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A real-time TaqMan® RT-PCR assay with an internal amplification control for rapid detection of transmissible gastroenteritis virus in swine fecal samples

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A B S T R A C T

A TaqMan probe-based real-time RT-PCR assay was developed for simultaneous detection of RNA of transmissible gastroenteritis virus (TGEV) in pig fecal samples and RNA of enhanced green fluorescent protein (EGFP) added exogenously as an internal amplification control. The TGEV primers and probe were designed to be specific to a portion of the S gene sequence conserved in all TGEV isolates, but absent in the closely related porcine respiratory coronaviruses. The optimized TaqMan assay detected a minimum of 2.8 copies of in vitro transcribed RNA of the target S gene and RNA extracted from 1 $TCID_{50}$/ml of TGEV. Using 113 clinical samples received at our diagnostic laboratory over a 4-year period, the performance of the assay was tested and compared with that of a previously described nested RT-PCR assay. All the fecal samples which tested positive for TGEV by the nested RT-PCR assay also tested positive by the TaqMan assay. However, approximately 9% of the samples that tested negative by the nested RT-PCR assay tested positive by the TaqMan assay. These results indicate that the developed TaqMan assay is a highly sensitive diagnostic test for rapid detection of TGEV in pig fecal samples.

1. Introduction

Transmissible gastroenteritis (TGE) is an acute enteric viral disease of pigs of all ages (Saif and Wesley, 1999). The disease is caused by TGE virus (TGEV), an enveloped virus with a large, single positive-strand RNA genome that belongs to *Coronaviridae* family. TGEV replicates in intestinal enterocytes and shed in the feces of infected pigs. The infection is transmitted by fecal–oral route. Clinical signs of the disease include watery diarrhea, vomiting, dehydration and high mortality in unweaned piglets (Saif and Wesley, 1999). TGEV infections can cause severe economic losses to swine industry. Because of the highly contagious nature of TGE, availability of rapid diagnostic methods that are highly specific and sensitive for detection of TGEV in fecal samples is very useful for timely implementation of disease management practices.

Natural mutants of TGEV with deletions in spike (S) gene, known as porcine respiratory coronavirus (PRCV), show tropism towards respiratory tissue and cause mild or subclinical respiratory infections (Rasschaert et al., 1990; Kim et al., 2000b). Some pigs infected with PRCV can shed the virus in their feces (Saif and Wesley, 1999; Kim et al., 2000a; Costantini et al., 2004). Therefore, any diagnostic assay for TGE should be able to differentiate it from PRCV. The conventional diagnostic methods used for TGE include, cell culture isolation of the virus, identification of coronavirus in feces by electron microscopy, fluorescent antibody test on cryosections of intestines, antigen-capture enzyme-linked immunosorbent assay (ELISA) for detection of TGEV in feces, and detection of serum antibodies to TGEV by virus neutralization assay or ELISA (Dulac et al., 1977; van Nieuwstadt et al., 1988a,b; Sirinarumitr et al., 1997; Saif and Wesley, 1999; Carman et al., 2002). These methods are not suitable for the rapid detection of clinical TGE because of either their low specificity and sensitivity or the prolonged time required to perform the assay. A previously developed nested reverse transcriptase-polymerase chain reaction (nRT-PCR) that can differentially detect TGEV and PRCV has been very useful for the diagnostic testing of TGE cases (Kim et al., 2000a). However, this assay is also time-consuming because of the need to perform two PCR amplifications and detect the amplified products by agarose gel electrophoresis. In addition, nested PCR assays are generally more prone to give false positive results because of cross-contamination of the amplified DNA products, especially when processing large number of samples (Aslanzadeh et al., 1996; Belák and Thorén, 2001; Khlif et al., 2009).

Probe-based real-time PCR assays are better suited for rapid, accurate and sensitive detection of pathogens in clinical samples (Espy et al., 2006; Belák, 2007). This technology also makes it easier to carryout high-throughput testing of samples. In this study, a TaqMan probe-based real-time RT-PCR was developed for specific detection of TGEV in pig fecal samples. The assay included

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an internal amplification control to monitor the presence of PCR inhibitors in RNA extracted from pig feces. Performance of the assay was tested with field clinical samples and compared with that of nRT-PCR.

2. Materials and methods

2.1. Primers and probe design

In comparison to TGEV, all PRCV strains have a deletion of 621–681 nucleotides within the amino terminus encoding part of the S gene (Kim et al., 2000b). Multiple alignment of the TGEV and PRCV S gene sequences showed that a 561 nucleotide-long region, corresponding to sequence between nucleotides 105 and 666 of the TGEV S gene open reading frame, is absent from all PRCV strains (Kim et al., 2000b). Nucleotide sequences of S gene of 15 TGEV strains available in the databases at GenBank (China strain TH-98, AF494337; China strain TS, AY335549; Japan strain TO14, AF302263; Korea strain KT2, AF481360; Russia strain PPS, Y15449; Spain strain NEB72-RT, M94099; Taiwan strain TFI, Z35758; The Netherlands isolate, M21950; UK isolate TGEV FS772/70, X53128; UK strain 96-1933, AF104420; USA isolate BW021898B, AF179882; USA isolate CHV-TGE, U26215; USA strain Miller, S51223; USA strain PUR46-MAD, AJ271965; USA strain Purdue-115, X05695) were retrieved and aligned using ClustalW multiple alignment algorithm (MegAlign program, LaserGene sequence analysis software, DNASTAR, Inc., Madison, WI) to identify the conserved sequences within the region that is absent in S gene of PRCV. A set of primers (forward primer: 5′-TCTGCTGAAGGTGCTATTATATGC-3′; reverse primer: 5′-CCACAATTTGCCTCTGAATTAGAAG-3′) and a TaqMan probe (5′-C/TAAGGGCTCACCACCTACTACCACCA-3′) targeting a conserved 145 bp region (corresponding to the region between nucleotides 370 and 515 of the TGEV S gene open reading frame) were designed using Beacon Designer 3 software (PREMIER Biosoft International, Palo Alto, CA). *In silico* analysis was performed to confirm the TGEV-specificity of the primers and probe by BLAST search of the nucleotide sequences present in databases at Genbank. The TGEV TaqMan probe contained the fluorescent dye 6-carboxyfluorescein (FAM) at the 5′-end and a non-fluorescent quencher (Black Hole Quencher 1, BHQ1) at the 3′-end. Previously described primers and probe were used for detection of synthetic RNA of enhanced green fluorescent protein (EGFP) gene (forward primer: 5′-GACCACTACCAGCAGAACAC-3′; reverse primer: 5′-GAACTCCAGCAGGACCATG-3′; probe: 5′-AGCACCCAGTCCGCCCTGAGCA-3′) (Hoffmann et al., 2006). The EGFP TaqMan probe contained hexachlorofluorescein (HEX) at the 5′-end and BHQ1 at the 3′-end. The primers and probes were custom synthesized at a commercial source (Operon Biotechnologies, Inc., Huntsville, AL).

2.2. Viruses and fecal samples

Cell culture grown TGEV (Purdue reference strain) with 50% tissue culture infectious dose ($TCID_{50}$)/ml of 2.4×10^7 was obtained from National Veterinary Services Laboratory, Ames, IA. Field isolates of TGEV, PRCV, bovine coronavirus (BCV) and feline infectious peritonitis virus (FIPV) were obtained from our virology culture collection. Fecal samples collected from 24 healthy 10-week-old pigs of an unrelated experimental study were used as true negative samples in the development of the assay. Some of the fecal samples from pigs with diarrhea submitted over a 4-year period (2003–2006) to our Animal Disease Diagnostic Laboratory for detection of diarrheogenic pathogens (viral and bacterial) were used to evaluate the performance of the TaqMan assay.

2.3. Synthetic RNA

A 874 bp DNA product encompassing the TGEV-specific S gene sequence that was the target for the TaqMan assay was amplified by RT-PCR using previously described F2 and R2 primers (Kim et al., 2000a), cloned in pCR2.1 vector (Invitrogen, Carlsbad, CA), and sequenced to confirm the nucleotide sequences. The insert was excised from pCR2.1 with XbaI and BamHI digestion and cloned into the same sites of pCDNA3.1 (Invitrogen). For an internal control, EGFP gene from pEGFP-N1 (Clontech, Mountain View, CA) was excised with XbaI and BamHI digestion and cloned into the same sites of pCDNA3.1. The pCDNA3.1 plasmids with the inserts were linearized with BamHI and in vitro transcribed from the Sp6 promoter using RNAMaxx High Yield Transcription kit (Stratagene, Cedar Creek, TX). The transcribed RNA was extracted using TRIzol reagent (Invitrogen) and digested with RNase-free DNase to remove any contaminating DNA, and the RNA was purified again using RNeasy column as per the RNeasy kit protocol (Qiagen, Valencia, CA). Quant-iT RiboGreen RNA assay kit (Invitrogen) was used to quantify the purified RNA transcripts. RNA copy numbers were calculated based on the exact length and nucleotide composition of the RNA molecules. Tenfold serial dilution of the transcripts were prepared in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) with 0.5 U/μl of RNasin (Promega, Madison, WI) and stored at −20 °C.

2.4. RNA extraction from feces

After initial evaluation of different RNA extraction methods, a hybrid protocol that uses TRIzol LS reagent (Invitrogen) and RNeasy spin column (Qiagen) was developed to reduce the time required for RNA extraction with no reduction in the RNA yield. A 250 μl of 20% (w/v) fecal suspension in DNase- and RNase-free water was added to a 1.5 ml microcentrifuge tube with 750 μl of TRIzol LS reagent, mixed thoroughly by vortexing for 15 s, and incubated at room temperature for 5 min. To this, 200 μl chloroform was added, vortexed briefly and incubated for 3 min at room temperature. The tube was centrifuged at 12,000 × *g* for 10 min and 600 μl of the aqueous phase was transferred to a clean tube containing 600 μl of 70% ethanol. After mixing by inverting, the entire contents, 600 μl at a time, were passed through a RNeasy spin column following the RNeasy kit protocol (Qiagen). The spin column was washed, once with 700 μl of RW1 buffer and twice with 500 μl of RPE buffer, and the RNA was eluted using 30 μl of DNase- and RNase-free water. The extracted RNA samples were stored at −20 °C until use.

2.5. TaqMan assay

Reagents of the One-Step RT-PCR kits from either Qiagen or Invitrogen were used for the assay. The assays were performed in a 25 μl reaction volume using SmartCycler II (Cepheid, Sunnyvale, CA). The assays were performed in a single-target format for detection of TGEV only and in a double-target (duplex) format for detection of TGEV and simultaneous amplification of the EGFP RNA, the internal amplification control. The assays were optimized for primer, probe and $MgCl_2$ concentrations as well as the thermal cycling parameters. The final optimized duplex assay contained 3 mM $MgCl_2$, 0.2 mM dNTP mix, 13 U of RNasin (Promega), 0.4 μM of TGEV F primer, 1.2 μM of TGEV R primer, 0.3 μM of TGEV probe, 0.4 μM each of EGFP primers, 0.3 μM of EGFP probe and 10 fg of EGFP RNA template. In the single-target format assay for detection of TGEV, the EGFP-specific primers, probe and the synthetic RNA template were omitted. In both assay formats, 5 μl of RNA extracted from samples or serially diluted TGEV RNA or the synthetic transcripts was used as template; DNase- and RNase-free water was used in the no template controls. The amplification parameters were: reverse transcription at 50 °C for 30 min followed