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# RT-PCR-based dot blot hybridization for the detection and differentiation between porcine epidemic diarrhea virus and transmissible gastroenteritis virus in fecal samples using a non-radioactive digoxigenin cDNA probe

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**Abstract**

Multiplex reverse transcription-polymerase chain reaction (RT-PCR)-based dot blot hybridization was developed to increase the sensitivity for the detection and differentiation between porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV) in fecal samples. Fecal samples found positive by RT-PCR-based agarose gel electrophoresis were always found positive by RT-PCR-based dot blot hybridization. In addition, 5 out of 10 fecal samples which were negative for PEDV by RT-PCR-based agarose gel electrophoresis were positive for PEDV by RT-PCR-based dot blot hybridization. This RT-PCR-based dot blot hybridization increased 1000-fold in sensitivity for PEDV and 100-fold for TGEV; weakly positive bands in the agarose gel electrophoresis gave a clear positive result with dot blot hybridization.
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*Keywords:* Dot blot hybridization; Polymerase chain reaction; Porcine epidemic diarrhea virus; Transmissible gastroenteritis virus

## 1. Introduction

Porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV) are members of the family *Coronaviridae*, order Nidovirales (Pensaert and de Bouck, 1978; Cavanagh, 1997). PEDV and TGEV cause acute enteritis in swine of all ages, and it is often fatal for neonatal piglets (Pensaert and de Bouck, 1978; Chae et al., 2000). Clinical signs of PEDV and TGEV infection include anorexia, vomiting, diarrhea, and dehydration (Kim and Chae, 2002a, 2003). Histologically, two viruses cause destruction of villous enterocytes and villous atrophy within the jejunum and ileum (Kim and Chae, 2002a, 2003). PEDV and TGEV are detected by in situ hybridization in the jejunal and ileal villous enterocytes (Kim and Chae, 2000, 2001, 2002a,b, 2003). Replication of two viruses also occurs in the host cell cytoplasm (Kim et al., 1999). Although these two viral diseases can be difficult to distinguish clinically and histopathologically, PEDV is unable to grow in porcine cell cultures permissive to TGEV (Hofmann and Wyler, 1988).

The diagnosis of PEDV and TGEV infection is based on viral detection by immunohistochemistry, in situ hybridization and reverse transcription-polymerase chain reaction (RT-PCR) (Kim and Chae, 2001, 2002b; Jung et al., 2003) since there are inherent difficulties in isolating PEDV in cell culture (Hofmann and Wyler, 1988; Kim and Chae, 1999). Among these diagnostic methods, the RT-PCR was demonstrated to be an easy, rapid, specific, and sensitive test for the detection of PEDV and TGEV from fecal samples (Kim et al., 2000; Kim and Chae, 2001, 2002b; Jung et al., 2003). Therefore, RT-PCR provides a practical method for the rapid diagnosis of clinical samples.

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In RT-PCR, the viral target nucleic acid (RNA) is first converted to complementary double stranded DNA (cDNA) in the reverse transcription step, followed by PCR amplification of the target cDNA sequences to a level detectable by gel electrophoresis or dot blot hybridization. However, agarose gel electrophoresis with ethidium bromide staining does not appear to be sensitive enough for visualization of small amounts of PCR products (Sander and Penno, 1999; Lingappa et al., 2002). However, dot blot hybridization used for the detection of the PCR-amplified DNA showed a 10-fold higher sensitivity than agarose gel electrophoresis with ethidium bromide staining (Luneberg et al., 1993; Anderson et al., 1994; Lingappa et al., 2002). The objective of this study was to develop multiplex RT-PCR-based dot blot hybridization with a non-radioactive digoxigenin cDNA probe for the differentiation between PEDV and TGEV directly from fecal samples and compared it with multiplex RT-PCR-based agarose gel electrophoresis with ethidium bromide staining.

## 2. Materials and methods

### 2.1. Experimental design

Forty colostrum-deprived piglets, 3 days of age, were divided randomly into four groups of 10 pigs each. Each group was randomly assigned to one of four treatments. The four treatments included intraoral inoculation with cell culture containing one of two viruses, PEDV strain SNUVR971496 (Kim and Chae, 1999) or TGEV strain SNUVR980473 (Kim and Chae, 2002a), with both PEDV and TGEV, or with uninfected cell culture medium.

Pigs in all the three groups were inoculated intraorally with 3 ml of tissue culture fluid containing $10^4$ tissue culture infective doses 50% ($TCID_{50}$)/ml of PEDV, 3 ml of tissue culture fluid containing $10^4$ $TCID_{50}$/ml of TGEV, or 3 ml of tissue culture fluid mixture containing $10^4$ $TCID_{50}$/ml of PEDV and TGEV (1:1). Ten control pigs were exposed in the same manner to uninfected cell culture supernatant. Fecal samples were collected of 10 pigs from each group at 12, 24, 36, 48, and 60 h postinoculation (hpi). The methods were previously approved by the Seoul National University, Institutional Animal Care and Use Committee.

### 2.2. Clinical fecal samples

A total of 20 fecal samples from each of 10 pigs naturally infected with either PEDV or TGEV were also used in the present investigation. Cases were selected on the basis of clinical signs, histopathological lesions and in situ hybridization. Additional 10 fecal samples that were negative for PEDV and TGEV by multiplex RT-PCR-based agarose gel electrophoresis were also selected to compare with the multiplex RT-PCR-based dot blot hybridization. The 10 herds from these 10 fecal samples were highly suspected of enteric viral infection because pigs with diarrhea showed typical clinical signs such as vomiting, high mortality and no response to antibiotic treatment.

### 2.3. RNA extraction

For extraction of RNA from fecal samples, suspensions of fecal samples were prepared by vortexing 2 g of intestines or feces with 2 ml of phosphate-buffered saline (0.1 M, pH 7.2). The suspensions were clarified at $9000 \times g$ for 10 min at 4 °C. Five hundred microlitres of supernatants was mixed with 500 μl of Trizol LS Reagent (Gibco BRL, Grand Island, NY, USA) for 10 min and then, 300 μl of chloroform was added for an additional 10 min at 4 °C. After centrifugation ($4000 \times g$, 15 min), isopropanol precipitation was carried out overnight at 4 °C. The precipitated RNA was pelleted by centrifugation for 10 min at $14000 \times g$, washed twice with 70% ethanol, and then dissolved in 30 μl of diethylpyrocarbonate (Sigma Chemical Company, St. Louis, MO, USA)-treated water.

### 2.4. Primers

The RT-PCR were carried out using primers as described previously (Kim et al., 2000), which amplified a 412-base pair (bp) region from membrane protein gene of PEDV and a 612-bp region from nucleocapsid protein gene of TGEV, respectively. For PEDV, the forward and reverse primers were 5′-GGGCGCCTGTATAGAGTTTA-3′ (nucleotides 927–946) and 5′-AGACCACCAAGAATGTGTCC-3′ (nucleotides 1319–1338). For TGEV, the forward and reverse primers were 5′-GATGGCGACCAGATAGAAGT-3′(nucleotides 1226–1245) and 5′-GCAATAGGGTTGCTTGTACC-3′ (nucleotides 1818–1837).

### 2.5. Reverse transcription-polymerase chain reaction

Twenty-five picomoles of oligo(dT) was added to 1 μg of extracted RNA in 7 μl of diethylpyrocarbonate (Sigma Chemical Company)-treated water, denatured at 75 °C for 10 min, and cooled on ice. The amplification was carried out in a 50-μl reaction mixture containing 1.25 mM $MgCl_2$, 1× PCR buffer, 0.2 mM of each dNTP, 1.00 μM of each primer, and 2.5 U of *Taq* DNA polymerase. Reaction was run in a thermocycler and required the same conditions: 40 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s and extension at 72 °C for 30 s. The PCR was ended with a final extension step at 72 °C for 10 min. The RT-PCR reactions were performed in triplicate. Control cDNA from reference PEDV (SNUVR971496) and TGEV (SNUVR980473) strains was included in each reaction.

### 2.6. Agarose gel electrophoresis for the detection of amplified cDNA

The amplified RT-PCR products were visualized by standard gel electrophoresis of 10 μl of the final reac-

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