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Research article

MOLECULAR CHARACTERIZATION OF PRRSV STRAINS CIRCULATING IN NORTH CENTRAL REGION, VIETNAM DURING 2023-2024

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Porcine reproductive and respiratory syndrome virus (PRRSV) is a major pathogen that poses a serious threat to the global pig industry. North Central Vietnam, comprising six provinces, is one of the country's largest pig-breeding regions; however, the information of molecular characterization of this virus circulating in this area have been limited. To address this gap, 130 samples were collected from these provinces between 2023 and 2024. The results showed a PRRSV-positive rate of 23.07% (30/130). Molecular characterizations of PRRSV were further analyzed based on sequences of the ORF5 and NSP2 hypervariable regions (HVRs), in comparison with reference strains from Vietnam and other countries downloaded from GenBank (NCBI). Phylogenetic analysis revealed that the sequenced strains belonged to PRRSV-2 (lineages 1, 5, and 8). Specifically, 28 PRRSV-2 strains were selected for ORF5 analysis and classified into sub-lineage 8.7 (HP-PRRSV), sub-lineage 5.1 (classical PRRSV), and the newly identified sub-lineage 1.8 (NADC30-like). The NSP2 HVRs of the identified strains showed amino acid identities ranging from 38.63% to 100%, while ORF5 identities ranged from 80.3% to 100%. Sequence analysis of ORF5 indicated that GP5 mutations were mainly concentrated in HVR1, HVR2, and virulence-associated sites. In summary, the detection of NADC30-like PRRSV for the first time in Vietnam is important, indicating that this HP-PRRSV and classical PRRSV strains reported previously are currently co-circulating among the pig population in North Central Vietnam. Additionally, these findings suggest that PRRSV strains in Vietnam are undergoing substantial genomic divergence.

Keywords: PRRS, phylogenetic analysis, NADC30-like, Vietnam

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INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) was first identified in the United States in 1987 and later emerged in Europe during the 1990s [1]. Since then, it has spread worldwide, becoming a major threat to the global swine industry. PRRS is a highly economically significant infectious disease in pigs, characterized by reproductive failure and respiratory illness in pigs of all ages. The virus was first reported in Asia in the early 1990s [2]. In 2007, a highly pathogenic (HP) variant of PRRSV emerged in China and Vietnam, characterized by severe morbidity and high mortality rates. This HP-PRRSV strain rapidly spread to other Asian countries, including Korea and Thailand, causing substantial economic losses across the region's swine industry [1,3,4]. PRRSV is an enveloped, positive-sense, single-stranded RNA virus belonging to the family *Arteriviridae*, order *Nidovirales* [5]. The complete PRRSV genome is approximately 15 kb in length and contains at least ten open reading frames (ORFs): ORF1a, ORF1b, ORF2a, ORF2b, ORF3, ORF4, ORF5, ORF5a, ORF6, and ORF7 [5]. ORF1a and ORF1b encode viral replicase polyproteins, which are subsequently cleaved into 16 nonstructural proteins (Nsps), including NSP1α, NSP1β, NSP2, NSP2TF, NSP2N, NSP3, NSP4, NSP5, NSP6, NSP7α, NSP7β, NSP8, NSP9, NSP10, NSP11, and NSP12 [5,6]. The remaining ORFs encode the viral structural proteins GP2a, E, GP3, GP4, GP5, ORF5a, M, and N, respectively [7,8]. PRRSV is divided into two distinct genotypes: Type 1 (European genotype), represented by the prototype Lelystad virus, and Type 2 (North American genotype), represented by the prototype strain VR-2332. Despite sharing only about 60% nucleotide identity, both genotypes cause similar clinical syndromes in pigs [9-11]. Among PRRSV genes, *NSP2* and *GP5* are the most variable and are commonly used in phylogenetic and molecular epidemiological analyses [11,12].

In Vietnam, PRRSV is believed to have been introduced via imported pigs from the United States in the late 1990s [13]. Molecular epidemiological studies conducted prior to 2021, primarily based on ORF5 gene sequencing, identified three PRRSV lineages currently circulating in the country: lineage 8 (sub-lineage 8.7, associated with highly pathogenic PRRSV), lineage 5.1, and lineage 1 (NADC30-like group, sub-lineage 1.4). Of these, sub-lineage 8.7 and lineage 5.1 have been present since 2007, while sub-lineage 1.4 was first detected in 2021 [3,14-16]. However, since 2021, no studies have examined the genetic characteristics of PRRSV in the North Central region of Vietnam. To address this gap and gain a more comprehensive understanding of PRRSV molecular epidemiology in the region, we collected samples from pigs across six provinces in North Central Vietnam. Positive samples were subjected to sequencing of the NSP2 hypervariable (HV) region and the ORF5 gene. Furthermore, the evolutionary characteristics of PRRSV strains circulating in this region were analyzed.

MATERIALS AND METHODS

Sample collection

Between 2023 and 2024, a total of 130 clinical samples from pigs suspected of PRRSV infection were collected from farms across six provinces in North Central Vietnam: Thanh Hoa, Nghe An, Ha Tinh, Quang Binh, Quang Nam, and Thua Thien Hue. These samples, which included lungs, lymph nodes, and serum specimens, were obtained from farms where morbidity associated with suspected PRRSV infection ranged from 5% to 10%. Most of the affected pigs also showed evidence of mixed bacterial infections. The samples were transported under low-temperature conditions and stored at −20°C. A summary of the sample names, collection date, types, and isolation regions is provided in Table 1.

Table 1. Statistics data of PRRSV positive samples

No	Province	Total samples	Years		(+) samples	Sequences	
			2023	2024		ORF5	Nsp2
1	Thanh Hoa	33	13	20	7	7	7
2	Nghe An	32	12	20	14	12	12
3	Ha Tinh	30	10	20	3	3	3
4	Quang Binh	13	0	13	2	2	2
5	Quang Nam	11	0	11	2	2	2
6	Hue	11	0	11	2	2	2
	Totals	**130**	**35**	**95**	**30**	**28**	**28**

RNA extraction, Realtime RT-PCR and RT-PCR amplification

Tissue samples were homogenized in 1 mL of PBS containing 2% penicillin–streptomycin. Total RNA was extracted using TRIzol reagent (Life Technologies, USA) according to the manufacturer's protocol. After RNA extraction, the samples were analyzed using real-time RT-PCR with primer pairs as recommended by the Department of Animal Health (TCVN 8400-21:2014)/WOAH [17], employing the commercial qScript® XLT One-Step RT-PCR kit (Quantabio, USA), following the manufacturer's instructions. The reaction components and thermal cycling conditions for PRRSV detection were conducted in accordance with the standard protocols of the TCVN/WOAH guidelines.

For cDNA synthesis, reverse transcription was carried out in a total reaction volume of 20 µL, containing 10.5 µL of total RNA, 4 µL of 5× reverse transcription buffer, 2 µL of a 10 mM deoxynucleoside triphosphate (dNTP) mixture (Thermo Scientific, USA), 1 µL of 9-mer random primers (50 pM), 2 µL of M-MLV reverse transcriptase (Promega, MA, USA), and 0.5 µL of RNase inhibitor (40 U/µL) (Thermo Scientific, USA). The mixture was gently mixed, incubated at 42°C for 1 hour, and then placed on ice for 2 minutes.

Primers used to amplify the NSP2 hypervariable (HVR) region were: Forward: 5'-TCCGTGGTGCAACAA-3' and reverse: 5'-GGCTTGAGCTGAGTAT-3'; Primers used to amplify the ORF5 gene were: forward: 5'-ATGTTGGGGAAGTGCTT-3' and reverse: 5'-GACGACCCCATTGTT-3' [18]. Amplification was performed under the following conditions: one cycle at 94°C for 5 min; 30 cycles at 94°C for 30 s, 57°C for 45 s, and 72°C for 30 s; followed by a final extension at 72°C for 10 min and a hold at 4°C. PCR products were visualized on a 1% agarose gel under ultraviolet light. Amplicons of the expected size were excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Purified PCR products were sequenced at Apical Scientific Sdn. Bhd. (Seri Kembangan, Selangor, Malaysia).

Sequence alignment and phylogenetic analysis

Amino acid (AA) of the ORF5 gene and NSP2 hypervariable region (HVR) were aligned using the MUSCLE algorithm implemented in MEGA version 7.0. The final alignments consisted of 200aa for ORF5 and 600aa for the NSP2 HVR. Representative reference sequences were retrieved from GenBank to encompass the major PRRSV-2 lineages and sub-lineages, including JXA1-like, SX2009-like, VR-2332-like, NADC30-like, NADC34-like, and QYYZ-like viruses. Phylogenetic trees were reconstructed using the Maximum Likelihood (ML) method with the Neighbor-joining and gamma distributed. Gaps and missing data were handled using the partial deletion option. Node support was evaluated using 1,000 bootstrap replicates, and bootstrap values ≥70% are displayed on the corresponding nodes.

Virus isolation and titration

The positive samples were further processed for virus isolation by inoculating them onto Marc-145 cell lines. The Marc-145 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, CA, USA) and antibiotics (100 units/mL penicillin, 10 mg/mL streptomycin, and 25 mg/mL amphotericin B; Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a 5% CO_2 incubator. Following inoculation, the cells were monitored daily for the development of cytopathic effects (CPE) up to 3 days post-inoculation (dpi). The supernatants from Marc-145 cells showing suspected CPE were collected and tested by real-time RT-PCR. Cultures demonstrating either visible CPE or positive RT-PCR results were subjected to second and third passages in fresh Marc-145 cells. When CPE was observed after the third passage, the cells were harvested, aliquoted, confirmed as PRRSV-positive by real-time RT-PCR, and stored at −80°C.

Virus titration was performed in Marc-145 cells according to the TCVN 8400-21:2014/WOAH [17] guidelines. Marc-145 cells were seeded in 96-well plates, and the positive samples from the third passage were serially diluted tenfold and inoculated in triplicate. Viral growth was assessed based on the development of CPE over 3–4 days.

The 50% tissue culture infective dose ($TCID_{50}$) was calculated using the Reed and Muench method [19]. Growth kinetics were evaluated descriptively. Virus titers are presented as the mean ± standard deviation of three independent technical replicates, and no formal statistical comparisons among isolates were performed.

RESULTS

Clinical sample detection

Between 2023 and 2024, a total of 130 clinical samples from pigs suspected of PRRSV infection were collected from farms in six provinces of North Central Vietnam, including Thanh Hoa, Nghe An, Ha Tinh, Quang Binh, Quang Nam, and Hue, as summarized in Table 1. Thirty samples (23.07%) tested positive for PRRSV by real-time RT-PCR. Of these, 28 samples were successfully amplified and sequenced for both the ORF5 and NSP2 genes. Two PRRSV-positive samples could not be sequenced because RT-PCR amplification of the target genes was unsuccessful, likely due to low viral RNA concentration and/or RNA degradation. The 28 sequenced strains included 13 JXA1-like, 8 SX2009-like, 4 US-like (VR-2332-like), and 3 NADC30-like PRRSV strains, which were subsequently subjected to phylogenetic analysis.

Sequencing alignment and phylogenetic analysis of ORF5 protein sequences

Phylogenetic analysis based on the ORF5 gene demonstrated that the 28 PRRSV strains obtained from six provinces in North Central Vietnam during 2023–2024 clustered into four distinct sub-lineages (Fig. 1). Among these, 21 strains belonged to sub-lineage 8.7, representing highly pathogenic PRRSV (HP-PRRSV) strains such as SX2009-like and JXA1-like; four strains belonged to sub-lineage 5.1, representing the classical PRRSV lineage (VR-2332-like); and three strains were grouped into sub-lineage 1.8, representing NADC30-like PRRSV (Fig. 1). Our findings indicate that the NADC30-like PRRSV strains—NCR23-26, NCR24-17, and NCR24-35-were reported for the first time in Vietnam.

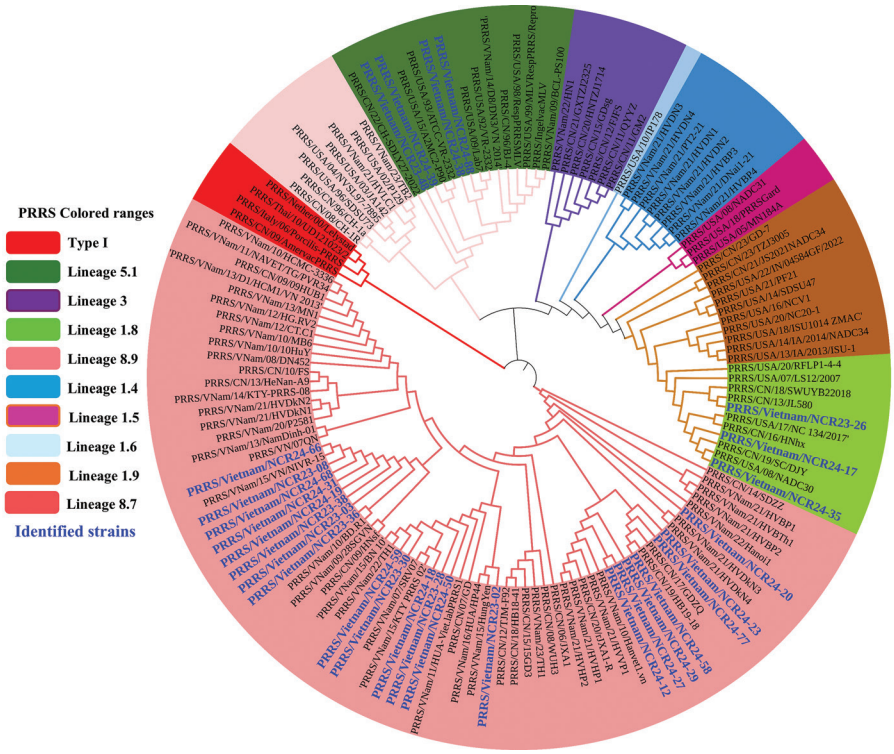

Figure 1. Phylogenetic analysis based on the ORF5 protein sequences. PRRSV strains identified in this study are shown in blue bold, and reference PRRSV strains are shown in black. The phylogenetic tree was constructed using the neighbor-joining method in MEGA software with 1,000 bootstrap replicates.

The amino acid identity and similarity of the ORF5 gene among the type 2 PRRSV strains isolated in this study are summarized in Table 2. The amino acid identity between PRRSV strains from North Central Vietnam and the representative reference strains JXA1-like, SX2009-like, VR-2332, QYYZ, NADC34-like, and NADC30-like ranged from 83.5–100%, 84.0–100%, 83.5–100%, 80.5–89.4%, 80.3–99%, and 83.5–100%, respectively. The corresponding amino acid similarities ranged from 88.5–100%, 89–100%, 87.5–100%, 83.5–100%, 85.3–95%, and 88–100%, respectively. Furthermore, the amino acid identity between the newly identified NADC30-like strains and the JXA1-like, SX2009-like, and VR-2332-like strains ranged from 83.5–99.5%, 85.5–95.5%, and 83–95.5%, respectively, while the corresponding similarities ranged from 88.5–99.5%, 90.5–96%, and 87–96%, respectively (Table 2). The amino acid identity and similarity between the Vietnamese NADC30-like isolates and the reference NADC30-like strains were 84–100% and 88–100%, respectively (Table 2). All three NADC30-like strains identified during 2023–2024 in North Central Vietnam were isolated from dead pigs on local farms.

Table 2. Nucleotide and deduced amino acid identity analysis based on ORF5 and Nsp2 HVR

Strains		Identified strains				Reference strains					
		ORF5 protein									
		JXA1-Like	SX2009 -Like	NADC30 -Like	VR-2332	JXA1 -Like	SX2009 -Like	VR-2332	QYYZ	NADC34 -Like	NADC30 -Like
JXA1-Like	Identified	84.5-100%				85-99.5%	84.5-100%	83.5-89.5%	81-85.4%	82.32-91.5%	84-94%
	Similary	89.5-100%				90-99.5%	89.5-100%	88-92%	85.5-88.5%	87.87-92.5%	88-95.5%
SX2009-Like	Identified	85.5-98.0%	98.5-100%			96-97.5%	96.5-98%	87.5-90%	82-86.4%	83.83-90%	85.5-87.5%
	Similary	90.5-98.5%	98.5-100%			96-97.5%	96.5-98%	90-92.5%	86-89.4%	89.89-94%	90-92%
NADC30-Like	Identified	83.5-99.5%	85.5-95.5%	82.5-100%		83.5-98.5%	84-97%	83.5-87%	82-85.5%	82.82-95%	84-100%
	Similary	88.5-99.5%	90.5-96%	88-100%		88.5-98.5%	89-97%	87.5-89.5%	85-89%	87.87-95%	88-100%
VR-2332	Identified	83-98.0%	88-100%	83-95.5%	88-100%	87.5-97.5%	87-98%	87.5-100%	80.5-84.9%	80.3-88.5%	83-86.5%
	Similary	87.5-98%	90.5-100%	87-96%	90.5-100%	90.5-97.5%	90-98%	90-100%	83.5-87.9%	85.35-93%	88-91%
		NSP2 HVR protein									
JXA1-Like	Identified	96.13-100%				96.13-100%	94.84-99.1%	69.09-71.2%	38.63-77.3%	47.23-53.4%	38.63-40.9%
	Similary	96.13-100%				96.13-100%	94.84-99.1%	73.39-75.5%	44.69-80.3%	55.21-60.1%	44.69-47.7%
SX2009-Like	Identified	96.13-98.7%	96.56-100%			96.13-98.7%	94.84-97.9%	69.09-72.1%	38.63-77.7%	47.85-52.8%	38.63-40.9%
	Similary	96.13-98.7%	96.56-100%			96.13-98.7%	94.84-97.9%	73.39-76.4%	44.69-80.7%	55.82-59.5%	44.69-47.7%
NADC30-Like	Identified	38.63-41.7%	38.63-42.4%	71.96-100%		38.63-41.7%	38.63-41.7%	47.72-50.8%	43.93-72%	44.69-46.2%	71.96-100%
	Similary	44.69-47%	44.69-47.7%	73.48-100%		45.45-47%	44.69-46.2%	53.03-56.8%	50.75-73.5%	51.51-53.8%	73.48-100%
VR-2332	Identified	68.66-70.4%	68.66-71.2%	47.72-49.2%	99.5-100%	69.52-70.4%	68.6-69.6%	98.85-99.2%	47.72-64.3%	46.62-49.1%	46.21-49.2%
	Similary	72.96-74.7%	72.96-75.5%	53.0-55.3%	99.5-100%	73.81-74.7%	72.96-73.8%	98.85-99.2%	53.03-67.7%	55.21-55.8%	53.78-55.3%

Sequence alignment and phylogenetic analysis using Nsp2 protein sequences

Phylogenetic analysis based on the Nsp2 hypervariable region (HVR) demonstrated that the 28 PRRSV strains obtained from six provinces in North Central Vietnam during 2023–2024 were grouped into three distinct sub-lineages (Fig. 2).

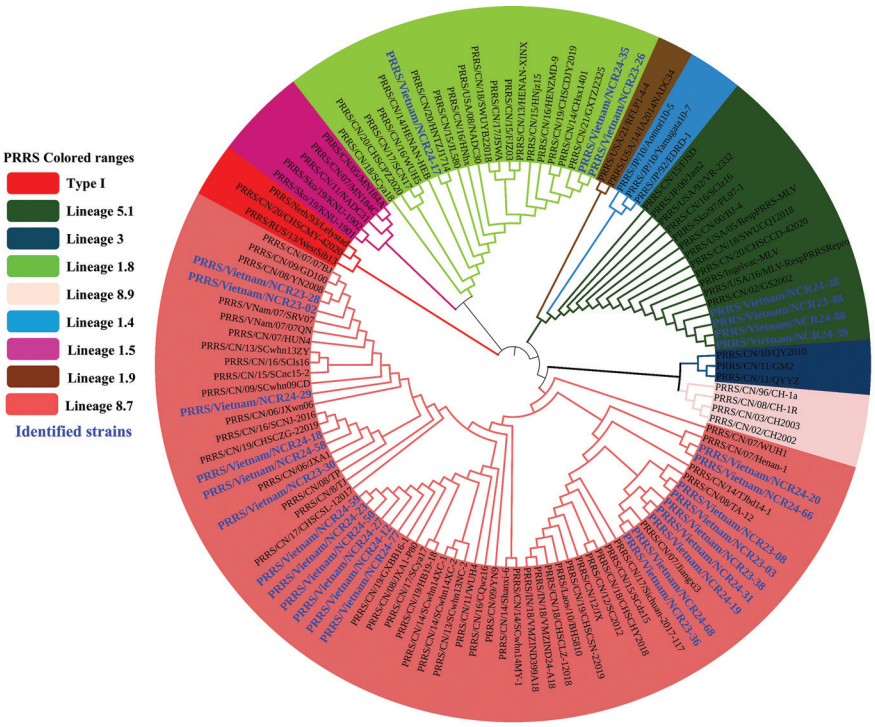

Figure 2. Phylogenetic analysis based on the NSP2 protein sequences. PRRSV strains identified in this study are shown in blue bold, and reference PRRSV strains are shown in black. The phylogenetic tree was constructed using the neighbor-joining method in MEGA software with 1,000 bootstrap replicates.

Among these, 21 strains belonged to sub-lineage 8.7, representing highly pathogenic PRRSV (HP-PRRSV) strains such as SX2009-like and JXA1-like; four strains belonged to sub-lineage 5.1, representing classical PRRSV (VR-2332-like); and three strains belonged to sub-lineage 1.8, representing NADC30-like PRRSV (Fig. 2). No type 1 PRRSV, QYYZ-like, or NADC34-like strains were detected in the current samples. The results of deduced amino acid identity and similarity analyses of the Nsp2 HVR of type 2 PRRSV strains are summarized in Table 2. The amino acid identity of the 21 sub-lineage 8.7 PRRSV strains from North Central Vietnam, compared with the reference strains JXA1-like, SX2009-like, VR-2332-like, QYYZ-like, NADC34-like, and NADC30-like, ranged from 96.13–100%, 94.84–99.1%, 69.09–71.2%, 38.63–77.3%, 47.23–53.4%, and 38.63–40.9%, respectively. The corresponding amino acid

similarity values ranged from 96.13–100%, 94.84–99.1%, 73.39–75.5%, 44.69–80.3%, 55.21–60.1%, and 44.69–47.7%, respectively.

For the four sub-lineage 5.1 PRRSV strains, amino acid identity with the same reference strains ranged from 69.52–70.4%, 68.6–69.6%, 98.85–99.2%, 47.72–64.3%, 46.62–49.1%, and 46.21–49.2%, respectively, while amino acid similarity ranged from 73.81–74.7%, 72.96–73.8%, 98.85–99.2%, 53.03–67.7%, 55.21–55.8%, and 53.78–55.3%, respectively (Table 2). The three sub-lineage 1.8 (NADC30-like) PRRSV strains showed amino acid identities of 38.63–41.7%, 38.63–41.7%, 47.72–50.8%, 43.93–72%, 44.69–46.2%, and 71.96–100% with JXA1-like, SX2009-like, VR-2332-like, QYYZ-like, NADC34-like, and NADC30-like reference strains, respectively. Corresponding amino acid similarity values ranged from 45.45–47%, 44.69–46.2%, 53.03–56.8%, 50.75–73.5%, 51.51–53.8%, and 73.48–100%, respectively (Table 2). Three NADC30-like PRRSV strains (NCR23-26, NCR24-27, and NCR24-35) were identified, showing amino acid identities of 38.63–41.7%, 38.63–42.4%, and 47.72–49.2%, and similarities of 44.69–47%, 44.69–47.7%, and 53.0–55.3% with the JXA1-like, SX2009-like, and VR-2332-like strains circulating in North Central Vietnam during 2023–2024 (Table 2).

Overall, these results indicate substantial variability in the Nsp2 amino acid sequences among different PRRSV strains circulating in North Central Vietnam during 2023–2024, suggesting ongoing viral evolution and genetic diversification in the region.

Amino acid mutation analysis of GP5 and Nsp2 HVR

There is one signal peptide, two hypervariable regions (HVR1 and HVR2), three transmembrane regions (TM1, TM2, and TM3), and five epitopes in the type 2 PRRSV GP5 protein (Fig. 3). The five epitopes include a decoy epitope, a neutralizing epitope (PNE), and three T-cell epitopes, as well as two virulence-related sites located at amino acid positions 13 and 151, respectively [18,20,21]. Representative strains of different lineages were selected to analyze amino acid variations encoded by ORF5 in comparison with PRRSV strains isolated from six provinces in North Central Vietnam during 2023–2024. Thirteen isolates belonging to the JXA1-like group showed highly conserved ORF5 sequences compared with the JXA1 and rJXA1-R reference strains, with only minor mutations observed among the isolates from the six provinces. These included substitutions such as S37→P37 in the HVR1 region, Q68→R/K68 in the HVR2 region, Q/R164→R/Q164 in epitope region 2, and L196→Q196 and L200→L200 in epitope region 3 (Fig. 3). All isolates in the SX2009-like group showed high sequence similarity to the Vietnamese strains SRV07 and 07QN, with only four amino acid mutations identified: N34→S34 in the HVR1 region, Q58→K58 in the HVR2 region, V124→G124 in T-cell epitope 1, and L200→P200 in T-cell epitope 3 (Fig. 3).

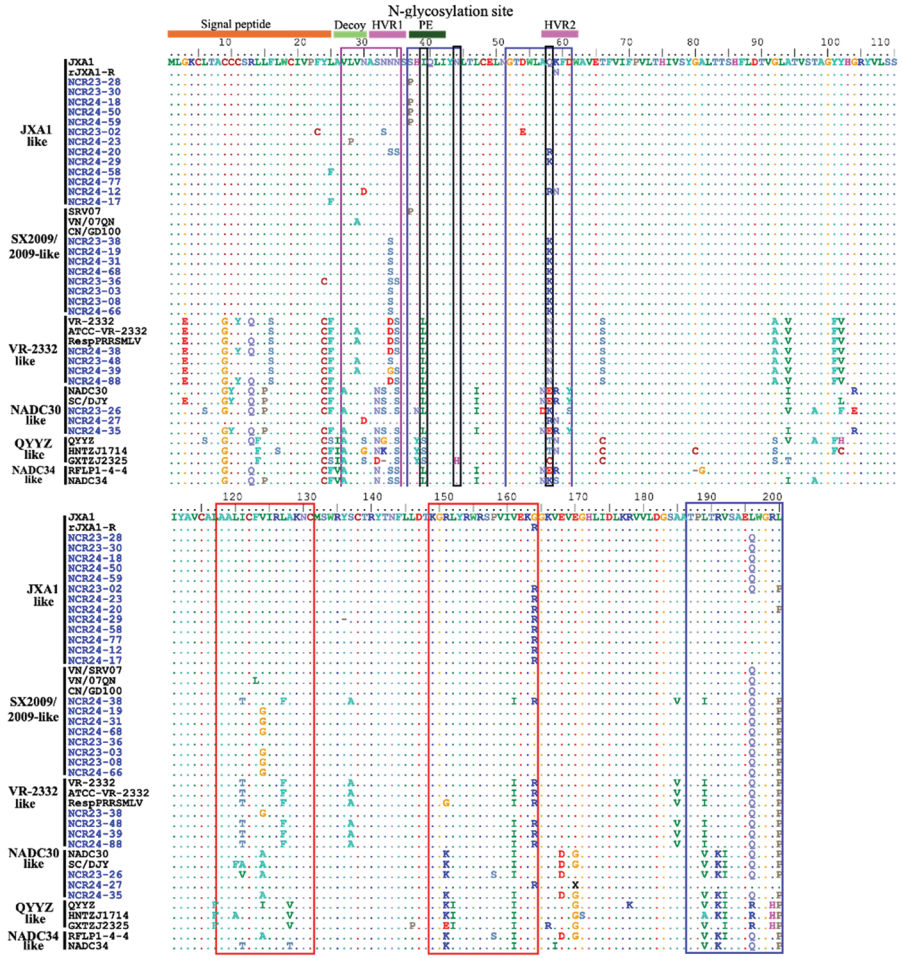

Figure 3. Amino acid sequence alignment of antigenic regions in the GP5 protein of PRRSV. Antigenic regions are framed. Amino acid sequences of GP5 from 28 field isolates (in blue bold) and reference strains are aligned; dots indicate amino acid identity, and letters denote amino acid substitutions. Purple, blue, and red boxes represent non-neutralizing, neutralizing, and T-cell epitopes, respectively. The black box indicates the position of the major linear epitope.

In contrast, the three NADC30-like isolates showed amino acid identities and similarities ranging from 85.5–100% and 87.5–100%, respectively, compared with reference NADC30-like strains. They were highly homologous to the Chinese NADC30 strain, with several amino acid substitutions, including E/R58→K/R/E58 in the HVR2 region, R109→E109 in TM2, A/V122→F122, and V159→ST159 in the T-cell epitope regions. The amino acid identity and similarity between the Vietnamese NADC30-like isolates and the NADC34-like reference strains ranged from 86–90% and 89.5–93.5%, respectively. Additionally, isolates in the VR-2332-like group showed highly conserved sequences compared with the VR-2332, ATCC-VT-2322,

and RespPRRS-MLV vaccine strains, with only one amino acid mutation detected among four isolates (NCR23-38, NCR23-48, NCR24-39, and NCR24-88) from the six provinces (D24→S/G/D24 in the HVR1 region) (Fig. 3).

As shown in Fig. 4, the amino acid sequences deduced from the hypervariable region (HV) of the Nsp2 protein were compared among six reference strains (SX2009, NADC34, NADC30, VR-2332, JXA1, and QYYZ) and 28 representative isolates collected from six provinces in North Central Vietnam during 2023–2024.

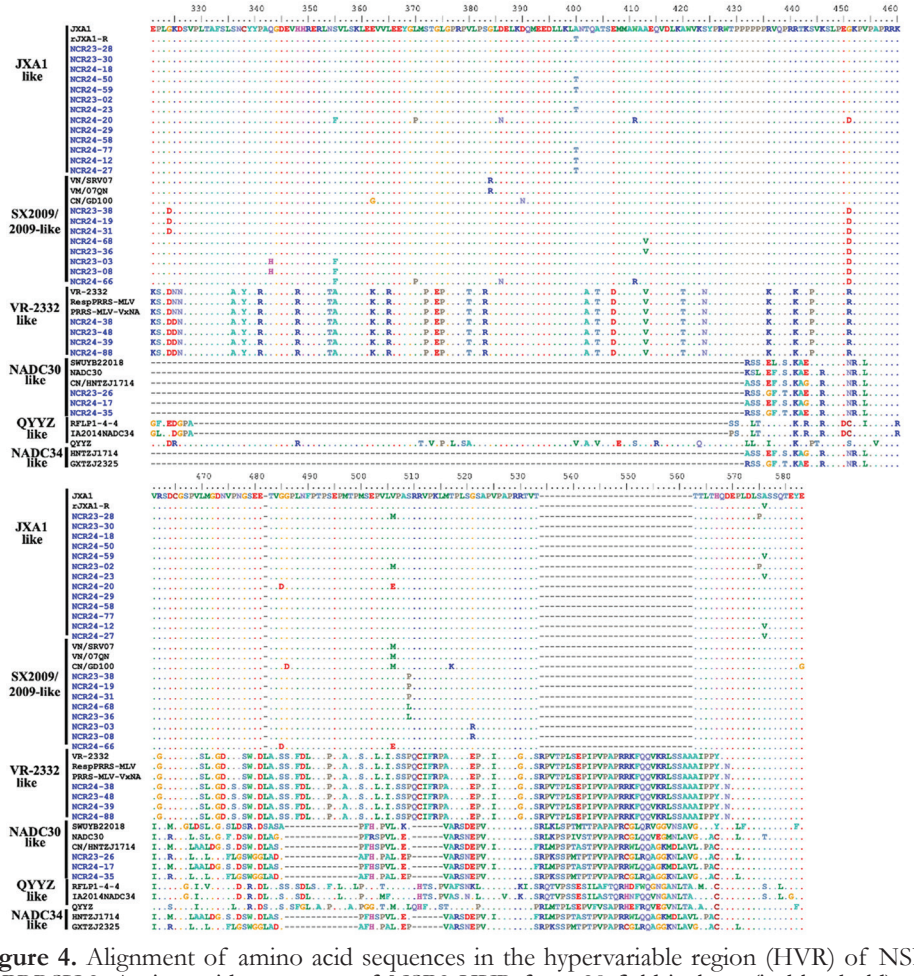

Figure 4. Alignment of amino acid sequences in the hypervariable region (HVR) of NSP2 in PRRSV-2. Amino acid sequences of NSP2 HVR from 28 field isolates (in blue bold) and reference strains are aligned; dots indicate identical amino acids, and letters denote substitutions.

The comparison showed that the three NADC30-like PRRSV strains (NCR23-26, NCR24-17, and NCR24-35) were highly conserved with the NADC30-like group in China and exhibited a characteristic 131-amino-acid deletion in the Nsp2 hypervariable region (positions 320–431, 486–499, and 511–515 aa) and a 30-amino-acid insertion (positions 482 and 534–562 aa) compared with the SX2009-like and JXA1-like strains

(Fig. 4). Comparison between the NADC30-like and VR-2332-like virus strains also revealed a 133-amino-acid deletion in the Nsp2 hypervariable region (positions 320–431, 486–500, and 510–515 aa) (Fig. 4). No amino acid deletions or insertions were observed in the three NADC30-like isolates when compared with the NADC34-like PRRSV strain (Fig. 4).

Our results indicate that the newly identified NADC30-like subtype isolated from North Central Vietnam during 2023–2024 is highly conserved and closely related to the NADC30-like subtype reported in China, represented by the PRRS/CN/20/HNTZJ1714 strain detected in 2020 [22]. These findings suggest that the newly detected NADC30-like strains in North Central Vietnam most likely originated from, or share a close genetic relationship with, the NADC30-like strains circulating in China in 2020.

Virus isolation

To assess the in vitro replication capacity of the PRRSV isolates, the growth kinetics of the viruses were analyzed in MARC-145 cells. We now state that 19 of the 30 PRRSV-positive samples were successfully isolated in MARC-145 cells. For the growth kinetics experiments, eight representative isolates were selected, including one SX2009-like strain (NCR24-19), five JXA1-like strains (NCR24-23, NCR24-18, NCR24-58, NCR24-12, and NCR24-17), one NADC30-like strain (NCR24-27), and one VR-2332-like strain (NCR24-88). These isolates represent all PRRSV-2 lineages identified in this study (sub-lineages 8.7, 5.1, and 1.8). Comparison between the NADC30-like and VR-2332-like virus strains showed that viral replication peaked between 96 – and 120-hours post-inoculation (hpi), with average peak titers ranging from 6.2 to 7.6 $\log_{10}$ $TCID_{50}$/mL (Fig. 5).

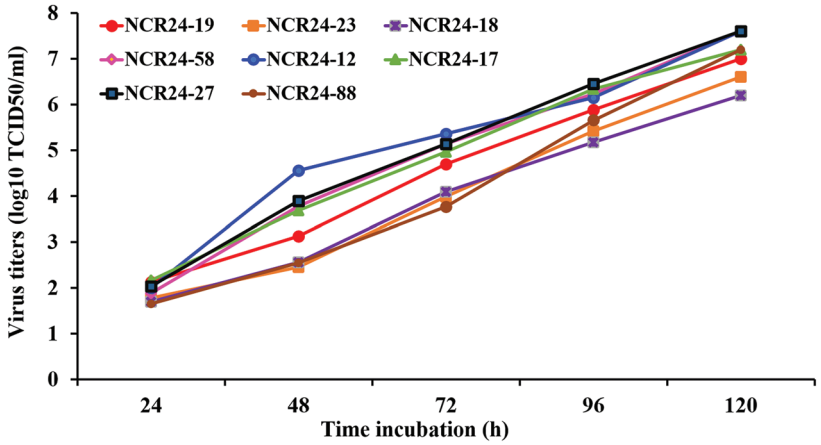

Figure 5. Growth kinetics of PRRSV isolates in mammalian cells. Marc-145 cell monolayers were infected with equal amounts of virus at an MOI of 0.01. Cell supernatants were harvested at 24, 48-, 72-, 96-, and 120-hours post-inoculation (hpi) and titrated in Marc-145 cells. Data are presented as mean virus titers ± SD ($\log_{10}$ $TCID_{50}$/mL).

DISCUSSION

Porcine reproductive and respiratory syndrome virus (PRRSV) were first reported in Vietnam in 1997 and has since continued to affect the country's pig industry [3]. Following the initial outbreak, vaccination programs were implemented as part of government efforts to prevent and control PRRSV infection in the swine population. Although vaccination has proven effective in reducing outbreaks, the uncontrolled use of highly pathogenic live-attenuated vaccines has resulted in the widespread dissemination of vaccine-derived viruses in pig farms. Recently, the emergence of new PRRSV mutants has been reported in Vietnam [16,23]. Phylogenetic analysis of 246 ORF5 sequences of PRRSV type 2 strains collected from Vietnam between 2007 and 2023 revealed that all belonged to the North American genotype, distributed among lineages 1, 5, and 8. Among these, highly pathogenic PRRSV (HP-PRRSV) strains of sub-lineage 8.7, represented by the SX2009-like and JXA1-like groups, were the predominant epidemic strains, while sub-lineage 5.1 (VR-2332-like or classical PRRSV) was sporadically detected in northern Vietnam [3,14-16,23]. In contrast, sub-lineage 1.4 (NADC-like) was first identified in 2021 [16]. The increasing prevalence of both HP-PRRSV and NADC-like strains likely reduces vaccine efficacy, posing a major challenge to disease management. Furthermore, sub-lineage 1.8 (NADC30-like), first reported in China in 2013, has since become dominant in several regions of that country [24]. In some areas, NADC30-like strains have even replaced HP-PRRSV as the predominant lineage [22,24].

In this study, we characterized PRRSV genotypes circulating in North Central Vietnam during 2023–2024. Of 30 PRRSV-positive samples and 28/30 positive samples were successfully amplified and sequenced for subsequent phylogenetic analysis, 21 belonged to HP-PRRSV sub-lineage 8.7, four to classical PRRSV sub-lineage 5.1, and three to NADC30-like PRRSV sub-lineage 1.8. The highest detection frequency was observed for HP-PRRSV sub-lineage 8.7. Both sub-lineages 5.1 and 8.7 were predominant during this period, while NADC30-like sub-lineage 1.8 strains were identified for the first time in this region. These findings suggest that multiple PRRSV sub-lineages are co-circulating among pigs in North Central Vietnam.

In PRRSV genome research, the *NSP2* and *ORF5* genes are known to exhibit the greatest genetic variability and are commonly used as key targets for molecular epidemiology and phylogenetic studies [20,25]. Previous research indicated that amino acids at positions R13 and R151 in the GP5 protein are associated with high virulence, whereas amino acid 137 differentiates attenuated vaccine strains (A137) from wild-type strains (S137) [18,20,21]. In our study, amino acids at positions 13 and 151 in the GP5 of JXA1 – and SX2009-like HP-PRRSV strains were arginine (R), consistent with reference strains. In contrast, PRRSV strains belonging to the VR-2332-like and NADC30-like lineages carried glutamine (Q) or lysine (K) at these positions, similar to their corresponding reference strains. Moreover, one SX2009-like HP-PRRSV strain (NCR24-38) and three VR-2332-like PRRSV strains (NCR23-48, NCR24-39, NCR24-

88) from piglets showing clinical symptoms possessed alanine (A) at position 137, characteristic of attenuated vaccine strains. These results suggest that most circulating PRRSV isolates are wild-type and potentially highly virulent in pigs from the six North Central provinces.

Previous studies have demonstrated that several critical antigenic regions of GP5 play essential roles in immune responses. Epitope A (amino acids 27–35) regulates early antibody production; epitopes B (37–45) and C (52–61) are associated with neutralizing antibody responses; and the region spanning amino acids 187–200 is linked to cross-neutralization between genotypes 1 and 2. Additionally, two T-cell epitopes, located at positions 117–131 and 149–163, contribute to cellular immune responses [26-28]. In this study, amino acid substitutions were detected within these epitopes. For example, changes such as N34→S34 in JXA1-like or SX2009-like HP-PRRSV and D34→S/G34 in VR-2332-like strains may promote rapid non-neutralizing antibody induction. The substitution I39→L39 observed in VR-2332 – and NADC30-like strains may influence vaccine-induced neutralizing immunity [29,30]. Similarly, mutations at positions 57–59 (C58→K/R/E58) within epitope C could contribute to antibody escape [27]. Furthermore, amino acid changes within the two T-cell epitopes (117–131 and 149–163) and at positions 187–200 (e.g., L189→I/V189, L191→Q191, L200→P200) were also observed, potentially affecting cross-neutralizing antibody responses [26,31]. Changes at these epitope sites may reduce the protective effectiveness of commercial vaccines against field NADC30-like strains [27,32].

NADC30-like PRRSV typically exhibits three discontinuous deletions (111, 14, and 6 amino acids) in *NSP2* compared to VR-2332 [22,24]. Sequence alignment revealed that the three NADC30-like PRRSV strains detected in North Central Vietnam during 2023–2024 possessed identical *NSP2* deletions to those of Chinese NADC30-like strains isolated between 2018 and 2020. This suggests that the newly identified NADC30-like strains in North Central Vietnam are closely related to the Chinese NADC30-like strains circulating during that period.

Limitations: This study has several limitations. First, the molecular characterization was based on sequencing of the ORF5 gene and the NSP2 hypervariable region (HVR), which are widely used for PRRSV genotyping, but do not capture the complete genomic diversity of the virus. Consequently, whole-genome phylogenetic analyses and recombination analyses were not performed, and potential recombination events could not be evaluated. Second, although samples were collected from six provinces in North Central Vietnam, the sample size was relatively limited and may not fully represent the genetic diversity of PRRSV circulating throughout Vietnam. Future studies incorporating larger sample sizes, broader geographic coverage, and whole-genome sequencing will provide a more comprehensive understanding of the molecular evolution, recombination patterns, and epidemiology of PRRSV in Vietnam.

CONCLUSION

In summary, this study provides a comprehensive molecular characterization of PRRSV epidemics and genetic evolution in North Central Vietnam during 2023–2024. Our findings offer valuable insights into the genetic diversity and epidemiological dynamics of PRRSV in this region, which will aid in developing more effective prevention and control strategies for the swine industry.

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Author Contributions

TAD, TTTH, and DVH contributed to conceptualization and methodology. TAD also contributed to formal analysis, data curation, writing – original draft preparation, and writing – review and editing. TTTH and DVH contributed to supervision and writing – review and editing. DVH, VTHL, and NDT contributed to conceptualization, methodology, and formal analysis. CTN, NPL, DQK, LVK contributed to methodology and data curation. All authors have read and agreed to the published version of the manuscriptAll authors read and approved the final manuscript.

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Declaration of conflicting interests

No potential conflict of interest relevant to this article was reported.

Ethics approval and consent to participate:

The study complied with the institutional rules for the care and use of laboratory animals and a protocol approved by the Ministry of Agriculture and Rural Development Vietnam (TCVN 8402:2010).

Data availability

All sequences generated in this study were submitted to GenBank under accession nos. PZ122696-PZ122723 for ORF5 genes and PZ126239-PZ126266 for NSP2 genes.

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MOLEKULARNA KARAKTERIZACIJA PRRSV SOJEVA KOJI CIRKULIŠU U SEVERNOM CENTRALNOM REGIONU VIJETNAMA U PERIODU OD 2023-2024

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Virus reproduktivnog i respiratornog sindroma svinja (PRRSV) je glavni patogen koji predstavlja ozbiljnu pretnju globalnoj industriji svinjogojstva. Severni centralni Vijetnam, koji obuhvata šest provincija, jedan je od najvećih regiona za uzgoj svinja u zemlji; međutim, informacije o molekularnoj karakterizaciji ovog virusa koji cirkuliše u ovom području su ograničene. Da bi se popunio ovaj jaz, 130 uzoraka je prikupljeno iz ovih provincija između 2023. i 2024. godine. Rezultati su pokazali stopu pozitivne PRRSV infekcije od 23,07% (30/130). Molekularne karakterizacije PRRSV virusa su dalje analizirane na osnovu sekvenci hipervarijabilnih regiona (HVR) ORF5 i NSP2, u poređenju sa referentnim sojevima iz Vijetnama i drugih zemalja preuzetim sa GenBank (NCBI). Filogenetska analiza je pokazala da sekvencionirani sojevi pripadaju PRRSV-2 (linije 1, 5 i 8). Konkretno, 28 PRRSV-2 sojeva je odabrano za ORF5 analizu i klasifikovano u podliniju 8.7 (HP-PRRSV), podliniju 5.1 (klasični PRRSV) i novoidentifikovanu podliniju 1.8 (NADC30-sličan). NSP2 HVR-ovi identifikovanih sojeva pokazali su identitet aminokiselina u rasponu od 38,63% do 100%, dok su se identiteti ORF5 kretali od 80,3% do 100%. Analiza sekvence ORF5 pokazala je da su mutacije GP5 uglavnom koncentrisane u HVR1, HVR2 i mestima povezanim sa virulencijom. Ukratko, detekcija PRRSV-a sličnog NADC30 po prvi put u Vijetnamu je važna, što ukazuje da ovaj HP-PRRSV i klasični PRRSV sojevi, o kojima je ranije bilo reči, tre-

nutno kocirkulišu među populacijom svinja u severno-centralnom Vijetnamu. Pored toga, ovi nalazi ukazuju na to da sojevi PRRSV u Vijetnamu prolaze kroz značajnu genomsku divergenciju.