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Cloning and expression of nucleocapsid protein gene of TGEV HB06 strain

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Abstract The nucleocapsid protein gene of transmissible gastroenteritis virus, 1 149 bp in length, was amplified by RT-PCR from isolated strain HB06 and cloned into pMD18-T. Sequence comparison with other transmissible gastroenteritis virus (TGEV) strains selected from the Gene Bank revealed that the homology of *N* gene complete sequence shares more than 97% in nucleotide. *N* gene was cloned into *Bam*HI and *Eco*RI multiple cloning sites of the prokaryotic expression vector pET 20 b, and named pETN. After being induced by isopropyl- β -D-thiogalactopyranoside (IPTG), the recombinant nucleocapsid protein was expressed. The result of SDS-PAGE and Western-blot showed that the recombinant nucleocapsid protein was 47 kDa and had strong positive reactions with TGEV-specific antibody.

Keywords transmissible gastroenteritis virus, *N* gene, cloning, expression

1 Introduction

Transmissible gastroenteritis (TGE) virus is a coronavirus that causes enteric disease in swine of all ages. The disease is especially severe in newborn animals less than two weeks old, in which mortality approaches 100% (Siddell et al., 1983; Sturman and Holmes, 1983; Saif and Bohl, 1986). Statistics suggest that 39.8% of dead pigs in China died of diarrhea and 10.3% were caused by TGEV. Transmissible gastroenteritis has become one of the severe viral diarrhea diseases in young pigs that leads to severe economic loss in pig farming. Therefore, it is important to prevent and control the disease. However, because of the similarity between TGE and porcine

epidemic diarrhea in clinical signs and pathology, there is no effective method in the country to distinguish one from the other accurately.

Transmissible gastroenteritis virus (TGEV) has a single-strand, positive-sense RNA genome of more than 20 kb (Brain et al., 1980; Hu et al., 1984; Rasschaert et al., 1987) and molecular mass 6.8×10^3 kDa (Julian et al., 1995; Hua et al., 2000). The viral particle is composed of three structural proteins (Kapke and Brian, 1986; Jacobs et al., 1987; Laude et al., 1987; Rasschaert and Laude, 1987; Correa et al., 1990; Eleouet et al., 1995; Tang et al., 2002) and *N* gene translates the nucleocapsid protein N (Britton and Garmes, 1986; Jacobs et al., 1986). Numerous studies have shown that the *N* gene of the TGEV is highly conserved (Jacobs et al., 1986) and high level of N protein-specific antibody is produced at the early stage of TGEV infection (Ksestak et al., 1999). Therefore, the *N* gene will be an ideal candidate for cloning and expression in the development of TGEV diagnostic antigen.

In this study, the RNA was extracted from TGEV HB06 strain isolated from the feces of piglets infected with TGEV on a pig farm in Hebei Province. *N* gene has been cloned and expressed in a prokaryotic expression system. The expressed fusion protein showed strong positive reactions with TGEV-specific antisera. These data are useful for the development of diagnostic antigen used for TGEV diagnosis.

2 Materials and methods

2.1 Enzymes and reagents

Enzymes (restriction endonucleases, polymerases and ligases) were purchased from TaKaRa Biotechnology (Dalian, China) Co., Ltd. Alkaline phosphatase-conjugated goat anti-swine immunoglobulin G antibodies were purchased from Sigma.

The TGEV strain HB06 was isolated from the feces of piglets suffering from severe diarrhea in Hebei Province and propagated in PK15 cell lines. The harvested virions were collected from cells disrupted by three cycles of freezing and thawing. Cellular debris was removed by low speed

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centrifugation. The supernatant was used for the extraction of the viral RNA using the Trizol reagent (Invitrogen USA) based on the manufacturer's instructions.

2.2 Primers for RT-PCR

A pair of oligonucleotide primers was designed to amplify the whole sequence of *N* gene from strain HB06 based on the published sequence. The sense primer UNP1 and antisense primer UNP2 were 5' TT GGATCC ATG GCC AAC C AGG GAC AG 3' and 5' CG GAATTC TTA GTT CGT TAC CTC ATC AA3', respectively.

2.3 RT-PCR

Reverse transcription was in 20 μ L volumes, using 5 μ L of viral RNA, 4 μ L of 5 $\times$ RT buffer, 4 μ L of dNTPs (2.5 mmol), 0.5 μ L of RNase Inhibitor (40 U/ μ L), 1 μ L of oligo dT18 (50 pmol) and 2 μ L of Avian myeloblastosis virus (AMV) reverse transcriptase (5 U/ μ L). Samples were incubated at room temperature for 5 min and then at 42°C for 90 min. The reaction was terminated by heating at 75°C for 5 min and then cooled to 4°C.

PCR was performed in 25 μ L volumes containing 5 μ L of the first-strand cDNA template, 2.5 μ L of 10 $\times$ PCR buffer, 3 μ L of 2.5 mmol dNTP mixture, 0.5 μ L of each 100 pmol sense and antisense primer and 0.5 μ L of Taq DNA polymerase (5 U/ μ L). The PCR parameters were 5 min at 94°C, and 30 cycles of 40 s at 94°C, 45 s at 55°C, 90 s at 72°C, and a final extension time of 8 min at 72°C. Following amplification, all reactions were extended at 72°C for 8 min and chilled at 4°C. The PCR product was visualized by gel electrophoresis.

2.4 Cloning and sequencing

The *N* gene fragments obtained after RT-PCR were separated by gel electrophoresis and purified using a Gel Extraction Mini Kit. The purified product corresponding to the *N* gene was cloned into pMD18-T plasmid DNA vector and named pMDN. The recombinant plasmid was used to transform competent *Escherichia coli* JM109. The positive clones were selected by their lacZ-negative phenotypes and verified by restriction enzyme digestion and DNA sequencing.

2.5 Sequence analysis

Nucleotide sequence analysis of the HB06 *N* gene with other TGEV strains was performed with DNAMAN software.

Expression of *N* gene fragment: The *N* target fragment was obtained from pMDN with the digestion of *Bam*HI and *Eco*RI, and inserted into pET20b expression vector. The recombinants were named pETN. Recombinant plasmids were introduced into the competent *Escherichia coli* BL21 by the GaCl_2 transformation procedure. The positive clones

were verified by restriction enzyme digestion. The *E. coli* strain BL21 with the pET20b plasmid containing the *N* gene fragment was cultured overnight and induced by the addition of 1 mmol isopropyl- β -D-thiogalactopyranoside (IPTG) at 37°C for 4 h. Bacteria cells were harvested by centrifugation for sonication, and the expressed proteins were analyzed by SDS-PAGE and immunoblotting.

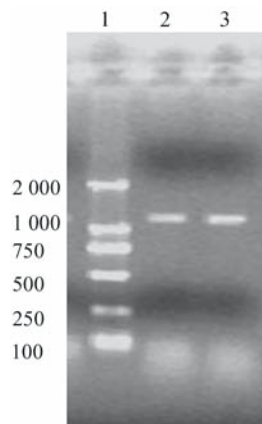
2.6 SDS-PAGE and Western blot

Samples from the lysate of transformed cells were dissolved in 2 $\times$ sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and were boiled for 10 min. Proteins separated by electrophoresis in 12% polyacrylamide gels were stained with Coomassie blue or electrophoretically transferred to a nitrocellulose membrane filter for Western blot analysis. After transfer, the nitrocellulose sheet was blocked overnight at room temperature with blocking buffer phosphate buffer solution (PBS) supplemented with 0.1% Tween-20 containing 5% bovine serum albumin. The membrane was incubated at 37°C for 1 h on a rocker platform with the TGEV-specific antisera. After three washings, the nitrocellulose paper was incubated with a 5 $\times 10^3$ -fold dilution of alkaline phosphatase-conjugated goat anti-swine immunoglobulin G antibodies, then another hour at 37°C. The membrane was soaked in 5'-bromo-4-chloro-3-indolylphosphate nitroblue tetrazolium (NBT) /5-bromo-4-chloro-3-indolylphosphate (BCIP) (Invitrogen) solution until development of color.

3 Results

3.1 Cloning of TGEV *N* gene

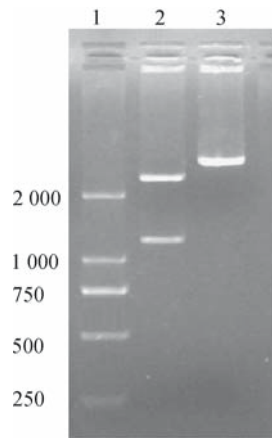
The target fragment of *N* gene with the expected size of 1 149 bp is amplified from cDNA template of TGEV strain HB06 by RT-PCR (Fig. 1) and cloned into pMD18-T vector.



Note: Lane 1, DNA Marker DL2000; Lane 2 and Lane 3, the target amplification of *N* gene.

Fig. 1 Identification of PCR product of TGEV *N* gene

The recombinant plasmid thus constructed, designated pMDN, is verified by restriction enzyme digestion (Fig. 2) and used as a template to determine the *N* gene sequences.



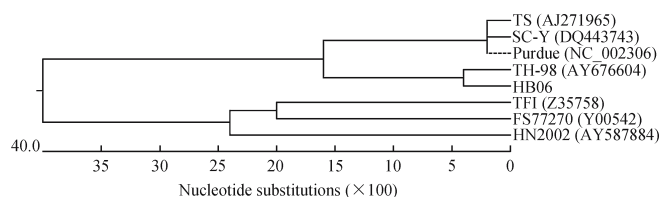
Note: Lane 1, DNA Marker DL2000; Lane 2, *N* DNA (1 149 bp) was identified from pMD18-T plasmid DNA (2 692 bp) after digestion of the recombinant DNA with *Bam*HI and *Eco*RI; Lane 3, pMDN (3 841 bp) was identified after digestion with *Eco*RI.

Fig. 2 Identification of recombinant plasmid pMDN with restriction digestion

3.2 Sequence analysis

Sequence analysis indicated that the nucleotide sequence of *N* gene Open reading frame (ORF) is 1,149 bp in length and encodes 382 amino acids. Sequence comparison with other TGEV strains revealed that the nucleotide sequence of HB06 is 99%, 99%, 99%, 97%, 97%, 97%, 97% and 97% identity with those of TH-98 (AY676604), SC-Y (DQ443743), Purdue (NC_002306), TS (AJ271965), HN2002 (AY587884), FS772/70 (Y00542), and TFI (Z35758) strain, respectively.

To analyze the phylogenetic relationships of HB06 with other TGEV strains isolated in various parts of the world, we constructed a neighbor-joining phylogenetic tree using the *N* gene sequences. A representative minimal tree for the *N* gene is shown in Fig. 3.

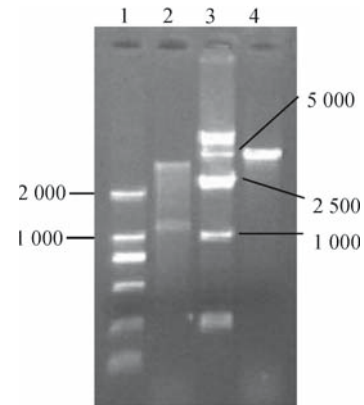


Note: The accession number for the *N* genes of TH-98, SC-Y, Purdue, TS, HN2002, FS772/70 and TFI are AY676604, DQ443743, NC_002306, AJ271965, AY587884, Y00542 and Z35758, respectively.

Fig. 3 Phylogenetic of TGEV strains based on the nucleotide sequences of *N* gene

3.3 Construction of recombinant plasmid pETN

Recombinant clone containing the *N* gene was *Bam*HI and *Eco*RI digested and cloned into the expression vector pET 20 b. The correct orientation was determined by restriction analysis (Fig. 4).

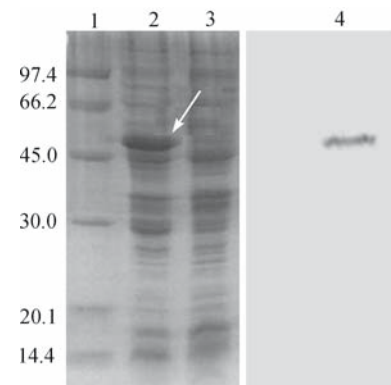


Note: Lane 1, DNA Marker 2000; Lane 2, *N* DNA (1 149 bp) was identified from pET20 plasmid DNA (3 176 bp) after digestion of the recombinant DNA with *Bam*HI and *Eco*RI; Lane 3, DNA Marker 15000; Lane 4, pETN (4 325 bp) was identified after digestion with *Eco*RI.

Fig. 4 Identification of recombinant plasmid PETN with restriction digestion

3.3 Expression and biological activity of *N* gene

Isopropyl- β -D-thiogalactopyranoside (IPTG) at concentration of 1mmol induced the expression of *N* gene in the pET20-N-BL21 system. After electrophoretic analysis and Coomassie blue-staining, a visible extra band of 47 kDa was detected in the extracts of BL21 cells transformed with the recombinant plasmid pETN (Fig. 5). The size observed corresponds to the expected size for the fusion protein. The



Note: Lane 1, molecular mass markers; Lane 2, crude extract from pETN transformed cells; Lane 3, Control; lane 4, Immunoblotting analysis of the expression protein with TGEV specific antiserum.

Fig. 5 SDS-PAGE and Western-blot analysis of the expression protein

identity of this protein was confirmed by immunoblotting. Specific antisera against TGEV gave a clear positive reaction with the 47 kDa protein (Fig. 5).

4 Discussion

The genome of TGEV consists of a single molecule of positive-sense, single-stranded RNA. Its encoding regions have been identified. The N protein forms the viral nucleocapsid (Martin Alonso et al., 1992; Song et al., 2006). Previous studies have demonstrated that the N protein has high immunoreactivity. High levels of N protein-specific antibodies will be produced in pigs in the initial stage of TGEV infection (Ksestak et al., 1999) and the level of antibody can reflect the level of neutralizing antibodies (Ksestak et al., 1999). The N protein, used as a candidate antigen for the diagnosis of TGEV infection, has advantages of high sequence conservation and large expression in TGEV infected cells (Jacobs et al., 1986).

In this study, we have successfully cloned the entire N gene of HB06 and determined its nucleotide sequence. The N gene has an ORF of 1,149 nucleotide coding for a 382 amino acid protein. Sequence comparison with other TGEV strains revealed that the N gene cDNA was significantly homologized with TH, SC-Y, Purdue, TS, HN2002, FS772/70, and TF1 strain, respectively. These results showed that the N gene of HB06 was conserved.

Recombinant plasmid pETN was successfully expressed using an *E. coli* expression system. In order to confirm the biological activity of the expressed protein, TGEV-specific antisera were used in immunoblotting. A clear positive reaction was observed in Western-blot analysis. These results showed the high immunoreactivity of the fusion protein.

In conclusion, the N gene of the HB06 strain was cloned and expressed, and the expressed protein was produced with biological activity in this study. Further studies are needed to use the fusion protein as an antigen for the diagnosis of TGEV infection in pigs.

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