Detection of Porcine Epidemic Diarrhea Virus by Immunohistochemistry with Recombinant Antibody Produced in Phages

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ABSTRACT. Several diagnostic methods including immunofluorescence, enzyme-linked immunosorbent assay, polymerase chain reaction and immunohistochemistry have been developed for the detection of porcine epidemic diarrhea virus (PEDV). An immunohistochemical method using a new recombinant antibody produced by a phage antibody system (PAS16) kit was investigated and compared with that using a monoclonal antibody for PEDV detection in PEDV-infected piglets. In both the immunohistochemical methods, PEDV antigens were detected in the cytoplasm of villous enterocytes and in the macrophages infiltrated in the lamina propria at 18 to 110 hr post inoculation. The positive signals with the recombinant PAS16 antibody were similar to those with the monoclonal antibody. This result suggests that the recombinant PAS16 antibody can be applicable for the rapid immunohistochemical diagnosis of PEDV infection.—KEY WORDS: monoclonal antibody, porcine epidemic diarrhea virus (PEDV), recombinant PAS16 antibody.

The outbreak of porcine epidemic disease (PED), which is characterized by watery diarrhea and emaciation in swine of all ages, causes a great economical loss in the pig industry [3, 5, 10, 17]. The causative agent of PED is coronavirus which is pleomorphic, spheroidal, and enveloped with size of 60–200 nm in diameter. The projections of envelope are 12–24 nm in length and they disperse evenly over surfaces [8, 16]. Porcine epidemic diarrhea virus (PEDV) is antigenically distinct from transmissible gastroenteritis (TGE) virus [12].

PED is transmissible mainly by the fecal-oral route after the introduction of infected animals or contaminated materials [3]. Several diagnostic methods including a direct immunofluorescence using cryostat sections of gut tissue and an enzyme-linked immunosorbent assay (ELISA) of fecal materials are the most commonly used for the detection of PEDV at present [2, 9]. Additional methods have been recently developed with potential for use in PEDV diagnostics, in particular, an immunohistochemical method using formalin-fixed and paraffin-embedded tissue and a polymerase chain reaction (PCR) for feces [9, 13, 18].

In this study, an immunohistochemistry method using a new recombinant antibody produced in a phage antibody system (PAS16) was evaluated and compared with that using a monoclonal antibody. PEDV antigens in the jejunum of colostrum-deprived piglets infected experimentally with Korean isolates were evidently detected by the immunohistochemistry using the PAS16 antibody.

Feces of pigs showing watery diarrhea were collected and the pathogen was diagnosed as PEDV by PCR [13]. The fecal samples of piglets were emulsified by 30% with phosphate buffered saline (PBS). The emulsions were centrifuged at 13,400 x g for 15 min. The supernatant was treated with antibiotics for 2 hr at 37°C and then passed through a 0.2-µm filter. Ten colostrum-deprived piglets were reared in three positive plastic isolators at 30°C. All piglets were fed with sterilized milk replaces. Seven piglets were inoculated per os with 5 ml of fecal samples of PEDV and one piglet was inoculated with KPEDV-9 (Korean strain) as a positive control. Two piglets were inoculated with PBS as negative controls. The animals were observed at regular intervals for signs of diarrhea. Piglets showing diarrhea were immediately killed by cutting the carotid blood vessels. Tissues were fixed in 10% phosphate-buffered formalin, dehydrated, embedded in paraffin wax, sectioned with 4 µm in thickness, and stained with hematoxylin and eosin (H&E).

Soluble recombinant PAS16 antibody was produced in mice immunized with cell-adapted KPEDV-9 strain according to the procedures of commercially available recombinant PAS16 kits (Pharmacia Biotech). Detection of specific epitope of PEDV by Western blotting was conducted for feces of piglets infected with a wild type of PEDV and cell-adapted KPEDV-9 as well. The wild type of PEDV was directly prepared from the small intestine of a neonatal pig. The small intestine was ground in PBS (pH 7.4). A 10% suspension of the ground intestine was then filtered using a 0.2-µm membrane filter (Acrodisc, Gelman) and concentrated by ultracentrifugation at 100,000 x g for 1.5 hr. The pellet was resuspended in 1/200 volume with TEC buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 150 mM NaCl). The cell culture supernatant from KPEDV-9 infected Vero cells was also concentrated as described above. Samples were subjected to SDS-PAGE, followed by
transfer to nitrocellulose membrane. The nitrocellulose membrane was blocked with TBS (150 mM NaCl, 50 mM Tris-HCl, pH 7.4) containing 10% skim milk for 60 min at room temperature. The membrane was then incubated for 1 hr with a 1:1000 dilution of anti-E tag antibody (Pharmacia Biotech). The membrane was washed with TBS containing 0.05% Tween 20 and then incubated with alkaline phosphatase conjugated goat anti-mouse serum for 1 hr at room temperature. After washing, the membrane was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyolphosphate. One SRA clone, designated PAS 16, reacted to 190–180 kDa spike protein of PEDV in Western blotting (Fig. 1a) and used throughout this study.

Monoclonal antibodies were produced by the following procedure. Four weeks old BALB/c mice were immunized with 200 µg of cell-adapted KPEDV-9 with Freund complete adjuvant. At regular three week-intervals, mice received twice the same antigen with Freund incomplete adjuvant and without adjuvant before the fusion of P3A6 cells with splenocytes. Monoclonal hybridomas were produced according to standard methods using P3 × 63 Ag 865311. Western blotting of monoclonal antibodies against PEDV was conducted in same procedure as described above except incubation with a 1:1000 dilution of monoclonal ascites, followed by incubation with horseradish peroxidase conjugated goat anti-mouse serum for 1 hr at room temperature. After washing, the membrane was developed with 4-chloro-1-maphtho for hybridoma ascites. The monoclonal antibody, designated 9–2–2, reacted with 32–30 kDa nucleocapsid protein of KPEDV-9 in Western blotting analysis (Fig. 1b).

For immunohistochemistry, paraffin-embedded jejenum tissue sections were deparaffinized with xylene and rehydrated through graded alcohols. Endogenous peroxidase was quenched with absolute methanol containing 3% hydrogen peroxide for 15 min. All slides were incubated with PBS containing 10% normal rabbit serum, 5% skim milk and 1% bovine serum albumin mixture for 60 min at 37°C to saturate non-specific protein-binding sites. The slides were incubated with PAS16 antibody (1:2) overnight at 4°C in a humid chamber. Following three washes with PBS, the sections were incubated with 1:1000 diluted anti-E Tag antibody (Pharmacia Biotech) at 37°C for 60 min and with biotinylated horse anti-mouse IgG (diluted as manufacturer’s instruction) at 37°C for 30 min. After thrice washing, the sections were incubated with avidin-biotin complex solution prepared according to the manufacturer’s instruction (Novocastra Laboratories, UK) for 30 min. After washing with PBS, the final reaction product was produced by immersing the sections in a solution of DAB kit (Vector Laboratories, U.S.A.) in distilled water for 3 min. The sections were lightly counterstained with Harris’ hematoxylin, dehydrated through graded concentrations of ethanol and xylene, and then mounted. For negative control, PAS16 antibody adsorbed to virus culture supernatants were used as primary antibody. For immunohistochemical evaluation with mouse anti-PEDV monoclonal antibody, jejunal sections were processed as described above. Negative control sections were obtained from colostrum-deprived piglets unexposed to PEDV and 10% normal mouse serum as a primary antibody was used.

Experimental infection of piglets with wild-type PEDV

![Fig. 1. Western blotting analysis for (a) PAS 16 antibody and (b) monoclonal antibody. M: molecular marker, Lane 1: PEDV before cell adaptation, Lane 2: Cell adapted KEDV-9.](image-url)
resulted clinically in watery diarrhea from 18 hr postinoculation (PI) to 110 hr PI. In the early stage of the disease, stools were profuse and watery. At 62 hr PI and 71 hr PI, piglets were severely dehydrated and they could not stand up. Foamy vomiting was observed in the PEDV-infected piglets. Two piglets died at 100 hr PI and 110 hr PI, respectively. At necropsy, gross lesions were limited to the small intestine, especially the distal portion of jejunum. Piglets, which died at the later stages of the disease, showed various degrees of watery feces. The small intestine of the piglets was gaseous and the wall was thin. The large intestine was filled with watery feces and mucoid.

General histopathological lesions of experimental animals were briefly summarized in Table 1. The villi of the small intestine of two control piglets were long and narrow (Fig. 2a). Mean ratio of villus to crypt in two mock-infected piglets was 7:1. At 18 hr PI, a few eosinophilic materials and some lymphocytes were infiltrated in the lamina propria.
of the jejunum, and the length of the villi and the depth of the crypts were close to those of control piglets. The villi of jejunum of piglets were atrophied in the late stage of infection between 37 hr PI and 62 hr PI (Fig. 2b). The length of intestinal villi was markedly shortened in the distal portion of jejunum. Exfoliation and vacuolation of enterocytes were seen on the tips of villi or spread over the entire villi. Small and large vacuoles were seen in these cells, especially in the terminal parts of the jejunum of some piglet (Fig. 2b). At 62 hr PI to 110 hr PI, lesions were constantly similar. In the jejunum and ileum of piglets infected with KPEDV-9 strain, a marked villous shortening was observed.

When PAS16 antibody was used as a primary antibody, PEDV antigens were detected in the enterocytes of the jejunum in the infected piglets (Fig. 3a). The antigens were confined to the cytoplasm, especially villus tip. Most infected cells were not continuously arranged in the epithelial layer and the borders between infected and uninfected cells were distinct. Infected cells were observed

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Fig. 3. Immunohistochemistry for the villi of PEDV-infected piglets at 100 hr post-inoculation using (a) PAS 16 antibody, (b) monoclonal antibody, and (c) PAS 16 antibody absorbed with purified viruses. The villi of the jejunum in PEDV-infected piglets were markedly atrophied. PEDV antigens (arrows) were detected in the cytoplasm of enterocytes with the immunohistochemistry using both PAS 16 antibody and monoclonal antibody. Magnification, ×200.
occasionally in the crypts and in the lamina propria. In the piglet infected with KPEDV-9, viral antigens were also detected in the cytoplasm of jejunal enterocytes. PEDV antigens were not detected in the jejunum of two mock-infected piglets. When monoclonal antibody was used as a primary antibody, the antigens were detected in the enterocytes of the jejunum, crypt cell and macrophages in lamina propria as shown by the method using a new recombinant PAS16 antibody (Fig. 3b). The antigen detection by monoclonal antibody was equally efficient to that by PAS16 antibody. Viral antigens were detected in the jejunal tissues of piglets at 18 hr PI to 110 hr PI. The control staining using PBS or PAS antibody adsorbed to virus culture supernatant, as a primary antibody, was negative (Fig. 3c).

In this study, two of eight infected animals showed the sign of diarrhea at 18 hr PI, although villus atrophy was not seen at the time. At 50 hr PI, all infected animals showed diarrhea. The diarrhea might be due to a functional impairment of the enterocytes, which precede shortening of villi [7]. In a piglet that showed diarrhea at 18 hr PI, villi were not totally naked. This finding was different from that of TGE in which exfoliation occurs severely and most enterocytes are detached [1, 14, 15, 20].

Histopathologically, villous shortening as determined by a villi-crypt ratio was a most pronounced lesion in the infected piglets. Atrophy of villi might be the result of exfoliation of enterocytes [15]. The majority of the epithelial cells on the villi were vacuolated in infected piglets. The vacuoles were fat-positive with the Scharlach stain [4]. The fatty degeneration might be due to an inability of the cell to transport fat to the lymphatics [15]. In this study, the colons of piglets experimentally infected with PEDV showed no obvious epithelial changes and the crypt epithelium was intact thereby preserving a regeneration capacity. The marked elongation and hyperplasia of the crypts might be an answer to villus atrophy as described in TGE [11]. PEDV, like TGE virus, replicates essentially in crypts might be an answer to villus atrophy as described in TGE [11]. PEDV showed no obvious epithelial changes and the crypt epithelium was intact thereby preserving a regeneration capacity. The marked elongation and hyperplasia of the crypts might be an answer to villus atrophy as described in TGE [11].

In a piglet that showed diarrhea at 18 hr PI, the PEDV-infected piglets, viral antigens could be detected as early as 18 hr PI before the onset of diarrhea. It was assumed that diarrhea was induced initially by malabsorption due to functional disorder of absorptive epithelial cells. ELISA, immunohistochemistry, immunofluorescence test, and RT-PCR have been used as diagnostic methods of PEDV infection [9, 13, 19]. Recently, Gussetti et al. [9] compared the immunohistochemical method to other methods for detection of PEDV in experimentally infected pigs. ELISA and PCR showed occasionally some negative results in cases, where other tests indicated the presence of virus. In contrast to the tests using fecal material, the antigen detection methods such as immunohistochemistry and immunofluorescence tests showed a very high sensitivity. They concluded that the immunohistochemical method using formalin-fixed tissue sections and the immunofluorescence method using cryostat sections were equivalent in reliability, but the former was more practical. In this study, we used both PAS16 antibody and monoclonal antibody as the primary antibody for immunohistochemical detection of PEDV. The immunohistochemical method using PAS16 antibody in paraffin embedded tissue sections was sensitive and reliable. In addition, the method using the PAS16 antibody were equally efficient as compared to that using the monoclonal antibody for detecting PEDV in the infected piglets. It is concluded that the use of the PAS16 antibody for the immunohistochemical method can be an alternative for the monoclonal antibody as a primary antibody.

REFERENCES