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Full Length Research Paper

Molecular cloning and phylogenetic analysis of the E gene of transmissible gastroenteritis virus (TGEV) isolated in China

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Porcine transmissible gastroenteritis caused by transmissible gastroenteritis virus (TGEV) induces acute, high-contact intestinal infectious diseases in pigs. TGEV encodes four structural proteins including spike (S), membrane (M), envelope (E) and nucleoprotein (N), of these, the genes of S, M and N respectively had been deeply elucidated. However, the real function of E protein was still unclear and sequence comparison of the E gene of different TGEV isolates had not been reported so far. In this study, E gene of TGEV strain isolated in China was molecularly cloned and sequenced. Phylogenetic analysis confirmed this gene was relatively conserved. All the China TGEV strains were brached in one clade together with the American strains, but showed relatively alien to the European strains.

Key words: Transmissible gastroenteritis virus, E gene, phylogenetic analysis.

INTRODUCTION

Transmissible gastroenteritis virus (TGEV) is identified as one of the most important pathogenic agents during swine enteric infection, leading to high mortality in neonatal pigs and severe annual economic loss in swine-producing areas (Zhou et al., 2007). As a member of the *Coronaviridae*, TGEV possesses a large 28.5 kb single-stranded sense RNA genome in which genes are arranged in the order 5'-Rep-S-3a-3b-E-M-N-7-3'(Park et al., 2008). Four of them, rep, 3a, 3b, and 7 encode non-structural proteins (Penzes et al., 2001). The others encode four structural proteins including spike (S), membrane (M), envelope (E) and nucleoprotein (N). In these four structural genes, the genes of S, M and N respectively are being deeply elucidated, and some characteristics have been ascertained and explained (Escors et al., 2001; Gebauer et al., 1991; Kapke and Brian, 1986; Krempl et al., 1997; Laude et al., 1987). Nevertheless, the functional action of E gene is poorlyunderstood currently.

E gene is a small structural gene that encodes an 82 amino acid membrane-associated protein called E protein (formerly called sM)(Baudoux et al., 1998). E protein is an integral membrane protein having a Cexo-Nendo orientation and binding with the envelope. The epitopes of this protein are localized within the last 21 C-terminal residues of the sequence, and the antibodies that are specific for E protein can be tested in pig sera infected TGEV (Godet et al., 1992). Previous research suggested that E protein is an efficient inductor of alpha interferon (IFN-alpha) synthesis *in vitro* (Baudoux et al., 1998; Riffault et al., 1997). Recently, it was reported that it is essential for viral reproduction and play a role in virion assembly or release (Ortego et al., 2007). However, the real function of E protein is still unclear. Moreover, to the best of our knowledge, sequence comparison of the E gene of different TGEV isolates have been not reported so far.

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Table 1. The characteristics of TGEVs used in this study.

Strain name	Place of Origin (abbreviation)	Genbank accession number
H16	China	FJ755618
attenuated H	China	EU074218
SC-Y	China	DQ443743
TS	China	DQ201447
TFI	Taiwan	Z35758
Miller M60	the United States of America (USA)	DQ811786
Miller M6	the United States of America (USA)	DQ811785
Purdue	the United States of America (USA)	DQ811789
Purdue P115	the United States of America (USA)	DQ811788
PUR46-MAD	the United States of America (USA)	AJ271965
96-1933	the United Kingdom of Great British (UK)	AF104420
FS772/70	the United Kingdom of Great British (UK)	Y00542

MATERIALS AND METHODS

Virus and *in vitro* growth

A Chinese TGEV strain named TGEs-1 was isolated from pigs in a small commercial pig farm that lies in Shanghai suburb. The swine testis (ST) cells were grown in Dulbecco's modified Eagle medium (DMEM, GIBCO, USA) supplemented with 10% foetal bovine serum (FBS, GIBCO, USA) and maintained in maintenance medium (DMEM supplemented with 2% FBS) at 37°C in a 5% CO_2 atmosphere.

The TGEs-1 strain was passaged 6 times on ST cells and the value of $TCID_{50}$ was evaluated. Virus infected ST cells showing 90% cytopathic effects were frozen and thawed and cellular debris was removed by centrifugation at 10×l0 ^{3}g at 4°C for 30 min. The clarified cell culture supernatant was collected and used for preparation of viral RNA.

RNA isolation and reverse transcription-polymerase chain reaction (RT- PCR)

Viral RNA was extracted by use of TRIZOL Reagent (Takara, China) according to the manufacturer's instructions. The RT reaction was performed using PrimeScript™ 1 st Strand cDNA Synthesis Kit (Takara, China) following the manufacturer's protocol. Briefly, the mixture containing 5 µl of RNA, 1 µl of random primers (50 µM), and 1 µl of dNTPs (10 mM) was heated at 65°C for 5 min. Then, 4 µl of 5×PrimeScript™ Buffer, 0.5 µl of RNase inhibitor (40 U/µl), 1 µl of PrimeScript™ RTase (200 U/ µl) and 7.5 µl of RNase free ddH2O were added to the mixture for a total volume of 20 µl. The RT reaction to synthesise cDNA was performed at 30°C for 10 min, at 42°C for 60 min, followed by heating at 70°C for 15 min total volume of 20 µl. The RT reaction to synthesise cDNA was performed at 30°C for 10 min, at 42°C for 60 min, fol lowed by heating at 70°C for 15 min.

The cDNA obtained was amplified with forward primer TGEV-EF (5'-TTTATGTATTTTCACAGGAGCCC -3') and reverse primer TGEV-ER (5'- TAGCACAATAGCGTTCTCCACAT -3') designed by ourselves. The PCR reaction mixture contained 25 µl of PCR premix (0.1U of Taq polymerase/µl, 500 µM of dNTPeach, 20 mM Tris-HCl, 3 M MgCl2, 100 Mm KCl) (TIANGEN, China), 1 µl of forward and reverse primers, and 5 µl of cDNA. A 50 µl total reaction volume was obtained by adding distilled water. The PCR was performed by 35 cycles of 60 s at 94°C, 1 min at 50°C and 1.5 min at 72°C; followed by a final extension time of 5 min at 72°C.

PCR products were electrophoresed in 2% agarose gels containing ethidium bromide (10 mg/mL) and confirmed using a UV-transilluminator (Bio-Rad, USA).

Cloning and DNA sequencing

The PCR products were gel purified using AxyPrep DNA Gel Extraction Kit (Axygene, USA) according to the manufacturer's instructions. The purified DNA was ligated into the pMD-18 cloning vector (Takara, China) and transformed into competent cells (TOP10, TIANGEN, China). The positive recombinants were selected with blue and white screening. In addition, the plasmids were extracted with extraction Kits (Axygene, USA) .Then the identified positive recombinants were sequenced using dideoxynucleotide chaintermination procedures (Shanghai, China), and the sequence of E gene of TGEs-1 strain was submitted to GenBank of NCBI.

Sequence analysis

The DNA sequences were analyzed with the MegAlign software (DNAStar Inc., USA), and then compared with other 12 published TGEV DNA sequences (Table 1) using CLUSTAL X v 1.82 programme (USA). A phylogenetic tree of the E gene was generated by the Neighbour-joining method with 100 bootstrap replicates in a heuristic search with the MEGA 4.1 software programme (USA). The origin places and Genbank accession numbers of TGEVs used in this study are presented in Table 1.

RESULTS AND DISCUSSION

The TGEs-1 strain was passaged serially 6 times in ST cells. The growth characteristics of TGEs-1 of each in vitro generation were almost the same. Cytopathic effects (CPE) occurred 48 h post-infection. During this time the cells rounded, condensated, and gradually peeled off. The $TCID_{50}$ of TGEs-1 strain was $10^{-8.2}$/0.2 ml. These results suggest that this isolated China strain is a virulent strain.

Then the complete E gene of TGEs-1 strain was amplified by RT-PCR, cloned, and sequenced. The

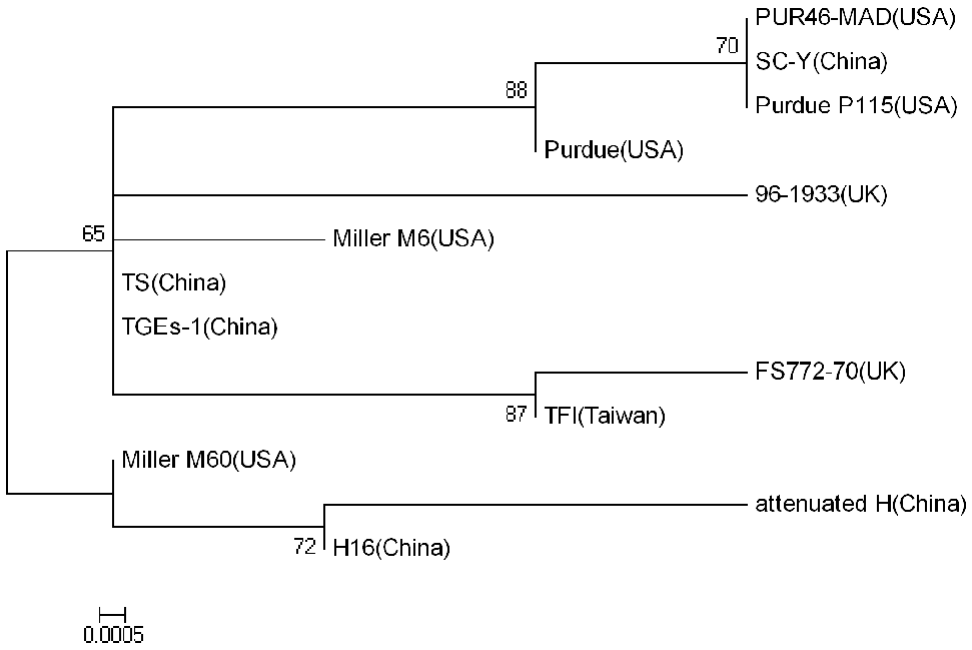

Figure 1. Phylogenetic relationships based on nucleotide sequences of the gene E region among TGEV strains used here. The phylogenetic tree was generated by the Neighbor-Joining method in a heuristic search with 1000 bootstrap replicates.

complete E gene sequence of TGEs-1 strain was 249 bp (GenBank accession number: GU250738). The nucleotide sequence comparison of TGEs-1 strain with other reference strains of TGEV showed that there was no deletion or insertion in the E gene of the TGEs-1 strain. Among these strains, TGEs-1 strain had high nucleotide identities of 98.4 -100% with other strains, specially had a 100% identity with China TS strain. Comparison of these strains, only the Taiwan strain TFI and the UK strain FS772/70 missing two bases in the E gene sequence, but these two strains are all virulent isolate, and whether this kind of point mutation will influence the viral reproduction, assembly, release or tropism of the virus need further study. Likewise, the amino acid sequence comparison showed that TGEs-1 strain had a similarity between 95.1% (attenuated H) and 100% (TS) with other reference strains. In general, there is little different between each strain in E gene, and the E gene of TGEV is relatively conserved.

The precise function of the E gene product is not clearly understood, but it has been suggested that this gene might play a role in the pathogenicity of TGEV (Baudoux et al., 1998; Ortego et al., 2007; Riffault et al., 1997). However, interestingly, here we found that virulent strains have little difference with attenuated virulent strains in E gene. This result is contradicted to the previous studies and remained to be explored. We speculate that the virulence of TGEV is related with E gene, but E gene is not the only determining factor.

To investigate the evolutionary relationships between TGEs-1 strain and others, a phylogentic tree was constructed based on their E gene sequences (Figure 1). The tree revealed the five China TGEV isolates were clustered into three distinct groups. As expected, based on genetic homology, TGEs-1 and TS were clustered together in a group. SC-Y strain (China) has closer relationship with Purdue P115 and PUR46-MAD strains (USA), H16 strain and attenuated H strain could be clustered together in a group. Notably, the phylogentic relationship of TGEV strains from China was closed with the American strains, but alien to the European strains.

In conclusion, the E gene sequences of TGEV isolated from different regions are relatively conserved. All the China TGEV strains possess extremely intimate relationship with the American strains and are relatively alien to the European strains.

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