Pathogenesis comparison between the United States porcine epidemic diarrhoea virus prototype and S-INDEL-variant strains in conventional neonatal piglets

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At least two genetically different porcine epidemic diarrhoea virus (PEDV) strains have been identified in the USA: US PEDV prototype and S-INDEL-variant strains. The objective of this study was to compare the pathogenicity differences of the US PEDV prototype and S-INDEL-variant strains in conventional neonatal piglets under experimental infections.

Fifty PEDV-negative 5-day-old pigs were divided into five groups of ten pigs each and were inoculated orogastrically with three US PEDV prototype isolates (IN19338/2013, NC35140/2013 and NC49469/2013), an S-INDEL-variant isolate (IL20697/2014), and virus-negative culture medium, respectively, with virus titres of $10^4$ TCID$_{50}$ ml$^{-1}$, 10 ml per pig.

All three PEDV prototype isolates tested in this study, regardless of their phylogenetic clades, had similar pathogenicity and caused severe enteric disease in 5-day-old pigs as evidenced by clinical signs, faecal virus shedding, and gross and histopathological lesions. Compared with pigs inoculated with the three US PEDV prototype isolates, pigs inoculated with the S-INDEL-variant isolate had significantly diminished clinical signs, virus shedding in faeces, gross lesions in small intestines, caeca and colons, histopathological lesions in small intestines, and immunohistochemistry staining in ileum. However, the US PEDV prototype and the S-INDEL-variant strains induced similar viraemia levels in inoculated pigs. Whole genome sequences of the PEDV prototype and S-INDEL-variant strains were determined, but the molecular basis of virulence differences between these PEDV strains remains to be elucidated using a reverse genetics approach.
PEDV prototype strain, the US PEDV variant strain has insertions and deletions (INDELs) in the spike gene and is also called the S INDEL strain (Vlasova et al., 2014). In this study, we describe PEDVs circulating in the USA as the US PEDV prototype strain and US PEDV S-INDEL-variant strain. Since the PED outbreak in the USA, detection of US prototype-like PEDV has been reported in Canada, Mexico, Taiwan, South Korea, Japan and Ukraine (Dastjerdi et al., 2015; Lee & Lee, 2014; Lin et al., 2014; Ojkic et al., 2015; Van Diep et al., 2015; Vlasova et al., 2014); detection of US S-INDEL-variant-like PEDV has been reported in South Korea, Germany, Belgium, France, Portugal, Japan, Italy and Austria (Boniotti et al., 2016; Grasland et al., 2015; Hanke et al., 2015; Lee et al., 2014; Mesquita et al., 2015; Stadler et al., 2015; Steinrigl et al., 2015; Theuns et al., 2015; Yamamoto et al., 2015).

The pathogenicity of the US PEDV prototype strain has been evaluated in gnotobiotic (Jung et al., 2014) Caesarean-derived colostrum-deprived (Liu et al., 2015; Madson et al., 2016) and conventional pigs (Jung et al., 2015a; Madson et al., 2014; Thomas et al., 2015); these studies experimentally confirmed that the US PEDV prototype isolates are highly virulent. Evaluation of the pathogenicity of the US S-INDEL-variant strain in experimentally infected pigs has not been reported until recently (Lin et al., 2015a), when we were in the process of revising this manuscript. In Lin et al. (2015a), pathogenesis of a US S-INDEL-variant PEDV was investigated in 3–4 day-old suckling piglets housed together with sows. The objective of the current study was to evaluate the pathogenesis of the US PEDV S-INDEL-variant strain and also compare it with the pathogenesis of three US PEDV prototype isolates in 5-day-old conventional piglets.

RESULTS

Isolation and sequence comparison of US PEDVs

Three US PEDV prototype isolates, USA/NC35140/2013, USA/IA49379/2013 and USA/NC49469/2013, were isolated in Vero cells. Typical PEDV cytopathic effects, including syncytial body formation and cell detachment, were observed and virus growth was confirmed by immunofluorescence staining. All isolates grew efficiently in Vero cells and the infectious titres ranged from $10^3$ to $10^6$ TCID$_{50}$ ml$^{-1}$ for the first ten passages.

The whole genome sequences of the three US PEDV prototype isolates NC35140, IA49379 and NC49469 were determined and compared with those of the previously described US PEDV prototype isolate USA/IN19338/2013 and the US PEDV S-INDEL-variant isolate USA/IL20697/2014, with results summarized in Table 1. Schematic diagrams of PEDV genome organization and putative functions of viral proteins are described in Fig. S1 (available in the online Supplementary Material). The prototype isolates IN19338, NC35140, IA49379 and NC49469 all had a genome 28038 nt in length and had 99.75–99.91% nt identity (26–69 nt differences) to each other at the whole genome level. The spike genes of these prototype isolates were all 4161 nt in length and had 99.54–99.88% nucleotide identity (5–19 nt differences) to each other. The S-INDEL-variant isolate IL20697 had a genome 28029 nt in length and had 99.08–99.22% nucleotide identity (220–259 nt differences) at the whole genome level to the four prototype isolates evaluated in this study. Among them, about 64–96 nt differences were located in the ORF1a/1b region, especially the nsp12 and nsp16 regions; however, a majority of these nucleotide changes on nsp12 and nsp16 were synonymous (silent) changes at the amino acid level (Table 1). Striking differences between the US PEDV prototype and S-INDEL-variant isolates were located in the spike gene (96.25–96.37% nucleotide identity; 151–156 nt differences), especially the S1 portion (93.14–93.32% nucleotide identity; 148–152 nt differences); the nucleotide changes in the S1 portion resulted in changes of the deduced amino acids (Table 1). Compared with the prototype isolates, the S gene of the variant isolate IL20697 had three characteristic deletions (a 1 nt deletion of G at position 167, an 11 nt deletion of AGGG-TGTCaAT at positions 176–186, and a 3 nt deletion of ATA at positions 416–418) and one insertion (a 6 nt insertion of CAGGAT between positions 474 and 475).

Phylogenetic analyses of the PEDV isolates described in this study and 45 PEDV reference sequences are provided in Fig. 1. In the whole genome sequence-based neighbour-joining tree (Fig. 1a), the US PEDV prototype-like strains clustered together, and could be further divided into clade 1 and clade 2; however, the US PEDV S-INDEL-variant-like strains clustered separately. In the whole genome sequence-based maximum-likelihood tree (Fig. 1b), the US PEDV prototype-like strains also clustered into clade 1 and clade 2; however, the S-INDEL-variant-like strains formed a separate sublineage within clade 2. In contrast, the phylogenetic clusters in the S1 sequence-based neighbour-joining tree (Fig. 1c) and the maximum-likelihood tree (Fig. 1d) were similar. In both (Fig. 1c, d), the US PEDV prototype-like strains clustered together and could be further divided into clade 1 and clade 2, similar to the whole genome sequence-based neighbour-joining tree (Fig. 1a); the US PEDV S-INDEL-variant-like strains formed a separate branch that was more closely related to some classical PEDV isolates such as Europe/CV777, South Korea/SM98 and China/SD-M, which had the same pattern of insertions and deletions in the S gene as the US PEDV S-INDEL-variant-like strains.

The prototype isolates IN19338 and IA49379 belonged to clade 1 and the isolate NC35140 belonged to clade 2 in both the whole genome-based trees (Fig. 1a, b) and the S1-based trees (Fig. 1c, d). However, the prototype isolate NC49469 belonged to clade 1 in the whole genome-based trees (Fig. 1a, b) but to clade 2 in the S1-based trees (Fig. 1c, d). Three prototype isolates (IN19338 in clade 1,
### Table 1. Nucleotide and amino acid differences between the US PEDV prototype and S-INDEL-variant isolates

<table>
<thead>
<tr>
<th>Genome region or ORF (nt position)*</th>
<th>Protein: region [length (aa)]</th>
<th>Difference among prototype isolates†</th>
<th>Difference between prototype isolates‡ and S-INDEL-variant‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole genome (1–28038)</td>
<td></td>
<td>Dnt, N (%)§</td>
<td>Ant, N (%)§</td>
</tr>
<tr>
<td>5' UTR (1–292)</td>
<td></td>
<td>26–69 (0.09–0.25)</td>
<td>220–259 (0.78–0.92)</td>
</tr>
<tr>
<td>Nonstructural proteins</td>
<td></td>
<td>Aaa, N (%)§</td>
<td>0–2 (0–0.68)</td>
</tr>
<tr>
<td>ORF1a/b (293–20637)</td>
<td>lab Polyprotein [6781]</td>
<td>0–2 (0–0.68)</td>
<td>0–2 (0–0.68)</td>
</tr>
<tr>
<td>nsp1: Met1–Gly110 [110]</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>nsp2: Asn111–Gly895 [785]</td>
<td></td>
<td>5–13 (0.21–0.55)</td>
<td>4–17 (0.17–0.72)</td>
</tr>
<tr>
<td>nsp3: Gly896–Gly2516 [1621]</td>
<td></td>
<td>5–22 (0.10–0.45)</td>
<td>6–24 (0.12–0.49)</td>
</tr>
<tr>
<td>nsp4: Ala2517–Gln2997 [481]</td>
<td></td>
<td>0–1 (0–0.07)</td>
<td>1–2 (0.07–0.14)</td>
</tr>
<tr>
<td>nsp5: Ala2998–Gln3299 [302]</td>
<td></td>
<td>0–2 (0–0.22)</td>
<td>0–1 (0–0.11)</td>
</tr>
<tr>
<td>nsp6: Ser3300–Gln3579 [280]</td>
<td></td>
<td>0–2 (0–0.24)</td>
<td>0–1 (0–0.12)</td>
</tr>
<tr>
<td>nsp7: Ser3580–Gln3662 [83]</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>nsp8: Ser3663–Gln3857 [195]</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>nsp9: Asn3858–Gln3965 [108]</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>nsp10: Ala3966–Gln4100 [135]</td>
<td></td>
<td>0–1 (0–0.25)</td>
<td>0–1 (0–0.25)</td>
</tr>
<tr>
<td>nsp12: Ser4101–Gln5027 [927]</td>
<td></td>
<td>0–6 (0–0.22)</td>
<td>23–27 (0.83–0.97)</td>
</tr>
<tr>
<td>nsp13: Ser5028–Gln5546 [519]</td>
<td></td>
<td>1–2 (0.06–0.12)</td>
<td>0–3 (0.12–0.19)</td>
</tr>
<tr>
<td>nsp14: Asn5547–Gln6141 [595]</td>
<td></td>
<td>0–3 (0–0.17)</td>
<td>1–2 (0.06–0.11)</td>
</tr>
<tr>
<td>nsp15: Gly6142–Gln6480 [339]</td>
<td></td>
<td>0–2 (0–0.20)</td>
<td>8–9 (0.79–0.88)</td>
</tr>
<tr>
<td>nsp16: Ala6481–Lys6781 [301]</td>
<td></td>
<td>0–1 (0–0.11)</td>
<td>12–13 (1.32–1.43)</td>
</tr>
<tr>
<td>Structural/accessory proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spike (20634–24794)</td>
<td>S [1386]</td>
<td>5–19 (0.12–0.46)</td>
<td>151–156 (3.63–3.75)</td>
</tr>
<tr>
<td>Spike 1 (20634–22847)</td>
<td>S1 [738]</td>
<td>4–13 (0.18–0.59)</td>
<td>148–152 (6.68–6.86)</td>
</tr>
<tr>
<td>Spike 2 (22848–24794)</td>
<td>S2 [648]</td>
<td>0–6 (0–0.31)</td>
<td>3–8 (0.15–0.41)</td>
</tr>
<tr>
<td>ORF3 (24794–25468)</td>
<td>NS3B [224]</td>
<td>0–3 (0–0.44)</td>
<td>1–3 (0.15–0.44)</td>
</tr>
<tr>
<td>Envelope (25449–25679)</td>
<td>E [76]</td>
<td>0–1 (0–0.43)</td>
<td>0–1 (0–0.43)</td>
</tr>
<tr>
<td>Membrane (25687–26367)</td>
<td>M [226]</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Nucleocapsid (26379–27704)</td>
<td>N [441]</td>
<td>1–2 (0.07–0.15)</td>
<td>3–4 (0.23–0.30)</td>
</tr>
<tr>
<td>3' UTR (27705–28038)</td>
<td>NA</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

NA, Not applicable.

*Nucleotides are numbered according to the sequence of US PEDV prototype isolate USA/IN19338/2013 (GenBank accession number KF650371).

†Prototype isolates IN19338, IA49379, NC49469 and NC35140.

‡S-INDEL-variant isolate IL20697.

§Percentage nucleotide (nt) and amino acid (aa) differences were calculated at each gene or protein level.
NC35140 in clade 2, and NC49469 in clade 1 or clade 2 depending on whole genome- or S1-based phylogenetic analyses) and one S- INDEL-variant isolate, IL20697, were selected to compare their pathogenesis in pigs (Table 2).

**Clinical assessment**

All pigs in groups 1 (G1, IN19338), 2 (G2, NC35140) and 3 (G3, NC49469) developed soft to watery diarrhoea starting from 1 day post-infection (p.i.) and continuing through 6 or 7 days p.i. In contrast, in group 4 (G4, IL20697), only one pig had mild diarrhoea with soft faeces at 1 day p.i. The mean diarrhoea scores are summarized in Fig. 2(a). Overall, pigs in G1 (P<0.001) and G3 (P<0.0001) had significantly higher mean diarrhoea scores than pigs in G2 when 0–7 days p.i. diarrhoea scores were analysed as described in Methods. Pigs in G1–G3, inoculated with the prototype PEDV isolates, overall had significantly higher mean diarrhoea scores than G4 (P<0.0001), inoculated with the PEDV variant isolate, and G5 (negative control, P<0.0001). The mean diarrhoea scores were not significantly different between G4 and G5 (P=1).

No vomiting was observed from any pig throughout the study. In G1–G3, inoculated with the prototype isolates: (1) almost all pigs lost their appetite during the study period and tube feeding had to be administered; (2) severe dehydration, rough hair, and flat or thin flanks were observed in all pigs with most severe body conditions at about 4 days p.i.; (3) various degrees of lethargy, including head down and recumence, were observed from 1 day p.i. to the end of the study. In contrast, in G4 inoculated with the variant isolate: (1) all pigs had normal appetite; (2) no dehydration or lethargy was observed; (3) 90% of pigs had mild flat flanks at 1 or 2 days p.i. but recovered to normal after 4 days p.i. All G5 pigs were active, without diarrhoea, dehydration, lethargy or anorexia during the study period.

From −1 to 3 days p.i., PEDV-inoculated pigs (G1–G4) had significantly lower mean daily weight gain (MDG, P<0.0001) than pigs in G5 (negative control), but there were no significant differences in MDG (P values ranged from 0.089 to 1) among G1–G4 (Fig. 2b). From −1 to 7 days p.i., G1–G3 (prototype isolates) had significantly lower MDG (P values ranged from 0.0 to 0.037) than G4 (variant isolate), although none of G1, G2, G3 or G4 had significant difference in MDG (P values ranged from 0.078 to 0.847) compared with G5 (negative control) (Fig. 2b).

**Virus shedding and distribution**

PEDV RNA was detected in rectal swab samples from all pigs in G1–G3 (prototype isolates) at 1 day p.i. until the end of the study. In G4 (variant isolate), PEDV RNA was detected in rectal swab samples from all pigs 1 day p.i. (Fig. 2b).
The mean numbers of genomic copies ml⁻¹ in rectal swabs are summarized in Fig. 2(c). Pigs in G1, G2, G3 and G5 had similar levels (P = 0.601) of faecal virus shedding from 1 to 7 days p.i., with the quantity ranging from 10⁷.² to 10⁹.⁰ genomic copies ml⁻¹, corresponding to C₅₀ values of 16–22. Pigs in G3 had the highest level of faecal virus shedding at 1–2 days p.i. (approx. 10⁹ genomic copies ml⁻¹, with C₅₀ of 16) and the faecal virus shedding from 1 to 7 days p.i., with the quantity ranging from 10⁷.² to 10⁹.⁰ genomic copies ml⁻¹, corresponding to C₅₀ values of 16–22. Pigs in G3 had the highest level of faecal virus shedding at 1–2 days p.i. (approx. 10⁹ genomic copies ml⁻¹, with C₅₀ of 16) and the faecal virus

### Table 2. Design of experiment in which 5-day-old pigs were inoculated with various PEDV isolates

<table>
<thead>
<tr>
<th>Group</th>
<th>No. pigs</th>
<th>US PEDV strain</th>
<th>Inoculum</th>
<th>3 days p.i.</th>
<th>7 days p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>10</td>
<td>Prototype isolate USA/IN19338/2013</td>
<td>10⁴ TCID₅₀ ml⁻¹; 10 ml</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>G2</td>
<td>10</td>
<td>Prototype isolate USA/NC35140/2013</td>
<td>10⁴ TCID₅₀ ml⁻¹; 10 ml</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>G3</td>
<td>10</td>
<td>Prototype isolate USA/NC49469/2013</td>
<td>10⁴ TCID₅₀ ml⁻¹; 10 ml</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>G4</td>
<td>10</td>
<td>S-INDEL-variant isolate USA/LI20697/2014</td>
<td>10⁴ TCID₅₀ ml⁻¹; 10 ml</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>G5</td>
<td>10</td>
<td>Virus-negative culture medium</td>
<td>10 ml</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

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**Pathogenesis comparison of US PEDVs in neonates**

**Fig. 1.** Phylogenetic analysis of the full-length genome and S1 portion nucleotide sequences. Three US prototype PEDV isolates obtained in this study (USA/NC35140/2013, USA/A49379/2013 and USA/NC49469/2013) and two previous isolates evaluated in this study (US PEDV prototype isolate USA/IN19338/2013 and US S-INDEL-variant isolate USA/IL20697/2014) are indicated with circles or a triangle, respectively. Forty-five PEDV sequences selected from GenBank were also included for analysis. The trees were reconstructed using the distance-based neighbour-joining (NJ) method (a, c) and the maximum-likelihood (ML) method (b, d) of the software MEGA 6. The US prototype-like PEDVs are shown in blue type, with clade 1 and clade 2 indicated. The US S-INDEL-variant-like PEDVs are shown in red type. Bars indicate nucleotide substitutions per site.
shedding gradually declined to approximately $10^{5.4}$ genomic copies ml$^{-1}$ at 7 days p.i., corresponding to a $C_t$ value of 28. In contrast, pigs in G4 had about $10^{2.3}$ genomic copies ml$^{-1}$ ($C_t$ 31.8) faecal virus shedding at 1 day p.i.; the faecal virus shedding gradually increased, peaked at 5 days p.i. ($10^{3.4}$ genomic copies ml$^{-1}$ with $C_t$ of 28.8) and then declined to approximately $10^{1.3}$ genomic copies ml$^{-1}$ ($C_t$ 36.3) at 7 days p.i. Statistical analyses indicated that G1–G3 (prototype isolates) had significantly larger amounts of viral RNA shedding in rectal swabs ($P_{v}<0.0001$) than did G4 (variant isolate).

PEDV RNA was detected in serum samples from all pigs in G1–G4 necropsied at 3 days p.i., with mean $C_t$ values of 33.2 (G1), 30.5 (G2), 31.9 (G3) and 28.4 (G4). There were no significant differences between mean PEDV genomic copies in sera of G1–G4 at 3 days p.i. ($P$ values ranged from 0.077 to 0.646, Fig. 2d). At 7 days p.i., PEDV RNA was detected in serum samples from three or four out of five pigs in G1–G4, with mean $C_t$ values (only for PCR-positive pigs) of 33.5 (G1), 34.2 (G2), 37.4 (G3) and 35.8 (G4). The mean number of genomic copies of PEDV in sera of G4 (variant isolate) showed no significant difference ($P$ values ranged from 0.050 to 0.717) from those in G1–G3 (prototype isolates) at 7 days p.i. (Fig. 2d).

Virus distributions in tissues are summarized in Table S1. At 3 days p.i., regardless of G1, G2, G3 or G4, mean PEDV RNA concentrations in ileums, caeca, colons and mesenteric lymph nodes were higher than the concentrations in other tissues within the same inoculation group. When viral RNA concentrations in each tissue type were compared across the four inoculation groups at 3 days p.i., viral RNA concentrations in caecum and

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**Fig. 2.** Clinical assessment and virus shedding of 5-day-old pigs inoculated with US PEDV prototype and S-INDEL-variant isolates. (a) Mean diarrhoea scores over the course of 7 days p.i. See Methods for scoring system. (b) Mean daily weight gain (MDG). Statistical analyses on MDG were performed among groups from −1 to 3 days p.i. and from −1 to 7 days p.i., respectively. Virus shedding in rectal swabs (c) and sera (d) of inoculated pigs was determined by a quantitative PEDV N-gene-based real-time RT-PCR. The virus titres [log$_{10}$ (genomic copies ml$^{-1}$)] at each time point were the mean values of all available pigs (both PCR-positive and -negative pigs) in each group. Error bars indicate SE. In (d), virusaemia levels among the groups were statistically analysed at 3 and 7 days p.i., respectively. Labels without the same letters indicate significant differences, for example A and B have significant difference, but A and AB have no significant difference.
colon of G4 (variant isolate) were overall significantly lower than in caecum and colon of G1–G3 (prototype isolates). However, viral RNA concentrations in other tissues of G4 were similar to those in the corresponding tissues of G1–G3; the same types of tissues in G1–G3 had similar levels of viral RNA. Data at 7 days p.i. overall supported similar conclusions to 3 days p.i., except that the numbers of viral genomic copies in caecum and colon were much lower than those in ileum and mesenteric lymph node in G4.

All rectal swabs, sera and tissue samples from G5 (negative control) were negative by PEDV real-time reverse transcription PCR (RT-PCR) throughout the study period.

**Gross pathology**

At 3 days p.i., thin and transparent walls, sometimes dilated by gas and/or yellowish fluid, were observed in small intestine, caecum and colon tissues in most pigs inoculated with the PEDV prototype isolates (G1–G3). In addition, almost all pigs in G1–G3 had watery contents in small intestines, caeca and colons. In contrast, only mildly thin walls could be observed in small intestines of 3/5 pigs inoculated with the PEDV variant isolate (G4); no apparent gross lesions were observed in caeca or colons of pigs in G4. Also, in G4, only 1/5 pigs had watery contents in the small intestine, caecum and colon; two other pigs had semi-watery contents in the caecum. Statistically, all G1–G3 (prototype isolates) had significantly higher small intestine, caecum and colon content scores (P values ranged from <0.0001 to 0.0127) than G4 (variant isolate) and G5 (negative control) (Fig. 3a). A significant difference was not observed among G1–G3 (P values ranged from 0.3722 to 1) for small intestine, caecum and colon content scores (Fig. 3a). For G4, only caecum content scores were significantly higher than for G5 (P=0.003), not small intestine or colon content scores (P values ranged from 0.5259 to 1; Fig. 3a). All prototype isolate-inoculated groups (G1–G3) had significantly higher tissue lesion scores on small intestine, caecum and colon (P values ranged from 0.0012 to 0.049) than the variant isolate-inoculated group (G4) and the negative control group (G5) (Fig. 3b). No significant differences were observed in small intestine, caecum and colon lesion scores among G1–G3; the same types of tissues in G1–G3 had significantly decreased mean villus heights, increased mean crypt depths, and lower mean villus/crypt ratios in duodenum, proximal jejunum, middle jejunum, distal jejunum and ileum compared with pigs in G4 (variant isolate) and G5 (negative control), with some exceptions (Fig. 5); exceptions include villus height and villus/crypt ratio in duodenum of G2 and G4 as well as crypt depth in middle jejunum of G1 and G4. The mean villus heights, crypt depths and villus/crypt ratios of small intestine sections at 3 days p.i. were, overall, similar across the three groups G1–G3 inoculated with the prototype isolates (Fig. 5). The mean crypt depths of all small intestinal sections at 3 days p.i. were not significantly different between G4 (variant isolate) and G5 (negative control); however, pigs in G4 had significantly decreased mean villus heights and lower mean villus/crypt ratios in duodenum, middle and distal jejunum, and ileum at 3 days p.i. compared with pigs in G5 (Fig. 5).

At 7 days p.i., most pigs in G1–G3 still had thin-walled and/or gas-distended small intestines, but about 50% of pigs in G1–G3 had thin-walled and/or gas-distended caeca and colons. All pigs in G1 and G2, and 4/5 pigs in G3 had watery small intestine contents. About 60–100% of pigs in G1–G3 had semi-watery or watery contents in caeca and colons. In G4, only 1/5 pigs had thin-walled small intestines and none of the pigs had gross lesions in caeca and colons. In G4, 5/5, 1/5 and 0/5 pigs had semi-watery or watery contents in small intestines, caeca and colons. Significant differences in tissue content scores and tissue lesion scores were observed between some groups (Fig. 3).

Rectal swabs collected at 3 and 7 days p.i. were confirmed negative for porcine deltacoronavirus (PDCoV), transmissible gastroenteritis virus (TGEV), and porcine rotaviruses (groups A, B and C) and negative for haemolytic *Escherichia coli* and *Salmonella* spp.

**Histopathology**

No remarkable microscopic lesions were observed in stomach, caecum, colon, tonsil, mesenteric lymph node, heart, lung, liver, spleen and kidney sections of all piglets (G1–G5) at either 3 or 7 days p.i.

Severe lesions consistent with viral enteritis (e.g. villous enterocyte swelling, villous atrophy, collapsed lamina propria) were observed in small intestinal sections (duodenum, jejunum and ileum) of all pigs in G1–G3 (prototype isolates) necropsied at 3 and 7 days p.i. Mild microscopic lesions consistent with viral enteritis were observed in small intestinal sections of pigs in G4 (variant isolate) necropsied at 3 days p.i.; however, microscopic lesions were not remarkable in small intestinal sections of pigs in G4 necropsied at 7 days p.i. Microscopic lesions were not apparent in small intestinal sections of pigs in G5 (negative control) at either 3 or 7 days p.i. Representative images of haematoxylin and eosin (H&E)-stained ileum sections of pigs necropsied at 3 days p.i. from G1–G5 are shown in Fig. 4(a–e).

Villus height, crypt depth and villus height/crypt depth ratio were measured and compared on small intestinal sections of the five inoculation groups. At 3 days p.i., pigs in G1–G3 (prototype isolates) had significantly decreased mean villus heights, increased mean crypt depths, and lower mean villus/crypt ratios in duodenum, proximal jejunum, middle jejunum, distal jejunum and ileum compared with pigs in G4 (variant isolate) and G5 (negative control), with some exceptions (Fig. 5); exceptions include villus height and villus/crypt ratio in duodenum of G2 and G4 as well as crypt depth in middle jejunum of G1 and G4. The mean villus heights, crypt depths and villus/crypt ratios of small intestine sections at 3 days p.i. were, overall, similar across the three groups G1–G3 inoculated with the prototype isolates (Fig. 5). The mean crypt depths of all small intestinal sections at 3 days p.i. were not significantly different between G4 (variant isolate) and G5 (negative control); however, pigs in G4 had significantly decreased mean villus heights and lower mean villus/crypt ratios in duodenum, middle and distal jejunum, and ileum at 3 days p.i. compared with pigs in G5 (Fig. 5).

At 7 days p.i., the mean villus heights and villus/crypt ratios of small intestinal sections were, overall, similar across the
three groups G1–G3 (Fig. 6a, c). Pigs in G1–G3, overall, had significantly decreased mean villus heights and lower mean villus/crypt ratios in small intestinal sections compared with pigs in G4 and G5 at 7 days p.i. (Fig. 6a, c). Interestingly, the mean villus heights at 7 days p.i. either were not significantly different between G4 (variant isolate) and G5 (negative control) or were significantly higher in G4 than G5 (Fig. 6a). The mean villus/crypt ratios at 7 days p.i. were not significantly different in proximal, middle and distal jejunum, and ileum between G4 and G3, although the mean villus/crypt ratios in duodenum were significantly different between G4 and G5 (Fig. 6c). Comparison of mean crypt depths at 7 days p.i. is presented in Fig. 6(b). The mean crypt depths were similar in all small intestinal sections between the prototype isolate-inoculated groups G2 and G3; both G2 and G3 had significantly greater crypt depths than the negative control group G5. Another prototype-isolate-inoculated group, G1, had mean crypt depth values that were between the negative control group G5 and the prototype-isolate-inoculated groups G2 and G3. The variant-isolate-inoculated group G4 had significantly increased mean crypt depths in duodenum, and proximal and distal jejunums compared with the negative control group G5, while G4 had mean crypt depths similar to those of G1, G2 and G3 in most of the small intestinal sections.

**Immunohistochemistry (IHC)**

At 3 days p.i., PEDV-specific IHC staining was performed on serial sections of ileum, caecum and colon of all five inoculation groups. None of the five pigs in G5 (negative control) was IHC positive in the ileum, caecum or colon. All five pigs in each of G1–G4 were IHC positive in the ileum, with mean IHC scores of 3.9 (G1), 3.7 (G2), 3.8 (G3) and 2.5 (G4). The mean IHC scores for ileum were

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**Fig. 3.** Examination of gross lesions of inoculated pigs at 3 and 7 days p.i. necropsies. Mean scores of contents of small intestine, caecum and colon are shown in (a). Mean scores of lesions of the same tissues are shown in (b). The scoring criteria are described in Methods. Statistical analyses were performed on various inoculated groups, but each time on one tissue type and at one time point. Labels without the same letters indicate significant differences, as explained for Fig. 2. Error bars indicate SE.
similar across G1–G3 and were significantly higher than for G4 (Fig. 5d). Regarding IHC staining of caeca, 5/5 (G1), 4/5 (G2), 5/5 (G3) and 3/5 (G4) pigs were positive, with no significant differences in mean IHC scores among G1–G4 (Fig. 5d). For colons, 5/5 (G1), 4/5 (G2), 4/5 (G3) and 2/5 (G4) pigs were IHC positive, but the mean IHC scores were not significantly different among G1–G4 (Fig. 5d). Representative PEDV IHC staining images are shown in Fig. 4(f–t).

At 7 days p.i., PEDV IHC staining was only performed on serial sections of ileum. Mild/scant IHC staining was observed in G1 and G2 but no staining was observed in G3, G4 and G5 (Fig. 6d).

**DISCUSSION**

Sequence analyses demonstrated that at least two genetically different PEDV strains have been circulating in the USA (Vlasova et al., 2014; Wang et al., 2014), and they are referred to as US PEDV prototype strain and US PEDV S-INDEL-variant strain. The US prototype PEDVs can be phylogenetically further divided into clade 1 and clade 2. In the whole genome sequence-based phylogenetic analyses, the US S-INDEL-variant-like PEDVs clustered separately from clade 1 and clade 2 in the neighbour-joining tree, but they formed a separate sublineage within clade 2 in the maximum-likelihood tree (Fig. 1a, b). This suggests that phylogenetic analysis tools and tree construction methods could result in some differences in the outcomes of the analyses; thus, conclusions should be drawn cautiously by clearly indicating the tools and methods used for phylogenetic analyses. Among US prototype PEDVs, some always belong to clade 1 or clade 2 regardless of whether whole genome-based trees or S1-based trees are used; however, some (e.g. NC49469 and Minnesota62) belong to clade 1 in whole genome-based trees but belong to clade 2 in S1-based trees (Fig. 1). This is probably because the S1 sequences of NC49469 and Minnesota62 PEDVs are more closely related to clade 2 whereas the remaining genome sequences are more closely related to clade 1 PEDVs. Our group has isolated various PEDVs in cell culture that fall into each category described above, enabling us to compare the pathogenesis of various US prototype and S-INDEL-variant PEDVs.

Previous studies have demonstrated that neonatal piglets are more susceptible than weaned pigs to PEDV infection,
and PEDV infection induces greater disease severity in neonates than in weaned pigs (Jung et al., 2015a; Thomas et al., 2015). Therefore, a sensitive 5-day-old neonatal piglet model was selected for pathogenesis comparisons in this study. Among three US PEDV prototype isolates (IN19338, NC35140 and NC49469), the mean diarrhoea scores induced by the NC35140 isolate were lower than those induced by the IN19338 and NC49469 isolates; however, three prototype isolates had similar virus shedding, gross lesions, histopathological lesions and IHC staining. Overall, we conclude that the three US PEDV prototype isolates evaluated in this study have similar pathogenicity in neonatal piglets regardless of their phylogenetic clades. In contrast, data in the current study clearly demonstrate that the US PEDV S-INDEL-variant isolate IL20697 had significantly diminished clinical signs, virus shedding in faeces, gross lesions in small intestines, caeca and colons, histopathological lesions in small intestines, and IHC scores in ileum, compared with three US PEDV prototype isolates, IN19338, NC35140 and NC49469. Recent experimental studies by other groups also demonstrated that S-INDEL PEDVs overall had lower pathogenicity than the US prototype strains in 3–4-day-old or 1-week-old pigs (Lin et al., 2015a; Yamamoto et al., 2015). However, Lin et al. (2015a) observed that three litters of piglets inoculated with a US S-INDEL Iowa106 strain had zero mortality but one litter of piglets inoculated with the same virus strain had 75% mortality. They hypothesized that the sows’ health condition can have a direct impact on colostrum/milk production and thus affect the infection outcome of their piglets (Lin et al., 2015a). The virulence of S-INDEL PEDVs observed in the field has variations among farms and countries. In the USA, the S-INDEL variant strain OH851 infection only caused minimal to no clinical signs in suckling piglets on the farm (Wang et al., 2014). In Germany, two sow farms were infected with an S-INDEL PEDV that has 99.4% nucleotide identity to the US S-INDEL-variant OH851 at the whole genome level; however, severity of clinical signs and mortality in suckling piglets varied significantly between the two farms (Stadler et al., 2015). Factors contributing to the contradictory findings have not been clearly identified, but source of viruses (WT or cell-culture-adapted viruses), inoculation/infection doses, and PEDV infection induces greater disease severity in neonates than in weaned pigs (Jung et al., 2015a; Thomas et al., 2015). Therefore, a sensitive 5-day-old neonatal piglet model was selected for pathogenesis comparisons in this study. Among three US PEDV prototype isolates (IN19338, NC35140 and NC49469), the mean diarrhoea scores induced by the NC35140 isolate were lower than those induced by the IN19338 and NC49469 isolates; however, three prototype isolates had similar virus shedding, gross lesions, histopathological lesions and IHC staining. Overall, we conclude that the three US PEDV prototype isolates evaluated in this study have similar pathogenicity in neonatal piglets regardless of their phylogenetic clades. In contrast, data in the current study clearly demonstrate that the US PEDV S-INDEL-variant isolate IL20697 had significantly diminished clinical signs, virus shedding in faeces, gross lesions in small intestines, caeca and colons, histopathological lesions in small intestines, and IHC scores in ileum, compared with three US PEDV prototype isolates, IN19338, NC35140 and NC49469. Recent experimental studies by other groups also demonstrated that S-INDEL PEDVs overall had lower pathogenicity than the US prototype strains in 3–4-day-old or 1-week-old pigs (Lin et al., 2015a; Yamamoto et al., 2015). However, Lin et al. (2015a) observed that three litters of piglets inoculated with a US S-INDEL Iowa106 strain had zero mortality but one litter of piglets inoculated with the same virus strain had 75% mortality. They hypothesized that the sows’ health condition can have a direct impact on colostrum/milk production and thus affect the infection outcome of their piglets (Lin et al., 2015a). The virulence of S-INDEL PEDVs observed in the field has variations among farms and countries. In the USA, the S-INDEL variant strain OH851 infection only caused minimal to no clinical signs in suckling piglets on the farm (Wang et al., 2014). In Germany, two sow farms were infected with an S-INDEL PEDV that has 99.4% nucleotide identity to the US S-INDEL-variant OH851 at the whole genome level; however, severity of clinical signs and mortality in suckling piglets varied significantly between the two farms (Stadler et al., 2015). Factors contributing to the contradictory findings have not been clearly identified, but source of viruses (WT or cell-culture-adapted viruses), inoculation/infection doses,
animal/environmental conditions, and nucleotide/amino acid variations among S-INDEL PEDVs could contribute to the observed discrepancies among various experimental studies and field outbreaks. In addition, PEDV pathogenicity can be age-dependent. Further investigations of pathogenicity of S-INDEL PEDV variants in weaned pigs, finisher pigs, gilts and sows are warranted.

Previous studies showed that viraemia can occur in the acute stage of infection with US PEDV prototype isolates (Jung et al., 2014; Madson et al., 2016). In the present study, we also detected PEDV RNA in serum samples. In addition, the PEDV variant isolate and three prototype isolates had similar viraemia levels under the conditions of this study. PEDV is an enteropathogenic coronavirus that infects the villous enterocytes, resulting in villous atrophy and malabsorptive diarrhoea. Some quantities of PEDV could be taken into the blood stream through mechanisms not fully understood. But PEDV is not believed to actively replicate in blood, and viraemia levels may not necessarily correlate to virulence/pathogenicity. In the current study, high levels of PEDV RNA were detected in small intestine, caecum, colon and mesenteric lymph nodes, while low levels of PEDV RNA were detected in non-enteric tissues (tonsil, heart, lung, liver, spleen, kidney and muscle) from pigs inoculated with either prototype or S-INDEL-variant PEDV. Previous studies indicated that PEDV viral antigen (US prototype isolates) could be detected in small intestine, mesenteric lymph node, and some colon and spleen tissues (Jung et al., 2014, 2015b; Madson et al., 2016), but other non-enteric tissues such as lung, heart, kidney and liver were all negative for PEDV antigen (Madson et al., 2016). Therefore, detection of PEDV RNA does not necessarily mean that PEDV replicates in all of these non-enteric tissues. Considering that the blood was not drained before collecting each organ, the possibility that virus in these tissues was from blood cannot be excluded.

In the current study, PEDV IHC staining was only performed on ileum, caecum and colon of inoculated pigs. Among four groups inoculated with PEDVs (three prototype isolates and one variant isolate), PEDV IHC staining
was observed in 100% of ileums, 60–100% of caeca and 40–100% of colons at 3 days p.i. The mean IHC scores in ileums were significantly lower in the variant isolate-inoculated pigs than in the prototype isolates-inoculated pigs, consistent with observations on gross pathology and histopathological lesions of small intestines. Although the mean IHC scores in caeca and colons were numerically lower in pigs inoculated with the variant isolate than in pigs inoculated with the three prototype isolates, the differences were not significant. However, PEDV variant isolate-inoculated pigs had fewer gross changes in caecum and colon than the prototype isolate-inoculated pigs. Thus, the correlations of caecal and colonic changes with PEDV virulence/pathogenicity may need to be further elucidated.

All four groups G1–G4 inoculated with PEDVs (three prototype isolates and one variant isolate) had significantly shortened villus heights compared with the negative control group G5 at 3 days p.i., and G1–G3 (prototype isolates) had significantly shortened villus heights compared with G4 (variant isolate). This indicates that both US prototype and variant PEDV isolates can infect and destroy villus epithelium of small intestines, but the US PEDV variant isolate caused less severe villous atrophy than prototype isolates. Intestinal crypt epithelial cells serve to replace the destroyed villous enterocytes. At 3 days p.i., the mean crypt depths of G4 (variant isolate) were not significantly different from G5 (negative control), but the mean crypt depths of G1–G3 (prototype isolates) were significantly greater than G4 and G5. This may suggest that mild villous atrophy caused by the US PEDV variant isolate had not triggered significant proliferation and elongation of intestinal crypt at 3 days p.i.; however, intestinal crypts had started to elongate to some degree to repair severe villous atrophy in prototype isolate-inoculated groups G1–G3. At 7 days p.i., prototype isolate-inoculated groups had greater mean crypt depth than the negative control group, suggesting that crypt elongation continued to replace the damaged villus enterocytes but the villus epithelium had not recovered back to normal. The mean crypt depths of some G4 (variant isolate) sections of small intestine were significantly greater than G5 (negative control) at 7 days p.i., suggesting that elongation of crypts occurred later in G4 than in the prototype isolate-inoculated groups G1–G3. The proliferated crypts eventually recovered the destroyed villus enterocytes apparent at 3 days p.i. in G4. IHC staining also supported these observations.

Some studies showed that the antibodies against US PEDV prototype and S-INDEL-variant strains can cross-react and cross-neutralize both strains in vitro (Chen et al., unpublished; Lin et al., 2015b). An in vivo study (Goede et al., 2015) showed that sows exposed to S-INDEL-variant PEDV infection 7 months previously could provide partial protection to newborn piglets challenged with a US PEDV prototype strain. Another in vivo study (Lin et al., 2015a) demonstrated that 3–4-day-old piglets exposed to S-INDEL-variant PEDV were partially protected against subsequent challenge with a US prototype PEDV. We also have unpublished data that demonstrates that both US PEDV prototype and S-INDEL-variant strains can provide homologous and heterologous protection against two virus strains in a weaned pig model. In the current study, it was demonstrated that US PEDV S-INDEL-variant strain is less virulent than US PEDV prototype strains in neonatal pigs. These data collectively suggest that US PEDV S-INDEL-variant strain could potentially be a modified live virus vaccine candidate against PED, although additional evaluation work is needed.

The striking sequence differences between US prototype and S-INDEL-variant PEDVs are located in the spike gene, especially the S1 portion. The sequence differences in the spike gene may be responsible for the virulence differences between US prototype and S-INDEL-variant PEDVs, but this remains to be confirmed using a reverse genetics approach.

METHODS

**Virus isolates and cells.** Isolation and characterization of the US PEDV prototype isolate USA/IN19338/2013 and S-INDEL-variant isolate USA/IL20697/2014 have been described elsewhere (Chen et al., 2014; Chen et al., unpublished). Three additional US PEDV prototype isolates, USA/NC35140/2013, USA/IA49379/2013 and USA/NC49469/2013, were obtained for this study, all from archived piglet faeces submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) for routine diagnosis following previously described virus isolation procedures (Chen et al., 2014). All PEDV isolation, propagation and titration were performed in Vero cells (ATCC CCL-81) as previously described (Chen et al., 2014). All PEDV isolates used in this study were confirmed negative for PDCoV, TGEV, porcine rotaviruses (groups A, B and C), porcine reproductive and respiratory syndrome virus and porcine circovirus.

**Virus sequencing, comparative sequence analysis and phylogenetic analysis.** The whole genome sequences of the PEDV isolates described in this study were determined by next generation sequencing technology using the Illumina MiSeq platform, and assembled with SeqMan Pro version 11.2.1 (DNASTAR) as described previously (Chen et al., 2014). The sequence data of these PEDV isolates were deposited in GenBank with the following accession numbers: USA/IN19338/2013 [KF650371], USA/NC35140/2013 [KM975735], USA/IA49379/2013 [KM975736], USA/NC49469/2013 [KM975737] and USA/IL20697/2014 [KT860508].

The whole genome sequences and individual gene sequences (nucleotide and amino acid sequences) of all PEDV isolates used in this study were aligned using Clustal X version 2.0 (Larkin et al., 2007) and BioEdit version 7.0.4.1 (Hall, 1999) to compare the genetic similarity. Phylogenetic analysis was conducted using the entire genome and the S1 portion (S gene nt 1–2205 according to the sequence KF650371) nucleotide sequences of the PEDV isolates described in this study as well as representative global PEDVs (in total, 50 sequences). Phylogenetic trees were constructed using the distance-based neighbour-joining method and maximum-likelihood method of MEGA6 (Tamura et al., 2013). Bootstrap analysis was carried out on a 1000 replicate dataset.

**Experimental design.** The animal study protocol was approved by the Iowa State University Institutional Animal Care and Use Committee (approval number 6-14-7821 S; approved on 10 July 2014). Fifty 5-day-old piglets were purchased from a conventional breeding...
farm and delivered to the Iowa State University Laboratory Animal Resources facilities. All pigs were intramuscularly injected with a dose of Excede (Zoetis) upon arrival and confirmed negative for PEDV, PDCoV, TGEV and porcine rotaviruses (groups A, B and C) by virus-specific PCRs on rectal swabs, and negative for PEDV antibody by a virus-specific indirect fluorescent antibody assay on serum samples at the ISU VDL. Pigs were blocked by weight and then randomly divided into five groups of ten pigs each, one group per room on a solid floor. Pigs were fed a mixture of Eshibac (PetAg, Hampshire, Illinois, USA) liquid milk replacer and yoghurt and had free access to water. After 1 day acclimation (after which piglets were 6 days old), pigs in groups G1–G5 were oroatraumatically inoculated with three US PEDV prototype isolates, USA/IN19338/2013 (G1), USA/NC35140/2013 (G2) and USA/NC49469/2013 (G3), one US S-INDEL-variant isolate, USA/IL20697/2014 (G4), or virus-negative culture medium (G5), respectively (10 ml per pig; all viruses were at the seventh passage in cell culture with a titer of 10^9 TCID₅₀ ml⁻¹) (Table 2).

Piglets were evaluated daily for presence of vomiting and clinical signs of diarrhea, lethargy and body condition. Diarrhea severity was scored with the following criteria: 0, normal; 1, soft (cowpie); 2, liquid with some solid content; 3, watery with no solid content. Lethargy levels were categorized as: normal, mild lethargy (slow to move, head down), moderate lethargy (stands but wants to lie down) or severe lethargy (recumbent, moribund). Body condition was categorized as: normal, mild loss (flat flank), moderate (flank tucked in) or severe (backbone/ribs prominent).

Body weights were recorded prior to inoculation (at -1 day p.i.) and then at 3 and 7 days p.i. The MDG was calculated for pigs from -1 to 3 days p.i.; and -1 to 7 days p.i. Serum samples were collected at 0, 3 and 7 days p.i. Rectal swabs were collected daily from each pig from 0 days p.i. to necropsy and were submerged into 1 ml PBS immediately after collection. Five pigs from each group were randomly selected for necropsy at 3 days p.i.; and the remaining pigs were necropsied at 7 days p.i. Fresh and formalin-fixed samples collected at necropsy included tonsil, heart, lung, liver, spleen, kidney, skeletal muscle from rear leg, stomach, mesenteric lymph node, duodenum, proximal jejunum, middle jejunum, distal jejunum, ileum, caecum and colon. Collection of different intestinal segments was performed as previously described (Madson et al., 2014).

At necropsy, the small intestine, caecum and colon were examined for gross lesions by veterinary pathologists blind to the treatment groups. Villus lengths and crypt depths were measured from three representative villi and crypts of duodenum, proximal jejunum, middle jejunum, distal jejunum and ileum, using a computerized image system following previously described procedures (Madson et al., 2014). Villus-height-to-crypt-depth (villus/crypt) ratio of each tissue was calculated as the quotient of the mean villus length divided by the mean crypt depth.

**Virus shedding as examined by a quantitative PEDV N-gene-based real-time RT-PCR.** Viral RNA was extracted from rectal swabs, serum and 10% tissue homogenates as previously described (Chen et al., 2014). Five microtiter plates of each RNA template was used in a PCR setup in a 25 μl total reaction using the Path-ID Multiplex One-Step RT-PCR kit (Thermo Fisher Scientific). The primers, probes and in vitro-transcribed RNA used to generate standard curves of a quantitative PEDV N-gene-based real-time RT-PCR have been previously described (Lowe et al., 2014; Madson et al., 2014; Thomas et al., 2015). Based on standard curves, the virus concentration (expressed as genomic copies ml⁻¹) in tested samples was calculated. The mean C₅₀ values were calculated based on PCR-positive samples, and the mean virus concentrations were calculated based on all pigs within the group (both PCR-positive and PCR-negative pigs).

**Histopathology.** Tonsil, heart, lung, liver, spleen, kidney, mesenteric lymph node, stomach, duodenum, proximal jejunum, middle jejunum, distal jejunum, ileum, caecum and colon tissues were fixed in 10% formalin, embedded, sectioned and stained with H&E, and examined by a veterinary pathologist blinded to individual animal identifications and treatment groups. Villus lengths and crypt depths were measured from three representative villi and crypts of duodenum, proximal jejunum, middle jejunum, distal jejunum and ileum, using a computerized image system following previously described procedures (Madson et al., 2014). Villus-height-to-crypt-depth (villus/crypt) ratio of each tissue was calculated as the quotient of the mean villus length divided by the mean crypt depth.

**Statistical analyses.** A generalized linear mixed (GLIMMIX) model was used for all statistical comparisons with Statistical Analysis System (SAS) version 9.3 (SAS Institute). A P value < 0.05 was defined as statistically significant. P values of overall faecal viral shedding level [log_{10}(genomic copies ml⁻¹)] were assessed among treatments from 0–7 days p.i., with days p.i. and treatment as interacting variables, and similarly for analysis of diarrhea scores.

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