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Application of a focus formation assay for detection and titration of *porcine epidemic diarrhea virus*

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Abstract

A focus formation assay (FFA) for detection and titration of *porcine epidemic diarrhea virus* (PEDV) in a micro-culture system using Vero cells and PAP staining technique was evaluated. A linear correlation between the virus dilution and virus titer determined by FFA was observed between the range of 10 and 30 foci per well. Comparative analysis between FFA and plaque assay showed no significant difference in estimating the titer of cell adapted PEDV. However, the culture time required for detecting the virus was considerably shorter for FFA. In addition, FFA had higher sensitivity for detecting field isolates of PEDV as well as positive identification of the virus with the antibody specific reaction. A broader range of dilutions and number of replicates may be used for titration. A FFA may be applied as an alternative method for detection and titration of PEDV.

Keywords: *Porcine epidemic diarrhea virus*; Focus formation assay; Plaque assay; Peroxidase–antiperoxidase staining

1. Introduction

Porcine epidemic diarrhea virus (PEDV) is a member of the family Coronaviridae and is closely related with Human coronavirus 229E, Transmissible gastroenteritis virus and Feline infectious peritonitis virus (Bridgen et al., 1993; Yaling et al., 1988). This virus causes porcine epidemic diarrhea, an enteric disease characterized by acute watery diarrhea and dehydration (DeBouck and Pensaert, 1980). The clinical and pathological symptoms are similar to transmissible gastroenteritis, making it difficult to differentiate between the two infections (Pospischil et al., 1981; Pritchard et al., 1999).

Current diagnostic procedures for confirming PEDV infection is based mostly on viral detection by immunohistochemistry, *in situ* hybridization, reverse transcription-polymerase chain reaction (RT-PCR) and dot-blot hybridization (Jung et al., 2003; Jung and Chae, 2005; Kim and Chae, 1999, 2000, 2002). Isolation and *in vitro* culture of PEDV using stable cell lines is difficult, and only a few strains have been cultured successfully in cell lines (Hofmann and Wyler, 1988; Kadoi et al., 2002; Kweon et al., 1999). Among the diagnostic methods used, the RT-PCR method is an easy, rapid, specific, and sensitive test for the detection of PEDV in Vero cells and from fecal samples and homogenized intestinal tissues (Kim and Chae, 2002; Song et al., 2006). However, RT-PCR only confirms the presence of the viral genome, and not necessarily the presence of infectious virions. Hence, it may not provide sufficient information for determining the presence of infectious virions. At present, plaque assay and immunofluorescence antibody tests have been used for measuring virus infectivity of PEDV *in vitro* (Hofmann and Wyler, 1988).

An alternative method that has not been considered yet for PEDV is the focus formation assay (FFA) using the peroxidase–antiperoxidase staining method. Estimating virus titer by FFA has been reported for several types of viruses like *Mumps*, *Japanese encephalitis*, *Dengue*, *Chikungunya*, *Renal syndrome* and *Influenza* and (Okuno et al., 1977, 1985a,b, 1990; Raharjo et al., 1986; Tanishita et al., 1984).

In this study, the use of the focus formation assay for determining the titer of KPEDV-9 and for detection of PEDV in specimens collected in the field was evaluated. The results were compared with an infectivity assay using the plaque assay.

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2. Materials and methods

2.1. Cells and virus

KPEDV-9, a cell-adapted strain of PEDV from the Korean National Veterinary Research and Quarantine Services was cultured in African green monkey kidney cells (Vero, CCL-81) as described previously (Hofmann and Wyler, 1988). Briefly, KPEDV-9 was inoculated onto Vero cells grown to confluence with minimum essential medium (MEM, Gibco BRL) containing 5% fetal bovine serum (FBS, Hyclone) in a 15 cm diameter TC dish (SPL Life Science) then maintained in virus medium (MEM with 10 μg/ml trypsin) at 37 °C for 24 h. Culture medium was collected and centrifuged at 10,000 × *g* for 10 min to remove cell debris while infected cells were harvested by scraping and placed in 1 ml 100 mM NaCl, 10 mM Tris–Cl, 1 mM EDTA (STE buffer, pH 7.4). Progeny virions trapped in intracellular vesicles were released by repeated freezing and thawing and recovered into the supernatant after centrifugation at 10,000 × *g* for 10 min. All virus suspensions were kept at −80 °C prior to use.

2.2. Focus formation assay (FFA)

KPEDV-9 stock virus was examined by focus formation assay (FFA) following the peroxidase–antiperoxidase (PAP) method described by Okuno et al. (1985a, b), with some modifications. Vero cells were grown to confluence with MEM containing 5% FBS in a 96-well TC plate at 37 °C, 5% CO_2 for 24 h. After washing twice with 10 mM phosphate buffered saline (PBS, pH 7.4), twofold serially diluted virus suspension was inoculated in quadruplicate wells and kept at 37 °C, 5% CO_2 for 2 h. Inoculum was removed carefully and the cell monolayer was overlaid with virus medium containing 0.5% methyl cellulose (Sigma–Aldrich) then placed at 37 °C, 5% CO_2 between 8 and 48 h. The overlay medium was removed carefully and cell monolayer was fixed with 5% formaldehyde in PBS for 20 min then washed three times with PBS. The cells were permeated with 1% Nonidet-P 40 (NP-40) in PBS for 20 min, washed three times with PBS then blocked with 4% skim milk (Difco) in PBS. Infected cells were detected by probing with mouse anti-PEDV polyclonal antisera (1:4000) at room temperature for 1 h, followed by biotinylated rabbit anti-mouse IgG (1:1,000, Vector Lab) at room temperature for 1 h, and strepavidin-biotinylated horseradish peroxidase (Vector Lab) for 30 min. Each step was preceded by washing the wells three times with PBS. Finally, the cell monolayer was incubated with 0.5 mg/ml 3,3′-diaminobenzidine tetrahydrochloride dihydrate containing NiCl and H_2O_2 in PBS (DAB solution, Vector Lab) for 10 min at room temperature. The reaction was stopped by removing the DAB solution and rinsing with deionized water. Clusters of infected cells (focus) stained dark-gray were counted under an inverted microscope (Zeiss) and reported as focus forming units (ffu).

2.3. Plaque assay

KPEDV-9 stock virus was titrated by plaque assay as described elsewhere (Vautherot, 1981). Tenfold dilution of the stock virus was inoculated in 35 mm TC dishes (SPL Life Science) containing confluent monolayer of Vero cells. After 2 h, the inoculum was removed and the cell monolayer was overlaid with virus medium containing 1% agarose (SeaKem) and kept at 37 °C, 5% CO_2 for 24 h. The culture was then overlaid with virus medium containing 0.01% neutral red and 1% agarose, and kept at 37 °C, 5% CO_2 for another 24–48 h while monitoring the appearance of plaques. The cell monolayer was fixed with 5% formaldehyde in PBS and stained permanently with 0.01% crystal violet solution (0.01% crystal violet, 2.5% ethanol in PBS).

2.4. FFA and plaque assay of field-collected specimens

Small intestines from five piglets (5–6-weeks-old) exhibiting clinical signs and symptoms of PEDV were collected from local farms in South Korea. Portions of the jejunum and ileum were homogenized in sterile PBS (1.5 ml PBS per 1 g tissue). The homogenized tissues were centrifuged at 6000 × *g* and the supernatant was aliquoted and stored at −80 °C prior to use. FFA and plaque assay were carried out as stated above.

2.5. Statistical analysis

Pearson's correlation coefficient was used to determine the range of ffu count that provided the most accurate estimate virus titer. The paired *t*-test with an $\alpha \leq 0.05$ was used to compare virus titer obtained by FFA and plaque assay.

3. Results

3.1. Immunostaining of cells infected with PEDV

KPEDV-9 infection of Vero cells was stopped at 8, 10 12 or 24 h post inoculation (p.i.) by fixing with 5% formaldehyde then processed for FFA. Clusters of cells, or foci, infected with KPEDV-9 were colored dark gray in contrast with non-infected cells after reaction of the bound immune complex with the DAB solution. At 8 h p.i., the infected cells did not exhibit discernable CPE (Fig. 1A). However, by 10 h p.i., several adjacent cells were infected with PEDV as shown by immunostaining and early signs of cell-to-cell fusion began to manifest (Fig. 1B). By 12 h p.i., syncytium formation was observed among several foci (Fig. 1C). Within 24 h p.i., several syncytia have fused making it difficult to identify individual syncytium (Fig. 1D).

3.2. Correlation of dilution and virus titer in FFA

Various dilutions of KPEDV-9 were used to determine the approximate virus titer by FFA. Based on the previous experiment, virus infection was arrested at 10 h p.i. and immunostained immediately using anti-PEDV polyclonal antisera and DAB. The number of stained clusters of cells, or foci, was used to calculate the titer of in focus forming units per milliliter of suspension (ffu/ml). As shown in Fig. 2, KPEDV-9 suspension