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Original article

Effects of porcine epidemic diarrhea virus infection on tight junction protein gene expression and morphology of the intestinal mucosa in pigs

Q.F. Zong[1†], Y.J. Huang[1†], L.S. Wu[1], Z.C. Wu[1], S.L. Wu[1, 2], W.B. Bao[1, 2]

[1] Key Laboratory for Animal Genetic, Breeding, Reproduction and Molecular Design of Jiangsu Province, College of Animal Science and Technology, Yangzhou University, Yangzhou 225009, China
[2] Joint International Research Laboratory of Agriculture and Agri-Product Safety, Yangzhou University, Jiangsu Yangzhou 225009, China

Abstract

Tight junction proteins are important for the maintenance and repair of the intestinal mucosal barrier. The present study investigated relationships among tight junction protein gene expression, porcine epidemic diarrhea virus (PEDV) infection, and intestinal mucosal morphology in piglets. We compared the expression of six tight junction proteins (ZO-1, ZO-2, Occludin, Claudin-1, Claudin-4, and Claudin-5) between seven-day-old piglets infected with PEDV and normal piglets, as well as in PEDV-infected porcine intestinal epithelial cells (IPEC-J2). We also evaluated differences in mucosal morphology between PEDV-infected and normal piglets. The expression of six tight junction protein genes was lower in PEDV-infected piglets than in the normal animals. The expression of ZO-1, ZO-2, Occludin, and Claudin-4 in the intestine tissue was significantly lower ($p<0.05$) in PEDV-infected than in normal piglets. The expression of Claudin-5 in the jejunum was significantly lower in PEDV-infected piglets than in the normal animals ($p<0.01$). The expression of *Claudin-1* and *Claudin-5* genes in the ileum was significantly higher in PEDV-infected piglets than in normal piglets ($p<0.01$). Morphologically, the intestinal mucosa in PEDV-infected piglets exhibited clear pathological changes, including breakage and shedding of intestinal villi. In PEDV-infected IPEC-J2 cells, the mRNA expression of the six tight junction proteins showed a downward trend; in particular, the expression of the *Occludin* and *Claudin-4* genes was significantly lower ($p<0.01$). These data suggest that the expression of these six tight junction proteins, especially Occludin and Claudin-4, plays an important role in maintaining the integrity of the intestinal mucosal barrier and resistance to PEDV infection in piglets.

Key words: pig, tight junction protein, PEDV, diarrhea

Correspondence to: W.B. Bao, e-mail: wbbao@yzu.edu.cn, tel.: 15952738587

† These authors contributed equally to this work and should be considered co-first authors.

Introduction

The monolayer columnar epithelial cells in the gastrointestinal tract form a selective barrier to the intestinal cavity, called the intestinal mucosal barrier (Smith et al. 2010). The intestinal mucosal barrier plays important roles in ion transport, selective absorption of essential nutrients, and blockade of harmful substances, such as toxins and microbes (Kunzelmann and Mall 2002, Blikslager et al. 2007, Barrett 2008). The integrity of the intestinal mucosa is essential to the intestinal barrier. Tight junctions (TJs) are a pattern of cell adhesion between epithelial cells and endothelial cells that form a boundary between the apical and basolateral plasma membrane domains and hinders the diffusion of solutes between cells. As an important part of the intestinal mucosal barrier, TJs are integral in maintaining the mechanical barrier and permeability of the intestinal mucosal epithelium (Tsukita et al. 2001, Dokladny et al. 2016). Three kinds of tight junction proteins, Zonula occludens (ZOs), Occludin, and Claudins, are important in maintaining the integrity of the intestinal mucosal barrier and in determining intestinal permeability (Rescigno et al. 2001). Suzuki and Hara (2009) found that quercetin can enhance intestinal barrier function by increasing expression of ZO-2, Occludin, and Claudin-1, suggesting that changes in intestinal tight junction proteins may affect the structure of TJs between mucosal cells, and can lead to changes in intestinal barrier permeability.

Porcine epidemic diarrhea virus (PEDV) is one of the main pathogens responsible for porcine viral diarrhea (Curry et al. 2017). PEDV can cause intestinal hemorrhage, intestinal villus atrophy, and intestinal wall thinning, ultimately resulting in vacuolation and shedding of the cytoplasm of intestinal wall cells, leading to watery diarrhea, dehydration, and mortality (Stevenson et al. 2013). Pigs infected with *Salmonella* demonstrate decreased expression of tight junction proteins ZO-1, Occludin, and Claudin-1 (Guttman and Finlay 2009). Moreover, the infected pigs exhibited intestinal barrier dysfunction, increased intestinal permeability, intracavitary bacteria, toxins, and antigens transferred to epithelial tissue, which caused mucosal and systemic inflammatory responses (Guttman and Finlay 2009). Recent studies have revealed that damage to intestinal junction protein integrity in piglets may lead to damage of the intestinal epithelial barrier and loss of its protective function (Bruewer et al. 2005).

In the present study, we explore the relationship among the expression levels of intestinal tight junction protein genes, PEDV infection, and intestinal mucosal morphology in piglets. We employed a qPCR technique to detect the expression level of six tight junction proteins (ZO-1, ZO-2, Occludin, Claudin-1, Claudin-4 and Claudin-5) in seven-day-old pigs infected with PEDV and in normal piglets. Differences in mucosal morphology between PEDV-infected piglets and normal piglets were observed histologically. The expression levels of these genes were also quantified in the PEDV-infected porcine intestinal epithelial cell line (IPEC-J2) and in uninfected control cells. Our findings lay the foundation for further studies of the regulatory mechanisms mediating the role of tight junction proteins in the intestinal mucosal barrier and anti-virus responses, and provide guidance for the prevention and treatment of viral diarrhea in piglets.

Materials and Methods

Ethics statement

All experiments were conducted in the Animal Hospital of Yangzhou University according to the regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised in June 2012). The procedures were approved in the experimental animal use permit No. SYXK (Su) 2012 0029.

Experiment material

Six seven-day-old piglets with clinicopathological features of porcine epidemic diarrhea (vomiting, diarrhea, dehydration, and watery diarrhea) were selected from a pig farm. At the same time, six normal seven-day-old piglets from the same feeding environment were selected as controls. Tissues, including duodenum, jejunum, and ileum were collected for follow-up evaluation.

Identification of the PEDV genome

Three intestinal segments (duodenum, jejunum, and ileum) from the 12 piglets were rinsed with physiological saline. Total RNA was extracted and reverse transcribed into cDNA to generate the template for the qPCR reactions. The *M* gene of the classical PEDV strain, CV777, plays an important role in the assembly and budding of PEDV, and is the main gene used to identify PEDV serodiagnosis antigens (Jinghui and Yijing 2005). The primers for the *M* gene were designed by Primer Express 5.0 software. Primer sequences are listed in Table 1. The target fragment was amplified, the products were separated by 1% agarose gel electrophoresis, and sequenced.

Table 1. Primer sequences

Genes	Accession number	Primer sequences (5'→3')	Length (bp)
M	AF353511.1	F:AGGTCTGCATTCCAGTGCTT R:GGACATAGAAAGCCCAACCA	216
ZO-1	XM_021098856.1	F:AGCCCGAGGCGTGTTT R:GGTGGGAGGATGCTGTTG	147
ZO-2	NM_001206404.1	F:ATTCGGACCCATAGCAGACATAG R:GCGTCTCTTGGTTCTGTTTTAGC	90
OCCLUDIN	NM_001163647.2	F:ATCAACAAAGGCAACTCT R:GCAGCAGCCATGTACTCT	157
CLAUDIN-1	NM_001244539.1	F:ACCCCAGTCAATGCCAGATA R:GGCGAAGGTTTTGGATAGG	155
CLAUDIN-4	NM_001161637.1	F:CAACTGCGTGGATGATGAGA R:CCAGGGGATTGTAGAAGTGG	140
CLAUDIN-5	NM_001161636.1	F:CCTTCCTGGACCACAACATC R:CACCGAGTCGTACACCTTGC	110
ACTB	XM_00312428.3	F:TGGCGCCCAGCACGATGAAG R:GATCCAGGGGCCGGACTCGT	149
GAPDH	AF017079.1	F:ACATCATCCCTGCTTCTACTGG R:CTCGGACGCCTGCTTCAC	187

Infection of IPEC-J2 cells with PEDV

IPEC-J2 cells were cultured onto 6-well plates at ~1×10^6 cells/plate and incubated in complete DMEM medium containing 10% fetal bovine serum. After 24 h incubation, 300 µL PEDV and 2.5 µg/mL trypsin were added to each well. Virus was allowed to adsorb to the cells for 1 h. Afterward, the unattached virus was discarded, and fresh culture medium was added and cells were cultured for an additional 36 h.

Differential expression of tight junction protein in intestinal tissues and IPEC-J2 cells

Primer design and synthesis

Primers for qPCR were designed by Primer Express 5.0 software, based on the sequences of porcine *ZO-1*, *ZO-2*, *Occludin*, *Claudin-1*, *Claudin-4*, *Claudin-5*, *ACTB*, and *GAPDH* genes from the NCBI (http://www.ncbi.nlm.nih.gov/) database. The primers were synthesized by Bioengineering Co., Ltd. (Shanghai, China). Primer information is provided in Table 1.

Total RNA extraction

Total RNA was extracted from intestinal tissues and cells using Trizol Reagent (TaKaRa Biotechnology Dalian Co., Ltd) according to the manufacturer's instructions. The positive control used in this study was PEDV-infected Vero cells, and total RNA of the positive control was extracted using the same method. Total RNA purity and concentration was detected by 1% formaldehyde denatured agarose gel electrophoresis and by a NanoDrop-1000 microarray nucleic acid/protein concentration analyzer (General Electric Company, USA). RNA samples were stored at -70°C for later use.

cDNA synthesis

The prepared total RNA was reversely transcribed into cDNA using a TAKARA kit (Dalian, China), according to manufacturer's instructions. The cDNA synthesis reaction system (10 µL) contained 500 ng of total RNA, 2 µL 5×Primer Script RT Master Mix, and RNase Free ddH_2O up to 10 µL. The reaction conditions were 25°C for 10 min, 50°C for 30 min, and 85°C for 5 min.

qPCR reaction

The 25 µL qPCR reaction system contained 2 µL cDNA, 0.5 µL of each primer (10 µmol/L), 0.5 µL ROX Reference Dye II (50×), 12.5 µL SYBR Premix Ex TaqTM (2×), and 9 µL ddH2O. The PCR reaction conditions were 95°C for 30 s, 95°C for 5 s, and 60°C for 34 s, for a total of 40 cycles. Melting curve analysis was performed after amplification.

Histological evaluation of tissue slices

The middle part of three intestinal segments from 12 piglets were collected, rinsed with phosphate buffer

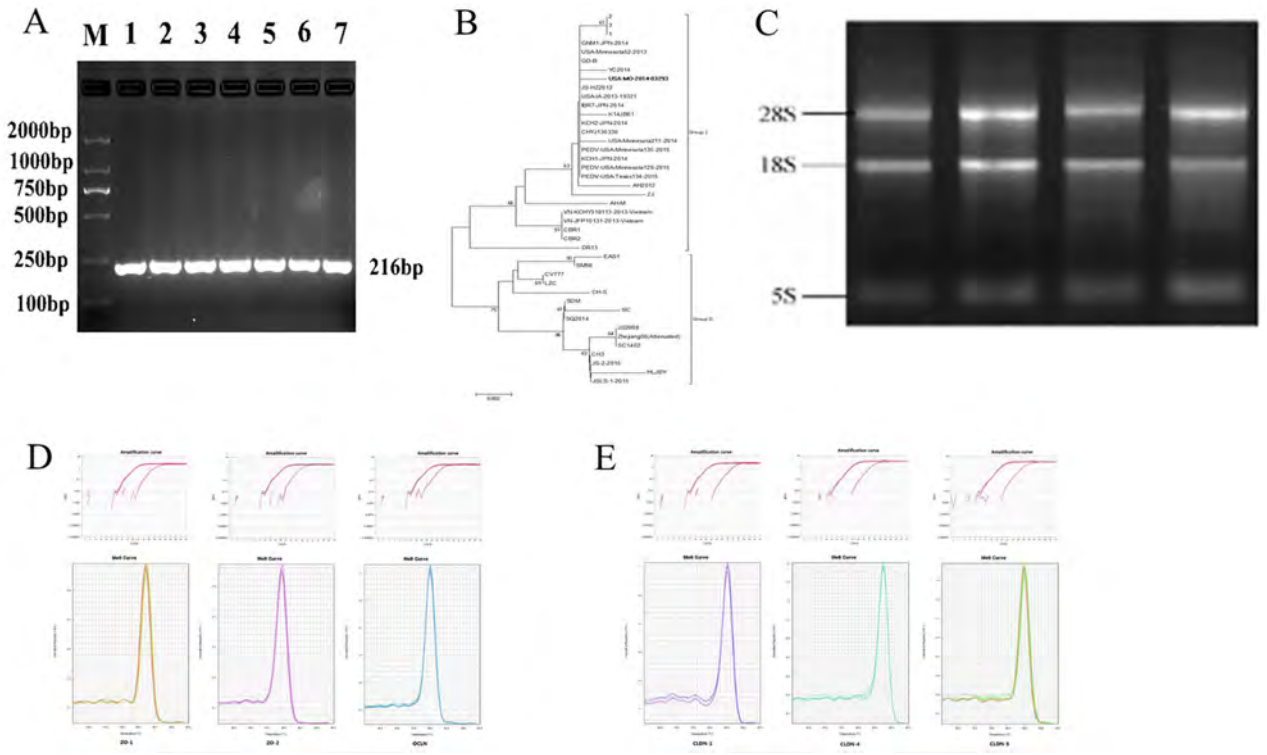

Fig. S1. Sequencing and polyacrylamide gel electrophoresis. A. Agarose gel detection map of the signature *M* gene of the PEDV classic strain CV777. M represents the D2000 molecular Marker; 1-6 represent samples of PEDV-infected piglets, and 7 represents a positive control. B. Phylogenetic tree based on *M* gene sequences. 1, 2, and 3 represent the duodenum, jejunum, and ileum of piglets infected with porcine epidemic diarrhea virus. C**.** Separation of RNA samples by 1% formaldehyde denaturing agarose gel electrophoresis. D and E. Tight junction gene amplification curve (upper) and melting curve (lower).

saline (PBS), and fixed in 4% paraformaldehyde for 24 h. After a series paraffin embedding, slicing, salvaging, baking at 60°C for 1 h and staining with hematoxylin-eosin (HE), the morphological differences in the mucosa of three intestinal segments between PEDV-infected piglets and normal piglets were observed under a light microscope. Images were taken by Motic photographic processing software and analyzed with a pathological image analysis system.

Data processing and analysis

Gene expression levels were compared using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and analyzed by SPSS 16.0 software. Data are expressed as X ± SD. Differences in relative expression levels between different samples were compared by independent sample t-test. *P* values of < 0.05 were considered significant, p values of < 0.01 were considered extremely significant.

Results

Virus genome identification

The PCR amplification products obtained from the 6 PEDV-infected piglets were 216 bp in length, which were consistent with those of the positive controls (Fig. S1A). The sequencing results showed that the viral sequence amplified from the PEDV-infected piglets was the same as viral *M* gene while the normal piglets lacked the corresponding amplification band (Fig. S1A). A phylogenetic tree was established based on the *M* gene sequence (Fig. S1B), revealing that the viral strain had extensive homology with the prevalent viral strains regionally and globally. Based on these results, we concluded that the six piglets with the typical clinical symptoms of viral diarrhea were indeed infected with the PEDV, while normal piglets were not infected with the associated pathogen.

Analysis of RNA purity and integrity

Analysis of the total RNA samples revealed the bands corresponding to 28S, 18S, and 5S and showed no DNA contamination or degradation, indicating that the extracted total RNA was of high quality (Fig. S1C). The RNA concentration and purity analysis showed that samples had a ratio of absorbance (A260/A280) between 1.8 and 1.9, indicating that both the concentration and purity of RNA met the requirements for quantitative analysis.

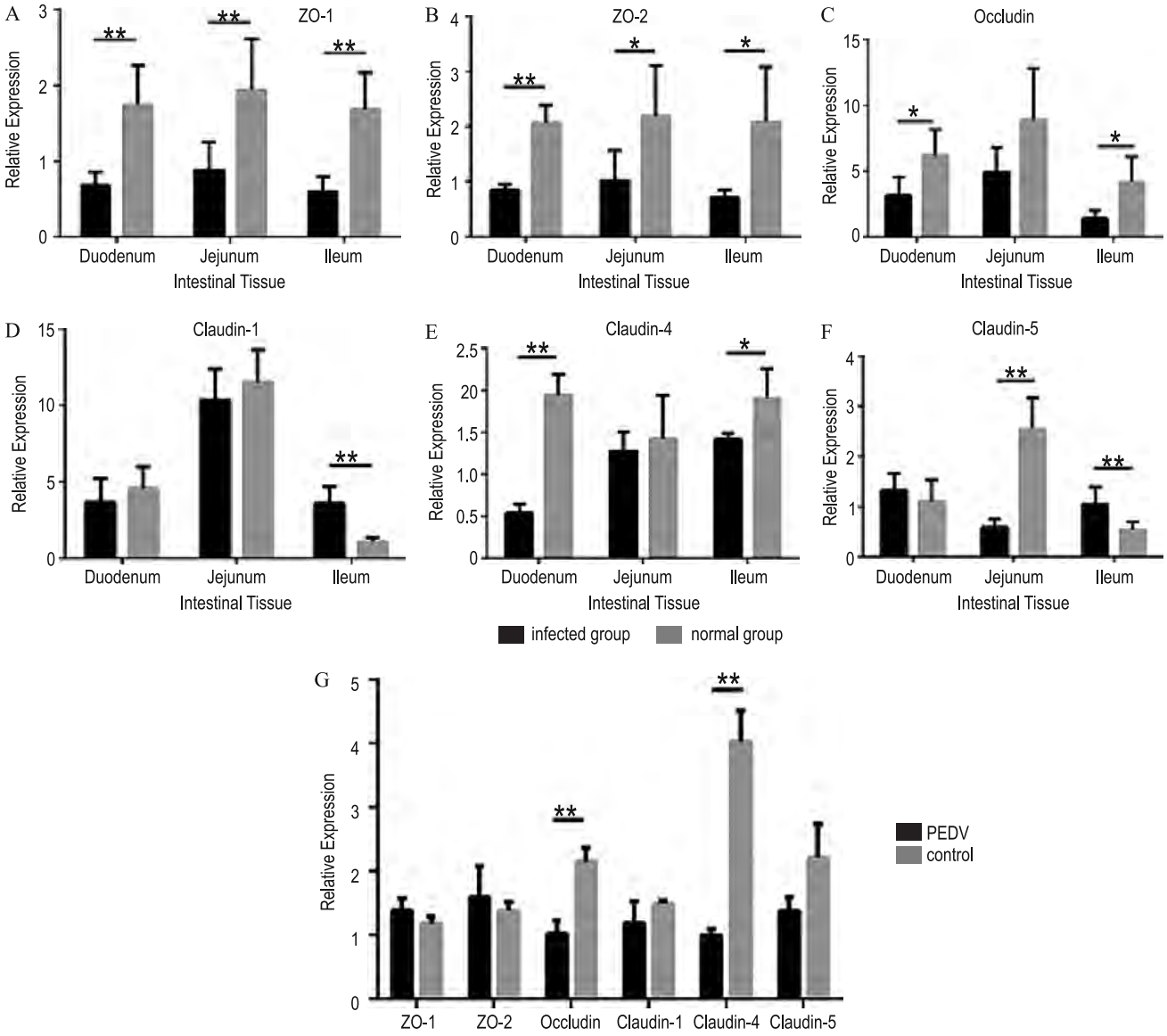

Fig. 1. Expression profiles of six tight junction protein genes. A-F. Expression of *ZO-1, ZO-2, Occludin, Claudin-1, Claudin-4, Claudin-5* genes in piglet duodenum, jejunum, and ileum tissue. G. Expression of tight junction protein genes in IPEC-J2 cells.

Amplification and melting curves from quantitative PCR

Melt curve analysis revealed a single, smooth peak for the PCR product, indicating the amplification of a specific product and demonstrating that the gene expression can be quantitatively compared by following the fluorescence signal (Fig. S1D and S1E).

Analysis of tight junction protein gene expression in three segments from the porcine intestinal tissue

The expression of the tight junction protein genes *ZO-1, ZO-2, Occludin, Claudin-1, Claudin-4* and, *Claudin-5* was determined in duodenum, jejunum, and ileum tissue by qPCR, and was compared between PEDV-infected piglets and normal piglets. The expression of *ZO-1, ZO-2, Occludin,* and *Claudin-4* genes in duodenum, jejunum and ileum was significantly decreased in PEDV-infected piglets compared to the normal animals. Among these genes, the expression of the *ZO-1* gene was significantly decreased in duodenum, jejunum, and ileum tissue ($p<0.01$) (Fig. 1A). The expression of the *ZO-2* gene was significantly decreased in the duodenum ($p<0.01$) and in the jejunum and ileum ($p<0.05$) (Fig. 1B). The expression of the *Occludin* gene was significantly decreased in duodenum and ileum tissue ($p<0.05$) (Fig. 1C). The expression of the *Claudin-4* gene was significantly decreased in the duodenum ($p<0.01$) and in ileum ($p<0.05$) (Fig. 1E). The expression of the *Claudin-1* was decreased in the duodenum and jejunum, but was significantly increased in the ileum ($p<0.01$) (Fig. 1D). The expres-

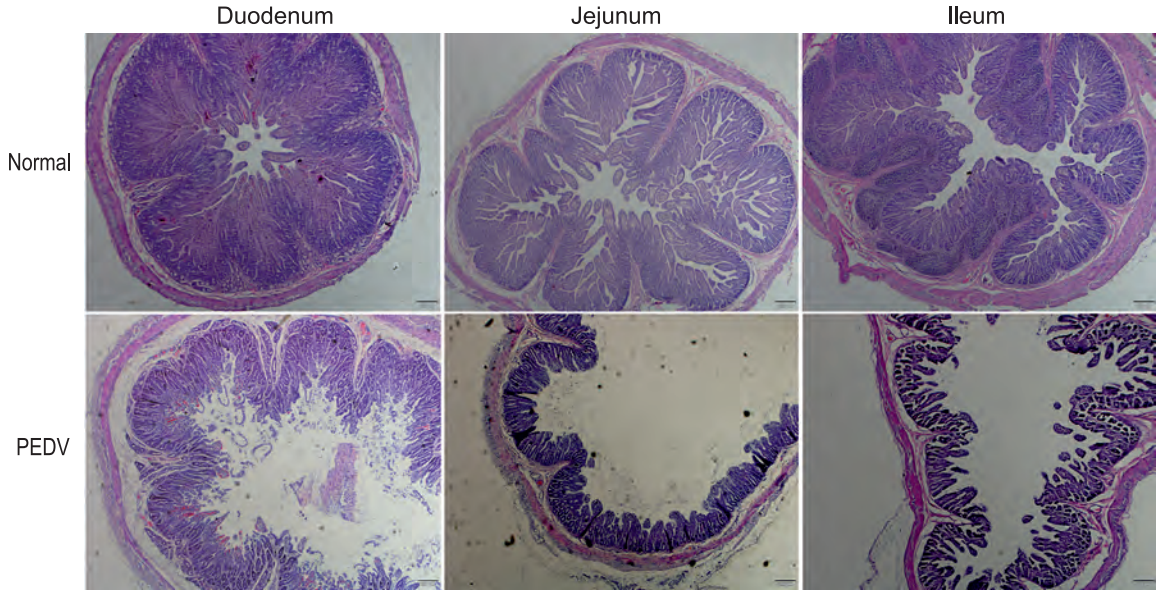

Fig. 2. Morphology of piglet duodenum, jejunum, and ileum intestinal tissue as viewed under a light microscope. Normal and porcine epidemic diarrhea virus indicate tissue from normal piglets or piglets infected with PEDV. All images are at 4×10 magnification.

sion of the *Claudin-5* was significantly decreased in the jejunum ($p<0.01$), but was significantly increased in ileum ($p<0.01$) (Fig. 1F).

Tissue slice identification

Analysis of HE staining of tissue preparations revealed that the morphology of the intestinal mucosa in healthy piglets was normal, and the villus structure was intact and distinct. The outline of the intestinal mucosa epithelial cells was clear and with regular arrangement. The stroma displayed no edema and no inflammatory cell infiltration. In contrast, the intestinal mucosa epithelium in PEDV-infected piglets exhibited pathological features including necrosis, shedding and damage of intestinal villi, decreased villi height, deepening of crypt depth, exposure of lamina propria, expanding of vasculature, and a large amount of neutrophil infiltration (Fig. 2).

Analysis of tight junction protein gene expression in IPEC-J2 cells

We performed qPCR to detect the expression of the tight junction genes *ZO-1, ZO-2, Occludin, Claudin-1, Claudin-4,* and *Claudin-5* in PEDV-infected IPEC-J2 cells and uninfected IPEC-J2 cells. Compared with the control group, the overall expression of these genes showed a downward trend in the PEDV treated cells, and in particular the expression of the *Occludin* and *Claudin-4* genes was significantly decreased ($p<0.01$) (Fig. 1G).

Discussion

In piglets, diarrhea is caused by many factors, including maternal factors, stress, nutrition, and disease (Leonardo 1992), among which PEDV is the most serious (Jung et al. 2014). PEDV infection can cause atrophic enteritis with viremia, which can lead to severe diarrhea and vomiting, and can destroy the integrity of pig intestinal mucosa (Jung et al. 2008). Therefore, maintaining the integrity of the intestinal mucosa is essential for piglets in order to resist the pathologies associated with PEDV infection. Tight junction proteins are an important part of the intestinal mucosal barrier, and not only maintain the function of the epithelial barrier, but also hinder the invasion of toxic macromolecules and microorganisms (Suzuki 2013). Tight junction proteins also selectively regulate entry of small molecules and ions into the body. As the most important tight junction proteins, the Zonula occludens, Occludin, and Claudin families have been widely studied. To illustrate this point, the expression of tight junction proteins, including ZO-1, Occludin, and Claudin-1 was significantly decreased in PEDV-infected porcine cells, indicating that PEDV infection is closely related to the expression of tight junction proteins (Gao et al. 2013, Zhao et al. 2014).

In this study, we found that the expression of *ZO-1, ZO-2, Occludin,* and *Claudin-4* genes in three intestinal segments of PEDV-infected piglets was significantly lower than that of normal piglets. It has previously been reported that ZO-1 is involved in the regulation of intracellular material transport, maintenance of epithelial polarity, and formation of cytoskeleton indirectly, and

also plays an important regulatory role in cell proliferation and differentiation, tumor cell metastasis, and gene transcription (Balda et al. 2003). When the intestinal barrier in piglets is destroyed, the expression of ZO-1 and Occludin are decreased, and the composition and function of tight junctions are also damaged. Probiotic strains can protect the intestinal barrier by increasing the expression and localization of ZO-1 and Occludin (Roselli et al. 2007, Yang et al. 2014). Previous studies have revealed the associations between the *Claudin-4* gene expression and the intestinal barrier integrity under mycotoxin and bacteria stimulation (Pinton et al. 2010, Lodemann et al. 2017). These findings indicate that PEDV infection can lead to a decrease in the expression of tight junction proteins in the intestine. The morphological structure of the intestinal mucosa is the structural basis of intestinal barrier function, and the integrity of the intestinal mucosa is indispensable for the integrity of the intestinal barrier. Therefore, in this study we compared the morphology of the intestinal mucosa between PEDV-infected piglets and normal piglets. Analysis of intestinal tissue preparations demonstrated that the intestinal mucosa was intact and morphologically normal in healthy piglets, while it exhibited obvious pathologies in PEDV-infected piglets, including shedding and breakage of villi. In addition, we compared the expression of six tight junction protein genes in PEDV-infected IPEC-J2 cells and uninfected control cells, and found that the overall expression in the PEDV treatment group trended downward; the expression of *Occludin* and *Claudin-4* genes was significantly lower ($p<0.01$). These results indicate that PEDV infection leads to a decrease in the expression of tight junction proteins, especially Occludin and Claudin-4.

Claudin-1 is one of the major transmembrane proteins that regulates epithelial cell permeability and is crucial for maintaining epithelial cell function (Wang et al. 2012). High expression of Claudin-5 in the intestinal tract leads to an increase in intestinal villi height, thereby reducing intestinal permeability and inhibiting diarrhea in weaning piglets (Yuan et al. 2012). These findings showed that high expression of *Claudin-1* and *Claudin-5* genes is conducive to maintaining the integrity of the intestinal mucosal barrier. It is worth noting that, in this study, the expression of *Claudin-1* gene in duodenum and jejunum tissue was decreased, and the expression of Claudin-5 in jejunum was significantly decreased in PEDV-infected piglets ($p<0.01$). However, the expression of *Claudin-1* and *Claudin-5* genes in the ileum of PEDV-infected piglets was significantly higher than that of normal piglets ($p<0.01$). Because of the inconsistency in the expression trends of these two genes with the other tight junction protein genes, we speculate that *Claudin-1* and *Claudin-5* may have unique regulatory mechanisms. Ishizaki et al. (2003) have shown that cyclic AMP can increase the expression of *Claudin-5* and decrease *Claudin-1* gene in porcine blood-brain barrier endothelial cells via protein kinase A-independent and -dependent pathways, respectively. As a viral entry factor, *Claudin-1* can facilitate the entry of dengue virus (Che et al. 2013). What's more, by examining the effect of expression changes of *Claudin-5* in human enteric coronavirus (HECV) cells, it was found that insertion of *Claudin-5* gene in HECV cells resulted in a significant less motility and less adhesion to matrix, revealing a link between *Claudin-5* and cell motility (Escudero-Esparza et al. 2012). Expression analysis of the *Claudin-1* and *Claudin-5* genes in malignant tumors indicated that the expression of *Claudin-1* gene increased in colorectal cancer and intestinal-type gastric cancer, but decreased in breast cancer (de Oliveira et al. 2005, Resnick et al. 2005, Morohashi et al. 2007). Moreover, the expression of *Claudin-5* gene increased in colorectal cancer and vascular tumors, but decreased in hepatocellular carcinoma and renal cell carcinoma (de Oliveira et al. 2005, Soini et al. 2006). Some researchers also found that the expression of *Claudin-1* and *Claudin-5* was associated with tumor invasion and lymph node metastasis (Miwa et al. 2001). These findings indicated the versatile roles of the *Claudin-1* and *Claudin-5* genes in different physiological and pathological processes. Therefore, the significant increase in the expression of *Claudin-1* and *Claudin-5* genes in the ileum of piglets with viral diarrhea may be related to the pathogenesis of other diseases. Combined with the morphological evaluation of the intestinal tissue slices, we found that the PEDV-infected piglets had a damaged intestinal mucosal barrier and a decreased expression of tight junction proteins, which indicated that the expression of tight junction proteins may be closely related to the integrity of the intestinal mucosa.

These data suggest that the expression of six tight junction proteins, especially Occludin and Claudin-4, plays an important role in maintaining the integrity of the intestinal mucosal barrier and resisting PEDV infection in piglets. Further functional studies are required to elucidate the molecular mechanisms underlying the roles of these tight junction genes in response to PEDV infection.

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References

Balda MS, Garrett MD, Matter K (**2003**) The ZO-1-associated Y-box factor ZONAB regulates epithelial cell proliferation and cell density. J Cell Biol 160: 423-432.

Barrett KE (**2008**) New ways of thinking about (and teaching about) intestinal epithelial function. Adv Physiol Educ 32: 25-34.

Blikslager AT, Moeser AJ, Gookin JL, Jones SL, Odle J (**2007**) Restoration of Barrier Function in Injured Intestinal Mucosa. Physiol Rev 87: 545-564.

Bruewer M, Utech M, Ivanov AI, Hopkins AM, Parkos CA, Nusrat A (**2005**) Interferon-gamma induces internalization of epithelial tight junction proteins via a macropinocytosis-like process. FASEB J 19: 923-933.

Che P, Tang H, Li Q (**2013**) The interaction between claudin-1 and dengue viral prM/M protein for its entry. Virology 446: 303-313.

Curry SM, Schwartz KJ, Yoon KJ, Gabler NK, Burrough ER (**2017**) Effects of porcine epidemic diarrhea virus infection on nursery pig intestinal function and barrier integrity. Vet Microbiol 211: 58-66.

de Oliveira SS, de Oliveira IM, De Souza W, Morgado-Díaz JA (**2005**) Claudins upregulation in human colorectal cancer. FEBS Lett 579: 6179-6185.

Dokladny K, Zuhl MN, Moseley PL (**2016**) Intestinal epithelial barrier function and tight junction proteins with heat and exercise. J Appl Physiol 120: 692-701.

Escudero-Esparza A, Jiang WG, Martin TA (**2012**) Claudin-5 participates in the regulation of endothelial cell motility. Mol Cell Biochem 362: 71-85.

Gao Y, Han F, Huang X, Rong Y, Yi H, Wang Y (**2013**) Changes in gut microbial populations, intestinal morphology, expression of tight junction proteins, and cytokine production between two pig breeds after challenge with Escherichia coli K88: a Comparative Study. J Anim Sci 91: 5614-5625.

Guttman JA, Finlay BB (**2009**) Tight junctions as targets of infectious agents. Biochim Biophys Acta 1788: 832-841.

Ishizaki T, Chiba H, Kojima T, Fujibe M, Soma T, Miyajima H, Nagasawa K, Wada I, Sawada N (**2003**) Cyclic AMP induces phosphorylation of claudin-5 immunoprecipitates and expression of claudin-5 gene in blood-brain-barrier endothelial cells via protein kinase A-dependent and -independent pathways. Exp Cell Res 290: 275-288.

Jinghui F, Yijing L (**2005**) Cloning and Sequence Analysis of the M gene of Porcine Epidemic Diarrhea Virus LJB/03. Virus Genes 30: 69-73.

Jung K, Kang BK, Kim JY, Shin KS, Lee CS, Song DS (**2008**) Effects of epidermal growth factor on atrophic enteritis in piglets induced by experimental porcine epidemic diarrhoea virus. Vet J 177: 231-235.

Jung K, Wang Q, Scheuer KA, Lu Z, Zhang Y, Saif LJ (**2014**) Pathology of US Porcine Epidemic Diarrhea Virus Strain PC21A in Gnotobiotic Pigs. Emerg Infect Dis 20: 668-671.

Kunzelmann K, Mall M (**2002**) Electrolyte Transport in the Mammalian Colon: Mechanisms and Implications for Disease. Physiol Rev 82: 245-289.

Leonardo M (**1992**) Diarrheal disease as a cause of malnutrition. Am J Trop Med Hyg 47: 16-27.

Livak KJ, Schmittgen TD (**2001**) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. Methods 25: 402-408.

Lodemann U, Amasheh S, Radloff J, Kern M, Bethe A, Wieler LH, Pieper R, Zentek J, Aschenbach JR (**2017**) Effects of Ex Vivo Infection with ETEC on Jejunal Barrier Properties and Cytokine Expression in Probiotic-Supplemented Pigs. Dig Dis Sci 62: 922-933.

Miwa N, Furuse M, Tsukita S, Niikawa N, Nakamura Y, Furukawa Y (**2001**) Involvement of claudin-1 in the beta-catenin/Tcf signaling pathway and its frequent upregulation in human colorectal cancers. Oncol Res 12: 469-476.

Morohashi S, Kusumi T, Sato F, Odagiri H, Chiba H, Yoshihara S, Hakamada K, Sasaki M, Kijima H (**2007**) Decreased expression of claudin-1 correlates with recurrence status in breast cancer. Int J Mol Med 20: 139-143.

Pinton P, Braicu C, Nougayrede JP, Laffitte J, Taranu I, Oswald IP (**2010**) Deoxynivalenol Impairs Porcine Intestinal Barrier Function and Decreases the Protein Expression of Claudin-4 through a Mitogen-Activated Protein Kinase-Dependent Mechanism. J Nutr 140: 1956-1962.

Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl JP, Ricciardi-Castagnoli P (**2001**) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol 2: 361-367.

Resnick MB, Gavilanez M, Newton E, Konkin T, Bhattacharya B, Britt DE, Sabo E, Moss SF (**2005**) Claudin expression in gastric adenocarcinomas: a tissue microarray study with prognostic correlation. Hum Pathol 36: 886-892.

Roselli M, Finamore A, Britti MS, Konstantinov SR, Smidt H, de Vos WM, Mengheri E (**2007**) The Novel Porcine Lactobacillus sobrius Strain Protects Intestinal Cells from Enterotoxigenic Escherichia coli K88 Infection and Prevents Membrane Barrier Damage. J Nutr 137: 2709-2716.

Smith F, Clark JE, Overman BL, Tozel CC, Huang JH, Rivier JE, Blikslager AT, Moeser AJ (**2010**) Early weaning stress impairs development of mucosal barrier function in the porcine intestine. Am J Physiol Gastrointest Liver Physiol 298: G352-G363.

Soini Y, Tommola S, Helin H, Martikainen P (**2006**) Claudins 1, 3, 4 and 5 in gastric carcinoma, loss of claudin expression associates with the diffuse subtype. Virchows Arch 448: 52-58.

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

Suzuki T (**2013**) Regulation of intestinal epithelial permeability by tight junctions. Cell Mol Life Sci 70: 631-659.

Suzuki T, Hara H (**2009**) Quercetin Enhances Intestinal Barrier Function through the Assembly of Zonula [corrected] Occludens-2, Occludin, and Claudin-1 and the Expression of Claudin-4 in Caco-2 Cells. J Nutr 139: 965-974.

Tsukita S, Furuse M, Itoh M (**2001**) Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol 2: 285-293.

Wang H, Wang P, Wang X, Wan Y, Liu Y (**2012**) Butyrate

Enhances Intestinal Epithelial Barrier Function via Up-Regulation of Tight Junction Protein Claudin-1 Transcription. Dig Dis Sci 57: 3126-3135.

Yang KM, Jiang ZY, Zheng CT, Wang L, Yang XF (**2014**) Effect of Lactobacillus plantarum on diarrhea and intestinal barrier function of young piglets challenged with enterotoxigenic Escherichia coli K88. J Anim Sci 92: 1496-1503.

Yuan L, Le Bras A, Sacharidou A, Itagaki K, Zhan Y, Kondo M, Carman CV, Davis GE, Aird WC, Oettgen P (**2012**) ETS-related Gene (ERG) Controls Endothelial Cell Permeability via Transcriptional Regulation of the Claudin 5 (CLDN5) Gene. J Biol Chem 287: 6582-6591.

Zhao S, Gao J, Zhu L, Yang Q (**2014**) Transmissible gastroenteritis virus and porcine epidemic diarrhoea virus infection induces dramatic changes in the tight junctions and microfilaments of polarized IPEC-J2 cells. Virus Res 192: 34-45.