Receptor usage and cell entry of porcine epidemic diarrhea coronavirus

Chang Liu [1, #], Jian Tang [2, #], Yuanmei Ma [3, #], Xueya Liang [3], Yang Yang [1], Guiqing Peng [4], Qianqian Qi [2, 5], Shibo Jiang [2, 5], Jianrong Li [3], Lanying Du [2, *], Fang Li [1, *]

[1] Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN 55455, USA

[2] Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY 10065, USA

[3] Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, Columbus, OH 43210, USA

[4] State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, Hubei, China

[5] Key Laboratory of Medical Molecular Virology of Ministries of Education and Health, Shanghai Medical College and Institute of Medical Microbiology, Fudan University, Shanghai, China

#These authors contributed equally to this work.

* Correspondence:
Fang Li (lifang@umn.edu); Lanying Du (ldu@nybloodcenter.org)

Key words: PEDV; spike protein; APN receptor; sugar co-receptor; virus infection; host range; tropism

Running title: Host range and tropism of PEDV

Number of words in text: 1303

Abstract

Porcine epidemic diarrhea coronavirus (PEDV) has significantly damaged America's pork industry. Here we investigated the receptor usage and cell entry of PEDV. PEDV recognizes protein receptor aminopeptidase N from pig and human, and sugar co-receptor N-acetylneuraminic acid. Moreover, PEDV infects cells from pig, human, monkey, and bat. These results support bats as an evolutionary origin for PEDV, implicate PEDV as a potential threat to other species, and suggest antiviral strategies to control its spread.

Text

Porcine epidemic diarrhea coronavirus (PEDV) causes large-scale outbreaks of diarrhea in pigs, and 80-100% fatality rate in suckling piglets (1-3). Since 2013, PEDV has swept throughout the US, wiped out more than 10% of America's pig population in less than a year, and significantly damaged the US pork industry (4-6). No vaccine or antiviral drug is currently available to keep the spread of PEDV in check. PEDV belongs to the α genus of the coronavirus family (7, 8), which also includes porcine transmissible gastroenteritis coronavirus (TGEV), bat coronavirus 512/2005 (BtCoV/512/2005), and human NL63 coronavirus (HCoV-NL63). Although PEDV and TGEV both infect pigs, PEDV is genetically more closely related to BtCoV/512/2005 than to TGEV, leading to the hypothesis that PEDV originated from bats (9).

Receptor binding and cell entry are essential steps in viral infection cycles, critical determinants of viral host range and tropism, and important targets for antiviral interventions. An envelope-anchored spike protein mediates coronavirus entry into cells.

The spike ectodomain consists of a receptor-binding subunit S1 and a membrane-fusion subunit S2. S1 contains two domains, N-terminal domain (S1-NTD) and C-terminal domain (S1-CTD), both of which can potentially function as receptor-binding domains (RBDs) (Fig. 1A) (10, 11). The capability of coronavirus RBDs to recognize receptor orthologs from different species is one of the most important determinants of coronavirus host range and tropism (8, 12-14). HCoV-NL63 S1-CTD recognizes human angiotensin-converting enzyme 2 (ACE2), whereas TGEV S1-CTD recognizes porcine aminopeptidase N (APN) and its S1-NTD recognizes two sugar co-receptors, N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) (15-18). Usage of sugar co-receptors is linked to the enteric tropism of coronaviruses (18, 19). It has been shown that PEDV uses porcine APN as its receptor (20). However, it is not known whether PEDV recognizes APN from other species or whether it uses sugar co-receptors. Addressing these questions will be critical for understanding the host range, tropism and evolutionary origin of PEDV, for evaluating its potential risk to other species particularly human, and for developing effective vaccines and antiviral drugs to curb the spread of PEDV in pigs and to other species.

To characterize the receptor usage of PEDV, here we identified the two S1 domains of PEDV based on the sequence similarity between PEDV and TGEV S1 subunits (Fig. 1B). The S1-NTD and S1-CTD of PEDV cover residues 19 to 252 and residues 509 to 638, respectively. However, expression of the two domains individually gave low yields. Instead, we expressed and purified a longer fragment (residues 19 to 638) using a previously described procedure (21, 22). This fragment contains both of the S1 domains and is termed S1-NTD-CTD (Fig. 2A). For comparison studies, we prepared

77 TGEV S1-NTD-CTD (residues 17 to 675) using the same procedure. We also expressed
78 and purified human and porcine APN as previously described (23, 24). These purified
79 recombinant proteins were subsequently used in biochemical studies.

80 We investigated the receptor binding capabilities of PEDV S1-NTD-CTD. First,
81 using a dot blot hybridization assay as previously described (24), we showed that PEDV
82 S1-NTD-CTD binds both porcine and human APN efficiently (Fig. 2B). Thus, both
83 porcine and human APN serve as efficient receptors for PEDV. In contrast, TGEV S1-
84 NTD-CTD binds porcine APN much more tightly than it binds human APN (Fig. 2B).
85 Second, using the dot blot hybridization assay as previously described (25, 26), we
86 demonstrated that PEDV S1-NTD-CTD binds bovine and porcine mucins both of which
87 contain a mixture of different types of sugar (Fig. 2C). Treatment of mucins with
88 neuraminidase removed part of the coated sugars, reducing the binding by PEDV S1-
89 NTD-CTD. Hence, sugar serves as a co-receptor for PEDV. As a comparison, TGEV S1-
90 NTD-CTD also binds these mucins. Third, using glycan screen array as previously
91 described (26), we identified Neu5Ac as the type of sugar most favored by PEDV (Fig.
92 2D, Table S1). Taken together, PEDV uses both porcine and human APN as its protein
93 receptors and Neu5Ac as a sugar co-receptor, whereas TGEV uses porcine APN and
94 sugar, but not human APN, as its receptors.

95 To further understand the receptor usage and also to investigate the cell entry of
96 PEDV, we performed a PEDV-spike-mediated pseudovirus entry (27). Retroviruses
97 pseudotyped with PEDV spike (i.e. PEDV pseudoviruses) efficiently entered MDCK
98 (canine kidney) cells exogenously expressing human or porcine APN, and these entries

could be blocked by anti-APN antibody (Fig. 3A). As a control, PEDV pseudoviruses could not enter MDCK cells not expressing human or porcine APN, consistent with a previous report that MDCK is non-permissive to PEDV infection (20). In contrast, TGEV pseudoviruses efficiently entered MDCK cells exogenously expressing porcine APN, but not those expressing human APN. Additionally, PEDV pseudoviruses efficiently entered both PK-15 (pig kidney) and Huh-7 (human lung) cells that endogenously express porcine and human APN, respectively (28, 29), and these entries could be blocked by anti-APN antibody and mucins (Fig. 3B, 3C). In contrast, TGEV pseudoviruses efficiently entered PK-15 cells, but not Huh-7 cells. These data collectively confirmed that human and porcine APN and sugar serve as receptors for PEDV and play important roles in PEDV-spike-mediated cell entry, whereas porcine APN and sugar, but not human APN, are receptors for TGEV.

To further examine PEDV entry into host cells, we carried out live PEDV infection in the following cell lines: PK-15 (pig kidney), ST (pig testis), Huh-7 (human liver), MRC-5 (human lung), Vero CCL-81 (monkey kidney), and Tb1-Lu (bat lung) cells. To this end, PEDV strain Ohio VBS2 was isolated from a piglet in Ohio, USA, and propagated in Vero CCL-81 cells using a procedure as previously described (30). Vero CCL-81-adapted PEDV was used to infect each of the above cell lines at a multiplicity of infection (MOI) of 1.0. The results showed that PEDV efficiently infects cells from pig, human, monkey, and bat (Fig. 4). It is worth noting that whereas pseudovirus entry is determined by receptor recognition and cell entry, the infection efficiency of live PEDV in cell culture is determined not only by receptor recognition and cell entry, but also by post-entry factors such as viral replication and release (31).

PEDV is a highly pathogenic and lethal pig coronavirus. This study investigated how PEDV recognizes host receptors from different species and how it infects cells from different species. First, we verified that PEDV recognizes porcine APN and infects pig cells. Second, for the first time to our knowledge, we showed that PEDV recognizes a sugar co-receptor Neu5Ac, which explains the enteric tropism of PEDV. Because TGEV also recognizes porcine APN and Neu5Ac, PEDV and TGEV are evolutionarily closely related despite the relative low sequence similarity in their spikes (Fig. 1B). Third, we demonstrated that PEDV infects bat cells, providing evidence that PEDV originated from bats. Finally, different from TGEV that does not use human APN as its receptor, PEDV recognizes human APN and infects human cells. Thus, neither receptor recognition nor other host cellular factors (e.g. cellular restrictions of viral replication) pose a hurdle for PEDV to infect humans. It remains to be seen whether systemic factors (e.g. host immune system) can prevent or timely clear PEDV infections in humans. Nevertheless, these results suggest that PEDV may be a potential threat to other species including humans. Overall, our study provides insight into the host range, tropism, and evolution of PEDV.

Our study also has implications for the development of antiviral strategies against PEDV. The S1-NTD-CTD fragment as identified in this study may serve as a subunit vaccine candidate. Monoclonal antibodies against S1-NTD-CTD may serve as immunotherapeutic agents to block PEDV attachment to both APN receptor and sugar co-receptor. In addition, sugar or sugar analogues may serve as antiviral drugs to block PEDV attachment to its sugar co-receptor. Development of these antiviral strategies are urgent because of the damaging impact that PEDV exerts on the US pork industry as well as the potential threat that PEDV poses to other species.

Acknowledgements

This work was supported by NIH grant R01AI089728. We thank the Consortium for Functional Glycomics for help in glycan screen arrays.

153 **References:**

154 1. **Song D, Park B.** 2012. Porcine epidemic diarrhoea virus: a comprehensive
155 review of molecular epidemiology, diagnosis, and vaccines. Virus Genes **44:**167-
156 175.

157 2. **Sun RQ, Cai RJ, Chen YQ, Liang PS, Chen DK, Song CX.** 2012. Outbreak of
158 porcine epidemic diarrhea in suckling piglets, China. Emerg Infect Dis **18:**161-
159 163.

160 3. **Pensaert MB, de Bouck P.** 1978. A new coronavirus-like particle associated with
161 diarrhea in swine. Arch Virol **58:**243-247.

162 4. **Mole B.** 2013. Deadly pig virus slips through US borders. Nature **499:**388.

163 5. **Stevenson GW, Hoang H, Schwartz KJ, Burrough ER, Sun D, Madson D,**
164 **Cooper VL, Pillatzki A, Gauger P, Schmitt BJ, Koster LG, Killian ML, Yoon**
165 **KJ.** 2013. Emergence of Porcine epidemic diarrhea virus in the United States:
166 clinical signs, lesions, and viral genomic sequences. J Vet Diagn Invest **25:**649-
167 654.

168 6. **Chen Q, Li G, Stasko J, Thomas JT, Stensland WR, Pillatzki AE, Gauger**
169 **PC, Schwartz KJ, Madson D, Yoon KJ, Stevenson GW, Burrough ER,**
170 **Harmon KM, Main RG, Zhang J.** 2014. Isolation and characterization of
171 porcine epidemic diarrhea viruses associated with the 2013 disease outbreak
172 among swine in the United States. J Clin Microbiol **52:**234-243.

173 7. **Perlman S, Netland J.** 2009. Coronaviruses post-SARS: update on replication
174 and pathogenesis. Nature Reviews Microbiology **7:**439-450.

175 8. **Li WH, Wong SK, Li F, Kuhn JH, Huang IC, Choe H, Farzan M.** 2006.
176 Animal origins of the severe acute respiratory syndrome coronavirus: Insight from
177 ACE2-S-protein interactions. Journal of Virology **80:**4211-4219.

178 9. **Huang YW, Dickerman AW, Pineyro P, Li L, Fang L, Kiehne R, Opriessnig**
179 **T, Meng XJ.** 2013. Origin, evolution, and genotyping of emergent porcine
180 epidemic diarrhea virus strains in the United States. Mbio **4:**e00737-00713.

181 10. **Li F.** 2015. Receptor Recognition Mechanisms of Coronaviruses: a Decade of
182 Structural Studies. J Virol **89:**1954-1964.

11. **Li F.** 2012. Evidence for a Common Evolutionary Origin of Coronavirus Spike Protein Receptor-Binding Subunits. Journal of Virology **86:**2856-2858.

12. **Li F.** 2013. Receptor recognition and cross-species infections of SARS coronavirus. Antiviral Res **100:**246-254.

13. **Li F, Li WH, Farzan M, Harrison SC.** 2005. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. Science **309:**1864-1868.

14. **Li WH, Zhang CS, Sui JH, Kuhn JH, Moore MJ, Luo SW, Wong SK, Huang IC, Xu KM, Vasilieva N, Murakami A, He YQ, Marasco WA, Guan Y, Choe HY, Farzan M.** 2005. Receptor and viral determinants of SARS-coronavirus adaptation to human ACE2. Embo Journal **24:**1634-1643.

15. **Delmas B, Gelfi J, Lharidon R, Vogel LK, Sjostrom H, Noren O, Laude H.** 1992. AMINOPEPTIDASE-N IS A MAJOR RECEPTOR FOR THE ENTEROPATHOGENIC CORONAVIRUS TGEV. Nature **357:**417-420.

16. **Schwegmann-Wessels C, Herrler G.** 2006. Sialic acids as receptor determinants for coronaviruses. Glycoconjugate Journal **23:**51-58.

17. **Krempl C, Schultze B, Laude H, Herrler G.** 1997. Point mutations in the S protein connect the sialic acid binding activity with the enteropathogenicity of transmissible gastroenteritis coronavirus. Journal of Virology **71:**3285-3287.

18. **Schultze B, Krempl C, Ballesteros ML, Shaw L, Schauer R, Enjuanes L, Herrler G.** 1996. Transmissible gastroenteritis coronavirus, but not the related porcine respiratory coronavirus, has a sialic acid (N-glycolylneuraminic acid) binding activity. Journal of Virology **70:**5634-5637.

19. **Krempl C, Laude H, Herrler G.** 1998. Is the sialic acid binding activity of the S protein involved in the enteropathogenicity of transmissible gastroenteritis virus? Adv Exp Med Biol **440:**557-561.

20. **Li BX, Ge JW, Li YJ.** 2007. Porcine aminopeptidase N is a functional receptor for the PEDV coronavirus. Virology **365:**166-172.

21. **Du L, Zhao G, Yang Y, Qiu H, Wang L, Kou Z, Tao X, Yu H, Sun S, Tseng CT, Jiang S, Li F, Zhou Y.** 2014. A Conformation-Dependent Neutralizing

Monoclonal Antibody Specifically Targeting Receptor-Binding Domain in Middle East Respiratory Syndrome Coronavirus Spike Protein. J Virol **88:**7045-7053.

22. **Ma C, Wang L, Tao X, Zhang N, Yang Y, Tseng CT, Li F, Zhou Y, Jiang S, Du L.** 2014. Searching for an ideal vaccine candidate among different MERS coronavirus receptor-binding fragments-The importance of immunofocusing in subunit vaccine design. Vaccine **32:**6170-6176.

23. **Chen L, Lin YL, Peng GQ, Li F.** 2012. Structural basis for multifunctional roles of mammalian aminopeptidase N. Proceedings of the National Academy of Sciences of the United States of America **109:**17966-17971.

24. **Liu C, Yang Y, Chen L, Lin YL, Li F.** 2014. A unified mechanism for aminopeptidase N-based tumor cell motility and tumor-homing therapy. J Biol Chem.

25. **Peng GQ, Sun DW, Rajashankar KR, Qian ZH, Holmes KV, Li F.** 2011. Crystal structure of mouse coronavirus receptor-binding domain complexed with its murine receptor. Proceedings of the National Academy of Sciences of the United States of America **108:**10696-10701.

26. **Peng GQ, Xu LQ, Lin YL, Chen L, Pasquarella JR, Holmes KV, Li F.** 2012. Crystal Structure of Bovine Coronavirus Spike Protein Lectin Domain. Journal of Biological Chemistry **287:**41931-41938.

27. **Yang Y, Du L, Liu C, Wang L, Ma C, Tang J, Baric RS, Jiang S, Li F.** 2014. Receptor usage and cell entry of bat coronavirus HKU4 provide insight into bat-to-human transmission of MERS coronavirus. Proc Natl Acad Sci U S A **111:**12516-12521.

28. **Weingartl HM, Derbyshire JB.** 1991. Antiviral activity against transmissible gastroenteritis virus, and cytotoxicity, of natural porcine interferons alpha and beta. Can J Vet Res **55:**143-149.

29. **Haraguchi N, Ishii H, Mimori K, Tanaka F, Ohkuma M, Kim HM, Akita H, Takiuchi D, Hatano H, Nagano H, Barnard GF, Doki Y, Mori M.** 2010. CD13 is a therapeutic target in human liver cancer stem cells. J Clin Invest **120:**3326-3339.

30. **Wicht O, Li W, Willems L, Meuleman TJ, Wubbolts RW, van Kuppeveld FJ, Rottier PJ, Bosch BJ.** 2014. Proteolytic activation of the porcine epidemic diarrhea coronavirus spike fusion protein by trypsin in cell culture. J Virol **88:**7952-7961.

31. **Shirato K, Matsuyama S, Ujike M, Taguchi F.** 2011. Role of proteases in the release of porcine epidemic diarrhea virus from infected cells. J Virol **85:**7872-7880.

Figure legends

Figure 1. PEDV spike protein. (A) Domain structure of PEDV spike. It contains a receptor-binding S1 subunit, a membrane-fusion S2 subunit, a single-pass transmembrane anchor (TM), and a short intracellular tail (IC). S1 contains an N-terminal domain (S1-NTD) and a C-terminal domain (S1-CTD). S2 contains the fusion peptide (FP), heptad repeat 1 (HR1), and heptad repeat 2 (HR2), all of which are essential structural elements for the membrane fusion process. (B) Amino acid sequence identities between PEDV spike and the spikes from TGEV, BtCoV/512/2005, and HCoV-NL63 in different regions. GenBank accession numbers are: AGO58924.1 for PEDV spike, CAA29175.1 for TGEV spike, ABG47078.1 for BtCoV/512/2005 spike, and AAS58177.1 for HCoV-NL63 spike.

Figure 2. PEDV spike binds porcine APN, human APN, and sugar receptors. (A) SDS-PAGE analysis of recombinant PEDV S1-NTD-CTD and TGEV S1-NTD-CTD. Both proteins were fused with a C-terminal human IgG1 Fc tag. The gel was stained using Coomassie Blue. (B) Dot blot hybridization assay showing the interactions between PEDV or TGEV S1-NTD-CTD (with a C-terminal human IgG1 Fc tag) and porcine or human APN (with a C-terminal His_6 tag) using a procedure as previously described (24). APN-binding S1-NTD-CTDs were detected using antibodies against their C-terminal Fc tag, and subsequently subjected to enzymatic color reactions. BSA was used as a negative control. (C) Dot-blot hybridization assay showing the interactions between PEDV or TGEV S1-NTD-CTD and sugar moieties on mucin-spotted nitrocellulose membranes using a procedure as previously described (25). Mucin was either mock-treated or treated with neuraminidase (New England BioLabs Inc). Sugar-binding S1-NTD-CTDs were

detected using antibodies against their C-terminal Fc tag, and subsequently subjected to enzymatic color reactions. (D) Glycan screen array that was performed to identify the type(s) of sugar most favored by PEDV S1-NTD-CTD (with a C-terminal Fc tag) using a procedure as previously described (26). A glycan library composed of 609 different natural and synthetic mammalian glycans (Table S1) was screened for PEDV S1-NTD-CTD binding. Glycan-binding S1-NTD-CTD was detected using antibodies against its C-terminal Fc tag. Readout was described arbitrarily as relative fluorescence unit (RFU). Among these glycans, N-acetylneuraminic acid (Neu5Ac) shows the highest binding affinity for PEDV S1-NTD-CTD.

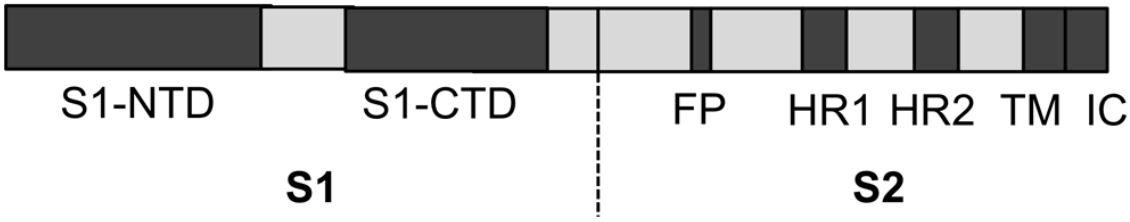
Figure 3. PEDV-spike-mediated pseudovirus entry into host cells. PEDV- and TGEV-spike-pseudotyped retroviruses were produced and used to infect cells using a procedure as previously described (27). Trypsin was not included in the pseudovirus entry assay. The cells being infected were MDCK cells exogenously expressing human APN (hAPN), porcine APN (pAPN) or empty vector (panel A), PK-15 cells (panel B), and Huh-7 cells (panel C). For antibody inhibition, cells were pre-incubated with 20 μg/ml anti-hAPN antibody (Santa Cruz Biotechnology) for 1 h at 37 °C before pseudovirus infection. For mucin inhibition, PEDV- or TGEV-spike-pseudotyped retroviruses were pre-incubated with 500 μg/ml porcine or bovine mucin before they were used to infect cells. The pseudovirus entry efficiency was characterized as luciferase activity accompanying the entry. Error bars indicate SEM (n = 4).

Figure 4. PEDV infections in cell culture. PEDV strain Ohio VBS2 was used to infect different cell lines at an MOI of 1.0 using a procedure as previously described (30). 5

μg/ml trypsin was included in the cell culture medium to facilitate live PEDV infections. After 24 h post-inoculation, cells were fixed with 4.0% (v/v) paraformaldehyde-0.2% (v/v). PEDV was detected with fluorescein isothiocyanate (FITC)-labeled mouse anti-PEDV N protein antibody, and observed under a fluorescence microscope.

Journal of Virology

A



B

PEDV spike	S1-NTD	S1-CTD	S1	S2
TGEV	19%	23%	31%	56%
BtCoV/ 512/2005	48%	36%	48%	74%
HCoV-NL63	21%	29%	36%	60%