

# Rapid differentiation of vaccine strain and Chinese field strains of transmissible gastroenteritis virus by restriction fragment length polymorphism of the N gene

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**Abstract** A strain of transmissible gastroenteritis virus (TGEV), designated H16, was isolated in PK-15 cells and passaged serially to level 165. Vaccines based on passages 155–165 in cell cultures are available commercially as vaccines for the prevention and control of infections with TGEV in China. Nucleoprotein (N) sequences of the virus at passages 155 and 165 were aligned and compared using a computer software program. The suitability of restriction fragment length polymorphism (RFLP) analysis for differentiation of the vaccine strain from the other TGEVs was investigated. The RFLP analysis identified a change in the cleavage sites of *AclI* at passages 155 and 165. This RFLP pattern of the N gene differentiated the Chinese vaccine strain from its parental strain, the 11 TGEVs studied and the other reported TGEVs in the GenBank. Using phylogenetic analysis, the Chinese TGEVs were divided into three groups (G1, G2, and G3). The G3 Chinese TGEVs possessed several specific nucleotides and amino acids that were not found in the G1 and G2 Chinese TGEVs or the other reference TGEVs. Analysis of the phylogenetic trees revealed that the G3 TGEVs represent a separate group that is distinct from the non-Chinese TGEVs and from Chinese TGEVs isolated previously.

These findings suggest that Chinese strains of TGEV are evolving continuously.

**Keywords** Transmissible gastroenteritis virus · Restriction fragment length polymorphism · N gene · RT-PCR

## Introduction

Transmissible gastroenteritis virus (TGEV), a member of the family Coronaviridae, is an enveloped, single-stranded RNA virus that causes severe enteropathogenic diarrhea in pigs and results in significant economic loss [1]. TGEV was identified as an etiological agent of transmissible gastroenteritis in swine in 1946 in the United States [2, 3]. In neonates, TGEV infects the epithelial cells of the small intestine, leading to potentially fatal gastroenteritis. Infection also occurs in the upper respiratory tract and, less often, in the lungs [4]. In adult pigs, TGEV causes mild disease.

TGEV was reported in many swine-producing countries between the late 1980s and the 1990s [5–8]. TGEV strains of varying virulence have been isolated and characterized worldwide [9–12]. Some strains have been developed successfully into modified live vaccines. In China, an outbreak of transmissible gastroenteritis (TGE) was first reported in the 1970s. Since then, the disease has been prevalent in the country. A TGEV was isolated from a field specimen in 1973, designated as H16, and passaged serially in PK-15 cells to level 165. Animal experiments demonstrated that pigs inoculated with H165 displayed no clinical signs of diarrhea or mortality [13]. Vaccines based on the cell cultures at passages 155–165 are available commercially for the prevention and control of infections with TGEV in China. Despite the current vaccination strategy,

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TGE still occurs in swine in China. Various methods have been applied to detect TGEV infection in clinical fecal specimens. These include virus isolation (VI) in cell/tissue cultures, enzyme immunoassay (EIA), and cDNA probes [14–18]. Virus isolation requires the isolation of the virus on cell cultures, followed by quantitative analysis of the virus and this method is time consuming [16]. Standard serological tests that are used to detect TGEV also require considerable time to complete, and have not been able to distinguish between different strains. The results can be obtained using RT-PCR more rapidly than with the VI method or serological tests, and the RT-PCR technique has been used increasingly as a supplementary method in TGEV diagnosis [19–27]. Paton et al. [25] detected TGEV in clinical fecal specimens using single-round PCR and was able to discriminate TGEV from PRCV. Using this method, they detected viral RNA from all of the 26 different strains of TGEV and PRCV examined, covering the period from 1946 to 1996. Kim et al. [23] detected TGEV in fecal samples of swine with diarrhea using nested RT-PCR. They detected TGEV in ten intestinal and nine fecal samples, and among the nine positive fecal samples, three samples were culture negative. Chen et al. [19] established a novel real-time RT-PCR assay with the LUX primer for the detection of TGEV from different TGEV strains and clinical specimens. They found that this method is more rapid, reliable and sensitive than a gel-based RT-PCR method. The above-mentioned studies indicate that RT-PCR can be useful as a routine diagnostic method for the detection of TGEV in fecal specimens, particularly during the early and late stages of infection when the viral load may be below the limit of detection by other less sensitive methods. Given that the tests employed currently to diagnose TGEV (VI, EIA, RT-PCR, etc.) lack the capacity to differentiate between the vaccine strain and the wild field strains, the use of RT-PCR followed by RFLP may be required. In the case of PEDV, which belongs to the family Coronaviridae with TGEV, Song et al. [28] used RT-PCR–RFLP to differentiate a Vero cell-adapted PEDV from Korean field strains by RFLP analysis of ORF3. The application of RT-PCR and subsequent RFLP analysis with the restriction endonuclease *HindIII* and *XhoI* enabled them to differentiate attenuated PEDV from other Korean field strains. Lee et al. [29] developed a RT-PCR–RFLP method to differentiate field strains of PEDV and a vaccine strain, J-vac. Furthermore, Lai et al. [30] used arms PCR and RFLP analysis with four separate restriction enzymes (*BstEII*, *Alw26I*, *DraIII*, or *MspAII*) to identify the genetic profiles of virulent, attenuated or vaccine strains of TGEV and PRCV.

The objective of the present study was therefore to apply RT-PCR to detect TGEV virus in porcine fecal specimens taken from piglets showing watery diarrhea and dehydration in China. Furthermore, RFLP analysis was performed

with the aim of differentiating between the Chinese vaccine strain and wild-type strains as well as the other reported TGEVs in the GenBank. The present study was also designed to investigate the diversity among Chinese strains of TGEV, according to the sequence analysis of the N genes. The analysis included Chinese and non-Chinese reference TGEVs as well as porcine respiratory coronavirus (PRCV) strain ISU-1 and was intended to identify the most prevalent TGEVs in China through sequence and phylogenetic analyses.

## Materials and methods

### Cells and viruses

The pig kidney cell line PK-15 (ATCC, CCL-33) was maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and 1 mM Na-pyruvate. The cell cultures at passages 155–165 and its parental strain H16 were obtained in our laboratory [13]. The Chinese virulent TGEV strain, H16, was propagated in neonatal piglets. The Chinese TGEV vaccine strain, H165, was derived from H16 by 165 serial passages and five plaque purification steps in PK-15 cells [13]. These cell cultures at passages 155–165 are used for the manufacture of live TGEV vaccines by the Chinese government.

### TGEV samples

A total of 11 porcine fecal samples were taken from piglets showing watery diarrhea and dehydration in eight different provinces of China. These fecal samples were confirmed to be positive for TGEV using the Anigen Rapid TGE Ag Test Kit (Animal Genetics Inc.). TGEV-positive fecal samples were handled as described previously [31]. The supernatants were collected for the amplification of the N gene by RT-PCR.

### Viral RNA extraction and purification

The RNA was isolated from the supernatant (200 µl) that contained TGEV using a Simply P Total RNA Extraction Kit (Bioer Technology Co., Ltd) according to the manufacturer's instructions. The RNA isolated was used for the following RT-PCR.

### RT-PCR amplification and RFLP analysis

Primers were designed based on the published sequences of the sM and ORF7 genes to cover the N gene of TGEV. The primers were N-U (forward), 5'-GCATTACCCAGCAGGA

C-3'; and N-L (reverse), 5'-AATAAATACAGCATGGA GGAGGAC-3'. The size of the amplified product was predicted to be 1,361 bp. Reverse transcription was carried out using N-L and M-MLV reverse transcriptase reagent kit (TaKaRa) according to the manufacturer's instructions. Three-step procedures were performed in a thermal cycler (TaKaRa) under the following conditions. A 5-min denaturation step at 94°C was used, followed by a number of amplification cycles: 30 s at 95°C, 30 s at the annealing temperature 64°C, an elongation step at 72°C for 1 min and 15 s, and an extra extension step of 10 min at 72°C. For amplification of the region of the N gene, reactions were carried out in a total of volume of 50 µl containing 25 µl of PerfectShot™ *Ex Taq* (containing 1.25 U TaKaRa *Ex Taq* HS, 0.4 mM dNTP Mixture, and 4 mM Mg<sup>2+</sup>), 1 µl of each specific primer (20 µM), about 2.5 ng of template, and sterile deionized water. A 5 µl volume of each PCR product was electrophoresed through 1.0% agarose gels and stained with ethidium bromide and observed using a UV transilluminator. The conditions for each sample that yielded a positive result of amplification were tested two to three times to ensure consistency of the results. A negative control was always included. The specificity of the PCR was evaluated using other common viral causative agents of diarrhea: porcine epidemic diarrhea virus (PEDV) and porcine rotavirus and the PCR oligonucleotides used to amplify bands were as described previously [31, 32].

Bands of the appropriate size (1,361 bp) were gel-purified using the QIAquick Gel Purification Kit (QIAGEN). Subsequently, 10 µl of each purified amplicon was digested by adding 1 µl (3 U) of *AcII* (New England Biolabs, Beverly, MA, USA) and incubated at 37°C for 5 min according to the manufacturer's instructions. Electrophoresis was performed as described above.

#### DNA sequencing and sequence analysis

The PCR amplicons were purified using the AxyPrep™ Gel Extraction Kit (Axygen Scientific, Inc., CA, USA) and cloned into the pGEM-T Easy Vector (Promega). Three to five independent clones of each TGEV amplicon were isolated and sequenced using M13 sequencing primers. The sequences were compiled and the ORFs of the N gene were determined using the Gene Runner program version 3.00 (<http://www.generunner.com>). Nucleotide and deduced amino acid sequences of the Chinese field TGEVs were aligned, edited and compared with the reference TGEVs in GenBank using MegAlign software. Phylogenetic trees were generated using an alignment of the nucleotide and deduced amino acid sequences of the N gene with reference TGEVs, as well as with PRCV-ISU-1, in DNASTar software. The N gene sequences of the 11 Chinese field TGEVs have been submitted to the GenBank database, and their

GenBank accession nos. are: CH/SDQ/08, GQ374565; CH/HLJT/09, GQ374564; CH/JSX/09, GQ374562; CH/GSJ/09, GQ374563; CH/JLY/09, GQ374560; CH/LNX/09, GQ374559; CH/HLJA/09, GQ374561; CH/SH/09, GQ374557; CH/HLJD/09, GQ374558; CH/HLJB/09, GQ374556; and CH/JLJ/09, GQ374555. The sequences of the reference TGEVs used for comparison were from the GenBank database and have GenBank accession nos.: virulent Purdue, DQ811789; Purdue P115, DQ811788; PUR46-MAD, NC\_002306; Miller M60, DQ811786; Miller M6, DQ811785; SC-Y, DQ443743; TS, DQ201447; TO14, AF302262; HN2002, AY587883; FS772/70, Y00542; 96-1933, AF104420; H16, FJ755618; H155, GQ374566; H165, EU074218; and PRCV-ISU-1, DQ811787.

## Results

### N gene sequencing and sequence analysis

The sequence of the N gene of the TGEV isolates was aligned and compared using MegAlign software. The PCR products were the expected size of 1,361 bp. No signal was detected with another porcine coronavirus (PEDV), with cells infected with porcine rotavirus, or with uninfected cells. Among the 1,361 nucleotides of the PCR product, only one nucleotide mutation (from C to T at 810) was observed in the Chinese attenuated virus (passages 155 and 165) in comparison with its parental H16 strain and the other TGEVs in GenBank. This nucleotide mutation caused the Chinese attenuated strain (from passages 155–165) to reveal one cleavage site of *AcII*. No cleavage site of the restriction enzyme *AcII* was found in the parental strain (H16), the 11 field strains, the previously reported Chinese TGEVs and the other TGEVs in GenBank.

The N protein gene of the 11 Chinese field TGEVs was sequenced to characterize the strains. The N genes of the 11 field TGEVs consisted of 1,161 nucleotides, and contained a single open reading frame (ORF) of 1,149 nucleotides, which encoded a peptide of 382 amino acids (aa). There was no deletion or insertion of nucleotides in the ORFs of the 11 strains. These strains had a transcription regulatory sequence (TRS) of 5'-CUAAAC-3', as previously recognized in Purdue strain [33]. The nucleotide and deduced amino acid sequences of these TGEVs were determined and compared with the sequences of the published TGEVs, as well as with PRCV-ISU-1. Three groups, group 1 (G1), group 2 (G2), and group 3 (G3), had significant differences in their nucleotide and deduced amino acid sequences. The G1 Chinese TGEV strain (CH/HLJD/09) had one specific change in the deduced amino acid sequence (from K to T at 296), which was produced by one change in the nucleotide sequence (from A to C at 887)

(Fig. 1). This change was not found in other Chinese TGEVs or in the other TGEVs in the GenBank or PRCV-ISU-1. The G2 Chinese TGEV strain (CH/LNX/09) had one specific change in the deduced amino acid sequence (from G to C at 119), which was produced by one change in the nucleotide sequence (from G to T at 355) that was not found in other Chinese TGEVs, the other TGEVs in the GenBank, or PRCV-ISU-1 (Fig. 1). The G3 TGEVs had three common specific changes in their deduced amino acid sequences (from N to S at 3, from K to R at 140, and from A to T at 252), which were produced by four changes in the nucleotide sequence (from A to G at 8, from AA to GA at 419 and 420, from A to C at 756) that were not found in other Chinese TGEVs or the other TGEVs in the GenBank. However, two specific changes in the deduced amino acid sequence (from N to S at 3, and from K to R at 140) were also found in PRCV-ISU-1 (Fig. 1).

The G3 TGEVs, except for CH/SDQ/08, had one common specific change in their deduced amino acid sequences (from R to I at 342), which was produced by one change in the nucleotide sequence (from G to T at 1,025) that was not found in the other Chinese TGEVs, the other TGEVs in the GenBank, or PRCV-ISU-1 (Fig. 1). In addition, the four G3 TGEVs (CH/JLY/09, CH/HLJB/09, CH/GSJ/09, and CH/HLJT/09) had two common specific changes in their deduced amino acid sequence (from V to A at 145, from D to G at 263), which were produced by two changes in the nucleotide sequence (from T to C at 435, and from A to G at 788) that were not found in other Chinese TGEVs, the other TGEVs in the GenBank, or PRCV-ISU-1 (Fig. 1). Moreover, the G3-1 TGEVs had one specific change in the deduced amino acid sequence (from I to T at 362), which was produced by one change in the nucleotide sequence (from T to C at 1,085) that was not found in other Chinese TGEVs, the other TGEVs in the GenBank, or PRCV-ISU-1 (Fig. 1).

Phylogenetic trees were generated on the basis of the nucleotide and deduced amino acid sequences. The phylogenetic tree shown in Fig. 2 was generated based on the nucleotide sequences, and that in Fig. 3 was based on the deduced amino acid sequences. Although these phylogenetic trees differed slightly, overall the Chinese TGEVs showed high similarity. In brief, all 11 field strains fell into three groups (G1, G2, G3), which included all the Chinese TGEVs. One group (G1) comprised CH/HLJD/09, Miller M6, Miller M60, TS, HN2002, the Chinese vaccine strain (H155 and H165) and its parental strain (H16). The second group (G2) consisted of CH/LNX/09, the Purdue strains, and one Chinese strain, SC-Y. The third group (G3) contained nine Chinese field TGEVs and had three subgroups (G3-1, G3-2, and G3-3). Strains CH/JLY/09, CH/HLJB/09, CH/GSJ/09, CH/JLJ/09, and CH/HLJT/09 formed one subgroup (G3-1), CH/SH/09, CH/HLJA/09, and CH/JSX/

09 formed the second subgroup (G3-2), and CH/SDQ/08 formed the third subgroup (G3-3).

The nucleotide and deduced amino acid sequence of the N gene of the Chinese field TGEVs are compared with those of the reference strains in Table 1. We found that the N genes of the G3 Chinese TGEVs had 99.7–100% DNA sequence identities with each other and they have 96.4–97.0 and 95.2–97.0% DNA sequence identities with the G1 and G2. Likewise, they have 99.2–100% identities with the deduced amino acid sequences of each other and they have 97.1–99.0, 96.1–99.0% identities with the deduced amino acid sequences of the G1 and G2.

#### RFLP analysis

The RT-PCR reaction on the amplicons that yielded a fragment of the expected 1,361 bp was analyzed further using the restriction enzyme *AcI*. The PCR products of the Chinese vaccine strain (passages 155 and 165) when digested by *AcI* generated two fragments, of 807 and 554 bp, but the PCR product of the parental stain (H16) was not cut by *AcI*. The 11 other field TGEVs used in this study revealed the same RFLP pattern as H16 (Fig. 4).

#### Discussion

In the present study, we employed the *AcI* restriction endonuclease to distinguish the Chinese TGEV vaccine strain from field strains. Investigation of 11 TGEV field strains indicated that the vaccine strain could be differentiated from the field strains. Although an attenuated vaccine against TGEV has been used widely to control TGEV in China, the disease has continued to be a problem. A total of 11 Chinese TGEV strains were analyzed in this study, first by RT-PCR–RFLP and then by nucleotide sequencing of the N gene. Although TGEV is thought at present to have only one serotype, the genome of TGEV shows considerable genetic diversity [9, 12, 34, 35]. Diversity among TGEVs and other reference TGEVs has been reported previously. However, this is the first report of genetic diversity in the N genes of Chinese TGEVs and reference TGEVs. Our findings show that Chinese TGEVs are genetically diverse in their N genes, both within the group and when compared with reference strains.

Coronaviruses, being single-stranded RNA viruses, are fully equipped to adapt to changing ecological niches because they exhibit high substitution rates of  $10^{-4}$  substitutions per year per site [36, 37]. Sequence analysis showed that several unique characteristics were identified among the 11 field TGEVs. The new reported G1, G2, and G3 TGEVs had 1, 19, and 26 unique nucleotides mutations when compared with the other group Chinese field strains,

**Fig. 1** Comparison of the N genes of new Chinese field TGEV strains with the previously TGEVs as well as PRCV-ISU-1. The *dashes* indicated regions where the sequences are identical to those of the sequences of the reference strains

H16	ATGGCCAACCAGGGACAACGTGTCAGTTGGGGAGATGAATCCACCAAAAACAGTGGTCCG	60
H155	-----	60
H165	-----	60
CH/HLJD/09	-----	60
HN2002	-----	60
SC-Y	-----T-----T	60
CH/LNX/09	-----T-----T	60
CH/GSJ/09	-----G-----T-----G-----	60
CH/HLJB/09	-----G-----T-----G-----	60
CH/HLJT/09	-----G-----T-----G-----	60
CH/JLY/09	-----G-----T-----G-----	60
CH/JLJ/09	-----G-----T-----G-----	60
CH/JSX/09	-----G-----T-----G-----	60
CH/HLJA/09	-----G-----T-----G-----	60
CH/SH/09	-----G-----T-----G-----	60
CH/SDQ/08	-----G-----T-----G-----	60
PRCV-ISU-1	-----G-----T-----TG-----A-----	60
H16	TCCAATTCCCGTGGTCGGAAGAGTAATAACATACCTCTTTCATTCTTCAACCCATAACC	120
H155	-----	120
H165	-----	120
CH/HLJD/09	-----	120
HN2002	-----	120
SC-Y	-----A-----	120
CH/LNX/09	-----A-----	120
CH/GSJ/09	-----	120
CH/HLJB/09	-----	120
CH/HLJT/09	-----	120
CH/JLY/09	-----	120
CH/JLJ/09	-----	120
CH/JSX/09	-----	120
CH/HLJA/09	-----	120
CH/SH/09	-----	120
CH/SDQ/08	-----T-----	120
PRCV-ISU-1	-----	120
H16	CTCCAGCAAGGTTCAAAATTTGGAACTTATGTCCGAGAGACTTTGTACCCAAAGGAATA	180
H155	-----	180
H165	-----	180
CH/HLJD/09	-----	180
HN2002	-----G-----	180
SC-Y	-----A-----C-----	180
CH/LNX/09	-----A-----	180
CH/GSJ/09	-----T-----G-----G-----G-----	180
CH/HLJB/09	-----T-----G-----G-----G-----	180
CH/HLJT/09	-----T-----G-----G-----G-----	180
CH/JLY/09	-----T-----G-----G-----G-----	180
CH/JLJ/09	-----T-----G-----G-----G-----	180
CH/JSX/09	-----T-----G-----G-----G-----	180
CH/HLJA/09	-----T-----G-----G-----G-----	180
CH/SH/09	-----T-----G-----G-----G-----	180
CH/SDQ/08	-----C-----T-----G-----	180
PRCV-ISU-1	-----G-----	180
H16	GGTAACAGGGATCAGCAGATTGGTTATTGGAATAGACAAAACCGCTATCGCATGGTGAAG	240
H155	-----	240
H165	-----	240
CH/HLJD/09	-----	240
HN2002	-----	240
SC-Y	-----A-----	240
CH/LNX/09	-----A-----	240
CH/GSJ/09	-----T-----C-----A-----	240
CH/HLJB/09	-----T-----C-----A-----	240
CH/HLJT/09	-----T-----C-----A-----	240
CH/JLY/09	-----T-----C-----A-----	240
CH/JLJ/09	-----T-----C-----A-----	240
CH/JSX/09	-----T-----C-----A-----	240
CH/HLJA/09	-----T-----C-----A-----	240
CH/SH/09	-----T-----C-----A-----	240
CH/SDQ/08	-----T-----C-----A-----	240
PRCV-ISU-1	-----T-----C-----A-----	240

Fig. 1 continued

H16	GGCCAACGTAAGAGCTTCCTGAAAGGTGGTTCTTCTACTACTTAGGTACTGGACCTCAT	300
H155	-----	300
H165	-----	300
CH/HLJD/09	-----	300
HN2002	-----	300
SC-Y	-----	300
CH/LNX/09	-----	300
CH/GSJ/09	-----C-----	300
CH/HLJB/09	-----C-----	300
CH/HLJT/09	-----C-----	300
CH/JLY/09	-----C-----	300
CH/JLJ/09	-----A-----C-----	300
CH/JSX/09	-----A-----C-----	300
CH/HLJA/09	-----A-----C-----	300
CH/SH/09	-----A-----C-----	300
CH/SDQ/08	-----A-----C-----	300
PRCV-ISU-1	-----C-----	300
H16	GCAGATGCCAAATTTAAAGATAAAATTAGATGGAGTTGTCTGGGTTGCCAAGGATGGTGCC	360
H155	-----	360
H165	-----	360
CH/HLJD/09	-----	360
HN2002	-----	360
SC-Y	-----	360
CH/LNX/09	-----T-----	360
CH/GSJ/09	-----C-----	360
CH/HLJB/09	-----C-----	360
CH/HLJT/09	-----C-----	360
CH/JLY/09	-----C-----	360
CH/JLJ/09	-----C-----	360
CH/JSX/09	-----C-----	360
CH/HLJA/09	-----C-----	360
CH/SH/09	-----C-----	360
CH/SDQ/08	-----C-----	360
PRCV-ISU-1	-----C-----T-----	360
H16	ATGAACAAACCAACCACGCTTGGTAGTCGGTGCTAATAATGAATCCAAAGCTTTGAAA	420
H155	-----	420
H165	-----	420
CH/HLJD/09	-----	420
HN2002	-----	420
SC-Y	-----	420
CH/LNX/09	-----	420
CH/GSJ/09	-----T--T-----G-	420
CH/HLJB/09	-----T--T-----G-	420
CH/HLJT/09	-----T--T-----G-	420
CH/JLY/09	-----T--T-----G-	420
CH/JLJ/09	-----T--T-----G-	420
CH/JSX/09	-----T--T-----G-	420
CH/HLJA/09	-----T--T-----G-	420
CH/SH/09	-----T--T-----G-	420
CH/SDQ/08	-----T--T-----G-	420
PRCV-ISU-1	-----T--T-----G-	420
H16	TTCGATGGTAAAGTGCCAGGCGAATTTCAACTTGAAGTTAACCAGTCAAGGGACAATTCA	480
H155	-----	480
H165	-----	480
CH/HLJD/09	-----	480
HN2002	-----	480
SC-Y	-----T--A--A-----	480
CH/LNX/09	-----T--A--A-----	480
CH/GSJ/09	-----C-----T-----C-----	480
CH/HLJB/09	-----C-----T-----C-----	480
CH/HLJT/09	-----C-----T-----C-----	480
CH/JLY/09	-----C-----T-----C-----	480
CH/JLJ/09	-----T-----C-----	480
CH/JSX/09	-----T-----C-----	480
CH/HLJA/09	-----T-----C-----	480
CH/SH/09	-----T-----C-----	480
CH/SDQ/08	-----T-----T--C-----	480
PRCV-ISU-1	-----T-----T--C-----	480

Fig. 1 continued

H16	AGGTCACGCTCTCAATCTAGATCTCGGTCTAGAAAACAGATCTCAATCTAGAGGCAGGCAA	540
H155	-----	540
H165	-----	540
CH/HLJD/09	-----	540
HN2002	-----	540
SC-Y	-----T-----	540
CH/LNX/09	-----T-----	540
CH/GSJ/09	-----G-----A-----	540
CH/HLJB/09	-----G-----A-----	540
CH/HLJT/09	-----G-----A-----	540
CH/JLY/09	-----G-----A-----	540
CH/JLJ/09	-----G-----A-----	540
CH/JSX/09	-----G-----A-----	540
CH/HLJA/09	-----G-----A-----	540
CH/SH/09	-----G-----A-----	540
CH/SDQ/08	-----G-----	540
PRCV-ISU-1	-----	540
H16	CAATCCAATAACAAGAAGGATGACAGTGTAGAACAAGCTGTCTTGGCCGACTTAAAAAG	600
H155	-----	600
H165	-----	600
CH/HLJD/09	-----	600
HN2002	-----	600
SC-Y	---T-----	600
CH/LNX/09	---T-----	600
CH/GSJ/09	-----	600
CH/HLJB/09	-----	600
CH/HLJT/09	-----	600
CH/JLY/09	-----	600
CH/JLJ/09	-----	600
CH/JSX/09	-----	600
CH/HLJA/09	-----	600
CH/SH/09	-----	600
CH/SDQ/08	-----	600
PRCV-ISU-1	-----T-----	600
H16	TTAGGTGTTGACACAGAAAAACACGCAACGCTCTCGTTCTAAATCTAAAGAACGTAGT	660
H155	-----T-----	660
H165	-----T-----	660
CH/HLJD/09	-----	660
HN2002	-----	660
SC-Y	-----	660
CH/LNX/09	-----	660
CH/GSJ/09	-----	660
CH/HLJB/09	-----	660
CH/HLJT/09	-----	660
CH/JLY/09	-----	660
CH/JLJ/09	-----	660
CH/JSX/09	-----	660
CH/HLJA/09	-----	660
CH/SH/09	-----	660
CH/SDQ/08	-----	660
PRCV-ISU-1	-----	660
H16	AACTCTAAAACAAGAGATACTACGCCTAAGAATGAAAACAAACACACCTGGAAGAGAACT	720
H155	-----	720
H165	-----	720
CH/HLJD/09	-----	720
HN2002	-----C-----	720
SC-Y	-----G-----A-----	720
CH/LNX/09	-----G-----A-----	720
CH/GSJ/09	-----C-----	720
CH/HLJB/09	-----C-----	720
CH/HLJT/09	-----C-----	720
CH/JLY/09	-----C-----	720
CH/JLJ/09	-----C-----	720
CH/JSX/09	-----C-----	720
CH/HLJA/09	-----C-----	720
CH/SH/09	-----C-----	720
CH/SDQ/08	-----C-----	720
PRCV-ISU-1	-----C-----	720

Fig. 1 continued

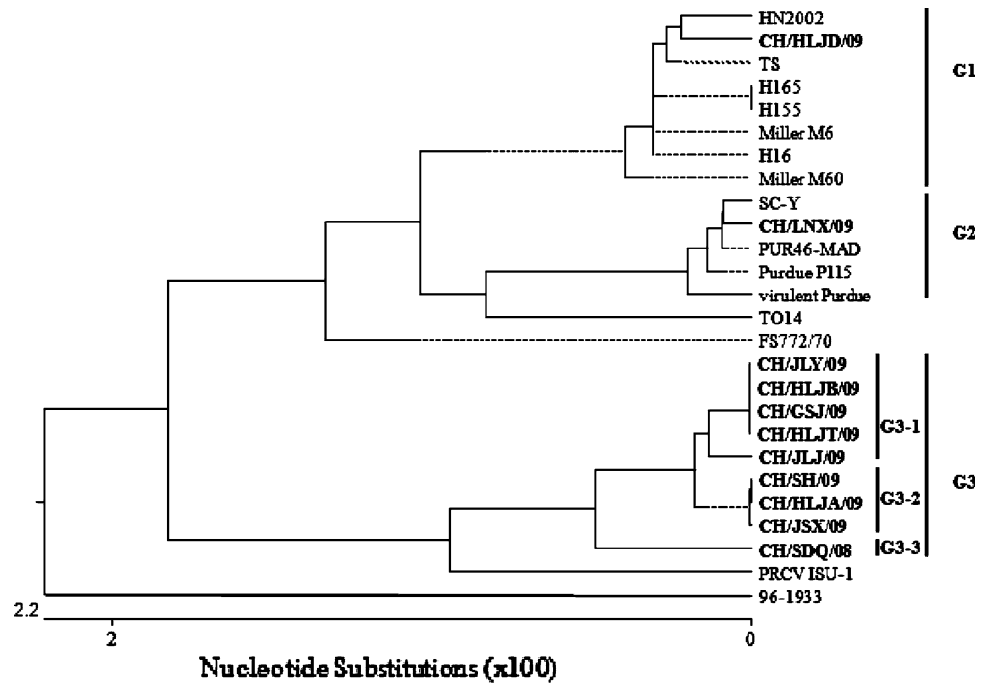
H16	GCAGGTAAAAGGTGATGTGACAAGATTTTATGGAGCTAGAAGCAGTTCAGCCAAATTTTGGT	780
H155	-----	780
H165	-----	780
CH/HLJD/09	-----	780
HN2002	-----	780
SC-Y	-----	780
CH/LNX/09	-----	780
CH/GSJ/09	-----C-----A-C-----T-----	780
CH/HLJB/09	-----C-----A-C-----T-----	780
CH/HLJT/09	-----C-----A-C-----T-----	780
CH/JLY/09	-----C-----A-C-----T-----	780
CH/JLJ/09	-----C-----A-C-----T-----	780
CH/JSX/09	-----C-----A-C-----T-----	780
CH/HLJA/09	-----C-----A-C-----T-----	780
CH/SH/09	-----C-----A-C-----T-----	780
CH/SDQ/08	-----C-----A-C-----	780
PRCV-ISU-1	-----C-----	780
H16	GACAGTGACCTCGTTGCCAATGGGAGCAGTGCCAAGCATTACCCACAATTGGCTGAATGT	840
H155	-----	840
H165	-----	840
CH/HLJD/09	-----	840
HN2002	-----	840
SC-Y	---C-----C-----	840
CH/LNX/09	---C-----C-----	840
CH/GSJ/09	---G---T-----C-----	840
CH/HLJB/09	---G---T-----C-----	840
CH/HLJT/09	---G---T-----C-----	840
CH/JLY/09	---G---T-----C-----	840
CH/JLJ/09	---T-----C-----	840
CH/JSX/09	---T-----C-----	840
CH/HLJA/09	---T-----C-----	840
CH/SH/09	---T-----C-----	840
CH/SDQ/08	---T-----C---G---	840
PRCV-ISU-1	-----T-----T-----C---G---	840
H16	GTTCACCTGTGTCTAGCATTITGTTGGAAGCTATTGGACTTCAAAGGAAGATGGCGAC	900
H155	-----	900
H165	-----	900
CH/HLJD/09	-----C-----	900
HN2002	-----	900
SC-Y	-----C-----	900
CH/LNX/09	-----C-----	900
CH/GSJ/09	-----C-----	900
CH/HLJB/09	-----C-----	900
CH/HLJT/09	-----C-----	900
CH/JLY/09	-----C-----	900
CH/JLJ/09	-----C-----	900
CH/JSX/09	-----C-----	900
CH/HLJA/09	-----C-----	900
CH/SH/09	-----C-----	900
CH/SDQ/08	-----C-----	900
PRCV-ISU-1	-----	900
H16	CAGATAGAAGTCACGTTCCACACACAAATACCCTTGCCAAAGGATGATCTAAAAGTCAA	960
H155	-----	960
H165	-----	960
CH/HLJD/09	-----	960
HN2002	-----T-----	960
SC-Y	-----G---G---	960
CH/LNX/09	-----G---G---	960
CH/GSJ/09	-----	960
CH/HLJB/09	-----	960
CH/HLJT/09	-----	960
CH/JLY/09	-----	960
CH/JLJ/09	-----	960
CH/JSX/09	-----	960
CH/HLJA/09	-----	960
CH/SH/09	-----	960
CH/SDQ/08	-----	960
PRCV-ISU-1	-----	960



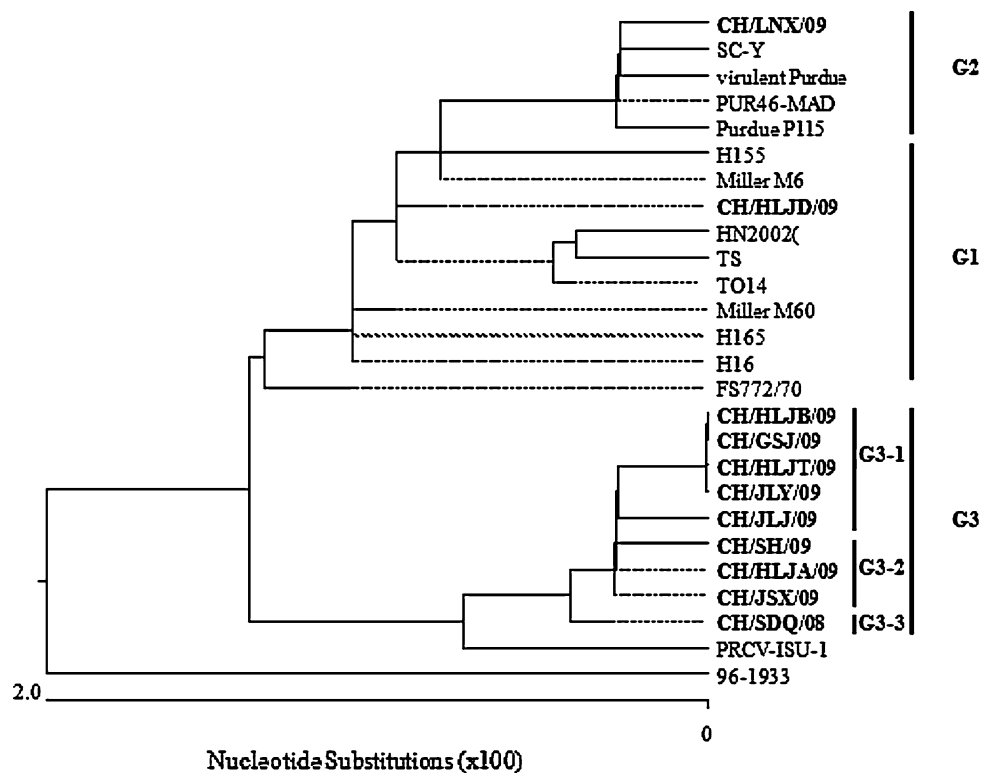
Fig. 1 continued

H16	CAATTCCTTCAGCAGATTAATGCCTATGCTCGCCATCAGAAAGTGGCAAAAAGAACAGAGA	1020
H155	-----	1020
H165	-----	1020
CH/HLJD/09	-----	1020
HN2002	-----G-----	1020
SC-Y	-----T-----	1020
CH/LNX/09	-----T-----	1020
CH/GSJ/09	-----	1020
CH/HLJB/09	-----	1020
CH/HLJT/09	-----	1020
CH/JLY/09	-----	1020
CH/JLJ/09	-----	1020
CH/JSX/09	-----	1020
CH/HLJA/09	-----	1020
CH/SH/09	-----	1020
CH/SDQ/08	-----	1020
PRCV-ISU-1	-----	1020
H16	AAAAGAAAATCTCGTCTAAATCTGCAGAAAGTCAGAGCAAGAGGTGGTACCTGATGCA	1080
H155	-----	1080
H165	-----	1080
CH/HLJD/09	-----	1080
HN2002	-----	1080
SC-Y	-----T-----	1080
CH/LNX/09	-----T-----	1080
CH/GSJ/09	---T-----T-----T---	1080
CH/HLJB/09	---T-----T-----T---	1080
CH/HLJT/09	---T-----T-----T---	1080
CH/JLY/09	---T-----T-----T---	1080
CH/JLJ/09	---T-----T-----T---	1080
CH/JSX/09	---T-----T-----T---	1080
CH/HLJA/09	---T-----T-----T---	1080
CH/SH/09	---T-----T-----T---	1080
CH/SDQ/08	---T-----T-----T---	1080
PRCV-ISU-1	-----T-T-----	1080
H16	TTAATAGAAAATTACACAGATGTGTTTGATGACACACAGGTTGAGATGATTGATGAGGTA	1140
H155	-----	1140
H165	-----	1140
CH/HLJD/09	-----	1140
HN2002	-----	1140
SC-Y	-----T-----A-----	1140
CH/LNX/09	-----T-----A-----	1140
CH/GSJ/09	C---C-----T-----C-----	1140
CH/HLJB/09	C---C-----T-----C-----	1140
CH/HLJT/09	C---C-----T-----C-----	1140
CH/JLY/09	C---C-----T-----C-----	1140
CH/JLJ/09	C---C-----T-----C-----	1140
CH/JSX/09	C---C-----T-----C-----	1140
CH/HLJA/09	C---C-----T-----C-----	1140
CH/SH/09	C---C-----T-----C-----	1140
CH/SDQ/08	C---C-----T-----C-----	1140
PRCV-ISU-1	-----T-----C-----	1140
H16	ACGAACTAA	1149
H155	-----	1149
H165	-----	1149
CH/HLJD/09	-----	1149
HN2002	-----	1149
SC-Y	-----	1149
CH/LNX/09	-----	1149
CH/GSJ/09	-----	1149
CH/HLJB/09	-----	1149
CH/HLJT/09	-----	1149
CH/JLY/09	-----	1149
CH/JLJ/09	-----	1149
CH/JSX/09	-----	1149
CH/HLJA/09	-----	1149
CH/SH/09	-----	1149
CH/SDQ/08	-----	1149
PRCV-ISU-1	-----	1149

**Fig. 2** Phylogenetic relationships based on the N nucleotide sequences of the Chinese field TGEVs in comparison with reference TGEVs and PRCV-ISU-1, obtained using DNASTar with the ClustalW methods. The Chinese field TGEVs are shown in *boldface* type



**Fig. 3** Phylogenetic relationships based on the N amino acid sequences of the Chinese field TGEVs in comparison with reference TGEVs and PRCV-ISU-1, obtained using DNASTar with the ClustalV methods. The Chinese field TGEVs are shown in *boldface* type



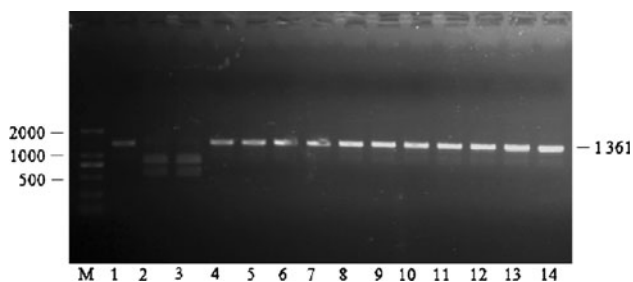
respectively. Moreover, these unique nucleotide mutations resulted in 1, 6, and 3 unique amino acid mutations when compared with the other group Chinese TGEVs. Sequence and phylogenetic analyses indicated that the 11 Chinese field TGEVs appeared to originate from different ancestors

and resulted from accumulation of mutations. Our results indicate the G3 TGEVs formed a unique group, which has not been previously reported. In the present study, the G3 strains were isolated from intensive swine farms, which contained an extremely large number of pigs, and most of

**Table 1** Nucleotide and deduced amino acid sequence identities of the N genes of Chinese field TGEVs in comparison with reference strains

Group	Percentage identity (%)				
	G1	G2	G3		
			G3-1	G3-2	G3-3
Percentage identity (%)					
G1	***	97.7–99.3	96.4–96.9	96.6–97.0	96.7–97.0
G2	97.4–100	***	95.2–96.6	95.4–96.7	95.5–97.0
G3					
G3-1	97.1–98.4	96.1–98.4	***	99.7–99.9	98.9–99.1
G3-2	97.9–98.7	96.9–98.7	99.2–100	***	99.2–100
G3-3	97.1–98.4	96.1–98.4	99.0–100	99.7–100	***

The upper right triangle is the nucleotide sequence identity and the lower left triangle is the deduced amino acid sequence identity



**Fig. 4** RFLP analysis of the PCR products of the TGEV N gene. Lane M DNA Marker DL 2000, lane 1 H16, lane 2 H155, lane 3 H165, lane 4 CH/SDQ/08, lane 5 CH/HLJT/09, lane 6 CH/JSX/09, lane 7 CH/GSJ/09, lane 8 CH/JLY/09, lane 9 CH/LNX/09, lane 10 CH/HLJA/09, lane 11 CH/SH/09, lane 12 CH/HLJD/09, lane 13 CH/HLJB/09, lane 14 CH/JLJ/09

these herds are maintained at a high density and exposed intensively to live vaccines. These findings also suggest that the Chinese TGEVs appear are evolving continuously.

Sequence and phylogenetic analyses of the N gene indicated that the G1 Chinese strain of TGEV (CH/HLJD/09) was very similar to HN2002, TS, Miller M6, Miller M60, H16, and Chinese vaccine strain. The G2 Chinese TGEV strain (CH/LNX/09) was very similar to SC-Y, virulent Purdue, PUR46-MAD, and Purdue P115. However, the G3 strains represented a separate group at the levels of the nucleotide and deduced amino acid sequences. It is notable that the most prevalent TGEV strains in China are not closely related to the Chinese strain SC-Y nor to the Chinese TGEVs HN2002, TS, H16, and the Chinese vaccine strain, even though they were isolated in China.

In summary, RT-PCR has distinct advantages in the rapid and reliable diagnosis of TGEV in fecal specimens. The RT-PCR–RFLP methodology described here is a simple and rapid technique (it can be performed within

3.5 h) to differentiate between the vaccine strain and wild-field strains. The present study provides sufficient data to suggest that this assay will be useful in clinical diagnosis and epidemiological studies of TGEV strains. These results have implications for the control of the disease as well as the study of its epidemiology. Further study will be needed to investigate whether the vaccine has been associated with clinical cases of disease (i.e., vaccine virus reversion) and whether or not these clinical cases can be attributed only to TGEV vaccine and not other viruses. The efficacy of our RFLP assay will need to be confirmed by subsequent animal experiments, if such reversions are observed.

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