J Vet Diagn Invest 12:385–388 (2000)

Development of a reverse transcription–nested polymerase chain reaction assay for differential diagnosis of transmissible gastroenteritis virus and porcine respiratory coronavirus from feces and nasal swabs of infected pigs

Lomi Kim, Kyeong-Ok Chang, Karol Sestak, Anil Parwani, Linda J. Saif

Abstract. Transmissible gastroenteritis virus (TGEV), a coronavirus, replicates in intestinal enterocytes and causes diarrhea in young pigs. Porcine respiratory coronavirus (PRCV), a spike (S) gene natural deletion mutant of TGEV, has a respiratory tissue tropism and causes mild or subclinical respiratory infections. Conventional antigen-based diagnostic tests fail to differentiate TGEV and PRCV, and a blocking ELISA test to serologically differentiate TGEV/PRCV-infected pigs is conducted on convalescent serum retrospectively after disease outbreaks. A reverse transcription (RT)-nested polymerase chain reaction (PCR) with primers targeted to the S gene deletion region to differentiate TGEV/PRCV was developed. The specificity of the RT-nested PCR was confirmed with reference and recent field strains of TGEV/PRCV, and its sensitivity was analyzed by testing nasal and fecal samples collected from pigs at various days postinoculation (DPI) with TGEV or PRCV. Specific PCR products for TGEV/PRCV were detected only with the homologous reference or field coronaviruses and for 10–14 DPI of pigs with TGEV (feces) or PRCV (nasal samples). The RT-nested PCR assay was more sensitive than antigen-based assays on the basis of duration of virus detection in experimentally infected pigs and was directly applicable to nasal as well as fecal specimens from the field.

Transmissible gastroenteritis virus (TGEV) is a member of the Coronaviridae family and is enveloped with a positive-stranded RNA genome.[3,7] Porcine respiratory coronavirus (PRCV) represents a natural deletion mutant of TGEV that appeared in 1983–1984 in Europe and in 1988 in the US.[3] Coronaviruses have 3 major structural proteins: the spike (S), the integral membrane glycoprotein, and the nucleocapsid protein.[3]

TGEV replicates primarily in small intestinal enterocytes, whereas PRCV replicates predominantly in the respiratory tract.[3,7] According to sequence comparisons of PRCV and TGEV, PRCV has a large deletion in the 5′ region of the S gene and minor deletions in genes 3 and 3-1.[3,11] These deletions are thought to influence the viral tissue tropism and virulence. The deletion size in the S gene ranges from 621 to 681 bp depending on the origin of the strain.[11]

Recently, strains of TGEV with reduced enteropathogenicity were reported in the field.[6] A similar suspect TGEV outbreak of reduced virulence (mild diarrhea and intestinal lesions, slow disease spread among pigs) in nursery pigs from a swine herd in the US Midwest was investigated. Diagnosis of TGEV in these pigs was sporadic and inconsistent and presumably complicated by the presence of antibodies to PRCV confirmed by a blocking differential ELISA test on sera from a number of pigs in this herd (L. J. Saif and P. Lewis, unpublished). However, this latter test showed inconsistent results for TGEV/PRCV differentiation with serially collected samples from the same pigs within the herd (inconsistent individual immune status), and some pigs in the herd tested only PRCV positive, whereas others were TGEV positive (inconsistent herd immune status). These new TGEV strains may represent naturally occurring recombinants with reduced virulence between TGEV and PRCV strains, or the presence of PRCV antibodies in these herds may have complicated the diagnosis and modulated the severity of conventional TGEV infections.

TGEV is a major cause of neonatal diarrhea and also causes enzootic diarrhea in older pigs.[7] It costs the swine industry in the US nearly $200 million a year.[7] PRCV causes infected swine to be diagnosed as TGEV positive in conventional serologic tests.[8] Several investigators have described the use of molecular assays to detect and differentiate TGEV/PRCV strains including reverse transcription–polymerase chain reaction (RT-PCR),[5] cDNA probes,[12,13] in situ hybridization,[10] and RT-PCR/restriction fragment length polymorphism.[2] To differentiate TGEV/PRCV with reference virus strains from tissue culture, an RT-PCR assay was developed with primers targeted to the S gene deletion.[5] These investigators used restriction endonuclease analysis to confirm the identity of their RT-PCR products. Use of the RT-nested PCR assay for detection and differentiation of TGEV/PRCV directly from nasal swabs or feces has not been reported. Therefore, the objective of this study was to develop and use RT-nested PCR assays to detect and differentiate TGEV/PRCV directly from fecal and nasal swab specimens from experimentally infected pigs and from field outbreak specimens.

Four field samples were obtained from a midwest swine herd with sporadic diarrhea cases in nursery pigs. The BW 021898B sample consisted of intestinal contents from a nursery pig with mild diarrhea (clinically suspect for transmissible gastroenteritis). Three nasal swab samples (BW126, BW154, and BW155) were obtained from normal TGEV-seronegative sentinel nursery pigs placed in contact with the diarrheic pigs in the same nursery. Swine testicular (ST) cells were used for virus isolation, growth, and cell culture

From the Food Animal Health Research Program, Department of Veterinary Preventive Medicine, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH 44691 (Kim, Chang, Parwani, Saif), and the School of Veterinary Medicine, Tufts University, North Grafton, MA 01536-1895 (Sestak).

Received for publication May 20, 1999.

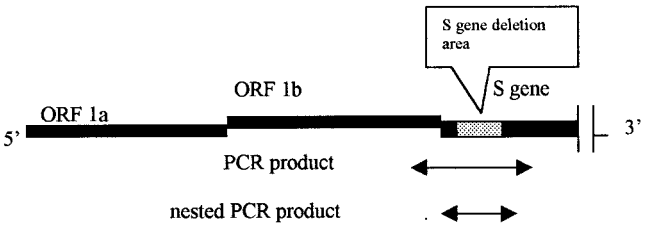
Table 1. Reference and field TGEV and PRCV strains.

Isolate	Isolation date	Location	P no. (PP)*	Source
TGEV reference strains				
M5C Miller	1965	Ohio	2 (2)	E. Bohl, OARDC,† Wooster, OH
M6 Miller	1965	Ohio	6 (2)	L. J. Saif, OARDC, Wooster, OH
P115 Purdue	1952	Indiana	115	E. Bohl, OARDC, Wooster, OH
PRCV reference strains				
ISU-1	1990	Indiana	8 (2)	H. Hill, Iowa State University
ISU-3	1990	North Carolina	6 (2)	H. Hill, Iowa State University
TGEV field strains				
T232	1988	Ohio	6 (0)	L. J. Saif, OARDC, Wooster, OH
T507	1988	Nebraska	3 (0)	R. Moxley, University of Nebraska
T988	1987	South Dakota	2 (0)	D. Benfield, South Dakota State University
U328	1989	Michigan	6	R. Maes, Michigan State University
W888	1990	North Carolina	6	North Carolina Diagnostic Laboratory
Coronavirus isolates				
BW 021898B	1998	Midwest	6 (2)	L. J. Saif, OARDC, Wooster, OH
BW126	1998	Midwest	3	L. J. Saif, OARDC, Wooster, OH
BW154	1998	Midwest	3	L. J. Saif, OARDC, Wooster, OH
BW155	1998	Midwest	3	L. J. Saif, OARDC, Wooster, OH

* Number of times passaged in cell culture (number of times plaque-purified).
† Ohio Agricultural Research and Development Center.

immunofluorescence tests (CCIF).[9] The viruses tested, including the four isolates from the midwest swine herd are summarized in Table 1. The BW 021898B strain was originally isolated from the large intestinal contents of a nursery pig. Strains BW126, BW154, and BW155 were isolated from the nasal swabs of the sentinel pigs in contact with the nursery pigs.

Four conventional 30-day-old nursery pigs (seronegative for TGEV/PRCV by virus serum neutralization tests) were infected with PRCV (ISU-1: 1×10^7 plaque-forming units [PFU]*) oronasally, and nasal swabs were collected at various time intervals from 0 days postinoculation (DPI) until 21 DPI. Another group of 4 11-day-old conventional pigs (seronegative for TGEV/PRCV by virus serum neutralization tests) was infected with virulent cell-passaged TGEV (M5C: titer 1×10^5 PFU), and rectal swabs were collected at various DPI from 0 DPI until 12 DPI.



F1 5' GGGTAAGTTGCTCATTAGAAATAATGG 3'
F2 5' TTGTGGTYTTGGTYGTAATKCC 3' [4]
R1 5' CTTCTTCAAAGCTAGGGACTG 3'
R2 5' GGCTGTTTGGTAACTAATTTRCCA 3' [4]

Figure 1. Schematic representation of the RNA genome of TGEV virus and expected PCR products. First RT-PCR products of S gene with primer F1, R1: TGEV, 1,006 bp; PRCV, 325–385 bp; nested PCR products of S gene with primer F2, R2: TGEV, 874 bp; PRCV, 193–253 bp.

Nasal swabs from experimental and field pigs were diluted in minimum essential medium (MEM) and tested by CCIF by previously described procedures.[9] Briefly, 4-fold serial dilutions of the nasal swab supernatants were inoculated onto ST cell monolayers in 96-well plates and incubated for 18 hours. The cells were fixed with 80% acetone, stained with hyperimmune porcine anti-TGEV serum conjugated to fluorescein isothiocyanate,[a] and analyzed by fluorescent microscopy.

Rectal swabs from TGEV (M5C) experimentally infected pigs and large intestinal contents from the field isolate (BW 021898B) were tested by double antibody sandwich (DAS)-ELISA with monoclonal antibodies to the S protein (25C9, 44C11) and N protein (25H7) for TGEV antigen capture by previously described methods.[4]

To differentiate TGEV from PRCV, RT-PCR primers F1, R1 and the nested PCR primers F2, R2 associated with the open reading frame 1b and the S gene deletion areas for US and European strains of PRCV (Fig. 1) were used. Viral RNA was extracted by previously described procedures.[5] TGEV/PRCV-infected cell culture lysates (Table 1), nasal/fecal swabs (diluted in MEM) from PRCV/TGEV experimentally infected and field pigs, were mixed with with 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, and 2 M sodium acetate, pH 5.0.[1] RNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol and purified with silicon matrix.[b] The RT-PCR reaction contained 10× PCR buffer,[c] 5 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTP, 20 U RNasin,[c] 5 U AMV-reverse transcriptase,[c] 2.5 U Taq DNA polymerase,[c] and 0.5 μl of 50 pmol of each primer in a total volume of 50 μl. Reaction mixtures were incubated at 55 C

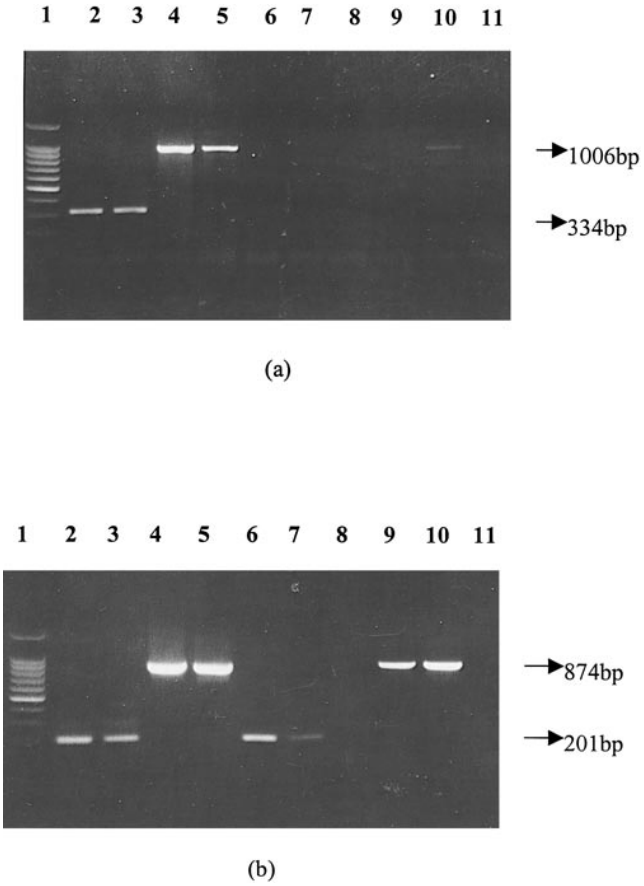


Figure 2. Differentiation of TGEV and PRCV strains by RT-PCR and nested PCR. First RT-PCR (**a**) and nested PCR (**b**) products. Lane 1, 100 bp marker[c]; lane 2, PRCV (ISU-1); lane 3, PRCV (ISU-3); lane 4, TGEV (Miller); lane 5, TGEV (Purdue); lanes 6 and 7, nasal swab (PRCV, ISU-1 inoculated); lane 8, nasal swab (negative control); lanes 9 and 10, feces (TGEV, M5C inoculated); lane 11, feces (negative control).

for 45 minutes, followed by 95 C for 5 minutes. The following parameters were used for amplification: 25 cycles at 94 C for 1 minute, 60 C for 1.5 minutes, 72 C for 2.5 minutes with final extension at 72 C for 10 minutes. Diluted amplicons were used as templates for nested PCR. For nested PCR, the pattern was 1 cycle of 95 C for 5 minutes, 25 cycles of 94 C for 1 minute, 62 C for 1.5 minutes, 72 C for 2.5 minutes, with a final extension at 72 C for 10 minutes. Ten microliters of the PCR products was analyzed on 1.5% agarose gels and then stained with ethidium bromide. Reference strains (Table 1) were used for standardization of the RT-nested PCR assay. The expected RT-PCR product sizes were 1,006 bp for TGEV and 325–385 bp for PRCV strains with F1, R1 primers (Fig. 2). The expected nested PCR product sizes were 874 bp for TGEV and 192–253 bp for PRCV strains with F2, R2 primers (Fig. 2). RT-PCR and nested PCR were conducted with the primers designated in Figure 1. After these primers were proven to differentiate TGEV and PRCV, nasal swab samples collected from a PRCV (ISU-1)-inoculated pig until 21 DPI and feces collected from a TGEV (M5C) inoculated pig until 12 DPI were assayed.

By the CCIF assay, PRCV-positive specimens were detected from nasal swabs (collected from PRCV [ISU-1: 1×10^7 PFU]-inoculated group) until 8 DPI; however, by the RT-nested PCR assay, PRCV-positive specimens were detected until 14 DPI. With DAS-ELISA, TGEV-positive specimens were detected from rectal swabs (collected from TGEV [M5C: 1×10^5 PFU]-inoculated group) until 7 DPI, but with RT-nested PCR, until 12 DPI. These data confirm the direct applicability and sensitivity of RT-nested PCR with nasal and fecal samples for the detection and differentiation of TGEV/PRCV.

The RT-nested PCR assay was used to test the original field samples from the midwest herd. The results showed that the BW 021898B intestinal sample was TGEV and the BW126, BW154, and BW155 nasal swab samples were PRCV (data not shown). These samples were adapted to ST cells for further characterization. The PCR products, including those from the TGEV cell culture isolate (BW 021898B) and the PRCV cell culture isolates (BW126, BW154, BW155), were purified with a commercial kit[b] and sequenced by dideoxynucleotide chain termination procedures with an automated sequencer. The PCR products were confirmed by sequence analysis of the partial S region with primers F1, R1 to confirm the validity of the RT-nested PCR methods. Sequence analysis confirmed the isolate BW 021898B as TGEV and the three isolates (BW126, BW154, BW155) as PRCV with different deletion areas and sizes (L. Kim, K. Chang, P. Lewis, I. Hayes, A. Parwani, and L. J. Saif, unpublished).

The nested PCR assay described here was rapid (1 day) and increased the sensitivity and specificity of detection and differentiation of TGEV/PRCV directly from nasal and fecal samples, including samples from both experimentally and field-exposed pigs. These tests would permit animals to be identified as to their TGEV or PRCV status directly from fecal or nasal specimens or on cell-passaged virus isolates and allow more rapid screening for initiation of control or isolation measures. Such rapid molecular assays are important for an enhanced understanding of the changes in the molecular epidemiology of TGEV/PRCV isolate strains from field outbreaks.

Acknowledgements. We thank Ms. Kathy Gadfield and Peggy Lewis for technical assistance. Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. This study was supported in part by the Pig Improvement Company, Franklin, KY, and HANOR Company, Inc., Spring Green, WI, and the National Pork Producers' Council on behalf of the National Pork Board, Des Moines, IA.

Sources and manufacturers

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b. Geneclean II, Bio101, Vista, CA.
c. Promega, Madison, WI.

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