Field Isolates of Transmissible Gastroenteritis Virus Differ at the Molecular Level from the Miller and Purdue Virulent and Attenuated Strains and from Porcine Respiratory Coronaviruses

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ABSTRACT. The diversity in selected regions of the transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV) genomes was analyzed among known TGEV and PRCV strains and field isolates. The N-terminal half of the spike (S) glycoprotein gene and open reading frames (ORF) 3, 3–1 and 4 were amplified by reverse transcriptase reaction and polymerase chain reaction (RT/PCR), and analyzed using restriction fragment length polymorphism (RFLP) patterns of the amplified DNA. Reference TGEV strains (Miller and Purdue) and a PRCV strain (ISU-1), and TGEV and PRCV field isolates were analyzed. Based on the size of the ORF 3, 3–1 and 4 RT/PCR products, TGEV and PRCV strains could be quickly and easily differentiated into three groups designated TGEV Miller, Purdue types and PRCV. By RFLP analysis of the N-terminal region of the S glycoprotein gene and ORFs 3, 3–1 and 4, TGEV and PRCV strains were differentiated into five groups using the restriction enzyme *Sau*3AI. Sequence analysis of a PCR product in the ORFs 3, 3–1 and 4 from virulent and attenuated Miller strains demonstrated additional differences in that region which have been correlated with a change in virulence of TGEV isolates. — KEY WORDS: coronavirus, PRCV, RT/PCR-RFLP analysis, TGEV.

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Transmissible gastroenteritis virus (TGEV) is the etiological agent of transmissible gastroenteritis (TEG), a highly contagious enteric viral disease of swine [34]. TGEV belongs to the family *Coronaviridae*. It is a pleomorphic enveloped virus with a large, single-stranded, positive RNA genome [40, 42].

At least eight virus-specified mRNAs are synthesized during TGEV replication [19, 39, 46]. These mRNAs consist of a 3' co-terminal nested set. TGEV virions contain three major structural proteins: the spike (S) glycoprotein, the integral membrane (M) glycoprotein, and the nucleocapsid (N) protein, which are the translation products from mRNAs 2, 6, and 7, respectively. The S glycoprotein forms the peplomers on the virion envelop and induces neutralizing antibodies [9, 10, 14, 20]. Unlike other coronaviruses, the S glycoprotein of TGEV, feline infectious peritonitis virus and canine coronavirus is not cleaved into two proteins [11, 18, 19]. The M glycoprotein is mostly embedded in the lipid virion envelope and the N phosphoprotein is associated with the genomic RNA to form the nucleocapsid [21, 26]. An additional small membrane protein (designated SM), translation product of ORF 4, was proposed as a viral membrane-associated minor structural polypeptide [13]. Other mRNAs, 3,5, and 8 are assumed to encode nonstructural proteins.

Porcine respiratory coronavirus (PRCV) has been isolated from pigs in Europe and more recently in the United States [29, 45]. Sequence data revealed that PRCV has a large deletion in the 5' region of the S glycoprotein gene. It has been proposed that the deletion may be related to the differences observed in tissue tropism or pathogenicity between PRCV and TGEV strains [3, 30, 49]. Researchers have reported that PRCV strains replicate almost exclusively in the respiratory tract [15, 48] and usually cause subclinical infections or only mild signs of respiratory disease.

Only one serotype of TGEV exists and the antigenicity of TGEV isolates appeared to be highly conserved [22, 31]. Recently, however, antigenic, genomic, and pathogenic diversity among TGEV strains has been described by using monoclonal antibodies and various molecular genetic techniques [17, 33, 36, 41, 44, 49, 51]. Although nucleotide sequence data has revealed the genomic diversity among TGEV isolates, this data has not been extensively correlated with antigenic variability among these viruses.

In this study, we used the polymerase chain reaction and restriction fragment length polymorphism (PCR/RFLP) analysis to further identify the genomic diversity among TGEV and PRCV isolates. Previous studies in our laboratory indicated that the 5' region of the S gene contained genetic diversity among TGEV and PRCV strains [1]. Using RFLP, TGEV and PRCV strains could be differentiated into several distinct groups. Also, a deletion in the ORF 3–1 of a high passage Miller strain of TGEV (M60) was observed.

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MATERIALS AND METHODS

Viruses and cells: The TGEV Miller strains (virulent M5C and M6, and attenuated M60), TGEV Purdue strains (attenuated P115 and virulent W184), TGEV Ambico vaccine strain (CC1861, received from Dr. Mark Walter, Ambico Inc., Dallas Center, Iowa), and PRCV strain (ISU-1) were used in this study as reference viruses. Additional TGEV field isolates (S387, T184, T232, T507, T517, T876, T988, U328, Zy, CC717 and PRCV field strains (ISU-3 and DD312) were analyzed in this study. The origin and passage history of TGEV and PRCV isolates were described elsewhere [41]. The enteropathogenicity of 5 of the TGEV field isolates (S387, T232, T876, U328 and Zy) was confirmed by passage in gnotobiotic pigs [41]. Strain CC717 was newly isolated from a fecal sample of a diarrheic pig diagnosed with TGE and identified as TGEV using PCR. Strain DD312 was isolated recently from the respiratory tract of a pig in the United Stated (Saif, Weilnau and Gadfield, unpublished) and identified as PRCV by PCR and serological methods in our laboratory. TGEV and PRCV strains were propagated *in vitro* on swine testicular (ST) cells that were grown in Dulbecco's modified Eagle medium (GIBCO BRL, Gaithersburg, MD) [27].

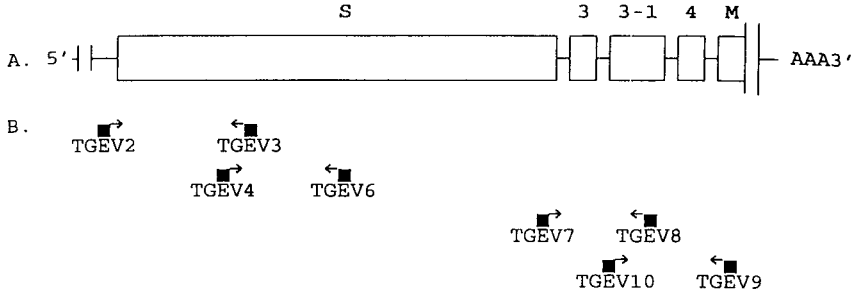
RNA extraction: Virus-infected ST cells showing cytopathic effects were frozen, thawed and the cell debris pelleted using centrifugation. The clarified cell culture supernatant was collected and used for preparation of viral RNA. RNA was extracted as described by Kwon *et al.* [23]. Briefly, sodium dodecyl sulfate (final concentration 2% wt/vol) and proteinase K (final concentration 250 μg/m*l*) were added to clarified cell culture supernatant, incubated for 5 min at 55°C, extracted with acid phenol (pH 4.0) and chloroform/isoamyl alcohol (49:1), and further purified using the RNaid kit (BIO 101, La Jolla, CA). Finally, the RNA was resuspended in diethyl-pyrocarbonate (DEP) treated water and stored at −70°C until used in the reverse transcriptase (RT) reaction.

Oligonucleotide primers: The nucleotide sequences of the oligonucleotide primers were selected with reference to the published TGEV sequences [31, 45, 46]. The primers were chosen to amplify the amino terminal half of the S gene, and ORF 3, 3–1 and 4. The sequences and locations of the primers used in this study are shown in Fig. 1.

Reverse transcriptase and polymerase chain reaction: The RT reaction contained 2 μ*l* of 10 X PCR buffer (500 mM KCl, 200 mM Tris, pH 8.4, 0.5 mg/m*l* nuclease-free bovine serum albumin), 2 μ*l* of 10 mM each dNTP, 250 *n*g antisense primer, 40 units RNasin (Promega, Madison, WI), 1.5 μ*l* of 60 mM $MgCl_2$, and 3–5 μ*l* of the RNA solution described above. A 20 μ*l* total reaction volume was obtained by adding sterile DEP-treated water. After heating at 65°C for 10 min, 200 units of Moloney murine leukemia virus RT (GIBCO BRL, Gaithersburg, MD) were added, and the mixture was inoculated for 1.5 hr at 45°C or 51°C. After incubation, the reaction was stopped by heating to 95°C for 5 min.

For the PCR reaction, 8 μ*l* of 10 X PCR buffer, 250 *n*g of the sense primer, 3.5 μ*l* of 60 mM $MgCl_2$, and 2.5 units of Taq DNA polymerase (Promega) were added to the RT reaction mixture. A 100 μ*l* total reaction volume was obtained by adding sterile distilled water.

PCR was performed by 35 cycles of denaturation at 94°C for 1 min, annealing at 45°C or 51°C for 2 min, and polymerization at 74°C for 5 min. The initial denaturation and polymerization steps were at 94°C for 5 min and 74°C



C. Primers
TGEV2 : 5'AAGGAAGGGTAAGTTGCTCA3'
TGEV3 : 5'GGTCCATCAGTTACGCCGAA3'
TGEV4 : 5'GTGCCACAGTGTTTTCATTG3'
TGEV6 : 5'GCAGTGCCACGAGTCCTATC3'
TGEV7 : 5'CGAACCTATTGAAAAAGTGC3'
TGEV8 : 5'ACTACGGGCTCCTGTGAAAA3'
TGEV9 : 5'ATGCAATCACACACGCTAAT3'
TGEV10 : 5'TGATTGGTGGACTTTTTC3'

Fig. 1. Schematic representation of A) the RNA genome of TGEV Miller strain, showing the location of spike glycoprotein gene, open reading frames 3, 3–1, and 4, and the N terminal M glycoprotein gene; B) the location of primers used in PCR; C) the sequence of the primers.

for 6 min, respectively. The final polymerization step was conducted at 74°C for 10 min. PCR products were analyzed on a 1% agarose gel containing ethidium bromide.

Restriction Fragment length polymorphism analysis: Amplified PCR products of the S glycoprotein and ORFs 3, 3–1 and 4 genes were digested with restriction endonucleases (RE) according to the manufacturer's recommendation (Promega; New England Biolabs, Beverly, MA). The REs used in this study were selected with reference to the published sequence of TGEV Miller and Purdue strains [31, 32, 45, 46]. If the nonspecific PCR products interfered with analysis of the restriction profile, the PCR product was purified before digestion. Briefly, the total PCR product was separated by electrophoresis on a 1% agarose gel. The DNA band with the expected size was cut from the gel and purified using the Geneclean kit (BIO 101) according to the manufacturer's recommendation. The RFLP patterns were observed following electrophoresis on a 2% agarose gel.

DNA sequencing: The PCR product from TGEV M60 amplified by the TGEV 7/9 primer pair (Fig. 1) was cut from the agarose gel and purified using the Geneclean kit (BIO 101) according to the manufacturer's recommendation. The purified DNA was ligated into the pCR™ II (Invitrogen Corp., San Diego, CA) cloning vector, and transformed into competent cells ($INV_{\alpha}F$) (Invitrogen Corp.). Cells carrying recombinant plasmid were selected on LB plates containing antibiotic and X-gal. Plasmid DNA was isolated by the alkaline lysis method [35], and further purified using the Geneclean kit (BIO 101) before sequencing. The cloned and purified PCR product was sequenced by the dideoxy chain termination procedure [35, 38] using the sequences version 2.0 kit (USB, Cleveland, OH). Also, the sequence of purified PCR product was confirmed by direct sequence analysis [23]. For sequencing, TGEV 7, 9 and 10 primers were used (Fig. 1). Nucleotide sequence data were analyzed using the computer program DNASIS (Hitachi Software Engineering).

RESULTS

Amplification of the S and ORFs 3, 3–1 and 4 genes: The 5' half of the S genes of reference strains and field isolates of TGEV including PRCV strains were amplified using TGEV 2/3, and 4/6 primer pairs. The size of the PRCV PCR product obtained using 2/3 and 4/6 primer pairs was distinguishable from TGEV strains M6 ad P115 following electrophoresis on 1% agarose gel (Fig. 2, data not shown for TGEV 4/6 primer pair). The size of the PCR product from PRCV strains obtained using the TGEV 2/3 primer pair (N-terminal end of S gene) appeared to be 581 base pairs which probably resulted from the large deletion (681 nucleotides for ISU-1) in the N-terminal end region. However, the PCR product of DD312, which is a recent PRCV field isolate, was slightly larger than the ISU-1 and ISU-3 PRCV strains. Using nucleotide sequence analysis, the deletion region of DD312 was determined to be shorter than ISU-1 and ISU-3 (Kwon *et al.*, unpublished observations). Using the TGEV 2/3 primer pair, PRCV strains could be easily differentiated from TGEV strains.

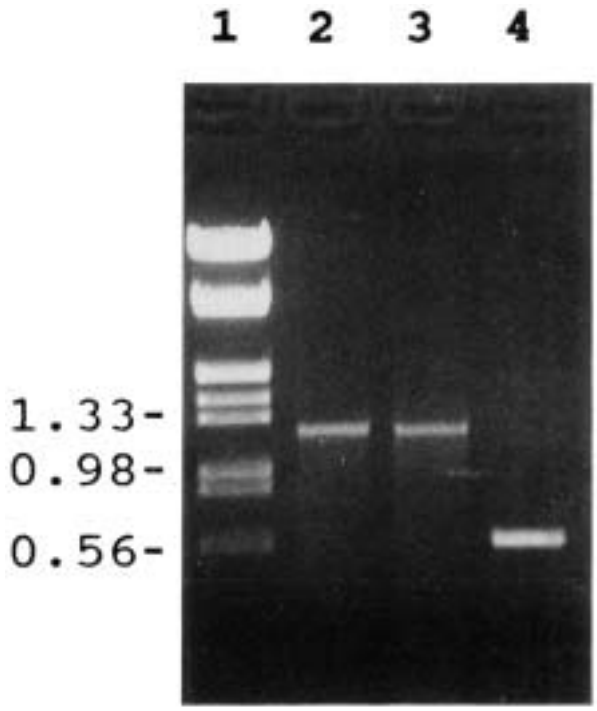


Fig. 2. Agarose gel electrophoresis of PCR products amplified using the TGEV 2/3 primer pair. Lane 1=molecular weight marker made from bacteriophage lambda DNA digested with *Hind*III and *Eco*RI; Lane 2=M6; Lane 3=P115; Lane 4=PRCV (ISU-1). The amplified region was the N-terminal end of the spike gene. Numbers at the left are molecular weight markers in kilobase pairs.

Except for M60, all TGEV and PRCV strains tested were amplified using the TGEV 7/8 primer pair (Fig. 3 and Table 1). The PCR products of PRCV ISU-1 and DD312 were smaller in length than TGEV strains, which made it possible to differentiate from TGEV strains. However, the size of PCR product of ISU-3 was similar to the Miller PCR product. Reference TGEV strain M6 was differentiated from the P115 strain by the length of the PCR products. The size of the PCR product from M6 was smaller than the P115 PCR product. Using this criterion, other TGEV strains could be grouped into M6 and P115 types (Table 1).

To amplify M60, another primer, TGEV 9, was designed (Fig. 1). M60 was amplified using this new primer and TGEV 7 primer (Fig. 1). However, the size of this PCR products was smaller than that observed in other TGEV strains using the TGEV 7/9 primer pair (Fig. 4). This indicated that M60 may have a deletion in ORFs 3, 3–1, and 4.

RFLP analysis: To test the genomic diversity of TGEV and PRCV strains, PCR amplified products from several TGEV and PRCV strains were analyzed using selected REs. Viruses M6, P115 and T517 were tested using each of the enzymes listed in Table 2. The other reference strains and

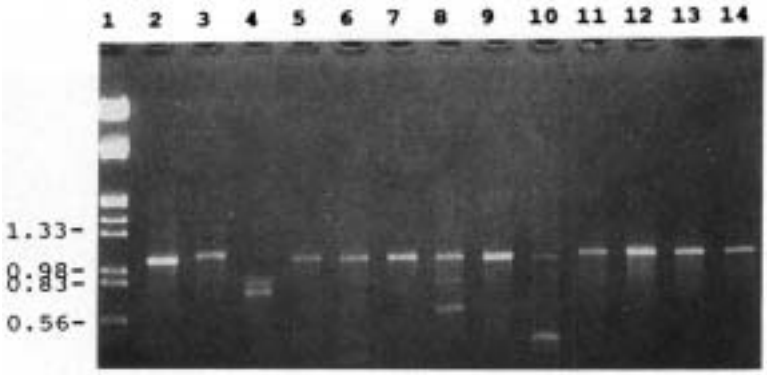


Fig. 3. Agarose gel electrophoresis of PCR products of single-stranded cDNA made from RNA extracted several TGEV strains by the TGEV 7/8 primer pair. Lane 1=molecular weight marker made from bacteriophage lambda DNA digested with *Hind*III and *Eco*RI; lane 2=M6; lane 3=P115; lane 4=PRCV (ISU-1); lane 5=T184; lane 6=T232; lane 7=U328; lane 8=S387; lane 9=M5C; lane 10=CC717; lane 11=W184; lane 12=CC1861; lane 13=T876; lane 14=Zy. Numbers at the left are molecular weight markers in kilobase pairs.

Table 1. Differentiation of TGEV strains based on the size of the ORFs 3 and 3-1 PCR products generated using TGEV 7/8 primer pair

Size (bp)[a)]	Association with strains
1059	M6, M5C, S387, T184, T232, T507, T517, T988, U328, CC717, ISU-3
1102	P115, T876, W184, Zy, CC1861
900[b)]	PRCV (ISU-1, DD312)

a) Sizes of the PCR products were determined by nucleotide sequence analysis (data not shown).
b) This PCR product represents the common band observed for ISU-1 and DD312. Other bands were also observed using TGEV 7/8 primer pair which have been shown to be located in ORF A and B but contain different size deletions (data not shown).

field isolates were not tested with each of the REs, but they were tested with a sufficient number of enzymes to assign each to a specific group. Although there were some exceptions in RFLP analysis, this assay differentiated TGEV and PRCV strains into the following groups: Miller group (M6, M60, M5C, S387, T232, T507, T517, T988 and U328), Purdue group (P115, T876, W184, Zy, and CC1861), and PRCV group (ISU-1, ISU3 and DD312) (Fig. 5, Table 2). The strain T184 amplified by the TGEV 2/3 primer pair was grouped into the Miller type by REs *Bst*EII, *Dra*III, or *Nsp*BII but grouped into the Purdue type by RE *Mnl*I. Strains T507 and T517 were mainly grouped into Miller type although they were grouped into Purdue type by RE *Bst*EII.

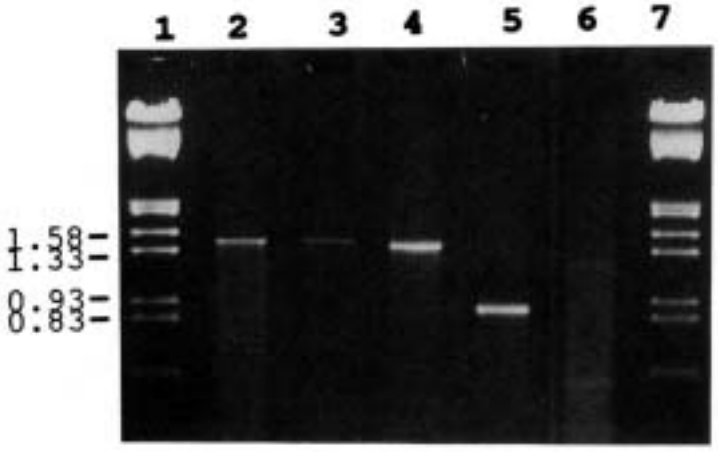


Fig. 4. Agarose gel electrophoresis of PCR products amplified using the 7/9 primer pair. Lane 1 and 7=molecular weight marker made from bacteriophage lambda DNA digested with *Hind*III and *Eco*RI; lane 2=P115; lane 3=W184; lane 4=M6; lane 5=M60; lane 6=PRCV (ISU-1). Numbers at the left are molecular weight markers in kilobase pairs.

Table 2. Restriction enzyme analysis profiles of PCR-amplified TGEV genome from several TGEV strains

Primers	Region	Restric. enzymes	Association with Group	Strains[a)]
TGEV 2/3	S gene	*Mnl*I	1.	M6, M60, M5C, T507, T517, T988, U328
			2.	P115, T184, T876, Zy
		*Bst*EII	1.	M6, M60, M5C, T184
			2.	P115, T507, T517, T876, U328, Zy
		*Dra*III	1.	M6, T184, T232, T507, T517, T876, T988
			2.	P115, Zy
		*Nsp*BII	1.	M6, T184, T232, T517, T988, U328, S387, CC717
			2.	P115, T876, W184, Zy
TGEV 4/6	S gene	*Sau*3AI	1.	M6, M60, PRCV (DD312)
			2.	P115, T876, Zy, CC1861
			3.	S387, T232, T507, T517, T988, U328, CC717
			4.	T184, W184
			5.	PRCV (ISU-1, ISU-3)
		*Ssp*I	1.	M6, T232, T507, T517, CC717, PRCV
			2.	P115, T876, W184
		*Dde*I	1.	M6, P115, W184, T517
			2.	S387, T232, T507, CC717
TGEV 7/8	ORF A, B	*Ase*I /*Bbv*I	1.	M6, M5C, T184, T232, T507, T517, T988, S387, U328, CC717
			2.	P115, T876, W184, Zy
		*Acc*I	1.	M6, M5C, T184, T232, T517, S387, CC717
			2.	P115, T876, W184, Zy
		*Sca*I	1.	M6, M5C, T184, T232, T507, T517, T988, S387, U328, CC717, W184
			2.	P115, T876, Zy, CC1861

a) Some strains were partially analyzed.

The PCR products of TGEV S gene amplified by the TGEV 4/6 primer pair were differentiated into 4 groups using the RE *Sau*3AI. A fifth *Sau*3AI group contained two of the PRCV isolates (ISU-1 and ISU-3). Using *Sau*3AI, TGEV strains S387, T232, T507, T517, T988, CC717, T184 and W184 were differentiated from the Miller and Purdue types viruses (Fig. 3, Table 2).

Differentiation of TGEV strains by RFLP analysis of ORFs 3, 3–1, and 4 was similar to differentiation of TGEV strains by PCR product using the TGEV 7/8 primer pair. The exception was TGEV W184 that was grouped into Miller type by RE *Sca*I but Purdue type by other REs (Fig. 3, Table 2).

Sequence analysis of ORFs 3, 3–1, and 4 of M60: PRFs 3, 3–1 and 4 of M60 were sequenced to confirm the deletion implied by the PCR product amplified by the TGEV 7/9 primer pair (Fig. 4). Comparison with M6 sequence showed that M60 had a 531 base deletion in ORF 3–1 (Fig. 6). A mismatch in the final 2nd and 6th bases of the 3' end of TGEV primer 8 to template RNA may explain why M60 was not amplified by the TGEV 7/8 primer pair.

DISCUSSION

The S glycoprotein gene and ORFs 3, 3–1, and 4 of TGEV and PRCV strains, were analyzed to study genomic diversity by PCR/RFLP analysis and sequencing. PCR amplification using specific primers could detect insertions or deletions in amplified DNA by the size of the PCR products. In some cases, two bands were observed for the PCR products (Fig. 3, lane 4). Nucleotide sequence analysis of these bands indicated that they were identical and thus an artifact of electrophoresis.

The N-terminal half of the S gene of several TGEV isolates had almost identical PCR product lengths. This indicates there were no detectable deletions or insertions in the amplified region. Although the P115 is reported to have 6 nucleotides deleted in the region amplified by the TGEV 2/3 primer pair and 4/6 primer pair compared with the Miller strain, these deletions were not detectable using 1% agarose gel electrophoresis [32, 45]. Based on the size of the S gene PCR product, PRCV strains could be differentiated from TGEV strains. It has been shown that PRCV strains have a large deletion in the N-terminus of the S gene. The U.S.A. and European PRCV isolates had 681 nucleotide (227aa) and 672 nucleotide (224aa) deletions, respectively [30, 37, 49]. However, their antigenicity was similar in studies with monoclonal antibodies [41, 50].

PRCV has been differentiated from TGEV by cDNA probes and monoclonal antibodies. Monoclonal antibodies against some epitopes of the S glycoprotein (antigenic site D) [4], which are absent in PRCV, could be used to distinguish between TGEV and PRCV strains [16, 37, 41]. Also, some probes originating from the 5' coding region of the S glycoprotein gene of TGEV only reacted with TGEV isolates [1, 47]. Since the monoclonal antibodies and probes used were specific for TGEV, the presence of PRCV must be confirmed before it can be differentiated from TGEV. In this study, the TGEV 2/3 primer pair could amplify both TGEV and PRCV, and the viruses could be differentiated immediately by comparing the PCR product lengths.

TGEV strains were grouped into Miller and Purdue types by comparing the lengths of the PCR products amplified with the TGEV 7/8 primer pair (Fig. 3 and Table 1). Sequence analysis of Miller and P115 strains revealed that the Miller strains had 2 large deletions between the 3' end of the S gene and ORF 3–1. As a result, the sequence of the Miller strains was 45 nucleotides shorter than the P115 strain [31, 46]. Two PRCV strains (ISU-1 and DD312) were differentiated using the TGEV 7/8 primer pair from Miller and Purdue strains by the length of their PCR products. Therefore, these PCR products could be used as a first step for characterization of TGEV and PRCV isolates.

The PCR in conjunction with RFLP analysis of the amplified product differentiated TGEV strains into several types. RFLP analysis using *Sau*3AI of PCR products

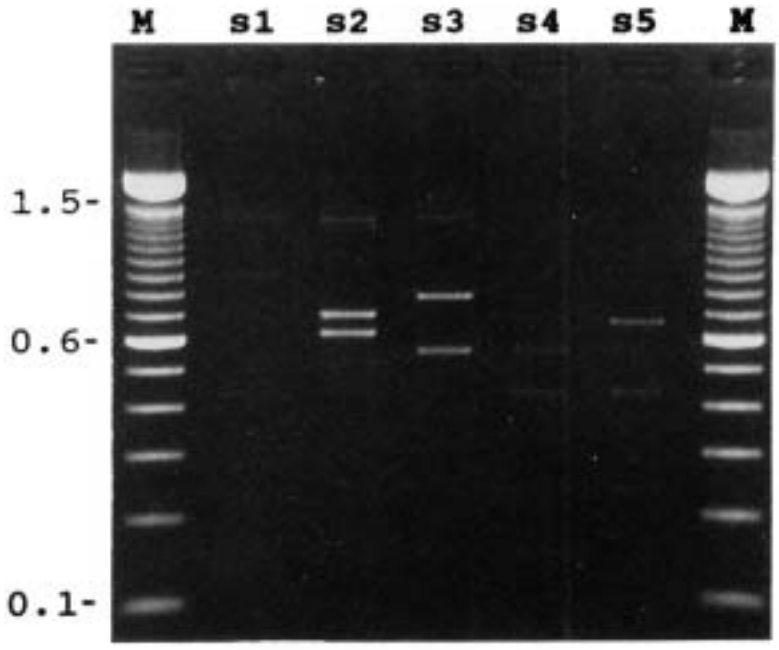


Fig. 5. Restriction fragment length polymorphism patterns of the PCR-amplified spike glycoprotein genes from several TGEV strains digested with restriction enzyme *Sau*3AI. Lane M=Molecular weight marker, 100 bp DNA ladder (GIBCOBRL, Gaithersburg, MD); lane s1=M6; lane s2=P115; lane s3=W184; lane s4=S387; lane s5=PRCV (ISU-1). Numbers at the left are molecular weight markers in kilobase pairs.

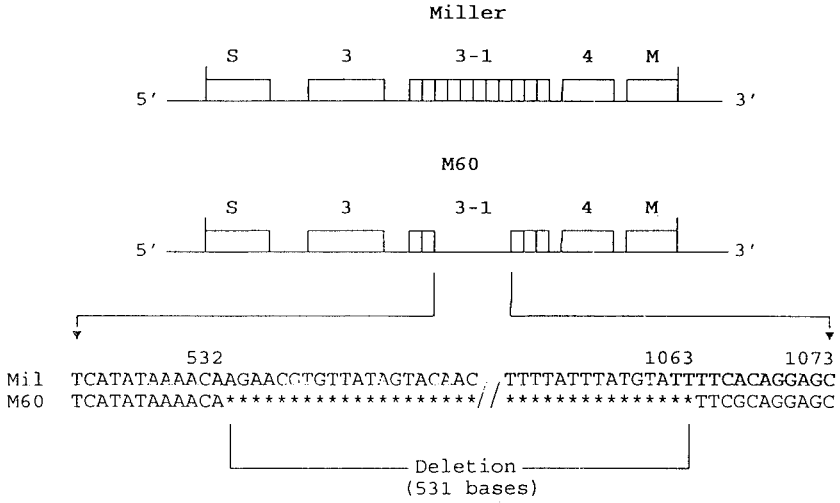


Fig. 6. Schematic representation of the sequence comparison between the spike and membrane genes of TGEV Miller (Mil) [46], and M60 strains. A deletion was found in open reading frame B of M60 TGEV strain. Asterisks indicate the deletion in TGEV M60. The boldface type indicates the sequences of TGEV primer 8.

amplified by the TGEV 4/6 primer pair differentiated TGEV strains into four groups and a fifth contained PRCV strains (ISU-1 and ISU-3) (Table 2). A recent PRCV field isolate (DD312) was placed in the Miller group using *Sau*3AI RFLP analysis but was recognized as PRCV by the deletion in the N-terminus of the S gene using RT/PCR analysis. Simpkins *et al*. [41] produced non-neutralizing monoclonal antibodies which were specific for the S glycoprotein of the TGEV Miller strain. Two of them (75B10 and 8G11) reacted with only TGEV Miller strains except that U328 was recognized by 75B10 (Table 3). Therefore, these Mabs could be used to confirm the results of PCR/RFLP analysis with primer pair 4/6 and RE *Sau*3AI.

The TGEV strains P115, T876, W184, Zy, and CC1861,

Table 3. Comparison of TGEV and PRCV strains by PCR/RFLP analysis using the 4/6 primer pair and *Sau*3AI with reactivity to monoclonal antibodies[a)]

	PCR/RFLP (*Sau*3AI)	MAbs[a)]
Group 1	M6, M60, PRCV (DD312)	75B10, 8G11, 11H8, 44C11, 45A8
Group 2	P115, T876, Zy, CC1861	44C11, 45A8
Group 3	S387, T232, T507, T517 T988, U328, CC717	44C11, 45A8
Group 4	T184, W184	44C11, 45A8
Group 5	PRCV (ISU-1, ISU-3)	11H8

a) Generated to S glycoprotein of the virulent Miller strain of TGEV [41].

which were placed in the Purdue group of viruses using PCR product length, were also grouped into Purdue types by PCR/RFLP analysis. There was some genetic heterogeneity within this group however, since the W184 strain was not placed in this group when the *Sca*I enzyme was used in the analysis of PCR products produced using the 7/8 primer pair. Furthermore, it was not categorized into the Purdue group by *Sau*3AI and formed an independent group with the field T184 strain. TGEV W184 strain is the virulent parental Purdue strain from which the high passaged, attenuated P115 Purdue strain was derived. The results observed may be due to point mutations in these RE recognition sites.

An independent group of recent isolates from TGE field outbreaks (S387, T232, T507, T517, T988, U328 and CC717) was different from the historical Miller and Purdue types. Although only one serotype of TGEV was described [22], it has been suggested that antigenic variation of TGEV may occur in nature [17, 25, 44, 52]. Delmas *et al*. [6] reported that four antigenic sites (A, B, C and D) of TGEV are located on the N-terminal half of the S glycoprotein. Most of the neutralization-mediating determinants were found in the domain A-B, which was highly conserved among TGEV strains [4]. The antigenicity of the S glycoprotein domains A and B is determined by carbohydrate-induced conformational dependent epitopes [5]. Site C was composed of largely conformation-independent epitopes and showed slight variation among TGEV strains [6]. Epitopes involved in the antigenic variation of TGEV strains have been found outside the major neutralization domain and induced non-neutralizing antibodies [25, 41]. However, these MAbs bound to wide range of TGEV strains. In this study, RFLP analysis of PCR products generated using the TGEV 4/6 primer pair showed variability among TGEV isolates. This region included antigenic sites A, B, and C. Although neutralization epitopes in regions A and B are known to be conserved among TGEV isolates, the RE *Sau*3AI could differentiate TGEV strains into several groups. Probably, *Sau*3AI recognized nucleotide sequences that were not critical for the formation of neutralization epitopes or that are located outside nucleotides forming neutralization epitopes. Some correlation was observed between the *Sau*3AI groups defined in the present study and MAb defined groups described previously [41]. Antigenic variation among other coronaviruses, such as infectious bronchitis virus (IBV) [12, 24], bovine coronavirus [7], and feline coronavirus [8] has been observed. Kwon *et al*. [23] used PCR/RFLP analysis to differentiate IBV strains. In that study, IBV serotyping by the PCR/RFLP method correlated with the serotype of IBV strains as determined by the virus neutralization test.

Sequence analysis of the TGEV M60 strain demonstrated a deletion of 531 nucleotides in ORF 3–1 (Fig. 6). Deletions within the ORF 3 and 3–1 have been found in a small plaque (SP) TGEV, TGEV vaccine strain 188-SG, and PRCV [2, 28, 48, 49]. The TGEV 188-SG strain contained a deletion of 250 nucleotides within the ORF-3a/3b (3 and 3–1) but had no deletion in the S gene. It was characterized by acid resistance, small plaque phenotype and decreased growth in cell culture compared to the virulent parent D-52 isolate [2]. An avirulent SP variant of TGEV contained a 462-nucleotide deletion within RNAs 3 and 4 that encode ORFs A and B respectively. The SP virus was characterized by small plaque size, low virulence, and persistence in swine leukocyte compared to virulent parent Miller strain of TGEV [48]. Unlike 188-SG strain and SP variant, the deletion of the M60 strain was located in only ORF 3–1 and was larger. These data suggest that ORFs 3 and 3–1 are not necessary for virus replication but could be involved in virulence and tissue tropism.

In conclusion, genomic variation of the TGEV and PRCV strains tested was revealed by PCR/RFLP analysis and sequencing. They could be differentiated into several groups by PCR/RFLP analysis.

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