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Identification and subcellular localization of porcine deltacoronavirus accessory protein NS6

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A B S T R A C T
Porcine deltacoronavirus (PDCoV) is an emerging swine enteric coronavirus. Accessory proteins are genus-specific for coronavirus, and two putative accessory proteins, NS6 and NS7, are predicted to be encoded by PDCoV; however, this remains to be confirmed experimentally. Here, we identified the leader-body junction sites of NS6 subgenomic RNA (sgRNA) and found that the actual transcription regulatory sequence (TRS) utilized by NS6 is non-canonical and is located upstream of the predicted TRS. Using the purified NS6 from an Escherichia coli expression system, we obtained two anti-NS6 monoclonal antibodies that could detect the predicted NS6 in cells infected with PDCoV or transfected with NS6-expressing plasmids. Further studies revealed that NS6 is always localized in the cytoplasm of PDCoV-infected cells, mainly co-localizing with the endoplasmic reticulum (ER) and ER-Golgi intermediate compartments, as well as partially with the Golgi apparatus. Together, our results identify the NS6 sgRNA and demonstrate its expression in PDCoV-infected cells.

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1. Introduction
Porcine deltacoronavirus (PDCoV) is an emerging swine enteric coronavirus that causes diarrhea in nursing piglets (Chen et al., 2015b; Hu et al., 2015; Jung et al., 2015; Ma et al., 2015). It was first detected in Hong Kong in 2012 (Woo et al., 2012). In early 2014, outbreaks of PDCoV were announced in swine populations in Ohio, Illinois, and Iowa, and it rapidly spread to multiple states in the United States (Li et al., 2014; Marthaler et al., 2014a; Marthaler et al., 2014b; Wang et al., 2014a, 2014b). Thereafter, PDCoVs were detected or caused outbreaks in Korea (Lee et al., 2016; Lee and Lee, 2014), China (Dong et al., 2015; Song et al., 2015; Wang et al., 2015) and Thailand (Janetanakit et al., 2016; Madapong et al., 2016), posing significant economic concerns and gaining considerable attention (Jung et al., 2016; Lorsirigool et al., 2016; Zhang, 2016).

PDCoV is an enveloped, single-stranded, positive-sense RNA virus belonging to the newly identified genus Deltacoronavirus within the family Coronaviridae. Its genome is approximately 25.4 kb in length, making it the smallest genome among the known coronaviruses (CoVs). The genome arrangement of PDCoV is similar to those of other CoVs with the typical gene order: 5′UTR-ORF1a-ORF1b-S-E-M-NS6-N-NS7-3′UTR (Song et al., 2015; Woo et al., 2012). Although the biological functions of PDCoV-encoded proteins have not been studied in detail, based on studies of other known CoVs, PDCoV ORF1a and ORF1b probably encode two viral replicase polyproteins, pp1a and pp1ab, which are predicted to be proteolytically cleaved into 15 mature nonstructural proteins responsible for viral replication and transcription; ORFs S, E, M, and N encode viral structural proteins, Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N), respectively (Chen et al., 2015a; Lee and Lee, 2014; Li et al., 2014; Woo et al., 2010). Additionally, NS6 and NS7 each encode a putative accessory protein (Fig. 1A).

Accessory proteins are genus-specific for coronavirus (Tan et al., 2006); however, different CoVs contain different numbers of accessory genes and proteins. For example, alphacoronavirus feline infectious peritonitis virus encodes five accessory proteins (p3a, p3b, p3c, p7a, and p7b), while only one accessory protein is encoded by porcine epidemic diarrhea virus (PEDV); betacoronavirus severe acute respiratory syndrome coronavirus (SARS-CoV) encodes a total of eight accessory proteins; and the most studied gammacoronavirus, infectious bronchitis virus (IBV), encodes four accessory proteins (Liu et al., 2014). Although coronavirus accessory proteins have generally been considered to be dispensable for viral replication in vitro (Hajjama et al., 2004; Yount et al., 2005), extensive functional studies have shown that many accessory...
proteins are involved in immune modulation (Kopecky-Bromberg et al., 2007) and viral pathogenesis in vivo (De Haan et al., 2002). The field of coronavirus accessory proteins has gained considerable attention in recent years. In the PDCoV genome, there are two putative accessory genes, NS6 and NS7. NS6 is predicted to be located in the genome between M and N and to encode a 94-amino acid peptide, while NS7 is predicted to be located within the N gene (Lee and Lee, 2015; Woo et al., 2012). To date, there are no reports regarding the expression of PDCoV accessory genes or the identification of an associated transcription regulatory sequence (TRS) for production of these subgenomic RNAs (sgRNAs) in virus-infected cells.

Here, we identified the leader-body fusion site and TRS of NS6 sgRNA. By using monoclonal antibodies (MAbs) that recognize the putative NS6 protein, we demonstrated that the predicted NS6 could be expressed and localized to the cytoplasm in PDCoV-infected cells, providing the first biochemical evidence for the existence of PDCoV NS6.
2. Results

2.1. Identification of PDCoV NS6 sgRNA

A vital feature of coronavirus transcription is the set of sgRNAs produced by discontinuous transcription. Each sgRNA contains a common 5’ leader sequence derived from the 5’ end of the viral genome and a so-called “body” sequence, which represents sequences from the 3’-poly(A) stretch to a position that is upstream of each genomic ORF encoding a structural or accessory protein (Sawicki et al., 2001, 2007). The fusion of leader and body sequences is processed, at least in part, by cis-acting elements termed transcription regulatory sequences (TRSs) (Hussain et al., 2005; Sawicki et al., 2007). Thus, determining the junction region of the leader and body sequences can verify the existence of the corresponding sgRNAs. To identify the possible NS6 sgRNA, total intracellular RNA was extracted from LLC-PK cells infected with PDCoV, and sgRNAs were amplified by leader-body junction RT-PCR with the primers Leader-F and NS6r (Fig. 1A) as reported previously (Dye and Siddell, 2005; Hussain et al., 2005). As shown in Fig. 1B, two specific RT-PCR products of approximately 1.0 kb and 500 bp were obtained and then isolated from the agarose gel, followed by cloning and sequencing. At least 10 independent clones were sequenced. The results reveal that the two specific RT-PCR products are sgRNAs M and NS6 (Fig. 1B). Our sequence analysis of these RT-PCR products indicates that the sgRNA M contains a leader sequence followed by the typical PDCoV TRS (ACACCA) (Woo et al., 2009, 2012), as expected for the predicted M sgRNA transcript. However, to our surprise, the leader-body fusion site (ACACCA) for sgRNA NS6 is 148 nucleotides, rather than the predicted 46 nucleotides, upstream of the AUG start codon of the NS6 gene (Fig. 1C). Furthermore, there is a nucleotide difference (underlined) in the TRS sequence (ACACC) compared with that of the M gene (ACACCA), indicating that the TRS utilized by the NS6 gene is non-canonical.

2.2. Prokaryotic expression and purification of NS6 and generation of anti-NS6 monoclonal antibodies (MAbs)

To further investigate the existence of NS6 at the protein level, we needed to prepare an NS6-specific antibody. To this end, a 285-bp cDNA fragment of the NS6 gene was amplified by RT-PCR and cloned into a prokaryotic expression vector, pET28a(+) leading to pET28a-NS6. This plasmid was then transformed into Escherichia coli Rosetta (DE3), and gene expression was induced with 0.8 Mm isopropylthiogalactopyranoside (IPTG). A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that the fusion protein His-NS6 was efficiently expressed in the form of inclusion bodies in E. coli, the size of which was consistent with the predicted molecular weight of the recombinant protein (Fig. 2A). The results of western blotting assays demonstrated that the recombinant NS6 protein was specifically recognized by anti-His MAb (Fig. 2B). Subsequently, the protein was purified (Fig. 2C), and the concentration of the purified NS6 protein was 0.46 mg/mL as measured by a Trace ultraviolet spectrophotometer.

Using the purified NS6 protein as an immunization antigen and the hybridoma technique, two hybridoma cell lines, named 4B9 and 2G3, were acquired through screening and subcloning. To further confirm the specificity of these two MAbs, a eukaryotic expression plasmid encoding NS6 was constructed by cloning the cDNA of the NS6 gene into pCAGGS with the primers NS6-F and NS6-R (Table 1), which was then transfected into LLC-PK cells; indirect immunofluorescence assays (IFAs) and western blotting assays were then performed with the obtained MAbs. As shown in Fig. 3A, specific fluorescence could be observed in cells transfected with pCAGGS-NS6, while neither MAb recognized cells transfected with the empty vector, pCAGGS. The results of western blotting assays also show that approximately 11-kDa specific protein bands, the size of which is identical to the size of the predicted NS6 protein (Fig. 3B), could be detected by both MAbs in the lysates of cells transfected with pCAGGS-NS6.

Table 1: Sequence of the primers used for identification of sgRNA and construction of plasmids.

| Primer | Nucleotide sequence* (5′–3′) | Nucleotide positionb |
|--------|-------------------------------|---------------------|
| Leader-F | AACATTATCTCCCTAGCTTCG | 40–60 |
| NS6r | AAGAATGACTTGGAGCGATGGTCGA | 23936–23960 |
| NS6-F | ACTGAAATCCATGGCAACTGCCAATCTGCAG | 23694–23714 |
| NS6-R | GCCCTCAGAGATTAAATCCATCTTCAGAATG | 23954–23975 |

a Restriction enzyme recognition sites are shown in bolded and underlined type.

b The number represents nucleotide position corresponding to the nucleotide sequence of the PDCoV CHN-HN-2014 (GenBank accession no KT316560).
2.3. Expression and subcellular localization of NS6 protein in PDCoV-infected cells

To test if NS6 is indeed expressed during PDCoV infection, LLC-PK cells were infected with PDCoV at a multiplicity of infection (MOI) of 5.0. The cells were fixed and either subjected to IFA or collected for western blotting at 12 h post-infection (hpi). For IFA, obvious immunofluorescence was observed in the cytoplasm in PDCoV-infected LLC-PK cells, while no fluorescence signals were observed in mock-infected cells (Fig. 4A). Also, specific protein bands similar to the size observed in pCAGGS-NS6-transfected cells could be detected by both MAbs in lysates of PDCoV-infected LLC-PK cells (Fig. 4B).

To further investigate the expression kinetics of NS6 protein in detail during PDCoV infection, LLC-PK cells seeded onto microscope coverslips in 24-well plates were mock-infected or infected with PDCoV at a MOI of 10. At 3, 6, 9, 12, and 15 hpi, cells were fixed for IFA with MAb 4B9. As shown in Fig. 5A, specific fluorescence could be observed at 6 hpi, and increasingly more fluorescence was readily detected as the infection progressed. Up to 15 hpi, obvious cytopathic effects characterized by cell shrinkage and detachment were observed.
Fig. 5. Expression kinetics and subcellular localization of NS6 protein in PDCoV-infected LLC-PK cells. LLC-PK cells were plated onto coverslips and then mock-infected or infected with PDCoV at a MOI of 10. (A) At the indicated times post-infection, cells were fixed for IFA with MAb 4B9 and Alexa Fluor 488-conjugated donkey anti-mouse IgG (green). (B) At 12 hpi, cells were fixed and stained with NS6-specific MAb (4B9) and rabbit anti-GRP94 (ER marker), rabbit anti-GS28 (Golgi marker), or rabbit anti-SEC31 (ER-Golgi intermediate compartment), followed by staining with secondary antibodies Alexa Fluor 488-conjugated donkey anti-rabbit IgG (green) or Alexa Fluor 594-conjugated donkey anti-mouse IgG (red). Nuclei were counterstained with DAPI (blue). Fluorescent images were acquired with a confocal laser scanning microscope (Fluoview ver. 3.1; Olympus, Japan).
The NS6 protein was localized in the cytoplasm during the entire experimental period (Fig. 5A).

To examine the precise subcellular localization of the NS6 protein in PDCoV-infected LLC-PK cells, the cells were fixed at 12 hpi and stained with both mouse anti-NS6 monoclonal antibody (MAb 4B9) and rabbit polyclonal antibodies against either the endoplasmic reticulum marker GRP94, the Golgi apparatus marker GS28, or the ER-Golgi intermediate compartment marker SEC31. NS6 protein was observed to predominantly colocalize with both the ER compartment and the ER-Golgi intermediate compartment, as well as to partially colocalize with the Golgi complex (Fig. 5B).

3. Discussion

Although coronavirus accessory proteins are not essential for virus replication in vitro (Casais et al., 2005; Yount et al., 2005), previous reports have made it clear that these accessory proteins are not redundant, but rather possess many functions, including modulating viral pathogenicity (De Haan et al., 2002) and acting as in vitro virus replication proteins. Although the molecular mechanisms of the leader-body fusion in coronavirus sgRNAs have not been elucidated fully, accumulating evidence suggests that most TRSs are derived from the leader TRS (TRS-L), which are considered canonical TRSs, while some non-canonical TRSs are derived from the body TRS (TRS-B) (Van Marle et al., 1999; Zuniga et al., 2004). The conserved hexanucleotide TRS used by PDCoV is ACACCA, as determined for M sgRNA by our study, while the TRS of NS6 sgRNA is ACACCT, which is identical to the core sequence of TRS-B, suggesting that NS6 uses a non-canonical TRS. Similar phenomena have also been found in sgRNAs 2-1 and 3-1 of SARS-CoV (Hussain et al., 2005). The differences in TRS sequence for different sgRNAs could play a regulatory role in controlling the abundances of different mRNAs (Bentley et al., 2013; Hussain et al., 2005; Zuniga et al., 2004). It is possible that the lower abundance of NS6 sgRNA is associated with its non-canonical TRS.

Although many sgRNA of different coronaviruses have been identified, their natural expression products in virus-infected cells are yet to be confirmed due to lack of specific antibodies. Here, we generated two MAbS against PDCoV NS6 and demonstrated that the NS6 protein could be detected in both PDCoV-infected cells and cells transfected with a eukaryotic expression construct of the NS6 gene. We also found that a similar intracellular localization distribution could be observed in both cells infected with PDCoV and those overexpressing NS6. Furthermore, the expression of NS6 could be detected at the early phase (6 hpi) of PDCoV infection. Subcellular localization analyses showed that NS6 localized partially with the Golgi, and predominantly with the ER complex and ER-Golgi intermediate compartment. Similar subcellular localization has also been reported for SARS-CoV ORF7a (also known as U122), an accessory protein incorporated into purified SARS-CoV particles (Fielding et al., 2004; Huang et al., 2006). It is well known that the ER-Golgi intermediate compartment is the site of coronavirus assembly and packaging (Klumperman et al., 1994; McBride and Fielding, 2012). The presence of NS6 in the ER-Golgi intermediate compartment suggests the possibility that NS6 plays a role in viral assembly and budding events. Indeed, our preliminary study found that NS6 could be detected in the purified virion by western blotting (data not shown); however, additional experimental evidence, especially confirmation with immunogold electron microscopy is required to support this idea.

Previous studies suggested that coronavirus accessory proteins are often dispensable for virus replication in vitro, but are required for optional replication and virulence in the natural host (McBride and Fielding, 2012; Zhao et al., 2009). PDCoV infection suppresses RIG-I-mediated interferon-β production (Luo et al., 2016), and previous studies suggested that some accessory proteins of coronavirus, such as SARS-CoV ORF3b (Freundt et al., 2009) and MERS-CoV ORF4b (Thornbrough et al., 2016), are interferon antagonists. Whether PDCoV NS6 possesses this property requires further study. In addition, BLAST search revealed that NS6 proteins share no amino acid similarities with other CoV accessory proteins or known host proteins. No putative transmembrane domain and functional domain were identified by TMHMM (www.cbs.dtu.dk/services/TMHMM) and InterProScan (www.ebi.ac.uk/interpro) analyses, respectively. Precise functions of NS6 protein need to be yet further explored. At present, we are making efforts to construct an infectious cDNA clone of PDCoV and hope to use a reverse genetics system to study the NS6 function in the PDCoV viral life cycle and pathogenesis.

In summary, we confirmed the existence of a separate NS6 sgRNA with a non-canonical leader-body fusion site. We also prepared two MAbs against PDCoV NS6 and demonstrated that NS6 proteins are indeed expressed in PDCoV-infected cells. The expression of NS6 could be detected as early as 6 hpi in the cytoplasm of PDCoV-infected cells. The expressed NS6 proteins in PDCoV-infected cells predominantly localize to the ER complex and ER-Golgi intermediate compartment. The identification of sgRNA NS6 and the expression and subcellular localization of NS6 protein lay the foundation for elucidation of the structure and function of NS6 in the PDCoV viral replication cycle.
4. Materials and methods

4.1. Cells, viruses, and reagents

LLC-PK and SP2/0 myeloma cells were respectively grown in Dulbecco’s Modified Eagle medium (DMEM) (Invitrogen) and RPMI 1640 Medium, each supplemented with 10% heat-inactivated fetal bovine serum, at 37 °C in a humidified 5% CO₂ incubator.

PDCoV isolate CHN-HN-2014 (GenBank accession no. KT336560), which was isolated from a suckling piglet with acute diarrhea in China in 2014, was propagated in LLC-PK cells in DMEM with which was isolated from a suckling piglet with acute diarrhea in PDCoV isolate CHN-HN-2014 (GenBank accession no. KT336560), the NS6 protein (100 4 weeks later, these mice were given a second immunization with the same amount of Freund’s incomplete adjuvant. Two weeks after the second inoculation, mouse antisera were collected, and the titers of antibody in the sera were assessed by performing indirect ELISA. Subsequently, mice with higher antibody titers were given a booster immunization of NS6 protein (200 μg) by intraperitoneal injection 3–5 days prior to their use in hybridoma production.

4.6. Production of anti-NS6 protein MAbs

Splenocytes from the mouse with the highest antibody titer following immunization with the NS6 protein were harvested and fused with SP2/0 myeloma cells under the condition of 50% polyethylene glycol (PEG4000) as a fusion agent. The resulting hybridoma cells were then cultured in 96-well plates at 37 °C in a humidified 5% CO₂ incubator in HAT screening culture medium with fetal bovine serum. One week later, positive hybridomas with coverage of one third to one half of the bottom of 96-well plates were filtered by indirect ELISA. Subsequently, the positive hybridoma cells were subcloned three times by the limiting dilution method. Finally, the stable hybridoma cells were passaged, and the resulting cell supernatants were collected for use in later experiments. Meanwhile, the stable hybridoma cells were stored in liquid nitrogen.

4.7. Indirect immunofluorescence assays (IFAs) and confocal microscopy

4.8. Western blotting analysis

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