

# Isolation and Identification of a New Strain of Porcine Transmissible Gastroenteritis Virus from Chongqing, Southwestern China

Zhenhui, S.,\* Xianjin, D., Xinzhi, C., Yue, Z., Yuntian, L., and Yong, L.

Department of Veterinary Medicine, Southwest University, Rongchang Campus, Chongqing 402460, P.R. China.  
Zhenhui S. and Xianjin D. have made an equal contribution to this article.

\* **Corresponding Author:** Assistant Professor Zhenhui SONG, PhD., Department of Veterinary Medicine, Southwest University, Rongchang Campus, Chongqing 402460, P.R.China. Tel: 00-86-23-46751087, Mobile: 00-86-15736158518, Fax: 00-86-23-46751732. E-mail: szh7678@126.com

## ABSTRACT

Porcine transmissible gastroenteritis virus (TGEV) infections continue to cause significant losses in the swine industry. The objective of the study was to document the isolation a new strain of TGEV, information which may also contribute significantly to understanding molecular epidemiology and evolution of TGEV in China. In this study, a suspected transmissible gastroenteritis virus (TGEV) strain was isolated from faeces of sick piglets in Chongqing (China) using swine testis (ST) cell lines. With the total RNA extracted from infected ST cells, the M gene of TGEV was amplified by polymerase chain reaction (PCR). The virus was named as the CQ strain, and was also identified by homology analysis, cell cytopathic effects (CPE), indirect fluorescent antibody test (IFA) and electron microscopy (EM). The results indicated that this virus was TGEV by a series of identification tests. The membrane proteins M gene was cloned and sequenced. Alignment with other 21 reference strains of TGEV and 1 strain PRCV in GenBank, showed that the homology was 94.3-99.6% for nucleotide sequence, 92.4-98.9% for amino sequence, respectively. The phylogenetic analysis has indicated that TGEV strains can be classified into two clusters, the CQ strain belonged to cluster I, and shared closest relationship with Purdue-P115.

**Keywords:** Identification; Isolation; Southwestern China; Transmissible gastroenteritis virus; Sick piglets

## INTRODUCTION

Transmissible gastroenteritis virus (TGEV) is the etiological agent of transmissible gastroenteritis (TGE), which is a condition associated with high morbidity in pigs of all ages and high mortality in sucking piglets. TGEV is a member of the coronaviridae family, which possesses a large 28.5 kb single-stranded sense RNA genome, and is comprised of four structural proteins encoded by the spike (S), membrane (M), envelope (sM), and nucleoprotein (N) genes. The S protein forms the peplomers on the virion envelope and carries major antigenic sites which induce neutralizing antibodies (1, 2). The M protein is embedded in the lipid envelope, taking part in virus particle assembly, and the N protein is associated

with the genomic RNA to form the nucleocapsid, inducing cell immunity to infected animals. The small sM protein is localized in the perinuclear region of infected cells, and is expressed on the cell surface, possibly causing apoptosis (3-6).

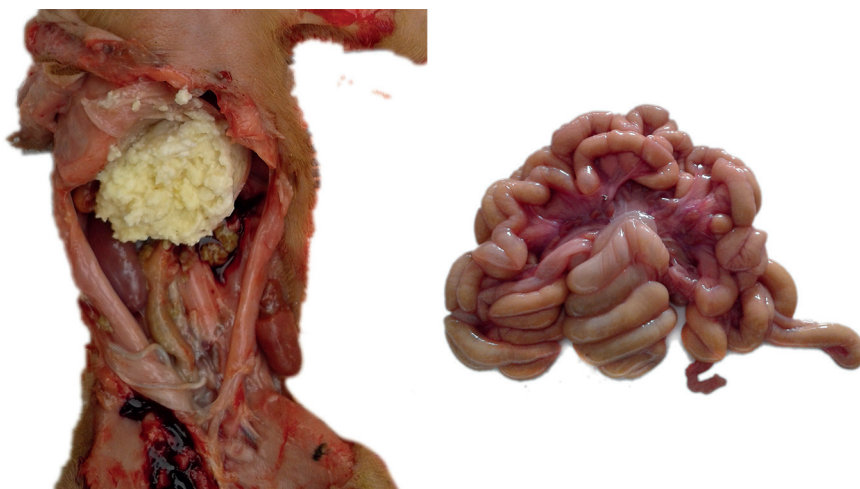
TGEV has been reported in many swine producing countries between the late 1980's and the 1990's (7-9). In China, an outbreak of porcine transmissible gastroenteritis was first reported in the 1970's. Since then, the disease has been prevalent in the country. Vaccination is an effective prophylactic measure. Although there are many commercial vaccines, the traditional inactivated vaccines have many deficiencies. Therefore, the disease is still a major problem in the swine industry in China (10, 11).

The main clinical symptoms of the disease in the infected piglets are diarrhea and vomiting. TGE occurs in most of swine-raising farms with death in 100% of suckling pigs less than 1 week old resulting in severe economic losses (12). Although TGE occurs frequently in China, the TGEV strains isolated from Southwest China had rarely been reported (13). Until now, the characterization of TGEV strains isolated from Hebei, Sichuan, Fujian, Jilin provinces have been reported in the years of 2010–2013 (14–16). Information about the history, incidence and prevalence of the TGE occurring southwestern China is limited. Here, we report the TGEV strain CQ isolated from infected piglets in Chongqing province in southwestern China by using isolation in cell cultures, fluorescent antibody assay, electron microscopy and PCR amplification. The key objective of this work was to detect TGEV from clinically suspected diseased piglets based on serological and molecular basis, which may help to reveal the epidemiology, diversity, and evolution of isolated strains in China, and potentially facilitate the development of more effective preventive measures for TGEV.

## MATERIALS AND METHODS

### Virus isolation

**Samples:** Thirty two samples of faeces were collected from sick piglets with symptom for diarrhea, vomiting and dehydration in the winter months, which may have been infected with TGEV according to clinical signs (Figure 1). A total of 1g of faeces was homogenized in 4 ml of phosphate-buffered saline (PBS, pH 7.2) and then freeze-thawed three times



**Fig 1:** Pathological changes from the intestines of a sick piglet with suspected TGEV.

and then centrifuged at 5000 rpm for 30 min. 1 mL of the supernatant (1:10 dilution of samples) was filtered and inoculated into monolayer cultures of swine testis (ST) cells for 1 hour. Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum up to 5ml was then added. The ST cultures were observed for 5–7 days for the presence of cytopathic effects (CPE). The cultures showing cytopathic effects were frozen and thawed, supernatants were collected after centrifugation with subsequent storage at  $-70^{\circ}\text{C}$  for virus isolation.

### Identification of the virus

TGEV was detected by indirect fluorescent antibody test (IFA) and electron microscopy (EM). For IFA, 100 TCID<sub>50</sub> of TGEV was inoculated to 24-well tissue culture plates containing confluent ST cell monolayer. The plates were incubated at  $37^{\circ}\text{C}$  with 5% CO<sub>2</sub>. When CPE was observed in these wells, the supernatant was removed and the cell sheet fixed in cold acetone for 20 min at  $-20^{\circ}\text{C}$  and stained by an IFA technique (17).

For electron microscopy, the virus released from cell culture was mounted on grids and negatively stained in 2% phosphotungstic acid and observed under transmission electron microscopy.

### RNA extraction, PCR amplification and sequencing

Total RNA was isolated from infected ST cells using TRIZOL reagent (*TaKaRa* Biotechnology (*Dalian* Co., Ltd., China)) according to the manufacture's protocol. In short, 200  $\mu\text{l}$  of infected ST cell was incubated with 1 ml of TRIZOL for 5 min at room temperature. 0.2 ml of chloroform was added. After vortexing, the mixture was incubated for 5 min at room temperature. The phases were separated by centrifugation ( $12,000\times g$  at  $4^{\circ}\text{C}$  for 15 min) and the aqueous phase was transferred to a new ependorf tubes.  $1\times$ volume of isopropyl alcohol and  $0.1\times$ volume of 3 M sodium acetate were added to this aqueous phase and incubated for 30 min at  $-20^{\circ}\text{C}$ .

The precipitated RNA was pelleted by centrifugation ( $12,000\times g$  at  $4^{\circ}\text{C}$  for 15 min), and after the removal of super-

nant, the RNA pellet was washed twice with 70% ethanol. After drying, the RNA was resuspended in 25  $\mu$ l of DEPC-treated water. Using mRNA as template, single-stranded cDNAs were generated by AMV (*TaKaRa* Biotechnology (*Dalian* Co., Ltd., China)) according to the manufacturer's directions. The primers sequences used in this study were as follows: Sense primer: 5'-GGGGGATCCCCACCATGAAGATTTTGTTAAT-3'; Antisense primer: 5'-GGGGzAATTCTTATACCATATGTAATAATTTTCTTG-3'. All primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The PCR conditions were 94°C for 2 min, followed by 30 cycles of DNA amplification (30 s at 94°C, 30 s at 55°C, and 1 min at 72°C) and 10 min incubation at 72°C. The PCR products were separated by electrophoresis, purified and cloned into the pMD18-T vector (*TaKaRa* Biotechnology (*Dalian* Co., Ltd., China)) and sequenced by using classical dideoxy Sanger sequencing (*TaKaRa* Biotechnology (*Dalian* Co., Ltd., China)).

### Analysis of sequence

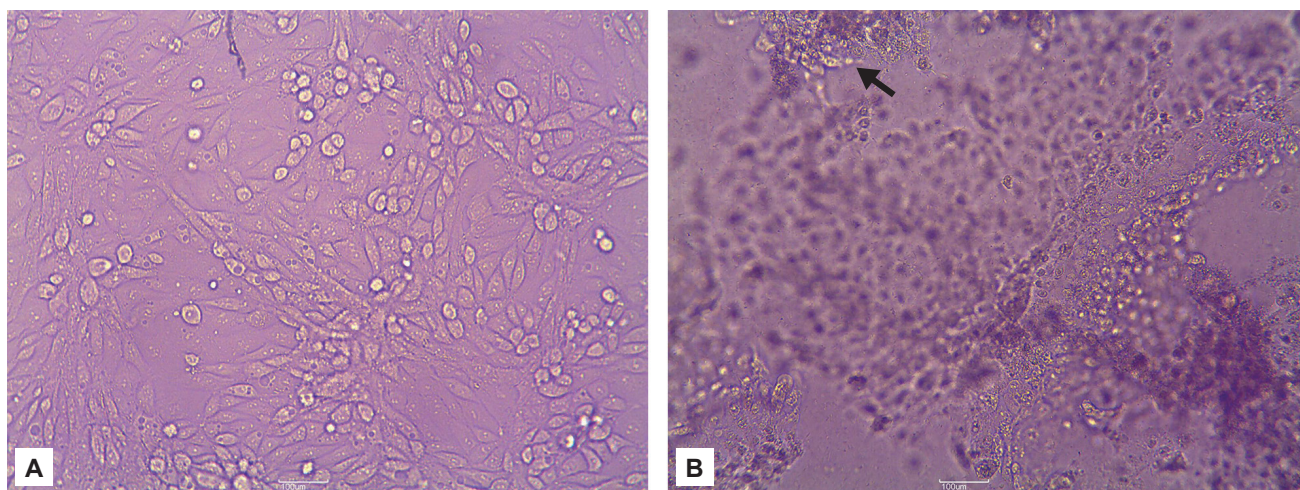
Homologous comparisons of the M gene nucleotide sequences and deduced amino acids of the CQ strain with those of other 23 virus strains (including TGEV 22 strains: 96-1993 (AF104420); 133 (AF481365); attenuated H (EU074218); AYU (HM776941); DAE (JQ693054); FJ (JQ700303); H16 (FJ755618); HN2002 (AY587883); HX (KC962433); KT2 (JQ693055); KT3 (JQ693056); Miller M6 (DQ811785); Miller M60 (DQ811786); PUR46-MAD (AJ271965);

Purdue P115 (DQ811788); SC-Y (DQ443743); TFI (Z35758); TO14 (AF302262); TS (AY335549); Virulent Purdue (DQ811789); WH-1 (HQ462571), PRCV 1 strain: PRCV ISU-1 (DQ811787) were performed with DNASTar software (<http://www.dnastar.com>). A phylogenetic tree was generated based on M protein sequences of reference TGEV strains, Bovine coronavirus 1 strain 179-07-11 (EU019216), Feline coronavirus 1 strain 79-1683 (AB086904) and Canine coronavirus 1 strain HF3 (AY864661) by applying the distance-based Neighbor-joining method in the software MegAlign (DNASTAR Inc., Madison, WI, U.S.A.).

## RESULTS

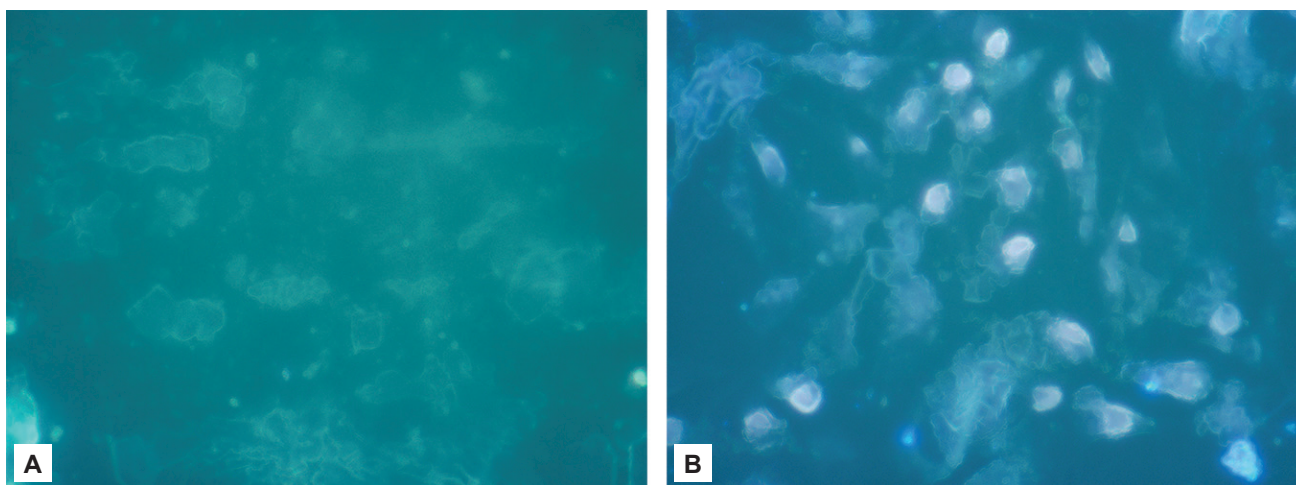
### Isolation and identification

Thirty two samples with a clinical diagnosis of suspected TGE were subjected to TGEV RT-PCR assays. Twenty samples tested resulted in the expected band sizes of amplified products (data not shown). The isolation of TGEV from one sample positive for RT-PCR testing was obtained by ST cell cultures. The primarily cytopathic effects (CPE) were observed in ST cell infected with 10 passages virus. In comparison to the control cells, cells with cytopathic changes showed cell rounding, strong refraction, cell aggregation and coalescence to form clusters after 48 h of post inoculation which gradually increased to affect 95% of cells which became completely detached. (Figure 2). The indirect fluorescent antibody test (IFA) showed that specific fluorescence appeared



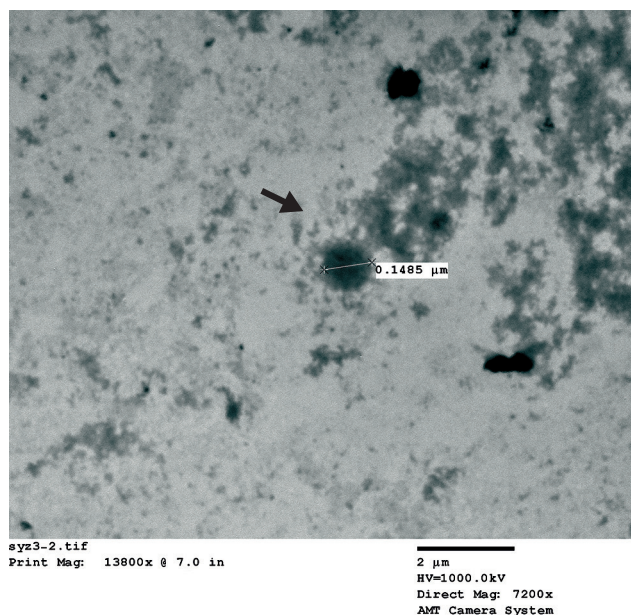
**Fig 2:** The CPE of TGEV. A: The ST cells as negative control; B: The CPE of CQ strain.

The primarily cytopathic effects (CPE) were observed in ST cell infected with 10 passages virus. The ST cells generated CPE cell lesions: cell rounding, refraction, aggregation and coalescence.



**Fig 3:** IFA identification in ST cells. A: Uninoculated ST control; B: Specific fluorescence was produced in infected ST cells. The indirect fluorescent antibody test (IFA) showed that the specificity fluorescence appeared in cytoplasmic infected ST cells.

in the cytoplasm of infected ST cells, which suggested that the isolated virus might be TGEV (Figure 3). The negatively stained virus particles extracted from the suspension of cell infected virus were approximately 149 nm in diameter when examined by the electron microscope (Figure 4), and appeared with morphology similar to TGEV.



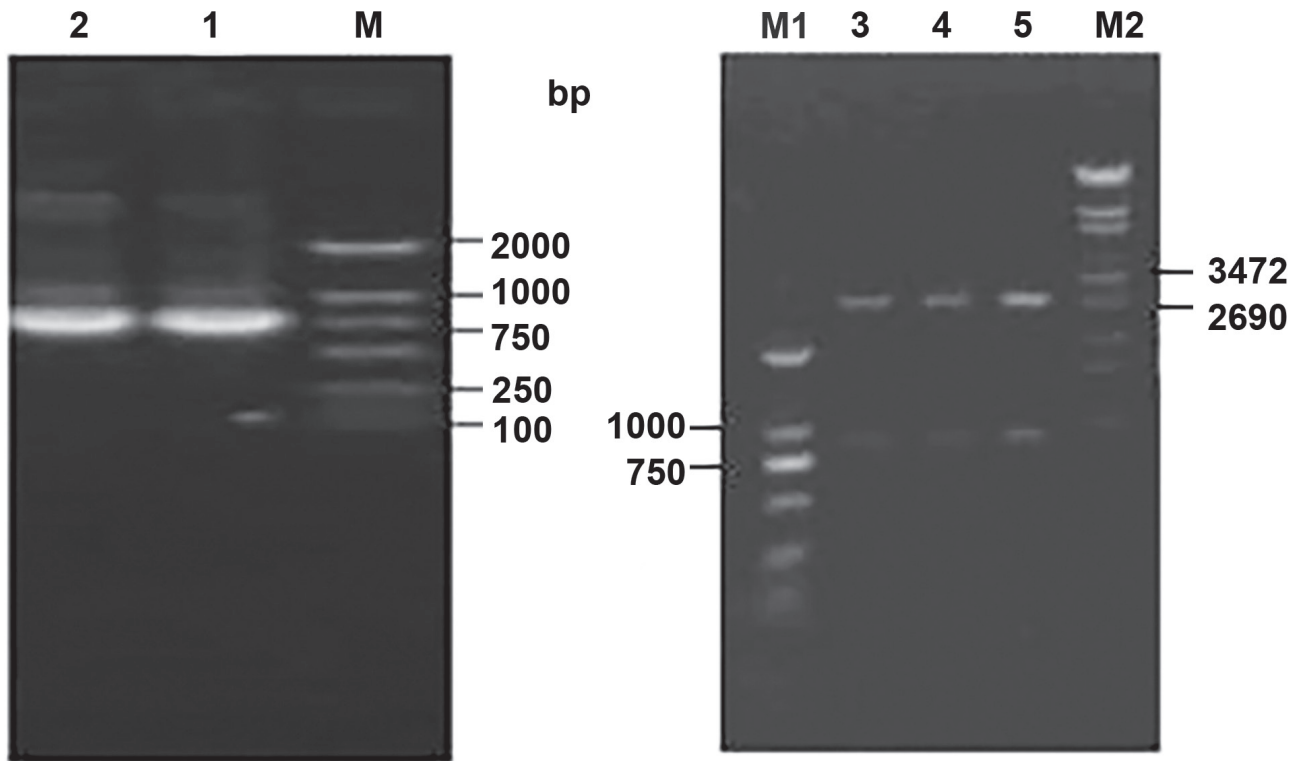
**Fig 4:** Electron microscopy: Negatively stained TGEV. The negatively stained virus particles extracted from suspension cell infected virus were approximately 149 nm in diameter.

### Amplification, sequencing and analysis of M gene

The ST infected cells with the isolate were positive by the RT-PCR assays, and the expected sizes 789bp of the PCR products were observed as a clear electrophoretic band (Figure 5). The obtained M genes segments by sequencing have been submitted for GenBank under accession No. KF273109. Alignment with other 21 reference strains of TGEV and 1 strain PRCV in GenBank, showed that the homology was between 94.3–99.6% for nucleotide sequence (Table 1) and 92.4–98.9% for amino sequence (Table 2), which shows highly homologous between the isolated strain and other TGEV strains.

### Phylogenetic analysis

To better understand the relationship of the isolate to other strains of TGEV, genetic sequences of M in GenBank were used to construct phylogenetic trees. The multiple sequence alignments showed that there is 3 nucleotide deviations, nt19(A→G), nt164(A→G), nt249(T→C), resulting in 2 differences (Ile6→Met6, Asn55→Ser55) in the M gene of CQ strain compared with other strains of TGEV. Genetic and phylogenetic analysis have shown that transmissible gastroenteritis virus have been divided into two clusters (I and II), cluster I included the American Purdue and cloned strains, partial Chinese isolates and strains isolated in Korea. Cluster II included the American Miller and attenuated Miller strain, partial Chinese strains TS, HN2002, FJ, H16 and



**Fig 5:** The amplified products by RT-PCR and enzyme identification of recombinant plasmids. Lane 1 and Lane 2: M gene from infected ST cell; Lane 3, Lane 4 and Lane 5: pMD18-M digested by BamHI and EcoRI.

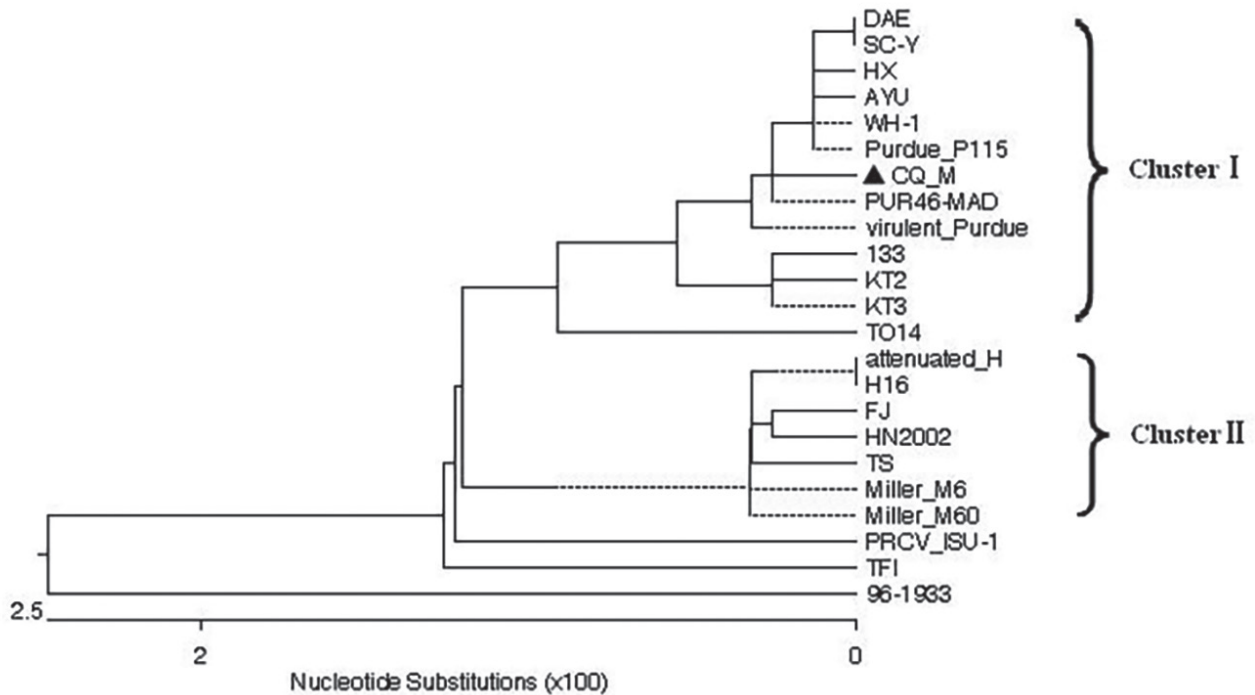
**Table 1:** Homology of M gene sequence of different TGEVs

		Percent Identity																								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23		
Divergence	1	■	94.2	98.1	99.0	98.7	99.0	97.7	98.1	98.0	99.0	99.5	99.7	98.2	98.2	97.1	99.1	99.1	99.0	96.8	98.1	98.0	99.2	99.1	1	133
	2	6.1	■	95.6	94.3	94.3	94.3	95.2	95.6	95.4	94.3	94.2	94.4	95.7	95.7	95.5	94.4	94.4	94.3	95.6	94.9	95.4	94.4	94.4	2	96-1933
	3	1.9	4.6	■	98.1	97.8	98.1	99.6	100.0	99.6	98.1	98.1	98.4	99.9	99.9	98.2	98.2	98.2	98.1	98.2	98.7	99.6	98.4	98.2	3	attenuated_H
	4	1.0	6.0	1.9	■	99.5	99.7	97.7	98.1	98.0	99.7	99.0	99.2	98.2	98.2	97.5	99.9	99.9	99.7	97.0	98.4	98.0	99.7	99.9	4	AYU
	5	1.3	6.0	2.2	0.5	■	99.5	97.5	97.8	97.7	99.5	98.7	99.0	98.0	98.0	97.2	99.6	99.6	99.5	96.7	98.1	97.7	99.5	99.6	5	CQ_M
	6	1.0	6.0	1.9	0.3	0.5	■	97.7	98.1	98.0	99.7	99.0	99.2	98.2	98.2	97.5	99.9	99.9	100.0	97.0	98.4	98.0	99.7	99.9	6	DAE
	7	2.3	5.0	0.4	2.3	2.6	2.3	■	99.6	99.5	97.7	97.7	98.0	99.5	99.5	97.8	97.8	97.8	97.7	97.8	98.4	99.2	98.0	97.8	7	FJ
	8	1.9	4.6	0.0	1.9	2.2	1.9	0.4	■	99.6	98.1	98.1	98.4	99.9	99.9	98.2	98.2	98.2	98.1	98.2	98.7	99.6	98.4	98.2	8	H16
	9	2.1	4.7	0.4	2.1	2.3	2.1	0.5	0.4	■	98.0	98.0	98.2	99.7	99.7	98.1	98.1	98.1	98.0	98.1	98.6	99.5	98.2	98.1	9	HN2002
	10	1.0	6.0	1.9	0.3	0.5	0.3	2.3	1.9	2.1	■	99.0	99.2	98.2	98.2	97.5	99.9	99.9	99.7	97.0	98.4	98.0	99.7	99.9	10	HX
	11	0.5	6.1	1.9	1.0	1.3	1.0	2.3	1.9	2.1	1.0	■	99.7	98.2	98.2	97.1	99.1	99.1	99.0	96.8	98.1	98.0	99.2	99.1	11	KT2
	12	0.3	5.8	1.7	0.8	1.0	0.8	2.1	1.7	1.8	0.8	0.3	■	98.5	98.5	97.3	99.4	99.4	99.2	97.1	98.4	98.2	99.5	99.4	12	KT3
	13	1.8	4.5	0.1	1.8	2.1	1.8	0.5	0.1	0.3	1.8	1.8	1.5	■	100.0	98.3	98.4	98.4	98.2	98.4	98.9	99.7	98.5	98.4	13	Miller_M6
	14	1.8	4.5	0.1	1.8	2.1	1.8	0.5	0.1	0.3	1.8	1.8	1.5	0.0	■	98.3	98.4	98.4	98.2	98.4	98.9	99.7	98.5	98.4	14	Miller_M60
	15	3.0	4.7	1.8	2.6	2.9	2.6	2.2	1.8	1.9	2.6	3.0	2.7	1.7	1.7	■	97.5	97.5	97.3	97.5	98.0	98.0	97.5	97.5	15	PRCV_ISU-1
	16	0.9	5.8	1.8	0.1	0.4	0.1	2.2	1.8	1.9	0.1	0.9	0.6	1.7	1.7	2.5	■	100.0	99.9	97.1	98.5	98.1	99.9	100.0	16	PUR46-MAD
	17	0.9	5.8	1.8	0.1	0.4	0.1	2.2	1.8	1.9	0.1	0.9	0.6	1.7	1.7	2.5	0.0	■	99.9	97.1	98.5	98.1	99.9	100.0	17	Purdue_P115
	18	1.0	6.0	1.9	0.3	0.5	0.0	2.3	1.9	2.1	0.3	1.0	0.8	1.8	1.8	2.6	0.1	0.1	■	97.0	98.4	98.0	99.7	99.9	18	SC-Y
	19	3.2	4.6	1.8	3.1	3.4	3.1	2.2	1.8	1.9	3.1	3.3	3.0	1.7	1.7	2.6	3.0	3.0	3.1	■	97.7	98.1	97.1	97.1	19	TFI
	20	1.9	5.3	1.3	1.7	1.9	1.7	1.7	1.3	1.4	1.7	1.9	1.7	1.1	1.1	1.9	1.5	1.5	1.7	2.3	■	98.6	98.6	98.5	20	TO14
	21	2.1	4.7	0.4	2.1	2.3	2.1	0.8	0.4	0.5	2.1	2.1	1.8	0.3	0.3	1.9	1.9	1.9	2.1	1.9	1.4	■	98.2	98.1	21	TS
	22	0.8	5.8	1.7	0.3	0.5	0.3	2.1	1.7	1.8	0.3	0.8	0.5	1.5	1.5	2.5	0.1	0.1	0.3	3.0	1.4	1.8	■	99.9	22	virulent_Purdue
	23	0.9	5.8	1.8	0.1	0.4	0.1	2.2	1.8	1.9	0.1	0.9	0.6	1.7	1.7	2.5	0.0	0.0	0.1	3.0	1.5	1.9	0.1	■	23	WH-1

**Table 2:** Homology of M amino acid sequence of different TGEVs

		Percent Identity																								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23		
Divergence	1	■	92.4	97.3	97.7	97.3	97.7	96.2	97.3	97.0	97.7	98.1	98.9	97.7	97.7	96.2	98.1	98.1	97.7	96.2	98.1	97.0	98.5	98.1	1	133
	2	7.6	■	93.9	92.0	92.4	92.0	92.8	93.9	93.5	92.0	92.4	93.2	94.3	94.3	94.3	92.4	92.0	93.9	93.2	93.5	92.8	92.4	2	96-1933	
	3	2.3	5.9	■	97.0	96.6	97.0	98.5	99.6	98.5	97.0	97.3	98.1	99.2	99.2	97.3	97.3	97.0	97.7	98.1	98.5	97.7	97.3	3	attenuated H	
	4	1.9	8.0	2.7	■	98.5	98.9	95.8	97.0	96.6	98.9	97.7	98.5	97.3	97.3	95.8	99.2	99.2	98.9	95.8	97.7	96.6	98.9	99.2	4	AYU
	5	2.3	7.6	3.1	1.1	■	98.5	95.4	96.6	96.2	98.5	97.3	98.1	97.0	97.0	95.4	98.9	98.9	98.5	95.4	97.3	96.2	98.5	98.9	5	CQ
	6	1.9	8.0	2.7	0.8	1.1	■	95.8	97.0	96.6	98.9	97.7	98.5	97.3	97.3	95.8	99.2	99.2	99.6	95.8	97.7	96.6	98.9	99.2	6	DAE
	7	3.5	7.2	1.1	3.9	4.3	3.9	■	98.5	98.1	95.8	96.2	97.0	98.1	98.1	96.2	96.2	96.2	95.8	96.6	97.0	97.3	96.6	96.2	7	FJ
	8	2.3	5.9	0.0	2.7	3.1	2.7	1.1	■	98.5	97.0	97.3	98.1	99.2	99.2	97.3	97.3	97.3	97.0	97.7	98.1	98.5	97.7	97.3	8	H16
	9	2.7	6.4	1.1	3.1	3.5	3.1	1.5	1.1	■	96.6	97.0	97.7	98.9	98.9	96.9	97.0	97.0	96.6	97.3	97.7	98.1	97.3	97.0	9	HN2002
	10	1.9	8.0	2.7	0.8	1.1	0.8	3.9	2.7	3.1	■	97.7	98.5	97.3	97.3	95.8	99.2	99.2	98.9	95.8	97.7	96.6	98.9	99.2	10	HX
	11	1.5	7.6	2.3	1.9	2.3	1.9	3.5	2.3	2.7	1.9	■	98.9	97.7	97.7	96.2	98.1	98.1	97.7	96.2	98.1	97.0	98.5	98.1	11	KT2
	12	0.8	6.8	1.5	1.1	1.5	1.1	2.7	1.5	1.9	1.1	0.8	■	98.5	98.5	96.9	98.9	98.9	98.5	97.0	98.9	97.7	99.2	98.9	12	KT3
	13	1.9	5.5	0.4	2.3	2.7	2.3	1.5	0.4	0.8	2.3	1.9	1.1	■	99.6	97.7	97.7	97.7	97.3	98.1	98.5	98.9	98.1	97.7	13	Miller M6
	14	1.9	5.5	0.4	2.3	2.7	2.3	1.5	0.4	0.8	2.3	1.9	1.1	0.0	■	97.7	97.7	97.3	98.1	98.5	98.9	98.1	97.7	14	Miller M60	
	15	3.5	5.6	2.3	3.9	4.3	3.9	3.5	2.3	2.7	3.9	3.5	2.7	1.9	1.9	■	96.2	96.2	95.8	96.2	96.9	96.9	96.6	96.2	15	PRCV ISU-1
	16	1.5	7.6	2.3	0.4	0.8	0.4	3.5	2.3	2.7	0.4	1.5	0.8	1.9	1.9	3.5	■	99.6	99.2	96.2	98.1	97.0	99.2	99.6	16	PUR46-MAD
	17	1.5	7.6	2.3	0.4	0.8	0.4	3.5	2.3	2.7	0.4	1.5	0.8	1.9	1.9	3.5	0.0	■	99.2	96.2	98.1	97.0	99.2	99.6	17	Purdue P115
	18	1.9	8.0	2.7	0.8	1.1	0.0	3.9	2.7	3.1	0.8	1.9	1.1	2.3	2.3	3.9	0.4	0.4	■	95.8	97.7	96.6	98.9	99.2	18	SC-Y
	19	3.5	5.9	1.9	3.9	4.3	3.9	3.1	1.9	2.3	3.9	3.5	2.7	1.5	1.5	3.1	3.5	3.5	3.9	■	97.7	97.3	96.6	96.2	19	TFI
	20	1.5	6.8	1.5	1.9	2.3	1.9	2.7	1.5	1.9	1.5	1.9	1.5	0.8	1.1	1.1	2.7	1.5	1.5	1.9	1.9	■	97.7	98.5	20	TO14
	21	2.7	6.4	1.1	3.1	3.5	3.1	2.3	1.1	1.5	3.1	2.7	1.9	0.8	0.8	2.7	2.7	2.7	3.1	2.3	1.9	■	97.3	97.0	21	TS
	22	1.1	7.2	1.9	0.8	1.1	0.8	3.1	1.9	2.3	0.8	1.1	0.4	1.5	1.5	3.1	0.4	0.4	0.8	3.1	1.1	2.3	■	99.2	22	virulent Purdue
	23	1.5	7.6	2.3	0.4	0.8	0.4	3.5	2.3	2.7	0.4	1.5	0.8	1.9	1.9	3.5	0.0	0.0	0.4	3.5	1.5	2.7	0.4	■	23	WH-1

**Table 1, 2:** Alignment with other 21 reference strains of TGEV and 1 strain PRCV in GenBank, showed that the homology were 94.3-99.6% for nucleotide sequence, 92.4-98.9% for amino sequence, respectively, which shows highly homologous between CQ strain and other TGEV strains.



**Fig 6:** Phylogenetic analysis of M gene sequence of different TGEV isolates.

Chinese vaccine strain attenuated H (18). Multiple sequence alignment in this study also indicated that the nt<sub>70</sub>, nt<sub>189</sub>, nt<sub>190</sub> in M gene of genotype cluster I TGEV strains were T, C, G, respectively. However, the cluster II TGEV strains was G, T, A, respectively. The isolated strain was found to belong to cluster I, showing a closer evolutionary relationship with the American Purdue-P115 strain (19) (Figure 6). Analysis of the phylogenetic trees revealed that the two clusters (I and II) of TGEVs were distinct from the non-Chinese TGEVs, 96-1933 (United Kingdom), TFI (Taiwanese), TO14 (Japan) and other coronaviruses HF3 (Canine coronavirus), WSV 79-1683 (Feline coronavirus), 179-07-11 (Bovine coronavirus) isolated previously. Therefore, based on the results of gene sequencing and homology analysis of M gene, the TGEV isolate was named as CQ strain, in combination with the results of CPE, IFA and EM.

## DISCUSSION

In this study, faeces samples were collected from sick piglets showing clinical signs during winter season in Chongqing, southern China. Twenty clinical samples showed positive results for RT-PCR assay. The field strain of the virus from one sample positive for RT-PCR testing was isolated from inoculated ST cells. The results of IFA, electron microscopic examination and RT-PCR tests all showed the virus was TGEV. CPE were observed after 10<sup>th</sup> passage. ST cell culture showed characteristic cytopathic effect with cell rounding, strong refraction, cell aggregation, coalescence to form clusters after 48 h of post inoculation and gradually increasing to affect 95% of cells which became completely detached. All the changes are consistent with CPE of TGEV infecting ST cell, which agrees with our previous reports (20-22).

The isolated CQ strain was identified by molecular cloning, homology analysis, IFA test and EM observation. Characteristic specific intracytoplasmic fluorescence appeared in IFA. Electron microscope examination showed the characteristic morphology of the virion. PCR was the test of choice for rapid detection and identification of the TGE outbreak causative agent. The PCR assay used in this work showed high specificity as a unique band of the expected size (~789bp) was obtained for RNA samples derived from infected ST cells by CQ strain.

The results of multiple sequence alignments showed that three nucleotide deviations existed in the M gene of the CQ

strain. Previously reported studies have suggested that amino acid mutations in the M protein affected its ability to induce IFN- $\alpha$  and M protein has a potential role in TGEV virulence (23, 24). Further studies will therefore be needed to ascertain the roles of the two amino acid mutations in M protein of the CQ strain. The M gene has been regarded as a highly conserved TGEV gene, but comparison of the sequences of TGEV M genes revealed that the M gene of M60 strain had a 6 nucleotide insertion, predicting a membrane protein 2 amino acids longer than in the other strains. PRCV ISU-1 had a 3 nucleotide deletion in the M gene compared with TGEV strains, predicting an M protein of 261 amino acids in length. The amino insertion in TGEV is suggestive of the M protein being subject to environmental selection pressure (25).

Genotype classification and clustering is based on divergence in the viral genome sequences revealed by phylogenetic analysis (26-30). Wang *et al.*, using phylogenetic analysis indicated the Chinese TGEVs were divided into three groups (G1, G2, and G3). Analysis of the phylogenetic trees revealed that the G3 Chinese TGEVs represented a separate group that was distinct from the non-Chinese TGEVs and from Chinese TGEVs isolated previously (31). Phylogenetic analysis based on 3a and 3b genes of TGEV strains showed that the Chinese strains were more closely related to TGEV strains H165, H16, Miller M6, Miller M60, TS, and CHV than other reference strains (32, 33). In this present study, based on phylogenetic comparison, the TGEV strains can be classified into the two main clusters: cluster I and cluster II. The isolated TGEV strains from China SC-Y, HX, AYU, WH-1 and CQ belonged to cluster I, while other Chinese strains H16, FJ, HN2002, TS and Chinese vaccine strain attenuated H belonged to cluster II. So it appears that there are two main clusters prevailing in China. Genetic and phylogenetic analysis based on M protein sequences showed that CQ strain shared the closest relationship with Purdue-P115 than other TGEV strains or PRCV ISU-1. Through comparing the obtained sequences with the known sequences listed in the GenBank database, we also found that three characteristic nucleotides including nt<sub>70</sub>, nt<sub>189</sub>, and nt<sub>190</sub> in the M gene of TGEV may be used to differentiate the genotype cluster I from cluster II.

In conclusion, the present study described the isolation and identification of TGEV isolated from sick piglets for the first time in Southwest China and named as the CQ strain.

According to the results of gene sequencing and homology analysis of M gene, this study has identified and analyzed a new strain of the TGEV isolated from southwest in China, which may be helpful to enrich the genetic information for molecular epidemiological investigation and evolution of TGEV. Furthermore it will be important to perform further studies to select appropriate vaccine virus for local TGE prevention.

## ACKNOWLEDGMENTS

Financial support for this work was provided by grants from the Chongqing Basic Research Program (2008bb5243), the Fundamental Research Funds for the Central Universities (XDJK2010C093), the Fundamental Research Funds for the xinjianguighur autonomous region universities (XJENU2012118), and Urumqi Research Program (Y121210005).

## REFERENCES

- Bernard, D., Denis, R., Murielle, G., Jacqueline, G. and Hubert, L.: Four major antigenic site of the coronavirus transmissible gastroenteritis virus are located on the amino-terminal half of spike glycoprotein S. *J. Gen. Virol.* 71: 1313-1323, 1990.
- Isabel, C., Gustavo, J., Carlos, S., Maria, J. B. and Luis, E.: Antigenic structure of the E2 glycoprotein from transmissible gastroenteritis coronavirus. *Virus Res.* 10: 77-93, 1998.
- Enjuanes, L., Brian, D. and Cavanagh, K.: *Virus Taxonomy. Classification and Nomenclature of Viruses-Coronaviridae.* Academic Press. New York, 2000.
- Laude, H. and Masters, P.S.: *The coronaviridae-The Coronavirus Nucleocapsid Protein.* Plenum press. New York, 1995.
- Murielle, G., Rene, L. H., Jean, F.V. and Hubert, L.: TGEV corona virus ORF4 encodes a membrane protein that is incorporated in virions. *Virol.* 188: 666-675, 1992.
- Li, D., Huang, Y., Du, Q., Dong, F., Zhao, X.M., Zhang, W.L., Xu, X.G. and Tong, D.W.: TGEV nucleocapsid protein induces cell cycle arrest and apoptosis through activation of p53 signaling. *Biochem. Biophys. Res. Comm.* 445: 497-503, 2014.
- Prichard, G.C.: Transmissible gastroenteritis in endemically infected breeding herds of pigs in East Anglia, 1981-1985. *Vet. Rec.* 120: 226-230, 1987.
- Wood, E.N., Prichard, G.C. and Gibson, E.A.: Transmissible gastroenteritis of pigs. *Vet. Rec.* 108: 41, 1981.
- Woods, R.D. and Wesley, R.D.: Immune response in sows given transmissible gastroenteritis virus or canine coronavirus. *Am. J. Vet. Res.* 47: 1239-1242, 1986.
- Johnson, M., Fitzgerald, G. and Welter, M.W.: Six most common pathogens responsible for diarrhea in newborn pigs. *Vet. Med.* 87: 382-386, 1992.
- Moxley, R.A. and Olson, L.D.: Clinical evaluation of transmissible gastroenteritis virus vaccines and vaccination procedures for inducing lactogenic immunity in sows. *Vet. Res.* 50: 111-118, 1989.
- Zhou, J.F., Huang, F., Hua X.G., Li, C., Zhang, W., Shen, Y., Yan, Y.J., Chen, P.R., Ding, D.Z., Mou, J., Chen, Q., Lan, D.L. and Yang, Z.B.: Inhibition of porcine transmissible gastroenteritis virus (TGEV) replication in mini-pigs by shRNA. *Virus Res.* 149: 51-55, 2010.
- Fan, J.H., Zuo, Y.Z., Zhao, Y.L., Li, T.Q. and Zhang, X.B.: Cloning and expression of nucleocapsid protein gene of TGEV HB06 strain. *Front. Agric. China.* 1: 357-360, 2007.
- Hou, Y.X., Yue, X.W., Cai, X.H., Wang, S.J., Liu, Y.G., Yuan, C.L., Cui, L. and Xiu, G.H.: Complete Genome of Transmissible Gastroenteritis Virus AYU Strain isolated in Shanghai, China. *J. Virol.* 86: 11935, 2012.
- Wang, C., Chen, J., Shi, H., Qiu, H., Xue, F., Liu, C., Zhu, Y., Liu, S., Almazan, F., Enjuanes, L. and Feng, L.: Molecular characterization of a Chinese vaccine strain of transmissible gastroenteritis virus: mutations that may contribute to attenuation. *Virus Genes.* 40: 403-409, 2010.
- Yu, F.S., Wang, S., Chen, S.L., Cheng, X.X., Huang, W.Q., Jiang, B., Chen, S.Y. and Shao, L.P.: Isolation and identification of Porcine Transmissible Gastroenteritis Virus FJ strain. *Fujian J. Agric. Sci.* 27: 1160-1164, 2012.
- Zaher, K.S. and Ahmed, W.M.: Investigations on Bovine Ephemeral Fever Virus in Egyptian cows and buffaloes with emphasis on isolation and identification of a field strain. *Global Veterinaria.* 6: 447-452, 2011.
- Sanchez, C.M., Jimenez, G., Laviada, M.D., Correa, I., Sune, C., Bullido, M.J., Gebauer, F., Smerdou, C., Callebaut, P., Escribano, J.M. and Enjuanes, L.: Antigenic homology among coronaviruses related to transmissible gastroenteritis. *Virol.* 174 : 410-417, 1990.
- Zhang, X.S., Mustafa, H., David, S., Rebecca, H., Wang, S.L., Sarah, S., Daniel, J., Nagesh, H., Tang, Y.X., Elodie, G. and Linda, S.: Complete genomic sequences, a key residue in the spike protein and deletions in nonstructural protein 3b of US strains of the virulent and attenuated corona viruses, transmissible gastroenteritis virus and porcine respiratory coronavirus. *Virol.* 358: 424-435, 2007.
- Song, Z.H. and Guo, W.Z.: Isolation and Genomic Sequence Analysis of Porcine Transmissible Gastroenteritis Virus. *Chin. J. Virol.* 24: 364-368, 2008.
- Yan, Q.G., Ou, Y., Guo, W.Z., Feng, T., Fan, W. Q., Lai, W.L., Cao, H.Z. and Li, B.: Isolation of SC-1 strain of transmissible gastroenteritis virus of swine and characteristic analysis of gene 7. *Chin. J. Vet. Sci.* 27: 613-617, 2007.
- Zhang, X., Hongyan, S., Chen, J.F., Shi, D., Dong, H. and Feng, F.: Identification of the interaction between vimentin and nucleocapsid protein of transmissible gastroenteritis virus. *Virus Res.* 200: 56-63, 2015.
- Baudoux, P., Carrat, C., Besnardeau, L., Charley, B. and Laude, H.: Coronavirus pseudoparticles formed with recombinant M and E proteins induce alpha interferon synthesis by leukocytes. *J. Virol.* 72: 8636-8643, 1998.
- Zuo, H., Dante, S.Z., Karol, S., Siqingaowa, S. and Ren, X.F.:



- Transmissible gastroenteritis virus: Identification of M protein-binding peptide ligands with antiviral and diagnostic potential. *Antiviral Res.* 99: 383-390, 2013.
25. Li, J. Q., Cheng, J. and Lan, X.: Complete genomic sequence of transmissible gastroenteritis virus TS and 3' end sequence characterization following cell culture. *Viol. Sin.* 25: 213-224, 2010.
  26. Evermann, J. and Ridpath, J.: Clinical and epidemiologic observations of bovine viral diarrhea virus in the northwestern United States. *Vet. Microbiol.* 89: 129-139, 2002.
  27. Sun, X.H., Fu, S.H., Gong, Z.D., Ge, J.Q., Meng, W.S., Feng, Y., Wang, J.L., Zhai, Y.G., Wang, H.Q. and Nasci, R.: Distribution of arboviruses and mosquitoes in northwestern Yunnan Province, China. *Vector Borne Zoonotic Dis.* 9: 623-630, 2009.
  28. Vilek, Paton, D., Durkovic, B., Strojny, L., Ibata, G., Moussa, A., Loitsch, A., Rossmanith, W., Vega, S. and Scicluna, M.: Bovine viral diarrhea virus genotype1 can be separated into at least eleven genetic groups. *Arch. Virol.* 146: 99-115, 2001.
  29. Wang, H.Y., Takasaki, T., Fu, S.H. and Liang, G.D.: Molecular epidemiological analysis of Japanese encephalitis virus in China. *J. Gen. Virol.* 883: 885-894, 2007.
  30. Wang, L.H., Fu, S.H., Zhang, H.L., Ye, X.F., Yu, D.S., Deng, Z., Yuan, J., Zhai, Y.G., Li, M.H. and Chen, W.X.: Identification and isolation of Genotype-1 Japanese Encephalitis virus from encephalitis patients. *Viol. J.* 7: 345-348, 2010.
  31. Wang, C.B., Chen, J.F., Shi, H.Y., Qiu, H.J., Xue, F., Liu, S.W., Liu, C.M., Zhu, Y.M., Fernando, A., Luis, E. and Feng, L.: Rapid differentiation of vaccine strain and Chinese field strains of transmissible gastroenteritis virus by restriction fragment length polymorphism of the N gene. *Virus gene.* 41: 47-58, 2010.
  32. Becher, P., Orlich, M., Kosmidou, A., Baroth, M. and Thiel, H.: Genetic diversity of pestiviruses: identification of novel groups and implications for classification. *Viol.* 262: 64-71, 1999.
  33. Couvreur, B., Letellier, C., Collard, A., Quenon, P., Dehan, P., Hamers, C., Pastoret, P. and Kekhofs, P.: Genetic and antigenic variability in bovine viral diarrhea virus (BVDV) isolates from Belgium. *Virus Res.* 85: 17-28, 2002.