First detection of NADC34-like PRRSV as a main epidemic strain on a large farm in Heilongjiang Province, China

Chao Li[1], Bangjun Gong[1], Qi Sun[1], Hu Xu[1], Jing Zhao[1], Lirun Xiang[1], Yandong Tang[1], Chaoliang Leng[2], Wansheng Li[1], Zhenyang Guo[1], Jun Fu[1], Jinmei Peng[1], Qian Wang[1], Guohui Zhou[1], Ying Yu[3], Fandan Meng[1], Tongqing An[1], Xuehui Cai[1], Zhijun Tian[1], and HongLiang Zhang[1]

[1]Chinese Academy of Agricultural Sciences Harbin Veterinary Research Institute
[2]Nanyang Normal University
[3]Qingdao Agricultural University

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Abstract

Newly emerged sublineage 1.5 (NADC34-like) porcine reproductive and respiratory syndrome virus (PRRSV) has posed a direct threat to the Chinese pig industry since 2018. However, the prevalence and impact of NADC34-like PRRSV on Chinese pig farms is unclear. In the present study, we continuously monitored pathogens, including PRRSV, African swine fever virus (AFSV), classical swine fever (CSFV), pseudorabies virus (PRV) and porcine circovirus 2 (PCV2), on seven fattening pig farms with strict biosecurity practices located in five provinces of China from 2020 to 2021. The results showed that multiple types of PRRSVs commonly coexisted on a single pig farm. NADC30-like PRRSV was the predominant strain on most pig farms. Importantly, NADC34-like PRRSV, detected during the period of peak mortality, was one of the predominant strains on one pig farm in northern China. Sequence alignment suggested that these strains shared the same 100-aa deletion in the Nsp2 protein as IA/2014/NADC34 isolated from the United States (U.S.) in 2014. Phylogenetic analysis based on open reading frame 5 (ORF5) showed that the genetic diversity of NADC34-like PRRSV on this farm was relatively singular, but it had a relatively high rate of evolution. Restriction fragment polymorphism pattern (RFLP) analysis showed that almost all ORF5 RFLPs were 1-7-4, with one 1-4-4. In addition, two complete genomes of NADC34-like PRRSVs were sequenced. Recombination analysis and sequence alignment demonstrated that both viruses, with 98.9% nucleotide similarity, were nonrecombinant viruses. This study reports the prevalence and characteristics of NADC34-like PRRSV on a large-scale breeding farm in northern China for the first time. These results will help reveal the impact of NADC34-like PRRSV on Chinese pig farms and provide a reference for detection and further prevention and control of NADC34-like PRRSV.

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[a] State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin 150001, China

[b] Henan Key Laboratory of Insect Biology in Funiu Mountain, Henan Provincial Engineering Laboratory of Insects Bio-reactor, China-UK-NYNU-RRes Joint Laboratory of Insect Biology, Nanyang Normal University, Nanyang 473061, China

[c] Department of Preventive Veterinary Medicine, College of Veterinary Medicine, Qingdao Agricultural University, Qingdao, 266109, China

[1] These authors contributed equally to this work.

* Corresponding author contact: Harbin Veterinary Research Institute, CAAS, No. 678 Haping Road, Xiangfang District, Harbin 150001, China.

Tel.: +86-13624503578

E-mail address: zhanghongliang01@caas.cn

Abstract: Newly emerged sublineage 1.5 (NADC34-like) porcine reproductive and respiratory syndrome virus (PRRSV) has posed a direct threat to the Chinese pig industry since 2018. However, the prevalence and impact of NADC34-like PRRSV on Chinese pig farms is unclear. In the present study, we continuously monitored pathogens, including PRRSV, African swine fever virus (AFSV), classical swine fever (CSFV), pseudorabies virus (PRV) and porcine circovirus 2 (PCV2), on seven fattening pig farms with strict biosecurity practices located in five provinces of China from 2020 to 2021. The results showed that multiple types of PRRSVs commonly coexisted on a single pig farm. NADC30-like PRRSV was the predominant strain on most pig farms. Importantly, NADC34-like PRRSV, detected during the period of peak mortality, was one of the predominant strains on one pig farm in northern China. Sequence alignment suggested that these strains shared the same 100-aa deletion in the Nsp2 protein as IA/2014/NADC34 isolated from the United States (U.S.) in 2014. Phylogenetic analysis based on open reading frame 5 (ORF5) showed that the genetic diversity of NADC34-like PRRSV on this farm was relatively singular, but it had a relatively high rate of evolution. Restriction fragment polymorphism pattern (RFLP) analysis showed that almost all ORF5 RFLPs were 1-7-4, with one 1-4-4. In addition, two complete genomes of NADC34-like PRRSVs were sequenced. Recombination analysis and sequence alignment demonstrated that both viruses, with 98.9% nucleotide similarity, were nonrecombinant viruses. This study reports the prevalence and characteristics of NADC34-like PRRSV on a large-scale breeding farm in northern China for the first time. These results will help reveal the impact of NADC34-like PRRSV on Chinese pig farms and provide a reference for detection and further prevention and control of NADC34-like PRRSV.

Keywords:

NADC34-like PRRSV, main epidemic strains, 100-aa deletion, RFLP

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most important pathogens affecting swine globally (Ramirez et al., 2019). PRRSV belongs to the genus Betaarterivirus, family Arteriviridae, and order Nidovirales (Dokland, 2010). PRRSV is currently classified into two distinct species, Betaarterivirus suid 1 (PRRSV-1) and Betaarterivirus suid 2 (PRRSV-2) (Brinton et al., 2021). Both PRRSVs have been circulating in China for decades and have caused substantial economic losses among the Chinese pig industry. PRRSV-2 has been the main epidemic strain in China, and is further classified into 9 lineages based on open reading frame 5 (ORF5) sequences (Shi et al., 2010). Four lineages (lineages 1, 3, 5 and 8) of PRRSV-2 circulate in the field (Guo et al., 2018). In recent years, the dominant lineages of PRRSV-2 have shifted from sublineage 8.7 (CH-1a-like and HP-PRRSV-like) to sublineage 1.8 (NADC30-like).

In 2014, the ORF5 RFLP 1-7-4 lineage was prevalent in the United States (U.S.) and was recognized to be the cause of dramatic abortion "storms" in sow herds and high mortality among piglets (Alkhamis et al., 2016; van Geelen et al., 2018). Subsequently, that sublineage was confirmed to be endemic in Peru (Ramirez et al., 2019). In China, NADC34-like PRRSV was first detected in Liaoning Province in 2017 (Zhang et al., 2018). Subsequently, the presence of such strains was also detected in southern China (Liu et al., 2019). In 2020 and 2021, the detection of sublineage 1.5 (NADC34-like) and research on its spread were successively reported (Bao and Li, 2021; Xie et al., 2020b; Xu et al., 2020). Unlike American NADC34-like PRRSVs, Chinese NADC34-like PRRSVs are currently mildly or moderately pathogenic in piglets (Song et

al., 2020; Xie et al., 2020a). However, the prevalence and impact of NADC34-like PRRSV in Chinese pig farms is unknown. During the epidemiological investigation of seven pig farms in five provinces in China (Heilongjiang, Xinjiang, Hebei, Henan, and Hubei), we found that NADC34-like PRRSV was one of the main endemic strains in a large pig farm in Heilongjiang Province. In this study, we monitored PRRSVs on this farm for 150 days and conducted a detailed study on the epidemic process and molecular characteristics of NADC34-like PRRSV.

Materials and Methods

A finishing pig farm (3400 heads) where five pathogens (ASFV, CSFV, PRV, PRRSV and PCV2) had been detected was selected for the study. The farm is located in Heilongjiang Province of China, and there is no neighboring pig farm within 3 kilometers. The pig farm employs a fully enclosed management model; personnel and materials entering and leaving the area are disinfected, and the manure used to produce fertilizer was treated appropriately. The internal layout of the pig farm is reasonable; the buildings are at least 6 meters apart, ventilation is good, disinfection is performed regularly, and necessary vaccines are administered in a timely manner. Livestock are vaccinated with the classical PRRSV modified-live virus (MLV) vaccine, classical swine fever MLV vaccine, pseudorabies virus MLV vaccine, foot and mouth disease (FMD) inactivated Vaccine and porcine circovirus 2 MLV vaccine. The pig farm employs professional management and veterinary personnel.

Approximately 15-45 lung and lymph node samples from livestock on the farm were submitted for laboratory testing approximately every 15 days during the study period. During the breeding period, dead pigs were dissected to collect samples, and the time of death and number of deaths were recorded. The number of dead pigs was counted every day and summed every 15 days. From September 2020 to January 2021, 283 clinical samples were collected from this farm. Tissue sample disposal, RNA and DNA extraction, cDNA preparation, RT-PCR analysis and genome sequencing were conducted as previously described (Leng et al., 2014; Leng et al., 2012; Zhang et al., 2015; Zhou et al., 2019). The primers used for virus detection (AFSV, CFSV, PCV2, PRRSV and PRV) and complete genome amplification have been reported previously (An et al., 2013; Cai et al., 2012; Gao et al., 2017; Torres et al., 2017; Zhang et al., 2015; Zhang et al., 2018).

All reference strains were downloaded from the NCBI, and the corresponding sequences were compared and intercepted. Deduced amino acid sequences were aligned by ClustalW with Lasergene software. All sequences were aligned using MAFFT version 7 (Katoh and Standley, 2013) with default parameters (https://mafft.cbrc.jp/alignment/software/) and manually adjusted in MEGA6 (Tamura et al., 2013). We followed the same rationale for the classification of sequences into lineages as previously published (Shi et al., 2010). To identify evolutionary relationships among strains on this farm, phylogenetic trees were constructed in MEGA 6.0 by the neighbor-joining method with a bootstrap value of 1,000 replicates and with the Kimura 2-parameter substitution model. The trees were annotated and modified using Evolview (version 2.0) (https://www.evolgenius.info/evolview/#login) (He et al., 2016). To analyze recombination events, similarity analysis was performed using SimPlot software (v3.5), with a 200-bp window and a 20-bp step (Yu et al., 2020).

The temporal signal in the phylogenetic datasets of NADC34-like PRRSV was first investigated using TempEst to confirm the appropriateness of the data for time-scaled phylogenetic tree reconstruction (Rambaut et al., 2016). To estimate the evolution rate of the NADC34-like PRRSV strain on this pig farm, a relaxed uncorrelated lognormal (UCLN) molecular clock was used, with a flexible Bayesian SkyGrid plot (BSP) demographic model and a general-time reversible model of nucleotide substitution with gamma-distributed rate variation among sites (GTR+Γ), allowing for partitions into codons in any of the three positions (Barba-Montoya et al., 2020). The Markov chain Monte Carlo (MCMC) algorithm was run for 200 million steps and sampled every 20,000 steps. Convergence was assessed with effective sample size (ESS) values, and ESS values over 200 were considered adequate. These analyses were performed using BEAST (v1.10.4). Three independent runs were performed in this study to prevent any local convergence. The BEAST results were entered into Tracer to evaluate model convergence and consistency between replicates.

GraphPad Prism 8.0 (San Diego, CA, USA) was used to perform the statistical data analyses.

Results and Discussion

In total, 412 pigs died over the course of 150 days. A total of 46 of 412 piglets died in the first 15 days (Fig 1). The main clinical symptoms were loss of appetite and fever, followed by acute death, and the main pathological changes observed during piglet necropsy were intestinal hemorrhage, abdominal hemorrhage or peritoneal effusion. Symptoms were preliminarily presumed to be caused by bacterial infection. Symptoms subsided after emergency antibiotics were administered for prevention and treatment on the 10th day. The peak mortality period (287/412) occurred from the 16th day to the 45th day (Fig 1). Most pigs showed obvious clinical respiratory symptoms, such as cough, wheezing or diaphragmatic breathing. Necropsy of dead pigs showed lung consolidation, partial intestinal bleeding, and abdominal hemorrhage. Antibiotics administered after 35 days, the number of dead pigs (79/412) decreased (Fig 1), and the majority of the deaths occurred in pigs previously isolated for respiratory symptoms. To explore the causes of death in the pigs, a total of 283 samples were collected from dead piglets and tested for AFSV, CFSV, PRRSV, PRV and PCV2. PRRSV and PCV2 were detected, while ASFV, CSFV and PRV were not. During the three different stages noted above, the detection rates of PRRSV and PCV2 were 17.39% (8/46), 33.13% (53/160), and 29.87% (23/77); and 89.13% (41/46), 81.88% (131/160), and 80.52% (62/77), respectively (Fig 1). The above results demonstrate that death in the early stage may have been mainly due to bacterial infection and that PRRSV contributed to the death curve but not PCV2 did not on this pig farm.

To explore the relationship between the PRRSV subtype and the death of pigs on the farm, we sequenced the NSP2 and ORF5 genes for all PRRSV positive samples. A total of 78 NSP2 sequences and 69 ORF5 sequences were obtained. Of these, 54 samples were NADC30-like PRRSV (64.29%), 17 were NADC34-like PRRSV (20.24%), 11 were HP-PRRSV-like (13.10%), 1 was CH-1a-like PRRSV (1.19%), and 1 was QYYZ-like PRRSV (1.19%) (Fig 3a). Therefore, NADC30-like PRRSV, NADC34-like PRRSV and HP-PRRSV-like were the main epidemic strains on this farm. Furthermore, the detection rates of the main epidemic strains were 12.50%, 71.70%, and 65.22% (NADC30-like PRRSV); 0.00%, 26.42%, and 13.04% (NADC34-like PRRSV); and 87.50%, 1.89%, and 17.39% (HP-PRRSV-like) in the three different stages, respectively. Surprisingly, the outbreak times of NADC30-like and NADC34-like PRRSVs were consistent with the peak periods of pig deaths on the pig farm (Fig 1). The above results demonstrated that NADC30-like PRRSV and NADC34-like PRRSV but not HP-PRRSV-like PRRSV were closely related to the deaths of pigs on this farm. In addition, a number of NADC34-like strains (14/17) were detected in the subsequent 10 days after initial detection in stage 2. The spread of NADC34-like PRRSV seemed to be faster than that of NADC30-like PRRSV. The pathogenicity of NADC34-like PRRSV on this farm remains to be studied.

NADC34-like PRRSV emergence was first reported in Liaoning Province in 2017 (Zhang et al., 2018). This strain subsequently emerged in other provinces of China (Bao and Li, 2021; Liu et al., 2019; Sun et al., 2020; Xie et al., 2020a; Xie et al., 2020b; Xu et al., 2020). All the NSP2 sequences of the NADC34-like strains shared the same 100 consecutive amino acid deletions between 328 and 427 as previously reported, compared with ATCC_VR2332 (accession number: U87392) (Fig 2). These deletions can be used as molecular markers to distinguish NADC34-like strains from other type 2 PRRSV strains in China, similar to the consistent Nsp2 protein deletion pattern in NADC30-like PRRSV (Brockmeier et al., 2012; Xu et al., 2020). The amino acid identities of NSP2 of the NADC34-like strains on this farm were between 99.2% and 99.9%. In the NCBI library, the highest identity was with IA/2014/NADC34 (accession number: MF326985), at 92.6%.

The nucleotide identity of the NADC34-like PRRSV ORF5 gene on this pig farm was 99.2%-100%, which also has the highest identity with the IA/2014/NADC34 strain in the NCBI, and the nucleotide similarity was 96.9%-97.2%. The consistency between these strains and the first NADC34-like strain, LNWK130, reported in China was 94.9%-95.0%. These indicate that NADC34-like PRRSV has evolved in China. Combined with the NSP2 analysis of NADC34-like PRRSV, NADC34-like PRRSV infection on this pig farm was caused by a single strain; this provides a good platform for studying the evolution rate of the NADC34-like strain (Barba-Montoya et al., 2020). Many methodological approaches previously used to study PRRSV ignored the fact that the evolutionary and epidemiological dynamics of rapidly evolving pathogens, such as PRRSV, occur

on approximately the same timescale. Thus, they must be studied under a unified methodological setting to be properly understood and to prevent biased conclusions, subsequently improving related decision-making processes (Alkhamis et al., 2016; Pybus et al., 2013). The NADC34-like strains on this farm showed a strong time signal (the correlation between the genetic difference and sampling time $r^2$was 0.52) and was thus suitable for phylogenetic analysis involving a molecular clock. The estimated viral substitution rates were 3.1×10^{-2} substitutions/site/year, which was higher than the evolution rate, which ranged from 6.6 x $10^{-3}$to 1.3 x 10^{-2} substitutions/site/year for all subtypes of lineage 1 previously reported in the U.S. (Alkhamis et al., 2016; Paploski et al., 2021). This is a dangerous signal that indicates that the time from the appearance of NADC34-like PRRSV in China to its peak in the population will be shorter than that in the U.S. (4.5 years on average) (Paploski et al., 2021). Moreover, surveillance of PRRSV showed that the number of NADC34-like PRRSVs has obviously increased since 2020, especially in 2021 (unpublished data). Therefore, we speculate that NADC34-like PRRSV has become dominant in parts of China.

We further classified the NADC34-like PRRSV strains on this farm according to restriction fragment length polymorphism (RFLP) of the ORF5 gene (Brar et al., 2011; Cha et al., 2004; Trevisan et al., 2021). The RFLP pattern of ORF5 of TZJ1277 is 1-4-4, while the others are 1-7-4. Compared with the newly emerged PRRSV lineage 1C variant (MW887655) in the U.S., TZJ1277 has a closer relationship with the earlier reported NADC34-like PRRSV (Fig 3a). RFLP typing has recognized shortcomings, which include an inability to represent genetic relationships between different RFLP types, the potential for distantly related viruses to share the same RFLP type, and instability of RFLP types over as few as 10 animal passages (Cha et al., 2004; Paploski et al., 2019). Partially due to these ambiguities in the interpretation of RFLP types, researchers in many countries have formulated their own naming conventions based on the epidemic situation in their countries (Paploski et al., 2019; Paploski et al., 2021), so the classification of virus strains in their home countries is crucial.

Whole-genome sequencing (WGS) can reveal more information about PRRSV than traditional Sanger sequencing analysis of ORF5 (Frias-De-Diego et al., 2021; Risser et al., 2021). WGS phylogenetic tree analysis showed that the lineage 1C variant (MW887655) and the two full-length sequences measured (TZJ864 and TZJ921) were clustered into the same branch (Fig 3b). The United States 1-4-4 lineage1C PRRSV variant is based on IA14737-2016 (NADC34-like) as the parent strain, and IA/2014/NADC34 and NADC30 strains provide recombinant fragmented recombinant virus. PRRSV recombination events have been the focus of researchers (van Geelen et al., 2018; Wang et al., 2019; Yu et al., 2020). Recombinant PRRSVs have been increasingly isolated since NADC30-like PRRSVs emerged in China (Chen et al., 2018; Ramirez et al., 2019; Sun et al., 2020). To explore whether the NADC34-like PRRSV isolated from this farm was a recombinant virus, we sequenced two complete genomes from NADC34-like PRRSVs from this farm. Recombination analysis and sequence alignment showed that they were not recombinant viruses and had relatively high identity (98.9%). However, five types of PRRSVs coexisting on the same farm will certainly increase the likelihood of recombination. Coincidentally, we have detected many recombinant NADC34-like PRRSVs from other pig farms since June 2021 (data unpublished). The characteristics and virulence of these recombinant NADC34-like PRRSVs need to be further studied.

Concerningly, the U.S. has reported high economic losses due to NADC34-like reorganization (van Geelen et al., 2018). Importantly, NADC34-like and NADC30-like PRRSVs recombine with strains of different subtypes, resulting in inconsistent virulence among the recombinant strains and causing great obstacles in the prevention of PRRSV (Chen et al., 2018; Chen et al., 2021). Considering that NADC30-like PRRSV has become the main epidemic strain in China (Jiang et al., 2020), outbreaks of NADC34-like PRRSVs in pig farms will inevitably lead to more frequent fragment exchanges between them. Therefore, we need to increase awareness of the importance of continuous monitoring of NADC34-like PRRSV strains, strictly control the selection of breeding pigs, and prevent the occurrence of multiple subtypes of PRRSV on pig farms.

Conclusion

In summary, multiple subtypes of PRRSV commonly coexist on single pig farms. NADC34-like PRRSV,

which correlated with the death curve, was first reported to be one of the main endemic strains on a pig farm in Heilongjiang Province, China. NADC34-like PRRSV from this farm had high nucleotide similarity and a 100-aa deletion in the NSP2 protein but no recombination. The genetic diversity of NADC34-like PRRSV is relatively singular, but it has a relatively high evolution rate. The prevalence of NADC34-like PRRSV on other pig farms in China needs further attention.

Ethical Approval

Sampling procedures were performed in accordance with the guidelines of the Animal Ethics Committee of the School of Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences. The Animal Ethics Committee Approval Number was SYXK (Hei) 2011022.

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Conflicts Of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship and publication of this article.

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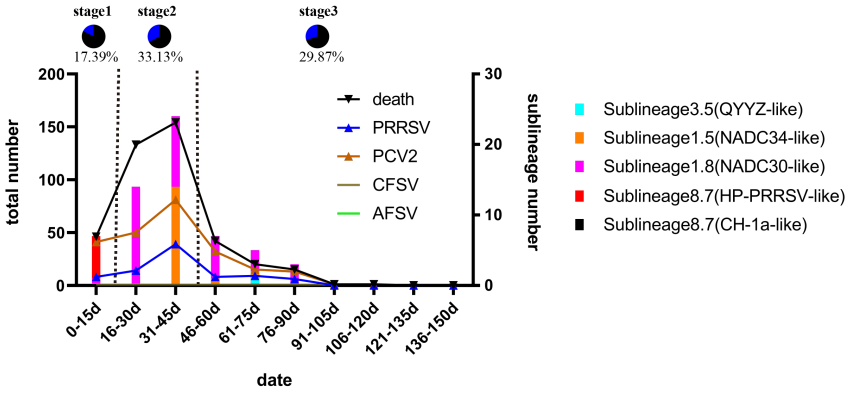

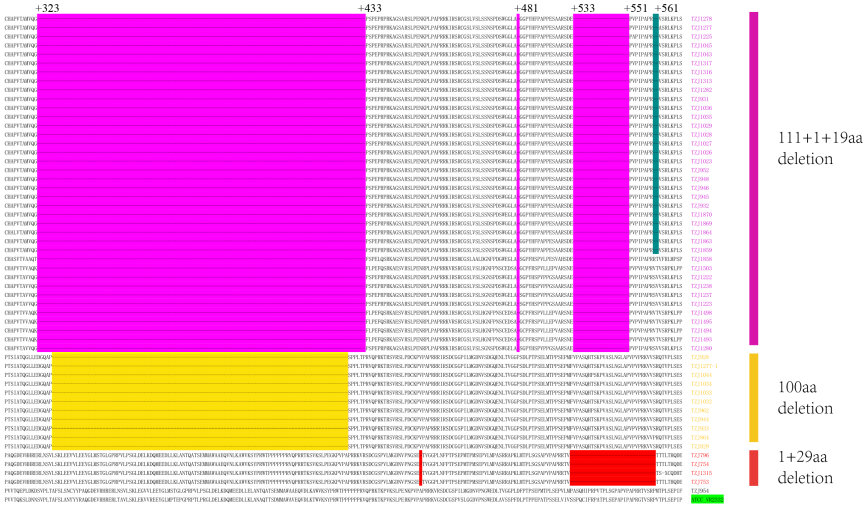

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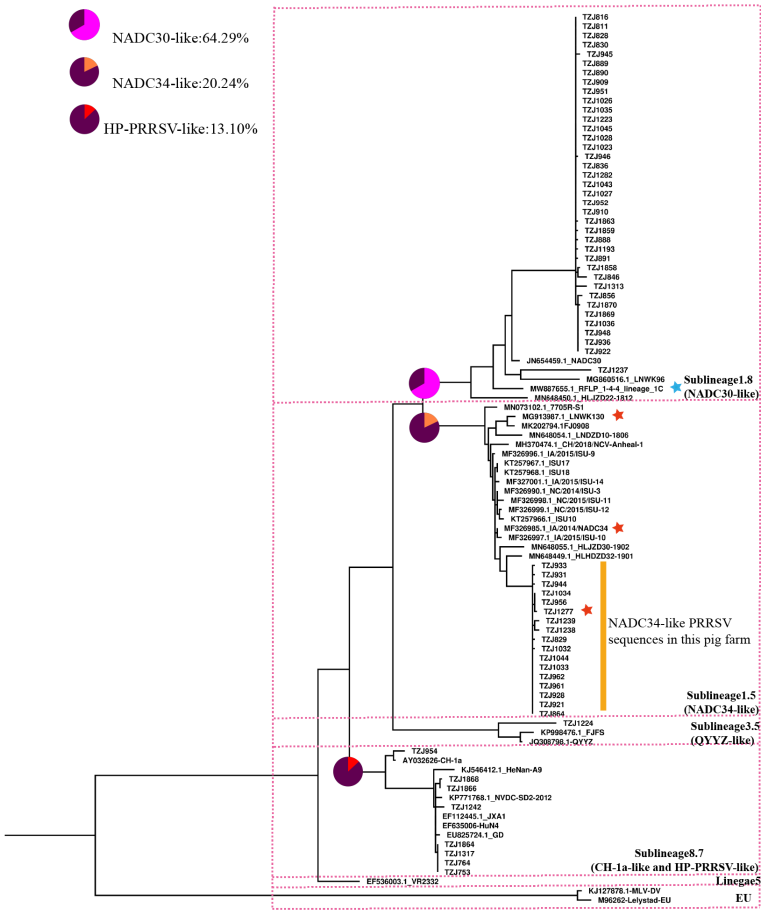

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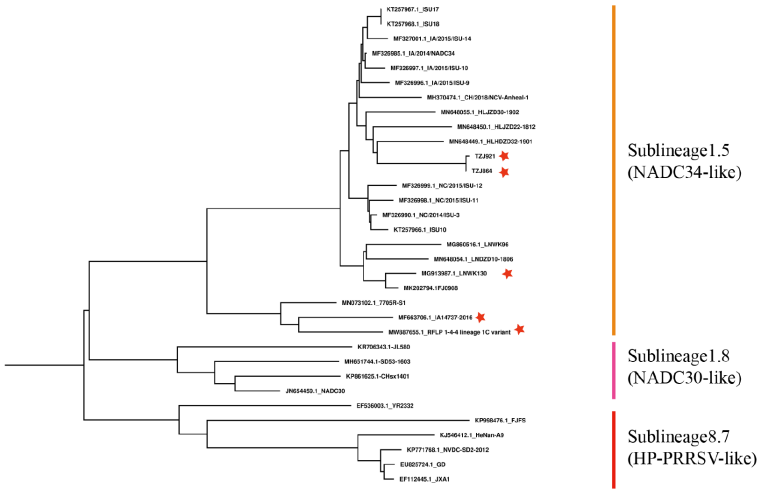

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