เชียงใหม่สัตวแพทยสาร 2561; 16(1): 37-46. DOI: 10.14456/cmvj.2018.5

เชียงใหม่สัตวแพทยสาร

Chiang Mai Veterinary Journal

ISSN; 1685-9502 (print) 2465-4604 (online)

Website; www.vet.cmu.ac.th/cmvj

Original Article

The comparison of villous damage at different ages of piglets infected with porcine epidemic diarrhea virus

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Abstract An outbreak of porcine epidemic diarrhea (PED) is spreading in Thailand since 2007, leading to the loss of economy to pig farmers. The causative agent of this disease is porcine epidemic diarrhea virus (PEDV). The objective of this study was to investigate gross lesions and histopathology of the small intestine of PEDV-infected piglets of different ages. The three parts of the small intestine: duodenum, jejunum, and ileum, were obtained from 3-day- and 10-day-old piglets infected with PEDV and normal piglets (control) for histopathological study and immunohistochemical staining using antibodies against PEDV. The diagnosis of PEDV infection was confirmed using Antigen Rapid TGE/PED Ag Test kit and reverse transcription polymerase chain reaction (RT-PCR). All PEDV-infected piglets showed positive results, while the normal piglets showed negative results. The immunohistochemistry indicated the presence of viral antigens in the cytoplasm of epithelial cells on the villi of the duodenum, jejunum, and ileum of piglets infected with PEDV of all ages. Moreover, the ratio of villous height (VH) to crypt depth (CD) was observed to decrease in all parts of the small intestine, particularly jejunum of 3-day-old PEDV-infected piglets, compared to that of normal piglets at the same age. A decreased ratio of VH:CD was observed in jejunum and ileum of 10-day-old piglets infected with PEDV. These findings indicated that younger piglets infected with PEDV showed more severe clinical signs, lesions, and villous damage than the older PEDV-infected piglets.

Keywords: villous damage, piglets, porcine epidemic diarrhea virus

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Article history; *received manuscript: 20 November 2017, accepted manuscript: 17 January 2018, published online: 26 January 2018*

บทความต้นฉบับ

การเปรียบเทียบความเสียหายของวิลไลในลูกสุกรติดเชื้อโรคท้องร่วงติดต่อในสุกรที่อายุแตกต่างกัน

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บทคัดย่อ โรคท้องร่วงติดต่อในสุกร (Porcine epidemic diarrhea, PED) เริ่มมีการระบาดในประเทศไทยตั้งแต่ปี พ.ศ. 2547 และสร้างความเสียหายทางเศรษฐกิจให้กับเกษตรกรผู้เลี้ยงสุกร สาเหตุของโรคนี้เกิดจากเชื้อไวรัส porcine epidemic diarrhea virus (PEDV) การศึกษาครั้งนี้เพื่อตรวจหารอยโรคด้วยตาเปล่าและลักษณะทางจุลพยาธิวิทยาที่ลำไส้เล็กของลูกสุกรติดเชื้อโรคท้องร่วงติดต่อที่อายุต่างกัน โดยเก็บตัวอย่างลำไส้เล็กส่วนต้น ส่วนกลาง และส่วนปลายของลูกสุกรที่ติดเชื้อโรคท้องร่วงติดต่อและลูกสุกรปกติที่ อายุ 3 และ 10 วัน เพื่อศึกษาลักษณะทางจุลพยาธิวิทยาและอิมมูโนฮีสโตเคมี โดยทำการตรวจยืนยันการติดเชื้อไวรัสที่ก่อโรคท้องร่วงติดต่อ ด้วยชุดทดสอบ Antigen Rapid TGE/PED Ag Test kit และยืนยันผลอีกครั้งด้วยวิธี reverse transcription polymerase chain reaction (RT-PCR) ซึ่งให้เป็นผลบวกทุกตัวในกลุ่มลูกสุกรป่วย และให้ผลเป็นลบในกลุ่มลูกสุกรปกติ เมื่อศึกษาด้วยอิมมูโนฮีสโตเคมีพบการติดเชื้อไวรัสที่ไซโทพลาซึมของเยื่อบุผิววิลไลลำไส้เล็กส่วนต้น ส่วนกลาง และส่วนปลาย ในลูกสุกรติดเชื้อไวรัสโรคท้องร่วงติดต่อ และเมื่อทำการวัดอัตราส่วนระหว่าง villous height (VH): crypt depth (CD) พบว่ามีอัตราส่วนลดลงในลำไส้เล็กทั้ง 3 ส่วน โดยพบชัดเจนที่สุดในลำไส้เล็กส่วนกลางของลูกสุกรอายุ 3 วันที่ติดเชื้อไวรัสโรคท้องร่วงติดต่อ เมื่อเปรียบเทียบกับลูกสุกรปกติที่อายุเดียวกัน ในขณะที่ลูกสุกรอายุ 10 วัน ที่ติดเชื้อโรคท้องร่วงติดต่อนั้น จะมีอัตราส่วน VH:CD ลดลงในลำไส้เล็กส่วนกลาง และส่วนปลาย ดังนั้นจากศึกษาในครั้งนี้สามารถสรุปได้ว่า ลูกสุกรอายุน้อยกว่าที่ติดเชื้อโรคท้องร่วงติดต่อในสุกรแสดงอาการทางคลินิก รอยโรคที่รุนแรง และเกิดความความเสียหายของวิลไลได้มากกว่าลูกสุกรที่อายุมากกว่าที่ติดเชื้อโรคท้องร่วงติดต่อ

คำสำคัญ ความเสียหายของวิลไล ลูกสุกร เชื้อไวรัสโรคท้องร่วงติดต่อในสุกร

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Introduction

An outbreak of porcine epidemic diarrhea (PED) is spreading in Thailand since 2007. The first outbreak was reported in affected farms at the Nakhon Pathom province (Puranaveja *et al.*, 2009). The spread of the outbreak in the country is still ongoing and has led to financial losses to porcine industries. The causative agent of this disease is the porcine epidemic diarrhea virus (PEDV), classified in genus *Alphacoronavirus,* family *Coronaviridae,* and order *Nidovirales* (Madson *et al.*, 2014; Jung *et al.*, 2015a). The suckling piglets infected with PEDV exhibit several clinical signs such as diarrhea, vomiting, and dehydration leading to increased chances of mortality, but the clinical signs in older pigs are less severe (Annamalai *et al.*, 2015). In addition, Olanratmanee *et al.* in 2010 reported the reduction of reproductive performance in gilts and sows infected with PEDV. PEDV infection causes the destruction of villous enterocytes within the small intestine, leading to reduced villous length and substantial decrease in the ratio of villous height to crypt depth (Sueyoshi *et al.*, 1995; Madson *et al.*, 2014).

The objective of this study was to investigate gross lesions and histopathology of the small intestine of PEDV-infected piglets of different ages.

Materials and methods

Experimental design

Twenty suckling piglets infected with PEDV were obtained from a two-site pig farm in Chonburi province, Thailand. Sows and gilts were housed in an open housing system facilitated with water sprinklers and fans. The farm status was stable of porcine reproductive and respiratory syndrome virus (PRRSV); this status was confirmed by negative RT-PCR results for PRRSV and positive results for PRRSV antibodies by ELISA test. Sows and gilts were vaccinated against swine fever virus (SFV) and pseudorabies virus (PRV) using commercial vaccines at four and three weeks before farrowing. After farrowing, they were vaccinated against foot-and-mouth disease virus (FMDV) and porcine parvovirus (PPV) at two and three weeks, respectively. In addition, they were vaccinated with modified live PRRSV vaccine every three months. They had never been vaccinated against PEDV. The suckling piglets were divided into four groups of five each: A (normal 3-day-old piglets), B (3-day-old piglets infected with PEDV), C (normal 10-day-old piglets), and D (10-day-old piglets infected with PEDV). The infected piglets were selected on the basis of clinical signs as well as positive results of Antigen Rapid TGE/PED Ag Test kit (BioNote, Inc.). The clinical signs were graded from 1 to 3, with '1' indicating no diarrhea, good body condition, and no dehydration; '2' indicating creamy diarrhea, mild emaciation, and mild dehydration; and '3' indicating watery diarrhea, emaciation, and severe dehydration. Piglets in

grade 1 were considered normal, while those in grade 3 were considered PEDV-infected.

All piglets were euthanized and a necropsy was performed for the observation of morphological changes. The tissues of the small intestine (duodenum, jejunum, and ileum) were collected for histopathological study. All experiments on animals were carried out in compliance with the Ethical Guidelines of Laboratory Animals in Research, National Research Council, and were approved by the Animal Care and Use Committee, Kasetsart University (ID# ACKU 02458)

Reverse transcription polymerase chain reaction (RT-PCR)

Only samples from jejunum were used for the detection of PEDV. The tissues were homogenized with 0.1 M phosphate-buffered saline (PBS) at pH 7.2 and were centrifuged for 10 min at 4,800 × g. Supernatants were collected and stored at –70°C till use.

For the extraction of RNA, 500 µL TRIzol and 300 µL chloroform were mixed with 200 µL supernatant. The resulting suspension was centrifuged at 1,200 × g for 10 min, and the supernatants were precipitated with isopropanol at –20°C for 20 min. The RNA pellets were washed twice with 1 mL of 75% ethanol and were centrifuged at 1,200 × g for 10 min. The resulting pellet was resuspended in 30 µL diethylpyrocarbonate (DEPC). RT-PCR was conducted using a previously described method (Kim *et al*., 2001).

Study of histopathology and immunohistochemistry

The duodenum, jejunum, and ileum of the small intestine were collected, fixed in 10% neutral buffered formalin, and embedded in paraffin. The samples of 5 µm thickness were cut and stained with hematoxylin and eosin (H&E). The sections were dehydrated in serially graded ethanol and xylene.

After the tissue slides were deparaffinized with xylene and rehydrated through graded alcohols, endogenous peroxidase was quenched using 3% hydrogen peroxide in PBS for immunohistochemistry. The slides were then subjected to the conventional antigen retrieval (AR) step in a microwave oven. Tissue slides were incubated at room temperature with 10% normal goat serum for 30 min. The slides were then stained with 1:200 (v/v) rabbit antiserum against PEDV at 4°C, and were left overnight. All slides were washed thrice with 0.1% PBS-Tween 20 (PBST). This was followed by incubation with 1:1000 (v/v) horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies (KPL, Gaithersburg, MD, USA) for 30 min at room temperature. After washing, the tissues were stained with 3,3'-Diaminobenzidine (DAB) substrate for peroxidase reaction and were counterstained with hematoxylin. The images were observed under Bio-Imaging Navigator FSX100 (Olympus)

Morphometric analysis

For the morphometric analysis, all tissue slides containing the sections of the small intestine

were taken from each of the PEDV-infected and normal piglets at different ages. Only transverse sections stained using H&E were used for morphometric analysis. Villous height (VH) and crypt depth (CD) were estimated by measuring 10 villi and crypts throughout the sections. The average VH and CD from each slide were used for statistical comparison of the two groups and calculation of VH:CD ratios.

Statistical analysis

VH, CD, and VH:CD were expressed as mean ± standard error of the mean (mean ± SEM). Statistical comparisons of these parameters between the groups were performed by Student's t-test or one-way ANOVA using the software GraphPad Prism. The *p*-values < 0.05 indicated a significant difference between the analyzed groups.

Results

Clinical observations and gross lesions

Ten PEDV-infected piglets (groups B and D) had yellowish watery diarrhea and vomiting, leading to dehydration and loss of body weight. All the PEDV-infected piglets showed thin and transparent intestinal walls and intestinal dilatation with an accumulation of yellowish fluid at necropsy (Figures 1B and 1D). The other internal organs appeared normal. Neither the clinical signs nor lesions were observed in normal piglets of both ages.

RT-PCR

The RT-PCR assay was standardized for the detection of PEDV in the jejunum of all experimental piglets. Positive results were observed only in piglets infected with PEDV (Figure 2).

Histopathology

The histological sections revealed shortened villi in the duodenum, jejunum, and ileum of piglets of group B, in comparison with piglets in group A (Figures 3A and 3B). On the other hand, piglets in group D exhibited shortened villi only in the jejunum. In addition, the erosion of epithelial cells at the tip of villi were also observed in piglets of both group B and group D. The epithelial lining of the crypts of Lieberkuhn was normal in all the groups.

Immunohistochemistry

Immunohistochemical staining using polyclonal antibodies specific for PEDV revealed abundant viral antigens in epithelial cells, predominantly on villi. The positive reaction was characterized by the development of brown color in the cytoplasm of the infected cells (Figure 3C). The jejunum of PEDV-infected piglets was observed to give stronger signal intensity, compared to the duodenum and ileum. Positive-stained cells were not observed in epithelial cells lining the crypts of Lieberkuhn.

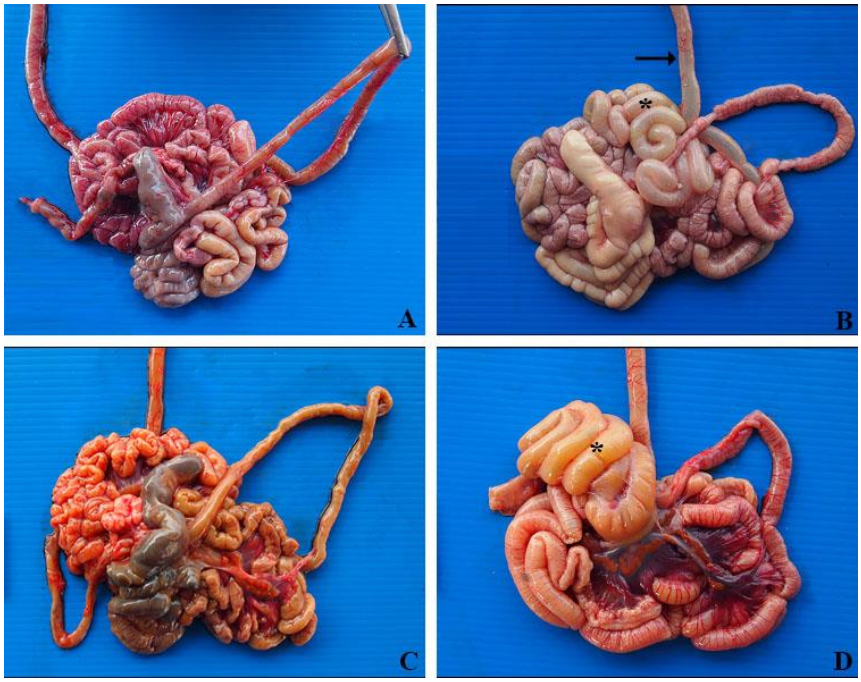

Figure 1 The necropsy results of piglets of different ages; 3-day old and 10-day-old piglets, (A); normal 3-day-old piglet, (B); 3-day-old PEDV-infected piglet, (C); normal 10-day-old piglet, (D); 10-day-old PEDV-infected piglet. The 3-day-old piglet infected with PEDV showing thin intestinal wall (B, arrow). Intestinal dilatation with fluid accumulation (*) is shown in 3-day old and 10-day-old PEDV-infected piglets (B and D, respectively).

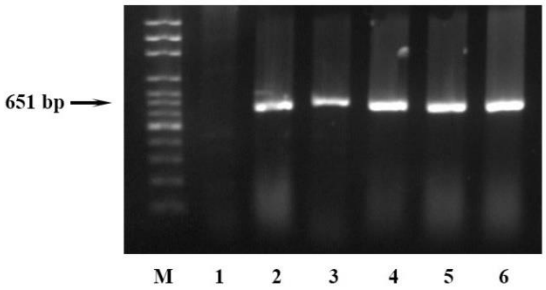

Figure 2 RT-PCR assay, lane M; 100 bp DNA ladder, lane 1; Negative control, lane 2–6; representative PEDV-positive samples (651 bp).

Morphometry

A decrease in the villous height was observed in the duodenum, jejunum, and ileum of piglets in group B and only in the jejunum of the piglets in group D. However, the difference in villous heights between PEDV-infected and normal piglets was not significant ($p > 0.05$). Crypt depth in all intestinal sections was similar across all groups (Figure 4). VH:CD ratios in all parts of the intestine were observed to decrease in piglets of group B. In the case of piglets in group D, the VH:CD ratios in jejunum and ileum were observed to decrease, but the differences were insignificant in comparison with the ratios observed in group C (Figure 5). All results are summarized in Figures 4 and 5, and in Table 1.

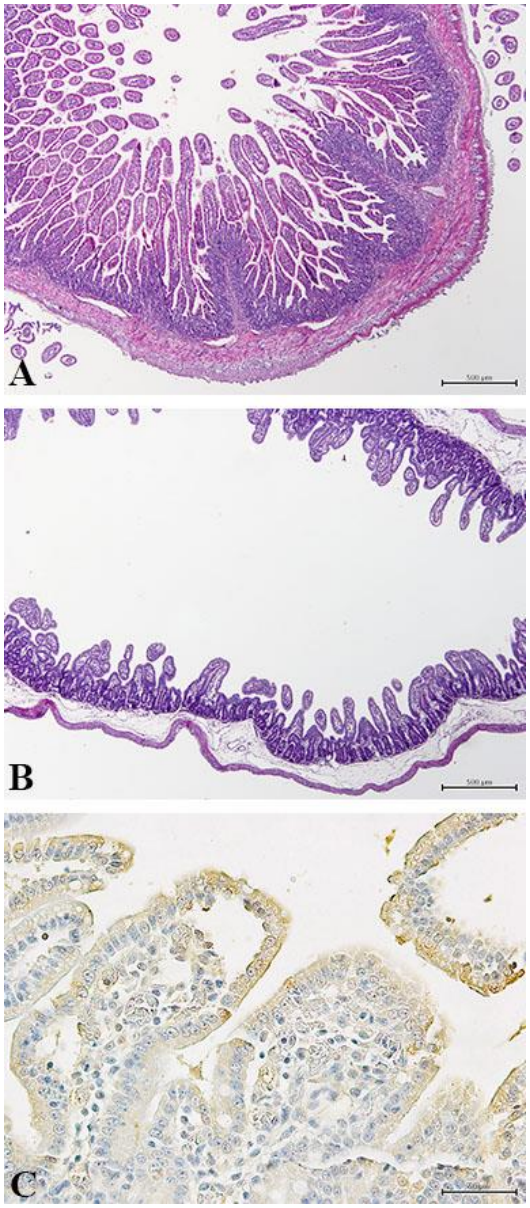

Figure 3 Histopathological appearance of the jejunum of PEDV-infected 3-day-old piglet shows villous atrophy and flattened epithelial lining (B) compared with normal 3-day-old piglet (A). Immunohistochemical staining for PEDV (C), the PEDV antigen (brown color) in the cytoplasm of epithelial cells on villi is shown in 3-day old piglet infected PEDV. Scale bar = 500 µm.

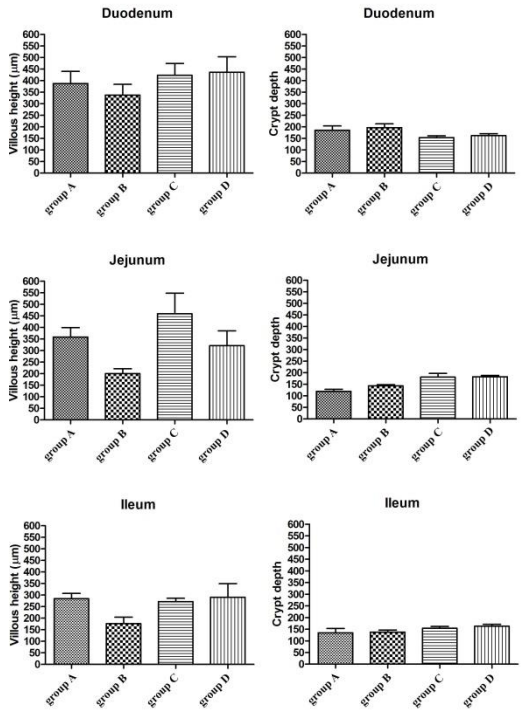

Figure 4 Morphometric analysis in the small intestine demonstrating villous height (µm) and crypt depth (µm) of normal and PEDV-infected piglets at three and ten days of age. Data are expressed as the mean ± SEM for five piglets per group.

Discussion

All the PEDV-infected piglets (3-day and 10-day-old) exhibited yellowish watery diarrhea, vomiting, and dehydration compared to the control group. They also showed positive results (band corresponding to 651 bp) in the RT-PCR assay, which was not observed in normal piglets of both groups. The histopathology indicated shortening and erosion of villi in the duodenum, jejunum, and ileum of piglets in group B. These findings were in line with the reports, which demonstrated PEDV affected villous atrophy in all segments of the small intestine of piglets

inoculated with PEDV, as well as those exposed to natural PEDV (Madson *et al.*, 2016; Stevenson *et al.*, 2013). In the case of piglets in group D, a decrease in the length of villi was observed only in the jejunum. In contrast, the crypt depth did not significantly differ between PEDV-infected piglets and normal piglets at all ages. A decrease in the VH:CD ratios in the small intestine was observed in this study, possibly because of the decrease in villous height. The crypt depth was found to increase in the jejunum of piglets in group B (Figure 4 and Table 1). It is possible that the higher numbers of proliferating crypt cells lead to increased turnover of enterocytes, as the intestinal stem cells located in crypts replace the apoptotic or necrotic enterocytes for maintenance of intestinal epithelium (Jung *et al.*, 2015a).

In the present study, PEDV antigens in the small intestine were detected by immunohistochemistry. Strongly positive results were obtained in the jejunum of PEDV-infected piglets, whereas fewer cells in duodenum and ileum were affected. This corroborates with the finding of Kim *et al.* in 1999, which showed the positive immunohistochemical staining of jejunal villi to be stronger than ileal villi. Jejunal and ileal villi are the main target for replication of PEDV, while viral replication at other sites may depend on the tissue specificity of PEDV strains (Kim and Chae, 2000). In addition, several reports indicated that the cells infected with PEDV were also found in colon and cecum (Jung *et al.*, 2014; Sueyoshi *et al.*, 1995). In contrast, other studies reported that histologic lesions did not appear in the large intestine (Jung *et al.*, 2015b). However, this study focused on changes in the small intestine and not the large intestine.

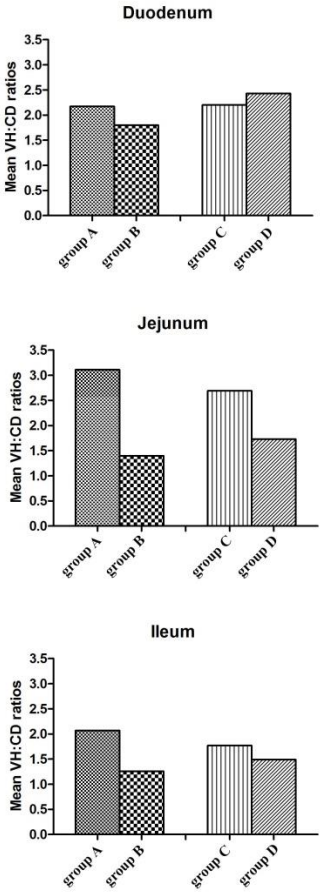

Figure 5 Morphometric analysis in the small intestine demonstrating villous height (μm) to crypt depth (μm) ratio of normal and PEDV-infected piglets at three and ten days of age (five piglets per group).

Table 1 Villous height (VH), crypt depth (CD) and ratio of VH to CD in small intestine of normal and PEDV-infected piglets at three and ten days of age.

	Group			
	A (n=5)	B (n=5)	C (n=5)	D (n=5)
Duodenum				
Villous height (μm)	387.6 ± 22.91	336.1 ± 18.63	423.3 ± 18.83	436.6 ± 23.86
Crypt depth (μm)	185.8 ± 8.99	196.8 ± 6.99	210.6 ± 10.08	171.4 ± 6.26
Villous: Crypt ratio	2.17:1	1.80:1	2.20:1	2.43:1
Jejunum				
Villous height (μm)	358.9 ± 24.00	200.5 ± 9.03	459 ± 31.51	321.1 ± 22.47
Crypt depth (μm)	119.7 ± 4.70	143.4 ± 4.58	181.5 ± 7.24	182.7 ± 4.95
Villous: Crypt ratio	3.11:1	1.41:1	2.69:1	1.73:1
Ileum				
Villous height (μm)	284.4 ±17.99	176.2 ±10.95	271.6 ±13.73	248.7 ±19.36
Crypt depth (μm)	134.2 ±7.88	137.2 ±4.31	153.8 ±5.41	162.5 ±4.50
Villous: Crypt ratio	2.07:1	1.26:1	1.77:1	1.49:1

Taken together, these findings indicated that the younger piglets infected with PEDV exhibited more severe clinical signs, lesions, and villous damage than the older piglets. These findings could be used for assessment and prediction of PEDV infection in pig farms. However, the appearance of histopathological lesions in piglets could vary with the infectious dose and/or age-dependent susceptibility to natural PEDV infection. Hence, colostrum management is very important, as the immunity of piglets is derived from mother's milk. In absence of adequate colostrum management, the sows having an epitheliochorial placenta cannot transfer maternal immunity during gestation. Moreover, the management of farrowing house should be done in an appropriate environment. The protective herd immune status in sows is important for appropriate control of the disease.

Conflict of interest

There is no conflict of interest.

Acknowledgement

This work was supported financially by Kasetsart University Research and Development Institute (KURDI). We would like to thank Assoc. Prof. Dr. Worawit Watchawalku who kindly provided specific polyclonal antibody for PEDV.

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