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A multiplex RT-PCR assay for rapid and differential diagnosis of four porcine diarrhea associated viruses in field samples from pig farms in East China from 2010 to 2012

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A B S T R A C T

Since October 2010, clinical outbreaks of diarrhea in suckling piglets have reemerged in pig-producing areas of China, causing an acute increase in the morbidity and mortality in young piglets. Four viruses, porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine group A rotaviruses (GAR), and porcine circovirus 2 (PCV2), are the major causative agents of enteric disease in piglets. A novel multiplex reverse transcription-polymerase chain reaction (mRT-PCR) was developed for simultaneous detection of the four viruses in field samples from piglets. A mixture of four previously published pairs of primers were used for amplification of viral gene, yielding four different amplicons with sizes of 481 bp for PCV2, 651 bp for PEDV, 859 bp for TGEV, and 309 bp for GAR, respectively. The sensitivity of the mRT-PCR using plasmids containing the specific viral target fragments was 2.17×10^3, 2.1×10^3, 1.74×10^4 and 1.26×10^4 copies for the four viruses, respectively. A total of 378 field samples were collected from suckling piglets with diarrhea in East China from October 2010 to December 2012, and detected by mRT-PCR. The PEDV-positive rates of the three years were 69.2%, 62.8% and 54.9%, respectively, suggesting that PEDV was a major pathogen in these diarrheal outbreaks. Taken together, all data indicated that this mRT-PCR assay was a simple, rapid, sensitive, and cost-effective detection method for clinical diagnosis of mixed infections of porcine diarrhea associated viruses.

1. Introduction

Acute infectious diarrhea is a major cause of high morbidity and mortality in piglets. Porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), and Group A rotaviruses (GAR) are recognized as the principal viruses causing diarrhea and malabsorption in piglets (Rodák et al., 2005; Pensaert and Yeo, 2006; Saif and Sestak, 2006; Collins et al., 2010). PCV2 has been shown to associate with a number of different syndromes and diseases such as postweaning multisystemic wasting syndrome, porcine dermatitis and nephropathy syndrome, porcine respiratory disease complex, reproductive failure and, possibly, exudative epidermitis (Chae, 2004, 2012). However, PCV2-associated enteritis (diarrhea and granulomatous enteritis) could be a distinct clinical manifestation of PCV2, and the clinical course of PEDV disease has been shown to be markedly affected by transplacental infection of PCV2 (Kim et al., 2004; Jung et al., 2006a,b).

Since October 2010, an outbreak of diarrhea in piglets reemerged in China, resulting in heavy economic losses to the pig industry and the increase in pork prices in China (Pan et al., 2012; Sun et al., 2012). Cases were characterized by watery diarrhea, dehydration and vomiting in suckling piglets. The morbidity and mortality of affected pigs on farms ranged from 90% to 100% and from 70% to 100%, respectively (Chen et al., 2010; Ge et al., 2013). We found that PEDV was a major diarrheal pathogen in these outbreaks, either alone or in conjunction with TGEV, GAR or PCV2. It was very difficult to make a diagnosis based solely on clinical symptoms of severe diarrhea. Therefore, it was important to develop an effective and rapid assay for the detection of mixed infections in pigs.

Multiplex polymerase chain reaction (mRT-PCR) is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction (Chamberlain et al., 1988). Since its first description in 1988, this method has been used to simultaneously identify and differentiate multiple viruses in a single sample on the basis of

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amplicon size, reducing time, labor and cost associated with traditional methods (Henegariu et al., 1997; Agüero et al., 2004; Huang et al., 2004). In several previous studies, a few PCR assays have been used to simultaneously identify and differentiate porcine infectious diarrhea viruses in a single sample, such as a nested RT-PCR method for detection of GAR (Elschner et al., 2002), a duplex RT-PCR method for detection of PEDV and TGEV (Kim et al., 2001), and a multiplex RT-PCR method for detection of PEDV, TGEV and GAR (Song et al., 2006). However, to our knowledge, there is no report on the use of mRT-PCR to simultaneously detect mixed infections with PEDV, TGEV, GAR and PCV2 in both the clinical and the research laboratories, even though these four viruses can co-infect piglets and cause diarrhea. In this study, mRT-PCR was developed and evaluated for simultaneous detection of these four viruses in pigs. This assay was a useful method for rapidly differentiating diarrhea associated viruses in field samples from pig farms in East China, 2010–2012.

2. Materials and methods

2.1. Viruses and cells

PEDV (CV777 Strain), TGEV (China Strain) and GAR (NX Strain) were obtained from the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences. PCV2 (SH Strain) was kindly provided by Jiangsu Nannong Hi-tech Co., Ltd. (Wuxi, China); PRRSV (JXA1-R vaccine strain), PRV (Bartha-K61 vaccine strain), CSFV (C Strain), PPV (NJ-1 Strain), JEV (NJ2008 Strain), *E. coli*, *Salmonella*, *Haemophilus parasuis*, PCV 1-free PK-15 cells, Vero cells, and MA-104 cells were held by our laboratory. PEDV were propagated in Vero cells, TGEV and PCV2 were propagated in PCV 1-free PK-15 cells, GAR were propagated in MA-104 cells.

2.2. Field samples

A total of 378 field samples (from pig farms) consisting of feces and intestinal contents were submitted to our laboratory from 4 provinces in East China between October 2010 and December 2012 (Table 1). All the samples were from sucking piglets with watery diarrhea and dehydration, which could not be cured with any antibiotic.

2.3. Extraction of RNA and DNA

Frozen feces and fresh intestinal contents were homogenized for 15–30 s with a homogenizer. Viral genomic DNA and RNA were simultaneously extracted from homogenized tissues and from lysates of infected cell cultures with MiniBEST Viral RNA/DNA Extraction Kit (Takara) according to the manufacturer's protocol.

2.4. Primers

Four previously published pairs of specific primers for detection of PEDV, TGEV, GAR and PCV2 (Table 2) (Ellis et al., 1999; Kim et al., 2001; Elschner et al., 2002) were used in the present study and obtained from a commercial source (Invitrogen).

2.5. Reverse transcription

The reverse transcription (RT) reaction was carried out using PrimeScript® RT Master Mix Kit (Takara) according to the manufacturer's protocol. This reaction was performed in a 10 μL PCR master mixture consisting of 2 μL 5× PrimeScript® RT Master Mix, 8 μL of mixture RNA/DNA and RNase free H_2O. The RT reaction was carried out at 37 °C for 15 min and inactivated at 85 °C for 5 s. The products were stored at −20 °C for single PCR and multiplex PCR.

2.6. Single PCR

Single PCR for PCV2, PEDV, TGEV, or GAR was carried out in a 50 μL mixture containing 5 μL of 10× PCR buffer (500 mM KCl, 100 mM Tris–HCl, pH 8.3), 4 μL of 200 μM dNTP (2.5 mmol/L), 3 μL of $MgCl_2$ (25 mmol/L), 1 μL of each primer (20 pmol), 0.5 μL of Takara Taq™ DNA Polymerase (5 U/μL), 5 μL of DNA or cDNA template and 30.5 μL of distilled water. In a negative control reaction, DEPC-treated water was used as a template. The amplifications were performed in a Takara PCR Thermo Cycler Dice (TP 600) amplifier using the following steps: one cycle at 94 °C for 5 min; followed by 30 cycles of denaturation at 94 °C for 30 s; annealing at 51 °C (TGEV), 51.5 °C (GAR), 53.4 °C (PCV2), 56 °C (PCV2) for 1 min, and amplification at 72 °C for 1 min; and a final extension step of 7 min at 72 °C. PCR products were analyzed by electrophoresing 5 μL aliquots through 2% agarose gel in 1× TAE buffer. Each specific viral target fragment was cloned into the plasmid pMD19-T vector (Takara) and then sequenced (Invitrogen).

2.7. Optimization of the multiplex conditions

The multiplex PCR reactions were optimized by varying a single parameter while other parameters were maintained as described above. The primer for each target from 5 to 20 pmol, $MgCl_2$ from 1.5 to 3.5 mM; dNTP from 100 to 400 μM, and the Takara Taq™ DNA Polymerase from 0.5 to 5 U. The annealing temperature (from 50 to 70 °C) and number of cycles (from 25 to 40) were also optimized experimentally. PCR products were analyzed by electrophoresing 5 μL aliquots through 2% agarose gel in 1× TAE buffer.

2.8. Sensitivity of the single PCR and multiplex PCR assays

To compare the sensitivity of mRT-PCR versus single RT-PCR, 10-fold serial dilutions of specific viral target fragments previously constructed were performed simultaneously using these two procedures. The following formula was used to calculate the number of gene copies per μL in each dilution: $\text{copies}/\mu L = (6.02 \times 10^{23}) \times (\text{Plasmid concentration } (ng/\mu L) \times 10^{-9})/(\text{DNA length(bp)} \times 660)$ (Yue et al., 2009).

2.9. Specificity of mRT-PCR assay

To verify the specificity of mRT-PCR assay, *E. coli*, *Salmonella*, *H. parasuis*, PPV, PRRSV, PRV, CSFV, JEV, PEDV, TGEV, GAR and PCV2 were tested using above primers. The size-specific PCR products (651, 859, 309 and 481 bp) obtained were analyzed manually, and the sequence similarity was checked against sequences

Table 1
Clinical samples used in this study.

Year	No.	Characteristic	Specimens	East area
2010	78	Sucking piglets	Feces, intestinal contents	Jiangsu, Anhui, Zhejiang, Shanghai
2011	156	Sucking piglets	Feces, intestinal contents	Jiangsu, Anhui, Zhejiang, Shanghai
2012	144	Sucking piglets	Feces, intestinal contents	Jiangsu, Anhui, Zhejiang, Shanghai