Epidemiological and genetic characteristics of swine pseudorabies virus in mainland China between 2012 and 2017 (#29677)

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Epidemiological and genetic characteristics of swine pseudorabies virus in mainland China between 2012 and 2017

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The outbreak of pseudorabies (PR) in many Bartha-K61 vaccinated farms in China in late 2011 has seriously damaged the pig industry of one of the largest producer of pork products in the world. To understand the epidemiological characteristics of the pseudorabies virus (PRV) strains currently prevalent in China, a total of 16,256 samples collected from large-scale farms suspected of PRV infection in 27 Provinces of China between 2012 and 2017 were evaluated for detection of PRV. Since the extensive use of gE-deleted PRV vaccine in China, the PRV-gE was applied for determining wild-type virus infection. Of the 16,256 samples detected, approximately 1,345 samples were positive for the detection of PRV-gE, yielding an average positive rate of 8.27%. The positive rates of PRV detection from 2012 to 2017 were 11.92% (153/1284), 12.19% (225/1846), 6.70% (169/2523), 11.10% (269/2424), 5.57% (147/2640), and 6.90% (382/5539), respectively. To understand the genetic characteristics of the PRV strains currently circulating, 25 PRV strains isolated from those PRV-gE positive samples were selected for further investigation. Phylogenetic analysis based on gB, gC, and gE showed that PRV strains prevalent in China had a remarkably distinct evolutionary relationship with PRVs from other countries, which might explain the observation that Bartha-K61 vaccine was unable to provide full protection against emergent strains. Sequence alignments identified many amino acid changes within the gB, gC, and gE proteins of the PRVs circulating in China after the outbreak compared to those from other countries or those prevalent in China before the outbreak; those changes also might affect the protective efficacy of previously used vaccines in China, as well as being associated in part with the increased virulence of the current PRV epidemic strains in China.

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Running title: Epidemiological characteristics of PRV

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Abstract

The outbreak of pseudorabies (PR) in many Bartha-K61 vaccinated farms in China in late 2011 has seriously damaged the pig industry of one of the largest producer of pork products in the world. To understand the epidemiological characteristics of the pseudorabies virus (PRV) strains currently prevalent in China, a total of 16,256 samples collected from large-scale farms suspected of PRV infection in 27 Provinces of China between 2012 and 2017 were evaluated for detection of PRV. Since the extensive use of gE-deleted PRV vaccine in China, the PRV-gE was applied for determining wild-type virus infection. Of the 16,256 samples detected, approximately 1,345 samples were positive for the detection of PRV-gE, yielding an average positive rate of 8.27%. The positive rates of PRV detection from 2012 to 2017 were 11.92% (153/1284), 12.19% (225/1846), 6.70% (169/2523), 11.10% (269/2424), 5.57% (147/2640), and 6.90% (382/5539), respectively. To understand the genetic characteristics of the PRV strains currently circulating, 25 PRV strains isolated from those PRV-gE positive samples were selected for further investigation. Phylogenetic analysis based on gB, gC, and gE showed that PRV strains prevalent in China had a remarkably distinct evolutionary relationship with PRVs from other countries, which might explain the observation that Bartha-K61 vaccine was unable to provide full protection against emergent strains. Sequence alignments identified many amino acid changes within the gB, gC, and gE proteins of the PRVs circulating in China after the outbreak compared to those from other countries or those prevalent in China before the outbreak; those changes also might affect the protective efficacy of previously used vaccines in China, as well as being associated in part with the increased virulence of the current PRV epidemic strains in China.

Key words: Pseudorabies virus, PCR detection, Virus isolation, Phylogenetic analysis, Sequence alignment

Introduction

Pseudorabies virus (PRV) mainly causes reproductive failure in sows as well as respiratory and neurological symptoms in piglets (Mettenleiter, 2000; Nauwynck et al., 2007). PRV possesses a double-stranded liner DNA genome which contains more than 70 functional genes encoding proteins participating in the formation of viral capsid, tegument, and envelope (Pomeranz et al., 2005). Among these proteins, the envelope component proteins gB and gC induce cellular and humoral immune responses (Ober et al., 1998; Ober et al., 2000), while gE acts as a major virulence determinant of PRV to pigs (Kimman et al., 1992; Wang et al., 2014). These three genes are commonly used for monitoring the evolution of PRV (Muller et al., 2011; Sozzi et al., 2014; Yu et al., 2014; Wang et al., 2015).

The first report of a PRV outbreak in China occurred in the 1950s. In the 1970s, an inactivated vaccine derived from PRV strain Bartha-K61 was imported from Hungary to China (Yuan et al., 1983; An et al., 2013). The widespread use of this vaccine in China was able to control outbreaks of pseudorabies between 1990 and 2011 (Tong and Chen, 1999). However, since late 2011, a pseudorabies (PR)-like disease has occurred in many Chinese pig farms that had been vaccinating pigs with the Bartha-K61 vaccine. PRV has been finally confirmed to be responsible for those outbreaks (An et al., 2013; Peng et al., 2013; Luo et al., 2014; Wang et al., 2014; Yu et al., 2014). A number of studies have noted that the Bartha-K61 vaccine appears to be unable to provide full protection against PRV strains isolated from those outbreaks (An et al., 2013; Wang et al., 2014; Yu et al., 2014). These findings suggest that there may be important changes in the PRVs currently

103 circulating in China. However, genetic information as well as epidemiological data about
104 PRV strains currently circulating in China is limited. Therefore, in this study, we report the
105 detection/genetic analysis of PRVs recovered from pigs in China between 2012 and 2017.
106 The aim of this study is to understand the epidemiological and genetic characteristics of
107 PRVs that are currently prevalent in China.

108

109 Materials and methods

110 Samples collection and virus isolation

111 A total of 16,256 samples including tissue from lungs, lymph nodes, brains, serums,
112 stillbirths, kidneys, and spleens were collected from large-scale farms suspected of PRV
113 infection in 27 Provinces in mainland China, excluding Ningxia and Tibet, between
114 January, 2012 and December, 2016 (Figure 1). The number of samples collected in 2012,
115 2013, 2014, 2015, 2016 and 2017 was 1284, 1846, 2523, 2424, 2640 and 5539,
116 respectively. After collection, tissues were minced, immersed with Dulbecco's modified
117 Eagle medium (DMEM), and homogenized using a QIAGEN TissueLyser II (QIAGEN
118 Germany). Sample homogenates were then frozen at -80 °C and thawed for three times.
119 Following centrifugation at 5000 rpm for 5 min, the supernatants were harvested for DNA
120 and/or virus isolation.

121

122 Template DNA for PCR detecting PRV was isolated using a Universe Genomic DNA Kit
123 (CWBIO, Beijing, China) following the manufacturer's instructions. For virus isolation,
124 homogenate supernatants were filtered using a 0.22 μm membrane, and inoculated into

PK-15 cells (Purchased from ATCC). Cells were then incubated in a 37 °C incubator supplemented with 5% CO2. Cell culture with obvious CPE was used for further plaque purification assays. Briefly, PK-15 cells were plated into a 6-well plate, and a series of 10-fold dilutions (from 10^{-1} to 10^{-6}) of virus was inoculated. The plate was incubated at 37 °C under an atmosphere containing 5% CO_2 for 2 h and was shaken one time every 15 min. After incubation, the virus was discarded and the cells were washed using DMEM for two times. Finally, DMEM medium containing 1% low melting agarose was added into each of the wells, and the plate was incubated at 4 °C until the medium solidified. The plate was then moved into a 37 °C cell incubator for plaque formation. Plaques with suitable size were selected and inoculated into 500 μL DMEM, frozen and thawed for three times, and then diluted 2-fold in DMEM for the second round of plaque purification assay. After that, plaque fluid was inoculated into PK-15 cells and cultured in flask.

PRV detection

Polymerase chain reaction (PCR) assays were designed to detect the presence of PRV gE gene from the clinical samples using the DNA isolated as template as well as the primers listed in Table 1. The gE gene was used as the target gene because the gE-deleted pseudorabies virus (PRV) vaccine has been used in China extensively, and the detection of the gene could be applied for determining wild-type virus infection. As shown in Table 1, primers gE1-F and gE1-R were designed for the detection of gE. PCR reactions were performed in a 25 μL volume mixture containing 12.5 μL 2×Taq Master mix (TAKARA, Japan), 8.5 μL nucleotide-free water, each of the forward and revise

primers 1 μL, 1 μL DMSO, and 1 μL template DNA. Thermocycler conditions used for PCR were 95 °C for 5 min ~~to inactivate transcriptase~~, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C, 30 s for gE, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min before storage at 4 °C. The PCR product was visualized using 1% agarose gel electrophoresis under ultraviolet light.

Sequencing and phylogenetic analysis

PCR assays were also designed for analysing the gB, gC, and gE genes of the PRVs currently circulating in China. The PCR volumes were the same as that used for PRV detection from the samples. Cycling conditions were 95 °C for 5 min ~~to inactivate transcriptase~~, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min before storage at 4 °C. The PCR product was visualized using 1% agarose gel electrophoresis under ultraviolet light.

After amplification, PCR products were purified using a TIANgel Midi Purification Kit (TIANgel, China) and cloned into a pMD19-T vector (TAKARA, Japan). Amino acid sequences deduced from the DNA sequences of the gB, gC and gE genes were subjected to sequence comparisons using the DNAStar and/or BioEdit software. Phylogenetic trees were generated through MEGA X, using neighbor-joining algorithm with 1,000 bootstrapping. Sequences of PRV strains listed in Table 2 retrieved from NCBI were used as references.

170

171 Results

172 PCR detection of PRV in mainland China

173 Of the 16,256 samples, approximately 1,345 samples were positive for the detection of
174 PRV-gE, yielding an average positive rate of 8.27%. The positive rates of PRV detection
175 from 2012 to 2017 were 11.92% (153/1284), 12.19% (225/1846), 6.70% (169/2523),
176 11.10% (269/2424), 5.57% (147/2640), and 6.90% (382/5539), respectively. Monthly,
177 higher positivity rates of PRV were detected in January, February, March, April, June,
178 October, November and December; and winter (December, January and February),
179 spring (March, April, and May) and autumn (September, October and November) were
180 the seasons with the high positivity rate of PRV detection during 2012 and 2017 (Figure
181 2A and 2B).

182

183 Mainland China is divided into seven parts including Northeastern China, Northern China,
184 Eastern China, Central China, Southern China, Northwestern China, and Southwestern
185 China (Figure 1). Among these graphic regions, the positive rates of PRV detection in
186 Southwestern China and Eastern China between 2012 and 2017 were higher than
187 10.00%, while the positive rates in other parts of China between 2012 and 2017 ranged
188 from approximately 7.00% and 10.00% (Figure 1). In addition, the positivity rate of PRV
189 detection in different graphic regions between different years displayed diversity. For
190 instance, the positive rates of PRV detection in Northern China in 2012, 2013 and 2015
191 were higher than 15.00%, but the positivity rates in 2014 and 2016 were lower than

192 5.00%; the positive rate of PRV detection in Southwestern China in 2012 was 26.15%,
193 while it was only 1.80% in 2013, and 6.90% in 2014, 5.97% in 2015, but zero in 2016; in
194 Northwestern China, the positivity rate of PRV detection in 2012 and 2013 were higher
195 than 20.00%, and approximately 10.40% in 2014 and 2015, but zero in 2016 (Figure 3).

196

197 PRV isolation

198 To understand the genetic characteristics of PRVs currently circulating in China, a total
199 of 25 PRV strains isolated herein were used for further analysis in this study (Table 3).
200 Most of those strains were isolated from lungs and their $TCID_{50}$/0.1 mL values ranged
201 from $10^{6.72}$ to $10^{7.96}$.

202

203 Phylogenetic characteristics

204 Phylogenetic analysis using gB, gC, and/or gE sequence showed that the PRV isolates
205 from China were located on a phylogenetic branch, which was distinct from the those
206 isolates from other countries of the world (Figures 4A, B, C). According to previous
207 study, those isolates from China belonged to genotype II, while those isolates from the
208 other parts of the world including Bartha, Becker, Kaplan, and NIA3 were genotype
209 strains. Interestingly, one isolate from China in 2016, which we designed HuB, had a
210 closer relationship to those genotype I strains when using gB to perform the
211 phylogenetic analysis (Figure 4A).

212

Analysis of gB, gC and gE

The maximal amino acid sequence divergence for gB, gC, and gE proteins of the 25 PRV isolates were 5.2, 2.7, and 2.6% within the isolates, and are 8.4, 9.2, and 5.5% compared to those isolates from the other countries, respectively. The maximal amino acid sequence divergence for the three proteins of the 25 isolates were 4.8, 9.9, and 2.8% compared to those strains prevalent in China before 2012, and were 4.8, 2.7, and 2.8%, respectively.

Alignment of amino acid sequences of gB found the isolates from China mainly had three types of mutations within the protein compared those strains from the other countries. The isolates from China harbored an-amino acid insertion at site 94 (G), a substitution of ten amino acids at sites 53 (A→T), 55 (P→T), 70 (T→A), 81 (N→D), 82 (D→G), 83 (V→F), 87 (A→E), 93 (E→D), 96 (F→V) and 102 (E→D), and a deletion of three-amino acids at sites 75-77 (S, P and G) compared to Bartha-Hungary, Kaplan-Hungary and NIA-3-Japan. In addition, there were also some different substitutions at different sites within the gB protein of the 25 isolates compared to Bartha-Hungary. For example, HuBHZ-China-2016 harbored a substitution of one amino acid at sites 11 (P→A) and 12 (R→G), while strains JSZL-China-2016, HuB-China-2016, and SDRZ-China-2016 had an amino acid substitution at sites 12 (R→H), 67 (A→V), and 228 (K→E), respectively (Figure 5).

For the gC protein, the isolates from China had an insertion of seven amino acids at sites 63-69 (A, A, A, S, T, P and A) within the protein compared to the genotype I strains; while strain LXB6-China-2009 harbored an insertion of six amino acids at sites 64-69 (A, A, S,

T, P and A). In particular, those isolates from China after 2012 contained a substitution of twenty-three amino acids substitutions at sites 14 (P→L), 16 (A→T), 52 (P→S), 55 (A→E), 57 (A→V), 59 (P→G), 60 (E→T), 76 (A→V), 87 (P→Q), 90 (N→G), 102 (A→S), 130 (F→V), 163 (S→P), 186 (T→A), 188 (V→A), 190 (E→V), 191 (D→V), 243 (S→H), 431 (L→M), 449 (A→T), 457 (S→T), 461 (V→T) and 467 (G→A) compared to the genotype I strains (Figure 6). In addition, some other substitutions were also found during the analysis: HuN-China-2016, SDRZ-China-2016, HuB-China-2016, HuN-China-2016, HuBAL-China-2016, and SDRZ-China-2016 harbored amino acid substitutions at sites 106 (K→T), 107 (R→C), 210/227 (A→T), 235 (A→V), 300 (L→P) and 386 (W→R), respectively.

The sequence alignments of the gE protein found also found some amino acid mutations within the protein of the genotype II strains compared to the genotype I isolates. Compared with Kaplan-Hungary and NIA3-Japan, the 25 isolates contained an insertion of one amino acid (D) at site 48 (Figure 7). Compared with Min-A-China-2002, the 25 isolates contained a deletion of one amino acid (D) at site 493. In particular, HeNJYG-China-2016, HuBWX-China-2016, HeNXY-China-2016 and GDHDYC-China-2016 deleted an amino acid (D) at site 491, while HuN-China-2016 and SDRZ-China-2016 had a deletion of one amino acid at sites 489 (Y) and 495 (D) compared to Min-A-China-2002, respectively; strain HuB-China-2016 deleted four amino acids (DLNG) at sites 61-64 compared to Min-A-China-2002. In addition to amino acid deletion, sequence alignments also identified amino acid substitutions within the gE protein of the strains isolated herein. For example, strains HeNJYG-China-2016, HuBHZ-China-2016, HuBLLP-China-2016,

HuN-China-2016, HuN-China-2016, and SX-China-2016 contained one amino acid substitution at sites 336 (D→G), 2 (R→G), 473 (T→M), 49 (L→R), 573 (A→T), and 526 (D→G) compared to the genotype I strains, respectively. Particularly, one strain, SDRZ-China-2016, contained four substitutions at sites 407 (V→L), 487 (E→D), 499 (E→D) and 535 (E→D) compared to the genotype I strains.

Discussion

PRV is a common threat to the pig industry worldwide and is particularly important in China. The outbreak of PR in China in late 2011 has seriously damaged the pig industry of one of the largest producer of pork products in the world (An et al., 2013; Yu et al., 2014). The present study reported the prevalence of PRV in China between 2012 and 2017. This report is the first large-scale etiological investigation of PRV involved in most regions of China following the outbreak. The data revealed an average positive rate of 8.27% for PRV detection during the six years, and higher than 6.9% positivity rate of PRV detection in different regions in China (Figure 1). While there is a lack of similar data from the other studies, a nationwide surveillance detecting the PRV-gE antibody revealed that the positive rate of PRV-gE antibody in China during 2013–2016 was higher than 13.74% (Liu et al., 2018). These findings confirm that the prevalence of PRV remains a problem in China.

It has been reported that PRV isolates are generally divided into two genotypes according to the gC gene; PRV strains prevalent in China belong to genotype II while PRV isolates

281 from the other countries belong to genotype I (Ye et al., 2015). In agreement with this
282 report, the isolates analyzed in this study were divided into two distinct clusters according
283 to their gC genes, with the previously reported genotype I strains Bartha, Backer, Kaplan
284 and NIA3 forming one cluster and the Chinese strains including the reported genotype II
285 strains TJ, JS, and HeN1 forming the second cluster (Figure 4B). Interestingly, similar
286 results were also obtained when using gB and gE to perform the phylogenetic analysis
287 (Figures 4A and C). Those findings again confirm that PRV strains circulating in China
288 harbor different genotypes from those spreading in the other countries. This observation
289 also might be the reason that the Bartha-K61 vaccine was unable to provide full protection
290 against these emergent strains. Interestingly, one isolate, HuB-China-2016, belonged to
291 genotype II according to gC and gE, but was identified as a genotype I strain when using
292 gB as a phylogenetic criterion (Figure 4). These findings suggest that a genetic
293 recombination might have occurred within the genome of this isolate. In the next step of
294 this investigation, we intend to do a follow up study in which the whole genome will be
295 sequenced in order to clarify what happened with this strain.

296

297 The gB protein is the most conserved glycoprotein in herpesviruses and allows PRV
298 strains to enter the target cells, thus contributing directly to cell-to-cell spread
299 (Mettenleiter, 2003). In addition, this gB protein is the principle immunogen of the virus,
300 stimulating the host to produce both complement-dependent and non-complement
301 dependent neutralizing antibodies (Okazaki, 2007). Alignments of the gB protein indicated
302 that the Chinese strains had amino acid insertions, deletions, and substitutions in
303 comparison with strain Bartha-k61 (Figure 5). These amino acid changes might lead to

304 the alteration of the neutralizing epitope of the gB protein and thus alter the protective
305 efficacy of previously used Bartha-k61vaccines in China.

306

307 The gC protein is another important neutralizing antigen and is the major virulent protein
308 of PRV, guiding the adsorption process between the virus and target cells (Karger et al.,
309 1998). Sequence alignments of the gC protein found that the most Chinese strains
310 contained a continuous insertion of seven amino acids (AAASTPA at sites 63-69) and
311 two-amino acid substitution within the protein compared to Bartha-k61 (Figure 6). These
312 changes might influence the structure of the gC glycoprotein of those strains, and
313 therefore influence the virus adhesion to host cells. The gE protein is another major
314 virulent protein of PRV (Mettenleiter et al., 1994). It has been reported that only a few
315 amino acids changes are required to alter the virulence of PRV isolates (Mettenleiter et
316 al., 1994). In this study, we found that the 25 PRV strains contained amino acid-
317 insertion/deletion within the gE protein either compared to the isolates from other
318 countries (Kaplan-Hungary and NIA3-Japan) or compared to the isolates from China
319 before 2012 (strain Min-A-China-2002); those changes might also have an effect on the
320 virulence of PRV isolates currently circulating in China.

321

322 ## Conclusion

323 In summary, this study reported a large-scale etiological investigation of PRV involved in
324 most regions of China after the outbreak of PR in late 2011. Our data revealed an average
325 positive rate of 8.27% for PRV detection between 2012 and 2017, indicating the risk of

pseudorabies prevalence in China. Phylogenetic analysis showed that the evolutionary relationship between the PRV isolates circulating in China and those from the other countries was remarkably distinct, suggesting that vaccination with foreign strains might be unable to provide full protection against currently epidemic isolates in China. In addition, PRV isolates currently circulating contained different types of mutations within their gB, gC, and gE proteins compared to those from other countries and/or those from China before the outbreak; these changes also might be associated with virulence changes of the virus. In the next step, we intend to evaluate the influence of those changes on the virulence/pathogenicity of the isolates.

Ethics approval and consent to participate

All samples used in this study were sent and provided by the farm owner. This study does not involve in human or animal use.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

ZP, HC and BW conceived and designed the experiments. YS, WL, QL, TZ, HZ, LH, XT, and DZ were responsible for sample detection, virus isolation, and data analysis. ZP, WL, CWS, YT, HC, and BW contributed to the manuscript writing and revision. All authors read and approved the final manuscript

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Figure legends

Figure 1 Samples collection from mainland China for PRV detection between 2012 and 2017

Figure 2 Positivity rate of PRV detection in different months (A) and seasons (B).

Figure 3 Positivity rate of PRV detection in different graphic parts of China.

Figure 4 Evolutionary relationships of PRV isolates based on gB (A), gC (B) and gC (C). The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 0.08532423 (gB)/ 0.11049107 (gC)/ 0.08878838 (gC) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. The analysis involved 50/34/33 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 293/462/570 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

442 **Figure 5 Alignment of partial amino acid sequences of PRV gB protein.** The
443 substitution regions are shown by the green boxes. The deletion region is shown by the
444 red box. The insertion region is shown by the blue box.

445

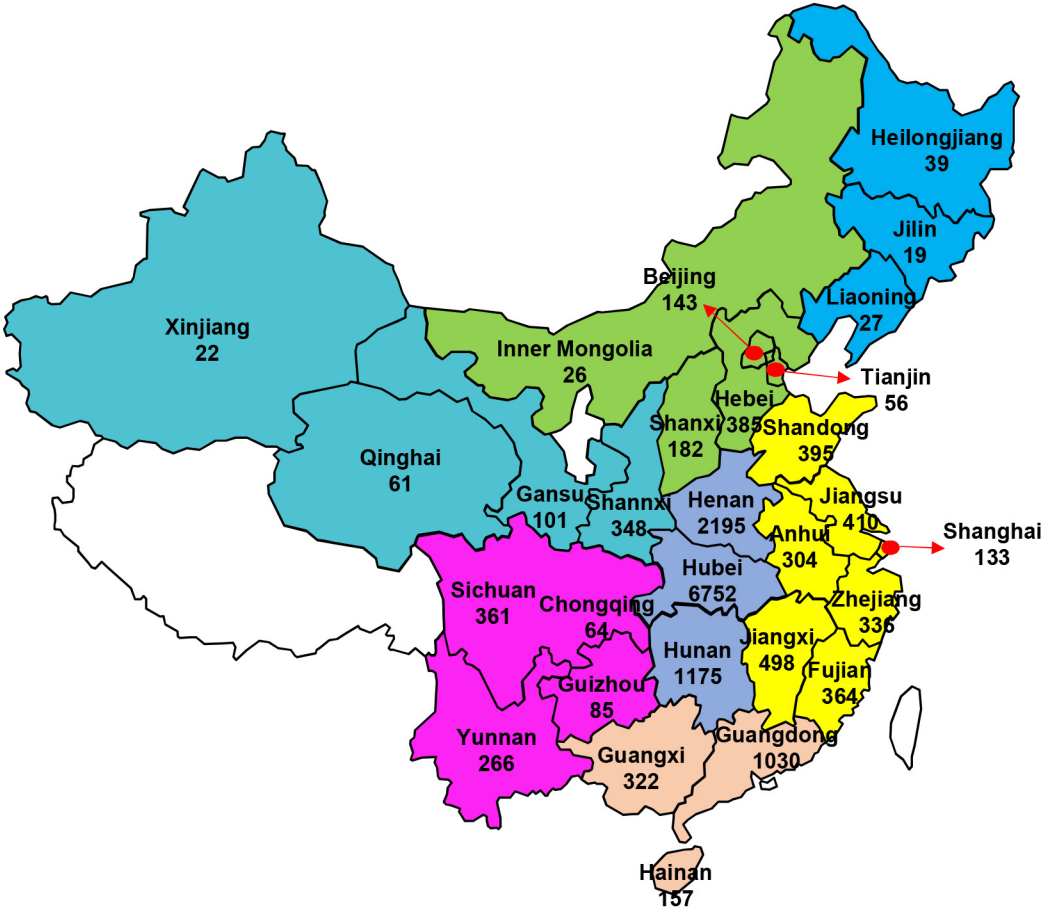
446 **Figure 6 Alignment of complete amino acid sequences of PRV gC protein.** The
447 substitution regions are shown by the green boxes. The insertion region is shown by the
448 blue box.

449

450 **Figure 7 Alignment of complete amino acid sequences of PRV gE protein.** The
451 substitution regions are shown by the green boxes. The insertion region is shown by the
452 blue box. The deletion regions are shown by the red boxes.

Figure 1(on next page)

Samples collection from mainland China for PRV detection between 2012 and 2017.



Graphic regions	PRV positive rate
Southwestern China	11.60%
Eastern China	10.90%
Northern China	8.59%
Northwestern China	8.27%
Northeastern China	8.24%
Southern China	7.16%
Central China	6.97%

Figure 2(on next page)

Positivity rate of PRV detection in different months (A) and seasons (B).

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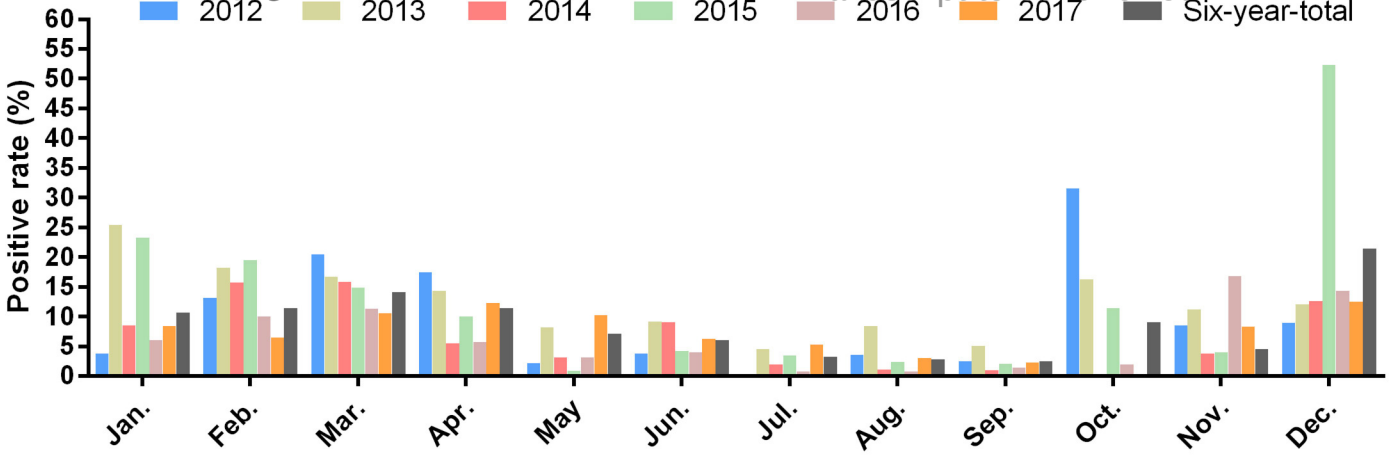
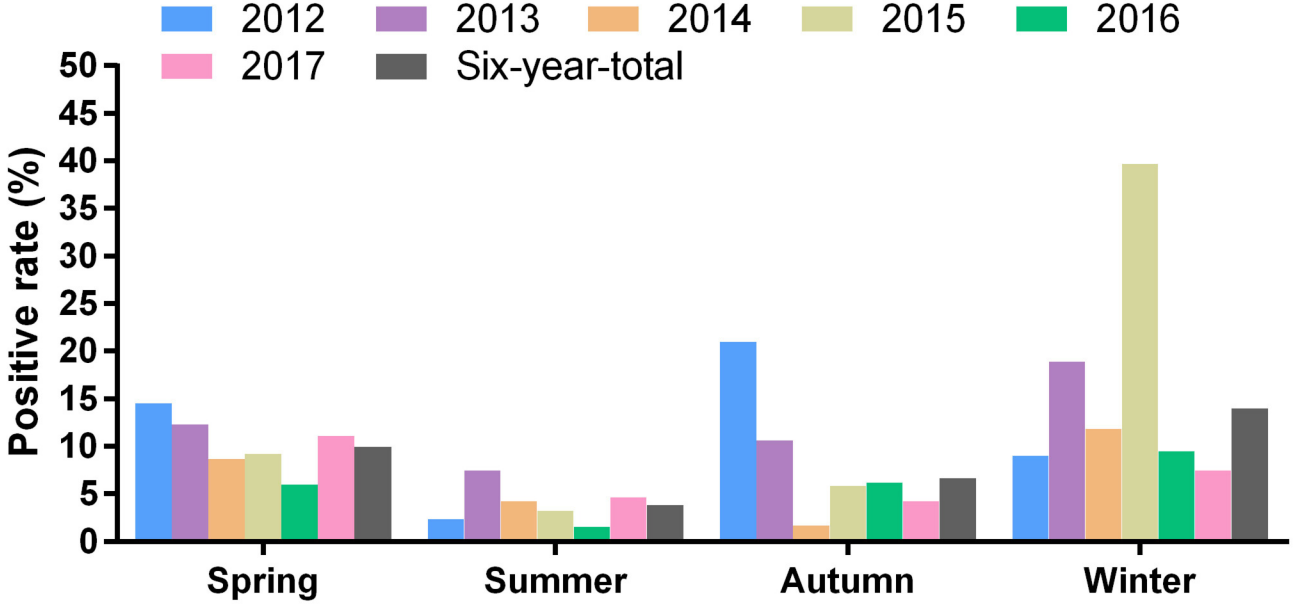


A.



B.

Figure 3(on next page)

Positivity rate of PRV detection in different graphic parts of China.

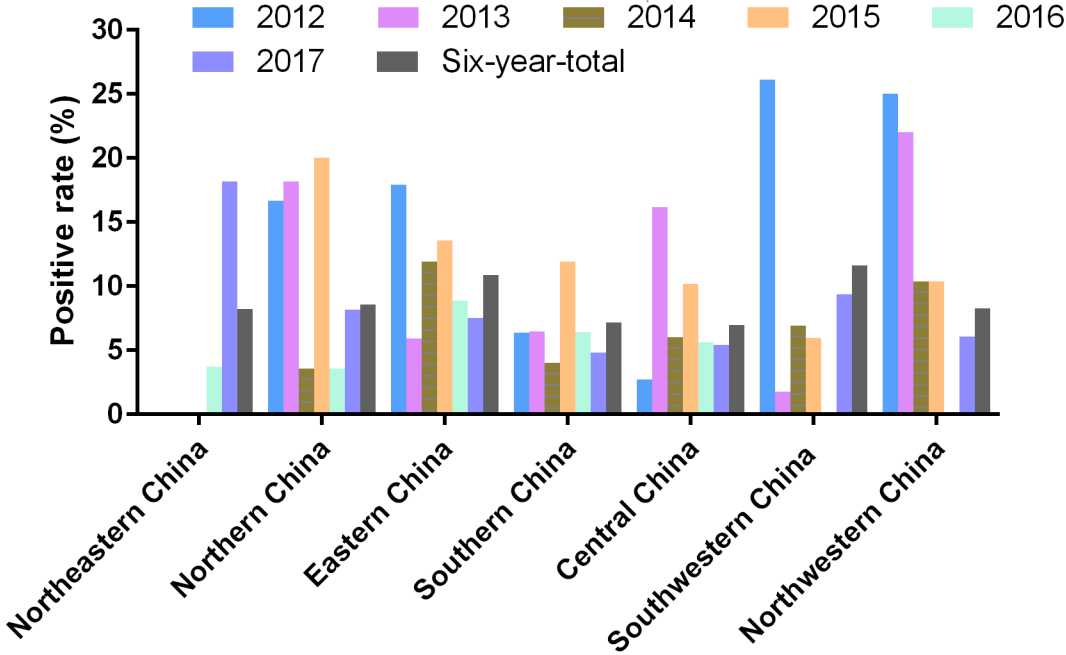

2012
2013
2014
2015
2016
2017
Six-year-total
Positive rate (%)
30
25
20
15
10
5
0
Northeastern China
Northern China
Eastern China
Southern China
Central China
Southwestern China
Northwestern China

Figure 4(on next page)

Evolutionary relationships of PRV isolates based on gB (A), gC (B) and gC (C).

The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 0.08532423 (gB)/ 0.11049107 (gC)/ 0.08878838 (gC) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. The analysis involved 50/34/33 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 293/462/570 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

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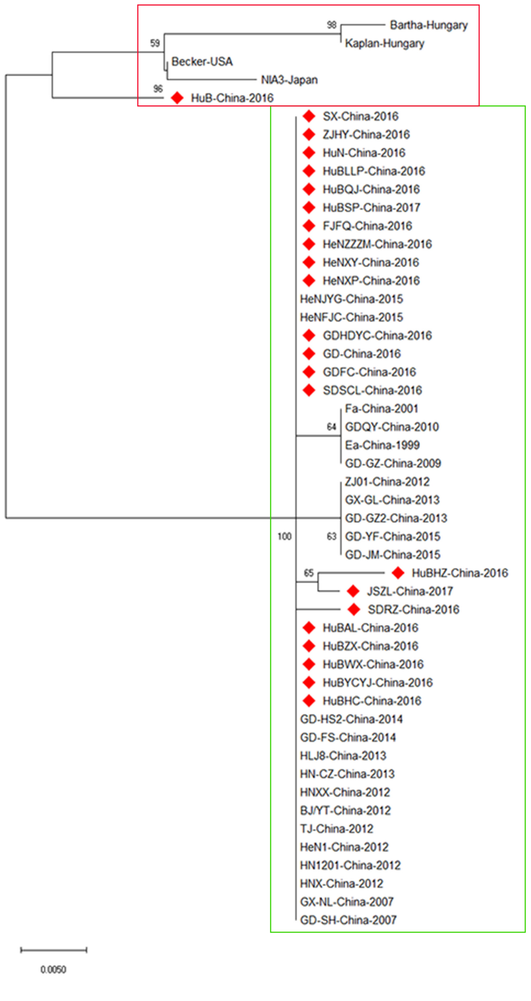
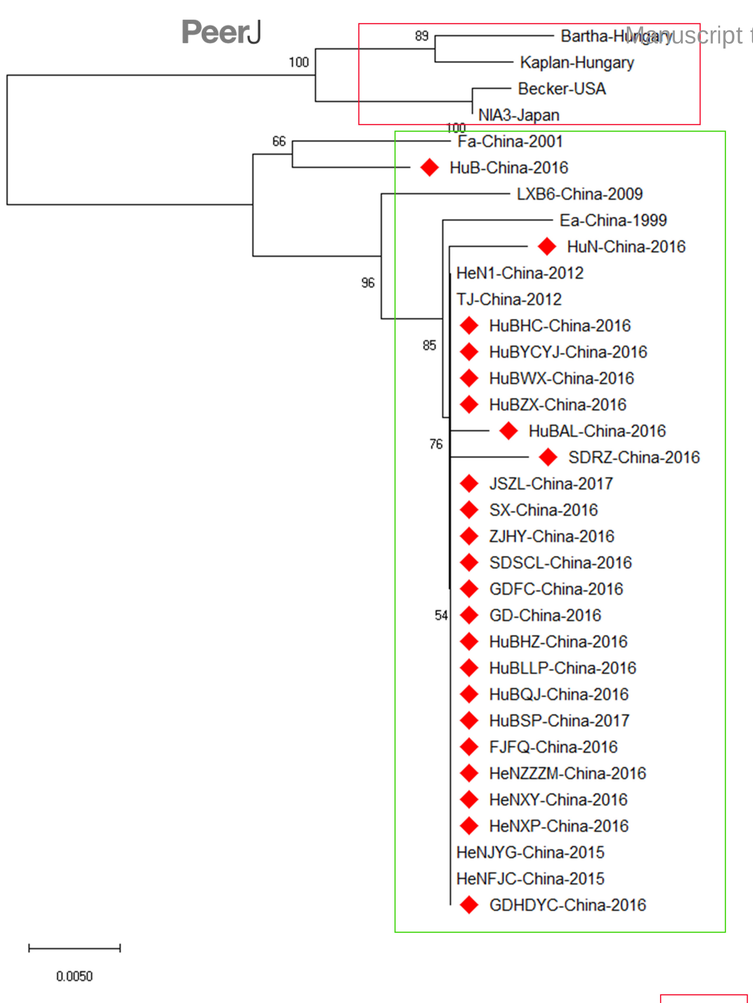
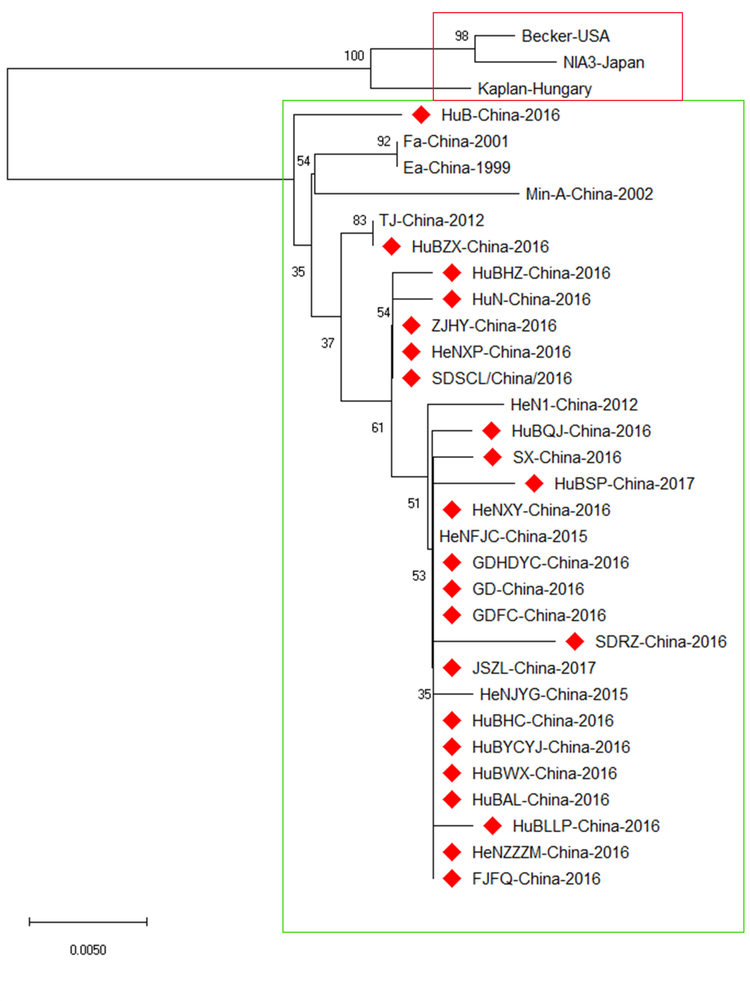


A.



B.



C.

Genotype I

Genotype II

Figure 5(on next page)

Alignment of partial amino acid sequences of PRV gB protein.

The substitution regions are shown by the green boxes. The deletion region is shown by the red box. The insertion region is shown by the blue box.

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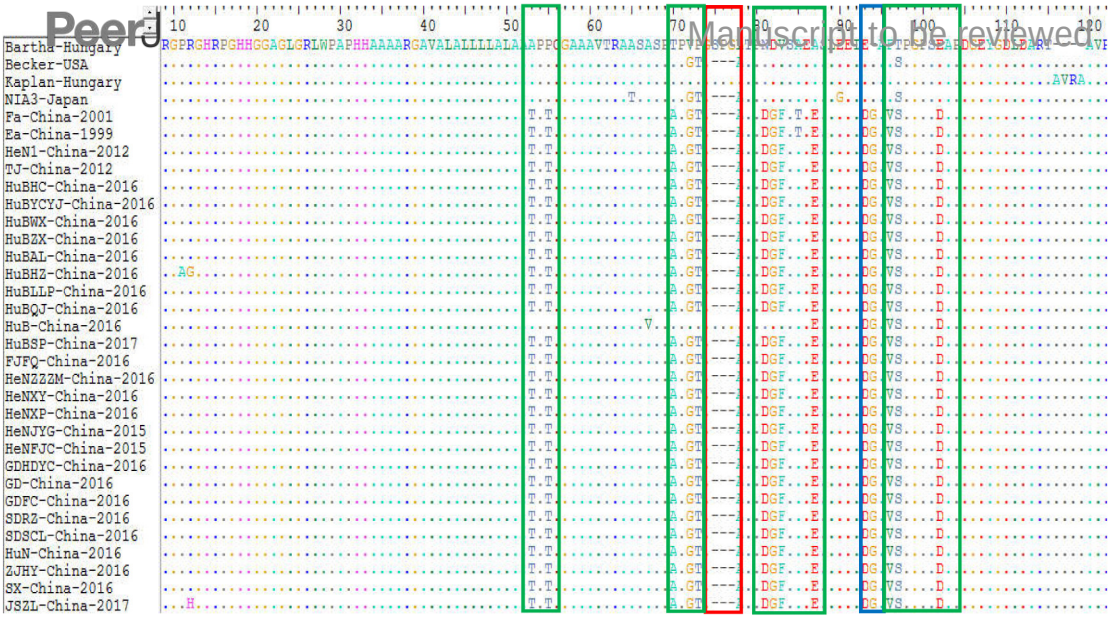

Figure 6(on next page)

Alignment of complete amino acid sequences of PRV gC protein.

The substitution regions are shown by the green boxes. The insertion region is shown by the blue box.

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Figure 7 (on next page)

Alignment of complete amino acid sequences of PRV gE protein.

The substitution regions are shown by the green boxes. The insertion region is shown by the blue box. The deletion regions are shown by the red boxes.

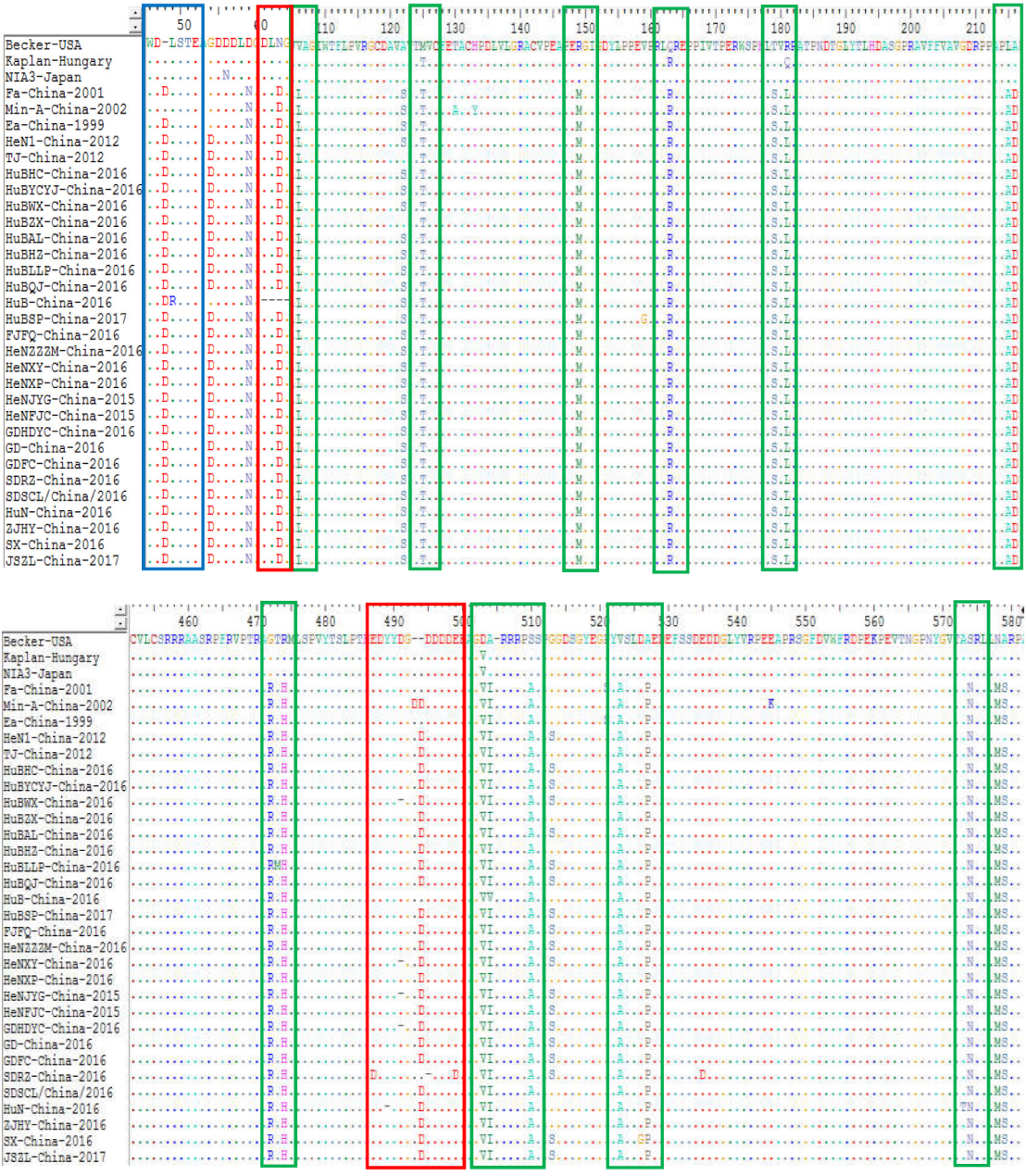
Becker-USA
Kaplan-Hungary
NIA3-Japan
Fa-China-2001
Min-A-China-2002
Ea-China-1999
HeN1-China-2012
TJ-China-2012
HuBHC-China-2016
HuBYCYJ-China-2016
HuBWX-China-2016
HuBZX-China-2016
HuBAL-China-2016
HuBHZ-China-2016
HuBLLP-China-2016
HuBQJ-China-2016
HuB-China-2016
HuBSP-China-2017
FJFQ-China-2016
HeNZZZM-China-2016
HeNXY-China-2016
HeNXP-China-2016
HeNJYG-China-2015
HeNFJC-China-2015
GDHDYC-China-2016
GD-China-2016
GDFC-China-2016
SDRZ-China-2016
SDSCL/China/2016
HuN-China-2016
ZJHY-China-2016
SX-China-2016
JSZL-China-2017

Table 1(on next page)

Primers used in this study

Table 1 Primers used in this study

Primers	Sequences (5'-3')	Products (bp)	Effects
gE1-F gE1-R	CGTGTGGCTCTGCGTGCTGT ATTCGTCACTTCCGGTTTC	342	Sample detection
gB2-F gB2-R	GGCTGGTGGCGGTGTTTGGCG AGGGCGAAGGAGTCGTAGGG	892	Amplifying gB
gC1-F gC1-R	CCATGTGYGCCACTAGCATT CGGTGCTGTTGGTCACGAAG	965	Amplifying the N-terminal of gC
gC2-F gC2-R	CAACGTCTCGCTCCTCCTGT GCCGTCGTCTCGTGTGGTT	921	Amplifying the C-terminal of gC
gE2-F gE2-R	GACCATGCGGCCCTTTCTGC GGTCCACCGGGCGCAGGCA	899	Amplifying the N-terminal of gE
gE3-F gE3-R	TTTACCGCCACGCTGGACTGGT CTTGGGGGCCAGCAGGACGT	1098	Amplifying the C-terminal of gE

Table 2(on next page)

PRV reference strains used in this study

Table 2 PRV reference strains used in this study

Strain	Year of isolation	Place of isolation	GenBank accession
Bartha	—	Hungary	JF797217.1 (complete genome)
Becker	—	United States	JF797219.1 (complete genome)
Kaplan	—	Hungary	JF797218.1 (complete genome)
NIA3	—	Japan	KU900059.1 (complete genome)
Fa	2001	China	KM189913.1 (complete genome)
TJ	2012	China/Tianjin	KJ789182.1 (complete genome)
BJ/YT	2012	China/Beijing	KC981239.1 (complete genome)
ZJ01	2012	China/Zhejiang	KM061380.1 (complete genome)
HN1201	2012	China/Henan	KP722022.1 (complete genome)
HNX	2012	China/Henan	KM189912.1 (complete genome)
HeN1	2012	China/Henan	KP098534.1 (complete genome)
HLJ8	2013	China/Heilongjiang	KT824771.1 (complete genome)
Ea	1999	China/Hubei	AF257079.1 (gB), AF158090.1 (gC), AF171937.1 (gE)
GX-NL	2007	China/Guangxi	KT948044.1 (gB), KU323908.1 (gC), KT936469.1 (gE)
GD-SH	2007	China/Guangdong	KT948054.1 (gB), KU323907.1 (gC), EF552427.1 (gE)
GD-GZ	2009	China/Guangdong	KT948042.1 (gB), KU323905.1 (gC), KT936466.1 (gE)
GD-GZ2	2013	China/Guangdong	KT948045.1 (gB), KU323903.1 (gC), KT936467.1 (gE)
HN-CZ	2013	China/Hunan	KT948049.1 (gB), KU323912.1 (gC), KT936465.1 (gE)
GD-FS	2014	China/Guangdong	KT948040.1 (gB), KU323909.1 (gC), KT936476.1 (gE)
GD-HS2	2014	China/Guangdong	KT948047.1 (gB), KU323911.1 (gC), KJ660063.1 (gE)
GD-JM	2015	China/Guangdong	KT948048.1 (gB), KU323899.1 (gC), KT936473.1 (gE)
GD-QY	2010	China/Guangdong	KT948053.1 (gB), KU323901.1 (gC)
GX-GL	2013	China/Guangdong	KT948046.1 (gB), KU323910.1 (gC)
GD-YF	2015	China/Guangdong	KT948041.1 (gB), KU323904.1 (gC)
P-PrV	2003	Malaysia	EU915280.1 (gC), FJ176390.1 (gE)
LXB6	2009	China/Heilongjiang	GQ926931.1 (gC), GQ926932.1 (gE)
SMX	2014	China/Henan	KR025920.1 (gC), KP192495.1 (gE)
GD-WH	2015	China/Guangdong	KU323902.1(gC), KT936468.1(gE)
HNXX	2012	China/Henan	KJ526436.1(gB), KJ526441.1(gC)
HS	2008	China/Sichuan	EU719636.1(gC)
Min-A	2002	China/Fujian	AY170318.1(gE)

Table 3(on next page)

Twenty-five strains of PRV isolated and analyzed in this study

Table 3 Twenty-five strains of PRV isolated and analyzed in this study

Strains	Place of isolation	Samples of isolation	Date of isolation	TCID50/0.1 mL
HeNFJC	Henan	Lung	2015/11	$10^{6.72}$
HeNJYG	Henan	Lung	2015/11	$10^{7.38}$
GDFC	Guangdong	Lung	2016/3	$10^{7.00}$
GD	Guangdong	Lung	2016/3	$10^{7.25}$
HeNZZZM	Henan	Lung	2016/3	$10^{7.25}$
HuBLLP	Hubei	Lung	2016/3	$10^{7.59}$
FJFQ	Fujian	Lung	2016/3	$10^{7.28}$
GDHDYC	Guangdong	Lung	2016/3	$10^{7.49}$
HuN	Henan	Brain	2016/3	$10^{7.96}$
SDRZ	Shandong	Lymph nodes	2016/3	$10^{7.36}$
HuBYCYJ	Hubei	Lymph nodes	2016/4	$10^{7.67}$
HeNXY	Henan	Lung	2016/4	$10^{7.08}$
HuBWX	Hubei	Lung	2016/4	$10^{7.25}$
SDSCL	Shandong	Lung	2016/5	$10^{7.80}$
HuBHC	Hubei	Lung	2016/5	$10^{7.59}$
HuBAL	Hubei	Brain	2016/9	$10^{7.12}$
HuBZX	Hubei	Brain	2016/9	$10^{7.40}$
ZJHY	Zhejiang	Lung	2016/10	$10^{7.25}$
SX	Shanxi	Lung	2016/10	$10^{7.57}$
HeNXP	Henan	Brain	2016/11	$10^{7.25}$
HuBHZ	Hubei	Lung	2016/11	$10^{7.12}$
HuBQJ	Hubei	Lung	2016/11	$10^{7.00}$
HuB	Hubei	Tonsil	2016/12	$10^{7.35}$
JSZL	Jiangsu	Brain	2016/12	$10^{7.43}$
HuBSP	Hubei	Brain	2016/12	$10^{7.54}$