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Genetic evolution and alterations in pathogenicity of highly pathogenic porcine reproductive and respiratory syndrome virus

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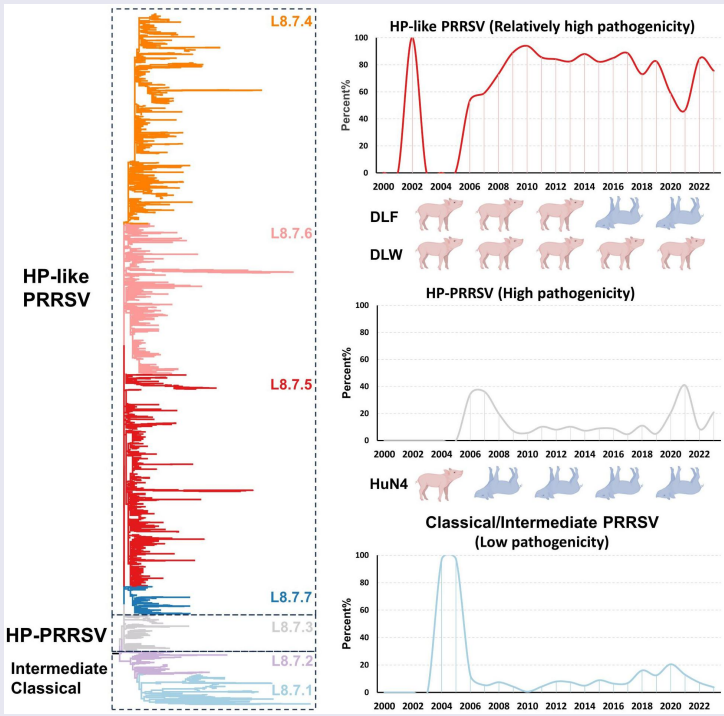
ABSTRACT

Highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) and HP-PRRSV variant strains remain prevalent in China and Southeast Asian countries. However, their epidemiological aspects, genomic characteristics, relationships with vaccine strains, and changes in pathogenicity remain unclear. In this study, 2509 global L8.7 ORF5 sequences were utilized for analysis, we classified L8.7 into 7 groups (L8.7.1-L8.7.7). L8.7.1-L8.7.3 strains corresponded to previously reported classical PRRSVs, intermediate strains, and HP-PRRSVs, respectively, whereas L8.7.4-L8.7.7 were designated HP-like PRRSVs. Statistical analysis revealed that HP-like PRRSVs were the most prevalent among L8.7 strains, and L8.7.5 and L8.7.6 strains accounted for the highest proportions in recent years. A comprehensive analysis of the genome revealed that the majority (72.15%) of L8.7 strains presented a wild-type phenotype. Evolution rate analysis revealed that the evolution rate of L8.7.3-L8.7.7 PRRSV in China was reduced by about 4.1 times after the use of HP-PRRSV MLV. Pathogenicity test results indicate that in comparison with HP-PRRSV (L8.7.3: HuN4), the HP-like PRRSV strains (L8.7.5: DLF; L8.7.6: DLW) presented reduced pathogenicity in piglets while maintaining relatively high virulence. In summary, this study systematically elucidated the epidemiological dynamics, evolutionary trends, relationships with vaccine strains, and changes in pathogenicity associated with L8.7 strains, providing crucial data to support prevention and control strategies against L8.7 PRRSV.



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Introduction

Porcine reproductive and respiratory syndrome (PRRS) has caused significant economic losses to the global swine industry since its emergence in the late 1980s. This viral infection leads to clinical manifestations characterized by reproductive failure in pregnant sows and acute respiratory illness in growing pigs, resulting in elevated preweaning mortality rates [1]. PRRSVs are classified into two distinct types on the basis of their genomic and antigenic diversity, namely, PRRSV-1 and PRRSV-2, which exhibit only approximately 60% sequence identity. The genome of PRRSV is a linear, positive-sense single-stranded RNA molecule of approximately 15.4 kb. The PRRSV genome encodes 11 open reading frames (ORFs) [2]. Nonstructural protein 2 (NSP2), encoded by ORF1a, frequently has amino acid deletions. Therefore, it is commonly employed as a molecular feature to differentiate PRRSV subtypes: 1 + 29 aa deletions (HP-PRRSV), 1 + 111 + 19 aa deletions (NADC30-like PRRSV), and 100 aa deletions (NADC34-like PRRSV) [3–5]. Among these ORFs, ORF5 encodes the major envelope glycoprotein GP5, which plays a crucial role in inducing virus-neutralizing antibodies and cross-protection among different variants of PRRSV. Owing to its significant genetic variation within a relatively short length, ORF5 is an ideal candidate for constructing phylogenetic trees [6].

According to phylogenetic tree analysis, PRRSV-1 can be classified into 4 lineages (L1-L4), whereas PRRSV-2 can be categorized into 11 lineages (L1-L11) [6–10]. In China, six sublineages of PRRSV-1 (L1.1, L1.2, L1.10, L1.11, L1.13 and L1.17) and five sublineages of PRRSV-2 (L1.5, L1.8, L3.5, L5.1 and L8.7) circulate in the field [9,11,12]. Since 2006, HP-PRRSV, which originated from CH-1a-like PRRSV, has caused atypical PRRS and has had a devastating impact on the swine industry, resulting in 20%-100% mortality in pigs [5,13]. A high-mortality swine pathogen, the HP-PRRSV variant, reappeared in some regions of China in 2009 [14]. HP-PRRSV continues to exist and evolve continually [12,15–18], and these strains display significant molecular differences [14–16,19]. Lineage 1, which was reported in 2013, includes L1.8 (NADC30-like PRRSV), which caused another PRRS pandemic, and L1.5 (NADC34-like PRRSV), which has recently caused an increased threat to pig farms [20,21]. Many studies reported conducted comprehensive analyses on the phylogenetic structure and sequence advantages of sublineages within lineage 1 of PRRSV-2 [22,23]. However, limited research has been published regarding the genetic variation and characteristics of the earliest strain CH-1a and the prominent HP-PRRSV in China [14–16]. Furthermore, there are notable discrepancies in sublineage classification results among different studies [12,19,24].

An updated and expanded sublineage classification system is imperative for monitoring L8.7 PRRSV diversity and pathogenicity. Such studies will increase our understanding of the potential factors driving lineage turnover and the pathogenic potential of novel strains. Furthermore, reports of L8.7 PRRSV in various regions worldwide, including India and Vietnam, highlight the urgent need for a comprehensive description of the macroevolutionary dynamics of PRRSV at broader geographic scales. Currently, there is a lack of research documenting the molecular distinctions of L8.7 PRRSV in these regions, necessitating the establishment of a comprehensive framework for clarification. In summary, in this study, we elucidated the classification of L8.7 by incorporating all recorded sequences until 2023 and monitoring data from our laboratory spanning 2014–2023. Furthermore, our findings provide clarity regarding the global geographical distribution of each subgroup within L8.7. Finally, leveraging insights gained from identifying predominant L8.7 clusters in China, we examined their pathogenicity.

Materials and methods

Source of sequences

NCBI database

We obtained all the available PRRSV sequences isolated from 1996 to 2023 from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov) until 1 December 2023 (FIG. S1). Some sequences were excluded because of duplicate strain names, cell passaging corresponding to the same original isolate, or missing collection time or geographic information.

Sample collection

From 2014 to 1 December 2023, a total of 4070 clinical samples (including lung, lymph node, and serum samples) suspected of PRRSV infection were collected from various pig farms in 21 provinces across China (TABLE S1). Tissue sample disposal procedures, RNA extraction and cDNA preparation were performed as previously described [25]. RT-PCR and genome sequencing techniques were employed with the primers mentioned earlier for PRRSV detection and amplification of complete gene sequences [19].

Phylogenetic analyses of L8.7 PRRSV

Data processing

All alignments were performed via MAFFT version 7 [26] with default parameters (https://mafft.cbrc.jp/alignment/software/) and further refined via MEGA 7.0 [27]. Detailed information regarding the sequence origin (time and location) can be found in TABLE S1.

Phylogenetic analysis of the L8.7 PRRSV genomes and ORF5 gene sequences

We applied the same rationale as previously described [6,7] for classifying sequences into lineages. Phylogenetic trees were constructed via maximum likelihood with 1,000 bootstrap replicates in IQ-TREE [28]. L8.7 was further stratified into groups via ClusterPicker software [29]. The trees were annotated and modified via Evolview (version 2.0) [30].

Evolutionary dynamic analysis of L8.7 PRRSV

Sequence cleaning

The duplicate sequences with a nucleotide consistency of greater than 98.5% were removed, while the ORF5 sequences collected at the earliest time point were retained. Additionally, any ORF5 sequences with more than 5 ambiguous nucleotides and incomplete ORF5 sequences were excluded from the analysis. Recombination analysis of both the genomic and ORF5 gene sequences was performed via RDP4 and Simplot software. Sequences showing evidence of recombination signals were also eliminated.

Molecular clock analysis of L8.7 PRRSV

Treetime was employed to eliminate sequences with weak temporal signals [31], and ModelFinder was utilized to identify the best fit model [32]. In summary, a relaxed uncorrelated lognormal (UCLN) molecular clock, incorporating a Bayesian skyline model and a general-time reversible model of nucleotide substitution with gamma-distributed rate variation among sites (GTR+γ), was applied, allowing for partitions into codons in any of three positions. The Markov chain Monte Carlo (MCMC) algorithm was run for 200 million steps and, the output was sampled every 1,000 trees. To ensure the avoidance of local convergence, three independent runs were conducted. Convergence and mixing were examined via the program Tracer with consideration of a burn-in of 10% of the total chain length [33]. The convergence of the MCMC chain was assessed on the basis of the effective sample size (ESS >200) of the parameters.

Differentiation between vaccine strains and wild-type strains

The deduced amino acid sequences were aligned with ClustalW via Lasergene software. On the basis of previous literature regarding the disparities between vaccines and their parental strains [24,34–38], a comparative analysis was performed to assess whole-genome nucleotide consistency, specific amino acid variations, and additional NSP2 deletions within the lineages encompassing JXA1-R, TJM, HuN4-F112, and GDr180.

A comparative analysis was performed via the Datamonkey webserver to examine stress-related GP5 selection sites of L8.7 PRRSV before and after vaccine administration (http://www.datamonkey.org/) with fixed effects likelihood (FEL) models [39].

Virus isolation and sequencing analysis

Isolation of the L8.7 mainstream strain

The HP-PRRSV HuN4 strain (GenBank accession: EF635006.1, belonging to L8.7.3) was isolated in 2006 and is maintained in our laboratory [40]. In 2020, the DLF (L8.7.5) and DLW (L8.7.6) strains were isolated from difference Dalian Farms in Liaoning Province, respectively, identification and IFA as previously reported

Sequencing analysis of DLF and DLW

Whole-genome sequencing, genome assembly, and sequence alignment were conducted following previously described protocols [21]. Detailed information regarding the primers used for whole-genome amplification can be found in a previous publication [38]. Recombination analysis was performed via RDP4 and Simpot software.

Animal experiments

Ethics statements

The sampling procedures and animal experiments were conducted in accordance with the guidelines of the Animal Ethics Committee of the School of Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The Animal Ethics Committee approval number is 230313–01-GR.

Animals and experimental design

PRRSV-free piglets were procured from the Laboratory Animal Center at Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, where the animal experiments were conducted. A total of eighteen

4-week-old PRRSV-free piglets were randomly allocated into four groups: the DLF challenge group ($n = 5$), DLW challenge group ($n = 5$), HuN4 challenge group ($n = 5$), and control group ($n = 3$). The piglets in the challenge and control groups were administered intramuscular (2 mL) and intranasal (2 mL) inoculations with DLF ($1 \times 10^{4.0}$ $TCID_{50}$/mL, 4 mL per piglet), DLW ($1 \times 10^{4.0}$ $TCID_{50}$/mL, 4 mL per piglet), HuN4 ($1 \times 10^{4.0}$ $TCID_{50}$/mL, 4 mL per piglet), or DMEM (4 mL per piglet), respectively. Clinical signs and rectal temperatures were recorded daily. The body weights of the piglets were measured at 0, 7, 14 and 21 days post infection (dpi). In cases of death, autopsies were performed immediately, and the remaining surviving pigs were euthanized at 21 dpi. Lung tissue and submaxillary lymph node tissue samples were collected and fixed in a solution of 10% neutral-buffered formalin. These samples were further processed for histopathological examination via haematoxylin-eosin (H&E) staining.

Viral distribution and viraemia detection

Blood samples were collected at 0, 3, 5, 7, 10, 14 and 21 dpi for the quantification of viraemia via a real-time quantitative PCR (RT-qPCR) method established by our laboratory [41]. To determine the distribution of the virus in piglets, various organs, including the heart, liver, spleen, lung, kidney, lymph nodes, tonsils, small intestine, stomach and brain, were collected from each piglet for fluorescence quantitative measurement via RT-qPCR.

Serological examination

PRRSV-specific antibodies were quantified via a commercially available ELISA kit (IDEXX PRRS X2, USA) in accordance with the manufacturer's instructions. According to the manufacturer's guidelines, sample-to-positive control (S/P) ratios ≥ 0.4 were considered indicative of positive results.

Data analysis

Significant differences between the two groups were assessed via t tests (nonparametric tests) in GraphPad 8.0 (San Diego, CA, USA). Significant disparities in weight gain and the viral load among the groups were identified. The level of significance was set at $p < 0.05$.

Results

Classification of L8.7 PRRSV

To investigate the evolution of PRRSV L8.7, a total of 2509 ORF5 sequences were utilized for analysis: 2159 sequences of L8.7 strains obtained from the NCBI database and 350 sequences of L8.7 strains collected in our laboratory from 2014 to 2023 (Figure 1(a)). As illustrated, L8.7 strains were further divided into seven groups (L8.7.1–8.7.7) (Figure 1(a,b)). All sequence information is listed in TABLE S1. As shown in Figure 1(b), the reference strains used to construct phylogenetic trees were selected on the basis of well-known strains and L8.7 PRRSVs reported in pathogenicity studies. The average genetic distance within and between groups is presented in Figure 1(c). The average genetic distance within groups was less than 5%, except for L8.7.2. Overall, a genetic distance of 4.3–10.4% was typically observed between groups (Figure 1(c)). Furthermore, L8.7.4–8.7.7 strains exhibited specific amino acid mutation patterns with different characteristics (TABLE S2) and were designated HP-like PRRSV. Among the 2509 sequences in the L8.7 group, 2.23% (56/2509) were classified as L8.7.1 (CH-1a-like PRRSV), 4.74% (119/2509) as L8.7.2 (Intermediate PRRSV), 11.48% (288/2509) as L8.7.3 (HP-PRRSV) and 81.54% (2046/2509) as HP-like PRRSV.

Sequential dominance of groups over time and global distribution of L8.7 PRRSV

In this study, all L8.7 sequences with known time and location were analysed. Notably, L8.7.4 was the most prevalent group and was present in 8 out of the 9 countries where L8.7 strains are found (Figure 2(a,b)). In Nepal, Laos, and Myanmar, only a single group of 8.7.4 is found, and no other groups have been discovered. L8.7.1, 8.7.3, 8.7.5, 8.7.6 and 8.7.7 strains were found in 2, 3, 4, 4 and 2 countries, respectively (Figure 2(a,b)). L8.7.2 strains have been reported only in China (Figure 2(b)). China has the highest quantity (2201/2509, 87.7%) and variety (7/7, 100%) of L8.7 PRRSV strains (Figure 2(b)).

In total, 2201 L8.7 PRRSVs from China were analysed. In all, 26 provinces reported L8.7 PRRSV, with Guangdong reporting the most cases, followed by Guangxi, Heilongjiang, Shandong, Hebei and Henan, all of which reported more than 40 cases (Figure 2(c,d)). In China, the prevalence of different PRRSV groups showed temporal variation (Figure 2(e)), with clear periods of higher prevalence for specific groups. The L8.7.1 and L8.7.2 groups are rarely detected and have been infrequently observed since 2006. Since L8.7.3 group caused an outbreak in 2006, it was predominantly prevalent from 2006 to 2009 and continues to persist.

HP-like PRRSVs (L8.7.4-L8.7.7) subsequently replaced HP-PRRSV as prevalent strains in China

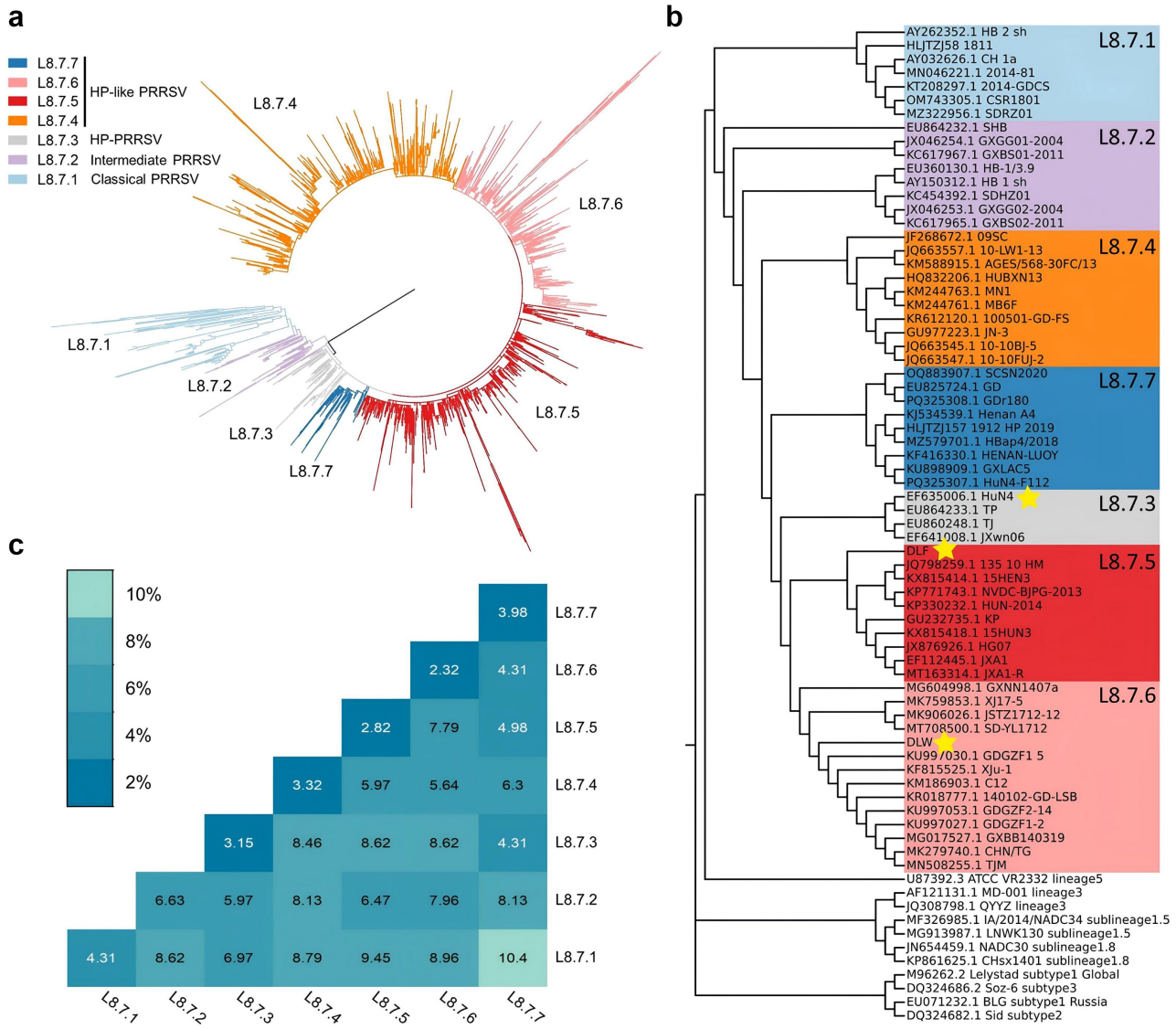

Figure 1. Phylogenetic tree and nucleotide consistency analysis of L8.7 strains. (a) Phylogenetic tree of L8.7 sequences classified into seven groups. (b) Phylogenetic tree constructed on the basis of the ORF5 gene of L8.7 PRRSV isolates and reference PRRSV strains from each lineage. The strains involved in animal experiments in this study are indicated with yellow five-pointed stars. (c) Genetic distance (% nucleotide difference) within and between groups among L8.7 strains.

(Figure 2(e)). The L8.7.4 group was first reported and monitored in China in 2006, and it constituted a significant proportion (41.3–79.6%) of the L8.7 strains during the period from 2009 to 2011 in China. Notably, some groups experienced a sudden surge in prevalence, including the L8.7.5 group, which first appeared in China in 2007 and remained consistently prevalent during the following time period; however, starting in 2011, there was a remarkable increase in its occurrence, as it accounted for approximately 17.1% to 51.6% of the L8.7 PRRSVs (Figure 2(f)). One notable observation is the recurring presence of L8.7.6 strains, which have exhibited a consistent prevalence over time. Initially identified in 2002 (EU709835.1, SH02), the number of detected L8.7.6 strains were initially limited, with only one strain recorded. Notably, no instances of L8.7.6 strains were found between 2003 and 2005; however, their prevalence rapidly increased in 2006 and gradually declined until 2009. Subsequently, from 2014 to 2023, the L8.7.6 group emerged as the predominant circulating strain, accounting for approximately 21.5–47.1% of the cases observed during this period. In China, the L8.7.6 group had the highest detection rate (612/2201, 27.8%) and was the most widespread (20/21, 95.2%) subtype detected (Figure 2(d,f)). Emergence of the L8.7.7 group was initially observed in 2008; however, its prevalence remained limited until 2011, when a gradual increase was noted. The detection rate of L8.7.7 strains rapidly increased to between 15.1% and 17.1% from 2022–2023.

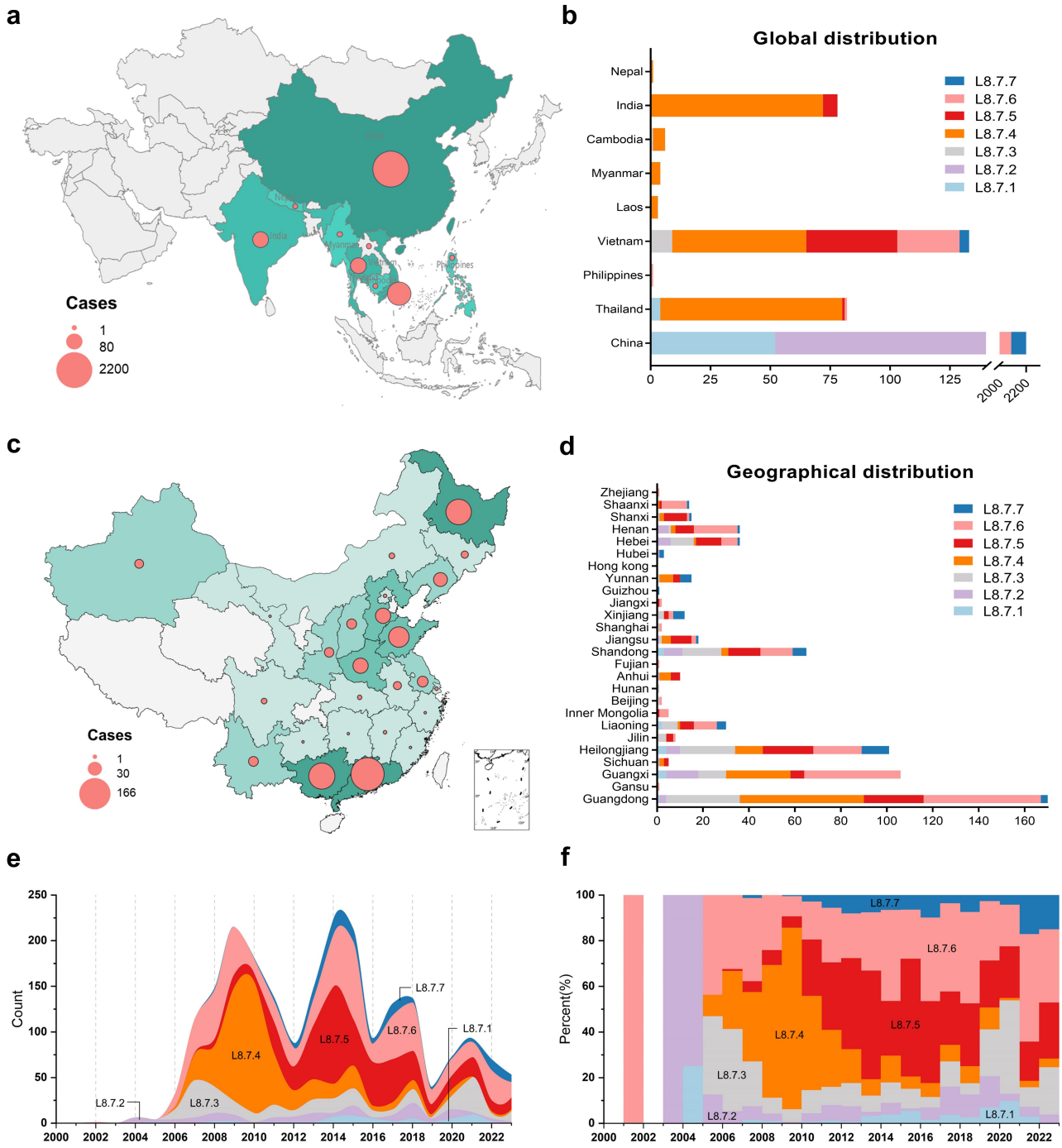

Figure 2. The temporal and geographical distributions of the L8.7 groups. (a) Geographical distribution of L8.7 strains across different global regions. (b) Number of L8.7 strains distributed in different countries. (c) Geographical distribution of L8.7 strains across different regions in China. (d) Number of L8.7 strains distributed in different provinces of China. (e) Number of L8.7 strains according to ORF5 sequences over time and (f) a stacked bar chart illustrating the relative frequency in China. This map was generated with https://www.edrawsoft.cng.

A subsequent analysis was conducted on 2201 sequences for which geographic information was recorded, revealing the presence of L8.7.5 and L8.7.6 strains in 18 and 20 provinces of China, respectively, indicating their widespread distribution throughout the country (Figure 2(c,d)). The above results revealed that the L8.7.5 and L8.7.6 strains were present in the greatest quantities and had the widest distribution in China over the past decade.

Evolutionary dynamics of L8.7 PRRSV

The time of emergence of L8.7 PRRSV was inferred by estimating the time to the most recent common

ancestor (TMRCA) for both L8.7 PRRSV and each group on the basis of ORF5 sequences. To generate more realistic evolutionary data, we excluded sequences with 98.5% consistency, sequences with potential recombination signals, and sequences without reliable time signals (FIG. S2A), leaving 734 sequences for easy analysis in Beast. The TMRCA for the L8.7 ORF5 gene was estimated to be approximately 1994 (95% highest posterior density [HPD] = 1988 to 2000). The estimated TMRCAs for the 7 groups, on the basis of analysis of the ORF5 gene, were as follows: 1996 (1989 to 2001, L8.7.1), 1997 (1991 to 2001, L8.7.2), 2001 (1999 to 2003, L8.7.3), 2003 (2001 to 2004, L8.7.4), 1997 (1990–2001, L8.7.5), 2001 (1997 to 2004, L8.7.6) and 2003 (2002 to 2004, L8.7.7) (FIG. S2B). According to analysis of the ORF5 gene, the mean nucleotide substitution rate (substitutions/site/year) was estimated to be 5.79 (5.16–-6.59) $\times 10^{-3}$ substitutions/site/year.

To mitigate the impact of HP-PRRSV on the swine industry, China developed the HP-PRRSV MLV in 2011. In this study, we further investigated the potential influence of the HP-PRRSV MLV on PRRSV evolution. The analysis of the ORF5 sequences of L8.7.3-L8.7.7 strains before and after the release of the vaccine revealed an evolution rate of 2.03 (1.16–3.42) $\times 10^{-3}$ substitutions/site/year prior to vaccine usage (1996–-2010), which subsequently decreased to 4.96 (3.53–-6.72) $\times 10^{-4}$ substitutions/site/year following vaccine implementation (2011–2023).

Relationship between the HP-PRRSV MLV and HP-like PRRSV

To investigate the correlation between the HP-PRRSV MLVs (JXA1-R, HuN4-F112, TJM-F92, and GDr180) and HP-like PRRSV, we analysed nucleotide consistency, NSP2 deletion features, and characteristic amino acid changes across all the whole genomes from 8.7.4 to 8.7.7 strains (Table 1). The TJM vaccine exhibits an additional deletion of 120 amino acids in NSP2; however, this characteristic is not observed in the majority of the NSP2 sequences of L8.7.6 strains (FIG. S2C). Initially, a statistical analysis was conducted on all full-length sequences of L8.7.6 strains, comprising a total of 79 sequences for evaluation (17 recombination sequences were removed, Table 1). The NSP2 sequences of these 79 strains were then compared, revealing that 22 sequences presented an additional deletion of 120 amino acids in addition to the previously identified 1 + 29 missing features (Table 1). We further compared the characteristic amino acids and genome-wide consistency between TJM-F92 and TJ with these 79 genomes (Table 1). Most strains exhibiting an additional 120 aa deletion presented more than 32 characteristic aa that were consistent with those of TJM (Table 1). However, even in the absence of the additional 120 aa deletion, their characteristic aa still exhibited a high degree of consistency with those of TJM (e.g. KP742986.1, KX815410.1) (TABLE S3). The results show that the key to distinguishing vaccine-like PRRSV from HP-like PRRSV could not be summarized by genome-wide nucleotide consistency or characteristic amino acid changes but could be determined on the basis of additional deletions in NSP2 (Table 1). A statistical analysis was conducted on L8.7.6 sequences, revealing that 22 out of 79 strains (27.85%) were associated with vaccines (TABLE S4).

Pathogenicity of HP-PRRSV and HP-like PRRSV strains in piglets

Isolation and identification of predominant strains within HP-like PRRSV

To gain a more comprehensive understanding of the pathogenicity of predominant groups (L8.7.5 and L8.7.6) within HP-like PRRSV, DLF (L8.7.5) and DLW (L8.7.6) strains were isolated in this study. These viruses were isolated from PAMs and Marc-145 cells. The expression of the PRRSV M protein was observed in both PAMs and Marc-145 cells following inoculation with the strains for IFA testing (Figure 3(a)). These findings indicate that DLF and DLW strains were successfully obtained.

Table 1. Relationship of genome-wide between HP-PRRSV MLV and HP-like PRRSV.

Groups	Number of HP-like PRRSVs	HP-PRRSV MLV	Number of HP-like PRRSVs with the same aa mutation[1]	Number of HP-like PRRSVs with nt consistency[2]	Number of HP-like PRRSVs with additional deletion of NSP2
L8.7.5	70	JXA1-R	53 (>32/43)	50 (>99%/99.23%)	\
L8.7.6	79	TJM	20 (>32/43)	15 (>99%/99.20%)	22 (additional 120 aa deletion)
L8.7.7	16	HuN4-F112	3 (>32/40)	6 (>99%/99.60%)	\
		GDr-180	0(>32/35)	0 (>99%/99.60%)	\

1. The number of HP-PRRSV MLV aa changes compared with that of the parental strains: 43 (JXA1-R/JXA1), 43 (TJM/TJ), 40 (HuN4-F112/HuN4) and 35 (GDr-80/GD).
2. Nt consistency of HP-PRRSV MLV compared with the parental strain: 99.23% (JXA1-R/JXA1), 99.20% (TJM/TJ), 99.60% (HuN4-F112/HuN4) and 99.60% (GDr-80/GD).

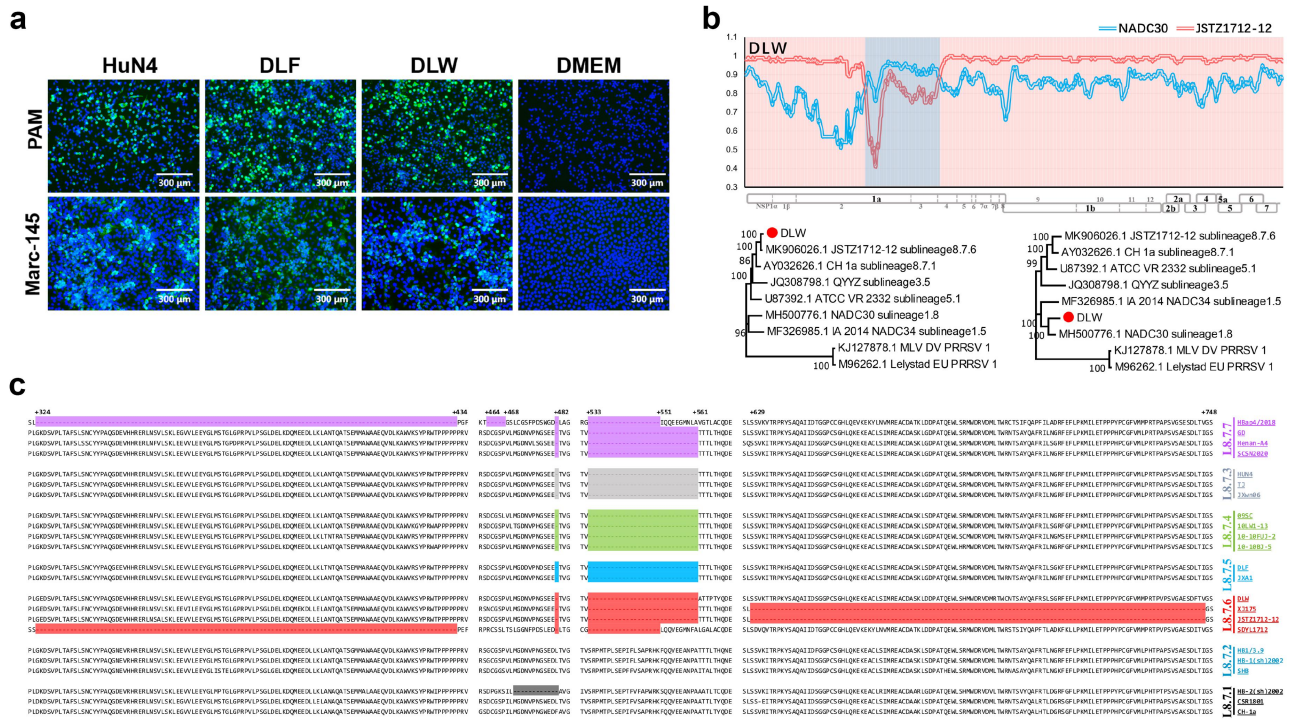

Figure 3. Strain isolation, recombination and characteristic NSP2 aa analysis of L8.7 strains. (a) Identification of DLW and DLF. The reactivity observed in immunofluorescence assays (IFAs) performed utilizing a monoclonal antibody targeting the PRRSV M protein in control, DLF-infected, DLW-infected, and HuN4-infected PAM and Marc-145 cells. Nuclei were counterstained with DAPI. Scale bar = 300 μm. (b). Recombination analysis of DLW. (c). Deduced NSP2 amino acid alignment of L8.7 strains.

Genomic characteristics of DLF and DLW

The complete genomes of DLF(PQ178809) and DLW (PQ178810) were 15,324 and 15,323 nucleotides (nt) in length, respectively, excluding the poly (A) tail. The genomic nucleotide similarity between HuN4/DLF, HuN4/DLW and DLF/DLW were 98.67%, 95.78% and 95.13%, respectively (Table 2). The NSP2 sequence alignment revealed that DLF and DLW exhibit

Table 2. Nucleotide and amino acid sequence similarity.

	HuN4/DLF		HuN4/DLW		DLF/DLW	
	Nucleotide similarity (%)	Deduced amino Gene acid similarity(%)	Nucleotide similarity (%)	Deduced amino Gene acid similarity(%)	Nucleotide similarity (%)	Deduced amino Gene acid similarity(%)
Whole genome	98.7	/	95.8	/	95.1	/
5'UTR	100	/	98.9	/	98.9	/
3'UTR	95.4	94	98	96	97.4	98
Nsp1a	98.7	99.4	98.9	99.4	98.3	98.9
NSp1B	98	95.5	98	97.5	97	95
Nsp2	98.3	96.7	89.1	88.1	88	86.5
Nsp3	99.4	100	82.6	76.3	82.6	76.3
Nsp4	99.2	99.5	98.5	99.5	98	99
Nsp5	99.2	99.4	98.8	99.4	98	98.8
Nsp6	100	100	97.9	100	97.9	100
Nsp7a	98.9	99.3	97.8	100	97.1	99.3
Nsp7B	99.4	99.1	98.5	99.1	97.9	98.2
Nsp8	98.5	97.8	99.3	100	99.3	97.8
Nsp9	98.9	99.4	98.8	99.2	98.3	99.5
NSp10	98.7	99.1	98.6	99.5	97.8	98.6
Nsp11	98.5	99.1	98.5	99.1	97.9	99.1
Nsp12	99.6	100	99.6	100	99.3	100
ORF2a	98.4	98.1	98.8	99.2	98.3	98.8
ORF2b	98.6	98.6	99.5	100	99.1	98.6
ORF3	98.7	97.6	98.3	98.8	97.5	97.3
ORF4	99.4	98.9	98.1	96.6	98.3	97.8
ORF5	98.3	98	99	97.5	97.5	96
ORF5a	96.8	94.2	98.7	100	95.5	94.2
ORF6	99.4	100	100	100	99.4	100
ORF7	99.5	99.2	98.1	99.2	97.6	98.4

discontinuous deletions of 30 amino acids (1 + 29), spanning residues 482 and 533–561 of CH-1a NSP2, which is consistent with the patterns observed in L8.7.3 (HP-PRRSV) (Figure 3(c)). To investigate the potential involvement of recombination events in the emergence of DLF and DLW, possible recombination events were examined via SimPlot and RDP4. The findings indicated that DLW exhibited recombination events (recombination position: 3500–5657 nt), whereas DLF did not (Figure 3(b)). Both DLF and DLW are wild-type strains, whether on the basis of previous studies [24,34] or our standard distinguishing vaccine-related strains from wild-type strains.

Clinical signs of infected piglets

The piglets were weighed at seven-day intervals, and blood samples were collected at 0, 3, 5, 7, 10, 14, and 21 dpi. The experimental procedure is illustrated in Figure 4(a). The piglets infected with HuN4 and DLF presented evident clinical symptoms at 2 dpi, including coughing, lethargy, dyspepsia, and chills. The DLW-challenged piglets presented typical clinical manifestations of PRRSV infection at 3 dpi, with 3 out of 5 infected piglets displaying symptoms such as coughing, lethargy, dyspepsia, and shivering. Piglets in the HuN4-challenged group showed high fever (≥40.5°C) for 4–6 days (Figure 4(b)) and began to die at 12 dpi, and the survival rate was 20% at 21 dpi (Figure 4(c)). DLF-challenged piglets began to die at 16 dpi and had a 60% survival rate at 21 dpi (Figure 4(c)), and although mortality was reduced in this group, DLF-challenged piglets presented a longer period of hyperthermia for 7-15 days (Figure 4(b)). The DLW-challenged piglets survived until the end of the experiment and presented a shorter fever duration (for 1–8 days) (Figure 4(b)). The control group piglets in this study did not exhibit any evident clinical manifestations and survived throughout the entire study period (Figure 4(b,c)).

The body weights of the piglets were measured at 0, 7, 14 and 21 dpi. The results of the statistical analysis revealed a significantly lower average daily body weight gain in the DLF-challenged piglets than in the uninfected piglets at 1–7 dpi, 8–14 dpi and 15–21 dpi (Figure 4(d)). Compared with uninfected piglets, HuN4-challenged piglets presented significantly lower average daily body weight gain from 8 to 14 dpi, and DLW-challenged piglets presented significantly lower average daily body weight gain from 8 to 14 dpi and 15-21dpi (Figure 4(d)).

Changes in PRRSV-specific antibodies

Blood samples were collected from all pigs at 0, 3, 5, 7, 10, 14 and 21 dpi for the purpose of measuring PRRSV-specific antibodies against the PRRSV N protein via a commercially available ELISA kit (IDEXX PRRS X2, USA). At 10 dpi, all pigs in both the DLF-challenged and HuN4-challenged groups presented detectable levels of PRRSV-specific antibodies (S/P ≥ 0.4). By 14 dpi, all five pigs in the DLW-challenged group had detectable PRRSV-specific antibodies (S/P ≥ 0.4). In PRRSV-challenged pigs, the S/P ratio gradually increased until the end of the experiment. No PRRSV-specific antibodies were detected in uninfected piglets (Figure 4(e)).

Evaluation of viraemia and the viral load in different tissues

The viral load and distribution in serum samples collected at 0, 3, 5, 7, 10, 14, and 21 dpi as well as in 10 organ tissues obtained during post-mortem examination were assessed via RT-qPCR. The level of viraemia in the PRRSV-challenged group initially increased at 3 dpi. The DLF- and DLW-challenged groups reached peak viraemia at 5 dpi, whereas the HuN4-challenged group reached peak viraemia at 7 dpi (Figure 4(f)). Subsequently, the viral load gradually decreased. At 7–10 dpi, there was a significant difference in the level of viraemia among the challenge groups (Figure 4(f)). Notably, no viraemia was detected throughout the entire study period in the control group. Although the viral load of different challenge groups in the same tissue was different, there was no significant difference (Figure 4(g)). The ORF7 gene was sequenced to confirm the presence of the original virus in these samples.

Macroscopic and histopathological lesions

All HuN4-infected piglets exhibited severe thymus atrophy (Figure 5(a)). Among the DLF-challenged group, four piglets displayed apparent thymus atrophy, whereas no thymus atrophy was observed in the DLW-challenged group (Figure 5(b,c)). In the HuN4-challenged group, five pigs presented severe lung consolidation (Figure 5(e)), and four pigs presented bleeding in the submandibular lymph nodes (Figure 5(m)). Among the 5 pigs in the DLF-challenged group, 3 had lung consolidation (Figure 5(f)), and 3 had bleeding in the submandibular lymph nodes (Figure 5(n)). In the DLW-challenged group, consolidation of the lungs was observed in two out of five pigs (Figure 5(g)), whereas slight bleeding was detected in the submandibular lymph nodes of two pigs (Figure 5(o)). No evident pathological changes were noted in the tissues or organs of uninfected piglets (Figure 5(d,h,p)).

Severe interstitial pneumonia associated with haemorrhage, as depicted in Figure 5(i), was observed in HuN4-challenged pigs. This condition

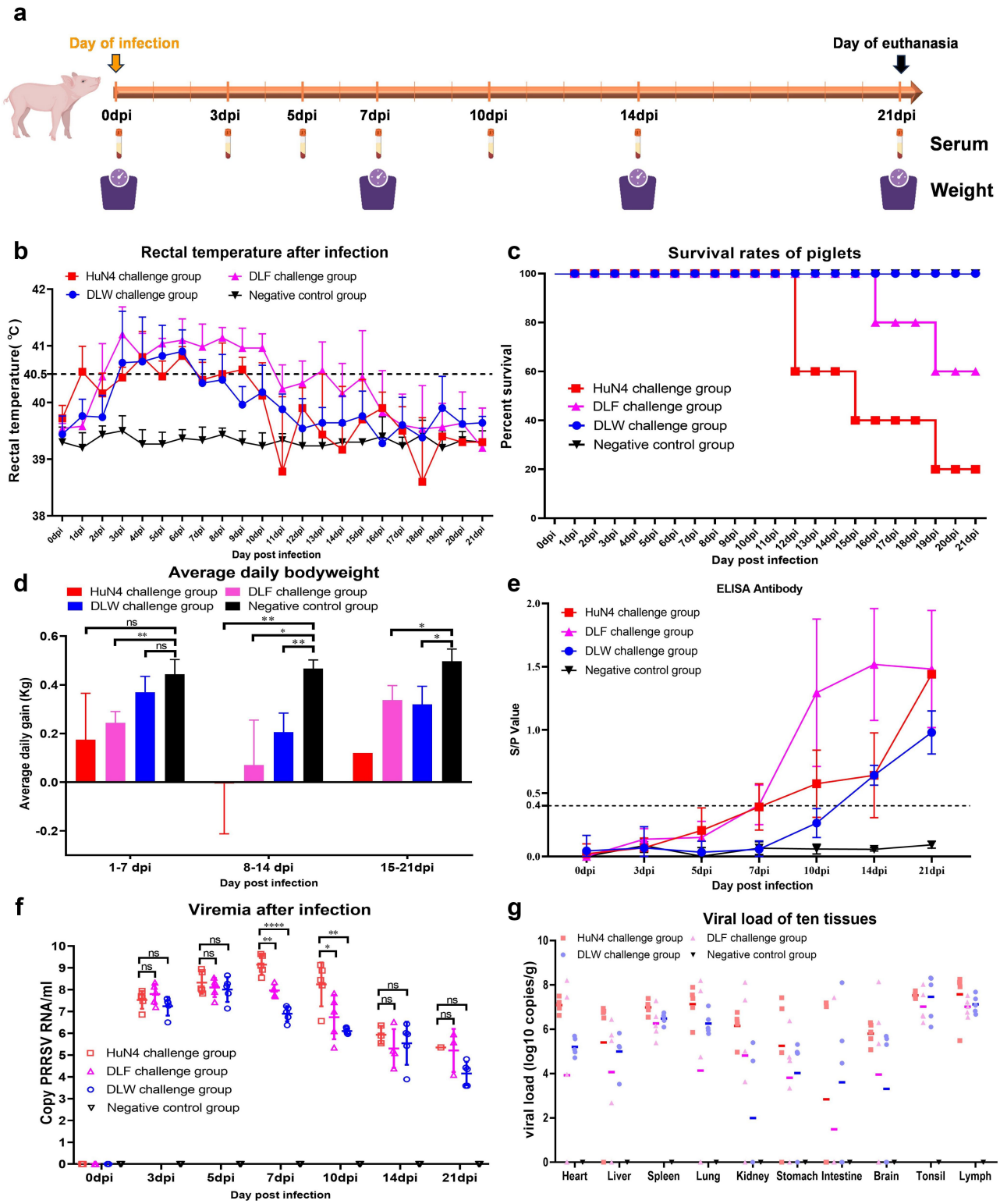

Figure 4. Study design, survival rate, rectal temperature, average daily weight gain, anti-PRRSV antibody titres, viraemia levels and viral loads in the experimental piglets. (a) Study design (as described in the materials and methods). (b) The rectal temperature changes associated with DLF, DLW, and HuN4 treatment. A rectal temperature ≥ 40.5°C was defined as fever. (c) Survival following challenge with DLF, DLW and HuN4. (d) Changes in the average daily weight gain in the DLF, DLW, and HuN4 groups. The mean ± SD (represented by error bars) of body weight gain is depicted. *, $p < 0.05$;**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, no statistically significant difference. (e) Anti-PRRSV antibody titre changes were induced by DLF, DLW, and HuN4. The seroconversion threshold was established at an S/P ratio of 0.4. (F) Viraemia changes were induced by challenge with DLF, DLW, or HuN4. (g) Viral loads in various tissues in the DLF-, DLW-, and HuN4-challenged groups.

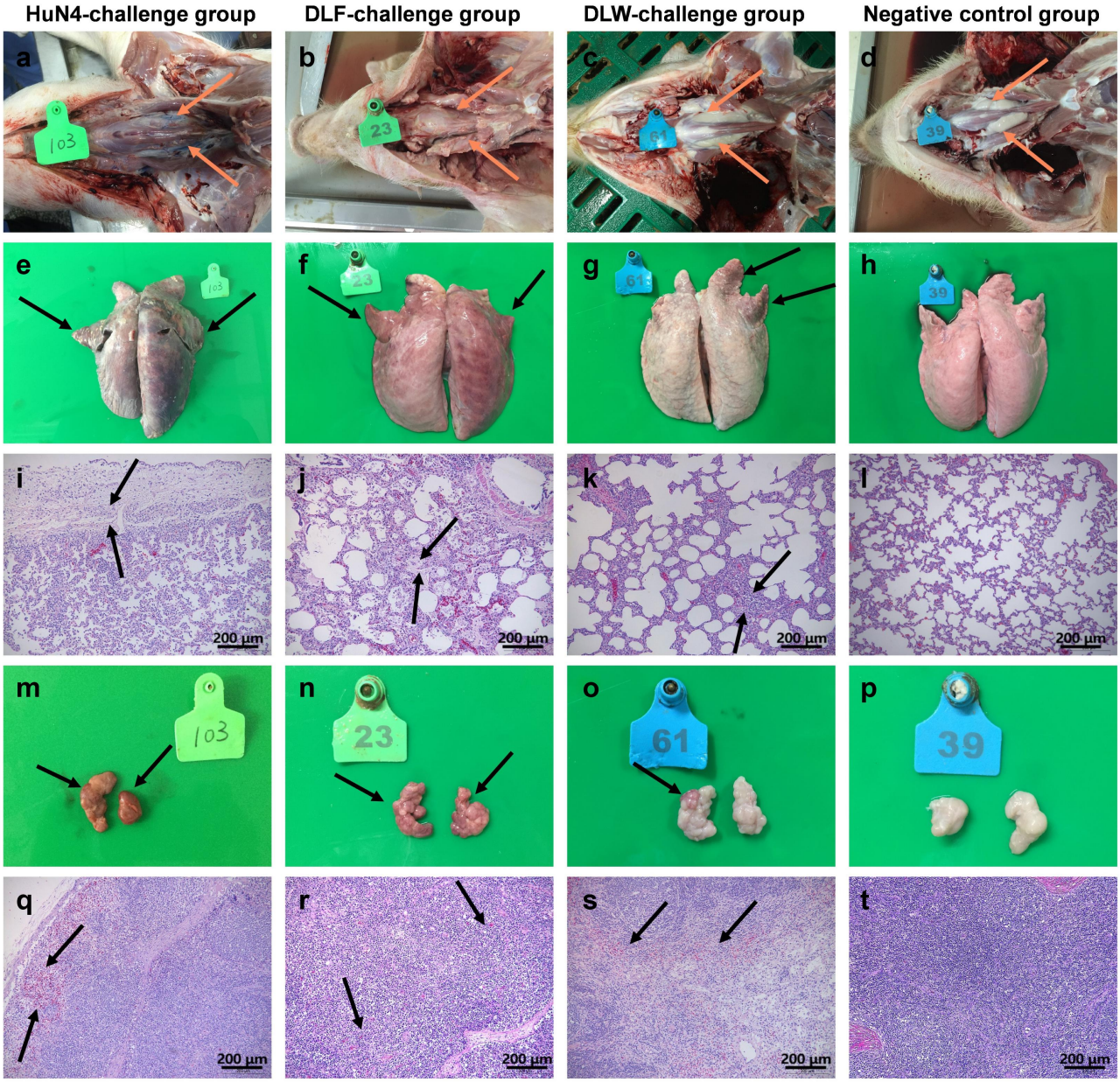

Figure 5. Gross and histological lung lesions in different challenged groups. The piglets infected with HuN4 or DLF presented varying degrees of thymic atrophy (a and b). In contrast with the control group, the HuN4 and DLF infection groups presented severe interstitial pneumonia with lung consolidation (e and f) and lymph node haemorrhage (i and j), whereas the DLW infection group presented milder interstitial pneumonia with lung consolidation (g) and lymph node haemorrhage (o). The challenge group exhibited varying degrees of pulmonary interstitial pneumonia severity, characterized by extensive infiltration of inflammatory cells, hyperplasia of the alveolar epithelium, and widening of the alveolar diaphragm (i-k). Additionally, submaxillary lymph node medullary haemorrhage was observed in the challenge group but not in the control group (q-t).

was characterized by thickening of the alveolar septa and infiltration of mononuclear cells. Microscopic lung lesions exhibited similar patterns between the DLF- and DLW-challenged groups but varied in terms of severity (Figure 5(j,k)). In the DLF-challenged group, extensive infiltration of inflammatory cells with serous exudation was observed, along with necrosis and shedding of alveolar epithelial cells as well as significant bronchiolar epithelial cell necrosis and shedding in DLF-challenged pigs (Figure 5(j)). In the DLW-challenged group, there was extensive infiltration by inflammatory cells and moderate widening of the alveolar diaphragm (Figure 5(k)). In addition, different degrees of submaxillary lymph node medullary haemorrhage were observed in the challenge groups, in contrast with the control groups (Figure 5(q–t)). Conversely, no pathological lesions

were observed in the aforementioned tissues of pigs in the control group (Figure 5(l,t)).

Discussion

The L8.7 lineage is the first lineage found in China and has been present for over 25 years. In 2006, an outbreak of infection with HP-PRRSV, which evolved from classical PRRSV, was characterized by high fever, high morbidity, and high mortality in China [40,42–45]. HP-PRRSV has since spread extensively across China and multiple regions in Asia [12,15,46] and has exhibited significant mutations [11,12,14,15,19,47,48]. Notably, HP-PRRSV and its variants have become predominant in China. However, research on the epidemiology, molecular evolution, and pathogenicity of novel L8.7 PRRSV is limited. Therefore, this study aimed to provide a systematic and comprehensive analysis focused on L8.7 PRRSV.

Currently, the genetic diversity of L8.7 PRRSVs is more extensive than that in previous periods. Several studies have proposed a preliminary framework for further subtyping L8.7 strains [12,19,24]. However, the primary data sources for these frameworks consist primarily of Chinese L8.7 sequences, and their classification method does not adhere to the currently accepted international classification of PRRSV. Therefore, previous studies have encountered challenges in characterizing L8.7 strains on a global scale. In this study, we built upon the currently internationally recognized PRRSV classification and further expanded our analysis database (the NCBI database and our laboratory database as of December 2023). Additionally, each group was subsequently characterized, and the differences in nucleotide consistency within and between groups were computed. Notably, the current L8.7 strains can be classified into 7 primary groups that have predominantly evolved and spread in China, exhibiting a wide geographical distribution. Compared with that of L8.7.3 strains (HP-PRRSV), the nucleotide consistency of 8.7.4–8.7.7 strains significantly differed (Figure 1(c)), so we designated L8.7.4-L8.7.7 strains as HP-like PRRSV. Notably, HP-like PRRSV has an advantage in terms of geographic range, particularly in Asia. Although the L8.7.4 group was the most widely distributed and had the highest number or proportion of detected strains, its temporal fluctuation was the most obvious, and the detected strains were concentrated mainly from 2009–2012. At present, the most prevalent L8.7 strains are L8.7.5 and L8.7.6 strains, which are isolated mainly in China. In the future, if new variants of L8.7 strains emerge, further differentiation can be pursued on the basis of the existing framework.

Commercial L8.7 MLV vaccines are commonly utilized for controlling PRRSV infection in China. For the first time, we calculated and compared the evolutionary rates of L8.7.3–8.7.7 strains before and after the use of vaccines. We found that the evolutionary rate was lower after the use of vaccines than before. This observation coincides with a study showing that even if vaccination against H7N9 does not completely eliminate the highly pathogenic avian influenza H7N9 virus in poultry in a short period, vaccination still significantly slows its evolution [49]. The GP5 selection pressure of L8.7 was analysed before and after the administration of the vaccine to understand the immune impact exerted by the L8.7 vaccine. The results revealed the presence of 5 sites under positive selection and 33 sites under negative selection prior to vaccine administration, whereas after vaccination was implemented, 22 sites presented positive selection, and 92 sites presented negative selection (FIG. S3). The vaccine induced more negative selection sites after administration, which indicates that the L8.7 strain underwent immune selection after the administration of the L8.7 vaccine. These findings imply that vaccination exerts a certain impact on the control of PRRSV.

There is no established ORF5 nt identity cut-off value to differentiate vaccine-like viruses from wild-type viruses [10]. To clarify the proportion of vaccine-associated strains among L8.7 PRRSVs, this study distinguished them on the basis of previous reports [24,34]. With the increase in the number of genome-wide analyses, it has become difficult to define vaccine-associated and wild-type viruses on the basis of either genome-wide consistency analysis or characteristic aa changes. Furthermore, we found that some “vaccine-like” strains (GU232735.1, JF748717.1) existed prior to vaccine use/development (TABLE S3). Given that TJM has an additional 120 aa deletion in NSP2, this strain was selected as a key study object to assess the proportion of vaccine-related strains within its branch. Our findings revealed that the prevalence of vaccine-related strains among the 8.7.6 group was approximately 27.85% (22/79). The comparison of nt consistency and characteristic aa changes within this branch revealed that these two parameters were unable to effectively distinguish vaccine-related strains, as there were instances in which strains, such as KP742986.1 and KX815410.1, met both criteria (nt consistency > 98.0% or same aa mutation > 30) but lacked the additional 120 amino acid deletion in NSP2 (TABLE S3). The analysis of selection pressure also revealed that wild strains from the same lineage experience immune selection in pigs vaccinated with vaccines from the same lineage. These “domestications” further reduce the diversity of

Table 3. Comparative analysis of the pathogenicity of L8.7.1-L8.7.7 PRRSV.

Group	Infecting PRRSV strain	Accession no.	Isolation date	Country	Recombination	Age of pigs (weeks)	Days post-inoculation (dpi)	Inoculation dose (TCID50/ml)	Parameters evaluated	Challenge group	Reference
8.7.1	CSR1801	OM743305.1	2018	Shandong, China	None	4	21	2×10^5	Days of fever Lesions in thymus Mortality	2 days (>39.7 °C) Not described 0%	Xu et al. [47]
	Ch-1a	AY032626.1	1996	China	None	4–5	14	$3 \times 10^{5.12}$	Days of fever Lesions in thymus Mortality	2 days (>40.2 °C) Not described 0%	Han et al. [48]
8.7.2	HB-1/3.9	EU360130.1	2002	China	None	4	16	2×10^5	Days of fever Lesions in thymus Mortality	Not described Not described 0%	Han et al. [43]
8.7.3	JXwn06	EF641008.1	2006	China	None	4	16	2×10^5	Days of fever Lesions in thymus Mortality	16 days (>40.0 °C) Not described 60%	Song et al. [44]
	TJ	EU860248.1	2006	Tianjin, China	None	3	21	10^3	Days of fever Lesions in thymus Mortality	9 days (>41°C) Not described Not described	Li et al. [45]
	HuN4	EF635006.1	2006	China	None	4–5	21	$3 \times 10^{5.12}$	Days of fever Lesions in thymus Mortality	12 days (>40.0°C) Severe thymus atrophy (5/5) 80%	Han et al. [48], [50]
8.7.4	10FUJ–2	JQ663547.1	2010	Fujian, China	L8.7+L8.7	10	21	$3 \times 10^{4.5}$	Days of fever Lesions in thymus Mortality	At least 3 days (>41°C) Not described 40%	[55]
	MB6	KM244761.1	2009	Vietnam	Not described	3	14	$3 \times 10^{5.5}$	Days of fever Lesions in thymus Mortality	12 days (>40.5°C) Not described 60%	[51]
	MN1	KM244763.1	2013	Vietnam	Not described	4	28	$3 \times 10^{5.5}$	Days of fever Lesions in thymus Mortality	6 days (>40.0°C) Not described 0%	[51]
	AGES/568-30FC/13	KM588915.1	2013	Vietnam	Not described	\	28	2×10^5	Days of fever Lesions in thymus Mortality	None Not described Not described	[17]
	09SC	JF268672.1	2009	Sichuan, China	Not described	4	21	$3 \times 10^{4.5}$	Days of fever Lesions in thymus Mortality	At least 4 days (>41.0°C) Not described 100%	[18]
	10BJ–5	JQ663545	2010	Beijing, China	Not described	10	21	$3 \times 10^{4.5}$	Days of fever Lesions in thymus Mortality	At least 4 days (>41.0°C) Not described 40%	
	LW1–13	JQ663557.1	2010	Vientiane, Laos	Not described	10	21	$3 \times 10^{4.5}$	Days of fever Lesions in thymus Mortality	At least 4 days (>41.0°C) Not described 60%	
8.7.5	JXA1	EF112445.1	2006	China	None	4	21	4×10^5	Days of fever Lesions in thymus Mortality	10 days (>40.0°C) / 40%	[55]
	DLF	PQ178809	2020	China	None	4	21	4×10^4	Days of fever Lesions in thymus Mortality	12 days (>40.3°C) Atrophy of the thymus in 4 pigs (4/5) 40%	this stay

(*Continued*)

Table 3. (Continued).

Group	Infecting PRRSV strain	Accession no.	Isolation date	Country	Recombination	Age of pigs (weeks)	Days post-inoculation (dpi)	Inoculation dose (TCID50/ml)	Parameters evaluated	Challenge group	Reference
8.7.6	SD-YL1712	MT708500.1	2017	Shandong, China	L8.7 (main) +L1.8	4	21	4×10^5	Days of fever Lesions in thymus Mortality	10 days (>40.0°C) Not described 0%	[56]
	XJ17–5	MK759853.1	2017	Xinjiang, China	None	4	14	2×10^5	Days of fever Lesions in thymus Mortality	4 days (>40.0°C) Not described 60%	[52]
	JSTZ1712–12	MK906026.1	2017	Jiangsu, China	None	4	14	2×10^5	Days of fever Lesions in thymus Mortality	None Not described 0%	
	DLW	PQ178810	2020	China	L8.7 (main) +L1.8	4	21	4×10^4	Days of fever Lesions in thymus Mortality	6 days (>40.3°C) Atrophy of the thymus (0/5) 0%	this stay
8.7.7	GD	EU825724.1	2007	China	None	5	35	2×10^5	Days of fever Lesions in thymus Mortality	4 days (>41.0°C) Not described Not described	[36]
	HBap4–2018	MZ579701.1	2018	Hebei, China	L8.7 (main) +L1.8	4	28	3×10^5	Days of fever Lesions in thymus Mortality	11 days (>40.5°C) Not described 60%	57
	SCSN2020	OQ883907.1	2020	Sichuan, China	None	4	14	2×10^5	Days of fever Lesions in thymus Mortality	6 days (>40.0°C) Not described 0%	[53]

aa sites, which could be a key factor contributing to the emergence of strains resembling vaccines rather than the emergence of true vaccine-like strains (FIG. S3). The NSP2 of other vaccine strains does not have additional aa deletions, so it is currently not possible to effectively distinguish between vaccine viruses and vaccine-related viruses (Figure 3(c)). Therefore, the present study partially elucidated the proportion of vaccine-associated strains in L8.7 PRRSV.

The concern with L8.7 strain sis primarily in their pathogenicity [48,50–53], particularly following the outbreak of L8.7.3 strains (HP-PRRSV) in 2006 [40]. Thus, we isolated and evaluated the pathogenicity of HP-like PRRSVs (L8.7.5:DLF and L8.7.6: DLW), which are currently the most prevalent among L8.7 strains. Although the virulence of HP-like PRRSV (DLF and DLW) decreased compared with that of HP-PRRSV (HuN4), which manifested as an increased survival rate of piglets, increased daily gain, fever temperature and duration decrease and varying degrees of atrophy of the thymus, these strains still showed substantial pathogenicity. The virulence of the virus strains in this study was correlated with the serum viral load of virus-challenged piglets at 7 dpi and 10 dpi, whereas no significant difference was observed at other time points. Therefore, on the basis of our results, the survival rate, fever temperature and duration, thymic atrophy are important indicators that objectively reflect the pathogenicity of PRRSV in piglets. As the L1 PRRSV became prevalent in China in 2016, an increasing number of recombinant strains were reported, and the Chinese recombination patterns were mainly L1 (L1.5 or L1.8) + L8.7 and L8.7+L1 (L1.5 or L1.8) [54–57]. In this study, DLW was a recombinant virus with a recombination pattern of L8.7 + L1.8, and its pathogenicity was lower than that of HuN4 and DLF. Although the pathogenicity of DLW to piglets was significantly reduced, the relationship between reduced virulence and recombination needs further study. In short, except the L8.7.1 (low pathogenicity) and L8.7.2 (low pathogenicity) strains, the L8.7.3 strains demonstrated robust pathogenicity in piglets (Table 3). The results of the previously reported 2006 L8.7.5 virus pathogenicity experiment and 2017 L8.7.6 virus pathogenicity experiment [35,56] revealed that, compared with L8.7.3 virus, HP-like PRRSV (L8.7.4–8.7.7) exhibited reduced pathogenicity in piglets while maintaining substantial virulence (Table 3).

In summary, this study is an epidemiological survey of L8.7 PRRSV in Chinese provinces from 1996 to 2023, and systematically studies the classification, characteristics and distribution of L8.7 PRRSV and its different populations around the world. Currently, L8.7 PRRSV has undergone diversification into seven distinct groups, with HP-like PRRSVs emerging as the predominant branches, which are widely distributed across China. Moreover, this study revealed that HP-like PRRSV preponderantly comprises wild strains rather than vaccine-related strains and that the utilization of the L8.7 vaccine has a certain efficacy in controlling L8.7 PRRSV in China. Compared with that of L8.7.3 PRRSV, the pathogenicity of HP-like PRRSV in piglets is slightly attenuated; however, these strains still exhibit a high level of virulence. This study has significant implications for comprehending evolution, changes in pathogenicity, and prevention and control strategies concerning global L8.7 strains.

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Author contributions

C.L., H.X. and J.H.L. designed and managed the study; C.L., H. X., J.H.L., B.J.G., Z.Y.G., G.H.Z., Y.D.T., Z.J.T. and H.L. Z. conducted the experiments; C.L., H.X., J.H.L. and B.J. G. analysed the data; L.R.X., S.Y.Z., Q.S., J.Z., M.L.Z., C.L.L., K.Z., J.N.W., Q.W., J.M.P., H.R.L., T.Q.A. and X.H.C. collect samples; C.L. and H.L.Z. wrote the manuscript. Y.D.T., Z.J. T. and H.L.Z. reviewed and edited the paper. All authors have read and agreed to the published version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Additional information

We have adhered to ARRIVE guidelines.

Data availability statement

The data and supplemental material that support the findings of this study are directly available in ScienceDB. ResearchData at https://doi.org/10.57760/sciencedb.17670.

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