. The PRV infections were found around the world and widely prevailed in China in the past decades, causing enormous economic loss in pig industry [1, 8–11]. The symptoms differed greatly from swine ages, such as 100% mortality rate in piglets, stillbirths, mummification and/or abortions in pregnant sows, and low growth rates in asymptomatic chronic infections in adult pigs [4–6, 8, 10, 12–14]. Although the infections has been controlled by an attenuated vaccines since 1970s, new

clinical features had developed in China, in terms of transmission diversity, infected area and rate, severe clinical signs, and coinfection. From a sudden break-out of pseudorabies, Gu et al. [15] collected 38 tissue samples from pigs with clinical signs of pseudorabies on 13 farms from 4 provinces in southern China in 2012–2013. After PCR detection, 29 samples (76%) showed wild-type PRV infection, indicating that a novel, highly virulent PRV strain with antigenic variance had spread widely in southern China. Therefore, new PR pathogenicity has been continuously revealed since late 2011 in China [5, 8, 10, 14–19).

There are a number of reports about the genetic information and the origin of the PRV genome [2, 10, 20–24]. The PRV genome is a double-stranded linear DNA molecule with 14.36 kb in length and contains 67–72 open reading frames (ORFs), which show significant variations when compared with previous isolates in most viral coat proteins including glycoproteins E, B, C, D, and I, which are critical factors that mediate viral attachment (gC and gD), viral spread (gE), and induction of host immunity (gG and gI). The glycoproteins play a major role for viral interaction and are of importance for pathogenesis [3, 4, 25]. Phylogenetic analysis based on the PRV genes was documented to reveal the origin and relationships between the PRV isolates [6, 8, 10, 12, 14]. In this study, we investigated the prevalence of PRV in Yunnan of China during the time period of 2010–2014 and performed a phylogenetic analysis of our isolates and some reference strains based on the PRV gD gene sequences attempting to investigate the genetic and variant features of PRV in Yunnan province.

Materials and methods

Sample collection and serological detection

Seven hundred ninety-one samples were collected including blood, semen, sera, and tissues from sows and boars in pig farms from 15 prefectures in Yunnan province and two adjoining provinces, China, during 2010–2014 Serological detection with Pseudorabies Virus gI Antibody (PRV gI Ab) Test Kit were performed to verify if the suspected sera samples were positive for PR virus according to the manufacturer's instructions (IDEXX Laboratories, Inc., Beijing Yuanxiang Biological Tech. Co. Ltd.).

Isolation of viral DNA and PCR amplification

The blood, tissue, and semen samples were subjected to DNA extraction according to the instructions of DNA extraction kit (Sangon Biological Engineering Co. Ltd.). The complete gD gene fragment of PRV was amplified by PCR from the extracted DNA using a pair of specific primers (Sangon Biological Engineering Co. Ltd.), which were designed according to the reference sequences in GenBank database (Accession Numbers AJ271966, BK001744 and JF797218). The primer sequences were 5′-CCCCAGGTTCCCATACACTC-3′ (forward, genomic position 118840–118859 nt) and 5′-TCATCATC GACGCCGGTACT-3′ (reverse, genomic position 120083–120102 nt), with the amplification resulting in a 1263 bp long product. PCR was performed in a 25-μL volume containing 2.5 U DNA polymerase, 2.5 μL buffer II, 10 μL GC enhancer, 10 pmol of each primer, 2 mM dNTP for each, 8.0 μL ddH_2O, and 1 μL extracted DNA containing 20–50 ng/μL. The PCR conditions consisted of an initial cycle at 94 °C for 5 min, 35 amplification cycles (94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min) and a final extension step at 72 °C for 7 min.

Cloning of gD gene and sequencing

After PCR, the products were subjected to 1.5% agarose gel electrophoresis for detecting the presence of gD gene fragment. Then, the PCR products were electrophoresed on 2.5% agarose gel for purification according to the instructions (Bio Teke Corparaion, Beijing, China). With random orientation, the PCR products were ligated into pMD18-T vectors (Sangon Biological Engineering Co. Ltd., Shanghai, China) at the EcoRV site, followed by transformation into *Escherichia coli* DH5a cells (Takara Biotch Co. Ltd.). The plasmids were extracted, with the inserts sequenced by Sangon Biological Engineering Co. Ltd.

Phylogenetic analysis of gD sequence

The resulting raw sequences were aligned and checked using DNAStar 6.0 (DNAStar Inc., Madison, WI). The complete gD gene (1203–1209 bp) were identified by comparison with the reference sequences of *Suid herpesvirus* 1 from GenBank database (Accession Numbers BK001744, JF797218 and AJ271966). Then, a total of 29 gD gene sequences from GenBank were used in the analysis (Table 1). Variant sites and haplotypes were determined using MEGA software, Version 4.0 [26] and DnaSP software, Version 3.00 [27]. The nucleotide homologies were analyzed using DNAman (version 6.0, Lynnon Co.). Phylogenetic tree was constructed using MEGA software under the Kimura 2-parameter substitution model by neighbor-joining method with 1000 bootstrap replicates.

Table 1 Information about the gD reference sequences from GenBank database

	GenBank Accession No.	Strains	Country	Isolation/submitted date	Length (bp)	gD (bp)	aa	Location	Source
1	JF797217	Bartha	Hungary	Nov. 2011	137,764	1203	400	119,436–120,638	PLOS Pathog. 2011, 7 (10), E1002282
2	JF797218	Kaplan	Hungary	Nov. 2011	140,377	1203	400	118,873–120,075	PLOS Pathog. 2011, 7 (10), E1002282
3	JF797219	Becker	USA	Nov. 2011	141,113	1209	402	119,647–120,855	PLOS Pathog. 2011, 7 (10), E1002282
4	KJ789182	TJ	Tianjin	Oct. 2014	143,642	1209	402	121,268–122,476	Vet. Microbiol. 2014, 174 (1–2),107–115
5	AY217094	Yangsan	South Korea	Mar. 2003	1306	1209	402	53–1261	Suui Kawahak Yongu Nonmunjip. 1996 38 (2), 240–250
6	AY174090	LA	Shandong	Jan. 2003	1203	1203	400	1–1203	Direct Submission
7	FJ477296	PRV-FZ	Fujian	Dec. 2008	1559	1203	400	33–1235	Direct Submission
8	AY169694	Min-A	Fujian	Dec. 2002	1250	1203	400	35–1237	Direct Submission
9	AF086702	Ea	Hubei	Sep. 1998	1300	1215	404	33–1247	Direct Submission
10	KJ717942	Kaplan	Hungary	Jul. 2014	143,423	1203	400	121,144–122,346	Genome Announc. 2016, 2 (4), e00628–14
11	JQ809330	DUL34Pass	Germany	May 2012	144,479	1203	400	122,228–123,430	J. Virol. 2012, 86(12): 6512–6521
12	AJ271966	Kaplan	Hungary	Jan. 2006	1590	1203	400	43–1245	J. Virol. 2000, 74 (9), 4004–4016
13	KT818620	qihe547	Shandong	Mar. 2016	1209	1209	402	1–1209	Direct Submission
14	GQ325660	Namyangju	South Korea	Dec. 2011	1203	1203	400	1–1203	J. Asia Pac. Entomol. 2011, 14 (1), 107–117
15	KF017337	QBA	Henan	Sep. 2013	1209	1209	402	33–1241	Direct Submission
16	KF017336	QXX	Henan	Sep. 2013	1209	1209	402	33–1241	Direct Submission
17	KF017335	QXY	Henan	Sep. 2013	1209	1209	402	33–1241	Direct Submission
18	KF017334	QYY	Henan	Sep. 2013	1209	1209	402	33–1241	Direct Submission
19	KU605805	SC-1-2015	Sichuan	Mar. 2016	1209	1209	402	1–1209	Direct Submission
20	KT824771	HLJ8	Heilongjiang	Mar. 2016	142,298	1209	402	120,420–121,628	Virology. 2016, 491, 56–63
21	KP315913	SD1404	Shandong	May 2015	1209	1209	402	1–1209	Direct Submission
22	AY196984	Fa	Guangdong	Mar. 2003	1258	1209	402	32–1240	Chin. J. Prev. Vet. Med. 2002, 24 (Supplement), 22–25
23[a]	/	XIN-W	Xinjiang	Jan. 2005	1209	1209	402	1–1209	Progress in Veterinary Medicine. 2005, 26(1), 66–70, 88
24	KP722022	HN1201	Henan	Apr 2016	144,174	1209	402	121,776–122,984	Genome Announc. 2016, 4 (2), e00149–16

[a] Note:/. no direct submission in GenBank. The sequences from GenBank shared 100% nucleotide sequence identity. EF622042, EF645837, and FJ477296 from Fz strain, Fujian of China, shared one haplotype. AJ271966 and BK001744 from Kaplan strain, Hungary, share one haplotype. And six isolates including YN1 (ZB10142374, this study), TJ (KJ789182), HLJ8 (KT824771), SD1404 (KP315913), HN1201 (KP722022), and qihe547 (KT818620) from China, shared one haplotype

Results

Positive rates of PRV from 2010 to 2014 in Yunnan

The prevalence of PRV in Yunnan province, China, is listed in Table 2. Ninety-nine positive samples were identified from 791 clinical samples during the period of 2010–2014, with an average rate of 15.43%. Declining tendency of PRV positive rates was observed through the 5 years, with the lowest rate in 2014 (0.35%), except for a slight increase in 2013 (5.22%).

Table 2 The positive rates of PRV in 791 samples from 2010 to 2014

Year	2010			2011			2012			2013			2014			Total
Sample type	Blood/serum	Semen	Tissue	Blood/serum	Semen	Tissue	Blood/serum	Semen	Tissue	Blood/serum	Semen	Tissue	Blood/serum	Semen	Tissue	
Total samples (N)	82	9	17	114	27	33	68	7	17	102	14	18	241	20	22	791
Numbers of positive rate of PRV (n)	39	1	12	14	3	20	1	0	1	1	1	5	0	0	1	99
Positive rates of PRV (%)	47.56	11.11	70.59	12.28	11.11	60.61	1.47	0.00	5.88	0.98	7.14	27.78	0.00	0.00	4.55	
Positive rates of PRV per year (%)	48.15			21.26			2.17			5.22			0.35			

PCR amplification, purification, and identification of PRV gD gene

After PCR amplification, the products were electrophoresed on 1.5% agarose gel, and the fragment of gD gene of PRV was about ~1300 bp, which was in accordance with the expected 1263 bp in length (Fig. 1a). The gD gene fragments of PRV were electrophoresed on 2.5% agarose gel for purification according to the manuals displaying ~1300 bp in size (Fig. 1b). The purified PCR products were ligated into pMD18-T vectors at the EcoRV site for transformation into *E. coli* DH5a cells. After analyzing the sequences of the pMD18-T vector and the amplicons from gD gene, *Hin*cII was found able to cut both pMD18-T vector and the amplicons from gD gene at single site, respectively, and therefore selected for digestion, which resulted in two digestion bands. The gel images of the products after digestion are shown in Fig. 1c, of which the fragments in length were as expected. Depending on the ligation orientation of the gD amplicons into pMD18-T vector, two possibilities could be seen after digestion of *Hin*cII: 3408 bp band and 547 bp band for samples 1–5, 3231 bp band and 724 bp band for sample 6, which are indicated in Fig. 1c. The plasmids were extracted, and the inserts were sequenced by Sangon Biological Engineering Co, Ltd.

Sequencing, variants, and phylogeny of gD gene of PRV

Six positive clones were sequenced, aligned, and compared with the complete gD gene counterparts of PRV available in GenBank database. The sequences of gD gene were identified in range of 1203–1209 bp in length, encoding 400–403 aa residues, and the GC content was 74.69%. Sixty-seven variant sites were found in the gD gene sequences, including 53 predominant transitions, 6 C/G (at the position 379, 468, 627, 828, 918, and 1023 nt) and 8 C/A (744, 808, 814, 820, 826, 1019, 1030, and 1129 nt) transversions, leading to 45 amino acid variations. The sequence variation of gD genes incurred as well in the gaps covering 829–834 nt which contained (GAACCC) insertions, coding $(PR)_{6-7}$ repeats residue (Figs. 2, 3).

The phylogenetic tree was constructed for the 29 complete gD genes together with some available sequences in GenBank using neighbor-joining (N-J) method. Clustering analysis revealed two independent clades, i.e., Asian clade and European–American clade (Fig. 4). The virus isolates from Henan, Tianjin, Heilongjiang, Sichuan, Shandong, Fujian, Xinjiang, Hubei, Guangdong, and Yunnan fell into Asian group, varying from 1203 to 1209 bp in length except for Ea strain (AF086702, 1215 bp), which were relatively closer to previously isolated strains, suggesting

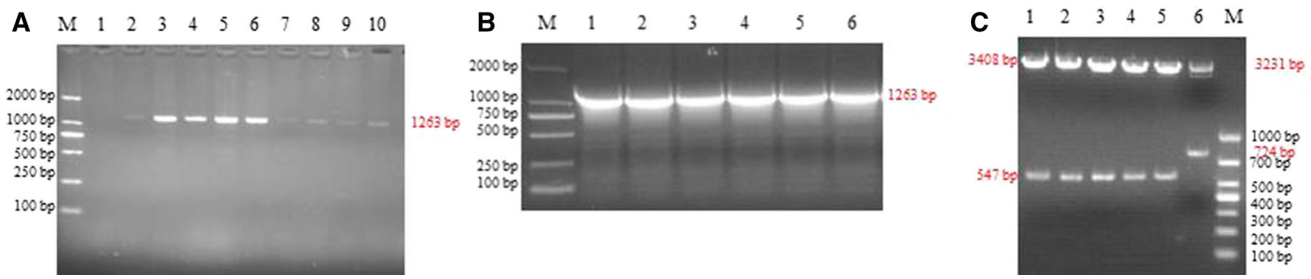

Fig. 1 PCR amplification, purification, and identification of PRV gD gene. **a** The PCR products of gD gene of PRV. *Lane 1* Negative control, *Lane 2–10* the PCR products of gD gene from nine representative suspected PRV samples. *M* DNA Marker DL2000. **b** The electrophoresis image of products of gD gene on 2% gel after purification from **a**. *Lane 1–6* the six clone samples; *M* DNA Marker DL2000. **c** Identification of recombinant plasmids digested by *Hinc*II. According to the sequences of pMD18T vector and the amplicons of gD gene, the cleavage sequence of *Hinc*II and the ligation orientation, the length of fragments from digestion is expected to be 3408 and 547 bp (*lanes 1–5*), or 3231 and 724 bp (*lane 6*). *M*: DNA Marker DL1000

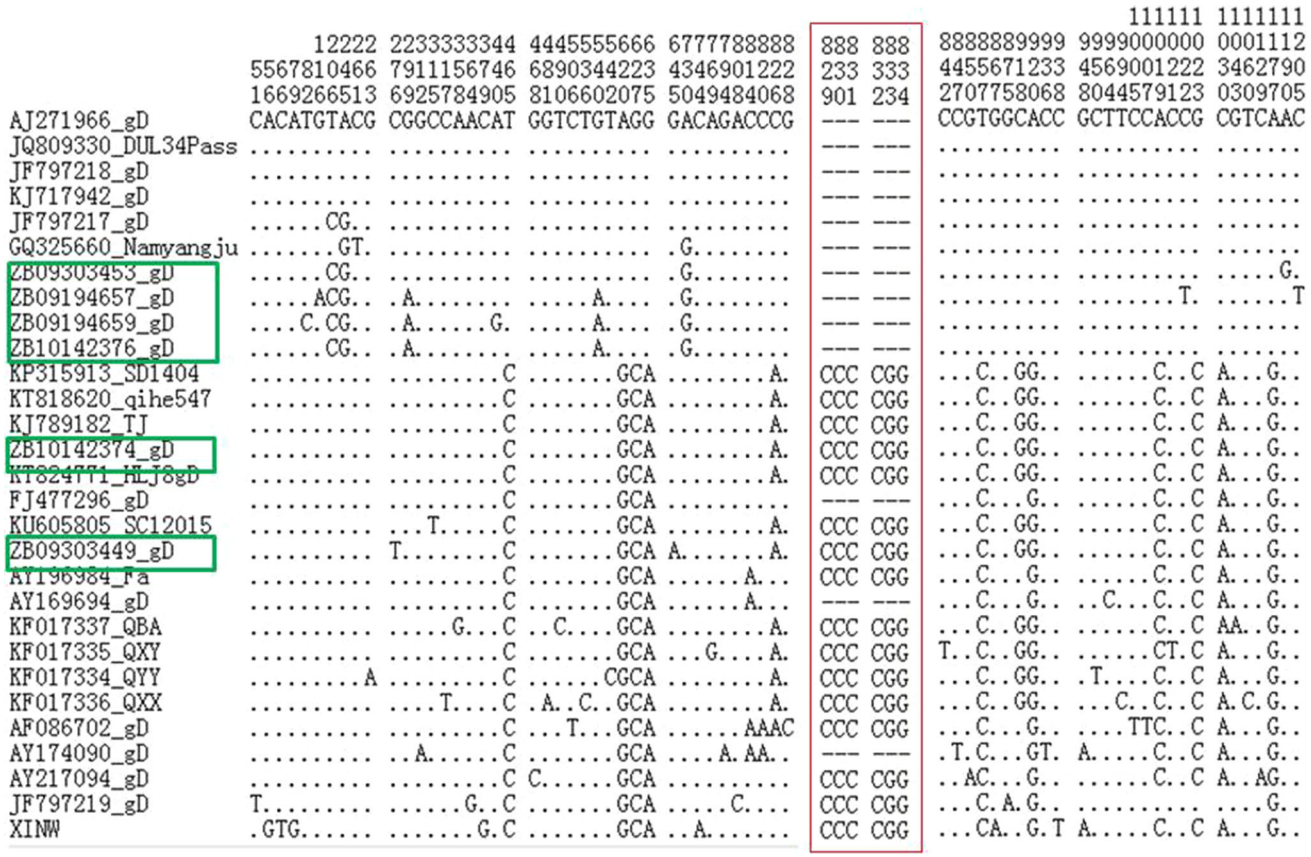

Fig. 2 Nucleotide variants of PRV gD gene. The names of the six gD genes cloned in this study are highlighted in *green squares*. Sixty-seven variant sites were found in the gD gene sequences, including 53 predominant transitions, 6 C/G (at the position 379, 468, 627, 828, 918, and 1023 nt), and 8 C/A (744, 808, 814, 820, 826, 1019, 1030, and 1129 nt) transversions. The sequence variance of gD genes incurred as well in the gaps covering 829–834 nt which contained (GAACCC) insertions (shown in the *red square*). Two independent clades, i.e., Asian clade and European–American clade can be seen based on the nucleotide acid sequence comparison. The nucleotide sequences shown here are not consecutive, with only sequence variants shown. The positions of the consensus nucleotide acids are shown above the sequence (Color figure online)

that there was a continuous evolutionary process. ZB10142374, SD1404, qihe547, HN1201, TJ, and HLJ8 strains from China shared 100% nucleotide identity with each other, as an Asian classical PRV in swine epidemiology, showing that PR was pandemic, and there was no clear sequence variation from the geographical zones between PRV virus isolates from China since late 2011. The typical isolates of European PRV, such as Kaplan, Batha, and Becker, together with the South Korean Namyangju, were clustered into the same group

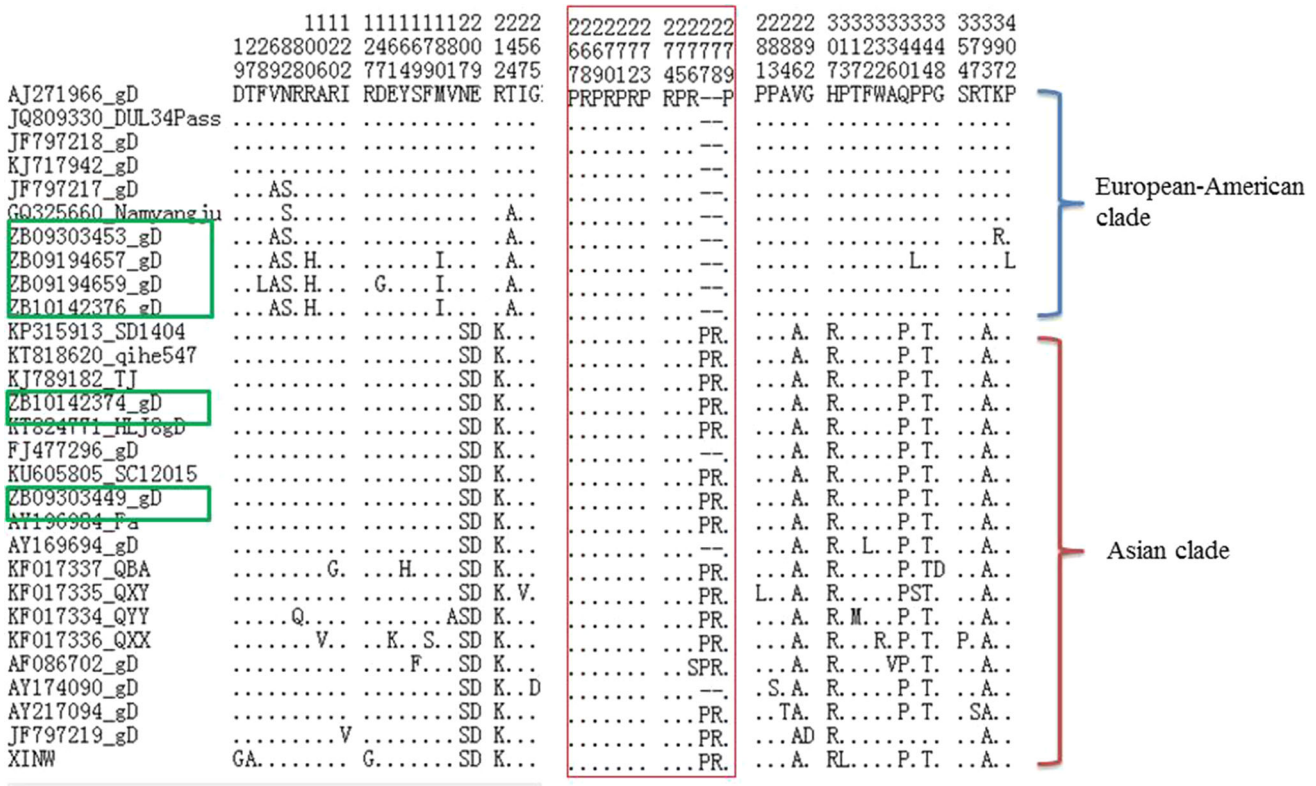

Fig. 3 Amino acid variants of PRV gD gene product. The names of the six gD genes cloned in this study are highlighted in *green squares*. The sixty-seven variant sites found in the gD gene sequences led to 45 amino acid variations $(PR)_{6–7}$ repeat insertion (shown in the *blue square*). Two independent clades, i.e., Asian clade and European–American clade can be seen based on the amino acid sequence comparison. The nucleotide sequences shown here are not consecutive, with only sequence variants shown. The positions of the consensus amino acids are shown above the sequence, in column (Color figure online)

characterized by 1203 bp in length, including 4 Yunnan virus isolates which formed a separated subclade (Fig. 4). Herein, the virus isolates shared one haplotype comprising of DUL34Pass (JQ809330) and Kaplan (KJ717942, JF797218, and AJ271966). The nucleotide sequences of gD gene were 97.8–100.0% homology between the PRV isolates. It was inferred that PRV isolates in Yunnan province after 2010 were extensively spread with the pig transmission and trade, and escaped from the protection from Bartha-K61 vaccine.

Discussion

Pseudorabies (PR) is caused by PRV infection and of economic importance in pig industry because this viral disease can lead to 100% mortality rate in piglets, stillbirths, mummification, and/or abortions in pregnant sows, as well as lower growth without obvious symptom in adult pigs as a natural reservoir and host. PR has been effectively controlled since 1970s using Bartha-K61 vaccine in China [1, 5, 9, 12, 14]. However, PR outbreak still occurs in China since late 2011, indicating that the vaccination cannot fully protect the pigs from PRV infection. Later, many cases with suspected animals, inoculated with PRV Bartha-K61, were collected to determine the presence and variants of PRV [5, 8, 10, 14, 15, 28]. In this study, we collected 791 suspected samples from pig farms mainly in Yunnan, China, to determine the positive rate by universal PRV gI Ab test kit and PCR technique. The average positive rate of PRV was 15.43% and showed a decline during the period of 2010–2014 (Table 2), except a slight increase in 2013 (5.22%). Some measures have been available for the control and eradication of the PRV, e.g., the application of inactivated vaccine before mating or artificial insemination and 4 weeks before delivery again for sows; intraocular-nasal vaccination for the piglets at the birth day; and vaccination by injection for the growing-finishing pig at the age of 70-day and breeding boars every 6 month.

Based on the gD gene analysis, the six PRV strains from Yunnan of China were separated into two independent branches, with only two strains into Asian clade and the other four into European–American clade which consists of a separated subclade. It inferred that the PR epidemiology in Yunnan was more complicated than other regions in China [5, 8, 10, 14, 28]. The recent isolates from Yunnan

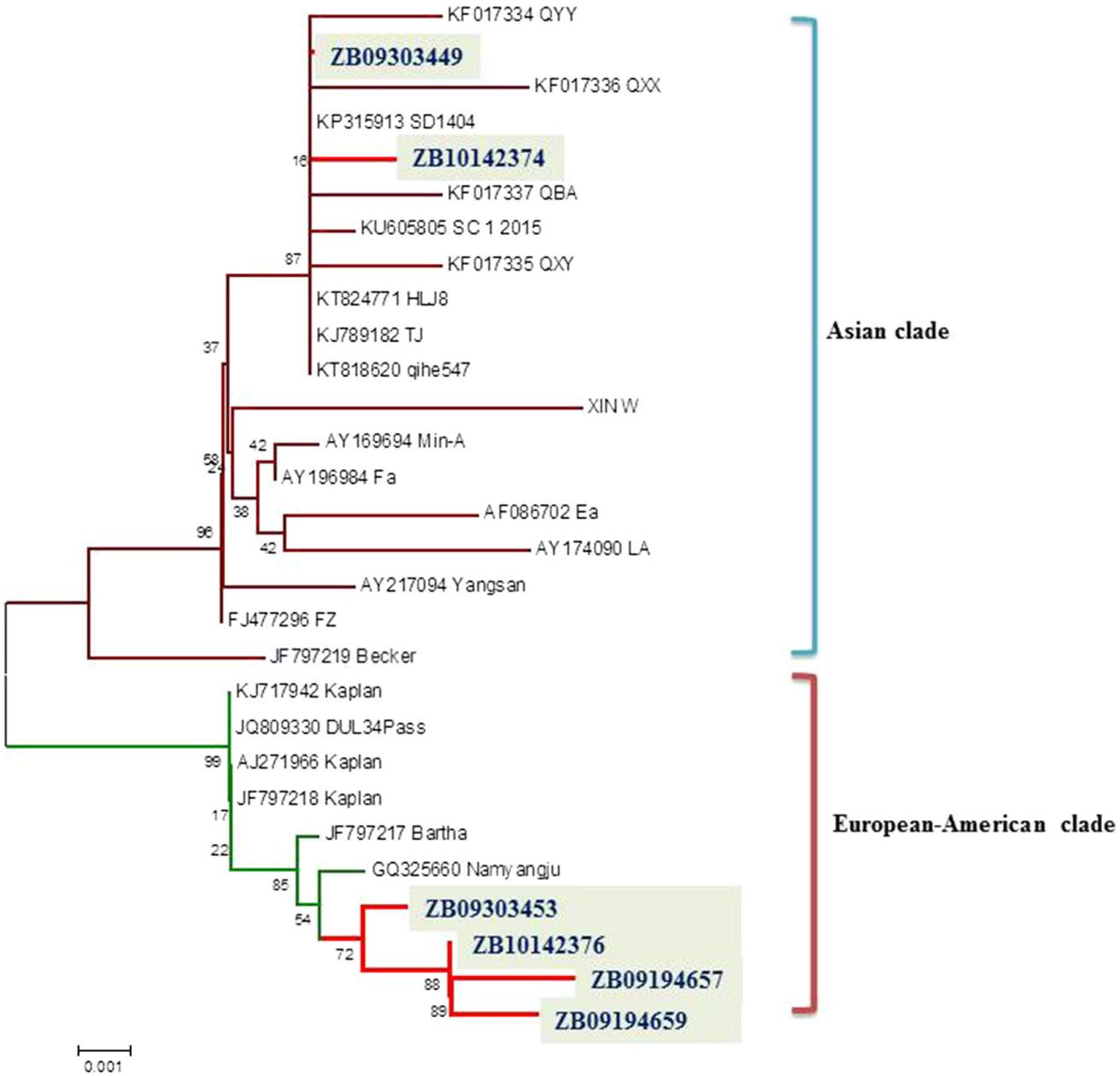

Fig. 4 Phylogenetic analysis with 29 complete gD genes and some reference sequences available in GenBank using neighbor-joining (N-J) method revealed two independent clades, i.e., Asian clade and European–American clade

intermingled and kept a continuous evolutionary history [10, 17, 18, 28], sharing one haplotype in Asian clade, as well as having an independent subclade in European–American clade. The nucleotide acid similarity of the PRV isolates was 97.6–100.0%. PR could be transmitted by commercial animals including swine and boar. The outbreak of PR in Yunnan since late 2010 might originate from PRV variants, animal introduction, or trade activities [15, 28]. It is of importance that PRV investigation is compulsory to monitor the presence and mutations of new PRV variants and epidemiology due to commercial transmission. More work on the prevalence and pathogenicity of PRV worldwide is needed for the control and eradication of PR.

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Author contributions G.F.Y. and X.H.S. conceived and designed the study. W.B.B. and X.Q.Z. collected the samples. C.L.S. and L.G. carried out the experiments. C.L.S., G.F.Y. and X.H.S. drafted the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors have declared no competing interests.

Human and animal consent This article does not contain any studies with human participants or animals performed by any of the authors.

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