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Retraction

Retracted: The Genomic and Pathogenic Characteristics of the Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus Isolate WUH2

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The paper titled "The Genomic and Pathogenic Characteristics of the Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus Isolate WUH2" [1], published in ISRN Virology, has been retracted as it was submitted for publication by the author Bin Li without the knowledge and approval of the coauthor Shaobo Xiao.

References

[1] B. Li, L. Fang, S. Liu, Y. Jiang, H. Chen, and S. Xiao, "The genomic and pathogenic characteristics of the highly pathogenic porcine reproductive and respiratory syndrome virus isolate WUH2," *ISRN Virology*, vol. 2014, Article ID 624535, 15 pages, 2014.

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Research Article

The Genomic and Pathogenic Characteristics of the Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus Isolate WUH2

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To fully understand the extent of genetic diversity and pathogenesis of the highly pathogenic PRRSV found in China, we determined the genomic sequence of PRRSV WUH2; the pathogenicity of WUH2 was compared to the classical PRRSV isolate CH-1a. Our results showed that the WUH2 genome had a discontinuous deletion of 30 aa in Nsp2, a 1 nucleotide deletion located in both the 5′ and 3′ UTRs, and point mutations within GP5. Experimental infection demonstrated that PRRSV WUH2 reproduced the phenotype and symptoms of porcine high fever syndrome. Importantly, we found that there were differences in viral burden in the serum and tissues when comparing infections of the pathogenic isolate WUH2 to those of the classical isolate CH-1a. These data provide insight into the genomic diversity and altered pathogenicity of Chinese PRRSV isolates and help elucidate the evolution and potential pathogenic mechanisms of PRRSV.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a relatively new viral disease of swine, that is, characterized by severe reproductive failure in sows and respiratory distress in piglets and growing pigs. Since PRRS was first reported in the United States in 1987 and Europe in 1990 [1, 2], the disease has devastated the swine industry by causing tremendous economic losses around the world; it is now considered one of the most important diseases in countries with intensive swine industries [3–5]. PRRS is caused by the PRRS virus (PRRSV), a member of a group of enveloped, positive-sense RNA viruses in the Arteriviridae family [6]. The genome of PRRSV is approximately 15 kb containing nine open reading frames (ORFs) [7]. ORF1a and ORF1b encode for the RNA-dependent RNA polymerase and nonstructural proteins (Nsps: Nsp1α, Nsp1β, and Nsp2–12), respectively [8–10]. ORF2a, ORF2b, and ORFs 3 through 7 encode the viral structural proteins GP2, E, GP3, GP4, GP5, M, and N, respectively [7, 11].

PRRSV can be divided into European and North American genotypes, which share only about 60% nucleotide identity at the genomic sequence level [12]. Isolates from each genotype exhibit significant sequence variation, particularly in Nsp2 and ORF5 which represent the most genetically variable regions in the PRRSV genome [4, 13, 14].

PRRSV was first reported in 1995 and the first isolate was identified in mainland China in 1996. Since then, PRRS has become one of the most important swine diseases worldwide. Many isolates have been obtained within China from different regions and times. A porcine high fever syndrome caused by a highly pathogenic PRRSV broke out in the south of China in 2006 and rapidly spread across the country, affecting more than 20 million pigs [15–17]. This syndrome was

characterized by a high continuous fever, anorexia, red discolorations on the body, and blue ears. The morbidity rate was 50%–100% and the mortality rate was 20%–100%. Genomic analysis showed that the etiologic virus was significantly different from previous isolates [15, 17–19].

In this study, we sequenced and analyzed the genome of the PRRSV isolate WUH2, a highly pathogenic PRRSV isolated in central China during the pandemic period of the porcine high fever syndrome. We then compared the pathogenic characteristics of WUH2 to the classical PRRSV isolate CH-1a. The reemergence of a highly pathogenic PRRSV and our study of its genome and pathogenic characteristics demonstrated the differences between highly pathogenic and classical isolates.

2. Materials and Methods

2.1. Virus and Cells. PRRSV strain WUH2 was isolated in MARC-145 from the brains of pigs suffering from the high fever syndrome in China at the end of 2006. The 5th passage of virus was purified, and other pathogens or microbial contaminants. PRRSV strain CH-1a, the first isolate identified in mainland China in 1996, was used as the classical PRRSV reference strain. MARC-145 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units of penicillin, and 20 units of streptomycin. Cells were maintained in a 37°C humidified chamber with 5% CO_2.

2.2. Primer Design, Preparation of RNA, RT-PCR, and Quantitative PCR. Oligonucleotide primers amplifying the WUH2 genome were designed based on sequence information from GenBank for PRRSV strains VR-2332 and CH-1a as well as sequences from highly pathogenic PRRSV isolates [20]. Tissue samples were weighed and an equal volume of PBS was added to the sample before they were homogenized. RNA was extracted from infected cells, serum, and tissue using the QIAamp viral RNA kit (Qiagen, Shanghai, China). Viral cDNA was synthesized by reverse transcription reactions using random hexanucleotide primers and SuperScript II reverse transcriptase (Gibco BRL) following the manufacturer's instructions. cDNA was then used as a template in subsequent PCR reactions with a final volume of 50 μL containing 25 pmol of each PRRSV-specific primer described above, 1x Pfu polymerase buffer, 200 μM dNTPs, and 2.5 units of Pfu DNA polymerase (Stratagene).

Viral load was detected by quantitative PCR. Primers and probes were designed targeting the ORF7 gene with the Primer Express software package. The probe (5′-TCCCGGTCCCTTGCCTCTGGA-3′) was labeled with FAM at its 5′ terminus (reporter) and TAMRA at its 3′ end (quencher). Primer sequences were 5′-TCAGCTGTGCCAAATGCTGG-3′ and 5′-AAATGGGGCTTCTCCGGGTTTT-3′. Amplification was performed using TaqMan mixtures (TaKaRa). The thermal cycler program was 60°C for 10 min, 95°C for 15 s, and 40 PCR cycles (90°C for 15 s and 60°C for 60 s). Known amounts of serially diluted *in vitro* transcribed RNA (10e12 copies/μL) were used to generate a standard curve for each plate run.

2.3. DNA Sequencing and Sequence Analysis. The 5′ terminal sequences of viral genomes were amplified and cloned as previously described [19]. PCR products were purified using the QIAquick gel extraction kit (QIAquick), cloned into the pMD18-T vector (TaKaRa), and confirmed by sequencing. For each amplified genomic region, three clones were sequenced from each of the three independent amplifications in both directions. Thus, nine determinations of the sequence of each region were performed.

Sequence data were assembled and analyzed using Lasergene software (DNASTAR). Multiple sequence alignments were done using Clustal W. A phylogenetic tree was constructed by the neighbor-joining method using MEGA 3.1 [21]. The reliability of the neighbor-joining tree was estimated by bootstrap analysis (1,000 replicates).

2.4. Experimental Infection. We performed experimental infections in pigs to compare the virulence and pathogenesis of isolate WUH2 to CH-1a. Thirty piglets, weaned at 5-6 weeks of age, were obtained from a PRRS-free farm in Hubei, China. The piglets were randomly separated into three groups that were housed in separate rooms. Piglets were inoculated intramuscularly with $10^{5.0}$ $TCID_{50}$/2 mL of either CH-1a or WUH2 (10 piglets per group). Control piglets were inoculated with DMEM. Experimental piglets were clinically examined daily. Rectal temperatures and mortality were recorded from 0 to 21 days after inoculation (dpi.); blood was collected at 0, 3, 7, 10, 14, and 21 d.p.i. to measure viral load by quantitative PCR. Two piglets from each group were euthanized and necropsied at 7, 10, and 21 dpi. Organs were collected including lung, brain, spleen, kidney, heart, liver, stomach intestines, and lymph nodes for viral load determination by quantitative PCR and histopathological examination [22]. The remaining 6 piglets in each group were observed for 21 days to determine death rates. Viral loads of the piglets, which were naturally death and two of live at 21 dpi, were detected too.

2.5. Statistical Analysis. Student's t-tests were used to compare viremia and viral load between the different groups. P values < 0.05 were considered statistically significant.

3. Results

3.1. The Complete Genomic Sequence of the PRRSV Strain WUH2. The complete WUH2 genome was found to be 15,350 nucleotides long excluding the poly(A) tail (GenBank accession number EU678352) with a 189 nucleotide long 5′ UTR and a 150 nucleotide long 3′ UTR. The genome also contained nine ORFs, two large overlapping ORFs (ORF1a and ORF1b) followed by the 5′ UTR, and seven ORFs encoding structural proteins (ORF2a, ORFs 2b to 7). Comparison of this genome to 6 representative genomic sequences from other PRRSV isolates showed that WUH2 shared 88.9% sequence identity with the representative North

Table 1: The amino acid identity of the proteins of PRRSV WUH2 with other strains (%).

ORF	Cleavage product	VR-2332	LV	RespPRRS MLV	CH-1a	HB-2(sh)/2002	JXA1
Genome		88.9	57.9	88.8	94.4	91.9	98.4
	Nsp1α	95.8	66.9	95.8	97.0	98.2	100.0
	Nsp1β	83.4	40.1	82.5	87.1	83.4	97.2
	Nsp2	74.9	29.1	74.8	84.7	80.9	97.3
	Nsp3	87.0	53.6	87.2	90.1	89.2	91.5
1a	Nsp4	88.7	59.3	88.7	90.7	90.2	94.1
	Nsp5	91.8	71.2	92.4	94.7	92.9	99.4
	Nsp6	93.8	75.0	93.8	100.0	100.0	100.0
	Nsp7	89.2	42.3	89.6	96.5	95.0	99.6
	Nsp8	97.8	66.7	97.8	97.8	95.6	97.8
	Nsp9	97.2	74.5	97.2	98.3	98.0	99.4
1b	Nsp10	96.4	64.7	96.6	98.2	97.5	99.1
	Nsp11	95.1	76.3	95.1	97.3	96.4	99.6
	Nsp12	94.1	36.0	94.1	96.1	95.4	98.7
2a	GP2	93.0	60.6	92.6	96.5	93.8	100.0
2b	E	90.4	64.5	89.0	94.5	94.5	100.0
3	GP3	85.8	54.5	85.8	92.9	90.9	98.0
4	GP4	88.8	67.8	88.8	96.1	93.8	96.6
5	GP5	88.5	56.6	87.5	93.5	91.5	99.5
6	M	96.6	79.9	96.0	96.6	97.1	98.9
7	N	95.1	58.0	95.1	95.1	91.9	100.0

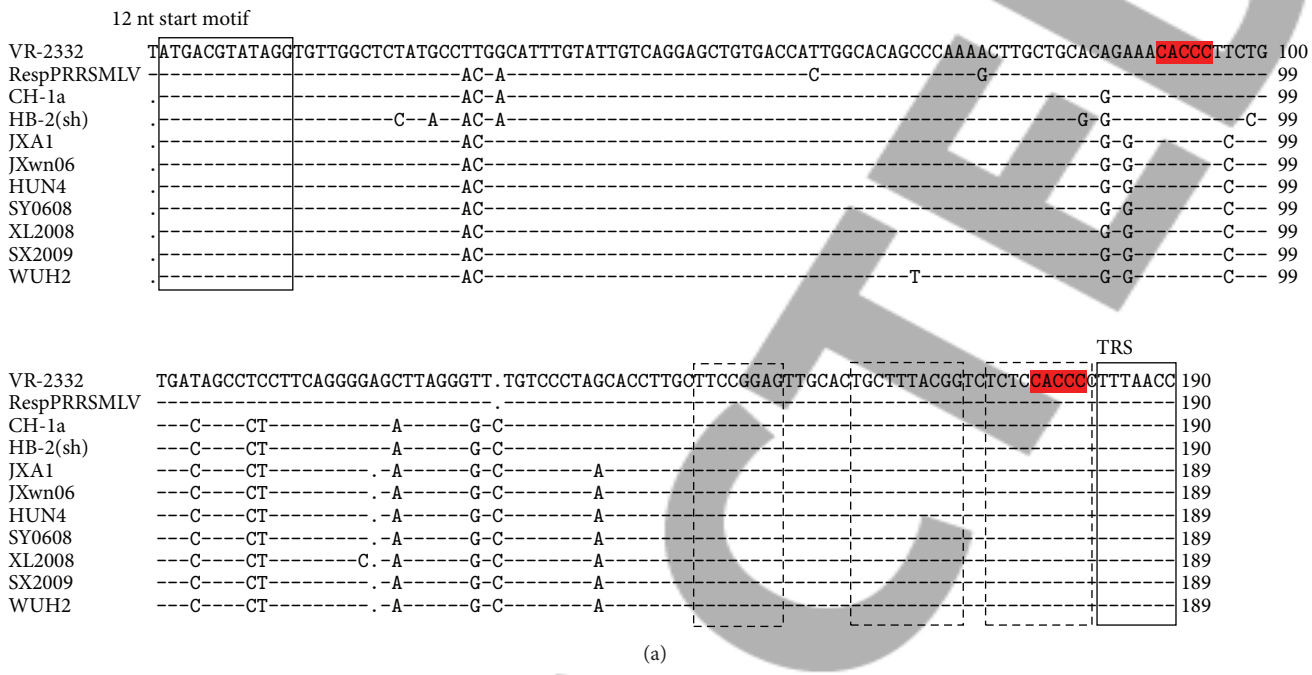
American strain VR-2332, 57.9% sequence identity with LV, and 94.4% nucleotide identity with CH-1a. WUH2 also had high sequence similarity to strain JXA1 (98.4%), the representative isolate from the highly pathogenic PRRSV in China in 2006; particularly notable was the presence of a discontinuous deletion of 30 aa (residues 481 and 533–561) in Nsp2 coding regions [16]. The detailed identities for each coding region of WUH2 compared to representative PRRSV isolates were summarized in Table 1.

3.2. Sequence Analysis of the UTRs. The 5′ UTR of WUH2 was 189 nts long and shared 91.1%–99.5% nucleotide identity with VR-2332 and other representative North American isolates. Sequence comparison revealed that the eleven strains contained a conserved 5′-leader start motif of 12 nt, the leader transcription regulatory sequence (TRS), and the CACCC motifs. The string of 8–11–9 conserved nucleotides were identical between VR-2332 and the Chinese isolates. There was a unique 1 nt deletion at position 119 in the 5′ UTR of WUH2 compared to VR-2332. This was a common characteristic of representative, highly pathogenic PRRSV isolates (JXA1, JXwn06, HUN4, SY0608, XL2008 and SX2009) (Figure 1(a)).

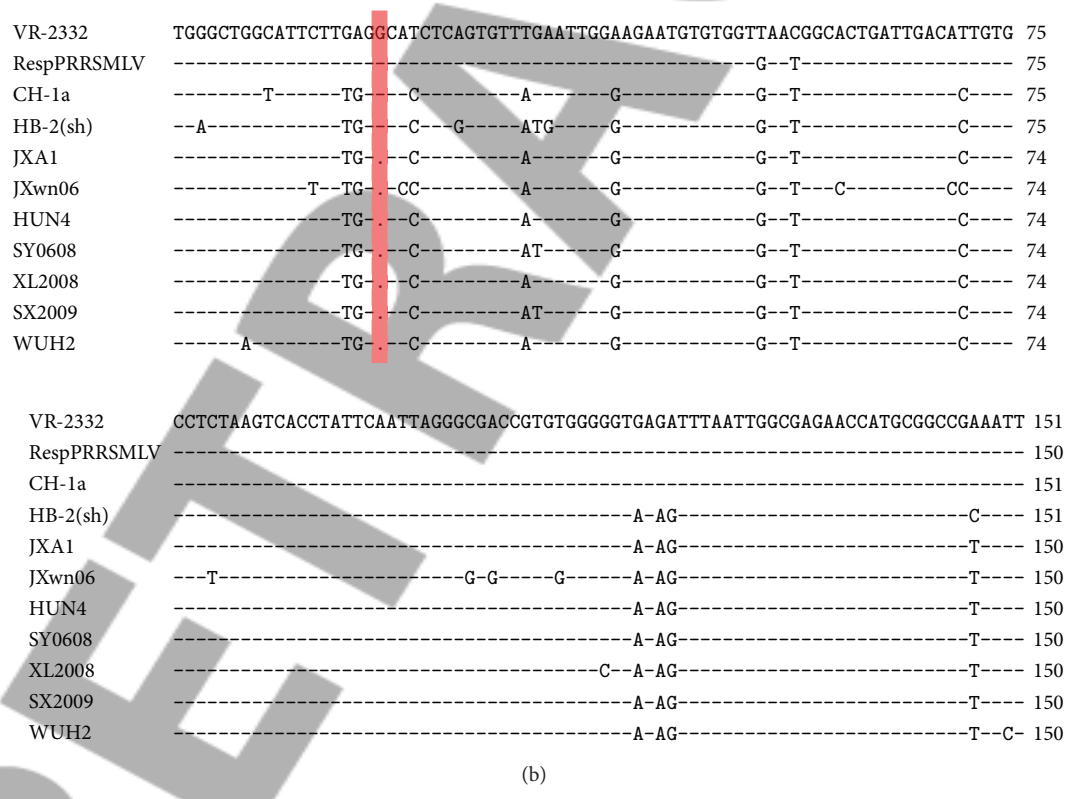
The 3′ UTR of WUH2 was 150 nucleotides long with a unique 1 nt deletion at position 19 compared to VR-2332 (Figure 1(b)). Similar to the 5′ UTR, the deletion was observed in all isolates of the highly pathogenic PRRSV. Genomic sequences were conserved at positions 82–104 in WUH2 and the representative isolates; a previous study similarly reported that a stretch of 23 bases (positions 82–104) was highly conserved between Lelystad and other PRRSVs [23]. The other nucleotide substitutions identified in WUH2 were also found in Chinese isolates.

3.3. Sequence Analysis of Nsp2 and GP5. The Nsp2 gene of PRRSV had the highest genetic diversity of all genes in the viral genome. In our study, analysis of sequence homology indicated that Nsp2 from WUH2 shared only 74.9% and 29.1% amino acid identity with VR-2332 and LV, respectively; the highest degree of sequence identity (97.3%) was found with JXA1. Further sequence analysis revealed that the Nsp2 gene from WUH2 was 2,850 bps long, encoding 950 amino acids with a discontinuous deletion of 1 and 29 amino acids at positions 481 and 533–561 compared to VR-2332 (Figure 2). These deletions in the Nsp2 region were identical and found at the same locations in the representative highly pathogenic PRRSV [16, 17].

The ORF5-encoded major envelope glycoprotein (GP5) was the least conserved structural protein [13, 24–26]. GP5 from WUH2 shared 88.5% and 56.6% amino acid identity with VR-2332 and LV, respectively. The GP5 gene shared 91.5%–99.5% sequence identity with representative Chinese isolates. The hypervariable region observed between WUH2 and other isolates includes two regions at the amino terminus (the signal peptide sequence and amino acid positions 30–36). Amino acid substitution tended to affect the position and number of potential N-glycosylation sites (Figure 3). There were five N-glycosylation sites in WUH2 and the other highly pathogenic PRRSV except SX2009. The variable of the primary neutralizing epitope (PNE, aa 37–44) was found at amino acid position 39. The L39I mutation was found in WUH2 and HP-PRRSV and was distinct from the L39F

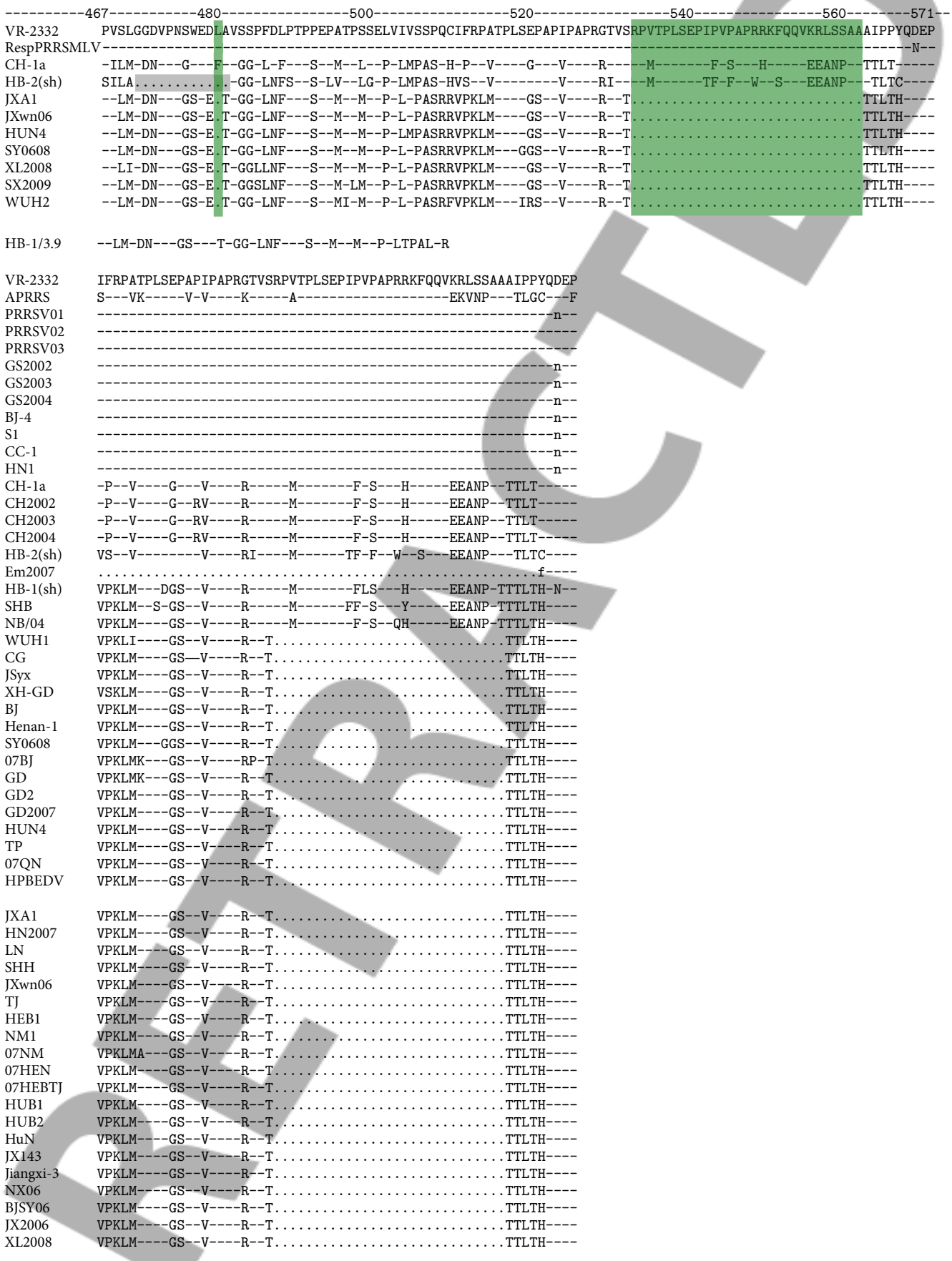


(a)



(b)

Figure 1: Alignment of the 5′ UTR (a) and 3′ UTR (b) of PRRSV isolates WUH2, VR-2332, RespPRRS MLV, CH-1a, HB-2(sh)/2002, JXA1, JXwn06, HuN4, SY0608, XL2008, and SX2009. (a) The 12 nt start motif and the leader TRS (black box) were absolutely conserved. The dashed region indicates the string of 8–11–9. The CACCC motifs were shown by the red boxes. (b) The dashed box indicates a stretch of 23 bases at positions 82–104. The two deleted regions are represented in red.



(a)

Figure 2: Continued.

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GS2008      VPKLM----GS--V----R--T.............................TTLTH----
SX2007      VPKLM----GS--V----R--T.............................TTLTH----
YN2008      VPKLM----GS--V----R--T.............................TTLTH----
BJPG        VPKLM----GS--V----R--T.............................TTLTH----
BJSD        VPKLM----GS--V----R--T.............................TTLTH----
CBB-1-F3    VPKLM----GS--V----R--T.............................TTLTH----
CWZ-1-F3    VPKLM----GS--V----R--T.............................TTLTH----
GDQJ        VPKLM----GS--V----R--T.............................TTLTH----
GDBY1       VPKLM----GS--V----R--T.............................TTLTH----
SD-CXA/08   VPKLM----GS--V----R--T.............................TTLTH----
SX-1        VPKLM----GS--V-T--R--T.............................TTLTH----
SX2009      VPKLM----GS--V----R--T.............................TTLTH----

HB-1/3.9    VPKLM---NGS--V----R-----M-------FLS---H-----EEANP-TTTLTH-N--
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(b)

Figure 2: Alignment of partial Nsp2 amino acid sequences of WUH2 with the North American prototype VR-2332 and the representative Chinese isolates. The deletions are shown in colors.

Figure 3: Amino acid sequence alignment of GP5 of WUH2 and the representative PRRSV isolates. The dash box identifies the signal peptide sequence and the hypervariable region. The key amino acid mutations were indicated with different colors.

mutation in CH-1a. The two amino acids R^{13} and R^{151} were altered during virus attenuation [7] and identified in our study in VR-2332. These data indicated that WUH2 possessed sequence characteristics of other pathogenic isolates. The A137S mutation was previously hypothesized to differentiate between vaccine strains [27] and was found in WUH2 and Chinese isolates.

3.4. Phylogenetic Analyses. To better understand the genetic characteristics of WUH2, a phylogenetic tree was constructed by the neighbor-joining method based on the representative genomic sequences of Chinese PRRSV isolated from 1996 to 2010. As shown in Figure 4, all Chinese PRRSV isolates belonged to the North American genotype and were further divided into four sub genotypes. WUH2 was defined in a group of the highly pathogenic PRRSVs that also contained JXA1, WUH1, JXwn06, SY0608, HUN4, HEB1, and HUB2; these strains have all been demonstrated to be highly pathogenic in artificial infection experiments [17, 20, 28–31].

3.5. Clinical Signs and Mortality. The negative control group showed no clinical signs of illness at any time during the experiment. Rectal temperatures of the control group were within normal range. Piglets inoculated with CH-1a showed only temporary fever, mild respiratory, and moderate anorexia. Death was not observed in this group. Obvious clinical signs including inappetence, lethargy, and high continuous fever (40.5–42.0°C) were observed in piglets inoculated with WUH2 beginning at 3 dpi (Figure 5(a)). These symptoms showed the rapid evolution of the disease caused by this isolate. A few areas of red discoloration on the body and blue ears were observed at 5 dpi and spread to the limbs

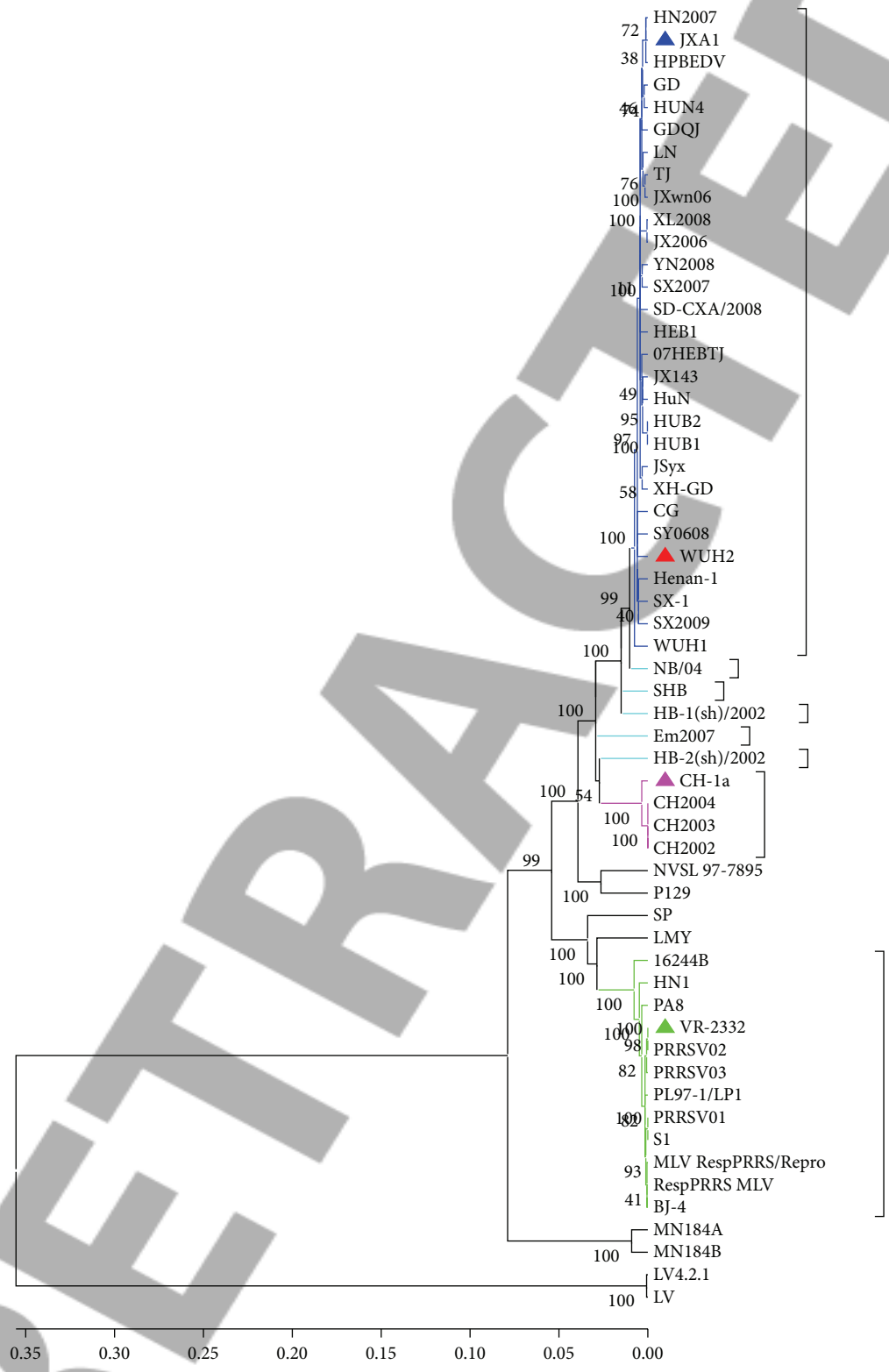

Figure 4: Phylogenetic tree of WUH2 and Chinese PRRSV isolates based on the complete genomic sequences. The phylogenetic tree was generated by the distance-based neighbor-joining method using the MEGA3.1 software. Bootstrap values were calculated on 1000 replicates of the alignment. The different subgenotypes are indicated by different colors. The different representative PRRSV isolates were marked with the triangle.

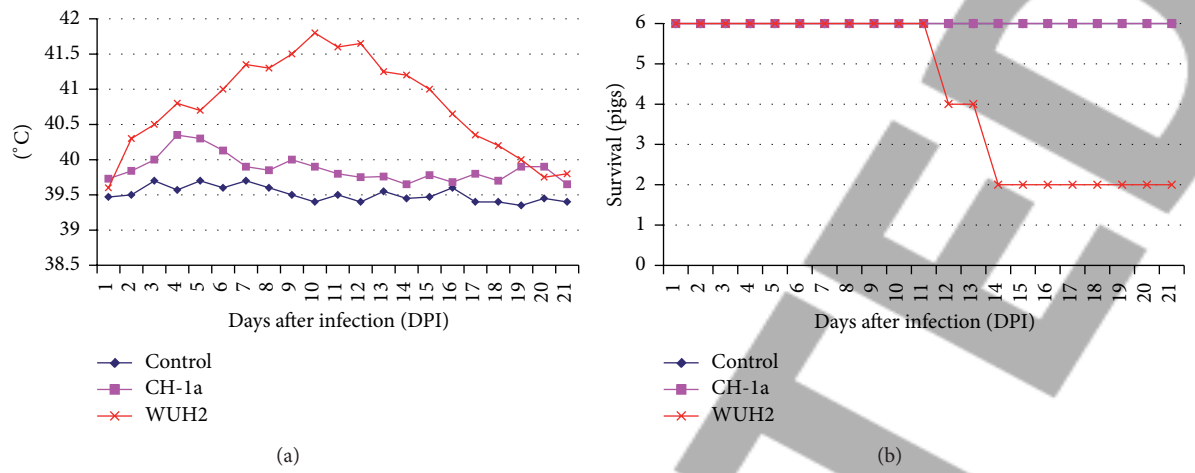

FIGURE 5: Pathogenicity comparison among the strains WUH2 and CH-1a. Forty-day-old PRRSV-free piglets were inoculated intramuscularly with $10^{5.0}$ $TCID_{50}$/2 mL of CH-1a and WUH2, respectively. Mean rectal temperature (a) and survival rate (b) of each group were recorded for 21 days after inoculation.

and belly at 7 dpi. The first death occurred at 12 dpi. Four of six piglets died within 21 dpi with WUH2 (Figure 5(b)).

3.6. Comparison of Gross Lesions and Histopathology. Two piglets from each group were euthanized and necropsied at 7, 10, and 21 dpi to compare gross lesions and histopathology. No gross lesions were observed at any time in the control group. Piglets in the CH-1a group showed mild lesions in organs: lungs had a typical pulmonary consolidation and mild tumescence, while intumescences were found in the submaxillary and inguinal lymph nodes. No obvious pathological changes were observed in the brain or spleen. In contrast, there were severe gross lesions in piglets inoculated with WUH2, including type and severity of pulmonary consolidation, severe intumescences, and hemorrhages in the lungs, brain, kidneys, and all lymph nodes (Figure 6(a)). Histopathology showed that piglets infected with WUH2 had severe interstitial pneumonia, thickening of the alveolar septa, increased infiltration of mononuclear cells in the lung and brain, and increased amounts of alveolar exudate and necrotic debris compared to piglets inoculated with CH-1a (Figure 6(b)). Nonsuppurative encephalitis also appeared in the brains of piglets inoculated with WUH2. Obvious perivascular cuffing and hemorrhages were seen in the vascular wall cells of the hippocampus (Figure 6(c)). Multifocal necrotic and histopathologic depletion were also found in the spleen, kidney, liver, and lymph follicles. There are obviously lymphoproliferative in the lymph nodes infected with WUH2.

3.7. Evaluation of Viremia. Blood was collected at 0, 3, 7, 10, 14, and 21 dpi to measure viremia by quantitative PCR. The mean PRRSV load in serum after challenge is shown in Table 2. PRRSV could not be detected in serum from animals in the control group at any time during the postchallenge period. All animals were PRRSV negative before inoculation (0 dpi). No significant differences ($P > 0.05$) were observed between the level of viremia for CH-1a and WUH2 inoculation groups before 7 dpi. Viral loads in the serum were approximately 10e3-10e4 RNA copies/mL. Viral loads in the serum were highest in all animals challenged at 10 dpi, equivalent to 10e4 RNA copies/mL in piglets inoculated with CH-1a. This difference was not statistically significant ($P > 0.05$) at 3, 7, or 10 dpi. Piglets from groups inoculated with WUH2 showed the highest viral loads at 10 dpi (10e7-10e9 RNA copies/mL). In piglets inoculated with WUH2, viral loads at 10 dpi were significantly higher ($P < 0.01$) than any other time, as well as all timepoints for piglets inoculated with CH-1a. There was a gradual reduction in the viral load from 10 dpi, and viral loads at 21 dpi were similar to those at 3 dpi.

3.8. Viral Burden in Tissues. Two piglets from each group were euthanized and necropsied at 7, 10, and 21 dpi; organs were collected for analysis of viral load by quantitative PCR. Table 3 represents individual values for viral load in selected tissues for each animal. PRRSV was undetectable in any tissues from piglets in the control group throughout the experimental period (data not shown). A low viral load for PRRSV was observed in selected organs of piglets inoculated with CH-1a throughout the duration of the experiment. In these same animals, a high viral load for PRRSV was found in the lungs (10e5 RNA copies/g), while the brain and stomach had relatively low viral loads (10e2 RNA copies/g).

A significant difference ($P < 0.05$) in viral load was observed at 10 dpi when comparing CH-1a to WUH2 groups. The viral load in select organs from WUH2-infected piglets was measured between 10e6-10e9 RNA copies/g. Viral load was the highest in the lungs (greater than 10e9 RNA copies/g), followed by the brain and lymph nodes (approximately 10e9 RNA copies/g). The stomach and intestines had viral loads between 10e6-10e7 RNA copies/g. There was a reduction in the viral load of PRRSV at 21 dpi in surviving piglets, similar to viremia.

We also measured the viral load in organs from piglets that died naturally during the experimental period (Table 4).

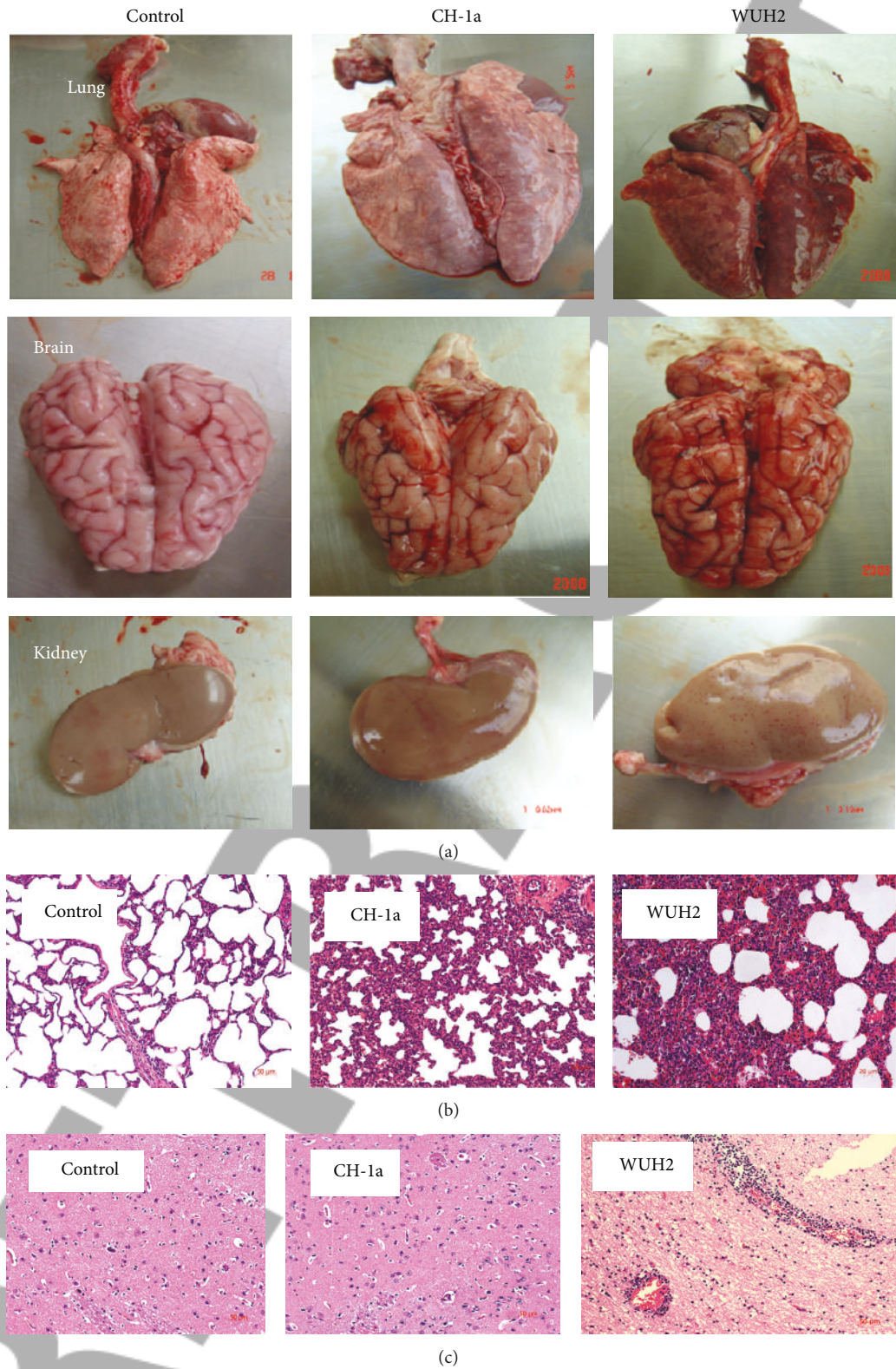

Figure 6: Gross lesions and histopathology in pigs experimentally infected with PRRSV CH-1a and WUH2. (a) Gross lesions of lung, brain, and kidney of pigs experimentally infected at 10 days after inoculation. Histopathological findings of HE stained lungs (b) and brains (c), 10 days after inoculation. Representative sections are shown.

Table 2: Viremia in serum measured by quantitative PCR.

Treatment	Animal ID number	Viremia					
		Day 0	Day 3	Day 7	Day 10	Day 14	Day 21
Control	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0
	5	0	0	0	0	0	0
CH-1a	6	0	3.43E + 03	4.23E + 03	9.40E + 03	Killed	
	7	0	2.58E + 03	5.94E + 03	2.71E + 04	Killed	
	8	0	2.38E + 03	7.51E + 03	3.21E + 04	2.98E + 04	6.10E + 02
	9	0	3.63E + 02	8.68E + 03	7.80E + 04	1.47E + 04	2.59E + 03
	10	0	1.42E + 03	7.48E + 03	1.29E + 04	5.25E + 03	4.32E + 03
	11	0	8.21E + 02	4.20E + 03	2.18E + 04	3.84E + 03	3.03E + 03
	12	0	2.25E + 03	3.32E + 03	8.40E + 04	2.45E + 04	1.17E + 03
	13	0	2.22E + 03	1.29E + 04	5.19E + 04	7.46E + 04	1.46E + 03
	14	0	2.51E + 03	1.32E + 04	Killed		
	15	0	2.63E + 03	1.79E + 04	Killed		
WUH2	16	0	7.26E + 03	4.50E + 04	1.51E + 09	Death	
	17	0	2.94E + 03	7.21E + 03	8.73E + 07	Killed	
	18	0	4.42E + 03	2.52E + 04	3.51E + 08	5.02E + 06	Death
	19	0	2.71E + 04	7.18E + 05	2.93E + 08	Killed	
	20	0	4.05E + 03	3.29E + 04	3.86E + 07	3.23E + 04	5.84E + 03
	21	0	4.49E + 03	2.61E + 04	4.09E + 07	3.04E + 04	2.10E + 03
	22	0	2.83E + 03	4.80E + 03	3.34E + 08	Death	
	23	0	5.25E + 03	8.37E + 03	1.54E + 09	Death	
	24	0	3.03E + 03	1.13E + 04	Killed		
	25	0	3.54E + 03	9.56E + 03	Killed		

The viral load in all organs at natural death was higher than viral loads in organs from piglets euthanized at 7, 10, and 21 dpi. Terminal viral loads were nearly 10e8 RNA copies/g, and the viral load in the lungs was greater than 10e9 RNA copies/g.

4. Discussion

In 2006, an unknown "high fever" syndrome emerged among swine herds in the Jiangxi Province of China. This syndrome rapidly spread to neighboring provinces, overwhelming most of the swine herds in mainland China in half a year. The disease affected more than 2,000,000 pigs with about 400,000 fatal cases, causing enormous economic losses for the pork-producing community. Studies considered the highly pathogenic PRRSV to be the causative agent of the "high fever" syndrome [16, 29]. The most remarkable genetic markers of the new isolates were two discontinuous deletions of 30 amino acids in the gene Nsp2. In a recent study, we also found two 1 nt deletions in the 5′ and 3′ UTRs [20]. The highly pathogenic PRRSV exhibits unusually high mortality in affected pigs of all ages that was different from the classical PRRSV. Therefore, it was necessary to perform a detailed comparison of the genome and pathogenesis of the highly pathogenic PRRSV to the classical strain.

In this study, we determined the complete nucleotide sequence of WUH2, characterizing its genome at a molecular level. Phylogenetic analyses based on the full-length genome sequence indicated that WUH2 belonged to a group of highly pathogenic PRRSVs. Analysis of genomic sequence data showed that WUH2 had a discontinuous deletion of 30 aa in Nsp2, a 1 nt deletion located in both the 5′ and 3′ UTR, and some important mutations in GP5.

The 5′ and 3′ UTRs are known important regulatory elements in the PRRSV genome that play critical roles in replication, transcription, and translation [19, 32, 33]. Interestingly, in addition to a discontinuous deletion in Nsp2, there was a 1 nucleotide deletion located in the 5′ and 3′ UTRs in WUH2 that was also found in nearly all other highly pathogenic PRRSV isolates. The significance of these mutations for PRRSV replication, transcription, and virulence remains to be determined.

The Nsp2 gene has been shown to be highly variable among arteriviruses [12, 13, 34, 35]. In particular, the large middle section of Nsp2 (VR-2332 Nsp2 amino acids 148–880) is highly variable in length. Previously, studies noted all instances of insertion or deletion in this hypervariable middle region [12, 26, 36–38]. These studies also showed that different amino acid insertions or deletions did not affect the survivorship of the virus, but their data were ambiguous on whether other biological characteristics of the virus were

Table 3: Viral load in selected tissues measured by quantitative PCR.

DPI	Treatment	Animal ID number	Viral burden in tissues									
			Heart	Liver	Spleen	Lung	Kidney	Brain	Stomach	Intestines	Submaxillary lymph nodes	Inguinal lymph nodes
7	CH-1a	14	$2.03E+03$	$3.58E+03$	$7.12E+03$	$3.43E+04$	$1.01E+04$	$3.98E+02$	$1.43E+02$	$4.38E+02$	$8.26E+03$	$9.55E+03$
		15	$1.59E+03$	$7.02E+03$	$1.88E+04$	$9.24E+04$	$3.96E+04$	$2.21E+03$	$7.86E+02$	$5.62E+02$	$2.74E+04$	$2.58E+04$
	WUH2	34	$3.19E+05$	$5.41E+05$	$5.43E+06$	$1.05E+08$	$7.73E+05$	$6.39E+06$	$5.32E+06$	$1.44E+05$	$5.31E+06$	$6.17E+06$
		35	$3.52E+05$	$2.91E+05$	$2.19E+06$	$6.92E+07$	$4.37E+05$	$8.66E+06$	$2.18E+06$	$3.84E+05$	$4.59E+06$	$4.31E+06$
10	CH-1a	6	$5.32E+04$	$5.55E+04$	$2.57E+04$	$1.02E+05$	$7.44E+04$	$2.62E+03$	$5.61E+03$	$2.03E+04$	$3.83E+04$	$2.36E+04$
		7	$1.25E+04$	$1.33E+04$	$5.85E+04$	$3.61E+05$	$1.18E+04$	$1.26E+03$	$2.01E+03$	$1.51E+04$	$5.79E+04$	$7.12E+04$
	WUH2	27	$5.76E+06$	$1.39E+07$	$6.17E+07$	$1.94E+09$	$1.07E+08$	$2.43E+08$	$7.88E+06$	$5.91E+06$	$3.85E+08$	$9.41E+08$
		29	$8.92E+06$	$3.19E+07$	$1.21E+07$	$9.92E+08$	$9.64E+07$	$6.31E+08$	$1.24E+07$	$4.22E+06$	$1.05E+07$	$2.69E+07$
21	CH-1a	9	$5.68E+04$	$1.30E+04$	$3.69E+05$	$3.61E+05$	$4.63E+04$	$2.63E+03$	$1.24E+03$	$2.71E+03$	$5.66E+04$	$7.93E+04$
		12	$1.97E+03$	$2.26E+05$	$1.76E+05$	$3.59E+05$	$5.52E+03$	$2.98E+03$	$1.05E+04$	$1.93E+04$	$3.32E+04$	$2.03E+04$
	WUH2	30	$3.86E+05$	$5.45E+05$	$5.75E+05$	$8.46E+06$	$4.24E+04$	$2.63E+05$	$3.93E+04$	$3.23E+04$	$7.56E+05$	$2.52E+05$
		31	$9.88E+05$	$1.48E+05$	$1.67E+05$	$1.25E+07$	$4.95E+03$	$1.11E+05$	$8.41E+04$	$3.62E+04$	$1.22E+06$	$9.66E+05$

Table 4: Viral load in selected tissues of piglet that died naturally measured by quantitative PCR.

DPI	Treatment	Animal ID number	Viral burden in tissues									
			Heart	Liver	Spleen	Lung	Kidney	Brain	Stomach	Intestines	Submaxillary lymph nodes	Inguinal lymph nodes
12	WUH2	33	$7.07E+08$	$2.03E+08$	$3.91E+09$	$4.95E+09$	$2.72E+08$	$6.56E+08$	$1.54E+08$	$1.81E+08$	$2.16E+08$	$1.85E+08$
14	WUH2	26	$9.37E+07$	$1.46E+08$	$2.01E+08$	$2.15E+09$	$1.03E+08$	$1.87E+08$	$1.81E+07$	$9.86E+06$	$2.87E+08$	$1.42E+08$
		32	$6.97E+08$	$4.59E+07$	$1.25E+08$	$2.31E+09$	$5.05E+07$	$1.38E+08$	$1.81E+07$	$7.60E+07$	$1.01E+08$	$1.02E+08$
17	WUH2	25	$4.11E+07$	$4.19E+07$	$1.76E+08$	$7.61E+09$	$8.97E+07$	$1.47E+07$	$1.34E+07$	$6.42E+07$	$5.21E+07$	$1.10E+07$

altered [18, 35]. The variable regions of Nsp2 may also be associated with cell and tissue tropism and may be involved in species-specific functions for arteriviruses [6].

The highly pathogenic PRRSV isolated in the 2006 Chinese outbreak contained a discontinuous deletion of 1 and 29 amino acids at positions 481 and 533–561 in Nsp2, respectively. The deletions were used as a defining characteristic of the highly pathogenic strain of PRRSV at the incipient stage of porcine "high fever syndrome." However, Zhou et al. [39] demonstrated that the discontinuous deletion of 30 amino acids in Nsp2 of the emerging highly pathogenic PRRSV is not related to its virulence by utilizing a reverse genetics assay system. Regardless, the Nsp2 gene may be an ideal marker for monitoring the genetic variation of PRRSV, facilitating research on its molecular epidemiology and the development of diagnostic tests [34, 36].

In addition to deletions in Nsp2 and both UTRs, extensive sequence changes were found in GP5. The ORF5-encoded major envelope glycoprotein (GP5) is essential for viral infectivity and contains the primary neutralization epitope. GP5 is also one of the most genetically variable structural proteins of PRRSV. Due to its immunological significance and polymorphic nature, GP5 has also been a target for analysis of the genetic diversity of PRRSV [40–42]. Residues 13 and 151 of GP5 were believed to be associated with virulence. R^{13} and R^{151} were altered with Q^{13} and G^{151}, respectively, when VR-2332 was attenuated to the RespPRRS/Repro vaccine strain [43]. The $^{A}137^{S}$ mutation was assumed to differentiate between the two vaccine strains [27]. The key amino acids found in GP5 of WUH2 (R^{13}, R^{151} and S^{137}) were identical to those found for VR-2332. In our analysis, the appearance of these characterized amino acids (which were hypothesized to be involved in PRRSV's virulence) suggested that the virulence of WUH2 could be higher when compared to classical isolates. Furthermore, the substitution of a critical amino acid in the PNE may allow these field isolates to escape antibody neutralization induced by the attenuated RespPRRS/Repro vaccine strain, resulting in incomplete immune protection by vaccines. These results suggest that a more effective vaccine against the Chinese PRRSV is urgently required, especially to neutralize the emerging, highly pathogenic PRRSV.

In experimental infection of piglets, we observed that PRRSV WUH2 reproduced a disease state where the clinical signs and mortality were virtually identical to what was seen in the field. Mortality was approximately 70%, and the high continuous fever (over 40.5°C) was observed from 3 dpi until death. Cyanopathy in the ears, internal hemorrhaging, and petechiae were common and significant. Typical interstitial pneumonia and nonsuppurative encephalitis were identified by histopathology. Clinical signs, gross lesions, and histopathology were more severe in the WUH2 group compared to the CH-1a group.

We identified important pathogenic characteristics of highly pathogenic PRRSV, including viremia and viral load in tissues, by comparing experimental infection with different isolates to infection with CH-1a by quantitative PCR. The viral load in serum and tissues of animals inoculated with WUH2 exhibited peak levels (10e8 RNA copies/mL) at 10 dpi, and these viral loads were significantly higher ($P < 0.01$) than viral loads on other days and in piglets inoculated with CH-1a. The peak viral load at 10 dpi also correlated with high mortality. Comparison of viral burden in tissues indicated that the tissue tropism of PRRSV may have been changing [30]. The brain, typically the representative organ for identifying a change in viral tropism, was rarely infected by CH-1a as determined by the lack of gross lesions and histopathology. In contrast, the brains of piglets inoculated with WUH2 presented nonsuppurative encephalitis, including obvious perivascular cuffing and hemorrhages. At 10 dpi, the viral load in the brains of piglets inoculated with WUH2 (10e8 RNA copies/mL) was significantly higher compared to levels in the brains from the CH-1a group (10e2 copies RNA/mL; $P < 0.01$). It was also notable that there was a change in the viral burden in the stomach and intestines.

In addition to the change in viral burden in tissues, the highly pathogenic PRRSV emerging in Chinese isolates seems to adapt to MARC-145 cells more easily than the classical isolates. Further exploration of virulence-determining regions or points in the PRRSV genome is essential to improve our understanding of pathogenic determinants.

5. Conclusion

We analyzed the genomic and pathogenic characteristics of the highly pathogenic Chinese PRRSV isolates in this study. Our data clearly showed that important variations were detected in the 5′ and 3′ UTRs, as well as the Nsp2 and GP5 sequences. There were apparent differences in the viral burden in serum and tissues of animals infected with Chinese isolates compared to classical isolates. These results provide insight into the genomic diversity and changes involved in the pathogenicity of Chinese PRRSV isolates and help to elucidate the evolutionary and pathogenic mechanisms of PRRSV.

Conflict of Interests

The authors declare no potential conflict of interests with respect to the research, authorship, and/or publication of this paper.

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