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ORF3a deletion in field strains of porcine-transmissible gastroenteritis virus in China: A hint of association with porcine respiratory coronavirus

X. Zhang[1†] | Y. Zhu[1†] | X. Zhu[1] | J. Chen[1] | H. Shi[1] | D. Shi[1] | H. Dong[1,2] | L. Feng[1]

[1]State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences, Harbin, Heilongjiang, China

[2]Molecular Biology, Gembloux Agro-Bio Tech, University of Liège, Liège, Belgium

Correspondence
L. Feng, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences, Harbin, Heilongjiang, China.
Emails: fl@hvri.ac.cn, fengli_h@163.com

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Summary

Porcine-transmissible gastroenteritis virus (TGEV) is a pathogenic coronavirus responsible for high diarrhoea-associated morbidity and mortality in suckling piglets. We analysed the TGEV *ORF3* gene using nested polymerase chain reaction and identified an *ORF3a* deletion in three field strains of TGEV collected from piglets in China in 2015. Eight TGEV *ORF3* sequences were obtained in this study. Phylogenetic tree analysis of *ORF3* showed that the eight TGEV *ORF3* genes all belonged to the Miller cluster. CH-LNCT and CH-MZL were closely correlated with Miller M6, while CH-SH was correlated with Miller M60. These results thus indicate that the existence of Miller, as well as the Purdue cluster, in Chinese field strains of TGEV. Furthermore, we found the first evidence for a large deletion in *ORF3* resulting in the loss of *ORF3a*, previously reported in porcine respiratory coronavirus, in three field strains (CH-LNCT, CH-MZL, and CH-SH) of TGEV. The results of the present study thus provide important information regarding the underlying evolution mechanisms of coronaviruses.

KEYWORDS
deletion, *ORF3a*, transmissible gastroenteritis virus

1 | INTRODUCTION

Porcine diarrhoea can lead to weight loss and death in piglets. Coronaviruses (CoVs) are a major cause of porcine diarrhoea in pig herds worldwide, leading to important economic losses (Zuniga, Pascual-Iglesias, Sanchez, Sola, & Enjuanes, 2016). Coronaviruses comprise four genera: alpha-, beta- (lineages A–D), gamma-, and delta-CoVs. The CoV antigen responsible for causing diarrhoea in swine has been identified (Ma et al., 2015). Porcine-transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhoea virus (PEDV), and a natural TGEV deletion mutant porcine respiratory virus (PRCV) are alpha-CoVs. Porcine hemagglutinating encephalomyelitis virus is a beta-CoV, and porcine deltacoronavirus is a delta-CoV. Coronaviruses have high mutation rates resulting in changes in tissue tropism, transmission pathways, and host specificities (Ma et al., 2015). Transmissible gastroenteritis virus is a member of the *Coronaviridae* family responsible for severe diarrhoea in suckling piglets (about 2 weeks old). It is an enveloped virus with a positive-sense RNA genome of 28.5 kb, that was first reported in the USA in 1946 (Doyle & Hutchings, 1946). Transmissible gastroenteritis virus is currently divided into two distinct genogroups: the Miller cluster and the Purdue cluster (Hu et al., 2015). Transmissible gastroenteritis virus occurs in suckling piglets in swine-producing areas in China (Weiwei et al., 2014). The TGEV genome was first sequenced by the analysis of polymerase chain reaction (PCR) products in 1993 (Chen, Pocock, & Britton, 1993).

The TGEV genome encodes nine proteins, including four structural proteins (spike, membrane, nucleocapsid, and envelope) and

[†]These authors contributed equally to this paper.

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five non-structural proteins (polyprotein 1a, polyprotein 1ab, ORF3a, ORF3b, and 7). Porcine respiratory virus is an *S* gene deletion mutant of TGEV first identified in Belgium in 1984 (Pensaert, Callebaut, & Vergote, 1986). Porcine respiratory virus can replicate in respiratory tissues in infected piglets and demonstrates selective tropism with very little replication in intestinal tissues (Wang & Zhang, 2015). Porcine respiratory virus was identified as a TGEV mutant because of their antigenic and genetic relationships, though sequence analysis showed heterogeneity in the *S*, *ORF3a*, and *ORF3b* genes between TGEV and PRCV (Vaughn, Halbur, & Paul, 1995). Sequence analysis of TGEV strains also showed that large deletions or insertions in the *ORF3* gene region (Kwon, Saif, & Jackwood, 1998). Given the potential involvement of the TGEV *ORF3* gene in virus virulence or tropism, it is necessary to establish a method for its characterization.

Reverse transcription PCR (RT-PCR) was used to detect TGEV in 1991 (Page, Mawditt, & Britton, 1991), while nested PCR (nPCR) has been used to detect nucleic acids in members of the family *Coronaviridae* including PEDV (Chen et al., 2013), feline infectious peritonitis virus (Gamble, Lobbiani, Gramegna, Moore, & Colucci, 1997), canine coronavirus (Pratelli, Tempesta, Greco, Martella, & Buonavoglia, 1999), and TGEV (Rodriguez et al., 2012). Multiplex nPCR has also been used to differentiate between PEDV and TGEV infections (Jung, Kim, Kim, Kim, & Chae, 2003). In this study, we developed an nPCR-based diagnostic field test for the rapid detection of TGEV infection in farmed pigs. Furthermore, we detected TGEV field strains belonging to Miller cluster in China, and also provided the first evidence for a large deletion in ORF3 resulting in the loss of *ORF3a* deletion in China.

2 | MATERIALS AND METHODS

2.1 | Detection of viral RNA and sequencing

Samples or contents of the small intestine were collected from piglets with watery diarrhoea and dehydration at 44 pig farms in China in 2015. Samples were diluted with phosphate-buffered saline to make 10% (v/v) suspensions. The suspensions were vortexed for 1 min and clarified by centrifugation for 10 min at 5,000 *g* and the supernatants were collected for RT-nPCR. A volume of 30 μl of viral RNA was extracted from 140 μl of supernatants using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), and first-strand cDNA was synthesized with M-MLV reverse transcriptase (Promega, Madison, WI, USA) using Oligo d(T)18 (TaKaRa, Dalian, China), according to the manufacturers' instructions. Two primer pairs, TGE-ORF3-F1 and TGE-ORF3-F2, and TGE-ORF3-F1n and TGE-ORF3-F2n (Table S1), were designed and synthesized for RT-nPCR according to the corresponding sequence of TGEV strain H16 (GenBank No. FJ755618). First PCR was performed in a total volume of 25 μl containing 10 ng of cDNA template, 1× EmeraldAmp PCR Master Mix (TaKaRa), and a 0.5 μM concentration of each primer. After initial denaturation at 95°C for 5 min, amplification was performed for 30 cycles, each consisting of denaturation at 95°C for 30 s, annealing at 50°C for 45 s, and elongation at 72°C for 60 s. Second PCR was performed in a total volume of 25 μl containing 1 μl of product of the first PCR, 1× EmeraldAmp PCR Master Mix (TaKaRa), and a 0.5 μM concentration of each primer. After initial denaturation at 95°C for 5 min, amplification was performed for 30 cycles as the described for the first PCR. The outer span, including primers TGE-ORF3-F1 and TGE-ORF3-F2, was 1602 base pairs (bp), and the inner span, including primers TGE-ORF3-F1n and TGE-ORF3-F2n, was 1417 bp. Fragments containing the full-length TGEV *ORF3* gene were amplified from samples using these primers. Furthermore, a pair of primers designated TGEV-d-F1 and TGEV-d-F2 was created to allow the rapid detection of TGEV field strains. PCR products were excised from 1.0% agarose gels, purified using an AxyPrep™ DNA Gel Extraction Kit (Axygen Scientific, Inc., CA, USA), cloned into the pMD18-T, and introduced into *Escherichia coli* DH5α competent cells (TaKaRa) by transformation, according to the manufacturer's instructions. Eight recombinant DNA clones were sequenced by Comate Bioscience Company Limited (Jilin, China) and aligned using the MUSCLE method in MEGA5 software. A Construct/Test Maximum Likelihood Tree was obtained in Phylogeny analyse (a function of the MEGA software). Virus sequences used in this study are shown in Table S2.

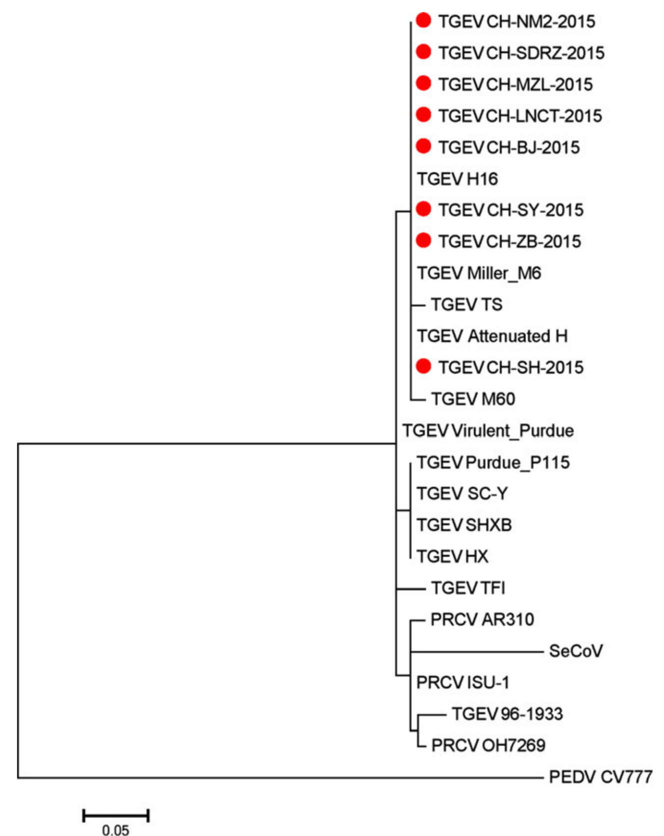

FIGURE 1 Phylogenetic tree based on the *ORF3* gene sequence of TGEV virus strain. Sequences were analysed with a Construct/Test Maximum Likelihood Tree using the MUSCLE method in MEGA5. ●Represents TGEV virus field strains in China in 2015. Scale bar indicates nucleotide substitutions. [Colour figure can be viewed at wileyonlinelibrary.com]

3 | RESULTS AND DISCUSSION

Eight TGEV *ORF3* sequences were obtained from eight farms in this study (Figure 1). The overall detection rate of TGEV in the samples by RT-nPCR was 8/44 (18.2%). All the sequences in this study have been deposited in the GenBank database. The field strains and their accession numbers are shown in Table S3. The identities among the sequences ranged from 98.6% to 99.9%. Phylogenetic tree analysis of *ORF3* showed that the eight TGEV *ORF3* genes all belonged to the Miller cluster (Figure 1). CH-SY and CH-ZB were closely correlated with Miller M6, which is a virulent TGEV strain, while CH-SH was correlated with Miller M60, which is an attenuated TGEV strain. Previous reports showed that the Purdue cluster also occurred in China (Weiwei et al., 2014). The current results thus indicate that the Miller, as well as the Purdue cluster, exists in Chinese field strains of TGEV. A large deletion (81 nucleotides [nt]) in *ORF3* was found in three strains (CH-LNCT, CH-MZL, and CH-SH) (Figure 2a). This may indicate that the *ORF3*-deleted and non-deleted TGEV field strains were both detected at farm in China.

We used the TGEV-d-F1 and TGEV-d-F2 primers to allow the rapid detection of TGEV strains with the 81-nt deletion. The PCR products of TGE-ORF3-F1 and TGE-ORF3-F2 were used as a template. The PCR products of CH-SDRZ, CH-SY, CH-ZB, and CH-BJ were 420 bp indicating no deletion, while those of CH-LNCT, CH-SH, CH-MZL were 339 bp, indicating the presence of the 81-nt deletion (Figure 2b). This result validated the previous sequencing results. Transmissible gastroenteritis virus is difficult to grow in cell culture, and virus lacking 3a replicates particularly poorly in cell cultures (Paul, Vaughn, & Halbur, 1997). We tried to isolate the *ORF3a*-deleted TGEV virus from samples prepared as above, but unfortunately were unable to do so (data not shown).

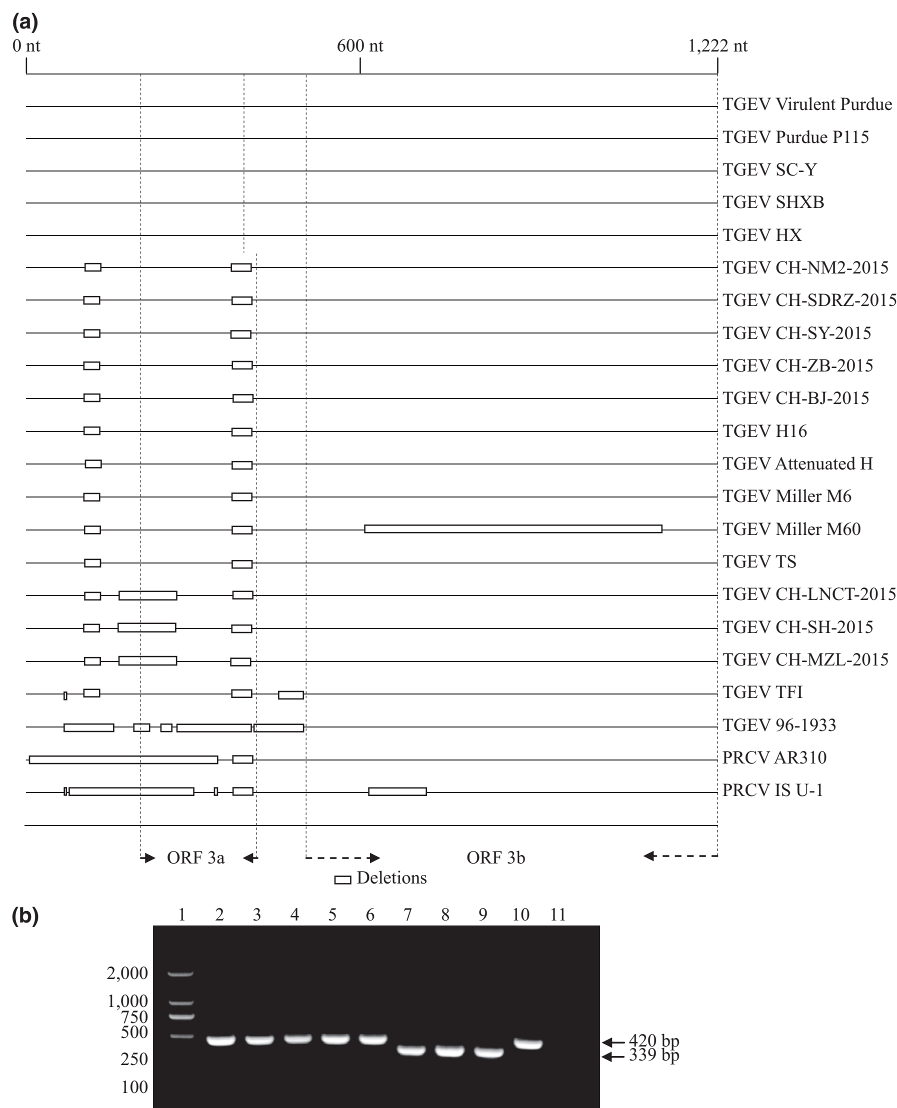

FIGURE 2 Deletions in *ORF3* (3a and 3b) in TGEV field strains and PCR confirmation. (a) Deletions in *ORF3* (3a and 3b) in TGEV field strains and other TGEV and PRCV strains. (b) PCR results of TGEV field strains with and without *ORF3a* deletion. Lane 1: DNA marker; lane 2: CH-SDRZ; lane 3: CH-SY; lane 4: CH-ZB; lane 5: CH-BJ; lane 6: CH-NM2; lane 7: CH-LNCT; lane 8: CH-SH; lane 9: CH-MZL; lane 10: Positive control (attenuated H); lane 11: negative control

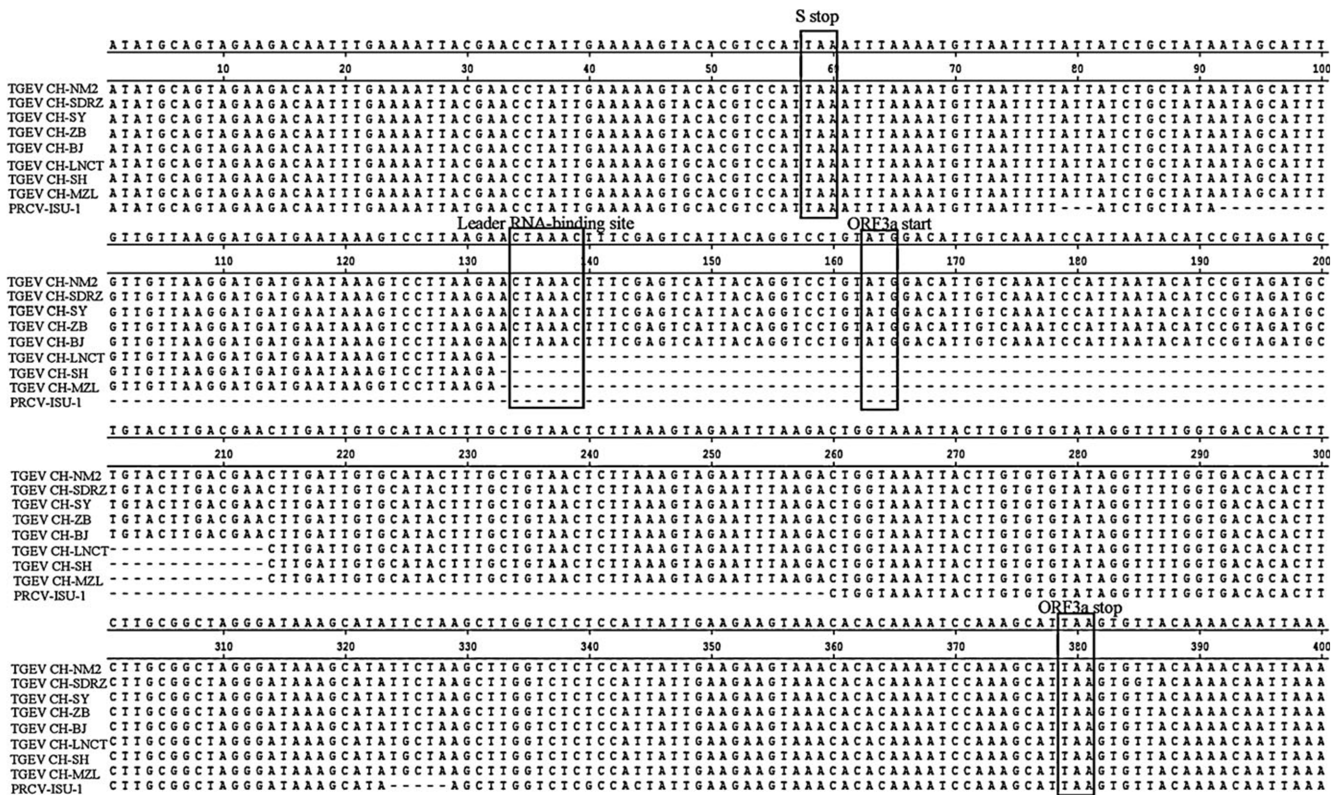

FIGURE 3 Deleted regions of leader RNA-binding site and ORF3a start site in TGEV virus field strains in China in 2015, and in PRCV strain ISU-1.

Previous reports detected a series of large deletions downstream of the TGEV *S* gene, resulting in loss of the transcription signal and the initiation codon of *ORF3a*. (McGoldrick, Lowings, & Paton, 1999). Similarly, a small plaque variant and two non-pathogenic cell culture-adapted strains of TGEV have deletions or insertions that alter *ORF3* (Zhang et al., 2007). These results suggest that the *ORF3* region of the TGEV genome is an important determinant for regulating viral virulence (Zuniga et al., 2016). Transmissible gastroenteritis virus strain 96-1933 is a virulent variant isolated in England in 1996 (Jones & Paton, 1996), including an intact *S* gene, but with a deletion in *ORF3a*. There have been no other relevant reports of *ORF3a*-deleted TGEV until the current study, which demonstrated an 81-nt deletion in *ORF3a*. However, the locations of the current and 96-1933 deletions differed; gene loss in 96-1933 was due to a discontinuity in *ORF3a*, while the deletion of TGEV *ORF3a* in the current study was continuous, which has not previously been reported in TGEV.

Some TGEV viruses belonging to the Miller group have been found in China (attenuated H and TS) (Li et al., 2010; Wang et al., 2010), while Purdue strains, such as TGEV HX and SHXB strains, are also prevalent in China (Hu et al., 2015; Weiwei et al., 2014), thus providing increased opportunities for reorganization. The leader RNA-binding site and *ORF3a* start site were included in the deleted region identified in TGEV the current study (Figure 3), and in the sequences of PRCV-ISU-1 (Lai, Welter, & Welter, 1995; Zhang et al., 2007). Previous studies on the genetic structures of PRCV and TGEV revealed two unique characteristics. First, the PRCV *S* gene lacks nt 621–681, resulting in a smaller S glycoprotein in PRCV than TGEV. Second, TGEV and PRCV differ in the *ORF3* region; the leader RNA-binding site (CTAAAC) in PRCV is altered or partially deleted before the *ORF3a* gene (Wang & Zhang, 2015).

A deletion occurring at a common break point was previously found in non-structural protein 3b of the naturally attenuated PRCV-ISU-1 and TGEV Miller M60 (Zhang et al., 2007). Sequence comparison showed that TGEV Miller strains had a closer relationship with PRCV-ISU-1 compared with Purdue strains. In this study, eight ORF3 sequences belonging to Miller cluster were identified in field strains of TGEV. Furthermore, a novel type of deletion within ORF3a was found in three TGEV field strains, suggesting that some of the differences among ORF3a sequences maybe with regard to PRCV. However, we have not yet succeeded in isolating *ORF3a*-deleted TGEV, and have therefore been unable to evaluate its pathogenic mechanisms in pigs to date. However, further studies are planned to isolate this virus.

Chimeric viruses termed swine enteric coronaviruses (SeCoVs) were recently found in Central Eastern Europe (Belsham et al., 2016) and Italy (Boniotti et al., 2016) in 2016. We examined the relationship between *ORF3* in SeCoVs and the *ORF3* detected in this study by comparing the TGEV *ORF3* sequences with the *ORF3* gene of SeCoVs. As shown in Figure 1, the TGEV *ORF3* sequences were not closely related to the *ORF3* gene of SeCoVs, which was in turn closely related to PRCV AR310 and ISU-1.

4 | CONCLUSIONS

In summary, we characterized eight ORF3 genes in TGEV field strains from China. We also provided novel evidence for the existence of Miller cluster TGEV field strains in Chinese pig populations, indicating the complexity of TGEV field strains in China. Interestingly, we also identify a novel deletion in within ORF3a in three field strains of TGEV, similar to the deletion found in PRCV, thus reflecting the relationship between the TGEV and PRCV. The detected-*ORF3a* deletion in the CH-LNCT, CH-MZL, and CH-SH strains provides the basis for further evaluation pathogenicity and replication of TGEV, and further studies are needed to determine the role of TGEV *ORF3a*-deletion field strains in porcine diarrhoea using an animal model. The results of the present study thus provide important information regarding the evolution of coronaviruses.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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