Two critical N-terminal epitopes of the nucleocapsid protein contribute to the cross-reactivity between porcine epidemic diarrhea virus and porcine transmissible gastroenteritis virus

Wenting Xie,1,2,3 Chaojie Ao,1,2 Yilin Yang,1,2,3 Yinan Liu,1,2,3 Rui Liang,1,2,3 Zhe Zeng,1,2,3 Gang Ye,1,2,3 Shaobo Xiao,1,2 Zhen F. Fu,1,2,4 Wanyu Dong1,2,3,5,* and Guiqing Peng1,2,3,*

Abstract
Both porcine epidemic diarrhoea virus (PEDV) and porcine transmissible gastroenteritis virus (TGEV), which cause high mortality in piglets and produce similar clinical symptoms and histopathological morphology, belong to the genus Alphacoronavirus. Serological diagnosis plays an important role in distinguishing pathogen species. Together with the spike (S) protein, the nucleocapsid (N) protein is one of the immunodominant regions among coronaviruses. In this study, two-way antigenic cross-reactivity between the N proteins of PEDV and TGEV was observed by indirect immunofluorescence assay (IFA) and Western blot analysis. Furthermore, the PEDV N protein harbouring truncations of amino acids (aa) 1 to 170 or aa 125 to 301 was demonstrated to cross-react with the anti-TGEV N polyclonal antibody (PAb), whereas the truncation-expressing aa 302 to 401 resulted in a specific reaction with the anti-PEDV N PAb but not with the anti-TGEV N PAb. Mutants of the PEDV N protein were generated based on sequence alignment and structural analysis; we then confirmed that the N-terminal residues 58-RWRMRRGERIE-68 and 78-LGTGPHAD-85 contributed to the cross-reactivity. All the results provide vital clues for the development of precise diagnostic assays for porcine coronaviruses.

INTRODUCTION
Porcine epidemic diarrhoea virus (PEDV) and porcine transmissible gastroenteritis virus (TGEV) are two types of coronaviruses with enteric tropism that cause severe widespread economic losses in the swine breeding industry [1–4]. Coronaviruses are spherical, single-stranded, positive-sense, enveloped RNA viruses. The genomes mainly encode four structural proteins: the spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins [5]. PEDV and TGEV are classified into different species within the genus Alphacoronavirus [6]. Both are transmitted primarily via the faecal–oral route and show similar clinical symptoms of vomiting, diarrhoea, dehydration and high mortality in piglets [7–9]. The specific detection of these two pathogens is essential for controlling viral epidemic diarrhoea. Given the clinical and histopathological similarities between PEDV and TGEV, the specific identification of PEDV and TGEV mainly depends on laboratory diagnosis approaches, including PCR and serological methods [3, 10–14].

In general, antigen presentation serves as the initial stage in triggering the immune response. The amount of antigen present directly determines the level of antibody response. The N protein is the most abundant coronavirus antigen produced during viral infection [6, 15]. Compared with other structural proteins, the N protein, which is used as the common antigen in the serological diagnosis of coronaviruses, has a high expression level and produces stable, long-lasting antibodies. However, different cross-reactions between porcine coronaviruses induced by the N protein have attracted attention in recent years. One-way antigenic cross-reactivity between different PEDV strains and both TGEV Miller hyperimmune antiserum and an anti-TGEV N protein monoclonal antibody (14G9.3C) was detected by cell culture immunofluorescence (CCIF), enzyme-linked immunosorbent assays (ELISAs) and
immunoblotting, indicating that the N protein is crucial to the antigenic relationship [16]. Luis further supported this phenomenon between PEDV and TGEV, verifying the cross-reactivity at the whole virus (WV) particle, N protein and M protein levels [17]. This nonspecific binding affects the effective detection of these two pathogens, impeding the accurate differentiation of porcine coronaviruses.

To establish more specific diagnostic methods, studies on the precise position of porcine coronavirus epitopes recognized by monoclonal antibodies have attracted much attention [18–20]. However, the sites that mediate the cross-reactive binding of N protein antigens among swine coronaviruses need further research. Thus, the objective of this study was to further describe the nature of the antigenic relationship between PEDV and TGEV N protein, to map the regions of N protein involved in cross-reactions and to identify cross-reactive epitopes.

Here, we investigated the antigenic relationship between PEDV and TGEV by different serological methods. The cross-reactive regions and epitopes mapped on the PEDV N protein were further explored by Western blotting. All the findings are beneficial for the development of specific serological assays.

RESULTS

A two-way cross-reaction between PEDV and TGEV was observed by immunofluorescence assay (IFA) and Western blotting

To investigate the antigenic relationship between PEDV and TGEV, we performed IFA and Western blotting with cells infected with either PEDV or TGEV using PAbs against the full-length PEDV or TGEV N proteins and hyperimmune swine antisera. The anti-N protein TGEV PAb reacted with the TGEV strain and cross-reacted weakly with the PEDV strain, while the reaction of the anti-PEDV N protein PAb to the PEDV-infected and TGEV-infected groups was similar in terms of fluorescence staining patterns (Fig. 1a). The two-way cross-reaction of whole-virus lysates with the hyperimmune swine antisera and the corresponding anti-N protein PAbs was observed by Western blotting (Fig. 1b, c). The results were consistent with those from the IFA. All anti-N protein PAbs showed greater binding for the homologous virus than for the heterologous virus.

To further determine whether this cross-reaction was induced by the N protein, plasmids encoding either the PEDV or the TGEV N protein were transfected into HEK-293T cells. Intracellular proteins were extracted and subjected to Western blotting. The successful expression of the TGEV and PEDV N proteins was verified by Western blotting using the corresponding anti-TGEV N protein or anti-PEDV N protein PAb, which had predicted molecular weights of approximately 45 KD and 56 KD, respectively (Fig. 1d). Similar to the result in the virus-infected lysates, the anti-PEDV N protein and anti-TGEV N protein PABs were strongly immunogenic to the homologous N protein, and moderate cross-reaction with the heterologous N protein was observed (Fig. 1d). This finding indicated that the anti-N protein PABs showed a preference for the N protein from homologous viruses as opposed to the N protein from viruses of other genetic groups. All experimental results showed evidence of a two-way cross-reaction between the PEDV and TGEV N proteins.

Identification of the cross-reactive regions on the PEDV N protein

The cross-reactivity between PEDV and TGEV suggested the presence of common antigenic determinants on the N proteins. To further identify the cross-reactive regions, different truncations of the PEDV N protein were constructed (Fig. 2a). Based on the predicted secondary structure of the PEDV N protein, all truncation sites were located in the loop region to maximally maintain the original structures of the truncated proteins (Fig. S1, available in the online supplementary material).

Truncations of amino acids (aa) 1 to 196 and aa 197 to 441 were designed, and successful protein expression was detected by Western blotting. Both truncations were detected by the anti-TGEV N protein PAb (Fig. 2b), which indicated that both the N-terminus and C-terminus contained cross-reactive epitopes. To further narrow the cross-reactive region, three N protein truncations were constructed, comprising aa 1 to 124, aa 125 to 301 and aa 302 to 441. The truncation comprising aa 302 to 441 was recognized by the anti-PEDV N protein PAb but not by the anti-TGEV N protein PAb (Fig. 2c), indicating that this region did not induce cross-reactivity between PEDV and TGEV. The truncation comprising aa 125 to 301 was detected by both the anti-PEDV and anti-TGEV N protein PABs, which provided further accuracy in mapping the cross-reactive site. Due to the unsuccessful expression of the truncation of aa 1 to 124, different truncations of the N-terminus were constructed. The result showed that the minimum cross-reactive site in the N-terminus was composed of aa 1 to 170, while the truncation of aa 1 to 136 could not be expressed (Fig. 2d).

The sequence identity of the full-length PEDV and TGEV N proteins was 31.1–32.2 %, while the identities for the regions comprising aa 1 to 124, aa 125 to 301 and aa 302 to 441 were 42.6–43.3 %, 25.5–27.1 % and 28.2–29.6 %, respectively (Fig. 3c). The regions comprising aa 1 to 124, aa 125 to 301 and aa 302 to 441 were termed the N-terminal domain, the central domain and the C-terminal domain, respectively. The N-terminal domain of the PEDV N protein was the most conserved compared with the TGEV N protein sequence determined by multiple sequence alignment. The structure of the PEDV N protein has not yet been resolved. To further explore potential motifs on the surface of the PEDV N protein, the predicted structure of the full-length PEDV N protein was generated based on intensive modelling in Phyre2 (Fig. 3a, b). Based on the predicted structure, truncated segments comprising aa 1 to 124 and aa 125 to
301 were selected to further identify the cross-reactive epitopes between PEDV and TGEV.

**Identification of cross-reactive epitopes on the PEDV N protein by Western blotting**

Based on structural prediction and sequence alignment, epitopes suspected of being related to cross-reactivity were selected, and each residue in these candidate epitopes was replaced with alanine residues in the full-length PEDV N protein. The mutation sites were divided into two groups, namely, the N-terminal domain and the central domain, where the experimental results suggested that the cross-reactive epitopes were located. The cross-reactive levels of recombinant N proteins were assessed by Western blotting with the anti-TGEV and anti-PEDV N protein PAbs.

To identify the cross-reactive epitopes in the N-terminal domain, mutants of highly conserved motifs 44-GNKDQ-48, 78-LGTGPHAD-85 and 99-AKEGA-103 were constructed and named M1, M2 and M3, respectively (Fig. 4a, b). M2 showed significantly lower cross-reactivity than the wild-type PEDV N protein. Furthermore, mutation of this motif did not affect the immunogenicity of the PEDV N protein, as evidenced by the strong binding ability of M2 to the anti-PEDV N protein PAb (Fig. 4c). However, M1 and M3 showed no difference in cross-reactivity compared with the positive control (Fig. 4c).

Mutation 2, namely, the 78-LGTGPHAD-85 motif, was confirmed to be the dominant, but not the only factor contributing to the cross-reactivity. To further investigate other epitopes in the N-terminal domain, the conserved 49-QIGYWT-54 and 58-RWRM-61 motifs and the relatively conserved 58-RWRMRRGERIE-68 motifs were mutated and named M5, M6 and M7, respectively (Fig. 5a, b). The reaction of the anti-PEDV N protein PAb with these three mutants revealed the successful expression of the recombinant proteins. Compared with the positive control, M5 showed similar cross-reactivity. However, M6 and M7 exhibited greatly reduced reaction with the anti-TGEV N protein PAb (Fig. 5c).

To determine the cross-reactive epitopes in the central domain, we designed a series of plasmids expressing mutants of the conserved motifs 146-NSRSMSR-152, 201-RNQSK-205, 229-ALKSLGI-235 and 243-KQQQK-247, and these mutants were named M8, M9, M10 and M11, respectively (Fig. 6a, b). The successful expression of the mutants was verified by Western blotting with the anti-PEDV N protein PAb (Fig. 6c). Unexpectedly, no mutant exhibited a decrease in binding to the anti-TGEV N protein PAb compared with the binding observed in the positive group (Fig. 6c). This result indicated that these motifs in the central domain were not dominant factors contributing to the cross-reactivity.

The 78-LGTGPHAD-85, 58-RWRM-61 and 58-RWRMRRGERIE-68 motifs were demonstrated to be cross-reactive epitopes (Fig. 7a, b). To further determine the precise cross-reactive sites in the 58-RWRMRRGERIE-68 motif, the relatively conserved 62-RGERIE-68 motif was mutated and named M12. The anti-TGEV N protein PAb had lower reactivity to M12 than to the wild-type PEDV N protein (Fig. 7c), which
showed that both the 58-RWRM-61 and 62-RRGERIE-68 motifs play an important role in the cross-reaction. Considering the potential for a synergistic effect to further reduce the cross-reactivity, we constructed a double mutant (both the 58-RWRMRRGERIE-68 and 78-LGTGPHAD-85 motifs) of the full-length PEDV N protein. However, no significant variation in the binding to the anti-TGEV N protein PAb was observed between the single mutants and the double mutant (Fig. 7c). In addition, M2 and M7 were further confirmed by the hyperimmune swine antiserum against TGEV. The results were consistent with those for anti-TGEV N PAb (Fig. 7d).

In summary, mutations were introduced into different residues (Table 1), and the 58-RWRMRRGERIE-68 and 78-LGTGPHAD-85 motifs were found to be the critical epitopes contributing to the cross-reactivity of the anti-TGEV N protein PAb with the PEDV N protein.

DISCUSSION

Antigenic cross-reactivity among TGEV, PEDV, porcine deltacoronavirus (PDCoV), feline infectious peritonitis virus (FIPV), human coronavirus (HCoV)-NL63 and severe acute respiratory syndrome (SARS)-CoV has been confirmed [16, 17, 21–25]. In general, the N protein of coronaviruses is an attractive target antigen with abundant expression throughout viral infection [12]. Therefore, the N protein is a suitable and stable structural protein for the early diagnosis of coronaviruses [3].

Previous studies showed that the cross-reactivity between TGEV and PEDV was mostly caused by the N protein [16], a finding that was consistent with our experimental results. Here, two-way cross-reactions were observed, instead of the one-way cross-reactions observed in previous studies [16], possibly because of the diversity between the different virus strains. Although an antigenic relationship between PEDV and TGEV has been reported, the details of the cross-reactive sites were virtually unknown.

Our research further confirmed the cross-reactive region and critical epitopes based on findings from previous studies. The N-terminus was strongly immunogenic, which is a common characteristic of the N protein in TGEV, PEDV and SARS [16, 18, 19, 26, 27]. The N-terminal region comprising aa 70 to 213 of the SARS N protein was suggested to be an important contributor to cross-reactivity with TGEV antiserum [23]. A monoclonal antibody (14G9.3C) against the TGEV N protein targeted the epitopes in the region comprising aa 1 to 205 and showed cross-reactivity with different PEDV strains [16, 23]. In this study, the anti-TGEV N PAb cross-reacted with the regions comprising aa 1 to 170 and aa 125 to 301 of the PEDV N protein in the Western blot analysis (Fig. 2c, d). The highly conserved QIGYWT motif was not exposed on the predicted structure (Fig. 5a), and the corresponding M5 showed strong binding ability to the anti-TGEV PAb, indicating that this motif was not the target epitope of the cross-reaction. The cross-reactivity of M13, the double mutant of the 58-RWRMRRGERIE-68 and 68-LGTGPHAD-78 motifs, was similar to that of M6, M10 and M12, rather than being significantly reduced. We speculated that this was possibly due to the steric hindrance of antigen-recognition sites. However, this assumption would need to be further confirmed. In addition, the highly conserved 78-LGTGPHAD-85 motif and the relatively conserved 58-RWRMRRGERIE-68 motif were the dominant factors (but not the only ones).
affecting the cross-reactivity between PEDV and TGEV, which was due to the presence of cross-reactive epitope(s) in the central domain. To date, the structure of the central region has not been resolved [28–34], which increases the difficulty of mapping the cross-reactive sites in the central domain. Thus, the details need to be further investigated. Furthermore, we demonstrated that the C-terminal domain comprising aa 302 to 441 was not the main contributor to the cross-reaction, similar to the previous finding of a non-cross-reactive region comprising aa 360 to 412 of the HCoV-SARS N protein in interaction with TGEV antiserum [23].

In conclusion, consistent with previous reports, our results provided evidence of cross-reactivity between PEDV and TGEV and showed that the N protein is the primary contributor to the antigenic relationship [16]. We further identified two regions and two dominant epitopes contributing to the cross-reaction. The 58-RWRMRGERIERE-68 and 78-LGTGPHAD-85 motifs were critical in affecting the cross-reaction between PEDV and TGEV, and mutations of these sites in the PEDV N protein were able to greatly attenuate this protein’s ability to bind to the anti-TGEV N PAb. All these results provide useful evidence for coronavirus immunoadsays development.

METHODS

Cells, viruses and reagent

African green monkey kidney cells (ATCC no. CCL-81) and porcine kidney-15 cells (ATCC no. CCL-33) were used for culturing PEDV and TGEV, respectively. Human embryonic kidney 293 cells stably expressing the SV40 large T antigen (HEK-293T, ATCC no. CRL-11268) were used for transient transfection. All cells were maintained at 37°C in a 5% CO2 incubator in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Waltham, MA, USA) supplemented with 10% (v/v) foetal bovine serum (FBS). The PEDV strain GD-1 and the TGEV strain WH-1 were isolated from suckling piglets. The PEDV GD-1 strain is clustered into a G2 genogroup (emerging Asian non-SINDEL) [35, 36]. The TGEV WH-1 strain is similar to the TGEV Purdue cluster [37]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific monoclonal antibody was purchased from Proteintech (Chicago, IL, USA).

Animals

Piglets were purchased from the experimental farm of Huazhong Agricultural University. Male Japanese white rabbits were purchased from the Experimental Animal Center of Huazhong Agricultural University, Hubei, People’s Republic of China. All experimental protocols involving
animals were approved by the Laboratory Animal Monitoring Committee of Huazhong Agricultural University and performed strictly according to their guidelines.

PAbs

PAbS were generated against the full-length TGEV or PEDV N protein. For the expression of N protein, TGEV strain WH-1 (GenBank: HQ462571.1) or PEDV strain GD-1 N (GenBank: JX647847.1) N gene was cloned into pET42b prokaryotic expression vector with a C-terminal His6 tag for protein purification. Then, the recombinant N protein was transformed into Escherichia coli strain BL21 (DE3), and cells were cultured at 37°C in Luria Bertani Miller broth (LB) medium until the OD600 reached 0.6. After this, 1 mM isopropyl- β-D-thiogalactopyranoside (IPTG) was added to induce protein expression for 6 h. The purity of the recombinant N proteins reached 80% by a Ni-NTA column. The initial subcutaneous immunization was performed with 0.5 mg of the N protein suspension mixed 1:1 (v/v) with Freund’s complete adjuvant into Japanese white rabbits. Additional boost inoculations were performed three times with 0.5 mg N protein suspension mixed 1:1 (v/v) with Freund’s incomplete adjuvant. Serum was collected with the highest serum titre to separate the polyclonal antibodies against the corresponding full-length N protein. The titres of the PAbs were evaluated by indirect ELISA using Hanet al.’s method [38] and found to be more than 1:12 800. Both of the PAbs were evaluated by Western blotting at a dilution of 1:3000.

Antisera

The hyperimmune antisera had been produced earlier from pigs according to the method described by Ma et al. [21] and preserved in our laboratory. Briefly, piglets were immunized twice (2 weeks apart) intramuscularly with inactivated PEDV (GD-1) and TGEV (WH-1) antigens, respectively. The
PEDV antiserum had a virus-serum neutralization (VN) titre of 1:256. The TGEV antiserum had a VN titre of 1:512.

**Plasmid construction and protein expression**

The pCAGGS vector was described previously [39]. The full-length PEDV N protein, along with eight truncations (residues 1–124, 125–301, 302–441, 1–136, 1–170, 1–196, 1–301 and 302–441), was cloned into a pCAGGS vector, and restriction enzyme sites (KpnI and Xhol) were engineered via PCR amplification. The alanine-substituted PEDV N protein mutants were designed according to the predicted three-dimensional structure. All recombinant plasmids were validated through DNA sequencing. The recombinant N protein plasmids were transiently transfected according to the manufacturer’s instructions for the Lipofectamine 2000 reagent (Invitrogen, Thermo Fisher Scientific, USA) as follows. Lipofectamine 2000 reagent and plasmids were diluted in Opti-MEM medium and incubated for 5 min at room temperature (RT; 18–22 °C). Dilutions were added at a 1:1 ratio and incubated for 20–30 min at RT. The DNA–reagent complex was added to 70–90% confluent HEK-293T cells for 6 h at 37 °C, after which the transfection mixture was removed, and the medium was replaced with DMEM supplemented with 10% (v/v) FBS for further incubation at 37 °C and 5% CO2. Total intracellular protein was extracted 30 h post-transfection. The treated cells transfected with the pCAGGS vector were considered to be the negative control group.

**IFA**

IFA was performed to determine the antigenic relationship between PEDV and TGEV. Confluent Vero CCL81 cells were inoculated with PEDV strain GD-1, and PK-15 cells were inoculated with TGEV strain WH-1 at a multiplicity of
infection (m.o.i.) of 0.01 and 0.1, respectively. Before inoculation, Vero cells were washed twice with DMEM containing 5 µg ml⁻¹ trypsin. When a cytopathic effect (CPE) was observed, the cells were gently washed twice with PBS and fixed with 4.0 % (v/v) paraformaldehyde for 15 min at RT. Membrane permeabilization in PBS containing 0.1 % (v/v) Triton X-100 (Sigma, 93426) for 15 min at RT was followed by blocking in PBS containing 2 % (w/v) bovine serum albumin (BSA) for 1 h. The anti-PEDV N protein PAb or the anti-TGEV N protein PAb was incubated with the infected cells as primary antibodies for 1 h at 37 °C. Staining was performed with Alexa Fluor 488-conjugated goat-rabbit IgG (Invitrogen) for 45 min, followed by nuclear counterstaining with DAPI for 5 min. Cells were washed three times with PBS between each step. Fluorescence images were captured with an Invitrogen EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific) at 20× magnification.

Western blot analysis
Cells inoculated with viruses or cells expressing the full-length N protein along with the truncations and mutants were collected by centrifugation at 3000 g for 5 min. Then, cells were treated with lysis buffer (Beyotime, People’s Republic of China) for 20 min at 4 °C. The supernatants were denatured by boiling for 10 min with 1×SDS-PAGE loading buffer containing 5% (v/v) beta-mercaptoethanol (2-ME). The treated samples were separated by 12% SDS-PAGE and transferred to Immuno-Blot PVDF membranes (Bio-Rad). After being blocked with 5% (w/v) skim milk for 3 h at RT, the membranes were incubated with the anti-PEDV or anti-TGEV N protein PAb for 3 h at RT. After unbound primary antibodies were rinsed off, the

\[ \text{Fold change in ratio of anti-TGEV/PEDV N PAb} \]

\[ \text{M8} \quad \text{M9} \quad \text{M10} \quad \text{M11} \quad + \quad - \]

\[ \text{Anti-TGEV N PAb} \quad \text{GAPDH} \]

\[ \text{Anti-PEDV N PAb} \quad \text{GAPDH} \]
membranes were immersed in a buffer containing HRP-conjugated goat anti-rabbit IgG (Boster) and incubated for 1 h at RT. Cells were washed three times with PBS between each incubation procedure. Immunoreactive bands were visualized by an enhanced chemiluminescence system (Amersham Imager 600, GE Healthcare). GAPDH was used as a loading control. The immunoreactive bands of target protein were quantified using ImageJ software by densitometric analysis. Three independent experiments were performed to obtain the data for statistical analysis.

**Three-dimensional structure prediction**

The predicted three-dimensional structure of the full-length PEDV N protein was generated as described in a previous report [18]. The Phyre2 server website (http://www.sbg.bio.ic.ac.uk/phyre2/) was used to predict the protein structure online. Based on heuristics to maximize confidence, percentage identity and alignment coverage, two templates (c5n4kA, aligned with the residues of aa 9 to 136; c5epwB, aligned with the residues of aa 274 to 386) were selected by Phyre2 to model the PEDV N protein. Structural figures were generated with the PyMOL molecular visualization system.

**Multiple sequence alignment and bioinformatics analysis**

To explore the conserved regions between PEDV and TGEV, the amino acid sequence of the PEDV N protein and the corresponding sequences in the TGEV and HCoV-NL63 genomes were aligned using MAFFT v7.037b. The GenBank accession numbers of these sequences are as follows: PEDV GD-1, JX647847.1; PEDV CV777, AF353511.1; PEDV TC Iowa106, KM392232.1; PEDV PC22A, KX683006.1; PEDV TC PC177-P2, KM392229.1; PEDV AJ1102, JX188454.1; PEDV YN144, KT021232.1; TGEV WH-1, HQ462571.1; TGEV Miller M6, DQ811783.1; TGEV virulent Purdue, DQ811789.2; TGEV Purdue P115, DQ811788.1; TGEV-HX, KC962433; and HCoV-NL63, DQ445912.1. The figure was rendered with ESPript 3.
Table 1. Mutations in the PEDV N protein affect cross-reactivity

<table>
<thead>
<tr>
<th>Mutation number</th>
<th>Sequence</th>
<th>Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation 1</td>
<td>44-GNKDQ-48</td>
<td>No</td>
</tr>
<tr>
<td>Mutation 2</td>
<td>78-LGTGPHAD-85</td>
<td>Yes</td>
</tr>
<tr>
<td>Mutation 3</td>
<td>99-AKEGA-103</td>
<td>No</td>
</tr>
<tr>
<td>Mutation 4</td>
<td>44-GNKDQ-48, 78-LGTGPHAD-85, 99-AKEGA-103</td>
<td>Yes</td>
</tr>
<tr>
<td>Mutation 5</td>
<td>49-QIQWTY-54</td>
<td>No</td>
</tr>
<tr>
<td>Mutation 6</td>
<td>58-RWRM-61</td>
<td>Yes</td>
</tr>
<tr>
<td>Mutation 7</td>
<td>58-RWRRMRRGERIE-68</td>
<td>Yes</td>
</tr>
<tr>
<td>Mutation 8</td>
<td>146-NSRMSR-152</td>
<td>No</td>
</tr>
<tr>
<td>Mutation 9</td>
<td>201-RNQSK-205</td>
<td>No</td>
</tr>
<tr>
<td>Mutation 10</td>
<td>229-ALKSLGI-235</td>
<td>No</td>
</tr>
<tr>
<td>Mutation 11</td>
<td>243-KQQQK-247</td>
<td>No</td>
</tr>
<tr>
<td>Mutation 12</td>
<td>62-RRGERIE-68</td>
<td>Yes</td>
</tr>
<tr>
<td>Mutation 13</td>
<td>78-LGTGPHAD-85, 58-RWRRMRRGERIE-68</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Statistical analysis

Statistical analysis was performed on the results of three independent experiments. The data in the histograms were calculated from the relative density obtained by ImageJ analysis of the technical duplicates in each individual experiment. The statistical results represent the ratio of the different recombinant proteins detected by the anti-TGEV and anti-PEDV N protein PAb normalized to GAPDH in the different groups. The data are the mean±SD. The differences in the means were analysed by Student’s t-test. P values of less than 0.05 were considered statistically significant (*P<0.05, **P<0.01 and ***P<0.001).

Funding information

This work was supported by the National Key R&D Plan of China (programme no. 2018YFD0500100) and the Huazhong Agricultural University Scientific and Technological Self-Innovation Foundation (programme nos 2662015JQ003 and 2662017PY028).

Acknowledgements

We thank Qianqian Liu and Liran Liu for the expression and purification of PEDV and TGEV N protein and the preparation of polyclonal antibodies.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

The authors declare no ethical issues relevant to this work.

References

23. Vlasova AN, Zhang X, Hasokszu M, Nagesha HS, Haynes LM et al. Two-way antigenic cross-reactivity between severe acute respiratory syndrome coronavirus (SARS-CoV) and group 1 animal CoVs.


---

**Five reasons to publish your next article with a Microbiology Society journal**

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.