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Molecular Cloning and Phylogenetic Analysis of ORF7 Region of Chinese Isolate TH-98 from Transmissible Gastroenteritis Virus*

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Abstract. Genomic RNA was extracted from a Chinese isolate of porcine transmissible gastroenteritis virus (TGEV) designated TH-98. Employing RT-PCR technique to amplify ORF7 sequence of TGEV, which located at the 3′ end of TGEV genome and is poorly understood functionally so far. A recombinant named pPROEX HTc-hp was constructed via inserting ORF7 gene into prokaryotic expression vector pPROEX HTc. The recombinant was sequenced and compared the DNA and its deduced amino acid (aa) sequences with that of some reference strains after restriction endonuclease and PCR analysis. The ORF7 gene named hp gene (Genbank accession number: AY337931) consists of 237 bp in length encoding a hydrophobic protein (HP) of 78 aa with a molecular weight of 9.1 kDa. The sequences of hp gene and Hp protein share 89%–97% and 87%–96% homologous identities compared with 11 TGEV reference strains derived from other regions or countries respectively, which revealed that there are significant variation within-strains, even though the ORF7 region is relatively conservative. In addition, a phylogenetic tree based on these ORF7 DNA sequences was generated, and the tree topology suggests that possible recombination events happened in the evolutionary history of TGEV.

Key words: AY337931, cDNA cloning, coronavirus, ORF7, phylogenetic analysis, TGEV

Introduction

Transmissible gastroenteritis virus of swine (TGEV) is an enveloped virus and belongs to a member of groups of coronaviruses, which is one of the most important causative agents of enteric infection. The infection is associated with high morbidity in animals of all ages and with high mortality in suckling piglets [1–3].

TGEV genome is a single-stranded, positive-sense 28.5-kb RNA. About two-thirds of the entire RNA comprises open reading frames (ORFs) 1a and 1b, encoding the replicase. The 3′ one-third of the genome comprises the genes encoding the structural and non-structural proteins [4–6]. The genes of TGEV are arranged in the order 5_-rep-S-3a-3b-E-M-N-7-3_. Four of them, rep, 3a, 3b, and 7, encode non-structural proteins [7]. At present, the functions of four structural proteins encoded by spike S, membrane M, envelope E, nucleoprotein N genes respectively are being deeply elucidated, and some characteristics have been ascertained and explained [8–14]. Nevertheless, the functional action of non-structural proteins is poorly understood currently.

The ORF7 we designated it as hp gene encoding a small hydrophobic protein (HP) and mapping at the 3′ end of TGEV genome was expressed during virus replication. A CYAAAC conserved

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intergenic sequence located the upstream of hp gene separates it from the N gene. The characteristics distinguished TGEV from other members of coronavirus family, such as murine hepatitis virus (MHV), bovine coronavirus (BCoV), infectious bronchtitis virus (IBV) and Human coronavirus (HCoV-OC43) [4,5]. HP is immunoprecipitable with porcine hyperimmuned anti-TGEV serum. The intracellular localization of HP suggests that it may play an important role in the process of membrane integrity of viral replication and/or virion assembly [15].

In this report, the hp gene was cloned by RT-PCR from a recently isolated TGEV in north China, the sequences of hp DNA and HP protein were compared with some other references strains derived from different regions and/or countries. Correspondingly, a phylogenetic tree was constructed and analyzed according to their ORF7 DNA sequence. The similarity and discrepancy among different strains have been demonstrated, which will contribute to the further functional exploration and genesis investigation of TGEV.

Materials and Methods

Viruses and Cells

TGEV strain named TH-98 was isolated from a suburb of Harbin, capital of Heilongjiang province, P.R. China, and Swine testicle (ST) cell line was grown as monolayer in Dulbecco's modified Eagle medium (DMEM) (GIBCO, USA) containing 10% fetal calf serum (GIBCO, USA) and 5% CO_2 in air. Viruses were harvested by three cycles of freezing and thawing, cellular debris was removed by low speed centrifugation at $10 \times l0^3$ g (HITACHI CR22E, Japan) at 4°C for 25 min, and virions in supernatant were pelleted by centrifugation at 100×10^3 g at 4°C for 1.5 h (HITACHI CR22E, Japan).

RNA Isolation

Total RNA was isolated from purified pellets using SDS-Protease K according to reference [16].

Reverse Transcription-Polymerase Chain Reaction (RT- PCR)

Extracted RNA was added into the below components: 5× reverse transcription buffer 1 μl, dNTP mixture (2.5 mM) 4 μl, RNase inhibitor 0.5 μl, primer P1 5 μl, AMV reverse transcriptase 2 μl (10 U), sterile water 3.5 μl, gently mixed in an Eppendorf tube and incubated at room temperature for 10 min, then transferred to a water incubator at 42°C for l h prior to stored at −20°C until use in PCR.

The cDNA obtained was amplified with the following primers P1 (5′-AATCTA-GATGCTCGTCCTCCT-3′) and P2 (5′-GAC-ATCGGGTACCCTTACATT-3′), the sequences of which were based on corresponding conservative region of hp gene from reference strain (Miller) and contained the full-length ORF7, in brief, the forward primer (P1) flanked at the 5′ end of ORF7 gene, which contained an artificial a *Xba*I site (blackened sequence) and the reverse primer P2 is complementary to 3′ end outside, in which has an artificial *Kpn*I sites (blackened sequence). The primers were designed with Oligo 4.1 version software and synthesized by Takara Biotechnology Company (Dalian, China). PCR amplification was performed using PE2400 PCR equipment (USA). PCR was in 100 μl volumes, using 10 μl of 10× buffer (50 mM KC1, 100 mM Tris–Cl pH8.3, 15 mM $MgCl_2$, 0.1% gelate, 16 μl dNTP mixture (10 mM), 1 μl cDNA, 5 μl of P1 and P2 and l μl *Taq* polymerase (0.5u Takara, Dalian, China). The temperature profile was 30 cycles of 60 s at 94°C, 1 min at 47.9°C and 1.5 min at 72°C, there was then a final extension time of 5 min at 72°C. A 2 μl aliquot of PCR product was visualized by agarose gel electrophoresis (2% agarose, 100 Vs for 20 min, 0.8 μg/ ml ethidium bromide included in gel) and subsequent U.V transillumination. The purified PCR product was named hp.

Expression Plasimid Construction

PCR product was gel purified and digested with restriction endonuclease (RE) *Kpn*I and *Xba*I and cloned into the corresponding sites of prokaryotic expression vector pPROEX HTc (GIBCO, USA) by transformation of competent cell JM109 (Takara, Dalian, China), and a positive

recombinant named pPROEX HTc-hp was selected with blue and white screening as well as the plasmid extracted with extraction Kits (Huashun, shanghai, China) was identified with RE. In addition, primers P1 and P2 were used for nested PCR identification of the recombinant.

Sequencing and Homologous Comparison

pPROEX HTc-hp was sequenced using dideoxynucleotide chaintermination procedures[17] by Sangon bio-company, Shanghai, China. Sequence of hp gene and its deduced amino acids were compared with that of TFI, TO-163, Ohira, Iga-1, Aomori, Ogawa, Miller, Purdue, 96–1933, and RM4 using Genedoc and Genestar softwares. The TGEV origin places and Genbank accession numbers of these ORF7 sequences are summarized in Table 1.

Phylogenetic Tree Analysis

A phylogenetic tree was generated using an alignment of ORF7 nucleotide sequences from the above-mentioned viruses by applying the Neighbor-joining method in the CLUSTAL X v1.82 program, which subsequently subjects to be edited manually.

Results

RNA Isolation

To isolate total RNA of TGEV purified pellets using SDS-Protease K and synthesize the cDNA containing hp gene with primer P1. The total RNA of TGEV was high concentration and purity, however, cDNA of TGEV was synthesized and could be detected through agarose gel electrophrosis (Fig. 1 illustrates).

PCR Amplification

The cDNA was used as template, and a fragment was amplified with forward primer P1 and reverse primer P2. The expected PCR product is 258 base pair (bp), meanwhile, a known about 260bp-sized fragment was amplified (data not shown), which served as positive PCR control and additional DNA Marker (Fig. 2 illustrates).

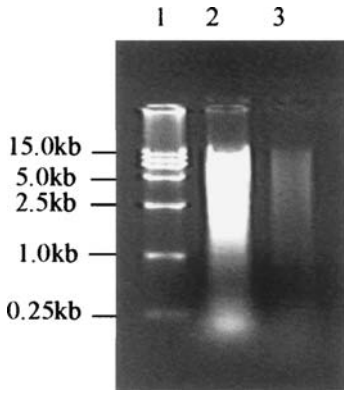

Fig. 1. Total RNA of TGEV and cDNA of hp gene on 0.8% agrose gel. Lane 1: DL15,000 molecular weight Marker; Lane 2: Total RNA of TGEV; Lane 3: cDNA of TGEV containing hp gene synthesized with primer P1.

Table 1. Source of TGEV ORF7 sequences used in the experiment

Strain name	Origin place (abbreviation)	Genbank accession number
TH-98	the Peoples Republic of China (CHN)	AY337931
TFI	Taiwan (TW)	Z35758
TO-163	Japan (JPN)	AB115401
Ohira	Japan (JPN)	AB115407
Iga-1	Japan (JPN)	AB115408
Aomori	Japan (JPN)	AB115409
Ogawa	Japan (JPN)	AB115406
Miller	the Unitied States of America (USA)	AB115411
Purdue	the Unitied States of America (USA)	AB115410
96-1933	the United Kingdom of Great British (UK)	AF104420
FS772/70	the United Kingdom of Great British (UK)	Y00542
RM4	France (FRA)	Z24675

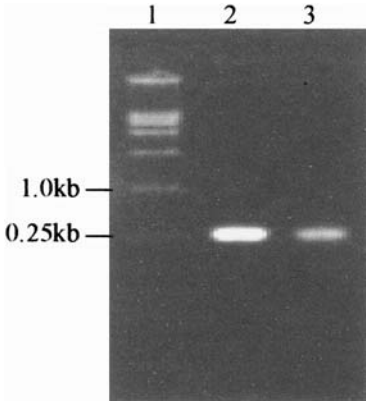

Fig. 2. Product of PCR on 2% gel. Lane l: DL 15,000 molecular weight Marker; Lane 2: Product of PCR, of 258 bp; Lane 3: Positive control of about 260 bp.

Identification of Recombinant with RE

The linear recombinant named pPROEX HTc-hp is 4779 bp in length, which consist of 4.7 kb-sized prokaryotic vector, pPROEX HTc and about 0.26 kb-sized hp gene. Bands with the expected size were detected were detected on agarose gel containing 0.1% ethidium bromide after digestion with *Kpn*I–*Xba*I (Fig. 3 illustrates) .

Identification of Recombinant by PCR

pPROEX HTc-hp circle plasmid was used as template for PCR identification with primer P1 and P2, the PCR product was about 260 bp as expected (Fig. 4 illustrates). Because the recombinant will be directly sequenced, we did not design new primers for nested PCR identification.

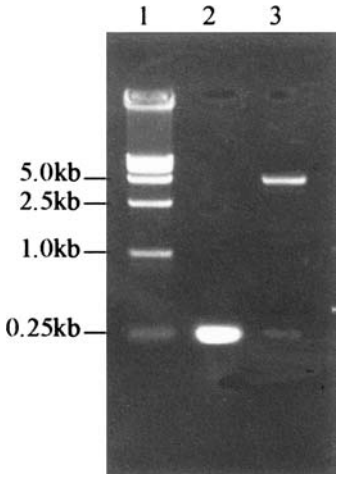

Fig. 3. Identification of recombinant with RE. Lane 1: DL15,000 molecular weight Marker; Lane 2: Product of PCR, 258 bp; Lane3: Two bands as expected, of about 4.7 kb (vector) and 0.26 kb (hp gene) digested with *Kpn*I and *Xba*I from pPROEX HTc-hp respectively.

Sequencing and Homologous Comparison

The DNA sequence of TH-98 ORF7 was compared with the others, the multiple sequences alignment was showed (Fig. 5 illustrates). TH-98 ORF7 shared 95%, 95%, 97%, 97%, 97%, 97%, 96%, 99%, 89%, 94% and 94% DNA sequence identities with that of TFI, TO-163, Ohira, Iga-1, Aomori, Ogawa, Miller, Purdue, 96-1933, FS772/70 and RM4, respectively. Likewise, they shared 94%, 94%, 96%, 96%, 96%, 96%, 94%, 98%, 87%, 93% and 92% homologous identities in terms of their deduced aa sequences. There were some point mutations in TH-98 isolate, and most of changes were T/C substitutions. Unexpectedly, the two Chinese isolate, TH-98 and TFI isolate showed that a high frequent mutation area (base 178-227), though they owned 95% DNA sequence identities.

Phylogenetic Tree Analysis

In order to further elucidate the phylogeny of TGEV strains, the Neighbor-joining method in the CLUSTAL X vl.82 program, a online tool provided by The European Bioinformatics Institute (EBI) was utilized to generate a phylogentic tree on the basis of the ORF7 DNA sequences (Fig. 6 illustrates). As for as the evolution distance was concerned that all the Asian TGEV

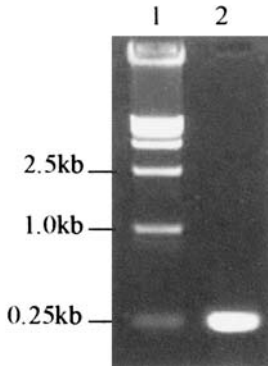

Fig. 4. Identification of recombinant by nested PCR. Lane 1: DL15,000 molecular weight Marker; Lane 2: The expected PCR product, about 260 bp.

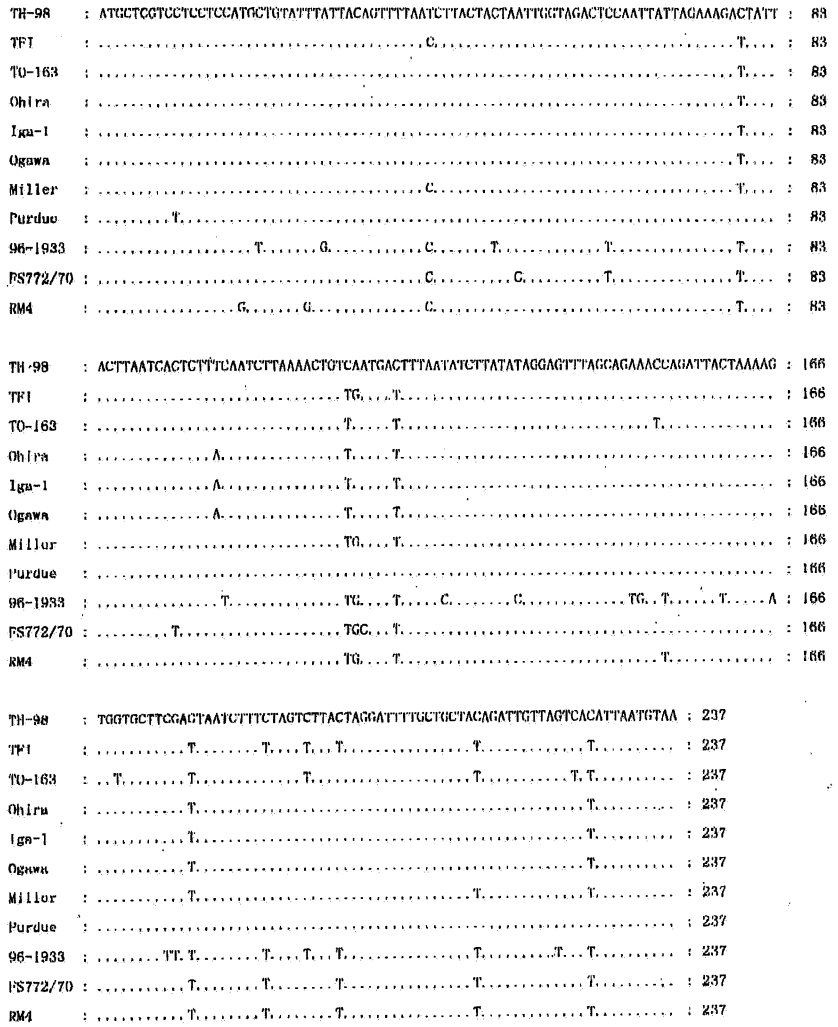

Fig. 5. Multiple sequence alignment of ORF7 among TGEV strains used here. The sequence of TH-98 isolate is displayed as consensus at the top, and the different nucleotides of other strains are indicated. Otherwise, there are symbols of the point for the consensus sequences in other strains.

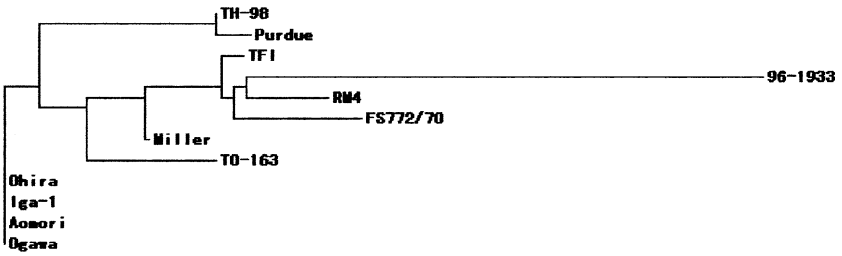

Fig. 6. Phylogenetic tree with Neighbor-Joining method was constructed and TH-98 was defined as outgroup (boostrapping is 1000).

strains closer to the American TGEV strains, but not to European strains. However, some strains isolated in the same country were clustered into different positions of the phylogenetic tree, such as TH-98, TFI, or Purdue and Miller etc. (Fig. 6 illustrates).

Discussion

HP protein of TGEV located in cell membrane, which may play a role associated with the process of membrane integrity and assembly of virion [18]. It was supposed that ORF7 gene might interact with viral RNA and/or other viral proteins of TGEV [13,15,19]. In addition to this point, it may influence virus pathogenesis [20]. However, the concrete integrity mechanism and the function in viral invasion course are not clear presently.

In this study, Chinese TGEV isolate was propagated on ST cells, and the cytopathic effects (CPE) was significant morphologically, the cells were beginning to round out, condensate and peel off gradually after challenged with TGEV. Finally, the typical CPE of cell monolayer is like a net formed by several cells and no syncytia formation (data not shown), so we further verified that ST cells were excellent permissive to TGEV infection. To consider economical reason, we purified TGEV viral pellets by different speed centrifugation and extracted RNA using common chemical reagent according to standard Manual of RNA manipulation [16]. The results showed that RNA extracted was high concentration and purity, however, cDNA of TGEV were synthesized without difficulty. The hp gene of TGEV Chinese isolate was cloned with RT-PCR and genetic recombination techniques for the first time. The recombinant can express the encoded protein directly in *E coli*. due to the utilizing a prokaryotic expression vector substituted for clone vector, which benefits to the further functional research and structural analysis.

The nucleotide sequence comparison showed that there was no deletion or insertion in the hp gene of all the TGEV strains used here. There were relatively high frequency changes from nucleotide T to C in the Chinese isolate TH-98 compared with the others, and if the kind of point mutation will influence the virulence or tropism of the virus will be further discussed in future. To analyze the sequence differences from the angle of geographical distribution, five Japanese strains' ORF7 sequences had the exact identity with the exception of TO-163, TGEV Chinese isolate, TH-98 and TFI shares high homology with these reference strains in term of hp gene (ORF7), which confirmed that 3′-terminal region of TGEV genome is relatively conservative. TH-98 isolate owned higher homologous identities with all the Asian and the American strains listed here. Nevertheless, there were more point mutations existed between Asian strains including TH-98 and the European strains.

In order to deeply investigate the evolutionary relationship between TH-98 and the other strains, a phylogenetic tree was generated on the basis of their nucleotide sequences of ORF7. In agree with the sequence comparison, the tree topology showed all the TGEV Asian strains posses extremely intimate relationship with the American strains and are relatively alien to the European strains. Interestingly, the TGEV strains that isolated in the same or close continent geographically were divided into different clusters on the tree respectively, for example, TGEV Chinese isolates TH-98 and TFI, American strains Miller and Purdue, TO-163 and other Japanese isolates etc. Combining the sequence identity data with consideration geographical and historical factors, the evolutionary analysis demonstrated that (i) the existence of geographically natural barrier has limited the transmission of TGEV to some extent; (ii) there were genetic mutation or recombination events happened in the origin of TGE, which derived from either the dissimilarly of regional environmental selective pressure or/and the immigration of foreign TGEV.

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