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Full Length Research Paper

New mutations existing in the nucleocapsid (N) gene of non-porcine TGEV strains isolated in China

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Transmissible gastroenteritis virus (TGEV) is the etiological agent of TGE, and non-porcine hosts are potential carriers of TGEVs. In this study, genomic RNA were extracted from TGEVs designated HYM-09, HYM-09-1 and HYM-09-2 isolated respectively from non-porcine hosts (dogs, cats and foxes) naturally infected with TGEVs. The nucleocapsid (N) genes of the three non-porcine TGEV strains were amplified by RT-PCR and cloned into pMD18-T vector respectively. Each N cDNA was sequenced and encompassed an open reading frame of 1149 nucleotides, encoding a 382-amino acids protein. Sequence analyses of the N genes were performed, including homologous comparison, phylogenetic tree analysis and homology modeling. The results showed that there existed some new point mutations in the three non-porcine TGEV isolates N genes. The phylogenetic tree analysis revealed that these non-porcine TGEVs N genes had closer genetic relationships and evolution distance to the N genes of Chinese TGEV strains. The three-dimensional structure of the N protein (32-154AA (amino acid)) of HYM-09-2 generated by homology modeling was different from that of other TGEV strains (including HYM-09 and HYM-09-1). These data serve as a foundation for further insight into the virulence or mutation rates of the non-porcine TGEVs.

Key words: *N* gene, cloning, TGEV, non-porcine, phylogenetic analysis, homology modeling.

INTRODUCTION

Transmissible gastroenteritis (TGE) is a highly contagious disease of pigs, and the disease is characterised by profuse diarrhoea and vomiting. The epizootic form of the disease has mortality as high as 100% in pigs younger than 2 weeks of age and infected suckling piglets may die of severe dehydration (Schwegmann-Wessels et al., 2003; Jones et al., 1997).

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Abbreviations: TGEV, Transmissible gastroenteritis virus; **AA,** amino acid; **STCs,** swine testicle cells; **NT,** neutralization test; **PRCV,** porcine respiratory coronavirus; **DMEM,** Dulbecco's modified Eagle medium; **RT-PCR,** Reverse transcriptase-polymerase chain reaction; ELISA, Enzyme-linked immunosorbent assay; **RT-PCR,** Reverse transcriptase-polymerase chain reaction.

Occurrences of TGE have become more sporadic. The disease is still reported on an occasional basis from parts of Europe, North America and Asia. Wild and domestic carnivores (foxes, dogs, possibly mink) and cats seroconvert to TGEV and are suggested as potential subclinical carriers of TGEV, serving as reservoirs between seasonal (winter) epidemics (Saif and Sestak, 2006).

Transmissible gastroenteritis virus (TGEV), a member of the *Coronaviridae* family, is a pleomorphic enveloped RNA virus with a single-stranded, positive-sense genome of 28.6 kb. The viral particle is composed of at least four structural proteins (Laude et al., 1990; Eleouet et al., 1995; Brian and Baric, 2005; Schwegmann-Wessels and Herrler, 2006; Ortego et al., 2007) and N gene encodes the nucleocapsid protein N (Britton and Garmes, 1986; Jacobs et al., 1986). Numerous studies have shown that the N gene of TGEV is highly conserved and high level of N protein-specific antibody is produced at the early stage

of TGEV infection (Sestak et al., 1999). Therefore, the N gene will be an ideal candidate for cloning and expression in the development of TGEV diagnostic antigen.

The aim of the present study is to generate new information in respect to TGEV strains isolated from non-porcine hosts. Since the N gene is one of the most conserved genes among coronaviruses, we can estimate genomic mutation rate and trend of TGEV through inspection the N gene sequences. In this report, the N genes were cloned by RT-PCR from a recently isolated TGEV HYM-09, HYM-09-1 and HYM-09-2 respectively in Heilongjiang province, China. The evolution distance and three-dimensional structures of non-porcine TGEV N proteins were analyzed, and several key site mutations were also detected in the present study.

MATERIALS AND METHODS

Viruses' identification and cells

Three suspect TGEV strains named HYM-09, HYM-09-1 and HYM-09-2 were all isolated from non-porcine animals respectively. All of these animals came from Harbin city, capital of Heilongjiang province, P.R. China. HYM-09 was isolated from domestic dogs' faeces at a dog farm. The dogs showed severe diarrhea, transient vomiting, weight loss and dehydration. HYM-09-1 and HYM-09-2 were isolated from faeces of domestic cats and silver foxes at a fox farm respectively. The cats and foxes did not show apparent clinical symptoms and signs of the disease, but piglets of a near pig farm were suffering from acute diarrhoea and vomiting caused by TGEV infection.

The identification of the suspect TGEV strains (HYM-09, HYM-09-1 and HYM-09-2) has been done with swine testicle cells (STCs) culture, fluorescent antibody test, neutralization test (NT), ELISA (Enzyme-linked immunosorbent assay) and RT-PCR. In addition, blocking ELISA tests were done to differentiate TGEV and porcine respiratory coronavirus (PRCV). Swine anti-TGEV serum produced by our lab and FITC-labeled Rabbit Anti-Swine IgG Antibody (Bipec Biopharma, USA) were used for identification of the non-porcine virus strains.

Viruses were cultured using STCs in our laboratory. The 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 10×10^3 g (BECKMAN Avanti J-30I, USA) at 4°C for 25 min, and the virions in the supernatant were pelleted by centrifugation at 100000 g at 4°C for 1.5 h (BECKMAN Avanti J-30I, USA).

RNA isolation

Total RNA was isolated from purified pellets using SDS-Protease K according to the reference (Sambrook and Russell, 2001).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The RT-PCR was performed to isolate the N genes using the pooled cDNAs from purified pellets above. The first-strand cDNA for N genes was carried out by RT using promega company reverse transcription reagent. The 25 µl reaction system was : 1.0 µl cDNA (100 ng/µl), 2.0 µl 2.5 mM mixed dNTPs (TaKaRa, Dalian), 2.5 µl 10× *Pyrobest* buffer II (TaKaRa, Dalian), 2.0 µl 10 µM forward primer, 2.0 µl 10 µM reverse primer, 0.5 µl *Pyrobest* DNA polymerase (5 U/µl; TaKaRa, Dalian), and 15µl sterile water. A pair of sense and antisense primer was designed and aligned based on nucleotide sequences of the N gene of H155 available in GenBank (GQ374566). The sense primer (Pu) 5'-TTATGGCCAACCAGGGACAAC-3' and antisense primer (Pd) 5'-TTAGTTCGTTACCTCATCAAT-3' were used to amplify the N gene coding sequences of the three TGEV strains. The PCR program initially started with a 94°C denaturation for 4 min, followed by 30 cycles of 94°C /1 min, 56°C /1 min, 72°C /1 min, then 72°C extension for 10 min, finally 4°C to terminate the reaction. PCR products of the expected size of 1.1 kb were obtained. The purified PCR products were cloned into the pMD18-T easy vector (TaKaRa, Dalian). The plasmids were transformed into *Escherichia coli* DH5α using standard molecular technique. Plasmid DNAs were extracted by alkaline-lysis from *E. coli* DH5α cultures and verified by using restriction enzyme digestion, and the digested products were analyzed by electrophoresis on a 1% agarose. Colonies with correct size were named pMD18-T-Ns and at least three independent plasmid clones were analyzed, confirmed and sequenced.

Sequencing

The nucleotide sequences of the N genes of HYM-09, HYM-09-1 and HYM-09-2 was determined by TaKaRa Biotechnology (Dalian) Co. Ltd.

Nucleotide sequence accession numbers

The complete nucleotide sequences of the TGEV HYM-09, HYM-09-1, HYM-09-2 N genes had been deposited in the GenBank Database and were assigned accession numbers GU356396, GU356397 and GU356398 respectively.

Sequences analyses

Homologous comparisons of the N gene nucleotide sequences and deduced amino acids of the three TGEV strains with those of other 18 virus strains (including TGEV (13 strains: hn2002 (AY587884); to14 (AF302264); h155 (GQ374566); purdue (NC_002306); 96-1933 (AF104420); ts (AY335549); AF298213 (AF298213); H16 (FJ755618); sc-y (DQ443743); FS772/70 (Y00542); TH-98 (AY676604); Miller M6 (DQ811785); TFI (Z35758)), PRCV (2 strains: ISU-1 (DQ811787); 86-137004 (X60056);) and Canine coronavirus (3 strains: 119-08 (EU924791); 430/07 (EU924790); 174/06 (EU856362)) were performed with DNAMAN software (http://www.lynnon.com). A phylogenetic tree was generated based on N protein sequences by applying the neighbor-joining methods in the CLUSTAL X version2.0 program, which subsequently was edited manually. Statistical significance of groups within phylogenetic tree was evaluated using the bootstrap method with 1,000 replications. The three-dimensional structures of N proteins were predicted by the amino acids homology modeling on http://www.expasy.org/swissmod/SWISSMODEL.html server (Arnold et al., 2006; Schwede et al., 2003; Guex and Peitsch, 1997).

RESULTS

STCs were infected by the three treated suspect TGE

isolates, and obvious cytopathic effects (CPE) were found at the 8th passage, however, the cells in the negative control group were in normal state. Fluorescent antibody tests showed that specific cytoplasmic fluorescence was found in ST cells using FITC-labeled Rabbit Anti-Swine IgG Antibody, and the three isolates were named HYM-09, HYM-09-1 and HYM-09-2 respectively. The $TCID_{50}$ values of HYM-09, HYM-09-1 and HYM-09-2 ($1\times10^{-3.85}$/0.05ml, $1\times10^{-3.92}$/0.05ml and $1\times10^{-3.87}$/0.05ml) were measured using Reed-Muench method, and their neutralization index (NI) were 71, 83 and 74 respectively. Blocking ELISA tests showed the experimental results of HYM-09, HYM-09-1 and HYM-09-2 were all positive, however, the results of control groups were negative. All the results demonstrated that the viruses were TGEVs.

RT-PCR products of approximate 1.1 kb were amplified from the samples and cloned into pMD18-T respectively. The complete nucleotide sequences of the three TGEV strain N genes had been deposited in GenBank, and accession numbers were GU356396 (HYM-09), GU356397 (HYM-09-1) and GU356398 (HYM-09-2). Sequence analyses indicated that all the open reading frames (ORFs) for the N genes of HYM-09, HYM-09-1 and HYM-09-2 consisted of 1149 nucleotides coding for a basic protein of 382 amino acids (Figure 1).

Sequence analysis and comparison revealed that the N gene sequences of the non-porcine TGEV were highly similar to those of other TGEV strains of porcine hosts and no deletion or insertion events were detected. The three non-porcine TGEV N DNA sequences shared over 96% identity with that of other porcine TGEV strains and PRCV strains (Figure 2). A representative minimal DNA homology tree for the N genes was shown in Figure 2. There were some new point mutations in the non-porcine TGEV N genes, and most of changes were A/G and C/T substitutions. As expected, the three non-porcine strains showed higher similarity to most Asia TGEV strains (over 97%) (hn2002; to14; h155; ts; H16; sc-y; TH-98) (Figure 2).

To evaluate the evolutionary relationships of the three non-porcine TGEV strains with other TGEV, PRCV and canine coronavirus strains isolated in various parts of the world, we constructed a phylogenetic tree using CLUSTAL X v2.0 program on the basis of the N amino acid sequences (Figure 3). Protein sequences from 21 strains were used for the unrooted phylogenetic tree and constructed by the neighbor-joining method. The N proteins from different strains were divided into three subgroups. The canine coronavirus strains (119-08, 430-07 and 174-06) were grouped into a cluster. The second group consisted of purdue, th-98 and sc-y with the HYM-09-1 isolate described here. The other TGEV and PRCV strains, including HYM-09 and HYN-09-2, formed the third group (Figure 3). The three N genes of non-porcine TGEVs had a closer genetic relationship with Chinese TGEV strains (hn2002; h155; ts; H16; sc-y; TH-98). The phylogenetic analysis showed that HYM-09, HYM-09-1 and HYM-09-2 may be three mutated strains of Chinese TGEVs.

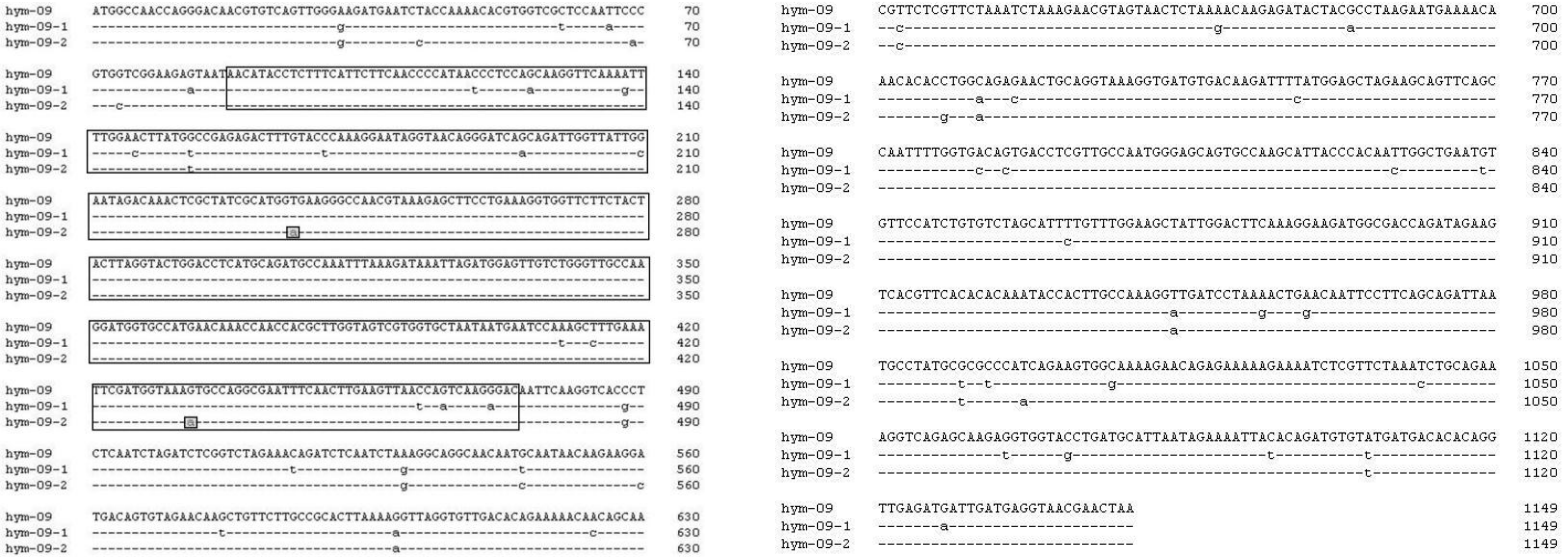
In order to better understand the detailed structures of N proteins from non-porcine TGEVs, the homology modeling of N proteins was performed to estimate its three-dimensional structure. The three-dimensional structures of the TGEV N (30-158AA) (including HYM-09 and HYM-09-1) by homology modeling were similar to that of the N-terminal RNA-binding domain of the SARS-CoV nucleocapsid protein (1sskA: 1-158AA) (Figure 4A). However, the three-dimensional structure of the HYM-09-2 N (32-154AA) by homology modeling was similar to that of MHV nucleocapsid protein NTD (3hd4A: 64-194AA) (Figure 4B). This difference may be due to the special mutations of HYM-09-2 at position 79AA (V to E mutation; T to A mutation at position 235nt) and 145AA (V to M mutation; G to A mutation at position 432nt)) in fox TGEV N amino acid sequence (Figure 1). Since three-dimensional structures are important for the protein function and virulence, the structure analysis may provide a basis for further studying the relationship between structure and virulence of N protein.

DISCUSSION

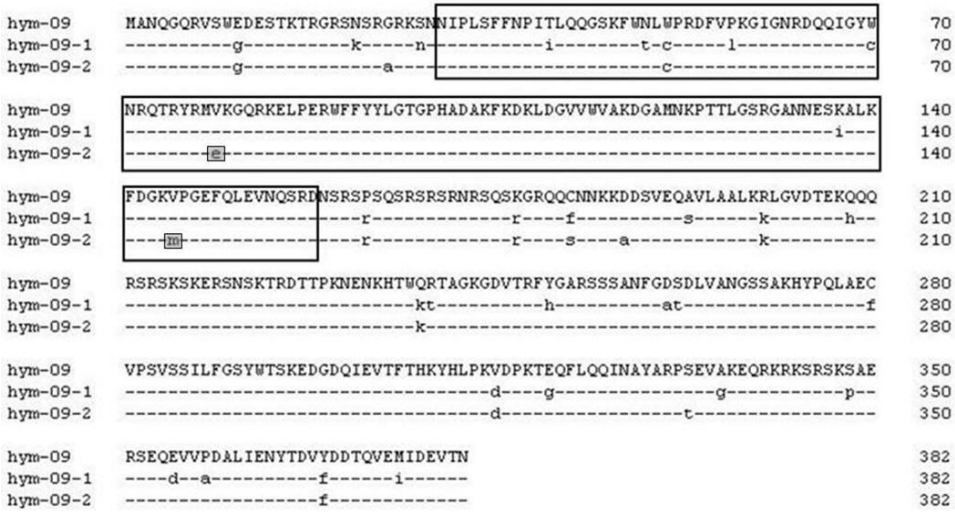
TGEV consists of several encoding regions in which one or more ORFs are identified. The N protein is a nucleocapsid protein and high level of N protein-specific antibodies is produced at the early stage of TGEV infection (Sestak et al., 1999). It has become one of the ideal candidate protein used for the development of reagents for serological diagnosis of TGE (Fan et al., 2007).

In this study, sequence and homology comparison among TGEVs (16 strains, including HYM-09, HYM-09-1, HYM-09-2), PRCVs (2 strains) and Canine coronaviruses (3 strains) showed that there existed some new point mutations in the three non-porcine TGEV isolates N genes which did not exist in the other isolates used here (Table 1). Homology modeling analysis showed that the three-dimensional structure of HYM-09-2 N protein (32-154AA) was different from that of other TGEV strains (Table.1). The most important cause of these own mutations and three-dimensional structure may come from selective pressure on the N gene in non-porcine hosts. We also found that there were relatively high frequency changes from nucleotide A, C or G to T, and A or C to G in the N genes of these strains used here, and if the kind of point mutations will influence the virulence or tropism of the non-porcine virus will be further studied in future.

In conclusion, this study indicates the presence of silent mutations in the N gene sequences of the three non-porcine TGEV isolates, and the three-dimensional structure of HYM-09-2 N protein is different from that of other non-porcine TGEV isolates and porcine TGEV



1a



1b

Figure 1. Multiple sequence alignment of N sequences among the three non-porcine TGEV strains (HYM-09, HYM-09-1 and HYM-09-2). Figure1a: nucleotide sequences; Figure1b: deduced amino acid sequences. The sequence of HYM-09 isolate is displayed as consensus at the top, and the different nucleotides of other strains are indicated. Dashes(-) indicate regions where sequences were identical to those of HYM-09. The amino acid sequence (30-158AA) used for homology modeling is boxed. Special mutation sites of HYM-09-2 are shaded.

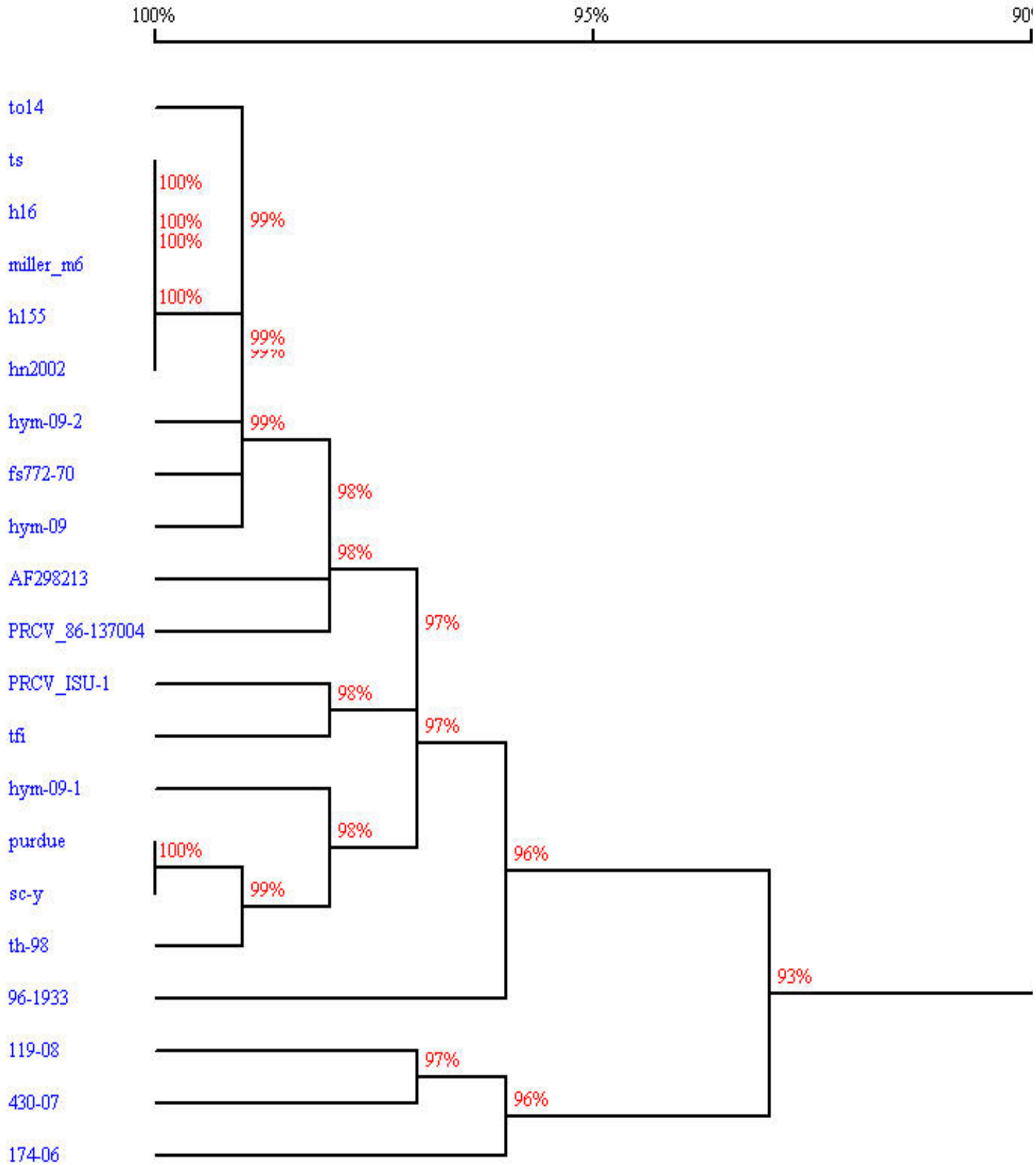


Figure 2. Homology tree of TGEV strains based on the nucletide sequences of N gene. Analysis was done using the DNAMAN software. hn2002 (China, AY587884); to14 (Japan, AF302264); h155 (China, GQ374566); purdue (USA, NC_002306); 96-1933 (England, AF104420); ts (China, AY335549); AF298213 (Korea, AF298213); H16 (China, FJ755618); sc-y (China, DQ443743); FS772/70 (England, Y00542); TH-98 (China, AY676604); Miller M6 (USA, DQ811785); TFI (China (Taiwan), Z35758); ISU-1 (USA, DQ811787); 86-137004 (England, X60056); 119-08 (Italy, EU924791); 430/07 (Italy, EU924790); 174/06 (Italy, EU856362).

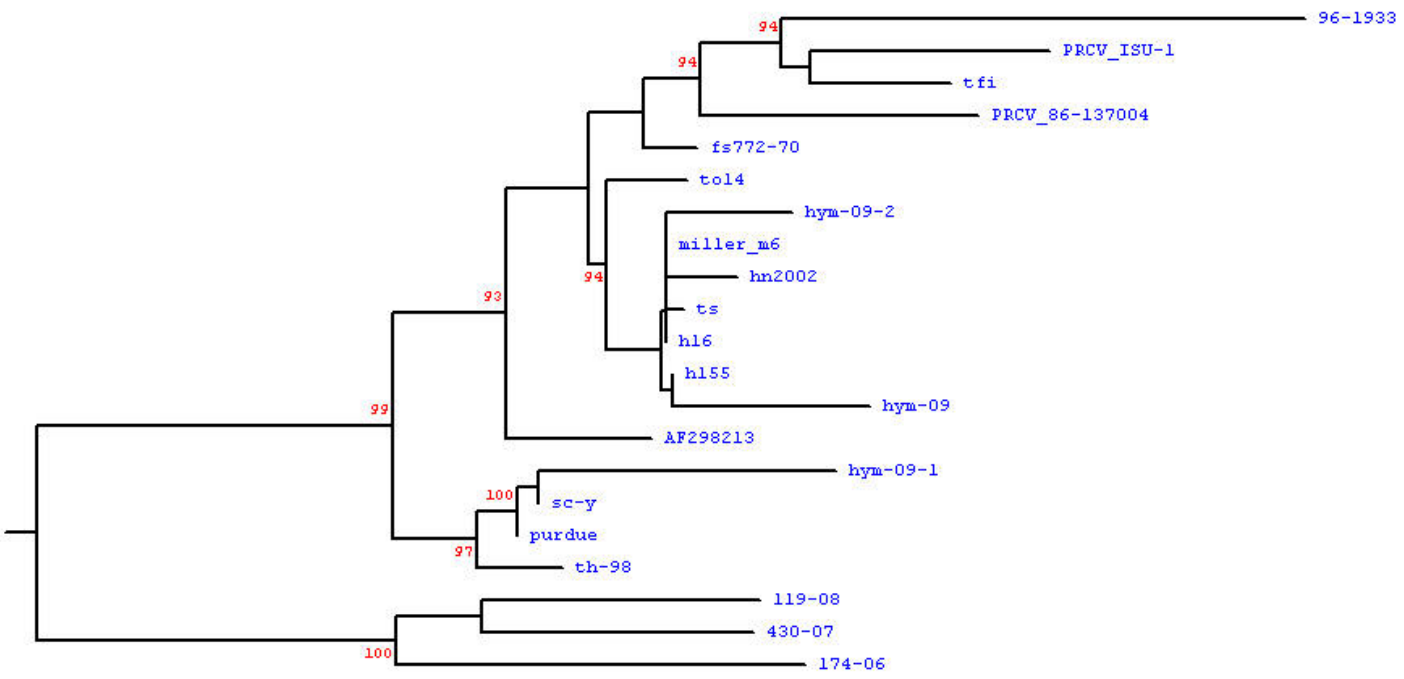


Figure 3. Phylogenetic tree of TGEV strains based on the nucleotide sequences of N gene. The tree was obtained by bootstrap analysis with the neighbor-joining method; Numbers on the branches represent bootstrap values for 1,000 replications. The values of bootstrap confidence level (BCL) of the nodes are indicated above the branch.

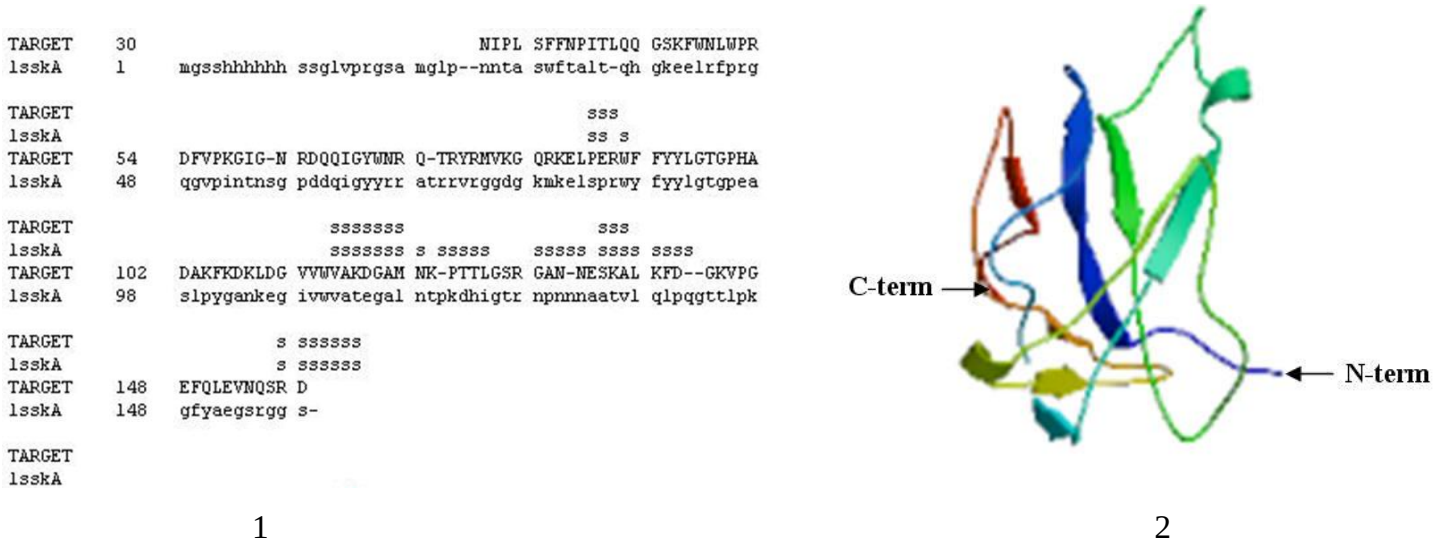


Figure 4A. Homology modeling of the N protein (30-158AA) of TGEV. Figure 4a: Homology modeling of N protein (30-158AA) of HYM-09 strain based on the crystal structure of the N-terminal RNA-binding domain of the SARS-CoV nucleocapsid protein (1sskA: 1-158AA). 1 and 2: Amino acid sequence alignments: ‘‘S’’ indicates the amino acid residues responsible for formation of the ‘‘bend’’. Diagram comparing the relative position of the amino acid residues of TGEV N proteins with that of the corresponding virus ortholog; Figure 4a-2 and Figure 4b-2: The three-dimensional structures of N protein partial sequences were constructed by comparative protein modeling program SWISS-MODEL.

strains. These results suggest that the non-porcine TGEV isolate (HYM-09-2) may have happened virulence changes.

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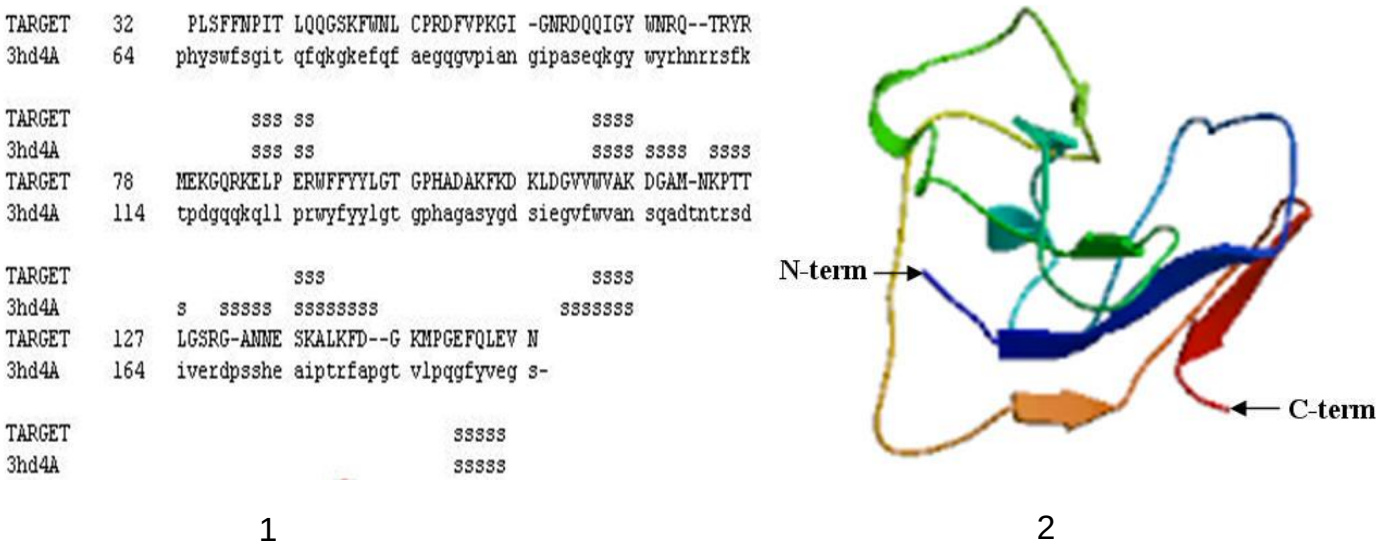

Figure 4B. Homology modeling of the N protein (30-158AA) of TGEV. Figure 4a: Homology modeling of N protein (30-158AA) of HYM-09 strain based on the crystal structure of the N-terminal RNA-binding domain of the SARS-CoV nucleocapsid protein (1sskA: 1-158AA). Figure 4b: Homology modeling of N protein (32-154AA) of HYM-09-2 strain based on the crystal structure of MHV nucleocapsid protein NTD (3hd4A: 64-194AA). Figure 4a-2 and Figure 4b-2: The three-dimensional structures of N protein partial sequences were constructed by comparative protein modeling program SWISS-MODEL.

Table 1. Summary of the main results in the experiment.

TGEV strain	Species name	Specific point mutations in non-porcine TGEV N gene sequence	TGEV N protein three-dimensional structure (32-154AA)
HYM-09	Dog	31nt (G to A); 31nt (T to G); 487nt (G to C); 529nt (G to A); 598nt (A to G); 711nt (A to C); 944nt (A to T); 990nt (T to G); 1106nt (T to A);	Similar to 1sskA (1-158AA)
HYM-09-1	Cat	65nt (T to A); 118nt (C to T); 137nt (A to G); 169nt (C to T); 219nt (G to C); 409nt (A to T); 413nt (T to C); 576nt (G to T); 623nt (A to C); 716nt (G to C); 747nt (T to C); 781nt (A to C); 839nt (G to T); 1007nt (C to G); 1042nt (T to C); 1072nt (C to G);	Similar to 1sskA (1-158AA)
HYM-09-2	Fox	68nt (C to A); 73nt (G to C); 235nt (T to A); 432nt (G to A); 559nt (G to A); 707nt (C to G); 997nt (T to A);	Similar to 3hd4A (64-194AA)

of Heilongjiang Province (ZJN0602-02).

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