Nucleocapsid protein from porcine epidemic diarrhea virus isolates can antagonize interferon-λ production by blocking the nuclear factor-κB nuclear translocation

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Abstract: Porcine epidemic diarrhea virus (PEDV) is a highly infectious pathogen that can cause severe diseases in pigs and result in enormous economic losses in the worldwide swine industry. Previous studies revealed that PEDV exhibits an obvious capacity for modulating interferon (IFN) signaling or expression. The newly discovered type III IFN, which plays a crucial role in antiviral immunity, has strong antiviral activity against PEDV proliferation in IPEC-J2 cells. In this study, we aimed to investigate the effect of PEDV nucleocapsid (N) protein on type III IFN-λ. We found that the N proteins of ten PEDV strains isolated between 2013 and 2017 from different local farms shared high nucleotide identities, while the N protein of the CV777 vaccine strain formed a monophyletic branch in the phylogenetic tree. The N protein of the epidemic strain could antagonize type III IFN-λ, but not type I or type II IFN expression induced by polyinosinic-polycytidylic acid (poly(I:C)) in IPEC-J2 cells. Subsequently, we demonstrated that the inhibition of poly(I:C)-induced IFN-λ3 production by PEDV N protein was dependent on the blocking of nuclear factor-κB (NF-κB) nuclear translocation. These findings might help increase understanding of the pathogenesis of PEDV and its mechanisms for evading the host immune response.

Key words: Porcine epidemic diarrhea virus; Nucleocapsid protein; Interferon-λ (IFN-λ); Nuclear factor-κB (NF-κB); Intestinal epithelial cells

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1 Introduction

Porcine epidemic diarrhea (PED), one of the most severe and globally widespread infectious diseases in all ages of swine (Song and Park, 2012), is caused by the porcine epidemic diarrhea virus (PEDV). As a member of the Coronaviridae family, PEDV infects mainly the epithelial cells of the porcine intestine, leading to acute diarrhea, vomiting, and dehydration (Jung and Saif, 2015). The high morbidity and mortality in newborn piglets caused by PEDV...
infection can lead to significant economic loss and serious animal welfare issues. Thus, a better understanding of the genetic characteristics and molecular pathogenesis of PEDV is urgently needed for vaccine development and PED control. PEDV possesses genes encoding structural spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins in the 3' one-third of the genome (Lee, 2015). Epitopes of the N envelope (E), and nucleocapsid (N) proteins in the 3' end of the genome (Lee, 2015) are known to be important in cell-mediated immunity (CMI) induction (Saif, 1993).

Interferons (IFNs), especially type I and III IFNs, play crucial roles in antiviral host defense (Ank et al., 2008). The IFN-α and IFN-β are produced in response to innate immune stimulation leading to the production of IFN-α and IFN-β from a nuclear factor-κB (NF-κB) and interferon regulatory factor 3 (IRF3) (Ding et al., 2014). Only N protein and papain-like protease 2 (PLP2) of PEDV have been demonstrated as IFN antagonists (Ding et al., 2014). However, it remains to be determined whether the N protein of PEDV functions as an IFN-λ antagonist. Therefore, in this study, we used IPEC-J2 cells to investigate the effect of PEDV N protein on the production of IFN-λ to give a better understanding of the molecular mechanisms involved in the porcine host-pathogen interaction, which could help to identify novel therapeutic targets and develop effective vaccines against PEDV.

2 Materials and methods

2.1 Cells and viruses

The Vero E6 cell line was used to isolate and passage virus. Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B) and 10% fetal bovine serum (FBS, Gibco, USA).

The IPEC-J2 cell line was kindly provided by Prof. Jie FENG (Zhejiang University, Hangzhou, China) and cultured in DMEM supplemented with antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B) and 10% fetal bovine serum (FBS, Gibco, USA).

The PEDV strains were isolated from clinically diseased pigs in China. Briefly, intestinal tissues were homogenized in Earle's balanced salt solution (Sigma, USA) and centrifuged to obtain supernatants. The supernatants were then filtered through a 0.22-μm pore filter and used as inoculum for virus isolation. The supernatants were used to inoculate Vero cells. After incubating for 4 h, the inoculum was replaced with DMEM supplemented with antibiotics and tryptophan (5 μg/ml). The Vero cells were frozen at −80 °C and thawed once when 80% of cells presented cytopathogenic effects (CPEs). The cells and medium were mixed, and then centrifuged to obtain supernatant for secondary inoculation. For serial passaging, virus was gradually increased. Finally, flasks were used for propagation and isolated PEDV strains as seed stock.
2.2 Plasmids

The sub-cloning vector pEASYBlunt (Transgene, China) was used to clone N genes from different PEDV strains. The luciferase reporter vectors pGL3 and pRL-TK were purchased from Promega (USA). Luciferase reporter vector pGL3-P$_{IFN-\lambda 3}$ was constructed by inserting the promoter region of IFN-\(\lambda 3\) into the pGL3 backbone vector. The 1517-bp promoter region was predicted by an online prediction server (http://www.cbs.dtu.dk/services/Promoter) using the 5-kb upstream sequence of swine IFN-\(\lambda 3\) gene. The eukaryotic expression vector pcDNA3.1-flag vector was constructed and maintained by our laboratory (Zhou et al., 2017). The 13SX0101 strain was chosen randomly from the isolated strains for N gene cloning. The N gene from strain 13SX1101 was cloned into the pcDNA3.1-flag using ClonExpress II One Step Cloning Kit (Vazyme, China) to produce the pcDNA3-N expression vector. All the primers used in this study are listed in Table 1.

2.3 Sequence analysis of N protein

The Viral Total RNA Kit (Labserv, China) and Reverse Transcription Kit (Vazyme, China) were used for virus RNA extraction and cDNA synthesis, respectively. Specific primers were designed to amplify N genes from cDNA of isolated PEDV strains. The purified PCR products were cloned into the pEASYBlunt plasmid for sequencing. The nucleotide sequences of N genes from ten isolates were submitted to GenBank (accession numbers MF118911–MF118920). Nucleotide sequences and amino acid sequences were aligned and an amino acid phylogenetic tree was constructed using MEGA6.0 software and the ClustalW method.

2.4 Transfection

For Luc-assays, IPEC-J2 cells were seeded in 24-well plates and co-transfected with 1 \(\mu\)g pRL-TK and 1 \(\mu\)g luciferase reporter vector pGL3-P$_{IFN-\lambda 3}$ After 24 h, cells were then transfected with 0.5 \(\mu\)g polyinosinic-polycytidylic acid (poly(I:C)) and 1 \(\mu\)g pcDNA3-N. For quantitative PCR (qPCR) assays and immunofluorescence assays, IPEC-J2 cells were seeded in 24-well plates and co-transfected with 0.5 \(\mu\)g poly(I:C) and 1 \(\mu\)g pcDNA3-N. Lipofectamine 2000 reagent (Invitrogen, USA) was used in transfection assays. Cells transfected only with lipofectamine, only with the pcDNA3.1 vector, and those without any treatment were taken as the lipofectamine control, vector control, and blank control, respectively.

2.5 Quantitative PCR

Cells were collected at 24 h post transfection with poly(I:C) and pcDNA3-N by 1 min centrifugation at 13 800 \(g\), and subsequently homogenized in 500 \(\mu\)l TRIzol (Tiangen, Beijing, China). Uniq-10 Total RNA Extraction and Purification Kit (Tiangen, Beijing, China) was used to extract total RNA. Before cDNA synthesis, the residual genomic DNA was digested with DNaseI (Promega, USA). The cDNA was synthesized using a Reverse Transcription kit (TOYOBO, Japan). To detect the transcriptional level of different IFNs and transcriptional factors, qPCR was performed in a 20-\(\mu\)l reaction mixture with SYBR qPCR mix (TOYOBO, Japan) and specific primers (Table 1) using the iCycler iQ5 real-time PCR detection system (Bio-Rad, USA). The transcriptional level of housekeeping gene \(\beta\)-actin was used to normalize that of the target genes.

| Table 1 Primers used in this study |
|-----------------------------------|
| Primer   | Sequence (5'→3') | Length (bp) |
| N-F      | ATGGCTTCTGTCAGCTTTCAGGATCTC | 1326 |
| N-R      | ATTTCCCTGTATCGAGGATCTCGTGATAAAT | 109 |
| pcDNA3-F | GGTGGTGACACGGCCGTTGAAAATATCAAAGGAGATCTTCGATACGGAAAT | 5415 |
| pcDNA3-R | AGTCCCTGAAGCTGACAGAACAGGAAAT | 90 |
| IFNa-F   | CTGGCTGTGAGGAAATAC | 116 |
| IFNa-R   | ATCCCTCTCCTCTCCAG | 109 |
| IFNβ-F   | GATCTCGCATCTCTCA | 109 |
| IFNβ-R   | GATGTCGCTCCAGTCTC | 109 |
| IFNg-F   | GCCATGCTGAACCTCATC | 90 |
| IFNg-R   | GCCATGCTGAACCTCATC | 90 |
| IFNg3-F  | CCAATTCAGTCTCCTTG | 89 |
| IFNg3-R  | AGTCCCTGAAGCTCACAAGAAGAATT | 173 |
| IFR3-F   | CCAACTGGGAGAGGAATT | 173 |
| IFR3-R   | CCAACTGGGAGAGGAATT | 173 |
| IRF7-F   | GCCATGCTGAGGCTGTG | 109 |
| IRF7-R   | CCAACTGGGAGAGGAATT | 109 |
| β-actin-F | GTCACCCACACGCTGGCCA | 160 |
| β-actin-R | CCGGAGATCGTGGGCGGCA | 160 |
2.6 Luciferase reporter assay

Cells were harvested 24 h after the secondary transfection. The Dual-Glo luciferase assay (Promega, USA) was used to measure luciferase activity. Briefly, cells were collected by centrifugation at 13 800 g for 5 min and homogenized in 100 μl 1× passive lysis buffer. Cell lysates (20 μl) were added to a 96-well fluoroscopy plate. Firefly luciferase activity and *Renilla* luciferase activity were measured using a luminometer (Thermo, USA) immediately after addition of luciferase assay reagent (LAR) II (Promega, USA) and Stop & Glo reagent (Promega, USA), respectively. The relative luciferase values were measured by the ratio of firefly luciferase and *Renilla* luciferase.

2.7 Immunofluorescence assay

The cells at 24 h post transfection were fixed in 4% paraformaldehyde at 4 °C overnight, and then washed with 1× phosphate-buffered saline (PBS). Fixed cells were permeabilized with 0.2% Triton X-100 at room temperature for 15 min and blocked with PBS containing 10% bovine serum albumin (BSA) for 1 h. The samples were labeled with mouse monoclonal anti-PEDV N protein IgG (1:100 (v/v) dilution) and rabbit monoclonal anti-NF-κB IgG (1:100 (v/v) dilution; CST, USA) at 37 °C for 1 h, and then labeled with the Alexa Fluor 568 goat anti-mouse IgG antibody (1:1000 (v/v) dilution, Life-technology, USA) and Alexa Fluor 488 goat anti-rabbit IgG antibody (1:1000 (v/v) dilution, Lifetech- nology, USA) at 37 °C for 1 h. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Beyotime, China) at 1:1000 (v/v) dilution. Immunofluorescence was visualized using a laser confocal microscope IX81-FV1000 (Olympus, Japan).

2.8 Western blotting

Cell lysates were prepared in 50 μl of radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime, China) containing 1 mmol phenylmethanesulfonyl fluoride (PMSF) (Beyotime, China). Protein concentrations were quantified using a bicinchoninic acid protein assay kit (Sangon Biotech, China). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% (0.1 g/ml)) was used to separate proteins, which were then electrotransferred onto polyvinylidene difluoride membranes (Millipore, USA). Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% nonfat milk was used to block the blots before they were probed with primary antibodies, mouse monoclonal anti-N IgG and rabbit monoclonal anti-NF-κB IgG (CST, USA) at 1:500 (v/v) dilution and rabbit monoclonal anti-β-actin IgG (Hua’an, China) at 1:2000 (v/v) dilution. Blots were subsequently washed with TBST and incubated for 1 h with secondary antibodies, goat anti-mouse horseradish peroxidase (HRP)-labeled antibody, and goat anti-rabbit HRP-labeled antibody (Sangon Biotech, China). West Pico chemiluminescent substrate (Thermo, USA) was used to visualize the Western blotting results. The Gel 3100 chemiluminescent imaging system (Sagecreation, China) was used to capture images.

2.9 Statistical analysis

All results are presented as mean±standard error (SE) of triplicate experiments. The data were analyzed using GraphPad-Prism (GraphPad Software, USA). Statistical significances were tested by one-way analysis of variance (ANOVA).

3 Results

3.1 Sequencing of N protein from different PEDV isolates

The N genes of PEDV were successfully amplified from the CV777 vaccine strain and ten strains isolated between 2013 and 2017 from different farms in Zhejiang Province, China. A phylogenetic tree was constructed based on multiple sequence alignment of 11 sequences (Fig. 1). The recent PEDV strains shared N protein amino acid identities of 98.2% to 100.0% (Fig. 1a). The N protein sequence of the CV777 strain formed a monophyletic branch, while those of different recent PEDV strains in China showed a close phylogenetic relationship (Fig. 1b).

3.2 Expression of N protein in IPEC-J2 cells after transfection

Expression of N protein was detected at 24 and 48 h after transfection with pcDNA3-N. Western blots revealed that the N protein began to be expressed in IPEC-J2 cells at 24 h post transfection (Fig. 2).
Immunofluorescence validated this result and showed that the N protein was expressed in the cytoplasm (Fig. 3a, white box). N protein immunofluorescence could be detected in the transfected cells, suggesting that they could successfully express nucleoprotein (Fig. 3a) unlike those in the pcDNA3.1 vector, lipofectamine, and blank control groups (Figs. 3b and 3c). These results indicated that N protein was successfully expressed in IPEC-J2 cells after transfection.

3.3 Inhibition of IFN-λ3 expression by PEDV N protein

To investigate the effect of PEDV N protein on the production of IFN-λ3, we used poly(I:C) as an inducer of IFN transcription in IPEC-J2 cells and analyzed the expression of IFNs and their transcription factors. We found that all three types of IFN expression could be significantly induced after poly(I:C) induction, but only IFN-λ3 expression was significantly reduced ($P<0.05$) after PEDV N protein transfection compared with expression in the pcDNA3.1 vector transfection group (Fig. 4). The IFN transcriptional factor IRF3, but not IRF7, showed a similar downward trend. These results indicated that PEDV N protein might inhibit the induction of IFN-λ3, but not of the other IFNs in IPEC-J2 cells.

3.4 Effect of PEDV N protein on IFN-λ3 promoter activity

To further confirm IFN-λ3 inhibition by PEDV N protein, we constructed a luciferase reporter vector...
Fig. 3  Expression of nucleoprotein in almost all the cells at 24 h post transfection
N protein expressed in N protein immunofluorescence could be detected in the transfected cells cytoplasm (in the white box), suggesting that transfected cells could successfully express nucleoprotein compared to that in control groups of vector, lipofectamine, and blank. (a) Transfect with pcDNA3-N; (b) Lipofectamine control; (c) Vector control; (d) Blank control

Fig. 4  IFN-λ3 expression inhibited by PEDV nucleoprotein
All the three types of IFN expression could be induced after poly(I:C) induction, but only IFN-λ3 expression was decreased after PEDV nucleoprotein transfection compared with that in pcDNA3.1 vector transfection group. Cells only transfected with pcDNA3.1 vector were taken as vector control. *P<0.05, **P<0.01, ***P<0.001. ns: not significant
containing firefly luciferase under the control of the IFN-λ3 promoter. Consistent with the qPCR results, poly(I:C)-induced relative luciferase activity was significantly reduced after PEDV N protein was expressed in IPEC-J2 cells, while it remained induced after the pcDNA3.1 vector was transfected (Fig. 5). These results further indicated IFN-λ3 inhibition by PEDV N protein in IPEC-J2 cells. This inhibition might contribute to virus escape from mucosal immunity in the intestine.

4 Discussion

Secreted IFNs are defined by their ability to combat viral infections. However, many viruses have evolved elaborate mechanisms to modulate IFN signaling or expression using various proteins that may affect different innate immune pathways. Previous studies revealed that the N proteins of coronaviruses exhibit an obvious capacity to inhibit the production of IFN-β in vitro (Ye et al., 2007; Lu et al., 2011; Ding et al., 2014). Coronavirus N proteins, which have been reported to be involved in viral RNA replication and transcription, share similar motifs, structural features, and functions (Laude and Masters, 1995; Schelle et al., 2005). In accordance with previous reports, our phylogenetic tree analysis found that the majority of PEDV strains recently isolated in China shared the same N amino acid identity (Fig. 1).

The recently discovered IFN-λ has been reported to play a critical role in antiviral defense at the mucosal surface (Ank et al., 2006; Shen et al., 2016; Li et al., 2017). Li et al. (2017) found that IFN-λ, expressed on the epithelial cells of the intestine, exhibited strong antiviral activity against PEDV infection in IPEC-J2 cells. To investigate the effect of N protein on IFN-λ, we first increased IFN production in IPEC-J2 cells by the addition of poly(I:C). The three types of IFNs, IFN-α, IFN-β, IFN-γ, and IFN-λ3, were notably increased after treatment with poly(I:C) as determined by qPCR. The plasmid of pcDNA3-N was subsequently transfected into IPEC-J2 cells, and the expression of N protein was detected at 24 h by Western blot and immunofluorescence assay (IFA) (Figs. 2 and 3). The mRNA levels of IFN-α, IFN-β, and IFN-γ in poly(I:C)-treated cells were similar to those in poly(I:C) and pcDNA3-N co-transfected cells, whereas the mRNA level of IFN-λ3 was significantly reduced (P<0.05) when the N protein was expressed in IPEC-J2 cells (Fig. 4). A similar trend of the IFN-λ transcription level in poly(I:C) and pcDNA3-N co-transfected cells was shown by the luciferase activity assay (Fig. 5). These data strongly indicated that the N protein of PEDV inhibited the transcription and expression of IFN-λ, but not type I IFNs, in IPEC-J2 cells.

The type I IFN inhibitory mechanism of PEDV was extensively investigated in previous studies (Guo et al., 2016). However, Ding et al. (2014) found that PEDV N protein significantly inhibited the production of type I IFN in HEK 293 cells. TLR2 mediates
PEDV N protein-induced NF-κB activation (Cao et al., 2015a), and PEDV N could inhibit double-stranded RNA (dsRNA)-induced type I IFN by blocking the retinoic acid-inducible gene I (RIG-I)-mediated pathway in intestinal epithelial cells (IECs) isolated from the newborn piglet ileum (Cao et al., 2015b). One explanation for this disagreement may be the different choice of cells. IPEC-J2 cells are one
of the target porcine cell types that PEDV infects. Thus, the data collected from IPEC-J2 cells may reflect a more realistic situation of host-pathogen interaction in vivo. The mRNA level of IRF3 showed a decreasing pattern (Fig. 4). However, due to the low levels of fold change in N-expressing cells, the mRNA level of type I IFNs was not affected by PEDV N protein. Possible explanations for this have been put forward by Zhang et al. (2015) who reported that the IRF3 mRNA level was not the only factor that can affect type I IFNs. It is necessary to determine the protein level and localization of IRF3 to investigate the mechanism of action of type I IFNs. We conducted Western blot and immunofluorescence assays on IRF3 (data not shown), but unfortunately, the commercial antibodies for human IRF3 did not work on IRF3 (data not shown), but unfortunately, the commercial antibodies for human IRF3 did not work efficiently on swine cells. Because of these limitations, we focused our experiments only on the inhibitory effect of PEDV N protein on IFN-λ.

Recent studies confirmed that activated IRF3, IRF7, and NF-κB were also required for the production of type III IFN (Thomson et al., 2009; Lazear et al., 2015). Once infected by the virus, NF-κB or IRFs of epithelial cells were activated and then moved into the nuclei and up-regulated the transcription level of IFN-λ (Osterlund et al., 2005; Seth et al., 2005; Odendall et al., 2014; Odendall and Kagan, 2015). Iversen et al. (2010) found that NF-κB had a significant effect on the expression of IFN-λ rather than on type I IFN, suggesting that the NF-κB pathway might play the dominant role in driving the IFN-λ response on epithelial surfaces of the intestine. This might also explain why induction of only type III, not type I or II IFNs, was blocked in our study. However, the swine IFN-λ regulation mechanism remains unknown. In accordance with this evidence, our results indicated that NF-κB moved into the nuclei after the cells were treated with poly(I:C), leading to a significantly increased transcription level of IFN-λ. However, NF-κB failed to move into the nuclei in the PEDV N protein-expressing cells, resulting in a significant decrease in the mRNA level of IFN-λ (Fig. 6). Taken together, our study identified that PEDV N protein caused a blockade of IFN-λ induction through activation of NF-κB in IPEC-J2 cells. The knowledge gained from this study may assist in the development of antiviral reagents and vaccines.

Compliance with ethics guidelines

Ying SHAN, Zi-qi LIU, Guo-wei LI, Cong CHEN, Hao LUO, Ya-jie LIU, Xun-hui ZHUO, Xing-fen SHI, Wei-huan FANG, and Xiao-liang LI have declared no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文摘要

目：猪流行性腹泻病毒流行毒株核衣壳蛋白能够通过阻断核因子κB干预表达的活性β干扰素的产生，抑制由聚肌胞苷酸(poly(I:C))诱导表达的III型IFN。这种拮抗作用是通过阻断核因子κB对IFN-λ的表达实现的。
方法：利用 poly(I:C) 刺激 IPEC-J2 细胞使其 IFN 诱导表达。实验组转染 N 蛋白真核表达载体，对照组转染空载体；利用定量聚合酶链反应（qPCR）、荧光素酶报告基因等技术，检测 N 蛋白对 I 型、II 型及 III 型 IFN 表达抑制情况。利用间接免疫荧光技术，检测 NF-κB 在细胞内的分布情况，分析 NF-κB 入核与 N 蛋白抑制 IFN-λ 产生的关系。

结论：2013 年至 2017 年间从浙江省不同的农场分离的 10 个 PEDV 毒株的 N 蛋白具有较高的核苷酸同源性，而疫苗毒株 CV777 的 N 蛋白在系统发育树中形成单系分支（图 1）。流行病毒株的 N 蛋白可以在 IPEC-J2 细胞中成功表达（图 2 和 3），并拮抗由 poly(I:C) 诱导表达的 III 型 IFN，但不能拮抗 I 型或 II 型 IFN（图 4 和 5）。PEDV N 蛋白通过阻断 NF-κB 入核来对 poly(I:C) 诱导的 IFN-λ3 产生的抑制作用（图 6）。

关键词：猪流行性腹泻；核衣壳蛋白；λ 干扰素；核因子 κB；肠上皮细胞