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Preliminary screening of intestinal barrier genes associated with porcine epidemic diarrhea virus infection in pigs

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ABSTRACT - To screen intestinal barrier genes associated with porcine epidemic diarrhea virus (PEDV) infection, in the present study we first detected PEDV-infected piglets (*Sus scrofa*) with intestinal damage using quantitative real-time PCR (qPCR) and hematoxylin and eosin (HE) staining. Then, we used qPCR to identify expression differences of intestinal barrier genes between the PEDV-infected and control groups. The results showed that the expression levels of most genes were significantly different between the two groups. Hierarchical clustering and correlation analysis were performed for the expression levels of 25 candidate genes to reveal the key gene that may be involved in PEDV resistance. Two important candidate genes, *GLP2* (glucagon–like peptide 2) and *AQP3* (aquaporin 3), have their expression positively correlated (r = 0.84). We speculated that decreased expression of *GLP2* and *AQP3* might play an important role in the process of PEDV infection of piglets by reducing the expression of tight junction proteins and disrupting the junctions between intestinal epithelial cells. There may be an underlying biological interaction between the two genes, which together affect the functional integrity of the intestinal barrier.

Keywords: gene expression, piglet, quantitative real-time PCR

1. Introduction

Porcine epidemic diarrhea (PED) is a highly contagious intestinal infectious disease caused by porcine epidemic diarrhea virus (PEDV), with an epidemic peak time in cold climates in the late autumn and early winter, which is mainly characterized by vomiting, diarrhea, and dehydration of piglets (*Sus scrofa*), resulting in high morbidity and 80-100% mortality (Sun et al., 2012; Li et al., 2012). The PEDV is a coronavirus belonging to the alpha-coronavirus genus within the family of Coronaviridae, and is an enveloped single-stranded positive-sense RNA virus (Park et al., 2012). In addition to suckling piglets, weaned piglets, fattening pigs, and sows might also be affected. Individual death and decreased performance caused by this virus cause huge economic losses to the pig industry, while the use of drugs and vaccines and related biosafety problems incur further economic losses (Song and Park, 2012). The main means of prevention and control of PED is vaccination; however, the genetic variation of PEDV strains causes enhanced virulence of variant strains or make effective immunization using vaccines difficult (Li et al., 2012); it is, therefore, urgent to find a permanent cure. Genetic differences in disease resistance have been found between different pig breeds, as well as between different individuals within breeds (FAO, 2007; Lunney and Chen, 2010), and the genetic improvement of disease resistance in livestock is an effective and feasible approach. Therefore, genetically enhancing piglet resistance to PEDV might be the most effective and fundamental way to control PEDV infection in the long run.

The intestine is not just the main site of digestion and absorption in pigs, but also is the first line of defense of the body against foreign pathogens. The intestinal barrier prevents harmful substances, such as bacteria and viruses, from entering the blood circulation through the intestinal mucosa (Soderholm and Perdue, 2001). The intestinal barrier comprises mechanical, chemical, immune, and biological barriers (Baumgart and Dignass, 2002). Impaired or disturbed intestinal barrier function can lead to various intestinal diseases (Soderholm and Perdue, 2001; Camilleri et al., 2012). Impairment of the intestinal barrier is an important internal cause of diseases such as diarrhea and intestinal inflammation in piglets (Lallès et al., 2004). Therefore, screening of intestinal barrier genes associated with porcine PEDV from the perspective of molecular genetics would be of great value for pig anti-diarrheal disease research.

In the present study, we first confirmed that piglet diarrhea was caused by PEDV using quantitative real-time PCR (qPCR) in an eight-day-old piglet population, and then paraffin sections stained with hematoxylin and eosin (HE) were used to detect pathological changes in piglets after slaughter to provide experimental materials to screen key candidate genes related to PEDV infection in pigs. On this basis, 25 candidate genes related to the regulatory mechanism of the intestinal barrier were selected according to their biological function and literature mining (Table 1). The differential expression of candidate genes in each segment of the small intestine of PEDV-infected and control piglets was detected using qPCR to further identify the key candidate genes related to PEDV infection. Finally, we identified two potential candidate genes, *GLP2* and *AQP3*, through hierarchical clustering and correlation analysis for further study. The results of the present study provide an important experimental reference and basis for in-depth and systematic analysis of the regulatory mechanism of PEDV infection in pigs.

2. Material and Methods

2.1. Ethics statement and location of the experiment

Research on animals was conducted with approval from the institutional committee on animal use (case number: SYXK [Su] IACUC 2012-0029). All experimental procedures involving piglets were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals and were approved by the State Council of the People's Republic of China. The pig farm where the experiment was conducted is located in Taicang City, China (31°20' to 31°45' N and 120°58' to 121°20' E). The later experiments were completed in Yangzhou City, China (32°15' to 33°25' N and 119°01' to 119°54' E).

2.2. Animals

Six eight-day-old crossbred (Duroc × Landrace × Yorshire) piglets with clinicopathological features of PED (vomiting, dehydration, and watery diarrhea) were chosen from a pig farm. At the same time, six healthy eight-day-old piglets were selected from the same feeding environment with similar birth weight, body shape, and color. All piglets were fed by lactation before slaughter. The pigsty was equipped with an incubator. The environmental temperature was controlled at about 28 °C, and the relative humidity was controlled between 50 and 70%. During this period, no vaccine was injected or drugs were administered. Piglets were stunned electrically (300 V for 5 s) and bled by heart puncture under the left armpit. The duodenum, jejunum, and ileum tissues were sampled within 30 min of death. After cleaning with phosphate-buffered saline (PBS), each intestinal segment was cut transversely into two sections. One section was frozen in liquid nitrogen for RNA extraction, and the other section was fixed with 4% paraformaldehyde for paraffin sectioning.

2.3. Quantitative Real-time PCR primer design

Based on published GenBank sequences, the primers for the PEDV, PDCoV (porcine deltacoronavirus), RV (rotavirus), TGEV (transmissible gastroenteritis virus), and 25 intestinal barrier candidate

genes (Table 2) were designed using Primer Premier 5.0 software (PREMIER Biosoft International, San Francisco, CA, USA). *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *ACTB* (beta-actin) were used as internal control genes to normalize the threshold cycle (Ct) values of the other transcripts.

Table 1 - Intestinal barrier candidate genes

Gene name	Abbreviation	Function	Literature source
Zonula occludens	*ZO1* *ZO2*	Maintains and regulates epithelial barriers, participates in the regulation of cell material transport, and maintains epithelial polarity.	Neunlist et al., 2003
Occludin	*OCLN*	Promotes the formation of selective ion channels in tight junctions and is associated with intercellular adhesion, migration, and permeability.	Berkes et al., 2003
Claudins	*CLDN1* *CLDN4* *CLDN5*	Regulates the pore pathway, determines TER as well as paracellular charge selectivity.	Colegio et al., 2003
Cingulin	*CGN*	Regulates expression of TJ proteins through the RhoA signal pathway.	Hamard et al., 2010
Glucagon-like peptide 2	*GLP2*	Induces the proliferation and reduces the internalization of enterobacteriaceae, regenerates the intestinal mucosa, and improves the intestinal barrier function.	Cani et al., 2009
E-cadherin	*E-CDH*	Tight attachment of adjacent epithelial cells is ensured by allelic interactions at their basolateral adhesive junctions.	Uemura, 1998
Integrin	*ITG*	Establishes a physical connection between the extracellular matrix and the cytoskeleton that mediates the activation of cell adhesion signaling pathways.	Stupack and Cheresh, 2002
Nectin1	*NECTIN1*	Participates in intercellular adhesion and recruits TJ components.	Rikitake and Takai, 2008
Aquaporin 3	*AQP3*	Changes the ability of the intestinal tract to move water, causes diarrhea or constipation.	Li et al., 2008
Aquaporin 8	*AQP8*	Changes the intestinal mucosal water exchange capacity, disrupts the intestinal mucosal barrier, and causes chronic inflammation and ulcers.	Gao et al., 2014
Neonatal Fc receptor	*FcRN*	Specific receptor of IgG, which interacts with IgG to produce effects on the intestinal mucosa.	Dong et al., 2016
Mucins	*MUC1* *MUC2* *MUC4* *MUC5AC*	Lubricates and protects the intestinal mucosa, provides adhesion sites for probiotics, regulates intestinal bacteria balance, enhances intestinal barrier function, assists the intestinal immune system, and inhibits the production of proinflammatory factors.	Johansson et al., 2013
Trefoil factor 1	*TFF1*	Interacts with mucins such as MUC2, and preserves mucosal integrity.	Laukoetter et al., 2008
β-defensin 1	*PBD1*	Has broad-spectrum antimicrobial activity, induces the production of inflammatory mediators, and regulates innate and immune adaptive immunity.	Yang et al., 2004
β-defensin 2	*PBD2*	Prevents infection by bacteria and viruses, regulates immune factors, and participates in immune regulation.	Veldhuizen et al., 2008
Porcine myeloid antibacterial peptide 37	*PMAP37*	Inhibits gram-positive and -negative bacteria.	Tossi et al., 1995
Discs large homolog 5	*DLG5*	Stabilizes the apical protein complex and is associated with the maintenance of the integrity and polarity of epithelial cells.	Friedrichs and Stoll, 2006
Tumor necrosis factor-α	*TNFA*	Modulates the function of a tight junction between epithelial cells of the intestine, kidney, lung, and salivary glands.	Baker et al., 2008
Interferon-γ	*IFNG*	Modulates the function of a tight junction between epithelial cells of the intestine, kidney, lung, and salivary glands.	Baker et al., 2008

Table 2 - Real-time PCR primer sequences

Gene	Accession no.	Primer sequences (5′→3′)	Length (bp)
GAPDH	AF017079.1	F: ACATCATCCCTGCTTCTACTGG R: TCTCCTCCTGCTCCAATCCAG	187
ACTB	XM_00312428.3	F: TGGCGCCCAGCACGATGAAG R: GATGGAGGGGCCGGACTCGT	149
PEDV (M)	AF353511.1	F: AGGTCTGCATTCCAGTGCTT R: GGACATAGAAAGCCCAACCA	216
PDCoV (M)	JQ065042	F: ATCGACCACATGGCTCCAA R: CAGCTCTTGCCCATGTAGCTT	72
RV (VP6)	KJ559046	F: AAAGATGCTAGGGACAAAATTG R: TTCAGATTGTGGAGCTATTCCA	309
TGEV (M)	DQ811789	F: GCAATTCTTTGGGTTAGTGCAT R: AGCGTACAAATTCCCTGAAAGC	102
ZO1	XM_021098856.1	F: AGCCCGAGGCGTGTTT R: GGTGGGAGGATGCTGTTG	147
ZO2	NM_001206404.1	F: ATTCGGACCCATAGCAGACATAG R: GCGTCTCTTGGTTCTGTTTTAGC	90
OCLN	NM_001163647.2	F: ATCAACAAAGGCAACTCT R: GCAGCAGCCATGTACTCT	157
CLDN1	NM_001244539.1	F: ACCCCAGTCAATGCCAGATA R: GGCGAAGGTTTTGGATAGG	155
CLDN4	NM_001161637.1	F: CAACTGCGTGGATGATGAGA R: CCAGGGGATTGTAGAAGTGG	140
CLDN5	NM_001161636.1	F: CCTTCCTGGACCACAACATC R: CACCGAGTCGTACACCTTGC	110
CGN	XM_013997148.2	F: GTTAAAGAGCTGTCCATCCAGATTG R: CTTAGCTGGTCTTTCTGGTCATTG	123
GLP2	NM_214324.1	F: ACTCACAGGGCACGTTTACCA R: AGGTCCCTTCAGCATGTCTCT	150
E-CDH	NM_001163060.1	F: GAAGGAGGTGGAGAAGAGGAC R: AGAGTCATAAGGTGGGGCAGT	174
ITG	NM_213968.1	F: AATGTAACCAACCGTAGCA R: GAAGTCTGAAGTAATCCTCCT	261
NECTIN1	AF308632	F: CAGACAGGCAGAGACAAG R: TAGGCGGAGAAGAGGAAG	380
AQP3	HQ888860.1	F: TCGTGATGTTTGGCTGTGG R: GCCAGGTTGATGGTGAGGA	82
AQP8	NM_001112683.1	F: CCATTCTCCATCGGCTTCTC R: AGCAGGTCCAAAGGCTCGT	96
FcRN	NM_214197.2	F: CCTTGGATCTCTTTTCATGGGG R: CAGAGGCTCAGAAATACCAGC	199
MUC1	XM_001926883.1	F: ACACCCATGGGCGCTATGT R: GCCTGCAGAAACCTGCTCAT	123
MUC2	XM_002347185.2	F: ACGACTTTGACGGACACTGCT R: AGGGGACGTTCTCGGTGAT	99
MUC4	XM_001926442.1	F: GATGCCCTGGCCACAGAA R: TGATTCAAGGTAGCATTCATTTGC	89
MUC5AC	AF054584	F: CCCCCTCGTCTCCTTTTACC R: GGATGTCGCCAGAGACTGAGTA	71
TFF1	AM283538	F: CCATGGAGCACAAGGTGA R: AGGGTGGAAGCACCACGGGA	200
PBD1	NM_213838.1	F: CGCCTCCTCCTTGTATTCCTC R: GGTGCCGATCTGTTTCATCTT	144
PBD2	AY506573.1	F: CCAGAGGTCCGACCACTACA R: GGTCCCTTCAATCCTGTTGAA	168
PMAP37	NM_213863.1	F: CACCTGCAATGAGGGTGTCA R: GTCGCAACCGTGGTCTTCG	68
DLG5	XM_005671132.1	F: ATCCCTCTGTCATCGACCCA R: GTGCAGGTTCCCACCACATA	185
TNFA	X54859	F: CGACTCAGTGCCGAGATCAA R: CCTGCCCAGATTCAGCAAAG	402
IFNG	X53085	F: CAGCTTTGCGTGACTTTGTG R: GATGAGTTCACTGATGGCTTT	381

2.4. RNA extraction and identification of viruses

Total RNA was extracted from three intestinal segments (duodenum, jejunum, and ileum) (50-100 mg) using TRIzol (Takara Biotechnology Dalian Co., Ltd., Dalian, China). The precipitated RNA was suspended in 20 μL of RNase-free water and stored at –80 °C. Quality of RNA was assessed by 1.5% formaldehyde denatured gel electrophoresis. Concentration and purity of RNA were determined using a spectrophotometer (Nanodrop ND1000, NanoDrop Technologies Co., Ltd., Wilmington, DE, USA). Total RNA was reverse-transcribed into complementary DNA (cDNA) using a HiScript Q RT SuperMix kit (Vazyme Biotech Co., Ltd., Nanjing, China), and the products were stored at 4 °C. Subsequently, the target fragments of PEDV, PDCoV, RV, and TGEV were amplified using qPCR. The products were separated by 1% agarose gel electrophoresis and then sequenced. The target fragment of PEDV was sequenced, and the evolutionary tree based on the sequence of the *M* gene was established using the neighboring joining method to detect PEDV strains.

2.5. Histological evaluation of intestinal slices

The intestinal segments were fixed in 4% paraformaldehyde for 24 h. After paraffin embedding, slicing, salvaging, baking at 60 °C for 1 h, and staining with HE, the morphological differences in the mucosa of three intestinal segments between the PEDV-infected group and control groups were observed under a light microscope. Images were acquired using Motic photographic processing software (Kazemi et al., 2012) and analyzed with a pathological image analysis system.

2.6. Quantitative real-time PCR

Quantitative real-time PCR amplification was performed using a PCR kit (Vazyme Biotech Co., Ltd. Nanjing, China) in a 25-μL reaction mixture containing 2 μL of cDNA, 0.5 μL of forward and reverse primer (10 μM each), 0.5 μL of 50× ROX Reference Dye II, 10 μL of 2× SYBR Green Real-time PCR Master Mix, and double-distilled water. We used the 7500 Real-time PCR system (Applied Biosystems) to perform the reaction. The PCR conditions were set at 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Dissociation curve analysis was performed after amplification. A peak melting temperature (Tm) of 85±0.8 °C in the dissociation curve was used to determine the specificity of the amplification product. The Tm value for each sample was calculated using the average of triplicate technical samples.

2.7. Statistical and analysis

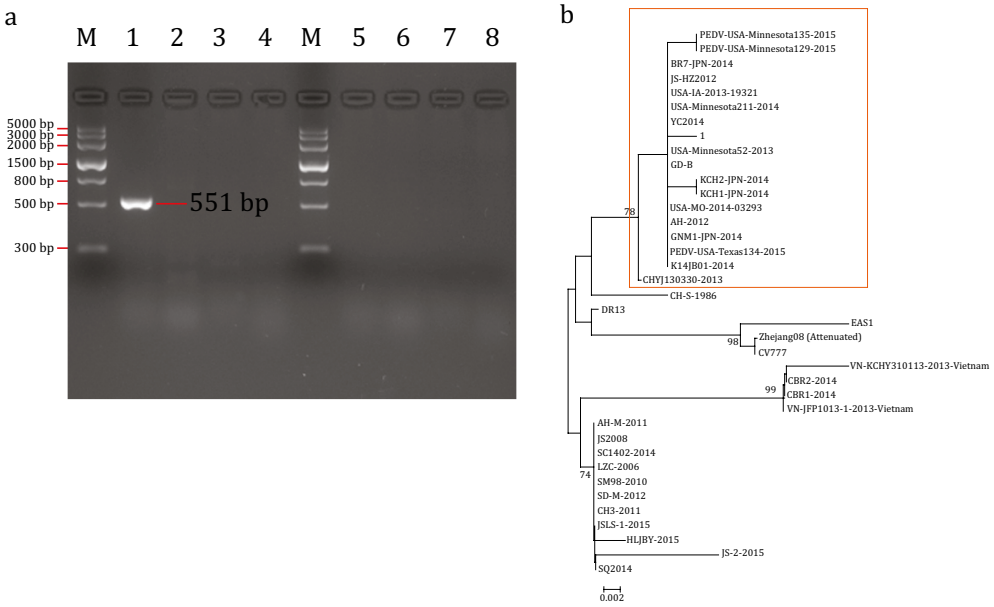
The original data were arranged using Excel 2007 software (Microsoft Corporation, Redmond, WA, USA). The relative quantitative expression results were calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001). Expression differences between the PEDV-infected and control groups were compared using an independent sample t test, and the heat map was drawn using HemI 1.0 (Deng et al., 2014) after log2 conversion using the mean of the expression levels (X = log2(expression data)). Correlation analysis was performed using SPSS 18.0 software (IBM Corp., Armonk, NY, USA) on the expression levels of all piglets in different tissues.

3. Results

3.1. Purity and integrity of total RNA and identification of coronaviruses

The RNA purification showed three bands, i.e., 28S, 18S, and 5S, with no DNA contamination and obvious degradation, and the A260/A280 (absorbance at 260 nm and 280 nm) ratio was 1.8-1.9. The results indicated that the extracted RNA had high integrity and purity and could be used in subsequent experiments. Agarose gel electrophoresis was used to detect the bands of PEDV, PDCoV, RV, and TGEV,

and the results showed that only a PEDV band at 551 bp was detected in the PEDV groups, while no bands were observed in the control group (Figure 1a). We established the evolutionary tree based on the sequence of the M gene (PEDV) using the neighboring joining method to detect PEDV strains. Strains in porcine intestines had high homology with the current domestic and foreign representative PEDV strains, confirming that these piglets with diarrhea were infected only with PEDV (Figure 1b).

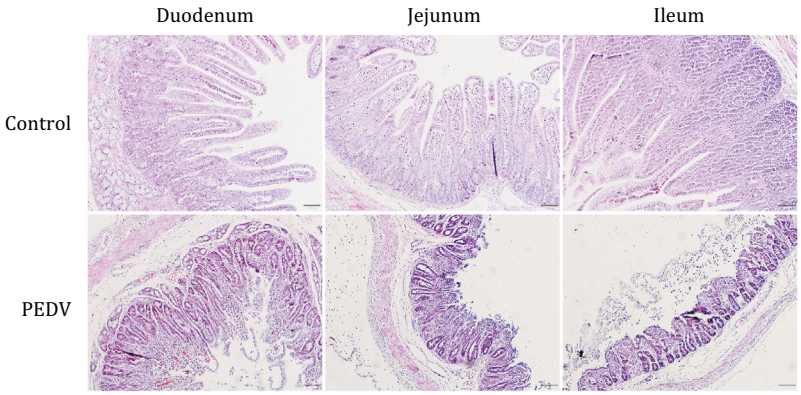


PEDV - porcine epidemic diarrhea virus; PDCoV - porcine deltacoronavirus; RV - rotavirus; TGEV - transmissible gastroenteritis virus.
a: Gel electrophoresis detection of multiple coronaviruses. M represents markerIII, lanes 1 to 4 and 5 to 8 represent electrophoretic bands of PEDV, PDCoV, RV, and TGEV in the PEDV-infected and control groups, respectively.
b: Adjacency based method for neighboring phylogenetic trees. The orange square represents some domestic and foreign PEDV epidemic strains that were closely related to the detected strains.

Figure 1 - Pathogen identification of piglet diarrhea.

3.2. Morphological characterization of intestinal mucosa

The results of HE staining showed that the intestinal mucosa of the control group had an intact structure and clear hierarchy, while the outline of epithelial cells was clear and regularly arranged. In the PEDV group, the intestines showed significant lesions, with shedding and damage of villi, exposure of the lamina propria, and atrophy of the intestinal glands (Figure 2).



PEDV - porcine epidemic diarrhea virus.
All images are shown at 10X magnification.

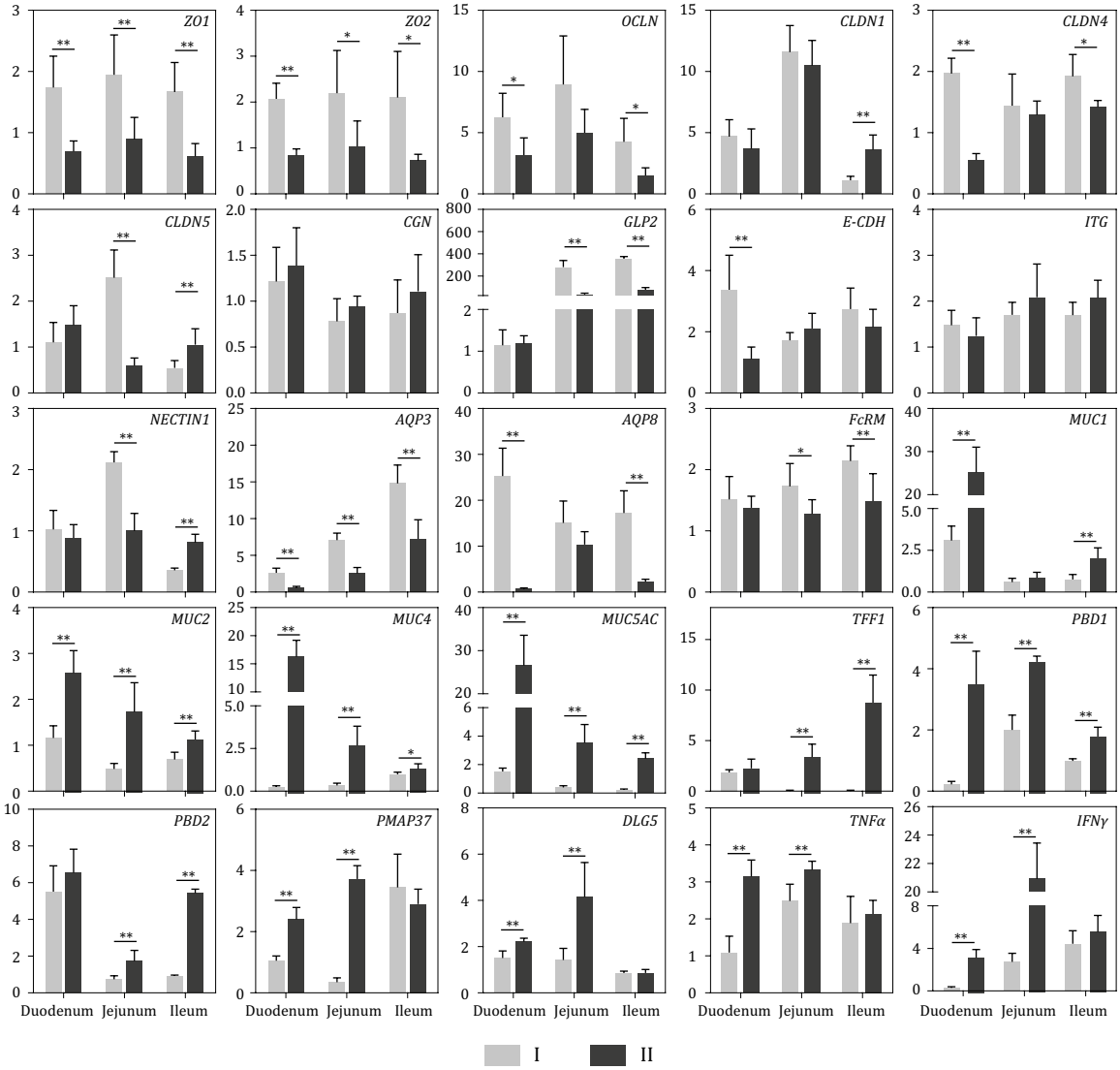
Figure 2 - Hematoxylin and eosin staining for different intestinal segments in PEDV-infected and control groups.

3.3. qPCR amplification and melting curves

The qPCR amplification and melting curves for the genes related to intestinal barrier function showed that the PCR products had only one definite peak, and no primer dimers or non-specific products.

3.4. Expression differences of intestinal barrier-related candidate genes between the PEDV-infected and control groups

The expression differences of intestinal barrier-related candidate genes in various small intestinal segments of piglets between the PEDV-infected and control groups were detected using qPCR (Figure 3). The results indicated that the expression levels of *ZO1*, *ZO2*, and *AQP3* in the control group were significantly higher than those in the PEDV-infected group in all small intestinal segments.



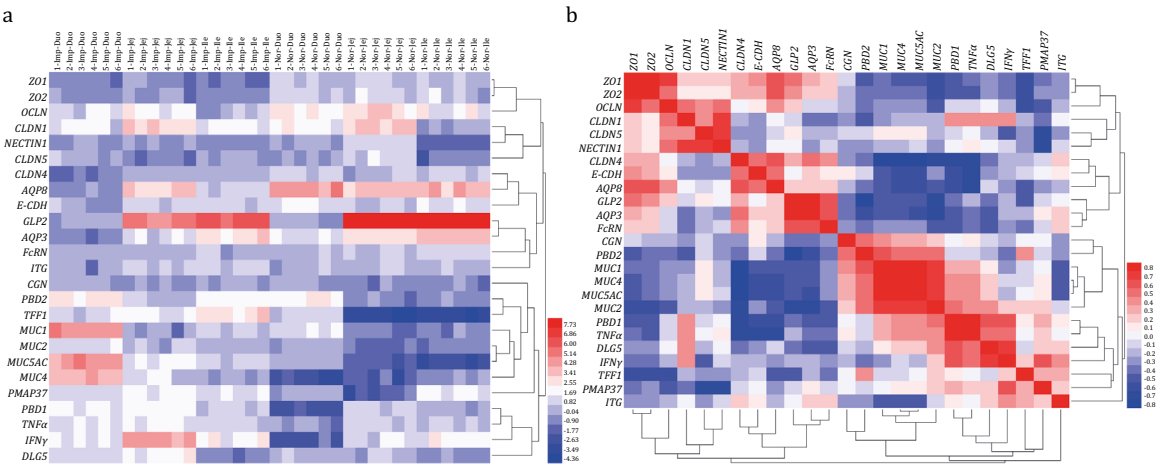
PEDV - porcine epidemic diarrhea virus.
The Roman numerals I and II represent control and PEDV-infected groups, respectively.
* Denotes a significant difference with P<0.05, while ** denotes a significant difference with P<0.01.

Figure 3 - Differential expression analysis of candidate genes related to the porcine intestinal barrier in porcine small intestine between PEDV-infected and control groups.

OCLN, *CLDN4*, and *AQP8* expression levels in the control group were significantly higher than those in the PEDV-infected group in the duodenum and ileum, and *GLP2* and *FcRN* expression levels in the control group were significantly higher than those in the PEDV-infected group in the jejunum and ileum. *E-CDH* expression levels in the control group were significantly higher in the duodenum than those in the PEDV-infected group. *CLDN5* and *NECTIN1* expression levels in the control group were significantly higher than those in the PEDV-infected group in the jejunum, but showed significantly lower expression in the ileum. *CLDN1* expression levels in the control group were significantly lower than those in the PEDV-infected group in the ileum (P<0.01). *MUC2*, *MUC4*, *MUC5AC*, and *PBD1* expression levels in the control group were significant or significantly lower than those in the PEDV-infected group in all small intestinal segments. *PMAP37*, *DLG5*, *TNFA*, and *IFNG* expression levels were significantly lower in the control group compared with those in the PEDV-infected group in the duodenum and jejunum, and *TFF1* and *PBD2* expression levels were significantly lower in the control group compared with those in the PEDV-infected group in the jejunum and ileum. *MUC1* expression levels were significantly lower in the control group compared with those in the PEDV-infected group in the duodenum and ileum. *CGN* and *ITG* expression levels in the small intestine were not significantly different between the two groups.

3.5. Hierarchical clustering and correlation analysis of the expressions of candidate genes

Hierarchical clustering of the expression of 25 candidate genes in 12 samples revealed that *GLP2*, *FcRN*, aquaporin genes (*AQP3* and *AQP8*), tight junction protein genes (*ZO1*, *ZO2, OCLN*, *CLDN1*, *CLDN4*, and *CLDN5*), and adhesion junction protein genes (*NECTIN1*, *E-CDH*, and *ITG*) were clustered together; and *CGN*, immune barrier-related genes (*TNFA* and *IFNG*), chemical barrier-related genes (*DLG5*, *PBD1*, *PBD2*, *PMAP37*, and mucin genes (*MUC1*, *MUC2*, *MUC4*, *MUC5AC*, and *TFF1*)) were clustered together (Figure 4a). The correlation analysis revealed that the clustering results were similar to those in Figure 4a, and divided into two categories. The genes within the two categories were mostly positively correlated with each other, whereas the genes between the two categories were mostly negatively correlated with each other (Figure 4b). Among them, there was a highly significant positive correlation between *ZO1* and *ZO2*; between *PBD1* and *TNFA*; among *MUC1*, *MUC2,* and *MUC5AC*; and between *GLP2* and *AQP3* (P<0.01, R>0.8) (Table 3). Notably, the *GLP2* expression was significantly higher than that of the other candidate genes (Figure 4a).



PEDV - porcine epidemic diarrhea virus.
a: Heat map of the relative expression of 25 candidate genes, which was plotted after log2 transformation, according to the methods described by Deng et al. (2014).
b: Heat map of the correlation between 25 candidate genes and PEDV infection.

Figure 4 - Hierarchical clustering and correlation analysis of the relative expression of candidate genes.

Table 3 - Correlation analysis of the expressions of candidate genes

Gene name	*ZO1*	*ZO2*	*OCLN*	*CLDN1*	*CLDN4*	*CLDN5*	*CGN*	*GLP2*	*PBD1*	*PBD2*	*MUC1*	*MUC2*
ZO1	1	0.866**	0.750**	0.224	0.370*	0.284	−0.024	0.502**	−0.462**	−0.436**	−0.382*	−0.572**
ZO2	0.866**	1	0.584**	0.115	0.370*	0.184	−0.115	0.499**	−0.447**	−0.383*	−0.323	−0.502**
OCLN	0.750**	0.584**	1	0.539**	0.031	0.421*	−0.126	0.284	−0.098	−0.351*	−0.259	−0.409*
CLDN1	0.224	0.115	0.539**	1	−0.131	0.460**	−0.234	−0.033	0.430**	−0.426**	−0.285	−0.148
CLDN4	0.370*	0.370*	0.031	−0.131	1	−0.243	−0.275	0.355*	−0.655**	−0.339*	−0.701**	−0.627**
CLDN5	0.284	0.184	0.421*	0.460**	−0.243	1	−0.024	0.123	−0.052	−0.062	0.179	−0.157
CGN	−0.024	−0.115	−0.126	−0.234	−0.275	−0.024	1	−0.399*	0.029	0.539**	0.436**	0.295
GLP2	0.502**	0.499**	0.284	−0.033	0.355*	0.123	−0.399*	1	−0.317	−0.736**	−0.437**	−0.663**
PBD1	−0.462**	−0.447**	−0.098	0.430**	−0.655**	−0.052	0.029	−0.317	1	−0.002	0.351*	0.545**
PBD2	−0.436**	−0.383*	−0.351*	−0.426**	−0.339*	−0.062	0.539**	−0.736**	−0.002	1	0.613**	0.531**
MUC1	−0.382*	−0.323	−0.259	−0.285	−0.701**	0.179	0.436**	−0.437**	0.351*	0.613**	1	0.754**
MUC2	−0.572**	−0.502**	−0.409*	−0.148	−0.627**	−0.157	0.295	−0.663**	0.545**	0.531**	0.754**	1
MUC4	−0.450**	−0.397*	−0.283	−0.198	−0.746**	0.113	0.388*	−0.418*	0.486**	0.513**	0.942**	0.745**
MUC5AC	−0.433**	−0.400*	−0.302	−0.187	−0.703**	0.114	0.376*	−0.457**	0.418*	0.512**	0.890**	0.772**
TFF1	−0.593**	−0.554**	−0.504**	−0.174	−0.169	−0.253	0.073	−0.456**	0.137	0.470**	−0.024	0.222
PMAP37	−0.385*	−0.272	−0.489**	−0.307	−0.097	−0.680**	0.064	−0.043	0.355*	−0.064	0.005	0.292
DLG5	−0.267	−0.261	0.071	0.427**	−0.319	−0.148	−0.002	−0.400*	0.656**	−0.116	0.102	0.439**
AQP3	0.315	0.269	−0.002	−0.345*	0.512**	−0.182	−0.345*	0.841**	−0.445**	−0.532**	−0.496**	−0.632**
AQP8	0.695**	0.696**	0.478**	0.052	0.669**	−0.042	−0.182	0.278	−0.596**	−0.372*	−0.492**	−0.458**
E-CDH	0.406*	0.363*	0.145	−0.223	0.543**	−0.267	0.1	0.065	−0.557**	−0.031	−0.429**	−0.299
ITG	−0.212	−0.251	−0.172	0.062	0.242	−0.263	−0.241	0.082	0.149	−0.202	−0.403*	−0.058
NECTIN1	0.253	0.162	0.530**	0.746**	−0.118	0.727**	−0.248	0.093	0.067	−0.236	−0.155	−0.266
FcRN	0.253	0.216	−0.006	−0.342*	0.448**	−0.14	−0.22	0.615**	−0.436**	−0.353*	−0.259	−0.438**
TNFα	−0.389*	−0.412*	−0.056	0.405*	−0.656**	0.082	0.106	−0.137	0.829**	−0.09	0.322	0.404*
IFNγ	−0.341*	−0.330*	−0.104	0.411*	−0.13	−0.419*	−0.152	−0.185	0.677**	−0.325	−0.224	0.232

Continues...

Table 3 (Continued)

Gene name	*MUC4*	*MUC5AC*	*TFF1*	*PMAP37*	*DLG5*	*AQP3*	*AQP8*	*E-CDH*	*ITG*	*NECTIN1*	*FcRN*	*TNFα*	*IFNγ*
ZO1	−0.450**	−0.433**	−0.593**	−0.385*	−0.267	0.315	0.695**	0.406*	−0.212	0.253	0.253	−0.389*	−0.341*
ZO2	−0.397*	−0.400*	−0.554**	−0.272	−0.261	0.269	0.696**	0.363*	−0.251	0.162	0.216	−0.412*	−0.330*
OCLN	−0.283	−0.302	−0.504**	−0.489**	0.071	−0.002	0.478**	0.145	−0.172	0.530**	−0.006	−0.056	−0.104
CLDN1	−0.198	−0.187	−0.174	−0.307	0.427**	−0.345*	0.052	−0.223	0.062	0.746**	−0.342*	0.405*	0.411*
CLDN4	−0.746**	−0.703**	−0.169	−0.097	−0.319	0.512**	0.669**	0.543**	0.242	−0.118	0.448**	−0.656**	−0.13
CLDN5	0.113	0.114	−0.253	−0.680**	−0.148	−0.182	−0.042	−0.267	−0.263	0.727**	−0.14	0.082	−0.419*
CGN	0.388*	0.376*	0.073	0.064	−0.002	−0.345*	−0.182	0.1	−0.241	−0.248	−0.22	0.106	−0.152
GLP2	−0.418*	−0.457**	−0.456**	−0.043	−0.400*	0.841**	0.278	0.065	0.082	0.093	0.615**	−0.137	−0.185
PBD1	0.486**	0.418*	0.137	0.355*	0.656**	−0.445**	−0.596**	−0.557**	0.149	0.067	−0.436**	0.829**	0.677**
PBD2	0.513**	0.512**	0.470**	−0.064	−0.116	−0.532**	−0.372*	−0.031	−0.202	−0.236	−0.353*	−0.09	−0.325
MUC1	0.942**	0.890**	−0.024	0.005	0.102	−0.496**	−0.492**	−0.429**	−0.403*	−0.155	−0.259	0.322	−0.224
MUC2	0.745**	0.772**	0.222	0.292	0.439**	−0.632**	−0.458**	−0.299	−0.058	−0.266	−0.438**	0.404*	0.232
MUC4	1	0.956**	−0.007	0.135	0.215	−0.489**	−0.586**	−0.529**	−0.426**	−0.142	−0.273	0.465**	−0.063
MUC5AC	0.956**	1	0.008	0.102	0.21	−0.516**	−0.558**	−0.519**	−0.403*	−0.096	−0.254	0.433**	−0.104
TFF1	−0.007	0.008	1	0.332*	−0.017	−0.148	−0.497**	−0.01	0.334*	−0.214	−0.370*	0.002	0.205
PMAP37	0.135	0.102	0.332*	1	0.272	0.183	−0.299	0.041	0.271	−0.688**	−0.039	0.251	0.601**
DLG5	0.215	0.21	−0.017	0.272	1	−0.499**	−0.092	−0.22	0.199	0.076	−0.483**	0.523**	0.722**
AQP3	−0.489**	−0.516**	−0.148	0.183	−0.499**	1	0.207	0.237	0.205	−0.26	0.649**	−0.306	−0.162
AQP8	−0.586**	−0.558**	−0.497**	−0.299	−0.092	0.207	1	0.586**	−0.115	0.064	0.214	−0.645**	−0.219
E-CDH	−0.529**	−0.519**	−0.01	0.041	−0.22	0.237	0.586**	1	0.134	−0.244	0.179	−0.646**	−0.105
ITG	−0.426**	−0.403*	0.334*	0.271	0.199	0.205	−0.115	0.134	1	−0.018	0.236	0.035	0.490**
NECTIN1	−0.142	−0.096	−0.214	−0.688**	0.076	−0.26	0.064	−0.244	−0.018	1	−0.078	0.157	−0.097
FcRN	−0.273	−0.254	−0.370*	−0.039	−0.483**	0.649**	0.214	0.179	0.236	−0.078	1	−0.343*	−0.242
TNFα	0.465**	0.433**	0.002	0.251	0.523**	−0.306	−0.645**	−0.646**	0.035	0.157	−0.343*	1	0.546**
IFNγ	−0.063	−0.104	0.205	0.601**	0.722**	−0.162	−0.219	−0.105	0.490**	−0.097	−0.242	0.546**	1

* P<0.05; ** P<0.01.

4. Discussion

Currently, PEDV variant strains have become the dominant strains of epidemic diarrhea in China, and the epidemic trend in pig farms should not be underestimated (Qin et al., 2015). Porcine epidemic diarrhea may become one of the most serious diarrheal diseases in piglets in the next few years in China. Therefore, there is an urgent need to develop new strategies to resist PEDV infection in piglets. The virus causes an increase in the severity of neonatal diarrhea, which in turn causes dehydration and death of piglets, partly resulting from a decrease in the rate of enterocyte replacement on the villi of the small intestine and insufficient innate defense in the small intestine after cell death caused by viral infection (Annamalai et al., 2015). In addition, the existing commercial vaccines were likely ineffective to PED. Therefore, from the genetics perspective, it would be valuable to screen functional candidate genes related to the structure or function of the intestinal barrier that might play an important role in PEDV virus infection in piglets.

In this study, we first verified that piglets with clinical features of epidemic diarrhea were indeed caused by PEDV infection, and confirmed that the intestines of PEDV-infected piglets were damaged. Then, we identified candidate genes according to their biological function and literature mining, and then determined differentially expressed genes between PEDV-infected and control groups. Most genes had expression differences between the two groups. The expression levels of tight junction protein genes, adhesion junction protein genes, and aquaporin genes in the control group were mostly higher than those in the PEDV-infected group, while the expression levels of intestinal immune and chemical barrier-related candidate genes were all lower than those in the PEDV-infected group. Tight junctions and adherent junctions are important mechanical couplers between intestinal epithelial cells and play a very important role in maintaining the mechanical intestinal barrier (Yang et al., 2005; Sander et al., 2005). Aquaporin genes play an important role in maintaining osmotic pressure and cell volume in epithelial cells and in altering the mucosal barrier (Matsuzaki et al., 1999; Fischer et al., 2001). We speculated that when piglets are infected by PEDV, the expression levels of genes related to transmembrane transport were decreased, and the junctions between intestinal epithelial cells were disrupted, further leading to impaired intestinal barrier function. Therefore, increasing the expression level of these genes might help to improve transmembrane transport and intestinal barrier abilities, enhance general disease resistance of piglets, which in turn would resist the invasion of exogenous pathogens. The chemical barrier is another important part of the intestinal barrier, which is formed by various digestive enzymes, lysozyme, bile, and mucopolysaccharides of the gastrointestinal tract; cytokines, inflammatory mediators, and antimicrobial peptides secreted by Paneth cells; and mucins secreted by goblet cells (Bischoff et al., 2014). This study speculated that the increased expression of chemical barrier-related genes after intestinal barrier damage would lead to increasing secretion of substances, such as antimicrobial peptides and mucus, and promote the recovery of the intestinal barrier. The immune barrier-related genes *TNFA* and *IFNG* encode key proinflammatory cytokines in the inflammatory response (Prasanna et al., 2010), and their increased expression might reflect the need for the body to mount an immune response to release inflammatory factors after the intestinal barrier is damaged.

The maintenance of intestinal barrier function and structure is achieved via a complex dynamic balance. Although the expression differences of most candidate genes between the PEDV-infected and control groups were very significant, it is insufficient to speculate on the identity of key genes in PEDV infection by solely relying on their expression differences. Therefore, we performed hierarchical clustering and correlation analysis of the expression levels of the 25 candidate genes to discover the possible interconnections among these candidate genes. The results showed that certain genes were correlated, but most of the correlations were moderate ($0.3<R\leq0.8$) or even weak ($R\leq0.3$). Only a few genes showed relatively significant and strong correlations ($P<0.01$, $R>0.8$). Among them, there was a strong correlation between *ZO1* and *ZO2*, possibly because they both belong to the zonula occludens family, which was similar to the strong correlation of mucin-like family members (Neunlist et al., 2003). *PBD1* has an important role in antibacterial and antiviral activities, can regulate immune factors, and is

involved in immune regulation, and *TNFα* acts as an important immune response factor, which might be responsible for the strong correlation between them. Not only was there a highly significant and strong correlation between *GLP2* and *AQP3*, but also the expression of *GLP2* was significantly higher than that of other candidate genes. In this study, *GLP2* expression in the control group was significantly higher than that in the PEDV-infected group in the jejunum and ileum, with up to 7.5- and 4.5-fold differences, respectively. *AQP3* also showed a very significant expression difference in the small intestine between the two groups. *GLP2* is an incretin hormone, and a reduced dosage of *GLP2* led to reduced expression of tight junction proteins (Moran et al., 2012). In addition, *GLP2* might reduce the expression of tight junction protein genes in the intestine of piglets via the mitogen activated protein kinase (MAPK) signaling pathway (Jiang et al., 2012). Furthermore, the levels of tight junction proteins *CLDN1* and *OCLN* were significantly reduced after inhibition of *AQP3* expression in Caco-2 cells (Zhang et al., 2011). Therefore, we speculated that decreased expression of *GLP2* and *AQP3* would reduce the expression of tight junction proteins and disrupt the junctions between intestinal epithelial cells, thereby affecting the permeability of the intestinal barrier, which in turn would promote PEDV infection of piglets via epithelial cells. There may be an underlying biological interaction between *GLP2* and *AQP3*, which together affect the functional integrity of the intestinal barrier.

5. Conclusions

The results of the present study suggested that *GLP2* and *AQP3* might play an important role in the process of PEDV infection in piglets by reducing the expression of tight junction proteins and disrupting the junctions between intestinal epithelial cells.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Data curation: X. Qi, Y. Xu and H. Wang. Formal analysis: X. Qi., Y. Xu and H. Wang. Investigation: X. Qi. Methodology: X. Qi. Project administration: X. Qi and W. Bao. Software: X. Qi and W. Qin. Validation: X. Qi. Visualization: X. Qi. Writing-original draft: X. Qi, Y. Xu and S. Wu. Writing-review & editing: X. Qi, S. Wu and W. Bao.

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