The Structural Proteins of Porcine Epidemic Diarrhea Virus

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Porcine epidemic diarrhea (PED) virus (PEDV) is one of the main pathogens causing diarrhea in piglets and fattening pigs. The clinical signs of PED are vomiting, acute diarrhea, dehydration, and mortality resulting in significant economic losses and becoming a major challenge in the pig industry. PEDV possesses various crucial structural and functional proteins, which play important roles in viral structure, infection, replication, assembly, and release, as well as in escaping host innate immunity.

Keywords: porcine epidemic diarrhea virus (PEDV) ; function ; S protein ; E protein ; M protein ; N protein

1. Introduction

Porcine epidemic diarrhea virus (PEDV), a member of *Alphacoronavirus*, is an enveloped, single-stranded, positive-sense RNA virus that can cause porcine epidemic diarrhea (PED) [1][2]. PEDV-infected piglets display clinical signs like vomiting, diarrhea with watery stools, and dehydration, which can cause a high death rate among newborn piglets and result in substantial financial losses for the pig industry [3]. In 1971, PED was first reported in the United Kingdom [4]. After rampant transmission in many countries, PEDV became prevalent in China in 2010 [5][6]. The PEDV pandemic strains in the United States have resulted in a mortality rate of over 80% among suckling piglets [7]. Additionally, these PEDV strains have been swiftly transmitted between farms in the United States since 2013, leading to the death of more than 8 million piglets in 2014 alone and accounting for nearly 10% of all farm piglets in the country [6]. The pig industry has suffered immense economic loss as a result of the widespread transmission of PEDV. With the emergence of PEDV variants, the prevalence of PEDV has increased significantly, from 50.21% to 62.10% [8]. Recently, the pig industry in China has faced severe economic losses as a result of the emergence of highly virulent and infectious strains of PEDV [9][10].

Although herd immunization and biosafety measures are currently the most effective methods to prevent PED, the continuous evolution of PEDV, a variety of highly pathogenic strains have emerged, and the lack of mucosal immunity caused by existing PED vaccines is likely to cause immune failure, which affects the prevention and control of PEDV [10] [11]. Consequently, there is an immediate requirement for a comprehensive investigation into PED and its pathogenesis, along with the production of efficient vaccines that are specifically designed for epidemic strains. Recent studies have focused on the epidemiology, diagnosis, and molecular evolution analysis of PED. However, the role of PEDV proteins in its pathogenesis still needs to be recognized [12]. Current knowledge indicates that the structural and nonstructural proteins of PEDV have a significant impact on the process of virus invasion into host cells, gene replication, transcription, translation, and immune escape [13]. **Table 1** provides a summary of the involvement of PEDV proteins in PEDV infection.

Table 1. The viral proteins involved in PEDV infection.

Classification	Viral Protein	Role in PEDV Infection	References
Structural proteins	S	Essential for viral entry, induces neutralizing antibodies, virulence, induces apoptosis	[14][15][16][17]
	E	Initiates ERS, activates NF-κB, inhibits IFN-β, ISGs, virulence	[18][19][20]
	M	Induce cell cycle arrest at the S-phase, acts as IFN antagonist	[21][22]
	N	Form ribonucleoprotein complex, induces S-phase arrest, induces ERS, upregulates the expression of IL-8, inhibits IFN-β and IFN-λ production	[23][24][25][26][27][28]
Accessory protein	ORF3	Arrests cells at the S-phase, triggers ERS, induces autophagy, inhibits IL-6 and IL-8 productions, upregulates IKBKB-meditated NF-κB promoter, downregulates IFN-β promoter	[29][30][31][32]

Classification	Viral Protein	Role in PEDV Infection	References
Nonstructural proteins	Nsp1	Acts as IFN antagonist, induces virulence, inhibits proinflammatory cytokine production, inhibits NF-κB activity	[22][33][34][35]
	Nsp2	Promotes the replication of PEDV	[36]
	Nsp3	PLpro, regulates the deubiquitination of RIG-I and STING, inhibits IFN-β and IFN-λ1	[37][38]
	Nsp4	Upregulates pro-inflammatory cytokines and chemokines expression (IL-1α, IL-1β, TNF-α, CCL2, CCL5, and CXCL8)	[39]
	Nsp5	3C-like protease，IFN antagonist	[40][41]
	Nsp7	Inhibits type I IFN	[42][43]
	Nsp8	Inhibits type III IFN	[22]
	Nsp9	Inhibits ERS-mediated apoptosis	[44]
	Nsp10	Essential for viral replication, upregulates IL-2, IL-4, IL-10, TNF-α, and IFN-γ	[45][46]
	Nsp12	RdRp, viral replication	[47]
	Nsp13	HEL, inhibits bidirectional IgG transport by FcRn	[48]
	Nsp14	ExoN, suppresses ER stress-induced GRP78, acts as NF-κB pathway antagonist, downregulates pro-inflammatory cytokines	[49][50]
	Nsp15	EndoU, inhibits IFN-β and IRF3, downregulates CCL5, CXCL8, CXCL10, OAS, MXs, STAT1, and IRF9	[51][52]
	Nsp16	2′-O-MTase, downregulates the activities of RIG-I/MDA5-mediated IFN-β and ISRE	[53]

2. The Function of PEDV S Protein

Situated on the surface of PEDV particles, the spike (S) protein functions as a type I transmembrane glycoprotein. It is responsible for the formation of distinct spikes and plays a significant role in viral attachment to target cells and the production of neutralizing antibodies [54]. During virus invasion, the PEDV S attaches to the host's receptors on the cell membrane and mediates the virus-host membrane fusion [14][15][55]. It is also the largest structural protein in PEDV, composed of 1383 amino acids, with about 150–220 kDa molecular weight [54]. Of all PEDV proteins, the S gene is a hypervariable region among different strains of PEDV. Therefore, the genetic variation of the S protein can serve as a phylogenetic indicator for assessing the genetic diversity of PEDV [56]. According to the genetic diversity of S, PEDV can be divided into two genomes, genome 1 (G1) and genome 2 (G2), which can be further divided into G1a, G1b, G2a, G2b, and G2c subgroups [57]. Among them, PEDV G1a includes the prototype strain, CV777, identified in Belgium, and all strains sharing high genetic identity with CV777 [58]. The G2 strain was highly virulent. Further homologous recombination between G1a and G2 strains led to the emergence of S-INDEL strains G1b and G2c. The G2c consisted of S-INDEL strains from America (e.g., OH851) and Europe (e.g., GER/L00862/2014) with high degrees of sequence similarity to the ZL29 strain from China, suggesting that S-INDEL strains from Europe may have originated from a common ancestor with strain ZL29 [59][60]. In the natural host, S has the capacity to elicit the production of neutralizing antibodies, so it can be used as the target for the development of neutralizing monoclonal antibodies [16]. Song et al. have generated a PED vaccine candidate by expressing the immunogenic PEDV S in an Ad5 vector, which can induce significant humoral immunity [61].

PEDV S is hydrolyzed by trypsin-like host cell proteases to produce S1 and S2 subunits [62]. Comprising two domains, the S1 subunit includes the N-terminal domain (S1 NTD, residues 21–324 based on PEDV CV777) for attaching to cellular carbohydrates, and the C-terminal domain (S1 CTD, residues 253–638) involved in binding to host [63]. The utilization of the core neutralization epitope region (COE) in the S1 region has been widespread in the development of subunit vaccines aiming to prevent viral infection [64][65]. A recent study demonstrated that the virulence of PEDV is reduced, and the antibody's virus-neutralizing abilities are affected when the S1 region of circulating PEDV is deleted [17].

Up to now, the receptor of PEDV is still controversial; the candidates include both host glycans and host proteins. A previous study showed that PEDV S can recognize porcine aminopeptidase N (pAPN) [66] and overexpression of exogenous pAPN increases the sensitivity of PEDV to infect cells [67]. Despite this, studies have shown that PEDV is

capable of infecting pAPN knockout pigs [68][69]. Further evidence indicates that the functional receptor APN is unnecessary for the infection of pigs by PEDV [70][71][72]. Many coronaviruses have been found to bind to sialic acid glycans using its S1 domain, and this also happens in PEDV [54][73][74]. Moreover, the host protein ATP1A1 could potentially serve as a functional receptor for PEDV, playing a role in PEDV attachment and co-localization with PEDV S1 protein during the initial phase of infection [75]. The PEDV S protein can induce apoptosis of Vero E6 cells, and it has been confirmed that S1 may be a key gene for PEDV-induced apoptosis [76].

The S2 subunit is related to virus fusion to host cell membrane, which contains a fusion peptide (FP, residues 891–908), two heptad repeat regions HR1 (residues 978–1117) and HR2 (residues 1274–1313), a transmembrane region (TM, residues 1328–1350), and a cytoplasmic domain (CP, residues 1351–1386) in the C-terminus [54][62][77][78]. A recent study found that the S2 protein possesses neutralizing epitopes, and the core sequence of the epitope is situated between amino acids 1261 and 1337 [79]. The infection of PEDV typically depends on the presence of trypsin. It is also a kind of tropism switching for the viruses to adapt to cultured cells if the cells are not primary or immortalized cells derived from a natural host. The available evidence indicates that three amino acid mutations, namely A605E, E633Q, and R891G, occurring in the S protein of the PEDV strain DR13att, which enable attenuated PEDV strain DR13 (DR13att) to infect Vero cells efficiently and productively, in contrast to the parental DR13 strain (DR13par). And have the potential to modify PEDV tropism by affecting the S2' cleavage site and the RBD structure [80]. Tan et al. verified the trypsin-dependent characteristic of the S protein by comparing two PEDV strains, the trypsin-enhanced strain YN200 and the trypsin-independent strain DR13. The S protein of YN200 exhibits a stronger ability to induce syncytium formation and to be cleaved by trypsin than that of DR13. Using a full-length infectious YN200 cDNA clone confirmed that the S protein is a trypsin dependency determinant by comparison of rYN200 and rYN200-SDR13. Moreover, their findings indicate that the trypsin dependence of PEDV is predominantly controlled by the S2 subunit, as opposed to a direct trypsin cleavage site [81].

In addition, the interaction between PEDV and its host is greatly influenced by the PEDV S. In their study, Zhou et al. observed that the host membrane protein HSPA5 is capable of interacting with the PEDV S protein through its N-terminal domain, consequently controlling attachment and playing a role in the internalization process of PEDV [82]. The PEDV S contains two nearby motifs in its cytoplasmic tail (CT): a tyrosine-based motif, YxxΦ (x is any residue and Φ is a bulky hydrophobic residue: F, M, I, L, or V), and an ER retrieval signal (ERRS), KVHVQ, which might regulate the amounts of PEDV S in the endoplasmic reticulum (ER)-Golgi intermediate compartments (ERGIC) or on the cell surface [83][84][85]. Hou et al. found that the deletion of YxxØ motif and ERRS can lead to the exposure of S protein to the cell surface, promote the fusion of cell membranes, enhance the recognition of immune cells, and reduce the pathogenicity of viruses. For the intact S protein, YxxØ motif and ERRS not only help the S protein be internalized from the cell surface and escape the recognition of extracellular immune cells but also assist the S protein to migrate to the ER and Golgi apparatus to assemble mature virus particles [86]. The data offer insightful information about the different roles played by the S protein in PEDV infection.

3. The Function of PEDV E Protein

Among the structural proteins in PEDV, the envelope (E) protein is the smallest, with about 7Da molecular weight of 7000–13,000, and is distributed on the surface of the virus envelope [87]. It also exhibits strong hydrophobicity [87]. The structure of E protein includes three parts: the short N-terminal hydrophilic region, the transmembrane region containing the α helix structure with a length of about 25aa, and the long C-terminal region [88]. There is still a lack of clear understanding regarding the function of PEDV E protein. Existing research has indicated that the E protein plays a vital role in viral assembly, budding, and the host immune response.

The E protein, which is situated in the endoplasmic reticulum (ER), triggers ER stress (ERS) by upregulating GRP78 and activating nuclear factor-κB (NF-κB), resulting in enhanced expression of IL-8 and Bcl-2 [18]. In light of previous research, it has been found that the E protein has the capacity to impede the activation of RIG-I signaling, significantly inhibit the transcription of IFN-β and ISGs, and interfere with the translocation of IRF3 from the cytoplasm to the nucleus through direct interaction [19]. Further research found that the E protein initiates ERS by activating the PERK/eIF2α pathway, thereby attenuating the translation of RIG-I signaling-related antiviral proteins, ultimately inhibiting type I IFN production [89]. Moreover, the E protein can inhibit the activation of β promoters and swine leukocyte antigen II DR (SLA-DR), a crucial MHC-II molecule that plays an important role in the initiation of $CD4^+$ T cell activation and antigen presentation [90][91]. The findings suggest that the E protein plays a crucial role in suppressing the immune response of the host.

Moreover, the E protein can affect the viral virulence. Li et al. found that the E protein functions as an interferon antagonist during infection and also identified it as a virulence factor of PEDV [20]. They further demonstrated that deletion of a 7-

amino-acid region in the E protein (EΔaa23–aa29) could be a promising approach for the development of live attenuated vaccines against PED [20]. Host factors can also affect the virulence of PEDV through E protein. Gao et al. found that karyopherin α 2 (KPNA2) binds to the PEDV E protein, leading to its degradation through autophagy as a means to inhibit PEDV replication [92].

Furthermore, the E protein serves as a diagnostic marker that could aid in the creation of innovative serological assays and the design of vaccines that enhance protective immunity [93].

4. The Function of PEDV M Protein

The M (membrane) protein, composed of 227 amino acids, is an essential membrane glycoprotein localized throughout the cytoplasm, which can affect cell growth, cell cycle progression, and interleukin 8 (IL-8) expression [21]. The expression of the M protein in an intestinal epithelial cell (IPEC) line can cause cell cycle arrest at the S-phase through activation of the cyclin A pathway [21]. Nevertheless, the PEDV M protein neither induces ERS nor activates NF-κB [21]. The coronavirus M protein has the same structural characteristics: three TM domains with N-exo-C-endo orientation flanked by a short-glycosylated N-terminal domain on the virion surface and a long C-terminal globular domain within the virion [94][95]. The high level of conservation observed in the M gene across diverse PEDV strains makes it a promising target for the establishment of PEDV detection methods, including ELISA, RT-qPCR, and RT-PCR [96][97][98]. Among different PEDV isolates, the linear B-cell epitope (195WAFYVR200) that spans the C-terminus of the M protein is remarkably conserved. Additionally, this specific epitope has the ability to distinguish between serum samples positive for PEDV and TGEV [99]. Recent studies have utilized a 3D model to forecast four linear B-cell epitopes, with the RSVNASSGTG and KHGDYSAVSNPSALT peptides being particularly noteworthy, six discontinuous B-cell epitopes, forty weak binding, and fourteen strong binding T-cell epitopes in the CV777 M protein [100].

The M protein serves as an essential structural protein in the processes of viral infection, replication, and assembly. Additionally, it can activate the immune response of the host for protection, although the precise mechanisms behind these functions are still not fully understood. The PEDV M protein has been recognized as an interferon antagonist [22]. Recent studies found that the 1–55 region of M protein is essential for disrupting IFN regulatory factor 7 (IRF7) function, suppressing the expression of type I IFN and various antiviral ISGs, enhancing viral replication, and determining the antagonistic mechanism used by M protein to target IFN [101]. Wang et al. found that M protein interacts with eukaryotic translation initiation factor 3L (eIF3L) during PEDV infection, downregulates the expression of eIF3L, and significantly increases virus replication [102]. Moreover, the M protein directly interacts with HSP70, thereby facilitating PEDV replication [103]. These observations indicate that M protein has the potential to serve as a candidate for the development of a differential diagnostic test. Additionally, it could be a valuable target in the design of multi-epitope vaccines and novel therapeutic approaches aimed at activating protective cellular mechanisms against PEDV.

5. The Function of PEDV N Protein

The N protein is a nucleocapsid protein composed of 1383 amino acids, which is highly conserved and undergoes alkaline phosphorylation. It has a relative molecular weight of about 56 kDa [104]. The N protein plays a crucial role in multiple aspects of the virus's life cycle, such as controlling viral RNA synthesis, packaging viral RNA into the helical nucleocapsid, and assembling viral particles [105][106]. A recent study has discovered that the PEDV SH strain N protein contains a distinctive deletion of 12 amino acids (aa 399–410), which includes an antigenic epitope. However, this deletion does not impact the immunogenicity or pathogenicity [107]. The N protein of PEDV has three conserved domains: N-terminal domain (NTD), linker region (LKR), and C-terminal domain (CTD). In addition, there is an intrinsically disordered region (IDR) at both ends [108]. The N protein is highly expressed during all stages of PEDV infection. Therefore, identifying the N protein enables the assessment of PEDV replication, rendering it useful for the early detection of PEDV infection [109][110]. The cytoplasm serves as the main location for the N protein; however, it can also be detected within the nucleolus. It possesses a nuclear localization signal (NLS) motif pat7 (261PKKNKSR267) and is actively transferred to the nucleolus during the time span of PEDV infection [111]. According to the study conducted by Shi et al., the translocation of N protein from the cytoplasm to the nucleolus is not reliant on the shuttle protein nucleolar phosphoprotein nucleophosmin (NPM1). However, N protein can interact with NPM1 to inhibit its proteolytic cleavage and improve cell survival, ultimately facilitating PEDV replication [112]. N protein has an RNA binding domain (RBD) that binds to RNA packaging signal (PS) and can associate with genomic RNA to form a ribonucleoprotein (RNP) complex, which participates in the replication and transcription process of the virus. It can also bind to M protein to promote the assembly of viral particles [23][108]. The N protein NLS (S71NWHFYYLGTGPHADLRYRT90) can interact with p53 and activate the p53-DREAM pathway, leading to S-phase arrest and finally promoting virus replication [24].

The N protein also actively participates in regulating host innate antiviral responses and regulates IFN signal transduction. The N protein has the ability to specifically impede the nuclear translocation of NF-κB, which relies on type III IFN while leaving the expression of type I or type II IFN induced by polyinosinic-polycytidylic acid (poly(I:C)) unaffected in IPEC-J2 cells [25]. During the interaction with host cells, the PEDV N protein exerts an inhibitory effect on cell proliferation by extending the duration of the S-phase cell cycle [26]. Xu et al. studied the subcellular localization and the effect of PEDV N protein on cell growth, cell cycle progression, cell survival, and IL-8 expression [26]. The N protein located in the ER can induce ERS, inhibit cell growth, and upregulate the expression of IL-8 in IPEC cells. The NLS sequence of the PEDV N protein is located within its amino acid region 71–90, with R87 and R89 being crucial for its biological function. Additionally, two nuclear export signals (NES) are at amino acids 221–236 and 325–364. However, only the NES within the 325–364 region is involved in the full-length N protein's functionality [26]. Ding et al. discovered that the N protein also hinders the production of IFN-β induced by the Sendai virus, the expression of the IFN-stimulated gene, as well as the activation of the transcription factors IFN regulatory factor 3 (IRF3) and NF-κB [27]. Moreover, by engaging in a direct interaction with TBK1, the N protein disrupts the TBK1-IRF3 association, resulting in the inhibition of both IRF3 activation and type I IFN production [27]. Likewise, Cao et al. observed that PEDV-infection in IECs impedes the dsRNA-induced IFN-β induction by interfering with IRF-3, which is associated with the RIG-I-mediated signaling pathway and its adapter molecule, IFN-β promoter stimulator 1 (IPS-1) [28]. However, it is not known whether it is caused by the N protein.

Furthermore, the N protein plays a crucial role in the interaction between the coronavirus and its host, as well as in the pathogenesis. The PEDV N protein serves as a multifunctional protein, playing a crucial role in facilitating PEDV replication by targeting specific host cytokines. For example, the N protein promotes the cyclization of viral mRNA through binding to PABPC1 and eIF4F proteins, thus promoting viral transcription and facilitating viral replication [113]. PEDV N protein can interact with TRIM28 to induce mitophagy, leading to inhibition of the JAK-STAT1 pathway to promote PEDV replication [114]. On the other hand, the host can use host cytokines to hijack the N protein and then degrade it to inhibit the replication of PEDV. The E3 ubiquitin ligase MARCH8 facilitates the interaction between EGR1 and the PEDV N protein, resulting in N protein ubiquitination and degradation. Additionally, EGR1 directly binds to the IRAV promoter, leading to the upregulation of IRAV expression and subsequent suppression of PEDV replication [115]. PTBP1 facilitates the recruitment of MARCH8, an E3 ubiquitin ligase, and NDP52, a cargo receptor, for the catalysis and degradation of N protein via selective autophagy, thereby inhibiting PEDV replication [116]. The activation of autophagy by HNRNPA1 promotes the degradation of PEDV N, while the N protein-mediated autophagy pathway triggered by PEDV infection also results in the degradation of HNRNPA1 protein [117]. PRPF19 can recruit the E3 ubiquitin ligase MARCH8 to the N protein for ubiquitination, and the ubiquitinated N protein is recognized by the NDP52 and transported to autolysosomes, thus inhibiting virus replication [118]. HnRNP K inhibits PEDV replication through autophagic degradation of viral N protein and upregulation of MyD88 expression, leading to IFN-1 production [119]. RALY can degrade the N protein through a RALY-MARCH8-NDP52-autophagosome pathway to suppress PEDV replication [120]. FUBP3, TARDBP, and BST2 are capable of initiating the degradation of the N protein via the MARCH8-NDP52-autophagosome pathway, resulting in the induction of IFN-I production, in an effort to hinder the replication of PEDV [121][122][123]. TRIM21 selectively directs the degradation of the N protein through a proteasome-dependent mechanism, resulting in the inhibition of PEDV replication [124]. Hence, the enhanced replication of PEDV in Vero E6 cells with overexpression of N protein could potentially be attributed to the suppression of host immune response induced by N protein [125].

In summary, the N protein performs various functions, while further exploration is required to fully comprehend its underlying mechanism. These data have improved our knowledge regarding the function of PEDV N protein.

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