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Research progress on the N protein of porcine reproductive and respiratory syndrome virus

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Porcine reproductive and respiratory syndrome (PRRS) is a highly contagious disease caused by the porcine reproductive and respiratory syndrome virus (PRRSV). PRRSV exhibits genetic diversity and complexity in terms of immune responses, posing challenges for eradication. The nucleocapsid (N) protein of PRRSV, an alkaline phosphoprotein, is important for various biological functions. This review summarizes the structural characteristics, genetic evolution, impact on PRRSV replication and virulence, interactions between viral and host proteins, modulation of host immunity, detection techniques targeting the N protein, and progress in vaccine development. The discussion provides a theoretical foundation for understanding the pathogenic mechanisms underlying PRRSV virulence, developing diagnostic techniques, and designing effective vaccines.

KEYWORDS

porcine reproductive and respiratory syndrome virus N protein, genetic evolution, protein interactions, detection techniques, vaccine development

1 Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a viral infectious disease adversely affecting the global pig farming industry with significant economic harm (Cao et al., 2014; Lunney et al., 2016). This disease causes severe damage in pigs at various stages of growth, with clinical symptoms primarily manifesting as respiratory distress in piglets, reproductive failure, and congenital infections in pregnant animals (Chand et al., 2012). Financial losses in the United States caused by porcine reproductive and respiratory syndrome virus (PRRSV) amounted to $664 million in 2013 alone (Holtkamp et al., 2005; Lalonde et al., 2020). According to economic estimates in Germany, the average farm profit loss due to PRRSV was −19.1%, reaching up to −41% in the worst-case scenario in 2021 (Renken et al., 2021). As a global swine pathogen, PRRSV causes significant economic losses and merits sustained and widespread attention (Du et al., 2017).

PRRSV was first identified in the Netherlands in 1991 (Wensvoort et al., 1991). The virus was subsequently discovered in the United States in 1992 (Benfield et al., 1992; Collins et al., 1992). China documented an inaugural instance of PRRS in 1995, and an outbreak of highly pathogenic PRRS (HP-PRRS) occurred in 2006. The NADC30 and NADC34 variants have become the predominant circulating strains of PRRSV in China in recent years (Li et al., 2016; Guo et al., 2018; Bao and Li, 2021). PRRSV strains exhibit remarkable genetic and antigenic heterogeneity and frequently undergo recombination events, resulting in the emergence of a plethora of novel strains (Wang L. et al., 2017; Yu et al., 2020, 2022; Sun et al., 2022). The complexity of PRRSV genetic variability and recombination increases challenges to epidemiology, prevention, and control of PRRSV (Zhao et al., 2015; Zhou et al., 2015; Wang et al., 2018).

PRRSV, a positive-sense RNA virus belonging to the family *Arteriviridae* within the order *Nidovirales*, has a genome length of approximately 15 kb with a 5′ cap and a 3′ poly-A tail (Balka et al., 2015; Lalonde et al., 2020). Over the years, increasing genetic differences have led to the classification of the virus into two distinct species: *Betaarterivirus suid-1* (PRRSV-1) and *Betaarterivirus suid-2* (PRRSV-2) (Brinton et al., 2018). PRRSV contains at least 11 open reading frames (ORFs), including ORF1a, ORF1b, ORF2a, ORF2b, ORFs3-7, ORF5a, and ORF2TF (Fang et al., 2012). ORF1a and ORF1b occupy approximately 75% of the viral genome at the 5′ end and encode two large polyprotein precursors, pp1a and pp1ab, processed into at least 16 non-structural proteins (Nsps), including Nsp1α, Nsp1β, Nsp2-6, Nsp2TF, Nsp2N, Nsp7α, Nsp7β, and Nsp8-12 (Fang et al., 2012; Li et al., 2019). ORF2a, ORF2b, ORFs3-7, and ORF5a are located at the 3′ end of the genome and encode glycoprotein (GP)2, envelope (E), GP3, GP4, GP5, ORF5a, membrane (M), and nucleocapsid (N) proteins (Fang and Snijder, 2010) (Figure 1). The largest difference in the sequence between PRRSV and other *Arteriviruses* lies in the N-protein domain (Meng, 2000).

The N protein is an immunogenic structural protein implicated in immune evasion (Music and Gagnon, 2010). This comprehensive review provides insights into the structural characteristics of the N protein, its genetic evolution, effects on PRRSV replication and virulence, interactions with other PRRSV and host proteins, modulation of host immunity, clinical detection and diagnosis, and applications of these findings in vaccine development. The information presented in this review lays the groundwork for enhancing our understanding of the pathogenic mechanisms associated with PRRSV and facilitating the development of commercially viable vaccines and diagnostic tools that specifically target the N protein of PRRSV.

2 Characteristics of the N protein

The alkaline phosphatase protein, known as the N protein, is versatile in various virus replication and pathogenesis mechanisms. This protein is encoded by the *ORF7* gene and has a molecular weight of approximately 15 kDa (Wootton et al., 2002). The N protein is a conserved peptide in PRRSV and is abundantly expressed owing to its discontinuous transcription mechanism, accounting for 40% of the total viral protein (Mardassi et al., 1994; Ke and Yoo, 2017). The amino acid residues at positions 105 (Y^{105}) and 120 (I^{120}) of the N protein are phosphorylation sites crucial for its functionality and involvement in viral replication (Chen Y. et al., 2018). The N protein has two domains: an RNA-binding domain at the N-terminus and a dimerization domain at the C-terminus. The N-terminal region, consisting of residues 1–57, is mainly disordered and contains several positively charged residues (Yoo et al., 2003). This region, rich in basic amino acid residues, promotes interactions between the N protein and RNA genome. The C-terminal region (residues 58–123) adopts a crystal structure comprising four antiparallel β-strands arranged in a dimeric form, surrounded by α-helices on its top and sides (Dokland et al., 2004; Dokland, 2010). This structure is crucial for maintaining the antigenicity of the N protein, the primary antigenic protein in PRRSV (Rowland and Yoo, 2003; Forsberg, 2005). The last 11 residues at the C-terminus of the N protein are essential for maintaining the structural integrity of the tertiary conformation (Wootton et al., 2001; Yoo and Wootton, 2001; Lee et al., 2005).

The N protein is distributed in both the cytoplasm and nucleus and regulates host cell processes within the nucleus (Rowland et al., 1999). Rowland et al. (2003) identified two potential nuclear localization signals (NLS) in the N protein of PRRSV-2 isolates. NLS-1 and NLS-2 are at amino acid positions 10–13 and 41–42, respectively. The transport of the N protein involves the participation of a single NLS, enabling its translocation from the cytoplasm to the nucleus. NLS-2 targets the N protein to the nucleolus. There is an NLS between 41–47 aa of the N protein, and these residues serve as highly conserved determinants (Wootton et al., 1998). You et al. (2008) found that the N protein exhibited a relatively higher distribution within the nucleus than in the cytoplasm, with a faster nuclear import rate than export.

The PRRSV N protein is involved in viral nucleocapsid formation, encapsulating the viral genome and enabling virus assembly. The N protein can form homodimers, indicating that it can interact with other N protein molecules to form a dimeric structure (Jourdan et al., 2011; Snijder et al., 2013). The N protein assembles into a spherical structure with a diameter of 20–30 nm as a dimer. Three conserved cysteine residues within the protein form disulfide bonds that stabilize the spherical structure (Doan and Dokland, 2003; Wootton and Yoo, 2003; Lee et al., 2004). This homodimeric structure is essential for binding to the viral genome and facilitating viral particle assembly.

The RNA-binding domain located at the N-terminus and the dimerization domain at the C-terminus of the N protein are vital for viral replication, maintaining N protein antigenicity, and maintaining protein tertiary conformation. Additionally, two potential NLS of the N protein facilitate its transport from the cytoplasm to the nucleus and then to the nucleolus. The N protein shows a dynamic distribution between the cytoplasm and the nucleolus, with quicker nuclear entry compared to nuclear export. Moreover, the N protein is crucial for the formation of viral nucleocapsids, and its homodimeric structure is necessary for binding to RNA genomes and assembling viral particles.

3 Genetic evolution of the N protein

Thirty-six PRRSV N sequences were selected from the NCBI nucleotide database to analyze the genetic evolution characteristics of the N protein (Table 1). The selected strains included isolates from different years, ranging from 1991 to 2023, vaccine strains, and widely referenced representative strains. Phylogenetic analysis was conducted on the selected 36 PRRSV N protein sequences (Figure 2). Among the PRRSV-1 strains, WestSib13-Russia-2013 and PRRSV-1-181187-2-2023 exhibited a greater genetic distance, while MLV-DV-Netherlands-1999 and Lelystad virus-Netherlands-1991 showed a closer genetic relationship. Among the PRRSV-2 strains, JS2021NADC34-China-2021 and wK730-China-2023 showed greater genetic distances, whereas NADC30-USA-2008 and TZJ3005-China-2023 exhibited a closer genetic relationship. In 1995, Meng et al. (1995) conducted an amino acid conservation study on the M and N genes of PRRSV isolates from the United States and Canada, leading to the classification of PRRSV

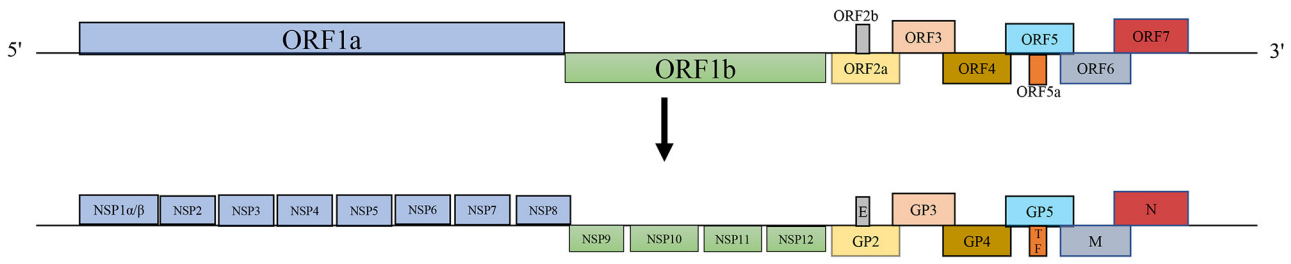

FIGURE 1
PRRSV genome structures.

isolates into two groups. Based on their evolutionary relationships, Figure 2 categorizes the PRRSV strains into two major branches, designated type 1 and type 2.

Nucleotide homology between PRRSV-1 N and PRRSV-2 N was 61.7%—100% (Figure 3). The strains with 61.7% nucleotide sequence homology were WK730-China-2023 and PRRSV-1-181187-2-2023. Strains with 100% nucleotide homology were Lelystad virus Netherlands 1991, MLV-DV Netherlands 1999, JXA1 China 2006, and HUN4 China 2007. Nucleotide homology within the PRRSV-2 strain was 61.7%—100%. The same applied to strains with a nucleotide homology of 61.7%. HUN4 China-2007 and GD China-2007 showed 100% homology. Nucleotide homology between the PRRSV-1 strains ranged from 83.7% to 100%. The strains with 83.7% nucleotide homology were WestSib13-Russia-2013 and PRRSV-1-181187-2-2023. Lelystad virus Netherlands 1991 and MLV-DV Netherlands 1999 strains showed 100% homology. Therefore, the N sequence has high nucleotide homology and conservation.

Amino acid homology analysis was conducted on the N sequences of the 36 strains of PRRSV (Figure 4). The amino acid homology between PRRSV-1 and PRRSV-2 was 55.4%—100%. JS2021NADC34-China-2021 and HLJB1-China-2014 strains showed 55.4% amino acid homology. SP-Singapore-1999 and BJ-4-China-2000, among others, showed 100% amino acid homology. The amino acid homology between the PRRSV-2 strains ranged from 55.4% to 100%, consistent with the amino acid homology between PRRSV-1 and PRRSV-2. Among the PRRSV-1 strains, amino acid homology ranged from 80.8% to 100%. Strains with 80.8% amino acid homology included WestSib13-Russia-2013 and TZJ226-China-2020. Strains with 100% amino acid homology included Lelystad virus-Netherlands-1991 and MLV-DV-Netherlands-1999. A relatively high conservation of N sequences at the amino acid level was inferred.

Amino acid mutation analysis was performed on the N sequences of the 36 selected PRRSV strains (Figure 5). The results revealed amino acid deletions in PRRSV. In PRRSV-1, deletions were at positions 14–17 aa and 35, whereas in PRRSV-2, deletions were at positions 44–46 aa and 50. Amino acid residues at positions 105 and 120 of the N protein are critical phosphorylation sites for the functionality and viral replication capacity (Chen Y. et al., 2018). The PRRSV-2 N protein is highly conserved at positions 90, 75, and 23 aa, which are responsible for crucial functions such as N-N interactions and the formation of disulfide bonds (Lee and Yoo, 2005). The mutation analysis validated the above conclusions, as indicated by the red markings in Figure 5, showing conserved amino acid positions within PRRSV-1 and PRRSV-2. The N proteins of PRRSV-1 and PRRSV-2 comprise 123 and 128 amino acids, respectively (Mardassi et al., 1995; Meulenberg et al., 1995). The yellow portion in Figure 5 indicates amino acid deletions in PRRSV-2 compared to those in PRRSV-1. Although multiple amino acid mutations exist in PRRSV, significant differences in the amino acid profiles of the two genotypes exist. Overall, there are relatively few amino acid mutations and a high level of conservation within the internal regions of both PRRSV-1 and PRRSV-2. Gall et al. (1998) demonstrated the minimal effect of the *in vivo* passage of PRRSV-1 on the ORF7 sequence. Only a few detectable amino acid substitutions were observed in ORF7, confirming its low variability. The sequence alignment results obtained by Zhou et al. (2006) revealed the high conservation of antigenic epitopes in the N protein among PRRSV strains.

Analysis of N sequences from 36 PRRSV strains identified two major branches, PRRSV-1 and PRRSV-2, through phylogenetic tree analysis. Homology analysis showed nucleotide homology ranging from 61.7%—100% and amino acid homology ranging from 55.4%—100% between PRRSV-1 and PRRSV-2. Amino acid mutation analysis of the N sequence indicated a low occurrence of mutations within PRRSV-1 and PRRSV-2, emphasizing their internal conservation. These results underscore the high conservation level in the N sequence, emphasizing the importance of timely monitoring genetic evolution in the N protein for effective PRRS prevention and control.

4 Function of the N protein in viral replication

The N protein serves as a phosphorylated protein. Mutations in the phosphorylation sites may affect the replication capacity of PRRSV within cells (Wootton et al., 2002; Chen L. et al., 2018). The N protein can interact with the 3′ end of the viral genome to regulate the synthesis of viral RNA and affect viral replication (Fahad and Kapil, 2001). Disulfide bond formation and the NLS of the N protein are crucial for viral replication (Wootton and Yoo, 2003; Lee and Yoo, 2005; Lee et al., 2005; Pei et al., 2008).

Poly (ADP-ribose) polymerase-1 (PARP-1) is a cellular factor that adds ADP-ribose moieties to proteins. Liu L. et al. (2014)

10.3389/fmicb.2024.1391697

TABLE 1 Information about the 36 selected PRRSV strains.

Year	Area	Strain	Genbank accession number	Genotype
1991	Netherlands	Lelystad virus	M96262	PRRSV-1
1996	Belgium	96V198	MK876228	PRRSV-1
1999	Netherlands	MLV-DV	KJ127878	PRRSV-1
2001	USA	SD-01-08	DQ489311	PRRSV-1
2003	Belgium	BE_03V140	MW053394	PRRSV-1
2006	China	BJEU06-1	GU047344	PRRSV-1
2008	Belarus	lena	JF802085	PRRSV-1
2009	Spain	Amervac PRRS	GU067771	PRRSV-1
2012	Germany	GER12-720789	OP529852	PRRSV-1
2013	Russia	WestSib13	KX668221	PRRSV-1
2014	China	HLJB1	KT224385	PRRSV-1
2016	South Korea	CBNU0495	MZ287327	PRRSV-1
2017	China	HENZMD-10	KY363382	PRRSV-1
2018	China	KZ2018	MN550991	PRRSV-1
2019	South Korea	JBNU-19-E01	MW847781	PRRSV-1
2020	China	TZJ226	OP566682	PRRSV-1
2022	Austria	AUT22-97	OP627116	PRRSV-1
2023	China	PRRSV-1-181187-2-2023	OQ856755.1	PRRSV-1
1996	China	CH-1a	AY032626	PRRSV-2
1999	USA	MLV RespPRRS-Repro	AF159149	PRRSV-2
1999	Singapore	SP	AF184212.1	PRRSV-2
2000	China	BJ-4	AF331831	PRRSV-2
2006	China	JXA1	EF112445	PRRSV-2
2007	USA	VR2332	EF536003	PRRSV-2
2007	China	HUN4	EF635006	PRRSV-2
2007	China	GD	EU109503	PRRSV-2
2008	USA	NADC30	JN654459	PRRSV-2
2008	China	CH-1R	EU807840.1	PRRSV-2
2010	China	FS	JF796180.1	PRRSV-2
2015	China	FJFS	KP998476	PRRSV-2
2019	China	GD1909	MT165636	PRRSV-2
2020	China	rJXA1-R	MT163314.1	PRRSV-2
2021	China	JS2021NADC34	MZ820388	PRRSV-2
2022	China	CH-HNPY-01-2022	OP716076.1	PRRSV-2
2023	China	WK730	OR826314	PRRSV-2
2023	China	TZJ3005	OR826313	PRRSV-2

demonstrated PARP-1′s involvement in viral replication. PARP-1 interacts with the N protein and affects PRRSV replication (Zhao et al., 2019). During PRRSV infection, the N protein interacts with the RNA helicase DExD/H-box helicase 9 (DHX9) and affects viral replication (Liu et al., 2016). Wang C. et al. (2017) demonstrated the interaction of the N protein with the unique SUMO E2 conjugating enzyme Ubc9 and their co-localization in the cytoplasm and nucleus. The SUMOylation characteristics of the N protein were shown. Ubc9 inhibited viral replication through its interaction with the N protein. MicroRNAs (miRNAs) affect viral replication by binding to mRNAs (Krol et al., 2010). An et al. (2020) found that miR-10a-5p overexpression decreased the level of the N protein and indirectly suppressed PRRSV replication. The PRRSV N protein promotes PRRSV proliferation by activating CCAAT/Enhancer Binding Protein β to induce the expression of Transcription Factor Dp-2 (TFDP2). PRRSV can utilize host proteins to regulate the cell cycle for its own benefit during infection (Zhu et al., 2021).

S100A9 belongs to the S100 protein family and has a damage-associated molecular pattern (DAMP). It inhibits PRRSV replication in a Ca^{2+}-dependent manner. Song et al. (2019) showed that the interaction between residues 36–37 aa of the N protein and amino acid residue 78 of S100A9 enabled their co-localization in the cytoplasm. This interaction restricted PRRSV proliferation.

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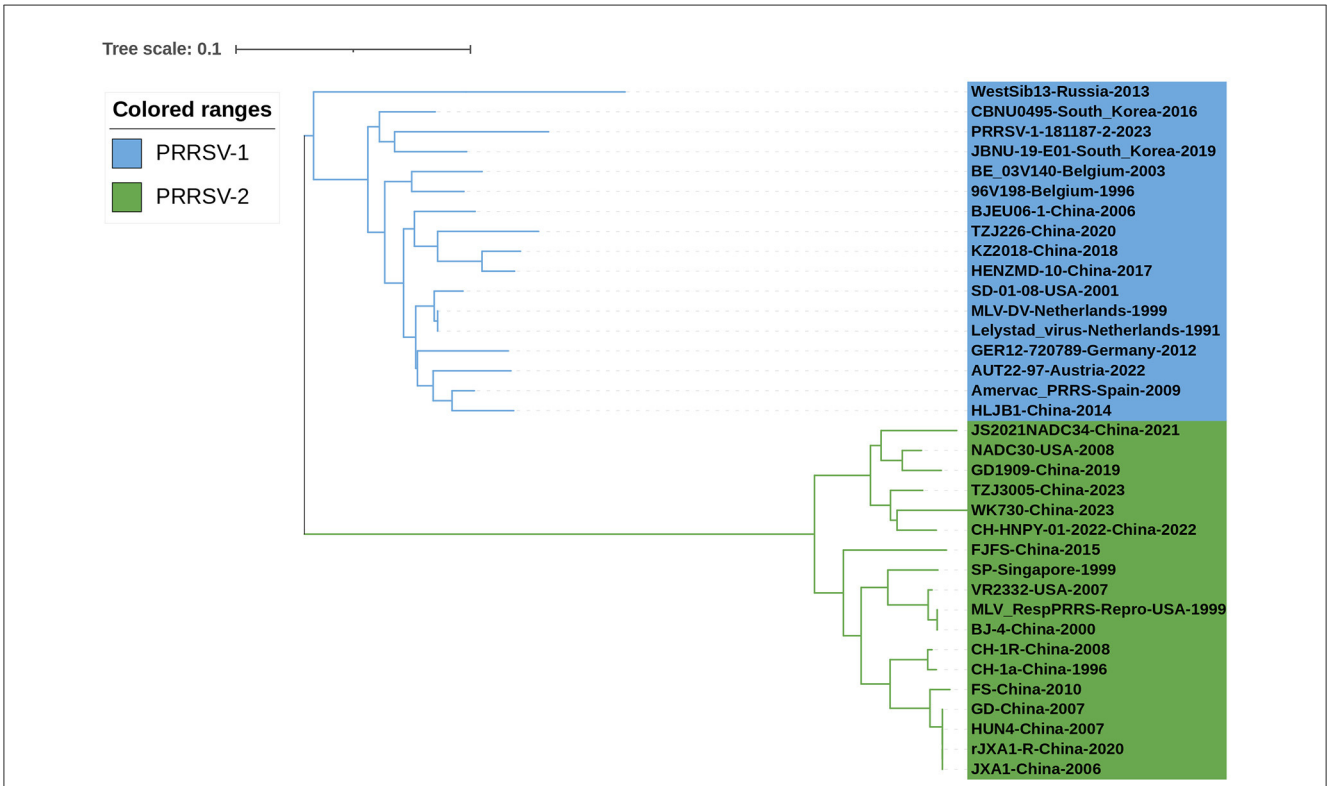

FIGURE 2
Phylogenetic analysis of the N gene. First, Clustal W alignment was conducted using the MegAlign feature in DNAStar software (version 7.0). Subsequently, the neighbor-joining method with 1000 bootstrap replicates was performed using the MEGA software (version 7.0). The resulting tree was visualized and annotated using the online "The Interactive Tree of Life" (https://itol.embl.de, accessed on 17 January 2024) software. PRRSV-1 and PRRSV-2 strains are represented in blue and green, respectively.

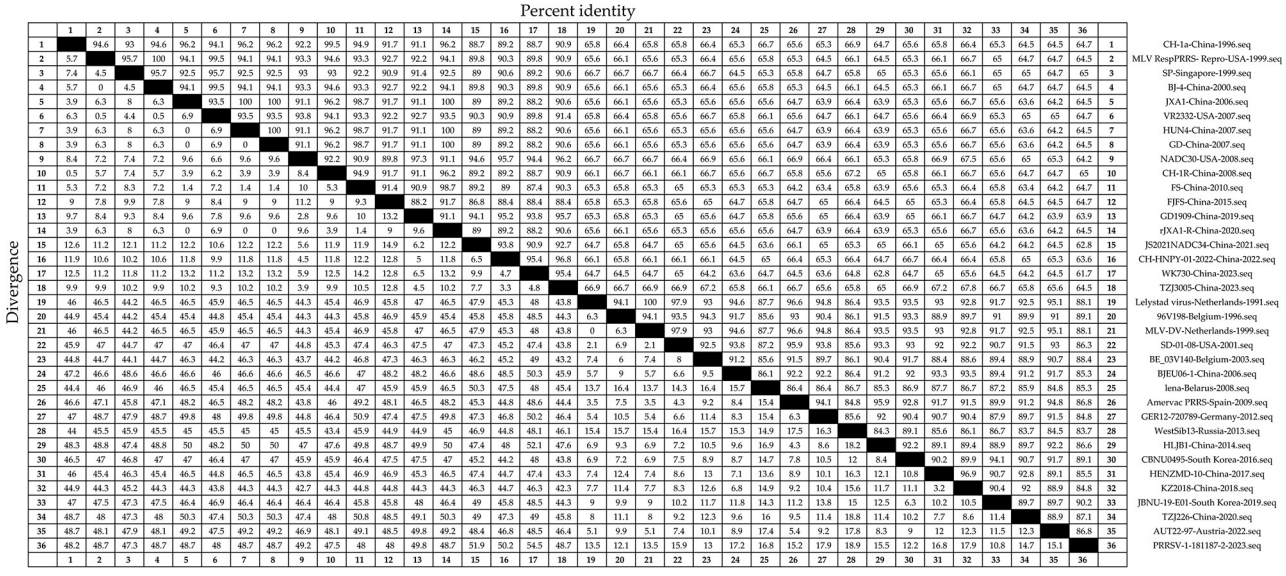

FIGURE 3
Thirty-six representative PRRSV strains were selected (Table 1). The N sequences were obtained and analyzed for nucleotide homology. The Clustal W algorithm in DNAStar software (version 7.0, Madison, WI, USA) was used to analyze the nucleotide homology of the N sequences.

S100A9 may restrict PRRSV proliferation by interacting with the viral N protein. Specific protein 1 (SP1) is a transcription factor regulating various biological processes (Vizcaíno et al., 2015). Chen J. et al. (2017) demonstrated that the N protein enhanced the expression of miR-373 through SP1, thereby suppressing IFN-β expression and PRRSV replication. Zhao et al. (2018), through

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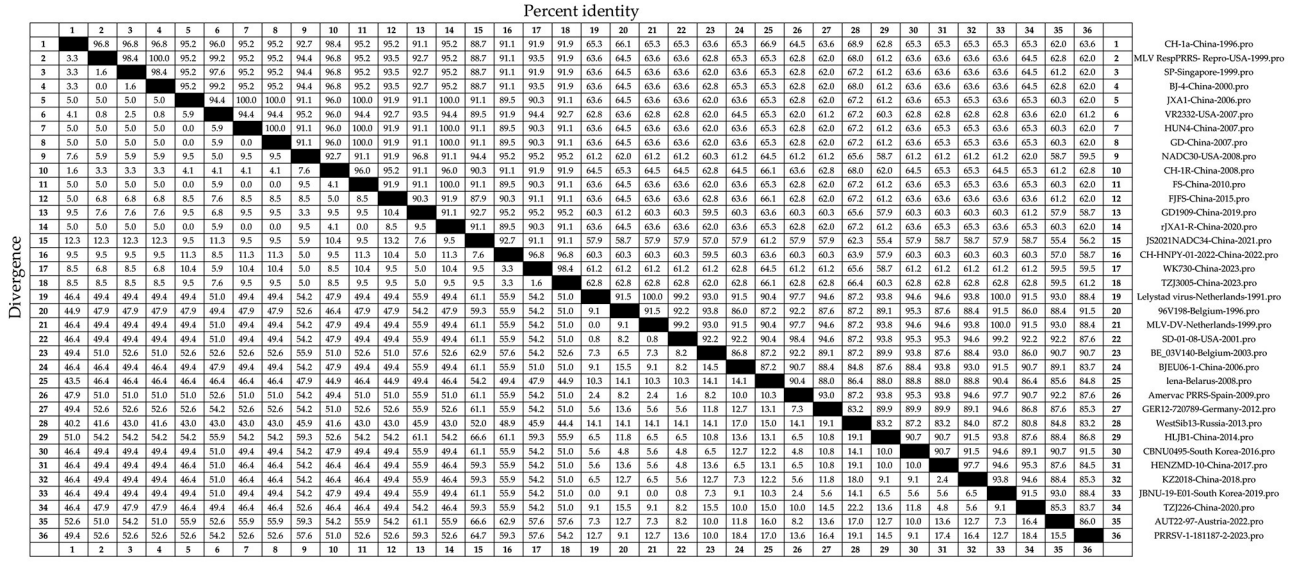

FIGURE 4
Thirty-six representative PRRSV strains (Table 1), including recently circulating strains, were selected. The N amino acid sequence homology was analyzed using DNAStar software (version 7.0, Madison, WI, USA) with the Clustal W algorithm in the MegAlign tool.

co-immunoprecipitation (Co-IP) and immunofluorescence co-localization, showed that Moloney leukemia virus 10 (MOV10) colocalized with the N protein in the cytoplasm. MOV10 causes cytoplasmic retention of the N protein and inhibits PRRSV replication by restricting its entry into the nucleus.

5 Effects of the N protein on viral virulence

Kwon et al. (2008) conducted an initial whole-genome scan using a model of reproductive failure in sows. Their results suggested that PRRSV's non-structural region (ORF1a and 1b) and structural region (ORF2-7) may contain virulence determinants, confirm the polygenic nature of PRRSV, and indicate that ORF7 is a potential determinant of virulence. This conclusion was supported by Wang et al. (2007). They constructed two chimeric PRRSV mutants by exchanging fragments of non-structural or structural genes. These gene fragments originated from attenuated vaccine strains and virulent strains. When pigs were inoculated with these two chimeric PRRSV mutants, they exhibited attenuated virulence. These findings unveil a strategy for the molecular generation of new attenuated vaccines.

Phosphorylation regulates the growth and virulence of various pathogens. However, whether phosphorylation of the N protein affects PRRSV virulence, remains unclear (Albataineh and Kadosh, 2016). Chen et al. (2019) demonstrated that certain mutations in the N protein reduced the replication capacity of PRRSV within cells. Experimental findings indicated that the mutant virus (A105-120) exhibited significantly lower pathogenicity than the parental virus strain (XH-GD). Taken together, phosphorylation of the N protein can affect virulence. No direct evidence suggests that the N protein affects PRRSV virulence. It is speculated that the N protein interacts with other structural or non-structural PRRSV proteins to affect viral virulence collectively. Additional studies are required to explore the effect of N proteins on viral virulence.

6 Interactions of the N protein with PRRSV proteins

PRRSV GP5 interacts with heparin sulfate glycosaminoglycan (HSGAG) and sialoadhesin/CD169 to facilitate viral entry (Veit et al., 2014; Shi et al., 2015). It interacts with the N protein to transport the viral RNA complex to specific sites (Montaner-Tarbes et al., 2019). Nuclear factor kappa B (NF-κB) is an inducible transcription factor. Lee and Kleiboeker (2005) found that PRRSV N protein and Nsp2 synergistically activated NF-κB. Lee and Yoo (2005) performed immunoprecipitation on cells co-expressing N and E proteins and demonstrated that the N protein did not co-precipitate with the PRRSV E protein under reducing or non-reducing conditions. A GST pull-down assay was conducted to confirm the specific interactions between N and N and between N and E, revealing the significance of non-covalent interactions between N and E proteins in PRRSV replication.

The above findings indicate that PRRSV GP5, Nsp2, and E can interact with the N protein. Understanding the interaction mechanisms between the N protein and other PRRSV proteins is crucial for developing drugs targeting the N protein.

7 N protein interacts with host proteins

The N protein performs diverse biological functions by interacting with multiple host proteins (Figure 6). Proteins of

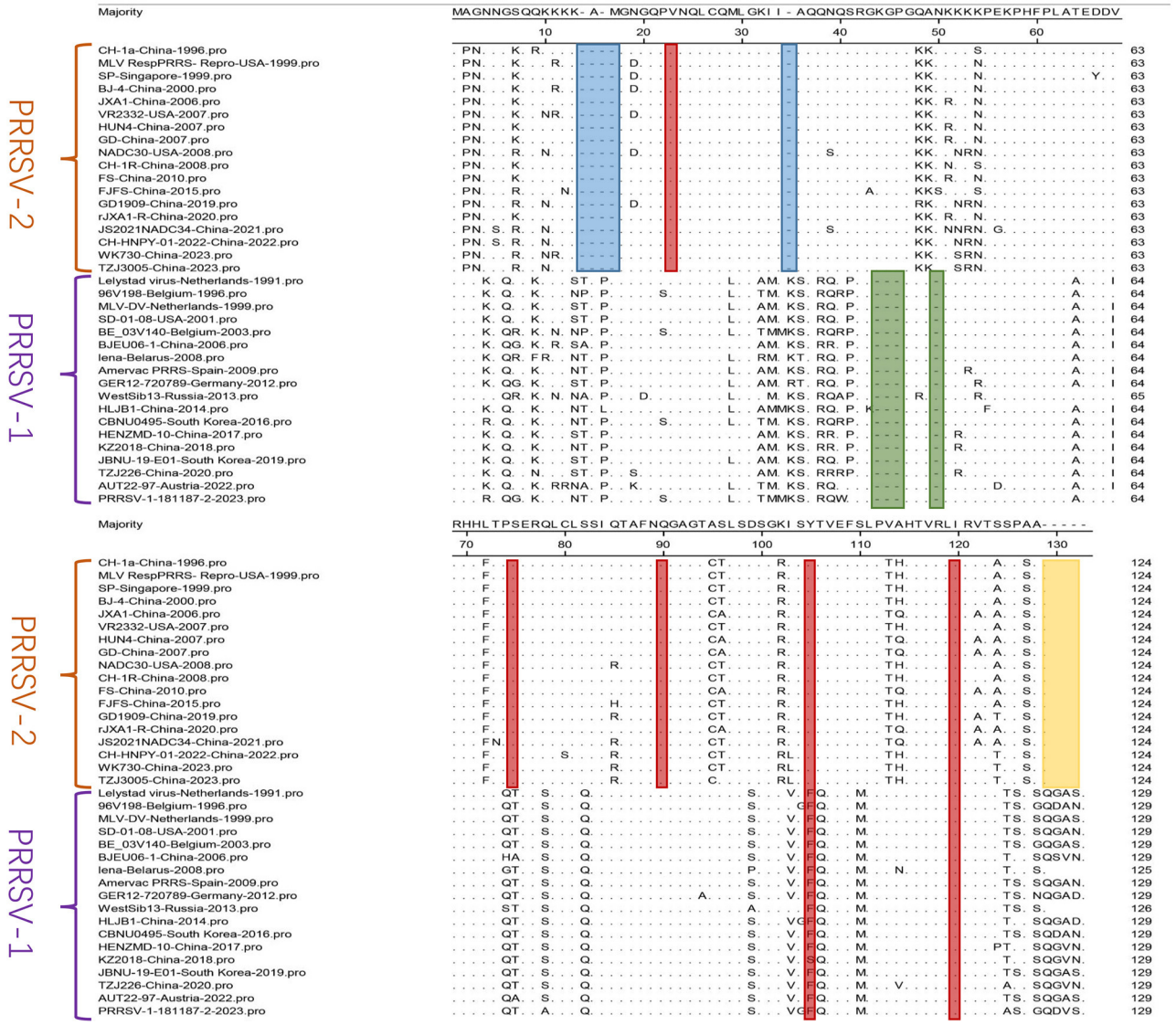

FIGURE 5
A comparison was performed for N sequences. The MegAlign sequence alignment editor in DNAStar software (version 7.0, Madison, WI) was used to analyze the amino acid sequences of N. The regions with multiple amino acid deletions are represented in blue, green, and yellow, and red indicates conserved amino acid positions.

the tripartite motif (TRIM) family, including TRIM22, TRIM25, and TRIM26, interact with the PRRSV N protein. Jing et al. (2019) found that the SPRY domain of TRIM22 interacted with the NLS2 motif of the PRRSV N protein. TRIM22 did not alter the N protein concentration. However, Chen et al. (2022) showed that TRIM22 enhanced lysosomal pathway activation by inducing lysosomal degradation of N proteins through interaction with LC3 and inhibited PRRSV replication. Zhao et al. (2019) revealed that PRRSV N protein interfered with the interaction between TRIM25 and RIG-I through competitive interactions to mediate RIG-I ubiquitination and counteract viral activity. Zhao et al. (2022) revealed that TRIM26 could bind to the N protein through its C-terminal PRY/SPRY domain and induce N protein degradation, thereby inhibiting PRRSV replication.

Poly(A)-binding protein (PABP) enhances the mRNA translation rate. Through yeast two-hybrid (Y2H) screening for cellular interactors of the N protein, the interaction region between the N protein and PABP was identified as within the 52–69 aa region. This interaction affects the viral replication (Wang et al., 2012). Sagong and Lee (2010) discovered that monkey viperin (mviperin), a homolog of mouse viperin, interacts with N protein at different cellular locations within the cytoplasm. Overexpression of mviperin inhibits viral genome replication, highlighting the significance of in-depth investigations on the interaction between mviperin and the N protein (Fang et al., 2016). Heterogeneous nuclear ribonucleoprotein F (HnRNP F) and hnRNP K affect protein expression. Knocking down hnRNP F effectively blocks the synthesis of the viral N protein (Zhang A. et al., 2022). The overexpression of hnRNP K

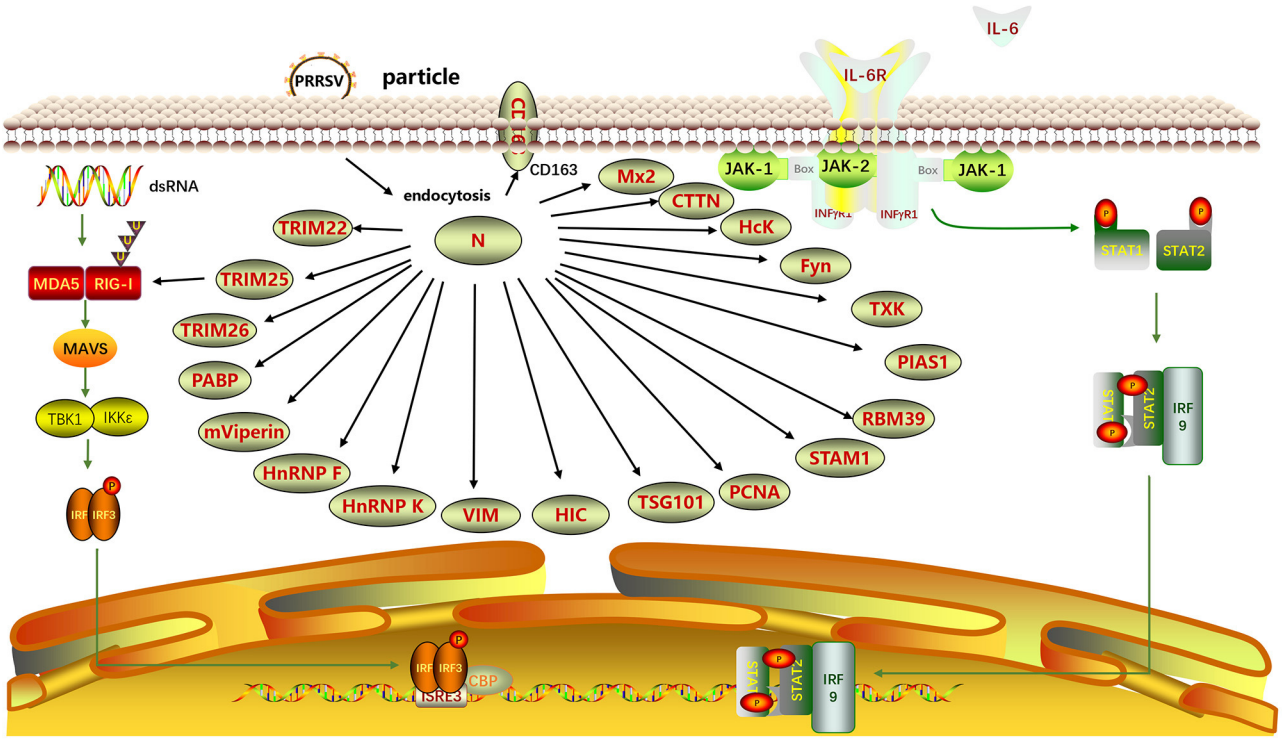

FIGURE 6
Interaction between N and host proteins. A protein-protein interaction network was generated using ScienceSlides software (version 2016). TRIM22, Tripartite motif-containing protein 22; TRIM25; TRIM26; PABP, Poly(A)-binding protein; mViperin, mouse Viperin; HnRNP F, Heterogeneous nuclear ribonucleoprotein F; HnRNP K; VIM, Vimentin; HIC, Hydrogenase-iron-only protein-like protein; TSG101, Tumor Susceptibility Gene 101; HcK, Hematopoietic cell kinase; PCNA, Proliferating Cell Nuclear Antigen; STAM1, Signal Transducing Adaptor Molecule 1; RBM39, RNA Binding Motif Protein 39; PIAS1, Protein Inhibitor of Activated STAT 1; TXK, Tec family kinase; Fyn, FYN proto-oncogene, Src family tyrosine kinase; CTTN, Cortactin; Mx2, Myxovirus resistance 2; CD163, Cluster of Differentiation 163.

inhibits PRRSV replication (Jing et al., 2023). These findings highlight the potential involvement of hnRNP F and hnRNP K in modulating PRRSV replication, enhancing our comprehension of host-PRRSV interactions.

Vimentin (VIM) is a major type III intermediate filament protein. It stabilizes the cytoskeleton and maintains cell integrity (Goldman et al., 1996). VIM is an important component of the PRRSV receptor complex, contributing to intracellular replication and dissemination of PRRSV (Wang et al., 2011). VIM can bind to the PRRSV N protein, and anti-VIM antibodies can block PRRSV infection in MARC-145 cells. VIM forms complexes with PRRSV Nsp2 and N proteins, which may be crucial for viral attachment and replication (Song et al., 2016). The initiator of chromosome condensation (HIC) is a protein containing an I-MyoD family inhibitor (I-mfa) domain, a recently discovered cellular transcription factor, and a homolog of human HIC. Song et al. (2009) experimentally confirmed that the N protein and the HIC-p40 isoform colocalize in the cell nucleus, whereas they colocalize with HIC-p32 in the cytoplasm, a truncated N-terminal product of HIC-p40, through mammalian two-hybrid analysis and immunoprecipitation assay. The interaction between the viral N protein and cellular transcription factors suggests that the N protein modulates host cell gene expression during PRRSV infection. The CD163 SRCR5 domain in macrophages confers resistance to PRRSV infection. Yu et al. (2019) discovered that the CD163 SRCR5 domain colocalized with the N protein within cells during the early stages of infection and participated in the viral invasion of the host.

Tumor susceptibility gene 101 (TSG101) is crucial in PRRSV infection. TSG101, a subunit of the ESCRT-I complex, interacts with the N protein to promote the formation of PRRSV viral particles. TSG101 is a cellular protein that can facilitate PRRSV assembly (Zhang Q. et al., 2022). Proliferating cell nuclear antigen (PCNA) is involved in DNA repair. Wang et al. (2023) discovered that PCNA interacted with the replication-associated proteins Nsp9, Nsp12, and N of PRRSV through immunoprecipitation and immunofluorescence co-localization assays. Region III (41–72 aa) of the N protein interacts with the IDCL region (118–135 aa) of PCNA. Therefore, the cytoplasmic translocation of PCNA and its effect on PRRSV RNA synthesis may represent potential targets for controlling PRRSV infection.

The Src homology 3 (SH3)-binding motif (SH3BM) PxxPxxP (PxxP) is conserved in the PRRSV N protein. In the study by Kenney and Meng (2015), five host cell proteins were identified to interact with SH3, including hematopoietic cell kinase (Hck), Tec family kinase (TXK), cortactin (CTTN), Fyn Proto-Oncogene, Src Family Tyrosine Kinase (fyn), and signal transducing adaptor molecule (STAM) I. The binding of SH3 proteins to the PRRSV N protein depends on at least one PxxP motif. The interaction of STAMI and Hck with PRRSV N protein requires an unobstructed

C-terminal structure. PRRSV promotes the expression of RNA-binding motif protein 39 (RBM39) within cells (Song et al., 2021). RBM39 interacts with PRRSV proteins, including Nsp4, M, and N (You et al., 2022). Ke et al. (2019) recently described the mechanisms of activation of NF-κB by PRRSV at the molecular level. PIAS1, a protein inhibitor of activated STAT1, interacts with N protein. The binding of the N protein to PIAS1 releases the p65 subunit of NF-κB, resulting in the activation of NF-κB. Myxovirus resistance 2 (Mx2) is a newly identified interferon-induced innate immune restriction factor that inhibits viral infections. Wang et al. (2016) reported the inhibitory effect of the porcine Mx2 protein on PRRSV replication, demonstrating the interaction between the Mx2 and N proteins of the virus leading to inhibition.

8 N protein's involvement in the host immune system

A PRRSV negatively regulates the host immune response, leading to persistent immunosuppression. PRRSV N protein is involved in this process by inhibiting the induction of type I IFN, IFN-β, and the phosphorylation of IRF3 (Sagong and Lee, 2011). The N protein has been demonstrated to suppress IFN-induced ISRE reporter expression and STAT2 elevation, while also hindering the nuclear translocation of STAT1. However, a comprehensive investigation into the specific functions and regulatory mechanisms of the N protein in the JAK/STAT signaling pathway requires the utilization of diverse experimental techniques and methods (Wang et al., 2013). PRRSV's inhibition of IFN by PRRSV is multifactorial. Several PRRSV proteins inhibit IFN, including Nsp1α, Nsp11, and N proteins (Yoo et al., 2010; Sun et al., 2012; Han and Yoo, 2014; Lunney et al., 2016).

PRRSV N protein is responsible for interleukin-10 (IL-10) production, and the non-covalent N-N domain is associated with this process (Hou et al., 2012; Liu et al., 2015; Yu et al., 2017). The N protein can trigger the expression of IL-10 in peripheral blood mononuclear cells (PBMCs) and monocyte-derived dendritic cells (MoDCs) (Wongyanin et al., 2012). Interleukin-15 (IL-15), a cytokine, promotes the production of various cells. The PRRSV N protein can induce the production of IL-15, which is mediated by multiple structural domains and activates NF-κB. The PRRSV N protein mediates NF-κB activation, leading to the induction of IL-15 production. This finding contributes to a deeper understanding of the mechanisms by which PRRSV infection induces IL-15 production (Fu et al., 2012).

The PRRSV N protein possesses multiple antigenic epitopes that form the basis for its involvement in the host immune system. Meulenberg et al. (1998) identified B-cell epitopes of the PRRSV-1 N protein at positions 2–12 and 25–30 aa using peptide scanning techniques. Plagemann et al. (2005) identified B cell epitopes of the PRRSV-2 N protein at positions 23–33, 31–50, and 43–56 aa using peptide scanning techniques. An et al. (2005) conducted a biopanning analysis using phage display and identified a B-cell epitope of the PRRSV II N protein at positions 78–87 aa. Identification of this epitope was based on monoclonal antibodies. Plagemann (2004) characterized epitopes at positions 24–32, 29–30, 31–39, 42–50, 50–60, and 54–92 aa, indicating that residues 23–92 aa may represent the dominant regions for B-cell epitopes. Wang et al. (2014) discovered that the B-cell epitope of the PRRSV I N protein was located at 1–15 aa. PRRSV infection increases the population of PRRSV-specific regulatory T lymphocytes (CD4+CD25+Foxp3+ Tregs) in infected pigs. Fan et al. (2015) reported that N proteins induce Treg proliferation. Further investigations have revealed three amino acid regions within the N protein, specifically 15–21 aa, 42–48 aa, and 88–94 aa, which play important roles in inducing Treg proliferation. Reverse genetics approaches have shown that the N^{15} and R^{46} residues in the N protein are crucial for inducing Treg proliferation. These findings contribute to our understanding of the involvement of PRRSV in host immune mechanisms.

PRRSV upregulates cytokine signaling 1 (SOCS1) to modulate the JAK/STAT signaling pathway (Wysocki et al., 2052). The PRRSV N protein can enhance the activity of the suppressor of SOCS1 through its NLS-2 (Luo et al., 2020). The dendritic cell (DC) marker, CD83, is associated with immune suppression, including DC activation and T-cell differentiation. Chen X. et al. (2017) showed that the infection caused by PRRSV triggered the upregulation of soluble CD83 (sCD83) in MoDCs derived from pigs by activating the NF-κB and Sp1 signaling cascades. The N, Nsp10, and Nsp1 proteins can enhance the promoter activity of CD83. Activation of the CD83 promoter relies heavily on two specific amino acids, namely R^{43} and K^{44}, within the N protein. The implications of this discovery establish a solid basis for expanding our understanding of the immunosuppressive effects of PRRSV.

NF-κB is important for host cell proliferation and innate immune response. The NF-κB pathway is activated during PRRSV infections. The mechanism underlying this phenomenon was investigated by Luo et al. (2011). They employed NF-κB DNA binding and luciferase activity assays to screen PRRSV structural proteins. NF-κB could be activated by PRRSV N protein. Moreover, the activation of NF-κB by the N protein depended on the sequence spanning from 30–73 aa. DExD/H-box protein 36 (DHX36) is an ATP-dependent RNA helicase. The N protein can enhance the activation of NF-κB through its interaction with the N-terminal tetramer of DHX36 (Jing et al., 2017). These findings provide a basis for understanding PRRSV infections and associated inflammatory responses.

9 Application of the N protein in clinical detection

PRRSV antigenic epitopes exist in many non-structural and structural proteins, including the N protein (Ostrowski et al., 2002; Rascón-Castelo et al., 2015). Antibodies against the N protein are easily detected during the initial stages of infection but these antibodies do not neutralize the virus and involved in antibody-dependent enhancement (Murtaugh et al., 2002; Mateu and Diaz, 2007). High levels of this antibody are maintained for several months. PRRSV detection methods rely primarily on assays that target N proteins (Plagemann et al., 2005).

Enzyme-linked immunosorbent Assay (ELISA) is the preferred method for assessing immune responses following vaccination and evaluating the dynamics of PRRSV antibodies over time. This confirms early PRRSV infection and enables the differentiation between antibodies induced by wild-type strains and vaccines

(Pan et al., 2023). Cai et al. (2009) developed a PRRSV antigen-capture ELISA method using monoclonal antibodies (mAbs) that have been extensively characterized against North American and European PRRSV N proteins. This antigen detection method can detect purified N proteins from two genotypes, with lower detection limits of 0.4 and 0.8 ng, respectively. Antigen detection methods are valuable field tools for the epidemiological control of PRRSV, allowing for rapid screening, particularly in asymptomatic animals. Zhang et al. (2019) generated three mAbs and identified three epitopes that were recognized by them. The 1G4 monoclonal antibody developed in this study is the first mAb targeting PRRSV-2 N, and amino acid residues 1–15 represent a newly identified epitope of the PRRSV-2 N protein. This discovery may open new perspectives for developing and expanding ELISA detection. Ye et al. (2022) used computational predictions to identify the major antigenic epitopes in the PRRSV N protein. They chemically synthesized three peptides targeting N protein antigenic epitopes as coating antigens and screened the optimal peptide to establish an indirect ELISA method for PRRSV antibody detection. The findings are important for routine diagnosis and epidemiological investigation of PRRSV. SDOW17 is an antibody that recognizes conformational epitopes in the N protein. It is used to detect PRRSV infection in cells. Serine, positioned at residue 90 within the N protein, is pivotal in recognizing conformational epitopes during interactions with SDOW17 mAb (Rappe et al., 2016).

ELISA is continuously being improved and enhanced as a key technology for the clinical detection of PRRSV. Chen et al. (2013) developed an effective indirect ELISA (iELISA) for detecting PRRSV antibodies using the recombinant fusion protein N-Gp5c produced in *Escherichia coli*. This method has been validated by comparison with immunofluorescence analysis and commercial iELISA methods, demonstrating its simplicity and ease of preparation and operation. Duan et al. (2021) developed a competitive ELISA using a nanobody-HRP fusion protein targeting the PRRSV-N protein specifically to detect anti-PRRSV-2 antibodies in pig serum. This method offers several advantages, such as a stable expression system and ease of operation. Sun et al. (2023) selected two high-affinity nanobodies to develop sandwich ELISA. The modified technique showed increased sensitivity. This detection method demonstrated high specificity and could detect all prevalent PRRSV-2 lineages in China.

In addition to ELISA, various detection methods based on the N protein have been established. Song et al. (2006), Chen et al. (2021), and Zhang L. et al. (2022) designed primers and probes for ORF7 of PRRSV to develop RT-qPCR-based detection methods. Hu (2021) developed an immunofluorescence assay (IFA) using the 5B4 monoclonal cell culture supernatant targeting the PRRSV N protein. However, a drawback of IFA is that it relies on viral replication. IFA is prone to nonspecific staining due to multiple interfering factors in the reaction. Luong et al. (2020) developed and conducted a comparative evaluation of the luciferase immunoprecipitation systems (LIPS) assay. The results obtained from LIPS were highly consistent with those of the commercial ELISA. This assay was used to measure the immune reactivity of the serum samples against the N protein and GP3. Compared to GP3, LIPS demonstrated higher diagnostic sensitivity for the N protein. Li et al. (2022) developed an immunochromatographic strip (ICS) based on latex microspheres. As the detection reagent, ICS uses latex microspheres labeled with a specific mAb 1H4 targeting the PRRSV N protein. These results demonstrate that the ICS assay accurately detects PRRSV and shows potential for clinical diagnostic applications.

10 Application of N protein-based vaccine

Current commercially available PRRS vaccines have less-than-ideal effectiveness (Nan et al., 2017). A major drawback of these vaccines is their lack of cross-protection. Developing a chimeric vaccine with multiple neutralizing epitopes is a promising strategy for addressing this challenge. Early studies revealed that chimeric viruses constructed by replacing ORF3-6 of VR2332 with the corresponding genes from JA142 or by introducing mutations in ORF2-7 of the FL12 strain to match the LMY sequence can induce cross-reactive neutralizing antibodies (Kim and Yoon, 2008). Targeting ORF7 for vaccine development is a promising strategy.

The N protein undergoes minimal changes during the *in vitro* passage (Liu X. et al., 2014; Chen et al., 2016). The CH-1R vaccine was prepared by inserting the porcine *IL-4* gene between the N and 3′-UTR sequences. This vaccine induces higher levels of IL-4 and a higher proportion of Tregs (Li et al., 2015b). The CH-1R vaccine can incorporate the porcine *GM-CSF* gene at the same position. The recombinant virus induces a higher proportion of Treg cells, elevated levels of IFN-γ, and lower viral viremia (Kimman et al., 2009; Li et al., 2015a). These findings have contributed to the development of novel recombinant vaccines. N proteins possess abundant antigenic epitopes and induce the production of long-lasting non-neutralizing antibodies after PRRSV infection (Plagemann, 2005; Ni et al., 2011), indicating their excellent immunogenicity and reactivity (Ren et al., 2010; Yu et al., 2010). Cheng et al. (2023) produced a mAb, N06, to target the N protein of PRRSV specifically. Using synthetic peptide fragments, they identified a sequential 16 aa pattern that characterized a remarkably immunogenic area within the N protein. mAbs exhibit strong immunogenicity. The immunomodulatory effects of the linearized and truncated forms of the PRRSV-N protein DNA vaccine (pORF7t) primarily manifest in preferentially inducing cellular immune responses against the PRRSV N protein. pORF7t modulates immune responses against PRRSV. Pigs immunized with this vaccine exhibit an increased number of specifically activated Tregs in response to PRRSV infection. This study reveals a novel concept for regulating PRRSV-specific immune responses by inducing cellular immune responses against the N protein, which contributes to developing PRRSV vaccines (Suradhat et al., 2015).

Predicting B and T cell epitopes is important for vaccine design and developing detection methods (El-Manzalawy et al., 2017). The N protein contains four or five antigenic regions (Dea et al., 2000). Although these antigenic regions have been studied extensively, most antigenic epitopes have not been precisely identified (Meulenberg et al., 1998; Plagemann, 2004). Inducing effective and protective B- and T-cell responses, specifically targeting the N protein, is essential for developing innovative vaccine formulations (Luo et al., 2023). Therefore, further experiments focusing on the antigenic epitopes of the N protein should be conducted.

Primary and booster immunizations of animals with DNA vaccines encoding truncated N proteins or antigens containing B- and T-cell epitopes from both PRRSV-1 and MLV can induce higher levels of T-cell and antibody responses (Sirisereewan et al., 2016; Bernelin-Cottet et al., 2019). This approach is considered a promising vaccination strategy to enhance the control of PRRS. Song et al. (2011) explored the application of replicon particles in vaccine development. They synthesized a self-replicating, non-propagating replicon RNA *in vitro* and transfected it into cells expressing the N protein. As a result, an infectious particle-packaging replicon RNA was obtained. These generated particles could target a single-round infection and lack intercellular spread. The transposon system targeting PRRSV provides a theoretical basis for studying antiviral vaccines based on viral replicons to prevent PRRSV infection and transmission.

Nanobodies have benefits, including affordability and ease of manufacturing. Duan et al. (2024) investigated the effects of a PRRSV-N-Nb1 nanobody in both laboratory settings and living organisms. Their findings demonstrated that the S105 residue within the PRRSV-N protein functioned as a crucial amino acid that could bind to the R97 residue of PRRSV-N-Nb1. PRRSV-N-Nb1 disrupted the self-interaction of the N protein following viral assembly. This discovery unveils the molecular foundation of N protein self-binding, serving as an essential element in viral replication, and highlights a promising target for advancing antiviral medications against PRRSV.

11 Summary and outlook

N protein is a basic phosphorylated protein, and phosphorylation sites are crucial for its biological functions. The N protein possesses an NLS that allows its distribution between the cytoplasm and nucleolus. The N protein can form homodimers participating in RNA genome binding and viral particle assembly. As a structural protein, the PRRSV N protein exhibits high conservation, which has been confirmed by genetic and evolutionary analyses. The PRRSV N protein demonstrates a high nucleotide and amino acid homology level and has been relatively conserved during evolution, with key amino acids being less prone to mutating. Furthermore, disulfide bond formation and the NLS of N proteins are crucial for viral replication. N protein affects PRRSV replication by interacting with PARP-1, DHX9, miR-10a-5p, S100A9, SP1, MOV10, and other factors. Currently, no direct evidence suggests that the N protein affects PRRSV virulence, and further investigation is needed to understand the effects of the N protein on virulence.

The N protein interacts with the PRRSV and its host proteins, enabling diverse biological functions. It interacts with its own GP5, Nsp2, and E proteins and host proteins (including TRIM22, TRIM25, and TRIM26), affecting viral invasion, replication, and signaling pathway regulation. The N protein contains multiple antigenic epitopes, including B- and T-cell epitopes, that form the basis of its involvement in the host immune system. PRRSV N protein regulates the host immune system by inhibiting IFN-I induction, mediating IL-10 production, and activating the NF-κB signaling pathway. Non-neutralizing antibodies induced by the N protein exhibit an early appearance and high levels. The existing PRRSV detection methods are primarily based on N proteins. ELISA methods established using the N protein have been widely used in clinical diagnostics and are continuously optimized and improved. In addition to ELISA, various detection methods based on the N protein have been developed, including RT-qPCR, IFA, LIPS, and ICS. The PRRSV N protein possesses abundant antigenic determinants and exhibits high immunogenicity and conservation, making it a suitable vaccine target. Approaches such as truncating the N protein, inserting exogenous genes, and producing mAbs with good immunogenicity can be used for vaccine development. Nanobody and transposon technologies are novel strategies for developing PRRSV vaccines.

The N protein, a structural protein of PRRSV, is crucial for viral RNA synthesis, viral entry, and the host immune system. Its clinical applications have been extensively studied, and various vaccines and detection technologies targeting the N protein have been developed. The N protein is an important component that should not be overlooked for preventing and treating PRRS. Their extensive biological functions merit further investigation and application in clinical practice.

Author contributions

YZ: Writing – original draft. GL: Writing – original draft. QL: Writing – original draft. HS: Writing – original draft. HZ: Writing – original draft. RW: Writing – review & editing. WK: Writing – review & editing. JL: Writing – review & editing, Funding acquisition. MZ: Funding acquisition, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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