J Vet Diagn Invest 18:278–281 (2006)

# Multiplex reverse transcription-PCR for rapid differential detection of porcine epidemic diarrhea virus, transmissible gastroenteritis virus, and porcine group A rotavirus

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**Abstract.** A novel multiplex reverse transcription polymerase chain reaction (multiplex RT-PCR) that can detect porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), and porcine group A rotavirus (GAR) was developed. The 3 viruses (PEDV, TGEV, and porcine GAR) are major agents in viral enteric diseases of piglets. As the clinical signs of these diseases are similar, including watery diarrhea, differential detection is required for etiologic diagnosis. A mixture of 3 pairs of published primers was used for amplification of viral nucleic acids, yielding 3 different amplicons with sizes of 859 bp, 651 bp, and 309 bp for TGEV, PEDV, and porcine GAR, respectively. A total of 157 specimens (78 fecal and 79 intestinal samples) from piglets with acute gastroenteritis were collected in Korea between January 2004 and May 2005. They were tested for the presence of 3 viruses by multiplex RT-PCR. Coinfections with PEDV and porcine GAR were identified in 16 farms (43.2%). PEDV, porcine GAR, and TGEV infection were 26.3%, 13.2%, and 2.7% respectively. The relative sensitivity and specificity of multiplex RT-PCR were evaluated, with results suggesting that this assay is equal in quality to conventional single-agent RT-PCR assays (sensitivity:100%, 92.9%, 100% for TGEV, PEDV, GARs; specificity: 100% for all 3 viruses). This multiplex RT-PCR is a simple assay and may be a potentially useful for rapid, sensitive, and cost-effective etiological diagnostic tool for acute viral gastroenteritis in piglets.

**Key words:** Multiplex reverse transcription-PCR; porcine enteric viruses.

Porcine epidemic diarrhea (PED) caused by PED virus (PEDV) is an infectious and highly contagious viral disease of pigs. This virus is a member of the genus Coronavirus, family Coronaviridae, order Nidovirales and is closely related to the transmissible gastroenteritis virus (TGEV). The 2 viruses induce similar clinical signs and lesions.[1,14,20] Along with TGEV and porcine group A rotavirus (GAR), PEDV is one of the most economically important viral causes of diarrhea in piglets.[18] In Asia (Korea, Japan, and China), mortality in suckling piglets infected with PEDV can be very high (~30%–80%).[22] Like TGEV, PEDV destroys villous enterocytes and causes villous atrophy in the small intestine.[2] However, PEDV and TGEV do not cross-react serologically and are antigenically distinct from each other.[3] Porcine GAR is the major cause of acute diarrhea in young piglets.[17] Subclinical infections with GAR are common, and it is believed that host and environmental factors may be important in the pathogenesis of disease by porcine GAR.[13]

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Etiologic diagnosis of viral gastroenteritis is best done by virus detection.[18] Virus isolation, immunohistochemistry, and electron microscopy are conventional techniques often used for detection of PEDV, TGEV, and GAR.[6,9,13,14,18,20] However, because these techniques are laborious and time-consuming, other techniques such as antigen capture ELISA and reverse transcription-PCR (RT-PCR) are gaining popularity.[9,10,11,13,14,17,18,19,20]

Current routine RT-PCR assays require 3 separate primer sets and 3 separate reactions to differentiate the 3 viruses. In contrast, multiplex RT-PCR makes it possible to amplify multiple target sequences in a single reaction tube by using multiple primer pairs.[9] The advantage of a multiplex RT-PCR for the simultaneous detection and differentiation among PEDV, TGEV, and GAR is that it combines the sensitivity and rapidity of PCR and avoids the need to test clinical specimens separately for each virus. This paper describes the development of a multiplex RT-PCR assay for the simultaneous detection and differentiation of PEDV, TGEV, and GAR in intestinal and fecal samples from pigs.

A total of 157 porcine samples (from 38 farms) consisting of feces or intestinal contents were submitted to the authors' laboratory from 8 provinces in Korea between January 2004 and May 2005. Cases were selected on the basis of clinical signs and lesions after necropsy. These herds were suspected to have enteric viral infection because pigs with diarrhea showed typical clinical signs such as vomiting, high mortality, and no response to antibiotic treatment. The farms each had 300 or more sows. Two to 10 fecal samples or intestinal contents were obtained from each outbreak of diarrhea. All the specimens were from piglets aged 1 to 14

days. When live piglets were submitted, intestinal samples and fecal samples were collected from each piglet at necropsy. Fecal and intestine samples submitted by swine practitioners were shipped in leakproof containers with ice packs. Fecal samples were diluted with phosphate buffered saline (PBS) to obtain 10% suspensions.

Experimental infections were carried out to produce fecal and intestinal samples for testing. Three reference viruses (SM98–1 strain of PEDV, NVRI and 175L strains of TGEV, and the Gottfried strain of porcine rotavirus) were provided[a] and used in this study. The propagation of PEDV and TGEV was carried out as previously described.[10] The viruses were infected in suspension at a multiplicity of about 0.01 50% tissue culture infective dose per cell. Porcine rotavirus was propagated in MA-104 cells[4] infected at a multiplicity of infection of about 0.01 50% tissue culture infective dose per cell. The titer of each virus was calculated following the Reed and Muench method.[16]

Twenty-eight 3-day-old piglets were divided randomly into 3 groups ($n = 8$) and a control ($n = 4$). Each piglet in the 3 groups was inoculated orally with 5 ml of cell culture supernatant fluid containing PEDV, TGEV (strain 175L), or porcine GAR at a titer of $10^{4.0}$ $TCID_{50}$/ml. Two pigs from inoculated groups and 1 from the control group were sacrificed at 12, 24, 48, and 72 hours postinoculation, and intestine and fecal samples were collected.

Viral RNA was extracted from the feces and intestinal contents using TRIzol LS[b] according to the manufacturer's instructions. The extraction of RNA from cell-cultured PEDV, TGEV, rotavirus, and fecal samples or intestinal samples was performed as previously described.[10,23] For reverse transcription, 10 μL of extracted RNA and 1 μL (1 μg/μl) of random primer (hexa-deoxyribonucleotide mixture)[c] were mixed. The mixture was denatured by heating to 95°C and was immediately placed on ice. The remaining reagents, which consisted of 10 μL of 5× first-strand buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DL-Dithiothreitol (DTT), 0.3 mM of each deoxyribonucleotide triphosphate (dNTP), and 100 units of M-MLV reverse transcriptase in a final volume of 50 μL, were added. The mixture was incubated at 37°C for 60 minutes, and the reaction was stopped by heating to 95°C for 2–3 minutes. The cDNA was either stored at −20°C or amplified immediately.

Three previously published pairs of specific primers for detection of PEDV, TGEV, and porcine GAR were used in the present study.[5,10] The primer pairs used were P1 (TTCTGAGTCACGAACAGCCA, 1466–1485) and P2 (CATATGCAGCCTGCTCTGAA, 2097–2116) for the S gene of PEDV, T1 (GTGGTTTTGGTYRTAAATGC, 16–35) and T2 (CACTAACCAACGTGGARCTA, 855–874) for the S gene of TGEV, and rot3 (AAAGATGCTAGGGACAAAATTG, 57–78) and rot5 (TTCAGATTGTGGAGCTATTCCA, 344–365) for the segment 6 region of group A rotavirus. In multiplex RT-PCR, 2 μL of cDNA was mixed with a reaction mixture containing 2.5 μL of 10× Taq DNA polymerase buffer,[d] 3 mM of $MgCl_2$, 2.0 μL of dNTPs (2.5 mM/μL), 0.5 μL of each primer (10 pmol each), and 1 μL of Taq DNA polymerase.[c] MilliQ water was added to make up a total volume of 25 μL. The amplification was carried out with a commercial amplification system.[e] The RT-PCR was performed at 94°C for 5 minutes, followed by 30 cycles of 94°C 30 seconds, 53°C 60 seconds, 72°C 60 seconds, and a final extension at 72°C for 5 minutes, and then held at 4°C. The RT-PCR products were analyzed by electrophoresis in 1.5% agarose gel containing ethidium bromide. For routine RT-PCR detection of PEDV, TGEV, and GAR, the same 3 sets of specific primers were used in separate tubes. The same volumes and concentration of primers, reagents, and thermal cycler conditions described above for multiplex RT-PCR were used.

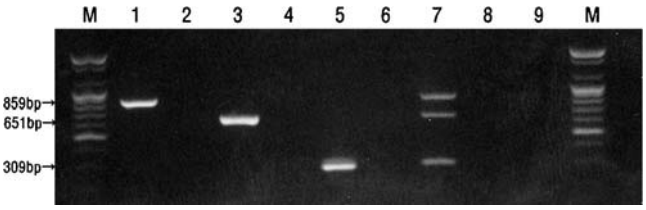


**Figure 1.** Specificity of the multiplex RT-PCR assay with a mixture of 3 primer pairs. Lane M; 100-bp DNA ladder, lane 1; TGEV, NVRI strain, lane 3; PEDV, SM98–1 strain, lane 5; porcine GAR, Gottfried strain, lane 7; TGEV+PEDV+porcine GAR, lanes 2,4,6,8; negative controls from uninfected cell cultures (ST, Vero, MA104, ST+Vero+MA104 cells), lane 9; blank.

The multiplex RT-PCR assay was standardized by testing the positive controls for the three viruses (PEDV, TGEV, and porcine GAR) in 2 ways: 1) the PCR mixture containing 3 primer pairs and 1 template, and 2) 3 primer pairs and all 3 templates. For the specificity test, negative controls consisted of porcine calicivirus,[f] bovine viral diarrhea virus, and porcine circovirus type 2. For these viruses, no amplicon was demonstrated (data not shown). RT-PCR products (Fig. 1: lanes 1, 3, 5, and 7) were sequenced and identified as corresponding viruses (data not shown). RT-PCR products of PEDV, TGEV, and GARs, from the intestinal and fecal samples at 72 hours postinoculation (4 samples/PEDV, 4 samples/TGEV, 2 samples/GARs), were sequenced to control the presence of potential false positive results.[14] Also, the clinical samples were sequenced among the positive samples (3 samples/PEDV, TGEV, and GARs positive samples). The sequences obtained were compared with all sequences of the GenBank and EMBL using the PubMed NCBI BLAST program. The sequences of the amplicon obtained from orally inoculated pigs were found to be identical to each virus. Sequence analysis of the RT-PCR products from clinical samples showed 96.7%∼99.6% of identity in PEDV, TGEV, and GARs-positive controls. To compare the analytical sensitivity of multiplex RT-PCR versus a routine RT-PCR, 10-fold serial dilutions of cell culture-derived TGEV, PEDV, and porcine GAR were performed in naïve small intestine and tested simultaneously using the 2 procedures. The sizes of amplified products were 859 bp for TGEV, 651 bp for PEDV, and 309 bp for rotavirus, which could be differentiated by agarose gel electrophoresis (Fig. 1). The detection limit of multiplex RT-PCR was $10^{1.0}$, $10^{2.0}$, and $10^{1.0}$ $TCID_{50}$/ml for TGEV, PEDV, and GAR, respectively. However, the minimum concentration de-

**Table 1.** Number of positive samples (out of 157) detected for each of the 3 viruses by routine RT-PCR and multiplex RT-PCR.

| Laboratory method | Target viruses | | | | | |
|---|---|---|---|---|---|---|
| | TGEV | | PEDV | | Porcine GAR | |
| | Intestine | Feces | Intestine | Feces | Intestine | Feces |
| Routine RT-PCR | 0 | 4 | 44 | 48 | 31 | 57 |
| Multilplex RT-PCR | 0 | 4 | 43 | 47 | 31 | 57 |

**Table 2.** Prevalence of viral enteropathogens alone or in combination in 157 piglets (1–14 days of age) from 38 farms between January 2004 and May 2005.

| Enteropathogens | No. of farms | % | No. of pigs | % |
|---|---|---|---|---|
| TGEV | 1 | 2.7 | 4 | 2.5 |
| PEDV | 10 | 26.3 | 21 | 13.3 |
| Porcine GAR | 5 | 13.2 | 17 | 10.8 |
| PEDV + porcine GAR | 16 | 43.2 | 71 | 45.2 |

tected by conventional RT-PCR was $10^{1.0}$ $TCID_{50}$/ml for all 3 viruses. Taken together, these results indicate that the multiplex RT-PCR is slightly less sensitive than single RT-PCR (Fig 2). Using the multiplex RT-PCR, all these viruses were detectable at a concentration of $10^{2.0}$ $TCID_{50}$/ml. However, the density of the band for PEDV and rotavirus was weaker than that of TGEV.

When 157 field samples (78 feces and 79 intestines) were tested for PEDV, TGEV, and porcine GAR using a routine RT-PCR and the multiplex RT-PCR (Table 1), the results were similar except for 2 samples that were positive for PEDV by routine RT-PCR but negative by multiplex PCR. Coinfection with PEDV and GAR was demonstrated in 71 specimens (45.2%) (Table 2). When intestinal and fecal samples from the 24 experimentally infected and 4 control piglets were tested by multiplex RT-PCR, none of the 4 control piglets was positive for any of the three viruses. In the TGEV-infected piglets, all 8 intestinal and fecal samples were positive for TGEV, and of the 8 positive fecal samples, 7 were also positive by virus isolation. In PEDV-inoculated pigs, 8 intestines and 7 fecal samples were multiplex RT-PCR-positive; of these, 5 and 4 respectively were positive by virus isolation. GAR was also detected in 7 intestines and 6 fecal samples, and among them 6 intestines and 5 feces were also positive by virus isolation.

In a previous study, the authors reported a duplex RT-PCR for the detection of PEDV and TGEV.[10] In the present study, a multiplex RT-PCR was developed for detection of those 2 viruses and GAR. A multiplex RT-PCR assay is a cost-effective diagnostic method because of the reduction in labor and reagent costs. However, in multiplex RT-PCR, pooling different primer pairs in 1 tube can create some difficulties.[15] Therefore, some modification was adopted to increase sensitivity with respect to the previous duplex RT-PCR. Annealing and extension time was adjusted from 30 seconds to 60 seconds to improve the sensitivity of the multiplex assay (data not shown). As a result of these modifications, this multiplex RT-PCR showed high analytical sensitivity on cell-cultured virus: limits of detection of $10^{1.0}$ $TCID_{50}$/ml for TGEV, $10^{2.0}$ $TCID_{50}$/ml for PEDV, $10^{1.0}$ $TCID_{50}$/ml for GAR. In mixed infection, this assay could detect all viruses at the concentration of $10^{2.0}$ $TCID_{50}$/ml. The sensitivity of multiplex RT-PCR compared to routine RT-PCR was the same or 10- to 100-fold lower.[7] In another study for the detection of PEDV and TGEV, RT-PCR–based dot blot hybridization increased the sensitivity by 100-~1,000-fold compared with agarose gel electrophoresis.[8] However, the present multiplex RT-PCR, which differentiates between TGEV, PEDV, and GAR, is more rapid than dot blot hybridization, virus isolation, or a routine RT-PCR. It is also easy to read, because the three amplicons (859 bp for TGEV, 651 bp for PEDV, and 309 bp for porcine GAR) are easily differentiated on agarose gel electrophoresis. Furthermore, the lack of amplification of heterologous viruses demonstrated the high specificity of the multiplex RT-PCR.

In Korea, outbreaks of acute gastroenteritis in piglets tend to be diagnosed as PEDV infection based on postmortem findings of distended thin-walled small in-

**Table 3.** Detection of specific viruses in intestinal and fecal samples from pigs inoculated orally with TGEV, PEDV, or GAR by multiplex RT-PCR (m-RT-PCR) and virus isolation (VI).

| Viruses | Samples | Methods | Results at intervals after inoculation (hours postinoculation) | | | |
|---|---|---|---|---|---|---|
| | | | 12 h | 24 h | 48 h | 72 h |
| TGEV | Intestines | m-RT-PCR | +/+* | +/+ | +/+ | +/+ |
| | | VI | +/+† | +/+ | +/+ | +/+ |
| | Feces | m-RT-PCR | +/+ | +/+ | +/+ | +/+ |
| | | VI | −/+ | +/+ | +/+ | +/+ |
| PEDV | Intestines | m-RT-PCR | +/+ | +/+ | +/+ | +/+ |
| | | VI | −/− | −/+ | +/+ | +/+ |
| | Feces | m-RT-PCR | −/+ | +/+ | +/+ | +/+ |
| | | VI | −/− | −/+ | +/+ | −/+ |
| GAR | Intestines | m-RT-PCR | +/+ | +/+ | +/+ | −/+ |
| | | VI | −/+ | +/+ | +/+ | −/+ |
| | Feces | m-RT-PCR | −/+ | +/+ | +/+ | −/+ |
| | | VI | −/+ | +/+ | +/+ | −/− |

* Piglet 1/ piglet 2 (+: RT-PCR positive, −: RT-PCR negative). † Piglet 1/ piglet 2 (+: cytopathic effect (CPE) positive, −: CPE negative).

testines with yellow and frequently foamy fluid containing flecks of curdled milk. All submitted samples in this study (157 samples from 38 farms) were initially suspected of PEDV infection by swine practitioners and producers. However, the PEDV infection rate was only 69.5%. Furthermore, the concurrent infection with PEDV and porcine GAR was 43.2%, indicating that porcine GAR is another major enteropathogen. The high prevalence of concurrent infection with PEDV and GAR could explain partial protection after PEDV vaccination in some swine farms. These results indicate that prevention of porcine GAR as well as PEDV might be necessary for protection of piglets against viral gastroenteritis; indeed, concurrent infections with multiple enteric viruses can produce synergistic or additive effects leading to more extensive villous atrophy throughout the intestine and more severe and prolonged diarrhea.[21] Diagnosis of the causal agents of viral gastroenteritis is a basic prerequisite both for introduction of immunoprophylactic measures and for evaluation of prevention measures.[4] The use of reliable methods allowing simultaneous detection of both rotavirus and coronavirus infections could contribute to this goal.[17] The RT-PCR described in this report would be a useful tool for this purpose.

*Acknowledgements.* This work was supported by Korea Research Foundation Grants (KRF-2002-070-C00069) and the Brain Korea 21 Project of the Ministry of Education & Human Resources Development, Republic of Korea.

## Sources and manufacturers

a. Division of Virology, National Veterinary Research and Quarantine Service (NVRQS), South Korea.
b. Invitrogen Corp., Carlsbad, CA.
c. Bioneer, Chungwon, Korea.
d. Promega, Madison, WI.
e. Applied Biosystems, Foster City, CA.
f. Dr. Linda J. Saif, Ohio State University, Columbus, OH.

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