Nucleocapsid proteins from other swine enteric coronaviruses differentially modulate PEDV replication

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ABSTRACT

Porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV) and porcine deltacoronavirus (PDCoV) share tropism for swine intestinal epithelial cells. Whether mixing of viral components during co-infection alters pathogenic outcomes or viral replication is not known. In this study, we investigated how different coronavirus nucleocapsid (CoV N) proteins interact and affect PEDV replication. We found that PDCoV N and TGEV N can competitively interact with PEDV N. However, the presence of PDCoV or TGEV N led to very different outcomes on PEDV replication. While PDCoV N significantly suppresses PEDV replication, overexpression of TGEV N, like that of PEDV N, increases production of PEDV RNA and virions. Despite partial interchangeability in nucleocapsid oligomerization and viral RNA synthesis, endogenous PEDV N cannot be replaced in the production of infectious PEDV particles. Results from this study give insights into functional compatibilities and evolutionary relationship between CoV viral proteins during viral co-infection and co-evolution.

1. Introduction

Porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV) and porcine deltacoronavirus (PDCoV) belong to the family Coronaviridae (Enjuanes, 2000). PEDV and TGEV have been classified into the alphacoronavirus genus, whereas PDCoV belongs to the deltacoronavirus genus (Jung et al., 2016a; Jung and Saif, 2015a). They share similar genome architectures, with a 25–30 kb positive-sense, single-stranded RNA genome. The 5′ two-thirds of the viral genome encodes non-structural proteins from open reading frames (ORF) 1a and 1b necessary for viral genome replication. The rest of the genome encodes a number of unique accessory proteins such as PEDV ORF3, TGEV 3a/3b/7, PDCoV NS6/NS7, and four common structural proteins, namely the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins (Kocherhans et al., 2001; Lee and Lee, 2014; Penzes et al., 2001). These enteric swine coronaviruses (CoVs) infect epithelial cells lining the small intestine and cause villous atrophy, resulting in malabsorption and severe diarrhea (Jung et al., 2016a). An outbreak of these viruses, especially PEDV, can lead to up-to-100% mortality in neonatal piglets, prompting huge economic losses in the swine production industry worldwide. Unless they are examined by laboratory-level diagnosis, these CoVs produce almost indistinguishable pathogenesis.

Co-infection of enteric pathogens are common. TGEV and PDCoV have been found to co-circulate with PEDV in the field (Song et al., 2015; Wang et al., 2016). In PDCoV-positive samples, the rate of PEDV co-infection as detected by RT-PCR varies from 33% to 50% (Jung et al., 2016a; Jung and Saif, 2015a). Although TGEV infection has become rarer nowadays, it has still been detectable in samples in China, and often together with PEDV and/or PDCoV (Dong et al., 2015; Wang et al., 2013). Despite substantial epidemiological evidence of co-infection, the effects of these events on disease outcomes have not yet been formally described. Since these enteric swine CoVs share cell tropism, co-infection of these viruses can theoretically cause mixing of viral components in the same cellular compartments, possibly leading to direct or indirect effects on viral replication kinetics or pathogenic outcomes. To the best of our knowledge, there are currently no reports on studies at molecular or cellular levels on how viral components from different CoV species interact with or affect other viruses. Investigation of possible molecular interactions between components of PEDV, PDCoV and TGEV and their influence on replication of each virus would provide a crucial insight into comprehensive understanding of these CoVs.

Of all viral proteins, we have chosen to start with the N protein, as it is among the most abundant and ubiquitous structural proteins in infected cells. The CoV N protein is functionally conserved across the family Coronaviridae (Chang et al., 2009; Cong et al., 2017), with its primary function being to form a scaffold for packaging viral genomic
RNA (gRNA) into the internal core of virions (de Haan and Rottier, 2005). Besides scaffolding, other functions of the CoV N protein (primarily based on studies of common representatives of the family like severe acute respiratory syndrome-CoV (SARS-CoV) or mouse hepatitis virus (MHV)) include acting as RNA chaperones (Zuniga et al., 2007, 2010), promoting viral genome transcription or replication (Hurst et al., 2010, 2013; Masters et al., 1994; Zuniga et al., 2007, 2010), facilitating viral assembly (de Haan and Rottier, 2005; Kuo et al., 2016), suppressing antiviral RNA-interference activity from their hosts (Cui et al., 2015), and suppressing host immunity (Ding et al., 2014, 2017; Xu et al., 2013; Zhang et al., 2018).

Based on sequence alignment and limited structural data from some representative CoVs, all CoV N proteins are predicted to consist of three structural domains: the N-terminal domain (NTD), linker region (LKR) and C-terminal domain (CTD) (Chang et al., 2014; McBride et al., 2010). NTD binds RNA through electrostatic interaction with its charged amino acids as well as interaction between conserved aromatic residues in the proteins and nucleotide bases in the RNA (Chang et al., 2014; Huang et al., 2004; Tan et al., 2006). LKR is a disordered domain between NTD and CTD. Studies of SARS-CoV N indicate roles for LKR in RNA binding, virion assembly and self-association (Chang et al., 2009, 2013; He et al., 2004a). Although it is also reported to have RNA binding capacity, CTD is a more hydrophobic domain mainly responsible for self-association to form stable dimers and subsequent oligomers of CoV N (Chen et al., 2007; Yu et al., 2006). Studies of N proteins from SARS-CoV and MHV revealed that Nimerization followed by multimerization is a common process in the formation of viral ribonucleoprotein that initiates viral genome packaging in the virion assembly process among CoVs (Cong et al., 2017; Fan et al., 2005; Ma et al., 2010; Yu et al., 2006).

Examples from other viruses suggest that cross-association between viral nucleocapsid proteins could determine different outcomes during co-infection. For instance, mixed infection between two types of plant tospoviruses results in interspecies interaction between their N proteins and more severe symptoms compared to single infections, suggesting synergy between these viruses (Bag et al., 2012; Tripathi et al., 2015). On the other hand, intertypic interference between types A and B influenza viruses could be partially explained by the inhibitory effect of type B influenza virus nucleoprotein exerted on its type A counterpart (Aoki et al., 1984; Jaru-ampornpan et al., 2014). Given structural similarities, we expect that CoV N proteins would likely cross-interact with each other during a co-infection event. Whether this cross-interaction has beneficial or detrimental effects on viral replication has never been explored. Investigation of the interaction between CoV N proteins could lead to insights into virus evolution, anti-CoV drug development and vaccine design (Chang et al., 2016; Lo et al., 2013).

2. Materials and methods

2.1. Biological materials

Human embryonic kidney (HEK) 293T cells, wild-type VeroE6 cells and VeroE6-based cell lines stably expressing CoV N were maintained in OptiMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C with 5% CO₂. The virus PEDV-AVCT12-mCherry (PEDV-mCherry), its infectious clone (pSMART-BAC-mCherry-PEDVAVCT12 (PEDV-mCherry)) and pCAGGS-PEDV N-Myc, a plasmid for C-terminally Myc-tagged PEDV N expression, have been described previously (Jaru-Ampornpan et al., 2017; Jengarn et al., 2015).

2.2. Plasmid construction

TGEV and PDCoV N encoding genes were codon-optimized for high expression in mammalian cells and to avoid expression of PDCoV NS7 embedded in the PDCoV N gene (Fang et al., 2017) (GenBank: AAG30228.1, AFD29191.1, respectively). PCR products of the corresponding N genes with indicated tags at the C-termini were ligated into pCAGGS vectors to give plasmids for expression of tagged CoV N (pCAGGS-TGEV N-FLAG and pCAGGS-PDCoV N-HA). pCAGGS-HA-PEDV N for N-terminally HA-tagged PEDV N expression was similarly constructed based on the same PEDV N DNA sequence in pCAGGS-PEDV N-Myc. pGEX-4T3-PEDV N was constructed by in-frame insertion of the PEDV N gene sequence, codon-optimized for bacterial expression based on the sequence from AVCT12 strain (accession number LC053455), into the pGEX-4T3 vector (Amersham) following the coding sequence of N-terminal GST. All plasmids were verified by DNA sequencing.

2.3. Western blot analysis and antibodies

Cells were lysed with lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol, supplemented with a protease inhibitor cocktail (Halt™ Protease inhibitor cocktail, Thermo Scientific)). Proteins in the cell lysate were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The membrane was blocked in 5% skim milk prior to incubation with indicated primary antibodies. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Biolegend) or (HRP)-conjugated donkey anti-rabbit IgG (BioLegend) was used as secondary antibodies. Primary antibodies used in this study include mouse-anti-Myc (Thermo Scientific), rabbit-anti-FLAG (Cell Science Technology), rabbit-anti-HA (Cell Science Technology), mouse-anti-S1 (a kind gift from Dr. Qigai He, Huazhong Agricultural University), and mouse-anti-PEDV N (SD 6–29, Medgene Labs).

2.4. Co-immunoprecipitation (Co-IP)

HEK293T cells were co-transfected with pCAGGS-PEDV N-Myc (or the empty pCAGGS vector for negative controls) and either pCAGGS-HA-PEDV N, pCAGGS-TGEV N-FLAG or pCAGGS-PDCoV N-HA using FuGENE HD (Promega) according to the manufacturer’s instructions. At 48 h post-transfection (hpt), cell lysates were prepared as described previously and pre-adsorbed to control agarose beads (Thermo Scientific) for 1 h at 4°C to reduce non-specific binding. Then, the treated lysates were incubated with anti-Myc-conjugated agarose beads (Thermo Scientific) at 4°C overnight. The mixtures were washed three times with lysis buffer supplemented with 250 mM NaCl. IP complexes were eluted by boiling the bead mixtures in 2× non-reducing sample buffer (Thermo Scientific) for 5 min and supplemented with 2 mM DTT before SDS-PAGE and western blot analysis.

2.5. GST-pulldown assay

GST and GST-PEDV N were expressed in BL21 (DE3*) cells. After 3-h protein induction by 0.5 mM isopropyl β-D-1-thiogalactopyranoside, bacteria were lysed by sonication in 1× phosphate-buffered saline (PBS). The lysates were clarified and stored in 100-μl aliquots at –80°C until use. HEK293T cells were transfected with plasmids as indicated and were lysed at 48 hpt with lysis buffer. The bacterial lysate containing the bait protein (GST/GST-PEDV N) and the HEK293T cell lysate containing the prey protein (HA-PEDV N, TGEV N-FLAG, PDCoV N-HA, or combinations thereof) were incubated with pre-equilibrated GSH FF resin (Amersham) overnight and washed twice in high-salt GST buffer and four times in low-salt GST buffer (20 mM Tris-HCl pH 8.0, 20% glycerol, 1 mM EDTA, 0.1% NP-40 and 1 M/100 mM NaCl) (Nguyen and Goodrich, 2006). The bound proteins were released by boiling the resin in 2× SDS-PAGE loading buffer and analyzed by Coomassie staining (for bait) and western blotting with indicated antibodies (for prey). Figures were representatives of three independent experiments. For Fig. 2, quantification of eluted protein bands in each GST pulldown reaction was performed using the BioRad Image Lab
Fluorescence images were taken under a fluorescence microscope (Olympus), and the supernatants were then used to transduce VeroE6 cells. A single clone of transduced cells expressing the corresponding N protein was selected based on similar levels of the transgene protein expression.

Because of variability between independent experiments, these ratios were normalized to the conditions without extra CoV N protein performed side-by-side in each experiment before statistical comparison. For the RNaseA experiment, the combined lysates were treated with 10 μg of RNaseA (Thermo Scientific) or PBS buffer for 30 min at 4°C to avoid protein precipitation at high temperature. To verify the absence of RNA after the treatment, total RNAs were purified from parts of treated lysates by RNA purification kit (Thermo Scientific) and were used at equal volumes as templates for RT-PCR. The RT-PCR products were visualized by Ethidium bromide staining.

2.6. Generation of VeroE6 cell lines stably expressing CoV N

VeroE6 cell lines expressing PEDV N-Myc, TGEV N-FLAG and PDCoV N-HA were constructed using the same protocol as VeroE6-PEDV N cells as described previously (Liwnaree et al., 2019). Briefly, lentiviruses carrying each CoV N gene were generated by co-transfection of pSIN-CSGW-UbEM carrying the inserted CoV N gene with a packaging plasmid encoding Gag, Pol, Rev and Tat (pCMV-ΔR8.91) and a plasmid expressing lentiviral VSV envelope glycoprotein (pMD2.G) into HEK293T cells. At 48 hpt, supernatants containing the lentiviruses were harvested and filtered through a 0.45-μm filter. The filtered supernatants were then used to transduce VeroE6 cells. A single clone of the transduced cells expressing the corresponding N protein was selected based on similar levels of the transgene protein expression.

2.7. Immunofluorescence analysis

Uniform expression of exogenous N proteins in the engineered VeroE6 cell lines was verified by immunofluorescence analysis. Briefly, cells were grown in an eight-well slide. At 24 h, cells were fixed with 80% cold acetone for 10 min. After washing twice with PBS, cells were blocked with 10% PBS, 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Cells were then incubated with mouse anti-PEDV N antibodies (SD 6–29) or anti-PDCoV N (SD55-197, Medgene Labs) or anti-TGEV N (211.56, Median Diagnostics, Republic of Korea). After washing twice with PBS, cells were incubated with goat anti-mouse IgG alkaline phosphatase antibodies (Abcam). Fluorescence was developed by adding alkaline phosphatase substrate (ImmPACT™ Vector® Red). Fluorescence images were taken under a fluorescence microscope (Olympus).

2.8. Construction of the N-deficient PEDV infectious clone (pPEDV-mCherry-ΔN)

The N-deficient PEDV infectious clone (pPEDV-mCherry-ΔN) was constructed using a strategy described previously (Jengarn et al., 2015; Wanitchang et al., 2019). A frameshift mutation was used to silence N expression by mutating the original start codon in the N ORF, ATATGT, to ATATGT in an intermediate plasmid, pTZ-GH, containing a PEDV genome fragment (designated ‘GH’; Fig. 6A) with the 5’ end of the N gene as described previously (Jengarn et al., 2015). The site-directed mutagenesis was performed in the pTZ-GH cloning vector. The mutated fragment was amplified by primers that facilitated subsequent In-Fusion ligation (In-Fusion HD, Clontech) into a pre-digested pSMART-BAC plasmid containing the rest of the PEDV genome (Jengarn et al., 2015) to yield pSMART-BAC-PEDV-mCherry-ΔN (designated pPEDV-mCherry-ΔN).

2.9. Rescue of reverse genetics-derived PEDV

HEK293T cells were transfected with 2 μg of the indicated infectious clone (or co-transfected with the indicated amount of a CoV N expressing plasmid) using FuGENE HD (Promega) according to the manufacturer’s instructions. At 72 hpt, cell lysates were prepared for western blot analysis as described previously, and supernatants were transferred for adsorption on VeroE6 cells for 1 h at 37 °C. For viral rescue of PEDV-mCherry-ΔN, VeroE6 cells stably expressing PEDV N was further transfected with 2 μg of pCAGGS-PEDV N for 24 h before virus adsorption to ensure sufficient expression of PEDV N in the infected cells. The inocula were then removed, and the cells were washed once with PBS and supplied with 2 ml fresh OptiMEM with 0.1% TrypLE (Thermo Scientific). At indicated time points, infected cells were imaged under a fluorescence microscope (Olympus), and the supernatants were collected for subsequent experiments or viral titer analysis by TCID50 assay.

2.10. Virus infection

VeroE6-based cell lines (5 × 10^6 cells/ml) were plated in a six-well plate. At 24 h, the cells were washed once with PBS and treated with 1 ml of virus at the indicated multiplicity of infection (MOI). At 1 h post-adsorption, the inoculum was removed, and the cells were washed once with PBS and supplied with 2 ml of fresh OptiMEM containing 0.1% TrypLE. Extent of PEDV infection was monitored by mCherry fluorescence under a fluorescence microscope. Cell lysates or
supernatants were harvested at the indicated time points for further analysis. For viral infection in the transient CoV N expression experiment, VeroE6 cells were transfected with 1 or 2 μg of pCAGGS-PEDV N-Myc, pCAGGS-PDCoV N-HA, pCAGGS-TGEV N-FLAG, or the empty pCAGGS vector and were incubated for 24 h to allow for protein expression. Cells were then infected with PEDV-mCherry (MOI = 0.0001) as described above.

2.11. TCID<sub>50</sub> assay

Monolayers of VeroE6 cells in 96-well plates were washed once with PBS. One hundred microliters of 10-fold serially diluted virus in OptiMEM with 0.1% TrypLE was added to the cells (8 wells per each dilution). At 72 h post-infection (hpi), the infected cells were scored by mCherry expression under a fluorescence microscope. TCID<sub>50</sub> titers were calculated using the Reed-Muench method (Reed and Muench, 1938).

2.12. RT-qPCR

For analysis of viral RNA synthesis, total RNA was extracted from VeroE6 cell lines expressing various N infected with PEDV-mCherry (MOI = 0.0001) at indicated time points using the RNA extraction kit (Thermo Scientific). DNaseI (Fermentas) was used to treat the RNA (15 min at 37 °C) before inactivation with EDTA (10 min at 65 °C). One-step RT-qPCR was performed with the Luna Universal One-Step RT-qPCR mix (New England Biolabs) as described previously (Liwnaree et al., 2019). Relative quantities of RNA accumulation were evaluated using the 2<sup>-ΔΔCt</sup> method normalized against viral RNA levels from infected VeroE6 cells at 24 hpi. Values are reported as averages ± SEM from three independent experiments.

2.13. Statistical analysis

GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA, USA) was used for figure preparation and statistical analyses of virus titers in Figs. 4 and 5. KaleidaGraph 4.1 (Synergy) was used for figure preparation and statistical analyses of GST pulldown in Fig. 2 and RT-qPCR results in Fig. 5.

3. Results

3.1. Cross-species interactions occur between swine enteric coronavirus nucleocapsid proteins

During co-infection, viruses can influence the course of infection of other viruses via direct physical interactions between viral components. To explore the possibility that swine CoV N proteins might affect replication of other co-infecting CoVs, we first investigated cross-species interaction between these N proteins. Specifically, we asked if N proteins from TGEV or PDCoV can interact with PEDV N and possibly affect PEDV replication. We first assessed interspecies protein-protein interaction by co-immunoprecipitation. HEK293T cells were co-transfected with pCAGGS-PEDV N-Myc, pCAGGS-PDCoV N-HA, pCAGGS-TGEV N-FLAG, or the empty pCAGGS vector and were incubated for 24 h to allow for protein expression. Cells were then infected with PEDV-mCherry (MOI = 0.0001) as described above.

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PDCoV N-HA or TGEV N-FLAG. At 48 hpt, lysates were prepared and incubated with agarose beads coupled to anti-Myc antibodies prior to elution and analysis by western blotting. As expected, HA-tagged PEDV N could be precipitated with Myc-tagged PDCoV N but not with empty beads, indicating specificity of the homo-oligomeric interaction (Fig. 1A). Interestingly, both N proteins from related CoVs also displayed specific interaction with PEDV N, both co-eluting with Myc-tagged PEDV N (Fig. 1A). Nevertheless, we observed slight non-specific binding in the case of PDCoV N-HA despite stringent washing conditions. Therefore, we utilized a GST pulldown assay as an alternative and independent verification of these results.

For GST pulldown, the bait proteins, GST and GST-PEDV N, were expressed in bacterial BL21 (DE3*) cells, and the prey proteins, HA-PEDV N, PDCoV N-HA and TGEV N-FLAG, were expressed in HEK293T cells. Bacterial and HEK293T cell lysates were incubated with GSH FF resin overnight to allow for bait-prey interaction. After removing non-specific binding by multiple rounds of washing, the bound proteins were released and analyzed by SDS-PAGE. Bait and prey proteins were detected by Coomassie staining and western blotting with indicated antibodies, respectively. HA-PEDV N could only be observed when pulled down with GST-PEDV N, while no HA-PEDV N was observed when pulled down with GST, demonstrating the specificity of the assay (Fig. 1B). Next, we performed the GST pulldown experiment with PDCoV N and TGEV N as prey proteins. Similarly, we found that PDCoV N-HA and TGEV N-FLAG only co-eluted with GST-PEDV N, indicating that N proteins from both TGEV and PDCoV bind to PEDV N specifically (Fig. 1B). Both co-immunoprecipitation and GST pulldown results indicated that interspecies hetero-oligomers of CoV N proteins are possible, suggesting conservation of the oligomerization motifs and function of nucleocapsid proteins among swine CoVs. These results encouraged us to further probe the factors affecting the nature of these interactions.

The cross-association capability of PDCoV N or TGEV N could possibly interrupt homo-oligomerization between PEDV N and lead to competitive binding between PEDV N and other CoV N proteins. Using the GST pulldown assay, we further probed the interplay between these CoV N proteins to see how PEDV N homo-oligomers would be affected in the presence of other CoV N proteins. HEK293T cells were co-transfected with pCAGGS-HA-PEDV N and varying amounts of pCAGGS-PDCoV N-HA or pCAGGS-TGEV N-FLAG. At 48 hpt, lysates were prepared and incubated with GST-PEDV N-containing bacterial lysate and GSH FF resin overnight. GST pulldown revealed that, upon increasing amounts of co-eluting PDCoV or TGEV N, co-eluting HA-tagged PEDV N decreased reciprocally (Fig. 2). This indicates, to a certain level, a competition between homo-oligomerization of PEDV N and hetero-oligomerization with nucleocapsid proteins from other CoVs, and suggests that these CoV N proteins utilize the same motifs or binding interfaces in oligomeric complex formation or harness common factors such as RNA that can mediate oligomeric interactions.

One of the primary functions of CoV N proteins is to bind and organize viral RNA genomes for viral assembly (McBride et al., 2014). However, there have been conflicting reports about the necessity of viral RNA in promoting or assisting CoV N self-oligomerization. Some reports observed RNaseA susceptibility of the oligomeric complex (Narayanan et al., 2003; Verheije et al., 2010), while others detected intact higher CoV N oligomers despite the absence of RNA (Cong et al., 2017; Jayaram et al., 2006; Ma et al., 2010). To investigate whether the presence of RNA influences interspecies association between these swine enteric CoV N proteins, GST pulldown experiments were performed with or without RNaseA treatment. First, however, we tested whether or not RNA affected PEDV N homo-oligomerization in this assay setup. HEK293T cell lysate containing HA-tagged PEDV N was mixed with GST-PEDV N bacterial lysate and split into two equal parts. The lysate mixtures were either treated with RNaseA or PBS buffer for 30 min at 4 °C. The absence of RNA after the treatment was assured by performing RT-PCR reactions with primers specific to the PEDV N gene (Fig. 3). The lysate mixtures were then incubated with GSH FF resin overnight to compare the amount of HA-PEDV N pulled down by GST-PEDV N in the presence or absence of RNA. Our results across three independent experiments consistently showed a slight but noticeable increase in GST-PEDV N in the elution fraction after RNaseA treatment (Fig. 3). Moreover, the amount of HA-PEDV N pulled down in the absence of RNA seemed to be greater than that observed in the presence of RNA, suggesting that RNA interferes with the ability of PEDV N to bind to each other. We also consistently observed a slight decrease in the level of tagged PEDV N in the input fraction after the lysates were treated with RNaseA, implying decreased overall stability of PEDV N in the absence of RNA. Similar decreases in protein levels were not observed with TGEV or PDCoV N proteins (see below), suggesting specificity of the phenomenon.

Interestingly, when the experiments were performed on lysates containing PDCoV N-HA or TGEV N-FLAG, a completely opposite trend...
was observed for hetero-oligomerization. While increased GST-PEDV N was still similarly observed in the eluted fraction in the absence of RNA, both PDCoV N and TGEV N were pulled down by GST-PEDV N much less efficiently in the absence of RNA (Fig. 3). It should be noted that the total amount of the tagged CoV N proteins did not decrease, arguing against the possibility that TGEV or PDCoV N proteins were more prone

Fig. 5. PEDV replication is promoted by TGEV N and suppressed by PDCoV N. (A) VeroE6 clones stably expressing CoV N from either PEDV, PDCoV or TGEV were generated by lentiviral transduction and selected based on relatively equal expression as determined by western blot and immunofluorescence analyses. The selected clones of VeroE6 cells were infected with PEDV-mCherry (MOI 0.0001) and monitored at the indicated time points for PEDV spread by (B) fluorescence microscopy and (C) production of infectious particle by TCID<sub>50</sub> assay. Values shown are averages ± SEM of three independent experiments. (D) Total RNA was extracted from infected cells in (B) to monitor subgenomic mRNA (top panel) and genomic RNA (bottom panel) synthesis by RT-qPCR with primer pairs specific to sgRNA of S or to ORF1a, respectively. Values are expressed relative to those from VeroE6 cells at 24 hpi (set to one) and are averages ± SEM of three independent experiments. *p < 0.05, **p < 0.01 compared to VeroE6 cells at the same time point.
to degradation without the bound RNA (Fig. 3, ‘input’ lanes). These results suggest that the presence of RNA immensely helps strengthen hetero-oligomerization between PEDV N and other CoV N proteins but renders homo-oligomerization between PEDV N weakened. Although not an absolute requirement for interspecies complex formation, RNA could act as a bridge for N proteins from different species to form the complex, implying the heterologous protein-protein interactions are significantly weaker or less specific than the homologous interaction. Even though the levels of RNA purified from RNaseA-treated lysates were below the detection limit of spectrophotometer (data not shown) and could not produce visible RT-PCR products, we could not rule out the possibility of leftover small RNA fragments mediating heterologous CoV N protein binding, giving rise to much fainter TGEV N or PDCoV N bands that co-eluted with GST-PEDV N. In summary, the results in this section demonstrate conserved association capability among different CoV N proteins but suggest some differentiating features, especially the influence of RNA on each complex, between homo- and hetero-oligomers involving PEDV N.

3.2. PEDV replication is promoted by TGEV N but suppressed by PDCoV N

Hetero-oligomer formation as demonstrated in the previous section formed a basis for our investigation into N-mediated virus-virus interaction. As it has been shown for other viruses, it is not unreasonable to assume that the specific and competitive binding between N proteins of PEDV and other CoVs could interrupt regular functions of PEDV N during viral growth. To investigate how this protein-protein interaction might affect PEDV replication, we transiently transfected VeroE6 cells with varying amounts of the pCAGGS plasmid expressing N proteins from either PDCoV or TGEV for 24 h before infection with PEDV-mCherry (MOI = 0.0001) and followed the course of viral replication for each condition. The levels of CoV N expression were verified by western blotting against tags attached to each N protein (Fig. 4A). Cell culture supernatants were collected at 24, 48 and 72 hpi to determine virus titers by TCID50 assay (Fig. 4B–D). Interestingly, VeroE6 cells transiently transfected with either PEDV N or TGEV N showed a dose-dependent increase, up to an order of magnitude, in PEDV-titer compared to infection in wild-type VeroE6 cells (Fig. 5B). In- terestingly, PEDV-mCherry titer levels were significantly higher in VeroE6-TGEV N cells than in VeroE6-PDCoV N cells, especially at high levels of PDCoV N expression (Fig. 4C).

Based on their ability to cross-associate and affect PEDV RNA synthesis and replication, we speculated that N proteins from other CoVs, especially TGEV, may have interchangeable functions with PEDV N in PEDV replication. In an attempt to answer which roles and functions of PEDV N could be replaced by other CoV N proteins, we constructed an N-deficient PEDV infectious clone, pPEDV-mCherry-ΔN, and determined whether supplying CoV N proteins in trans could replace the missing PEDV N protein from the PEDV’s viral genome. Since the nucleotide fragment of the N gene in the viral genome may play other important roles in viral genome packaging or transcription, we employed a frameshift mutation strategy to silence N expression from the infectious clone while minimally disturbing the nucleotide sequence of the N gene. The original start codon in the N ORF, ATGG, was mutated to ATATGCT, thereby shifting the start codon by two nucleotides, resulting in a +2 frameshift during translation (Fig. 6A). The mutated sequence theoretically produced a short unrelated peptide of 21 amino acids with early termination. We intentionally kept the mCherry gene in the construct as a visual indicator for sub-genomic mRNA transcription and protein expression in the virus rescue process. To verify the lack of N expression during PEDV reverse genetics rescue, we transfected the N-deficient infectious clone into HEK293T cells. Western blot analysis confirmed that N expression was completely diminished in cells transfected with the N-deficient infectious clone compared to the wild-type pPEDV-mCherry (Fig. 5B).

With the N-deficient infectious clone in hand, we next investigated PEDV reverse genetics rescue in the presence of other CoV N proteins.
HEK293T cells were co-transfected with pPEDV-mCherry-ΔN and a pCAGGS plasmid expressing CoV N protein. Expression of mCherry from the infectious clone was used as a visual proxy for production of viral RNA and proteins in HEK293T cells during the reverse genetics step. No detectable fluorescence was observed from co-transfection with the empty pCAGGS vector, confirming the role of PEDV N protein during at least the first round of viral RNA transcription and viral protein translation (Fig. 7A). Co-transfection with pCAGGS-PEDV N-Myc yielded a substantial fraction of red cells, while slightly lower amount of mCherry protein expression was observed from cells cotransfected with pCAGGS-TGEV N-FLAG. Co-transfection with pCAGGS-PDCoV N-HA resulted in very low, yet detectable, numbers of mCherry-positive cells (Fig. 7A). Cell lysates were prepared at 72 hpt and probed for S expression using anti-S antibody to corroborate visual inspection. As a negative control, cells transfected with pPEDV-mCherry-ΔN and pCAGGS vector displayed no detectable trace of S (Fig. 7B). Cells co-transfected with pCAGGS expressing CoV N proteins showed varying levels of S expression, with PEDV N-Myc giving the highest S expression and PDCoV N-HA giving the lowest (Fig. 7B). TGEV N-FLAG expression resulted in noticeably lower S expression compared to PEDV N-Myc but still higher than that of PDCoV N-HA (Fig. 7B). The levels of spike protein expression from the N-deficient clone in each condition correlated with the level of mCherry expression as observed by fluorescence microscopy (Fig. 7A and B). These results suggested that CoV N proteins from alphacoronaviruses can aid viral RNA and protein production from in-}

**4. Discussion**

Although the swine industry has focused on PEDV alone as one of its largest threats, few have attempted to understand the implications of swine enteric CoV co-infection despite multiple epidemiological studies. As the true impact of co-infection on disease outcomes still awaits further investigation, these viruses could potentially exert influence on each other, considerably shaping the course of their infection or evolution. Molecular interactions underlying these possible viral interactions also remain to be identified and characterized. In this work, we tried to understand how different enteric swine CoV N proteins, one of the most abundant structural proteins, interact and affect functions of PEDV N and replication kinetics of PEDV.

First, we provided evidence of cross-interaction between CoV N proteins despite low sequence similarity among them. Amino acid sequence analyses on PEDV, TGEV and PDCoV N proteins revealed that the proteins can be divided roughly into two structural domains (NTD...
and CTD) with relatively high conservation and three intrinsically disordered regions (IDRs) with relatively low conservation located in the middle and at the two termini (Fig. 8). This domain analysis is consistent with previous crystallographic studies on SARS-CoV and MHV N, which also suggested conserved general structural organization of CoV N (Chang et al., 2009; Cong et al., 2017; Ma et al., 2010; Yu et al., 2006). Moreover, immunological data documenting cross-reactivity of antibodies against N proteins from TGEV or PDCoV with PEDV N further implied shared topological similarity between these CoV N proteins (Gimenez-Lirola et al., 2017; Lin et al., 2015; Ma et al., 2016). Nevertheless, precise molecular mechanisms mediating interspecies oligomerization of CoV N proteins remain to be determined. Structural and biochemical studies of SARS-CoV N strongly suggest a role of CTD as a major mediator for CoV N dimerization. Moreover, CTDs of N proteins from SARS-CoV and porcine reproductive and respiratory syndrome virus (PRRSV), despite diverse sequences, could adopt well-conserved structures, implying similar approaches to oligomerization, at least at a gross structure level (Yu et al., 2006). However, since CoV N CTDs formed intricate molecular interaction networks during dimerization (Chang et al., 2014), it is unlikely that CoV N proteins from different species could replace all these specific molecular interactions. Consistent with our results that hetero-oligomer formation is highly sensitive to the presence of RNA, a more likely possibility is that RNA-protein interactions play a more dominant role in interspecies CoV N interactions than protein-protein interactions.

Next, we asked if N proteins from different CoVs can replace PEDV N function during viral RNA and protein synthesis. During the rescue of the N-deficient PEDV infectious clone, we showed that supplying N protein from any CoV in trans can initiate first-round sgRNA transcription and protein expression. Most likely, this function stems from the shared intrinsic ability of CoV N to bind viral RNA and act as an RNA chaperone that initiates RNA replication and/or transcription processes (Zuniga et al., 2007, 2010). These results support previous notions that CoV N proteins play important roles during CoV rescue. For instance, rescue of TGEV infectious RNA required at least co-transfection of mRNA encoding the N protein (You et al., 2000). Schelle et al. demonstrated that an N-deficient HCoV229E RNA vector replicon carrying the GFP gene can produce sgRNA and express GFP from the vector when co-transfected with N from PEDV, a closely related virus, but not from MHV, a more distantly-related one (Schelle et al., 2005). We noted...
a similar preference in our experiments. Between TGEV N and PDCoV N, the former is more effective in replacing the indigenous PEDV N than the latter. Since both PEDV and TGEV belong to the alphacoronavirus genus, while PDCoV is classified as a deltacoronavirus, TGEV N would be expected to be functionally and structurally closer to PEDV N, and hence able to form replication/transcription complexes and participate in PEDV replication with higher compatibility than PDCoV N.

Although both TGEV N and PDCoV N can oligomerize and perform some functions during g/sgRNA transcription, they alone cannot totally replace all PEDV N’s functions. Without native PEDV N, infectious PEDV virions could not be detected in the presence of TGEV or PDCoV N, implying that other functions are not interchangeable. These critical functions might involve viral core assembly as CoV N participates in many steps during viral core or virion assembly (McBride et al., 2014). For instance, several reports showed that CoV N is involved, directly or indirectly, with recognition of specific packaging signals for genome packaging (Hsieh et al., 2005; Hsin et al., 2018; Kuo et al., 2014). Since RNA packaging signals among CoVs are distinct in terms of length and sequence, even closely related CoV N might not be able to recognize PEDV packaging signals. Similar compatibility issues have been raised in the case of viral interference between different types of influenza viruses (Baker et al., 2014). Another critical step that could be disrupted by heterologous CoV N is viral assembly through interactions between N and M proteins. It is widely accepted that CoV N and M proteins interact to form the viral core, but different CoVs utilize different regions of N in binding to their own M proteins. For instance, while MHV utilizes the very C-terminal end of N to bind M, SARS-CoV N uses the middle disordered linker for the interaction (He et al., 2004b; Narayanan et al., 2000). In addition, although both TGEV and MHV N proteins used their C-termini to bind M, the N binding sites on TGEV M and MHV M are very distinct (Hurst et al., 2004b; Narayanan et al., 2000). In all, it is conceivable that not all of N functions could be replaced by the protein originating from other viral species, even though N proteins from PEDV, TGEV and PDCoV can cross-assemble, form high-order oligomers and aid in RNA synthesis processes. Notably, we observed completely opposite effects of TGEV and PDCoV N proteins on PEDV replication. We noticed that, in the presence of RNA, homologous binding between PEDV N itself is weakened and cross binding between PEDV N and the other N proteins is strengthened. During co-infection, where viral gRNA is ubiquitous, the RNA could act as a tethering bridge to stabilize the formation of heterologous oligomers, which may not be a productive core structure for PEDV virion assembly and may interrupt the optimal processes in viral

![Fig. 8. Analysis of CoV N sequences reveals low sequence similarity but conserved structural organization.](image)

Multiple amino acid sequence alignment was performed with T-coffee (Notredame et al., 2000). The color scheme denotes consistency between different alignment methods ranging from bad (low agreement; blue and green) to good (high agreement; pink). Bold and normal lettering denote ordered and disordered regions as predicted by the PrDOS program (Ishida and Kinoshita, 2007). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
replication that prefer the homo-oligomer of N. Such interruption could have resulted in similar suppression. Instead, we observed a small enhancement effect on PEDV replication in the presence of TGEV N. This could be the net outcome of other oligomerization-independent roles of TGEV N that indirectly enhance PEDV replication. A study by Zhao et al. showed that, when compared to single-virus infection, co-infection of TGEV and PEDV could synergistically enhance rupture to tight and adherens junctions of the intestinal epithelial cells IPEC-J2 by suppressing expression of proteins that help form the junction (Zhao et al., 2014). Such altered barrier integrity is possibly caused by CoV N and facilitate viral replication. Another possible role of PEDV or TGEV N that could lead to enhancement of PEDV replication is to act as a viral suppressor of RNA silencing (Cui et al., 2015). Using MHV N as a model, they found that CoV N can bind and suppress host antiviral RNAs, leading to increased viral protein expression and subsequent viral replication. More remarkably, they found that N proteins of alphacoronaviruses, including TGEV and PEDV, also have this ability. Indeed, among different CoV N proteins, they found that TGEV N was among the most active and MERS-CoV N was the least active (Cui et al., 2015). Therefore, N from TGEV may also employ this mechanism in promoting PEDV replication, while some other CoV N proteins might not be capable of this function. These are interesting hypotheses currently under investigation, which could reveal more complexity of co-infection between these viruses.

In summary, this work shows how other swine enteric CoV N proteins can substitute for endogenous PEDV N in some functions but not in others, demonstrating both conservation and specificity among re- teins can substitute for endogenous PEDV N in some functions but not in others, demonstrating both conservation and specificity among re- teins can substitute for endogenous PEDV N in some functions but not in others.


