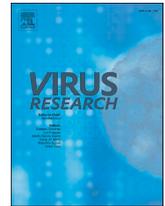




Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



Identification of a novel B cell epitope on the nucleocapsid protein of porcine deltacoronavirus

Shan Wei^{a,1}, Da Shi^{b,1}, Haoyang Wu^a, Haibo Sun^a, Jianfei Chen^b, Li Feng^b, Mingjun Su^{a,*}, Dongbo Sun^{a,*}

^a Laboratory for the Prevention and Control of Swine Infectious Diseases, College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing, 163319, China

^b State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, 150069, China

ARTICLE INFO

Keywords:
Porcine deltacoronavirus
N protein
Epitopes

ABSTRACT

Porcine deltacoronavirus (PDCoV) is an emerging coronavirus that causes vomiting, diarrhea, dehydration, and even death of piglets, resulting in significant losses to the pig industry worldwide. However, the epitopes of PDCoV remain largely unknown. In this study, a monoclonal antibody (mAb) against the PDCoV nucleocapsid (N) protein, termed 9G1, was prepared using the lymphocyte hybridoma technique, and was identified as a type IgG1 with a κ light chain and reacted with the native N protein of PDCoV. Furthermore, the epitope recognized by the 9G1 mAb was subjected to western blot and an ELISA using truncated recombinant proteins and synthetic polypeptides of the PDCoV N protein. The results indicate that 9G1 mAb recognized the epitope, G⁵⁹TPIP-PSYAFYY⁷⁰ (EP-9G1), a novel linear B cell epitope of the PDCoV N protein. A comparison analysis revealed that the EP-9G1 epitope was highly conserved among PDCoV strains, in which four residues (G⁵⁹—F⁶⁸YY⁷⁰) were observed among different coronavirus genera. These data demonstrate that the EP-9G1 epitope identified in this study provides some basic information for further characterization of the antigenic structure of the PDCoV N protein and has potential use for developing diagnostic reagents for PDCoV.

1. Introduction

Coronaviruses (CoVs) belong to the order *Nidovirales*, family *Coronaviridae*, and subfamily *Coronavirinae*, which is further classified into four genera: *alpha*-coronavirus, *beta*-coronavirus, *gamma*-coronavirus, and the newly assumed *delta*-coronavirus (Woo et al., 2012). Porcine deltacoronavirus (PDCoV) is a *delta*-coronavirus that causes vomiting, diarrhea, dehydration and can be fatal in piglets (Wang et al., 2014; Hu et al., 2015). PDCoV exhibits clinical signs similar to that caused by porcine epidemic diarrhea virus (PEDV) (Yang et al., 2020), transmissible gastroenteritis virus (TGEV) (Yuan et al., 2021), and swine acute diarrhea syndrome coronavirus (SADS-CoV) (Pan et al., 2017). PDCoV was first found from pig feces in Hong Kong in 2012 (Woo et al., 2012). The first outbreak of PDCoV was announced in the United States in 2014 (Wang et al., 2014). Thereafter, PDCoV was detected in China (Su et al., 2020), South Korea (Lee et al., 2016), and Thailand (Janetanakit et al., 2016), resulting in a significant loss to the pig industry worldwide (Song et al., 2015; Jung et al., 2016). Moreover, interspecific

transmission has also been reported in humans, cats, chickens, calves, and other domestic poultry (Jung et al., 2017; Li et al., 2018; Boley et al., 2020).

PDCoV is an enveloped, single-stranded, positive-sense RNA virus. The PDCoV genome is approximately 25 kb, which is similar to that of other porcine CoVs, and it encodes four similar major structural proteins: the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins (Li et al., 2014). The CoV N protein is highly conserved among different strains and is the predominant antigen in CoV-infected cells (Sturman and Holmes, 1983). The N protein is also involved in multiple stages of viral replication (McBride et al., 2014). Thus, serological assays based on the N protein have proven to be highly sensitive (Pradhan et al., 2014; Abdelwahab et al., 2015; Su et al., 2016). The antigenic epitope is a chemical group capable of stimulating the production of antibodies or sensitized lymphocytes, which plays an important role in the antiviral immune response. Thus, such epitopes can provide insight into the development of diagnostic methods and novel vaccines, as well as the design of antiviral immunization strategies. N protein epitopes

* Corresponding authors.

E-mail addresses: mingjunsu@163.com (M. Su), dongbosun@126.com (D. Sun).

¹ These authors contributed equally to this work.

have been identified in several CoVs, including PEDV, TGEV, SADS-CoV, avian infectious bronchitis virus (IBV), and mouse hepatitis virus (MHV) (Sun et al., 2008; Yu et al., 2010; Asano et al., 2011; Wang et al., 2016; Zhang et al., 2016; Han et al., 2019). However, to date, there is has been only one report on PDCoV epitopes (i.e., the epitope region of the PDCoV N protein is amino acids K³⁰⁹PKQKKPK³¹⁷ [EP-4E88]) (Fu et al., 2020). In the current study, a monoclonal antibody (mAb), termed 9G1, against the PDCoV N protein was produced, and was used to screen for epitopes. Sequence alignment revealed that the novel linear B epitope, EP-9G1, was highly conserved among different PDCoV strains, and four residues (G⁵⁹—F⁶⁸YY⁷⁰) were observed among different CoV genera. These data indicate that the 9G1 mAb and identified epitope could be used for the development of diagnostic methods and as a tool for the further study of PDCoV N protein structure and function.

2. Materials and methods

2.1. Ethics statement

The study protocol was approved by the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (approval no. Heilongjiang-SYXK-2006-032) and conducted in accordance with the institutional guidelines for the care and use of laboratory animals.

2.2. Cell lines, viruses and antibodies

Myeloma SP2/0 and ST cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen Corporation, Carlsbad, CA, USA) under a 5% CO₂/95% air humidified atmosphere at 37 °C. The myeloma SP2/0 cells and fused hybridoma cells were cultured in DMEM supplemented with 20% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and the ST cells were cultured in DMEM supplemented with 10% FBS. Ni-NTA His-Bind resin was purchased from Novagen Inc. Antibodies against the His-tag (SAB1305538) and GST tag (SAB1305540) were purchased from Sigma. PDCoV strain NH (GenBank accession no. KU981059), positive and negative PDCoV serum were kindly provided by the Division of Swine Digestive System Infectious Diseases, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

2.3. Expression and purification of the PDCoV N protein

A His-tagged recombinant plasmid (pET-32a-PDCoV-N) was constructed to obtain immune antigens for the preparation of mAbs, whereas a GST-labeled recombinant plasmid (pGEX-6p-1-PDCoV-N) was constructed to obtain screening antigens for the identification of the hybridoma cell lines. The primer sequences used in this study are listed in Table 1. Inserts in the recombinant plasmids were sequenced and those with correct sequences were transformed into competent *Escherichia coli* BL21 (DE3) cells. The expressed fusion proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The soluble recombinant protein encoded by pET-32a-PDCoV-N (His-N) was purified with Ni-NTA His•Tag resin and the insoluble recombinant protein encoded by pGEX-6p-1-PDCoV-N (GST-N) was stained with 0.25 M KCL and purified by cutting gels.

2.4. Screening of mAbs

Six-week-old female BALB/c mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd and housed in SPF isolators ventilated under negative pressure. The six-week-old female BALB/c mice were immunized by a subcutaneous injection with 50 µg purified recombinant His-N emulsified in complete Freund's adjuvant (Sigma-Aldrich Corporation). After 2 weeks, the mice received a booster immunization in the same manner, except that the protein was emulsified in incomplete Freund's adjuvant (Sigma-Aldrich Corporation). Two

Table 1

The primer sequences used in this study.

Primers	Sequences (5'–3')	Positions (aa)
His-PDCoV-N-U	GCGGATCCATGGCTGCACCAGTAGTCCCTACT	1–343
His-PDCoV-N-L	GCCTCGAGTCTACGCTGCTGATTCTGCTTTAT	
GST-PDCoV-N-U	GCGGATCCATGGCTGCACCAGTAGTCCCTACT	1–343
GST-PDCoV-N-L	GCCTCGAGTCTACGCTGCTGATTCTGCTTTAT	
N1-U	GCGGATCCATGGCTGCACCAGTAGTCCCTACT	1–115
N1-L	GCCTCGAGTAGTTGCGTTTGGCAACATGAGGCTT	
N2-U	GCGGATCCCCCAACAATCCTAAACATCAGCTG	116–185
N2-L	GCCTCGAGTACGCTTACGACGTACCGCAGCGGG	
N3-U	GCGGATCCCAACATCAAGCTCCCAAGCGGACT	186–343
N3-L	GCCTCGAGTACGCTGCTGATTCTGCTTTAT	
N1–1-U	GCGGATCCATGGCTGCACCAGTAGTCCCTACT	1–51
N1–1-L	GCCTCGAGTAACGCAGCCAGTAGCCATGGTTTTTC	
N1–2-U	GCGGATCCCGTTACACCAGACAAAAGCCAGGT	51–115
N1–2-L	GCCTCGAGTAGTTGCGTTTGGCAACATGAGGCTT	

weeks after the booster immunization, the mice were intraperitoneally injected with 100 µg recombinant His-N without adjuvant. The mice were euthanized three days later and the spleen cells fused with SP2/0 cells were harvested using standard procedures. The fused cells were cultured in 96-well plates containing DMEM supplemented with 20% FBS and selected in medium containing hypoxanthine-aminopterin-thymidine (Sigma-Aldrich Corporation) and hypoxanthine-thymidine (Sigma-Aldrich Corporation). Hybridoma supernatants were subjected to western blot analysis and an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of N protein-specific Abs. The selected positive hybridomas were subcloned three times using a limiting dilution assay. The hybridoma cells were injected into the abdominal cavity of the mice and stimulated with incomplete Freund's adjuvant to promote ascites production. An SBA Clonotyping™ System/HRP kit (SouthernBiotech, Birmingham, AL, USA) was used to identify the mAb subclasses.

2.5. mAb ELISA

The wells of 96-well plates were coated with 100 ng of the purified GST-N protein (100 µL/well in 0.05 M NaHCO₃, pH 9.6) as an antigen. After an overnight incubation at 4 °C, each well was blocked with 100 µL of 5% skim milk in phosphate-buffered saline (PBS) at 37 °C for 1 h. The culture supernatant of the hybridoma cells was added to each well and the plate was incubated at 37 °C for 1 h. Both non-immune and immune sera were used as negative and positive controls, respectively. Horse-radish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin (Ig) G was diluted to 1:10,000 and incubated at 37 °C for 1 h. The reaction was quantified by measuring the absorbance at 450 nm with a 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution in the dark at 37 °C for 15 min. Then, 100 µL of 2 M H₂SO₄ were added to each well to stop the reaction.

2.6. Western blot analysis

To verify the reaction between the screened mAbs and the natural virus, ST cells were inoculated with PDCoV strain NH and collected for western blot after 24 h. The samples were subjected to 12% SDS-PAGE and then transferred to nitrocellulose membranes, which were blocked with 5% skim milk in PBS containing 0.05% Tween-20 (PBST) at room temperature for 2 h. Afterward, the nitrocellulose membranes were incubated with the prepared PDCoV N mAb at 37 °C for 1 h. After washing three times with PBST, the membranes were incubated with goat anti-mouse IgG with a 1:10,000 dilution of HRP at 37 °C for 1 h, washed three times with PBST (10 min/wash), and stained with TMB substrate solution.

2.7. Immunofluorescence assay

ST cells were infected with PDCoV strain NH and fixed in 4% paraformaldehyde at 4 °C for 30 min. After washing three times with PBS, the cells were permeabilized with 0.2% TritonX-100 at room temperature for 15 min, washed three times with PBS, and probed with the 9G1 mAb (dilution, 1:500) at 37 °C for 1 h. After washing three times with PBS, the cells were incubated with goat anti-mouse IgG at 488 nm (dilution, 1:500) in the dark at room temperature for 1 h. The cells were washed three times with PBS, and images were captured using a fluorescence microscope.

2.8. Expression and verification of truncated N protein

The antigen epitopes were predicted using the website <http://imed.med.ucm.es/Tools/antigenic.pl>. For the first round, the PDCoV N protein was divided into three sections: N1 (amino acids [aa] 1–115); N2 (aa 116–185); and N3 (aa 186–343). For the second round, according to the 9G1 identification results, the N1 (aa 1–115) segment with a positive reaction was further divided into N1–1 (aa 1–51) and N1–2 (aa 51–115) segments to identify expression. The above truncated proteins were expressed by fusion with GST tags. Anti-GST Abs were used to verify fusion proteins expression. Antigenicity was verified using the serum from pigs immunized with PDCoV strain NH. In the last round, the N1–2 segment was further truncated into five polypeptides: N1–2–1 (R⁵¹YTRQKPGGTPIP-PSYAFYY⁷⁰); N1–2–2 (G⁵⁹TPIPPSYAFYYTGTGPRGN⁷⁸); N1–2–3 (T⁷¹GTGPRGNLKYGELPPNDTP⁹⁰); N1–2–4 (N⁸⁷DTPATTRVTWVKGSGADTS¹⁰⁶); and N1–2–5 (T⁹⁶WVKGSGADTSIKPHVAKRN¹¹⁵), which were synthesized by Genscript Co., Ltd. (Nanjing, China). However, due to the low amino acid coupling efficiency of the "TPATTRVT" fragment, the N1–2–4 peptide could not be synthesized.

2.9. ELISA for the detection of synthetic peptides

The wells of 96-well plates were coated with the synthetic peptides diluted to working concentrations of 6 µg/mL (100 µL/well in PBS, pH 7.4) at 4 °C overnight. The next day, each well was blocked with 100 µL of 1% bovine serum albumin in PBST at 37 °C for 1 h. Then, ascites with the 9G1 mAb (diluted 1:500) were added to each well at 37 °C for 1 h. HRP-labeled goat anti-mouse IgG diluted to 1:10,000 was added to each well and the plate was incubated at 37 °C for 30 min. The reaction was quantified by measuring the absorbance at 450 nm with a TMB substrate solution in the dark at 37 °C for 15 min. Finally, 100 µL of 2 M H₂SO₄ was added to each well to stop the reaction.

2.10. Biological information analysis

To analyze the conservation of the identified epitope among the CoV reference strains, the N protein epitope and flanking sequences were compared with other selected CoV strains using DNASTAR MegAlign software (DNASTAR, Inc., Madison, WI, USA) (Table 2). A divergence analysis of the N protein of PDCoV strains and other CoV strains was performed with WebLogo (<http://weblogo.threeplusone.com/>), online software for sequence logo generation (Crooks et al., 2004). The predicted tertiary structures of the N region were modeled using the open-source modeling server, SWISS-MODEL (<https://swissmodel.expasy.org/>) from the Swiss Institute of Bioinformatics (Biasini et al., 2014). Four templates in the Protein Data Bank (PDB) were selected to build the 3D structure model of the N protein of PEDV (Accession No. AF353511) (PDB ID: 5n4k), SARS-CoV 2 (Accession No. MW307301) (PDB ID: 4ud1), IBV (Accession No. AY044185) (PDB ID: 2gac), and PDCoV (Accession No. KU981059) (PDB ID: 1ssk), respectively. Illustrations of these modeled tertiary structures were obtained using the python-based molecular viewer, PyMOL (The PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC).

Table 2

CoV strains used to align the sequences of the PDCoV NH strain.

Strain Name	Accession No	Strain Name	Accession No
PDCoV/NH	KU981059	ALCCoV/Guangxi/F230/2006	ABQ39962
PDCoV/HeN/swine/2015	MN942260	Bulbul-CoV/HKU11-934	ACJ12039
PDCoV/USA/Nebraska137/2015	KX022604	Thrush-CoV/HKU12-600	ACJ12057
PDCoV/USA/Iowa136/2015	KX022602	Munia-CoV/HKU13-3514	ACJ12066
PDCoV/Swine/Taiwan33/2016	KY586148	White-eye-CoV/HKU16	YP_005352842
PDCoV/USA/Illinois136/2014	KJ601779	Sparrow-CoV/HKU17	YP_005352850
PDCoV/P1_13_ST1_0213/PDCoV/0213/Thailand	KX361343	Night-heron-CoV/HKU19	YP_005352867
PDCoV/CHGD/2016	MH715491	Wigeon-CoV/HKU20	YP_005352875
PDCoV/PDCoV/Swine/Vietnam/Binh21/2015	KX834352	Common moorhen-CoV/HKU21-8295	YP_005352885
PDCoV/P1_16_BTL_0115/PDCoV/2016/Lao	KX118627	Falcon-CoV/UAE-HKU27	BBC54826
PDCoV/Swine/CHJX/2015	KX083667	Pigeon-CoV/UAE-HKU29	BBC54846
PDCoV/Thailand/S5011	KU051641	Magpie-robin-CoV/HKU18-chu3	YP_005352858
PDCoV/CHJXN12/2015	KR131621	Sparrow-CoV/ISU690-4	AWV67111
PDCoV/SD-12-2018	MN173792	FECV	KU215419
PDCoV/PDCoV/CH-01	KX443143	HCoV NL63/BJ8081	DQ846901
PDCoV/USA/Ohio137/2014	KJ601780	HCoV 229E	NC_002645
PDCoV/Swine/Vietnam/HaNoi6/2015	KX834351	Quail-CoV/1101F	BBC54865
PDCoV/SD-08-2018	MN173788	CCoV/BGF10	AY342160
PDCoV/PDCoV/SD-05-2018	MN173785	SARS-CoV-2/human/IRN/IR-SARS2-24/2020	MW307301
PDCoV/PDCoV/SD-03-2018	MN173784	SADS-CoV/CN/GDWT/2017	MG557844
PDCoV/SD-01-2018	MN173783	SeACoV/CH/GD-01/2017/P2	MF370205
PEDV/CV777	AF353511	Bat-CoV/HKU2/HK/46/2006	EF203065
PEDV/AJ1102	JX188454	FIPV/79-1146	NC_002306
TGEV/Purdue_P115	DQ811788	MHV/A59	AY700211
TGEV/Miller_M6	DQ811785	BCoV/ENT	NC_003045
PRCV/ISU-1	DQ811787	HCoV-OC43/LRTI_238	KX344031
PHEV/VW572	DQ011855	PRCV/RM4	Z24675
PHEV/CC14	MF083115	PHEV/HEV-JT06	FJ009234
SARS-CoV/BJ202	AY864806	SADS-CoV/FarmA	MF094681
PEAV/GDS04_P12	MH697599	CRCoV/BJ232	KX432213
SeACoV/p10	MK977618	IBV/H52-GD	AY044185
		PDCoV/HKU15-155	NC_039208

2.11. Statistical analysis

All statistical analyses were performed using GraphPad Prism® 8.00 software (GraphPad Software, Inc., La Jolla, CA, USA). A one-way analysis of variance and *t*-test were used for data analysis and graph production. A probability (*p*) value < 0.05 was considered to be statistically significant and *p* < 0.01 was highly significant.

3. Results

3.1. Expression and purification of the recombinant PDCoV N protein

The recombinant His-fused protein, His-N, and GST-fused protein, GST-N, were successfully expressed in *E. coli* BL21 cells with an expected

molecular weight of approximately 56 kDa and 64 kDa, respectively (Fig. 1A and 1B). His-N was purified with Ni-NTA His•Bind resin and identified with anti-His-tag Abs (Fig. 1C), whereas GST-N was purified with cutting gels and identified with anti-GST-tag Abs (Fig. 1D). The purified His-N and GST-N both reacted with the positive serum of pigs immunized with the PDCoV strain NH (Fig. 1E), and did not react with the negative serum (Fig. 1F), indicating appropriate antigenicity.

3.2. Preparation and identification of the 9G1 mAb

Purified His-N was used to immunize 6-week-old female BALB/c mice to generate hybridoma cells secreting specific Abs. The Abs secreted in the supernatant of the hybridoma cells were screened with an indirect ELISA using GST-N as the coated antigen. Subsequently, the 9G1 mAb against the PDCoV N protein was identified and classified as IgG1/ κ . The western blot results revealed that the 9G1 mAb positively reacted with the natural PDCoV N protein, whereas the negative control group of ST cells without PDCoV infection did not respond (Fig. 2A). The titers of 9G1 ascites were $> 819,200$, as determined with an indirect ELISA (Fig. 2B). Furthermore, the results of the immunofluorescence assay revealed that the 9G1 mAb reacted with ST cells infected with PDCoV strain NH (Fig. 2C). Collectively, these results demonstrate that the obtained 9G1 mAb exhibited good immune activity and specificity.

3.3. Identification of the epitopes recognized by the 9G1 mAb

To identify the epitopes recognized by the 9G1 mAb, three truncated N proteins (N1: aa 1–115; N2: aa 116–185; and N3: aa 186–343) fused with GST tags were designed (Fig. 3), expressed (Fig. 4A), and subjected to western blot analysis with an antibody against the GST tag and positive serum from pigs immunized with PDCoV strain NH, respectively (Fig. 4B and 4C). As shown in Fig. 4D, the N1 protein (aa 1–115) contained the epitope recognized by 9G1. The truncated N1 protein was divided into two GST-fusion fragments (N1–1: aa 1–51; N1–2: aa 51–115), which were detected with the 9G1 mAb. The results showed that the N1–2 fragment (aa 51–115) was recognized by the 9G1 mAb.

Furthermore, the N1–2 fragment was truncated into five polypeptides (N1–2–1, N1–2–2, N1–2–3, N1–2–4, and N1–2–5). However, due to the low amino acid coupling efficiency of the 'TPATTRVT' fragment, the N1–2–4 peptide could not be synthesized. Subsequently, these four synthesized peptides were used as coating antigens for an ELISA and PBS was used as the negative control. The results showed that peptides N1–2–1 and N1–2–2 were both recognized by the 9G1 mAb (Fig. 4E). Based on above results, we concluded that the minimum linear epitope identified by the 9G1 mAb was the repeating region, G⁵⁹TPIPPSYAFY⁷⁰ (EP-9G1), of N1–2–1 and N1–2–2.

3.4. Homology analysis of the epitope

To determine whether the linear epitope recognized by the 9G1 mAb was conserved in the PDCoV isolates and other CoV strains, the alignments of the 20 most representative PDCoV strains were aligned and compared (Table 2). The results showed that the epitope of EP-9G1 was highly conserved in the different PDCoV strains, with the exception of two strains, PDCoV/Swine/Taiwan33/2016 (Y⁶⁶→F) and PDCoV/SD-03–2018 (A⁶⁷→T) (Fig. 5A). This finding indicated that the epitope EP-9G1 sequence represents a conserved epitope on the N protein of PDCoV. Furthermore, 15 delta-coronavirus strains were selected for sequence alignment. The results revealed that PDCoV and the other 15 delta-coronavirus shared a 50% – 100% sequence similarity in the position of epitope EP-9G1 (Fig. 5B). In addition, sequence alignment of the epitope EP-9G1 with PEDV, TGEV, SADS-CoV, porcine respiratory coronavirus (PRCV), and porcine hemagglutinating encephalomyelitis virus (PHEV) were performed to determine the level of conservation among porcine CoVs. The results showed a low sequence similarity in the position of epitope EP-9G1 (35.7%) (Fig. 5C). Furthermore, an analysis of the homologous sequences of the defined epitope from 20 different genera of CoV strains (including alpha-, beta-, gamma-, and delta-coronavirus) demonstrated that the epitope recognized by the 9G1 mAb shares a low homology among other CoVs (21.4% – 42.9%), and that only four residues G⁵⁹–F⁶⁸YY⁷⁰ were relatively conserved among all of the selected CoVs (Fig. 5D).

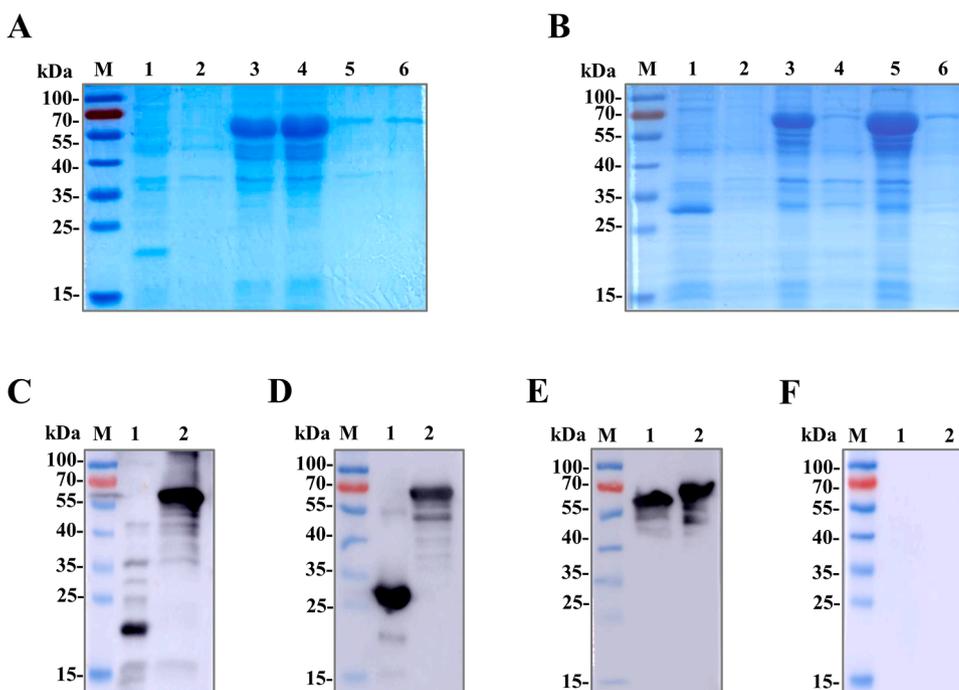


Fig. 1. (A) Expression of recombinant pET-32a-PDCoV-N (His-N) protein by SDS-PAGE. Lane 1: pET-32a as a negative control; Lane 2: His-N without IPTG induction; Lane 3: His-N induced with 1 mM IPTG for 6 h; Lane 4: His-N in the supernatant after ultrasonication; Lane 5: His-N in the precipitate after ultrasonication; Lane 6: purified His-N. (B) Expression of the recombinant pGEX-6p-1-PDCoV-N (GST-N) protein by SDS-PAGE. Lane 1: pGEX-6p-1 as a negative control; Lane 2: GST-N induced with 1 mM IPTG for 6 h; Lane 3: GST-N in the supernatant after ultrasonication; Lane 4: GST-N in the precipitate after ultrasonication; Lane 5: GST-N in the precipitate after ultrasonication; Lane 6: purified GST-N. (C) Expression of His-N was verified with His-labeled Abs by western blot. Lane 1: empty vector pET-32a as a negative control; Lane 2: purified His-N. (D) Expression of GST-N was verified with GST-labeled Abs by western blot. Lane 1: empty vector pGEX-6p-1 as a negative control; Lane 2: purified GST-N. (E) Antigenicity of the recombinant proteins was verified with positive serum from pigs immunized with PDCoV strain NH by western blot. Lane 1: purified His-N; Lane 2: purified GST-N. (F) Antigenicity of the recombinant proteins was verified with negative serum from pigs by western blot. Lane 1: purified His-N; Lane 2: purified GST-N.

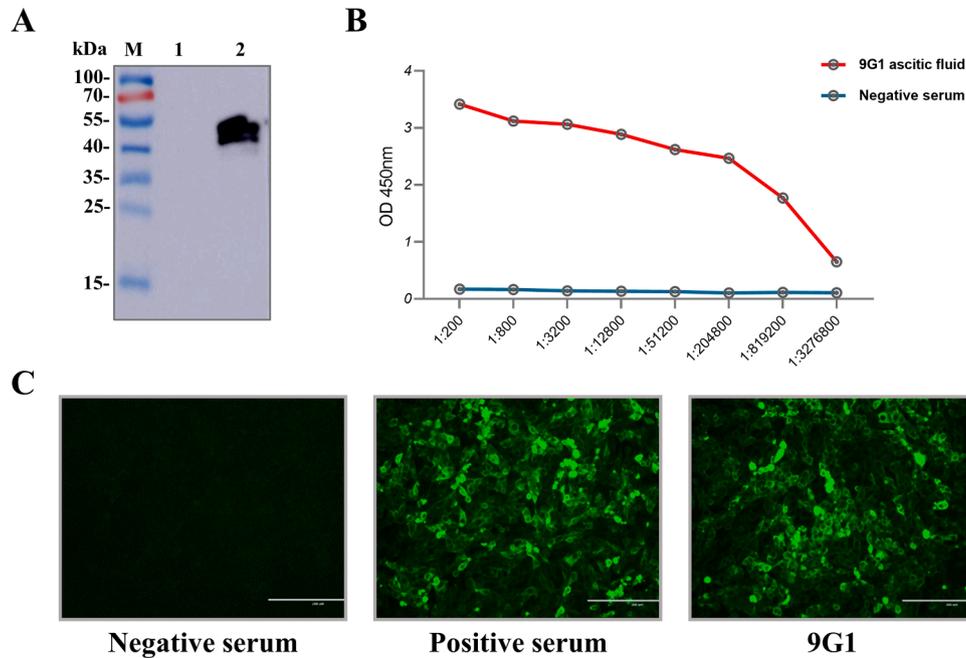


Fig. 2. Verification of the prepared 9G1 mAb. **(A)** The reaction between the 9G1 mAb with the natural virus by western blot. Lane 1: ST cells as a negative control; Lane 2: ST cells were infected for 24 h with PDCoV strain NH. **(B)** Determination of the 9G1 mAb titer in ascites by ELISA. **(C)** The reaction between the 9G1 mAb with the natural virus by an immunofluorescence assay.

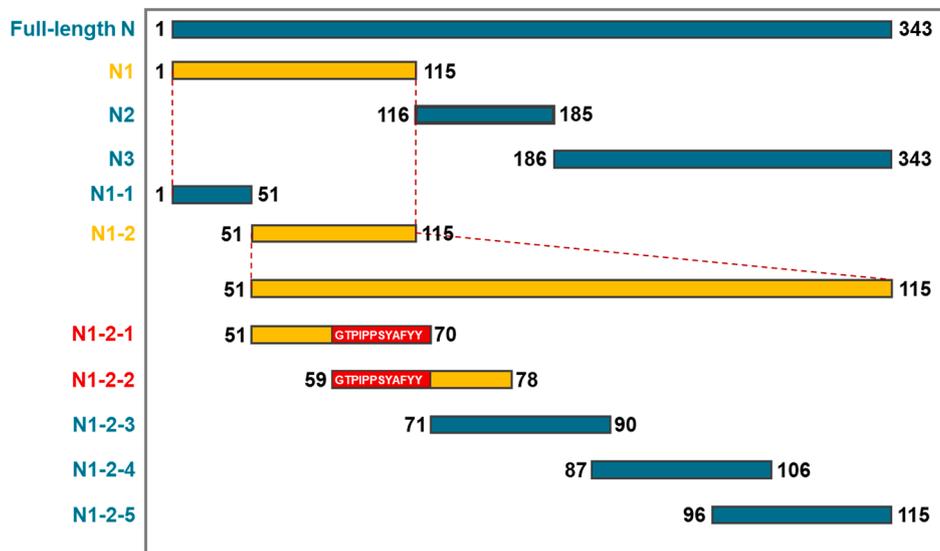


Fig. 3. Schematic representation of PDCoV N fragments used for B cell epitope mapping.

4. Discussion

Mapping epitopes of viral proteins and defining the degree of conservation of identified epitopes can enhance our current understanding of the antigenic structure, function, and virus-antibody interactions. Antigen epitopes are also commonly required for the development of effective vaccines and serological diagnostics (Kouzmitcheva et al., 2001; Westerink et al., 2001; Anandarao et al., 2006; Gómara et al., 2010; Sukupolvi et al., 2010; He et al., 2011). The CoV N protein is an important structural protein involved in various biological processes during viral replication and is also highly conserved among different CoV strains (McBride et al., 2014). In addition, studies have reported that CoV N proteins are highly expressed during the early infection stage and IgG predominantly targets the viral N protein in infected patients

(Leung et al., 2004). Therefore, it is important to identify epitopes on the CoV N protein for the development of the epitope-based vaccine and serological diagnostics.

To date, multiple B cell epitopes have been identified in several CoV N proteins, including PEDV (Wang et al., 2016, 2020), TGEV (Zhang et al., 2016), SADS-CoV (Han et al., 2019), IBV (Yu et al., 2010), and MHV (Asano et al., 2011). In addition, some epitopes have been further applied to the development of diagnostic methods. However, the epitopes present on the PDCoV N protein have been poorly characterized. In the present study, a novel B cell epitope on the N protein of PDCoV was identified by using a mAb. To produce mAbs against the PDCoV N protein, the His-tagged N protein (His-N) and GST-tagged N protein (GST-N) were expressed and identified by PDCoV-positive serum, indicating that both recombinant proteins exhibited good immunogenicity.

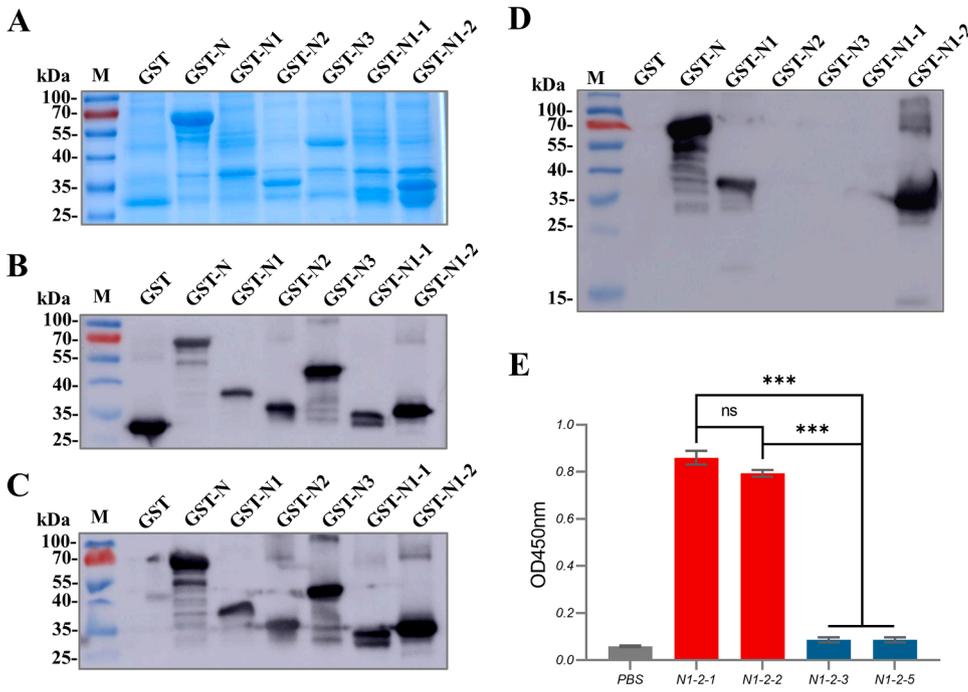


Fig. 4. Mapping of PDCoV N protein epitopes. (A) Expression of GST-fused truncated proteins. (B) GST-fused truncated proteins were verified with GST-labeled Abs by western blot. (C) Antigenicity of the GST-fused truncated proteins were verified with positive serum from pigs immunized with PDCoV strain NH by western blot. (D) Localization of GST-fused truncated proteins recognized with the 9G1 mAb. (E) The epitopes were precisely located using an ELISA via the reactivity of truncated peptides and 9G1 mAb.

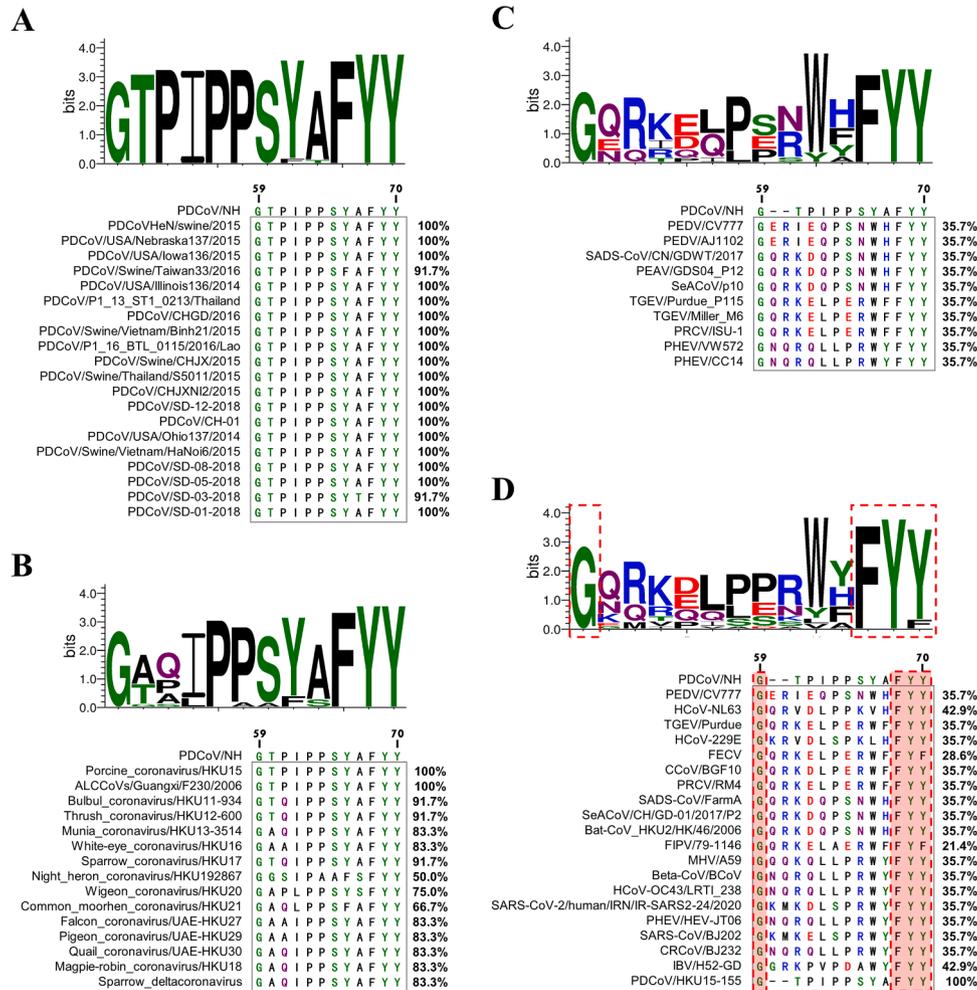


Fig. 5. Comparison of the amino acid sequence of the EP-9G1 epitope among different CoV strains. Alignment of the amino acid sequences of the EP-9G1 epitope and surrounding region with those of 20 PDCoV reference strains (A), 16 other delta-coronavirus strains (B), 10 porcine CoV strains (C), and 20 other CoV strains (D).

Next, the purified His-N was used as an immunogen to induce antibody production, and the purified GST-N was used for hybridoma screening to ensure that the envelope antigen has a different label from the immunogen. Ultimately, we found that the mAb, termed 9G1, specifically reacted to the natural N protein of the PDCoV NH strain and recombinant N protein. To map the epitopes on the N protein that bound to the 9G1 mAb, a series of truncated N proteins and polypeptides were generated. From these, one novel B cell linear epitope EP-9G1 present on the PDCoV N protein was identified.

To date, six porcine CoVs (PEDV, TGEV, PRCV, SADS-CoV, PHEV, and PDCoV) have been reported. Due to the similar clinical symptoms caused by a porcine enteric coronavirus infection (Hu et al., 2015; Wang et al., 2014), a serological method with a high diagnostic sensitivity and acceptable diagnostic specificity is required. Antigenic epitope-based methods have been used to detect antiviral antibodies, and have achieved good sensitivity and specificity (Anandarao et al., 2006; Gómaro et al., 2010; He et al., 2011). Thus, a highly conserved antigen epitope is critical to establish an epitope-based diagnosis method. For example, Asano et al. (2011) reported that two indirect ELISAs established based on the B cell epitopes of the MHV N protein were more sensitive than the commercial tests (Asano et al., 2011). Therefore, the use of highly conservative antigenic epitopes derived from the CoV N protein will make serological assays more specific and sensitive. In our present study, the epitope EP-9G1 was highly conserved among PDCoV strains, and shared a low sequence similarity with other five porcine CoVs. These findings indicate that the epitope EP-9G1 is unique to PDCoV and is a suitable antigen candidate for developing epitope-based serological diagnoses.

CoVs act as cross-species viruses and have the potential to spread rapidly into new host species and cause epidemic disease. Delta-coronaviruses have previously been found to infect both birds and mammals in China, and share similar genome characteristics and structure (Woo et al., 2012). Moreover, PDCoV was shown to efficiently infect cells with a broad host range, including swine, humans, and chickens (Li et al., 2018). In addition, studies have found that PDCoV can experimentally infect calves and chickens (Boley et al., 2020; Jung et al., 2017). The genome of the PDCoV N protein is closely related to avian trigonometric coronavirus (Woo et al., 2012). Recently, Fu et al. (2020) found that the epitope 4E88 located on K³⁰⁹PKQKKPK³¹⁷ of the N protein is highly conserved in PDCoV and has high similarity with sparrow CoV HKU17 (Fu et al., 2020). Similarly, the epitope EP-9G1 identified in our study is highly homologous with sparrow CoV HKU17 (91.7%) and other delta-coronaviruses. In addition, the sequence alignment showed that four residues (G⁵⁹—F⁶⁸YY⁷⁰) of EP-9G1 were highly conserved in different CoV genera. An N protein homology model showed that the G⁵⁹—F⁶⁸YY⁷⁰ residue was located in a similar region on the surface of different CoVs (Fig S1). These results suggest that the epitope EP-9G1 identified in this study may provide further insight into CoV evolution.

In conclusion, our study mapped a novel conserved B cell epitope, G⁵⁹TPIPPSYAFYY⁷⁰ (EP-9G1), on the PDCoV N protein using the 9G1 mAb. The epitope EP-9G1 was highly conserved among delta-coronavirus. Moreover, four residues (G⁵⁹—F⁶⁸YY⁷⁰) of EP-9G1 were identified to be conserved in different genera of CoVs. Our data may be useful for gaining a further understanding of the antigenic structure and its function in virus pathogenesis and provide a basis for designing clinical applications for epitope-mediated detection and diagnostic methods.

Author contributions

Conceptualization, D.B.S., M.S., and L.F., Formal analysis, S.W. and D.S., Funding acquisition, D.B.S., Investigation, S.W. and D.S., Methodology, S.W. and D.S., Project administration, S.W. and D.S., Resources, L.F., J.C., and D.S., Software, S.W. and D.S., Supervision, S.W. and D.S., Validation, S.W. and D.S., Visualization, S.W. and D.S., Writing

- original draft, S.W., D.S., H.W., H.S., and M.S., Writing - review & editing, M.S. and D.B.S.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Acknowledgements

This work was supported by Key Scientific Research Projects of Heilongjiang Province Farms & Land Reclamation Administration (201914) and the Heilongjiang Bayi Agricultural University Support Program for San Heng San Zong (grant no. TDJH201804). We also thank International Science Editing for editing the English text of a draft of this manuscript (<http://www.internationalscienceediting.com>).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.virusres.2021.198497](https://doi.org/10.1016/j.virusres.2021.198497).

References

- Abdelwahab, M., Loa, C.C., Wu, C.C., Lin, T.L., 2015. Recombinant nucleocapsid protein-based enzyme-linked immunosorbent assay for detection of antibody to turkey coronavirus. *J. Virol. Methods* 217, 36–41.
- Anandarao, R., Swaminathan, S., Fernando, S., Jana, A.M., Khanna, N., 2006. Recombinant multiepitope protein for early detection of dengue infections. *Clin. Vaccine Immunol.* 13 (1), 59–67.
- Asano, A., Torigoe, D., Sasaki, N., Agui, T., 2011. Identification of antigenic peptides derived from B-cell epitopes of nucleocapsid protein of mouse hepatitis virus for serological diagnosis. *J. Virol. Methods* 177 (1), 107–111.
- Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Schwede, T., 2014. SWISS-MODEL: modeling protein tertiary and quaternary structure using evolutionary information. *Nucleic. Acids. Res.* 42, w252–w258.
- Boley, P.A., Alhomo, M.A., Lossie, G., Yadav, K.K., Vasquez-Lee, M., Saif, L.J., Kenney, S.P., 2020. Porcine deltacoronavirus infection and transmission in Poultry, United States¹. *Emerg. Infect. Dis.* 26 (2), 255–265.
- Crooks, G.E., Hon, G., Chandonia, J.M., Brenner, S.E., 2004. WebLogo: a sequence logo generator. *Genome Res.* 14, 1188–1190.
- Fu, J., Chen, R., Hu, J., Qu, H., Zhao, Y., Cao, S., Wen, X., Wen, Y., Wu, R., Zhao, Q., Ma, X., Huang, X., 2020. Identification of a novel linear B-cell epitope on the nucleocapsid protein of porcine deltacoronavirus. *Int. J. Mol. Sci.* 21 (2), 648.
- Gómaro, M.J., Fernández, L., Pérez, T., Errilla, G., Haro, I., 2010. Assessment of synthetic chimeric multiple antigenic peptides for diagnosis of GB virus C infection. *Anal. Biochem.* 396 (1), 51–58.
- Han, Y., Zhang, J., Shi, H., Zhou, L., Chen, J., Zhang, X., Liu, J., Zhang, J., Wang, X., Ji, Z., Jing, Z., Cong, G., Ma, J., Shi, D., Li, F., 2019. Epitope mapping and cellular localization of swine acute diarrhoea syndrome coronavirus nucleocapsid protein using a novel monoclonal antibody. *Virus Res.* 273, 197752.
- He, J., Xiu, B., Wang, G., Chen, K., Feng, X., Song, X., Zhu, C., Ling, S., Zhang, H., 2011. Double-antigen sandwich ELISA for the detection of anti-hepatitis C virus antibodies. *J. Virol. Methods* 171 (1), 163–168.
- Hu, H., Jung, K., Vlasova, A.N., Chepogeno, J., Lu, Z., Wang, Q., Saif, L.J., 2015. Isolation and characterization of porcine deltacoronavirus from pigs with diarrhea in the United States. *J. Clin. Microbiol.* 53 (5), 1537–1548.
- Janetanakit, T., Lumyai, M., Bunpapong, N., Boonyapisitsopa, S., Chaiyawong, S., Nonthabenjawan, N., Kesdaengsakonwut, S., Amonsin, A., 2016. Porcine deltacoronavirus, Thailand, 2015. *Emerg. Infect. Dis.* 22 (4), 757–759.
- Jung, K., Hu, H., Saif, L.J., 2016. Porcine deltacoronavirus infection: etiology, cell culture for virus isolation and propagation, molecular epidemiology and pathogenesis. *Virus Res.* 226, 50–59.
- Jung, K., Hu, H., Saif, L.J., 2017. Calves are susceptible to infection with the newly emerged porcine deltacoronavirus, but not with the swine enteric alphacoronavirus, porcine epidemic diarrhea virus. *Arch. Virol.* 162 (8), 2357–2362.
- Kouzmitcheva, G.A., Petrenko, V.A., Smith, G.P., 2001. Identifying diagnostic peptides for Lyme disease through epitope discovery. *Clin. Diagn. Lab. Immunol.* 8 (1), 150–160.
- Lee, J.H., Chung, H.C., Nguyen, V.G., Moon, H.J., Kim, H.K., Park, S.J., Lee, C.H., Lee, G.E., Park, B.K., 2016. Detection and phylogenetic analysis of porcine deltacoronavirus in Korean swine farms, 2015. *Transbound Emerg. Dis.* 63 (3), 248–252.
- Leung, D.T., Tam, F.C., Ma, C.H., Chan, P.K., Cheung, J.L., Niu, H., Tam, J.S., Lim, P.L., 2004. Antibody response of patients with severe acute respiratory syndrome (SARS) targets the viral nucleocapsid. *J. Infect. Dis.* 190 (2), 379–386.
- Li, G., Chen, Q., Harmon, K.M., Yoon, K.J., Schwartz, K.J., Hoogland, M.J., Gauger, P.C., Main, R.G., Zhang, J., 2014. Full-length genome sequence of porcine deltacoronavirus strain USA/IA/2014/8734. *Genome Announc.* 2 (2) e00278-14.
- Li, W., Hulswit, R.J.G., Kenney, S.P., Widjaja, I., Jung, K., Alhomo, M.A., van Dieren, B., van Kuppeveld, F.J.M., Saif, L.J., Bosch, B.J., 2018. Broad receptor engagement of an

- emerging global coronavirus may potentiate its diverse cross-species transmissibility. *Proc. Natl. Acad. Sci. U. S. A.* 115 (22), e5135–e5143.
- McBride, R., van Zyl, M., Fielding, B.C., 2014. The coronavirus nucleocapsid is a multifunctional protein. *Viruses* 6 (8), 2991–3018.
- Pan, Y., Tian, X., Qin, P., Wang, B., Zhao, P., Yang, Y.L., Wang, L., Wang, D., Song, Y., Zhang, X., Huang, Y.W., 2017. Discovery of a novel swine enteric alphacoronavirus (SeACoV) in southern China. *Vet. Microbiol.* 211, 15–21.
- Pradhan, S.K., Kamble, N.M., Pillai, A.S., Gaikwad, S.S., Khulape, S.A., Reddy, M.R., Mohan, C.M., Kataria, J.M., Dey, S., 2014. Recombinant nucleocapsid protein based single serum dilution ELISA for the detection of antibodies to infectious bronchitis virus in poultry. *J. Virol. Methods* 209, 1–6.
- Song, D., Zhou, X., Peng, Q., Chen, Y., Zhang, F., Huang, T., Zhang, T., Li, A., Huang, D., Wu, Q., He, H., Tang, Y., 2015. Newly emerged porcine deltacoronavirus associated with diarrhoea in swine in China: identification, prevalence and full-length genome sequence analysis. *Transbound. Emerg. Dis.* 62 (6), 575–580.
- Sturman, L.S., Holmes, K.V., 1983. The molecular biology of coronaviruses. *Adv. Virus Res.* 28, 35–112.
- Su, M., Li, C., Guo, D., Wei, S., Wang, X., Geng, Y., Yao, S., Gao, J., Wang, E., Zhao, X., Wang, Z., Wang, J., Wu, R., Feng, L., Sun, D., 2016. A recombinant nucleocapsid protein-based indirect enzyme-linked immunosorbent assay to detect antibodies against porcine deltacoronavirus. *J. Vet. Med. Sci.* 78 (4), 601–606.
- Su, M., Li, C., Qi, S., Yang, D., Jiang, N., Yin, B., Guo, D., Kong, F., Yuan, D., Feng, L., Sun, D., 2020. A molecular epidemiological investigation of PEDV in China: characterization of co-infection and genetic diversity of S1-based genes. *Transbound Emerg. Dis.* 67 (3), 1129–1140.
- Sukupolvi-Petty, S., Austin, S.K., Engle, M., Brien, J.D., Dowd, K.A., Williams, K.L., Johnson, S., Rico-Hesse, R., Harris, E., Pierson, T.C., Fremont, D.H., Diamond, M.S., 2010. Structure and function analysis of therapeutic monoclonal antibodies against dengue virus type 2. *J. Virol.* 84 (18), 9227–9239.
- Sun, D., Feng, L., Shi, H., Chen, J., Cui, X., Chen, H., Liu, S., Tong, Y., Wang, Y., Tong, G., 2008. Identification of two novel B cell epitopes on porcine epidemic diarrhea virus spike protein. *Vet. Microbiol.* 131 (1–2), 73–81.
- Wang, K., Xie, C., Zhang, J., Zhang, W., Yang, D., Yu, L., Jiang, Y., Yang, S., Gao, F., Yang, Z., Zhou, Y., Tong, G., 2016. The identification and characterization of two novel epitopes on the nucleocapsid protein of the porcine epidemic diarrhea virus. *Sci. Rep.* 6, 39010.
- Wang, L., Byrum, B., Zhang, Y., 2014. Detection and genetic characterization of deltacoronavirus in pigs, Ohio, USA, 2014. *Emerg. Infect. Dis.* 20 (7), 1227–1230.
- Wang, X., Fang, L., Zhan, J., Shi, X., Liu, Q., Lu, Q., Bai, J., Li, Y., Jiang, P., 2020. Identification and characterization of linear B cell epitopes on the nucleocapsid protein of porcine epidemic diarrhea virus using monoclonal antibodies. *Virus Res.* 281, 197912.
- Westerink, M.A.J., Lesinski, G.B., 2001. Novel vaccine strategies to T-independent antigens. *J. Microbiol. Methods* 47 (2), 135–149.
- Woo, P.C., Lau, S.K., Lam, C.S., Lau, C.C., Tsang, A.K., Lau, J.H., Bai, R., Teng, J.L., Tsang, C.C., Wang, M., Zheng, B.J., Chan, K.H., Yuen, K.Y., 2012. Discovery of seven novel mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. *J. Virol.* 86 (7), 3995–4008.
- Yang, D., Su, M., Li, C., Zhang, B., Qi, S., Sun, D., Yin, B., 2020. Isolation and characterization of a variant subgroup GII-a porcine epidemic diarrhea virus strain in China. *Microb. Pathog.* 140, 103922.
- Yu, D., Han, Z., Xu, J., Shao, Y., Li, H., Kong, X., Liu, S., 2010. A novel B-cell epitope of avian infectious bronchitis virus N protein. *Viral Immunol.* 23 (2), 189–199.
- Yuan, D., Yan, Z., Li, M., Wang, Y., Su, M., Sun, D., 2021. Isolation and characterization of a porcine transmissible gastroenteritis coronavirus in northeast China. *Front. Vet. Sci.* 8, 611721.
- Zhang, X., Zhao, X., Dong, H., Zhu, Y., Shi, H., Chen, J., Shi, D., Feng, L., 2016. Characterization of two monoclonal antibodies that recognize linker region and carboxyl terminal domain of coronavirus nucleocapsid protein. *PLoS ONE* 11 (9), e0163920.