Characterization and evaluation of the pathogenicity of a natural recombinant transmissible gastroenteritis virus in China

Rongli Guo[a,b,c], Baochao Fan[a,b,c,d], Xinjian Chang[a,b,c], Jinzhu Zhou[a,b,c], Yongxiang Zhao[a,b,c], Danyi Shi[a,b,c,e], Zhengyu Yu[a,b,c], Kongwang He[a,b,c,**], Bin Li[a,b,c,f,*]

[a] Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, Key Laboratory of Veterinary Biological Engineering and Technology, Ministry of Agriculture, Nanjing, Jiangsu, 210014, China
[b] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou University, Yangzhou, Jiangsu, 225000, China
[c] Jiangsu Key Laboratory of Zoonoses, Yangzhou University, Yangzhou, Jiangsu, 225000, China
[d] Jiangsu Key Laboratory for Food Quality and Safety-State Key Laboratory Cultivation Base of Ministry of Science and Technology, Nanjing, 210014, China
[e] College of Animal Science and Technology, Hebei Normal University of Science and Technology, Qinhuangdao, 066004, China
[f] School of Food and Biological Engineering, Jiangsu University, Zhenjiang, 212013, China

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ABSTRACT

Porcine transmissible gastroenteritis virus (TGEV) is one of the major etiological agents of viral enteritis and fetal diarrhea in suckling piglets. In this study, a TGEV JS2012 strain was isolated from the feces of piglets in Jiangsu Province, China. The phylogenetic analysis showed that TGEV JS2012 was placed between the Purdue and the Miller clusters. Analysis of recombination confirmed that TGEV JS2012 is a natural recombinant strain between Miller M6 and Purdue 115. Similar to Miller M6, virulent Purdue and China strain TS, in S gene the JS2012 maintained genetic integrity and the characteristics of the TGEV virulent strains. In vivo, TGEV JS2012 caused 100% mortality in newborn piglets, indicating the strong pathogenicity of this isolate. These results reveal that the JS2012 is a novel natural recombinant TGEV with high virulence. Our findings provide valuable information about genetic diversity and infection mechanism of the coronavirus family.

1. Introduction

Transmissible gastroenteritis virus (TGEV) is a member of the family *Coronaviridae* and an enveloped, single-stranded RNA virus that was first reported in 1946 in the US(Doyle and Hutchings, 1946). Since then, outbreaks of the disease have been reported in many swine-producing countries, including England, Japan, China, Belgium, Africa and Australia (Kemeny and Woods, 1977; Pritchard, 1987; Wood et al., 1981). It causes viral enteritis and severe diarrhea with high morbidity in pigs of all ages, especially fetal diarrhea in neonates, leading to high mortality in suckling piglets (Enjuanes et al., 1995). TGEV causes significant economic losses at swine-raising farms (Enjuanes et al., 1995; Wesley et al., 1991). There have been many recent reports of TGEV infection in China (Hou et al., 2012; Li et al., 2010; Zhang et al., 2017). Epidemiological investigations have shown that TGEV is often present in mixed infection with porcine epidemic diarrhea virus (PEDV) (Lu et al., 2015; Zhang et al., 2013; Zhu et al., 2017).

Although serologically unrelated, TGEV and PEDV cause intestinal infections that are difficult to differentiate clinically (Pensaert et al., 1981; Siddell et al., 1983). Both viruses belong to the family *Coronaviridae* and genus *Alphacoronavirus*. The genomic sequence length of TGEV is about 28.5 kb (Vaughn et al., 1995). The genome contains nine open reading frames (ORFs) encoding four traditional coronavirus structural proteins: receptor-binding spike glycoprotein (S); envelope protein (E); membrane glycoprotein (M); nucleocapsid protein (N); and five nonstructural proteins (replicase 1a and 1b, 3a, 3b, and protein 7) in the order 5′- replicase polyproteins (pp1a and pp1ab)-S-3a-3b-E-M-N-7-3′(Enjuanes et al., 2008). The spike (S) gene of TGEV is about 4.3 kb in length. It encodes a protein of 1450 amino acids (aa), composed of S1 and S2 external domains, transmembrane domain, C-terminal cytoplasmic domain, and signal peptide. Four major antigenic sites, A–D, have been characterized at the N terminus of S protein (Delmas et al., 1990). The S protein plays a role in virulence, growth adaptation, receptor binding, and virus–cell membrane fusion (Ballesteros et al., 1997; Collins et al., 1982; Jimenez et al., 1986; Krempl et al., 1997; Sanchez et al., 1999; Xiao et al., 2008). Mutation in

S protein may affect virulence and tropism of TGEV (Ballesteros et al., 1997; Sanchez et al., 1999; Zhang et al., 2007). ORF3 consists of ORF3a and ORF3b. Deletions in ORF3a are found in many TGEV strains and respiratory coronaviruses (Kim et al., 2000; McGoldrick et al., 1999). Previous studies have suggested the gene 3 play a potential role in virulence of swine enteric TGEV and respiratory coronaviruses (Paul et al., 1997). Although some studies have suggested that ORF3a is not essential for virulence (McGoldrick et al., 1999; Sola et al., 2003).

In this study, we isolated swine enteric coronavirus TGEV JS2012. Genetic analysis showed that JS2012 may be a natural recombinant virus and S gene had the genetic characterization of a TGEV virulent strain. Mortality of 100% in piglets inoculated with isolated TGEV JS2012 indicated that the strain was highly pathogenic. Detailed analysis about the recombination, genetic variation and pathogenicity of this virus provides essential information for further understanding the evolution of TGEVs. **te ev.**

2. Materials and methods

2.1. Clinical samples, cell and virus isolation

Porcine intestine samples were removed from piglets showing watery diarrhea and dehydration on a swine-raising farm in Jiangsu Province, China. Intestinal samples were confirmed as positive for TGEV by RT-PCR, and placed in phosphate-buffered saline (PBS; 0.1 M, pH 7.2) to form 10% (v/v) suspensions. The suspensions were repeatedly frozen and thawed three times, vortexed by centrifugation for 10 min at 12000 rpm, filtered through a 0.22-μm syringe filter (Millipore, Billerica, MA, USA) and used as inocula. ST cells (American Type Culture Collection) were grown as a monolayer in growth medium, which was Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FBS; Life Technologies). The cells were cultured at 37 °C in a 5% CO_2 incubator. Growth medium was removed from confluent monolayer cells; the cells were washed twice with DMEM and inoculated with a mixture of the above inoculum and DMEM containing 20 μg/ml trypsin at a ratio of 1: 1. After adsorption for 60 min at 37 °C, the cells were washed with DMEM, and maintenance medium consisting of DMEM supplemented with 10 g/ml trypsin was added. The inoculated cell cultures were observed for CPE for 3–5 days, harvested, and blindly passaged for several generations. After CPE was observed, the virus was purified by picking CPE plaques three times and passaged. Finally, viral titers were determined using the Reed–Muench method.

2.2. Immunofluorescence microscopy

ST cells infected with TGEV JS2012 on 24-well plates were washed twice with PBS, fixed with acetone, and blocked with PBS containing 5% bovine serum albumin. A TGEV-specific monoclonal antibody (produced in our laboratory, and the immune-purified prokaryotic expression protein was N protein of TGEV SHXB strain; GenBank: KP202848) at 1:1000 dilution was added to the cells in each well and incubated for 1 h at room temperature. The cells were washed three times with PBS followed by incubation with secondary antibody (goat anti-mouse FITC, diluted 1:500) for 45 min. Finally, the cells were washed and visualized by fluorescent microscopy.

2.3. Electron microscopy

Electron microscopy for the detection of TGEV particles was carried out as previously described (Fan et al., 2017b). Supernatants from purified TGEV JS2012-infected cell cultures showing CPEs were negatively stained with 2% ammonium molybdate, and examined with an electron microscope (Hitachi H7500, Tokyo, Japan).

Table 1
The primers of 12 overlapping fragments used for amplification of JS2012 whole genome. The location corresponds to position within the WH-1(HQ462571.1)genome.

Primers name	Sequence
1-527-F	5′- ACTTTTAAAGTAAAGTGAGTGTAGC-3′
1-527-R	5′-ATCACTTACTCCGTTACCAA-3′
367-2561-F	5′-GTGCCTAGTCTTCCTATTCG-3′
367-2561-R	5′-CAGCCATCACAAACTACTTTCACA-3′
2419-4768-F	5′-AACATTCTTTGCCCTGATCCTCT-3′
2419-4768-R	5′-GTAACATTGACAGGGATAGAACA-3′
4710-7667-F	5′-CAGGGGATTGAATGTCTTCGTAT-3′
4710-7667-R	5′-CATGAAATGGTATCGTCAAAAGG-3′
7400-10477-F	5′-ACTCACATTTAATGCTGTAGGTT-3′
7400-10477-R	5′-AGTCAGATACAAAAGCAGATGGC-3′
10382–13539-F	5′-GTCTTACTACAAAGGATTGGATG-3′
10382–13539-R	5′-GACCTGGTTTTACTGTCTGATAT-3′
13469-16953-F	5′-CCAGCGTACTGTCTGTTTCTCCA-3′
13469-16953-R	5′-GTGGTATGTATTGTTCGCTCTTC-3′
16701-20472-F	5′-TCCGCTCAGGGTAGTGAGTATGA-3′
16701-20472-R	5′-ATGAGATTCCACTGGTTGCCTAT-3′
19388-23333-F	5′-ATTGGATTACTAAGGAAGGGT-3′
19388-23333-R	5′-TCAGCTATGTCATAACCACC-3′
23193-25077-F	5′-ATACATACTTCCGTCCCA-3′
23193-25077-R	5′-CACTTAATGCTTTGGGTTTT-3′
24806-28099-F	5′-TACGAGTCATTACAGGTC-3′
24806-28099-R	5′-ATAAATACAGCATGGAGG-3′
27911-28584-F	5′- CCATCAGAAGTGGCAAAAGAACA -3′
27911-28584-R	5′- TTTGTATCACTATCAAAAGGAAA -3′

2.4. Extraction of genomic TGEV RNA and reverse transcription

Viral RNA was extracted from TGEV JS2012 using TRIzol LS reagent (Invitrogen, USA). Viral cDNA was obtained by reverse transcription using Prime Script Reverse Transcriptase (TaKaRa,Japan).

2.5. Extraction of the viral genome sequence

The PCR primers (Table 1) were designed based on the sequence of TGEV strain WH-1 (HQ462571.1) using Primer 5. The total genome was divided into 12 segments with 100–300 bp overlap between each fragment. The PCR was performed using the Prime STAR GXL DNA Polymerase kit (TaKaRa, Japan). PCR products were visualized on 1% agarose gel electrophoresis and purified with the AxyPrep DNA Gel Extraction Kit (Axygen, Hangzhou, China), and cloned into the pEASY-Blunt Zero vector (Trans, Beijing, China). Three positive clones were selected and sequenced by a commercial service provider (Invitrogen, Shanghai, China).

2.6. Sequence analysis

The overlapping sequences of the PCR products were combined to obtain the complete genomic sequence of the TGEV JS2012 strain. Nucleotide sequences were analyzed using the CLUSTAL W program by MEGA version 6.0. Phylogenetic trees were constructed with MEGA 6.0 software using the neighbor-joining method. Bootstrap values were estimated for 1000 replicates, and the evolutionary distances were calculated using the Jukes–Cantor method.

2.7. Recombination analysis

We used RDP4 software, including RDP, Bootscan and SiScan, for recombination analysis to detect likely parental isolates and recombination breakpoints under default settings. Criteria for determining recombination and breakpoints were $P < 10^{-6}$ or a recombination score > 0.6.

2.8. Pathogenicity of TGEV JS2012 in newborn piglets without colostrum

We used 10 newborn piglets of both sexes without colostrum, without prior exposure to TGEV and free of TGEV antibodies. Ten newborn piglets were randomly allocated into the control group (n = 5) and challenged group (n = 5). The groups were separated by room and ventilation system within the same facility. TGEV-challenged newborn piglets were inoculated orally with 2 ml (10^6TCID$_{50}$/0.1 ml) of TGEV JS2012. The control group was inoculated orally with 2 ml DMEM. All animals were monitored daily for clinical signs of disease, including diarrhea and vomiting. Rectal swabs were collected for scoring fecal density (scores: 0 normal; 1 pasty stool; 2 semiliquid diarrhea; and 3 liquid diarrhea) and for enumerating fecal viral RNA shedding by quantitative RT-PCR. The primers and probes targeting conserved regions of the TGEV nucleocapsid protein gene were designed (accession no. DQ811786). The sequences of primers and probe were as follows: forward, 5′-AGGGACAACGTGTCAGTT-3′; reverse, 5′-TCTGCATGAGGTCCAGTA-3′; probe, 5′-FAM-TCTTTCATTCTTCAAC CCCATAACCCTCCA- TAMRA-3′. The piglets were necropsied after the appearance of severe diarrhea or becoming moribund, and intestinal material from each piglet was collected and stored at −80 °C.

Some of the intestinal material was analyzed by quantitative RT-PCR. The specific primers were designed for the TGEV S gene and the sequence of the PCR product was compared with that of the TGEV JS2012 S gene. At the same time, intestinal material was analyzed for TGEV and PDCoV by virus-specific RT-PCR. For each jejunal section, VH/CD ratio was calculated as previously described (Jung et al., 2014). A portion of the jejunum was fixed in 10% neutral buffered formalin for histopathological and immunohistochemical examinations. The fixed tissue sections were evaluated for TGEV antigen by immunohistochemistry using a TGEV-specific monoclonal antibody (1:500 dilution, produced by our laboratory). **Lion of TGEVs.**

3. Results

3.1. Virus isolation and identification

A distinct cytopathic effect (CPE) was noted after two passages at 5 days in ST cells. The virus isolate designated JS2012 was Purified by picking CPE plaques three times in ST cells. As the number of passages increased, the time of CPE formation became shorter, and finally stabilized at 24 h post infection (hpi). At passage 12, the CPE was characterized by cell fusion, Cell rounding and shrinkage, and eventual detachment from the plastic surface (Fig. 1a). Infected cells were tested positive by fluorescent antibody staining (Fig. 1b). Electron microscopy of a negatively stained sample revealed that the viral particles were 80–120 nm in diameter. Surface projections characteristic of coronaviruses were observed (Fig. 1c). We serially passaged isolate JS2012 to 30 passages in ST cells. With the increase in the number of passages, the time of CPE formation was reduced from 72 to 24 hpi and the virus titers were measured. The infectious titer of the isolate gradually increased from 10^4 to $10^{8.125}$ TCID$_{50}$/ml (Fig. 1d).

3.2. Complete genome sequence of TGEV JS2012

A total of 28542 nucleotides (nt) were determined for TGEV JS2012 (GenBank accession number: KT696544), excluding the polyadenylated sequences, and exhibited the genomic organization typical of all previously sequenced TGEV strains. The genome consisted of a 314-nt 5′ nontranslated regions (NTR); 20345-nt ORF1a/1b gene (nt 315–12368 for 1a and 12326–20368 for 1b); 4350-nt S gene (nt 20365–24714); 1014-nt ORF3a/b gene (nt 24817–25035 for 3a and 25097–25831 for ORF3b); 249-nt E gene (nt 25818–26066); 789-nt membrane (M) gene (nt 26077–26865); 1149-nt N gene (nt 26878–28026); 237-nt ORF7 (nt 28032–28268); and 274-nt 3′ NTR. The ORF1a/1b gene encoded the viral RNA-dependent RNA replicase.

3.3. Phylogenetic tree and homology analysis

The complete genome sequence of the JS2012 strain was compared with that of 22 other TGEV reference strains. The phylogenetic tree of the complete genome and S gene indicated that the TGEV strains could be divided into variant and traditional TGEV groups (Fig. 2a and b, respectively) that revealed two distinct genotypes: variant and traditional TGEV strains. The recent strains from the US made up the variant TGEV group. All Chinese TGEV strains and representative early US strains belonged to the traditional TGEV group. The traditional TGEV group could be further divided into the Purdue and Miller subgroups. The Purdue subgroups included Purdue, Purdue P115, AYU, WH-1, SHXB, SC-Y, HX and AHHF. The isolated TGEV JS2012 strain as well as TS, H16, Attenuated H, Miller 60, Miller M6 and CN12 belonged to the Miller subgroups. Phylogenetic analysis of the complete genome sequence showed that the JS2012 strain was more closely related to the Miller subgroup than to the Purdue subgroup and more distant evolutionarily from the variant TGEV group, which included the recent US strains (Fig. 2a). Phylogenetic analysis of the S gene showed that the JS2012 strain was located in the Purdue strain subgroup (Fig. 2b), which indicated that recombination may have occurred in S gene.

Alignment of the TGEV JS2012 genome sequence with 22 TGEV reference strains showed that the whole genome of JS2012 shared the highest levels of nucleotide identity (99.9%) with US Miller M6, and it had 99.4%–99.9% and 98.6%–99.1% genome nucleotide identity with the Miller and Purdue genotypes, respectively. However, it shared only 96.8%–97.2% nucleotide identity with variant genotypes.

3.4. Genomic characteristics

The genomic nucleotide sequence alignments of the 22 TGEV strains revealed that there were 2 major regions of insertion or deletion in the whole genome of JS2012. A 16-nt deletion occurred between the S and ORF3a genes and a 29-nt deletion occurred in the ORF3a gene. These insertions or deletions also occurred in the genome of Miller and variant strains (Fig. 3). ORF1a, ORF1b, S, ORF3a,ORF3b, E, M, N and ORF7 genes of TGEV JS2012 strain encoded predicted proteins containing 4017, 2680, 1449, 72, 244, 82, 262, 382 and 78 aa, respectively. Each gene encodes the same number of amino acids as Miller M6, TS (Table 2).

In traditional Miller strains, in addition to the above 2 major regions of insertion or deletion, a 3-nt deletion in the S gene was found in H16, Attenuated H, Miller M60, and CN12 (Fig. 3). Similarly, a 3-nt deletion also occurred in the S gene of Purdue strain AHHF. In traditional Purdue strains, 1 major deletion and insertion also occurred in S gene. A 6-nt deletion in the S gene was found in the Purdue strains, except for Purdue and AHHF. No deletions or insertions were found in the ORF3a gene of the Purdue strains (Fig. 3), but the predicted ORF3a-encoded protein was shorter than that in other TGEV strains (Table 2).

In the variant group, there were 8 major regions of insertion or deletion. Three deletions occurred within ORF1a; 2 deletions occurred between the S and ORF3a genes; 1 deletion occurred in the ORF3a gene; 1 deletion occurred between the ORF3a and ORF3b genes; and there was also a single deletion in the M gene (Fig. 3); all of which were consistent with previous reports. Three deletions within ORF1a and 1 in the M gene led to loss of 4 aa within pp1a protein and 1 aa within M protein compared with other traditional TGEV strains (Table 2).

3.5. Comparative analysis of the S gene

The genomic nucleotide sequence alignments showed that there were no insertions or deletions in S gene of strain JS2012. The S gene of strain JS2012 was 4350 nt in length, encoding a predicted protein of 1449 aa. The S protein of JS2012 had the same length as that of Miller M6, Purdue, TS and all variant strains (Table 2). However, some amino acid mutations were found. There were 2 unique amino acid
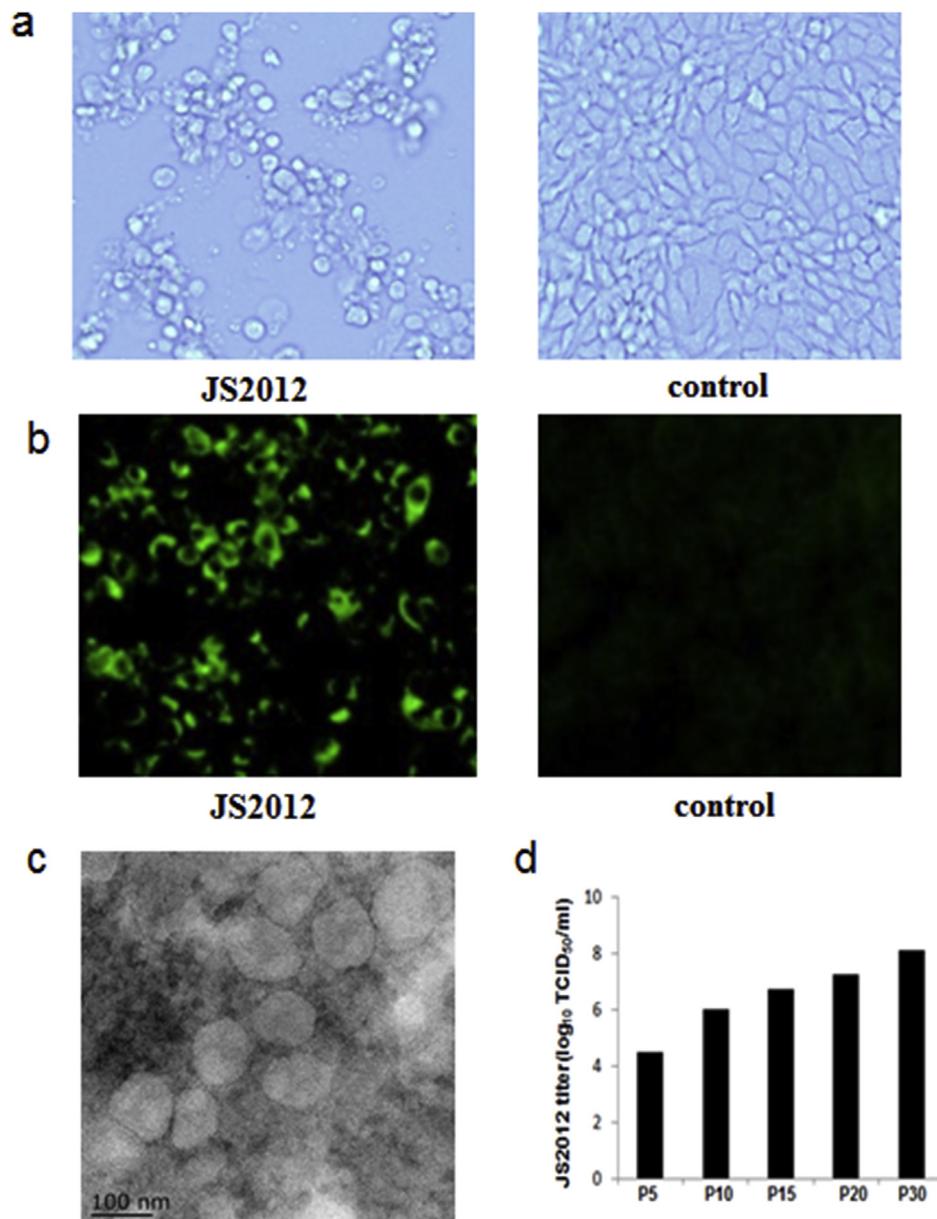
Fig. 1. Virus isolation and identification of TGEV strain JS2012. (a) The cytopathic effect of JS2012 on ST cells. (b) Fluorescence microscopy of JS2012 infection on ST cells. (c) Electron microscopy observation of JS2012. Scale bars = 50 nm. (d) Growth kinetics of JS2012 with different passages in ST cells.

substitutions at aa 35 and 397 among the S gene coding regions between JS2012 and all the reference strains (Fig. 4a). The isoleucine (I) residue at aa 418 was the same as that in TS and Miller M60 strains, but was different from that in other reference strains (Fig. 4a). It is worth noting that at nt 1753, a T-to-G mutation caused a serine-to-alanine mutation at aa 585, which may have weakened the virus virulence (Zhang et al., 2007). The nucleotide at position 1753 in the S gene of JS2012 was T, the same as in Miller M6, Purdue and TS, whereas it was G in the other TGEV strains (Fig. 4b). Therefore, there was no serine-to-alanine mutation at aa 585 in the S protein of TGEV JS2012, as in the three virulent strains Miller M6, Purdue, and TS (Fig. 4a).

3.6. Recombination analysis of TGEV JS2012

To analyze further the association between TGEV JS2012 and the existing isolates, genetic recombination analysis was completed using RDP4 software. According to TGEV genome organization (Fig. 5a), breakpoints for potential recombination zones were found at nt

23,240–24,307 of the complete genome, which was located at the end of S gene (Fig. 5b). The major parent strain was Miller 6, and the minor parent strain was Purdue P115 (Fig. 5b). The P value was 3.520×10^{-12} (Fig. 5b), and the recombination score was = 0.638 (Fig. 5c). These data met Criteria for determining recombination and breakpoints. These results indicate that TGEV JS2012 resulted from a natural recombination event between the Miller and Purdue clusters.

3.7. Clinical assessment

Ten newborn piglets without colostrum were active and fleshy prior to inoculation and had normal fecal consistency. At 12 h after inoculation, the TGEV-challenged group had clinical symptoms: eating less, diarrhea and depressed mood. As the disease developed, the piglets showed different degrees of diarrhea (Fig. 6a), accompanied by depression, Fasting, severe dehydration, difficulty breathing, vomiting, fecal stench and eventually death. Reverse transcription polymerase chain reaction (RT-PCR) of fecal samples revealed that all piglets in the
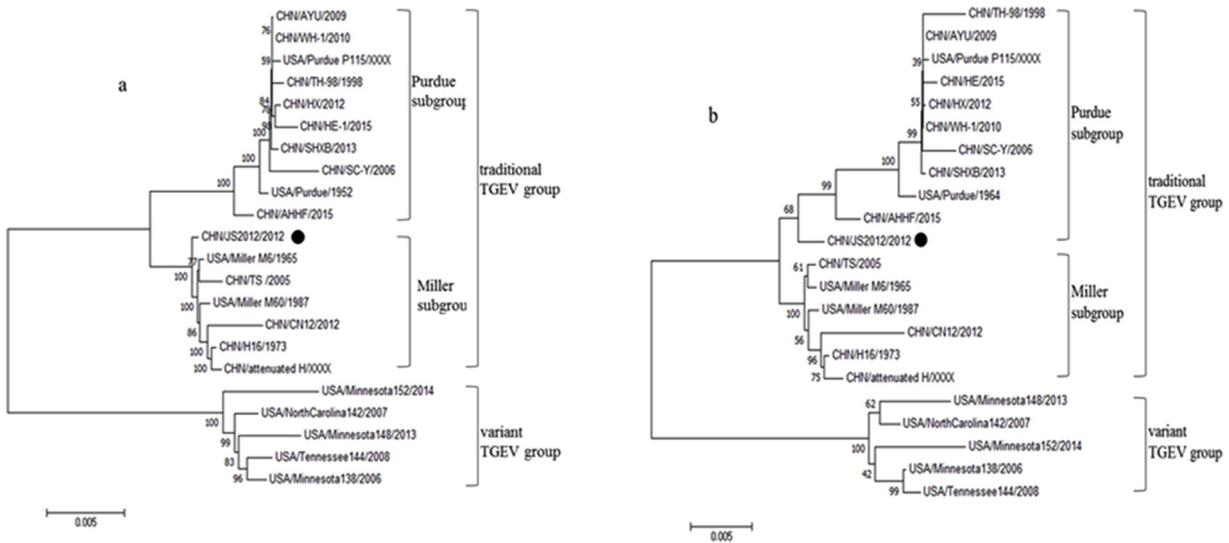
Fig. 2. Phylogenetic analysis based on the nucleotide sequence of the complete genome and S gene of TGEV strains. (a) Complete genome. (b) S gene. The trees were created using the neighbor-joining method in MEGA version 6.0, with 1000 bootstrap replicates to measure confidence of the groupings. The black origin indicates the new JS2012 reported in this study.

inoculated groups were positive for TGEV (3–11 log10 genomic equivalents/mL at 12–72 hpi) (Fig. 6b). Negative piglets remained active with normal feces, and fecal shedding of TGEV remained undetected throughout the study period. All piglets challenged with TGEV JS2012 had died by 72 hpi (Fig. 6c). These results reveal that TGEV strain JS2012 causes significant mortality in newborn piglets.

The infected and deceased piglets were dissected. Discernable salient features of viral infection were apparent in five challenged piglets. The small intestine was extremely inflated, full of yellow stench liquid, and the small intestine wall was thin and transparent. The stomach was distended and filled with curdled and undigested milk. The piglets were necropsied after the appearance of severe diarrhea or

Fig. 3. Visualization of genomic insertion and deletion regions of the 22 TGEV strains. The nucleotide sequences of 22 TGEV strains were analyzed using the CLUSTAL W program by MEGA version 6.06.

Table 2
Length in amino acids of the predicted structural and nonstructural proteins of 22 TGEV strains.

group	strain	Replicase 1a	Replicase 1b	S	ORF3a	ORF3b	E	M	N	ORF7
Traditional TGEV group Miller cluster.	CHN/JS2012/2012	4017	2680	1449	72	244	82	262	382	78
	CHN/TS/2005	4017	2680	1449	72	244	82	262	382	78
	USA/Miller M6/1965	4017	2680	1449	72	244	82	262	382	78
	CHN/H16/1973	4017	2680	1448	72	244	82	262	382	78
	CHN/AttenuatedH/XXXX	4017	2680	1448	72	244	82	262	382	78
	USA/Miller60/1987	4017	2680	1448	72	67	82	262	382	78
	CHN/CN12/2012	4017	2680	1448	50	244	82	262	382	78
Traditional TGEV group Purdue cluster	CHN/AHHF	4017	2680	1448	71	244	82	262	382	78
	USA/Purdue/1952	4017	2680	1449	71	244	82	262	382	78
	USA/PurdueP115/XXXX	4017	2680	1447	71	244	82	262	382	78
	CHN/AYU/2009	4017	2680	1447	71	244	82	262	382	78
	CHN/SHXB/2013	4017	2680	1447	71	244	82	262	382	78
	CHN/SC-Y/2006	4017	2680	1447	71	244	82	262	382	78
	CHN/WH-1/2010	4017	2680	1447	71	244	82	262	382	78
	CHN/HX/2012	4017	2680	1447	71	244	82	262	382	78
	CHN/HE-1/2015	4017	2680	1447	71	244	82	262	382	78
	CHN/TH-98/1998	4017	2680	1447	71	244	82	262	382	78
Variant TGEV group	USA/Minnesota152/2014	4013	2680	1449	72	244	82	261	381	78
	USA/Minnesota148	4013	2680	1449	72	244	82	261	381	78
	USA/Tennessee144/2008	4013	2680	1449	72	244	82	261	381	78
	USA/NorthCarolina142/2007	4013	2680	1449	72	244	82	261	381	78
	USA/Minnesota138/2006	4013	2680	1449	72	244	82	261	381	78
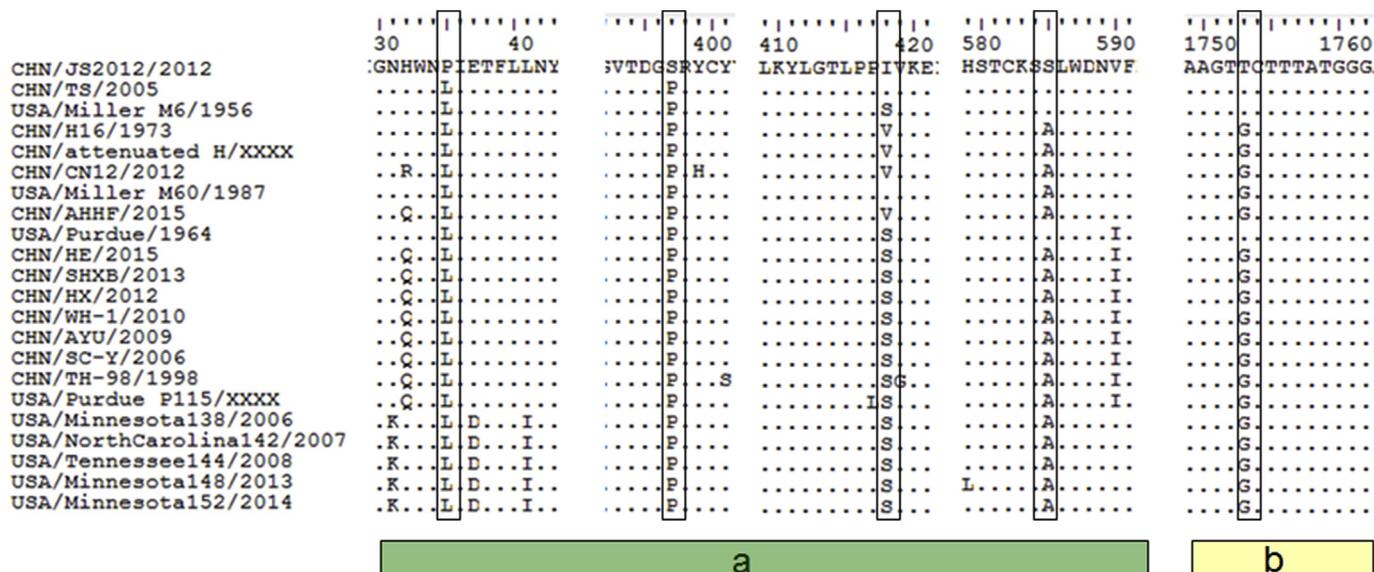
Fig. 4. Mutation in S protein and S gene of TGEV JS2012. (a) Mutation in S protein of TGEV JS2012. (b) T residue at nucleotide 1753 of JS2012 strains Virulent M6, Virulent Purdue, and TS, and G in the other strains. The amino acid and nucleotide sequences of the complete S gene of TGEV JS2012 and other TGEV strains were analyzed using the CLUSTAL W program by MEGA version 6.06.

becoming moribund, and intestinal material from each piglet was collected and stored at -80 °C. The intestinal material from each deceased piglet was analyzed by RT-PCR. The PCR product of TGEV S gene shared the same sequences as TGEV JS2012 S gene. The intestinal material was confirmed as negative for PEDV, porcine rotavirus and porcine deltacoronavirus (PDCoV) by virus-specific RT-PCR. Photomicrographs revealed necrosis and shedding of intestinal cells; and villous atrophy and fusion in challenged pigs (Fig. 7a). Immunohistochemistry for TGEV-N confirmed the presence of the virus in the cytoplasm of epithelial cells of atrophied villi in segments of the small intestines (Fig. 7b). Virus was also found in the intestinal crypts (Fig. 7b). The mean villous height/crypt depth (VH/CD) ratio of the jejunum of control piglets (7.02 ± 1.0) was higher than that of piglets inoculated with TGEV JS2012 (1.79 ± 0.5), and this difference was significant.

4. Discussion

TGEV infection in pigs was reported globally between the late 1980s and the 1990s (Zuniga et al., 2016; Kemeny and Woods, 1977; Pritchard, 1987; Wood et al., 1981; Woods and Wesley, 1986). In recent years, mixed infections have occurred frequently with TGEV and PEDV (Lu et al., 2015; Zhang et al., 2013; Zhu et al., 2017). In the present study, TGEV JS2012 strain was obtained from a farm with a diarrhea outbreak, in which TGEV and PEDV co-infection was detected by RT-PCR. Experimental infection indicated that the piglets had severe diarrhea with vomiting after TGEV JS2012 infection. Both infections cause destruction of villous enterocytes and villous atrophy in the jejunum and ileum (Kim and Chae, 2001; Madson et al., 2016). In TGEV JS2012 infection, viral nucleic acid was detected as early as 12 h after inoculation, as described previously (Kim and Chae, 2002). Piglets showed characteristic clinical symptoms at 12 h after inoculation and anal swab detection was positive, which indicated that virus had
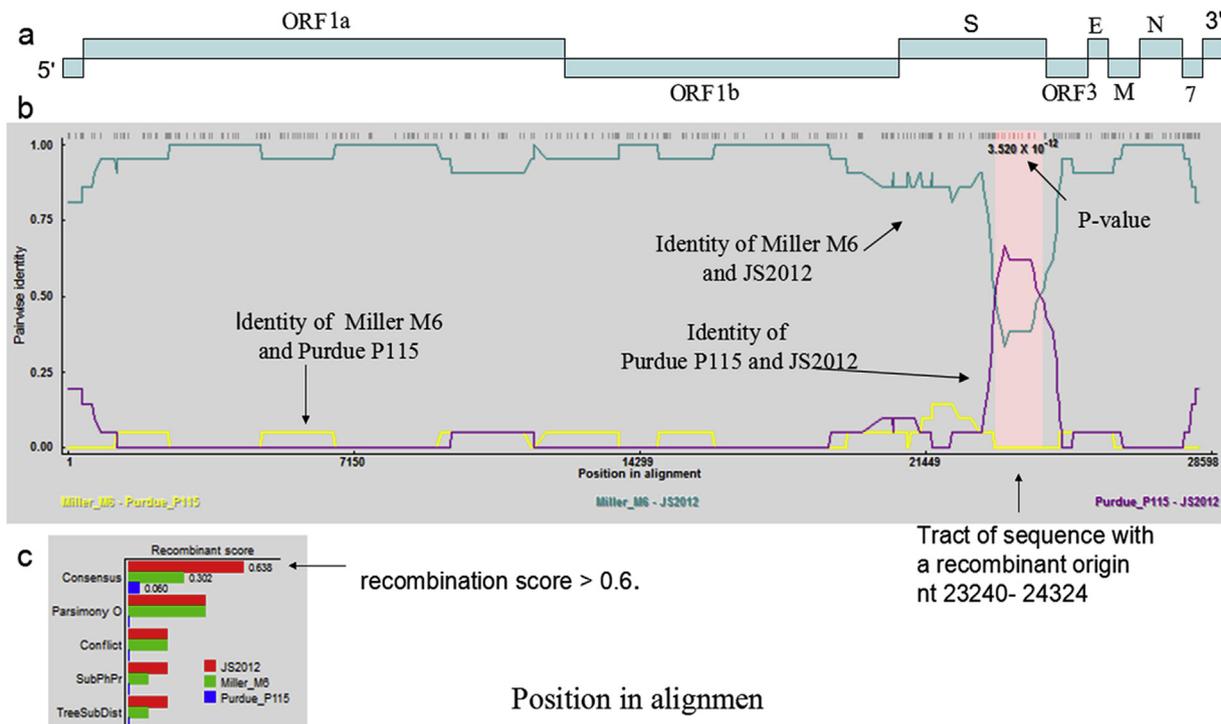
Fig. 5. Recombination analysis of TGEV JS2012 with other TGEV strains. (a) TGEV genome organization. (b) Recombination of Miller M6 and Purdue P115 located at nucleotides 23240–24324 of S gene. (c) Recombination score = 0.638.

colonized the small intestine 12 h after challenge. This phenomenon was similar to recent infections with virulent PEDV strains (Fan et al., 2017a; Lin et al., 2015; Madson et al., 2016). The significant differences showed that virus-positive cells are present in villous epithelial cells as well as the jejunal crypt enterocytes.

Our study found new genetic characteristics in the process of elucidating the molecular characterization and phylogeny of the virus. This classification indicates significant genetic diversity within TGEV strains. The traditional TGEV group could be further divided into two subgroups: Miller and Purdue. The phylogenetic analysis based on the genome demonstrated that JS2012 was clustered into the Miller subgroup. However, in the phylogenetic trees based on the S gene, TGEV JS2012 belonged to the Purdue subgroup. The recombinant analysis showed TGEV JS2012 is a recombinant virus between Miller M6 and Purdue P115. Purdue strain group viruses with high homology to attenuated strain Purdue 115, such as TGEV SHXB, HX, WH-1 and AYU are also prevalent in China, which providing increased opportunities for reorganization. The recombination breakpoints of TGEV JS2012 was located at the end of the S gene. The binding site of host cell receptor aminopeptide N and four major antigenic sites, A–D, are present at the N terminus of S protein (Delmas et al., 1990; Reguera et al., 2012),

which influences the antigenicity, growth adaptation, receptor binding, and cell membrane fusion of TGEV (Ballesteros et al., 1997; Collins et al., 1982; Jimenez et al., 1986; Krempl et al., 1997; Sanchez et al., 1999; Xiao et al., 2008). Meanwhile, the results of animal experiments suggest that this recombination does not affect the virulence of the virus.

The whole gene sequence analysis showed that there were two large deletions (16 and 29 nt, respectively) in the ORF3a gene of the Miller subgroup, which may distinguish the Miller and Purdue subgroup. Previous studies have suggested the gene 3 play a potential role in virulence of swine enteric TGEV and respiratory coronaviruses (Paul et al., 1997). However, animal experiments have shown that JS2012 has strong pathogenicity in piglets. These data proved that the two large deletions in the ORF3a gene are not necessary for viral virulence. This view is consistent with the report of McGoldrick and Sola (McGoldrick et al., 1999; Sola et al., 2003).

The S gene is another hypervariable region. The T to G mutation at nt 1753 of the S gene leads to the serine-to-alanine mutation at aa 585, which is located in the main major antigenic sites A/B of the TGEV S protein. This mutation may significantly influence receptor binding or interaction with neutralizing antibody and have a significant effect on
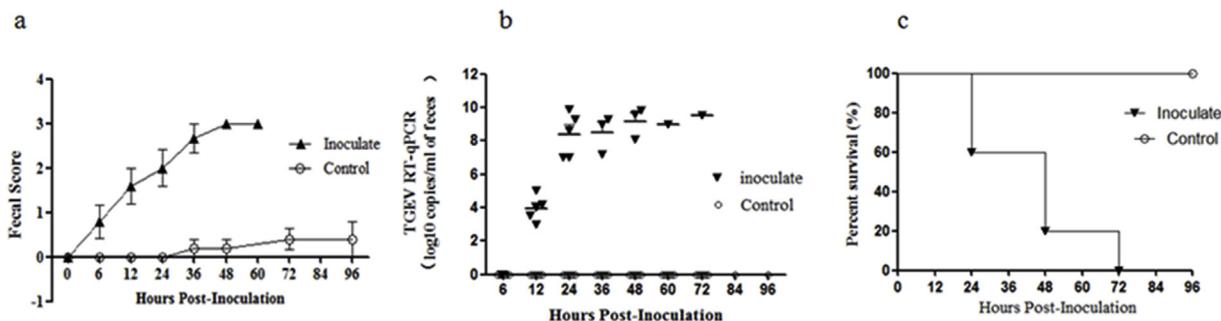
Fig. 6. Transmissible gastroenteritis virus JS2012 infection in newborn piglets. (a) Mean fecal scores after viral inoculation. (b) Mean quantitative reverse transcription polymerase chain reaction titers of fecal samples. (c) Survival curves of JS2012-infected clusters.
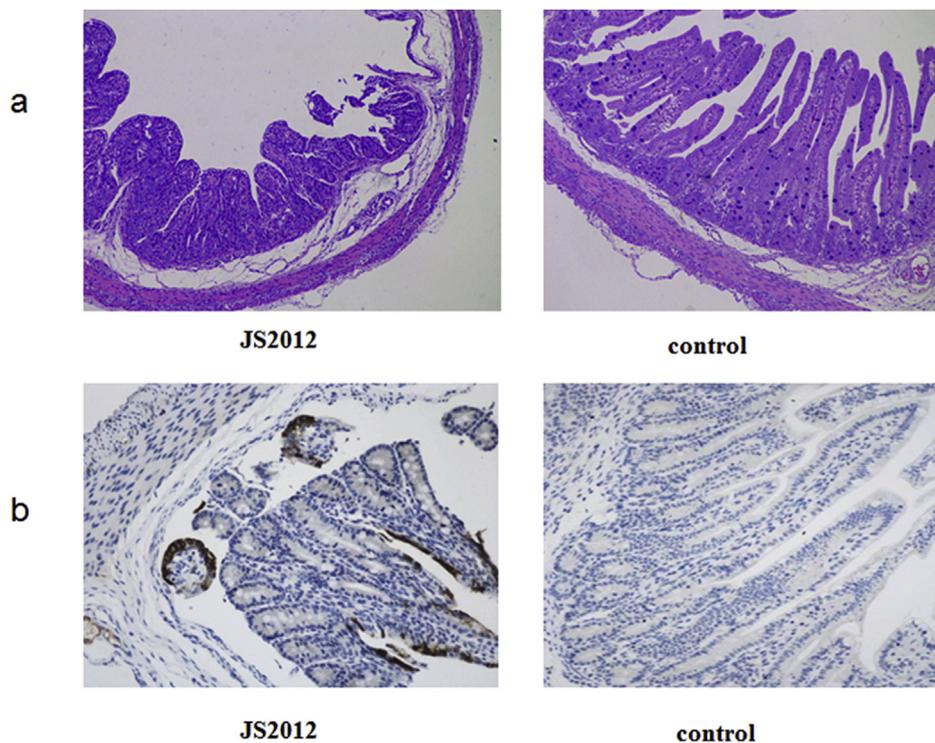
Fig. 7. Histology and IHC staining of TGEV JS2012-infected pig intestines. (a) HE-stained small intestines of piglets inoculated with JS2012 strains. (b) For IHC assays, the intestinal tissue sections were stained with a TGEV N monoclonal antibody (1:500 dilution). Positive cells presented in villous epithelial cells and the crypt enterocytes of Jejunum.

antigenicity, which is also considered to be a sign of attenuation (Li et al., 2010; Zhang et al., 2007). A 6-nt deletion is considered to play a role in viral attenuation (Penzes et al., 2001). There was no deletion or mutation in the S gene of JS2012. This predicted that JS2012 retained the genetic characteristics of the TGEV virulent strain and integrity of the S gene, and suggested that JS2012 was a virulent strain, which was further confirmed by the high pathogenicity in piglets in this study. Although there were 3 other amino acid mutations in S protein of JS2012, they were not located within the four major antigenic sites, regions A–D. The effect of these 3 amino acid mutations on this virus needs further research.

In conclusion, an epidemic field strain of TGEV JS2012 was isolated from a pig farm in China that had reports of severe diarrhea. The whole genome was obtained and analyzed. Characterization of the molecular biology and animal experiments confirmed that TGEV JS2012 is a novel natural recombinant virulent strain. These studies about the recombination, genetic variation and pathogenicity of this virus provide essential information for further understanding the evolution of TGEV, as well as other coronaviruses. tolution of TGE.

Ethics statement

The study and study protocol were approved by the Science and Technology Agency of Jiangsu Province. Approval was also granted by the Jiangsu Academy of Agricultural Sciences Experimental Animal Ethics Committee (approval ID NKYVET, 2015–2016). All efforts were made to minimize animal suffering. The immunization and challenge assays and the collection of serum and colostrum samples were performed in strict accordance with the guidelines of the Jiangsu Province Animal Regulations (Government Decree no. 45).

CRediT authorship contribution statement

Rongli Guo: Formal analysis, Writing - original draft. **Baochao Fan:** Formal analysis, Writing - original draft. **Xinjian Chang:** Methodology. **Jinzhu Zhou:** Methodology, Validation. **Yongxiang Zhao:** Investigation. **Danyi Shi:** Data curation. **Zhengyu Yu:**

Resources. **Kongwang He:** Supervision. **Bin Li:** Formal analysis, Writing - original draft.

Declaration of competing interest

The authors declare that they have no competing interests.

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