

Express Transmissible Gastroenteritis Virus Spike Gene B and C Antigen Sites in Multiple Expression Systems^{*}

Tian-fei Yu, Shu-li Shao, Xing-jun Xu, Jian-wei Lv, and Ming Li

College of Life Science and Agriculture Forestry University of Qiqihar
Qiqihar, Heilongjiang Province, China
{yutianfei2001,shshl32}@163.com, fiona001lee@yahoo.cn

Abstract. In order to illuminate the antigenicity of porcine transmissible gastroenteritis virus (TGEV) spike protein B and C antigen sites, the truncated spike gene including B and C antigen sites of Chinese isolate TH-98 was expressed respectively in *E.coli*, *baculovirus* and *pichia pastoris* expression systems. Dot enzyme-linked immunosorbent assays (Dot-ELISA) based on these three recombinant proteins were developed preliminarily. Ten sera obtained correspondingly from ten piglets two months old which showed up clinical symptom were used for examination. The study indicates that the assays are rapid, reliable and sensitive and it has the potential for use as serological methods for TGEV diagnosis.

Keywords: Transmissible gastroenteritis virus, Spike protein, Expression, Dot-ELISA.

1 Introduction

Transmissible gastroenteritis (TGE) is a highly contagious viral disease of swine characterized by vomiting, diarrhea, and dehydration. Its causative agent is transmissible gastroenteritis virus (TGEV), considered the principal etiologic agent responsible for dramatic outbreaks of diarrhea and high mortality of newborn pigs, in which mortality approaches 100% [1-2]. Porcine respiratory coronavirus (PRCV) is believed to be a mutant of TGEV, as it has been shown to be genetically related to TGEV but has a selective tropism for respiratory tissue with very little to no replication in the intestinal tissue of infected swine [3]. TGEV and PRCV share high homology in genome and can generate the full cross-reaction neutralizing antibody [4-6], so it is difficult to discriminate TGE in clinical diagnosis with traditional serological methods.

* This work is supported by National High-Tech Research and Development Plan of China #2008AA10Z326 to Y Dai and National Natural Science Foundation of China #20776112 to S Jia.

The truncated spike gene including B and C antigen sites which is absent in PRCV was expressed in *E.coli*, *baculovirus* and *pichia pastoris* in this research. Dot-ELISA assays based on these three recombinant proteins were developed to detect TGEV antibodies and could avoid antibody cross-reaction from PRCV theoretically.

2 Materials and Methods

2.1 Viruses and Cells

TGEV strain named TH-98 was isolated from a suburb of Harbin, 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% CO₂ in air. Viruses were harvested by three cycles of freezing and thawing, cellular debris was removed by low speed centrifugation at 1.0×10⁴g (HITACHI CR22E, Japan) at 4°C for 25 min, and virions in supernatant were pelleted by centrifugation at 1.0×10⁵g at 4°C for 1.5 h (HITACHI CR22E, Japan).

2.2 Oligonucleotide Primers

All the three pairs of primers were prepared according to the sequence of TGEV strain TH98 from a data base, GenBank (Accession no.AF494337). PGu/PGL contained *EcoR I* or *Sal I* restriction enzyme site respectively, PHu/PHI contained *Pst I* or *Sal I* restriction enzyme site respectively, and PYu/PYI contained *EcoR I* or *Not I* restriction enzyme site respectively. All the primers contained artificial start codon or termination codon (Table. 1).

Table 1. Nucleotide sequence of PCR primers for amplification of each expression system

Expression system	Sequence(5'-3')	Primer annealing temperature (°C)
<i>E.coli</i>	PGu: CCCgAATTCATgACCTTCCTTCTAAACTAT	55
	PGL : CCggTCgACTTATAACCTgCACTCACTAC from 115nt to 472nt of spike gene.	
<i>Baculovirus</i>	PHu: ggCTgCAGTATgTgTTCTAAATTgAC	53.5
	PHI: CggTCgACACgATTATACggTAC-3' from 61nt to 741nt of spike gene	
<i>pichia pastoris</i>	PYu: GTAGAATTCATGTGTTCTAAATTGACT'	56
	PYI: TTTGCGGCCGCTTAACCATTTTTATTATTA from 61nt to 744nt of spike gene	

2.3 RNA Isolation and Reverse Transcription-Polymerase Chain Reaction

RNA was extracted as described by Yin et al [7], meanwhile the ST cell RNA was also extracted as the negative control. Extracted RNA 4 μ l was added into the below components: 5 \times reverse transcription buffer 1 μ l, dNTP mixture (2.5 mM) 4 μ l, RNase inhibitor 0.5 μ l, primer PY1 5 μ l, AMV reverse transcriptase 2 μ l (10 U), sterile water 3.5 μ l, gently mixed in an Eppendorf tube and incubated at room temperature for 10 min, then transferred to a water incubator at 42 $^{\circ}$ C for 1 h prior to stored at -20 $^{\circ}$ C until use in PCR. PCR amplification was performed using PE2400 PCR equipment (USA). PCR was in 100 μ l volumes, using 10 μ l of 10 \times buffer (50 mM KCl, 100 mM Tris-Cl pH8.3, 15 mM MgCl₂, 0.1% gelate, 16 μ l dNTP mixture (10mM), 1 μ l cDNA, 5 μ l of primers and 1 μ l ExTaq polymerase (0.5u Takara, Dalian, China). For the PCR the mixture was submitted to 30 cycles of amplification involving heating at 95 $^{\circ}$ C for 30s, 55 $^{\circ}$ C (*E.coli* expression), 53.5 $^{\circ}$ C (*baculovirus* expression), 56 $^{\circ}$ C (*pichia pastoris* expression) for 30s, and 72 $^{\circ}$ C for 60s, there was then a final extension time of 10min at 72 $^{\circ}$ C. A 2 μ l aliquot of PCR product was visualized by agarose gel electrophoresis (2% agarose, 100 Vs for 20 min, 0.8 μ g/ ml ethidium bromide included in gel) and subsequent U.V transillumination. The purified PCR product was named ts, tg or ty respectively.

2.4 Expression Plasmids Construction

The amplified S gene (ts) DNA was digested with *EcoR I*, *Sal I* and the resulting fragment was inserted into the *EcoR I*, *Sal I* sites of the vector pGEX-6P-1 (Pharmacia Biotech, Inc., USA) to place the cDNA under the control of the tac promoter. The amplified S gene (tg) DNA was digested with *Pst I*, *Sal I* and the resulting fragment was inserted into the *Pst I*, *Sal I* sites of the transfer vector pBlueBacHis2A (Invitrogen Co., USA) to place the cDNA under the control of the polyhedrin promoter. The amplified S (ty) gene DNA was digested with *EcoR I*, *Not I* and the resulting fragment was inserted into the *EcoR I*, *Not I* sites of the transfer vector pPIC9K (Invitrogen Co., USA). The three recombinant vectors were sequenced by Sangon bio-company, Shanghai, China.

2.5 Expression of B and C Antigen Sites in *E.coli*, *Baculovirus* and *Pichia Pastoris*

Expression of B and C antigen sites in *E.coli* and recombinant proteins purification was followed the procedures offered by the description of *Glutathione S-transferase (GST) Gene Fusion System* (amersham pharmacia biotech, third edition, revision 2).

Expression of B and C antigen sites in baculovirus and pichia pastoris were followed Manual of Bac-N-Blue Transfection and Manual of Expression Guide and Methods for Expression of Recombinant Proteins in *Pichia pastoris* (Invitrogen Co., USA).

Twelve percent SDS polyacrylamide gel was used to analyze the three recombinant proteins. After electrophoresis, one of the gels was stained with Coomassie Brilliant Blue R250 to visualize the protein bands. The proteins of the other gel were transferred onto the NC membrane for the Western blot analysis. The diluted protein samples were spotted on the NC membrane for the Dot-ELISA. The membrane was blocked with 5% non-fat dried milk powder in PBS with 0.05% Tween20 (blocking solution). The membrane was probed with the TGEV immunized rabbit serum, in blocking solution (1:100). A peroxidase conjugated sheep anti-rabbit IgG (Promega, USA) was used as the secondary antibody (1:1000) and the signal was detected with H_2O_2 and 4-Chlor-1-naphthol as a chromogenic substrate.

3 Results

3.1 Expression of B and C Antigen Sites in *E.coli*

IPTG induced the *E.coli* BL21 (DE₃) pLysS transformed with the pGEX-6P-ts plasmid to express the recombinant fusion protein of about 40KD (Fig. 1. I illustrate). This was consistent with the expected molecular mass of the fusion protein of pGEX-6P-ts which consisted of the GST (26KD) and S gene B and C antigen sites subunit (14KD).

3.2 Expression of B and C Antigen Sites in *Baculovirus* Expression System

Two different MOIs, 5 and 10 were tested. Seed two 6-well plates with 10^6 cells in each well. The final volume in each well should be 1-1.5 ml. The harvest (post infection) was, 24 hours, 48 hours, 72 hours, and 96 hours. As shown in Fig. 1. II illustrate and Fig. 1. III illustrate, recombinant baculovirus expressed 33 KD protein corresponded to the molecular weight of TG of TGEV at 24 hours post-infection and the protein was accumulated in high amount till 96 hours post-infection. No specific band was detected in the culture medium by Coomassie brilliant blue staining.

Sf9 cells were inoculated recombinant virus with MOIs, 5. Infected cells were harvested after 96h. The cell sediments were resuspended in PBS (PH7.4). After interruptible ultrasonic treatment, the lysate and supernatant were analyzed by SDS-PAGE. The result showed that recombinant protein was soluble bulk in supernatant (The result was not given).

3.3 Expression of B and C Antigen Sites in *Pichia Pastoris*

The recombinant B and C antigen sites protein expressed into the yeast culture supernatant was identified on the bases of its molecular weight. Numerous bands were observed in the 14-116 KD molecular mass range. A sharp band was observed at molecular masses of approximately 40 KD by SDS-PAGE. The recombinant protein is the major protein component observed in the culture supernatant (Fig. 1. IV illustrate).

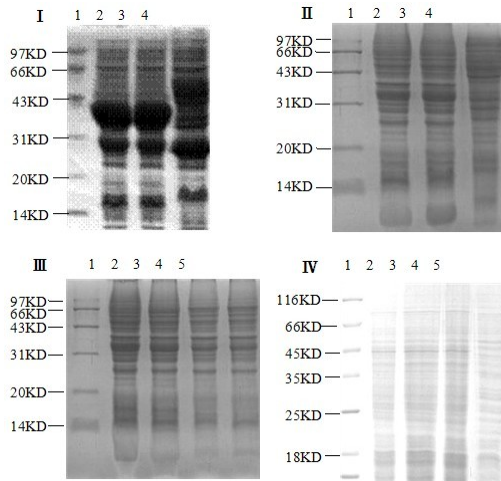


Fig. 1. SDS-PAGE analysis of expressed B and C antigen sites recombinant proteins. I . Expression of B and C antigen sites in E.coli. The vector or recombinant plasmid transformed E. coli was cultured in LB medium and induced with IPTG. Lane1: Protein molecular marker; Lane2 and Lane3: pGEX-6P-ts with IPTG induction for 4h; Lane4: pGEX-6P-1 with IPTG induction for 4h. II . Expression of B and C antigen sites in baculovirus expression system. Lane1: Protein molecular marker; Lane2: The sediment of Sf9 cell infected by recombinant baculovirus with MOIs, 10; Lane3: The sediment of Sf9 cell infected by recombinant baculovirus with MOIs, 5; Lane4: Uninfected Sf9 cells control. III . Sediments of Sf9 cell infected by recombinant baculovirus in different times. Lane1: Protein molecular marker; Lane2~Lane5: The sediments of Sf9 cell infected by recombinant baculovirus with MOIs, 5 after 96h, 72h, 48h and 24h. IV . Expression of B and C antigen sites in pichia pastoris. Lane1 was the protein molecular mass range. Samples were taken 24 h (Lane2), 48 h (Lane3) and 72 h (Lane4) after induction with methanol. Culture supernatant (20 μ l) from each time-point was analyzed by SDS-PAGE. Lane5 was the GS115 cells supernatant transformed with pPIC9K plasmids.

3.4 The Antigenicity of the Recombinant Proteins

The antigenicity of the three recombinant proteins was analyzed by Dot-ELISA assay. When the amount of spotting is 750ng, the recombinant protein expressed in prokaryotic system shows the positive reaction in contrast with GST (Fig. 2. I illustrate).

When the amount of spotting is 100ng, the ultrasonic lysis supernatant of Sf9 cell infected by recombinant baculovirus show the positive reaction in contrast with ultrasonic lysis supernatant of Sf9 cell (Fig. 2. II illustrate).

When the amount of spotting is 50ng, the recombinant B and C antigen sites protein expressed into the yeast culture supernatant show the positive reaction in contrast with the GS115 cells transformed with pPIC9K plasmids (Fig. 2. III illustrate).

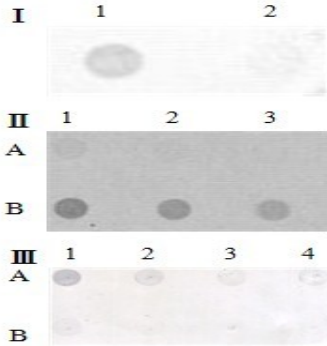


Fig. 2. The antigenicity of the recombinant proteins by Dot-ELISA assays. I . Analysis of the antigenicity of recombinant protein expressed in prokaryotic system. Lane1. Purified recombinant protein (750ng); Lane2. Purified GST protein (750ng). II . Analysis of the antigenicity of recombinant protein expressed in baculovirus system. Dots A₁ (1000ng), A₂ (100ng), A₃ (10ng) were ultrasonic lysis supernatant of Sf9 cell; Dots B₁ (1000ng), B₂ (100ng), B₃ (10ng) were ultrasonic lysis supernatant of Sf9 cell infected by recombinant baculovirus. III . Analysis of the antigenicity of recombinant protein expressed in *pichia pastoris* expression system. Dots A₁ (100ng), A₂ (50ng), A₃ (25ng), A₄ (12.5ng) were the positive yeast culture supernatant; Dots B₁ (100ng), B₂ (50ng), B₃ (25ng), B₄ (12.5ng) were the GS115 cells supernatant transformed with pPIC9K plasmids.

3.5 Detection of Transmissible Gastroenteritis Virus Antibody by Dot-ELISA

Ten field sera obtained from ten piglets about two months old which showed typical signs of epizootic TGE used for examination and the ten corresponding sera were positive detected by Dot-ELISA (Fig. 3).

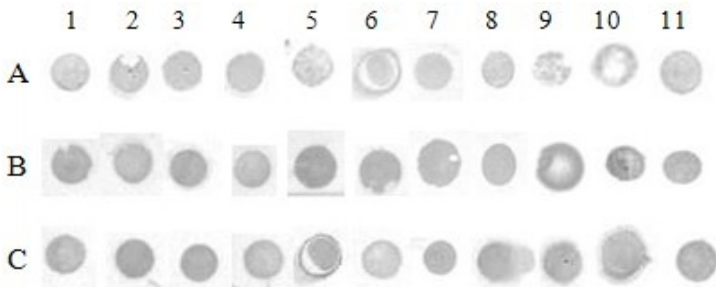


Fig. 3. Detection of transmissible gastroenteritis virus antibody by Dot-ELISA. The coating antigen in A, B, C rows were the recombinant protein expressed in *E.coli*, *baculovirus* and *pichia pastoris* respectively. The 11th column was the positive TGEV antibody control.

4 Discussion and Conclusion

The spike protein of TGEV has been shown to contain four major antigenic sites (A, B, C, and D). Site A is the main inducer of neutralizing antibodies and has been previously subdivided into the three subsites Aa, Ab, and Ac. Site A contains the residues 538, 591, and 543, which are essential in the formation of subsites Aa, Ab, and Ac, respectively. The peptide 537-MKSGYGGQPIA-547 represents, at least partially, subsite Ac which is highly conserved among coronaviruses. This site is relevant for diagnosis and could be of interest for protection. Other residues contribute to site B (residues 97 and 144), site C (residues 50 and 51), and site D (residue 385). Site C can be represented by the peptide 48-P-P/S-N-S-D/E-52 but is not exposed on the surface of native virus [8]. Site B is dependent on intracellular glycosylation and is complex and conformation-dependent. This site is formed by at least three epitopes. Although site B is conformation dependent, MAbs specific for this site can bind TGEV spike protein by immunoblotting providing that the samples were not treated with 2-mercaptoethanol. Most probably, renaturation of spike protein occurs during the blotting of the protein to nitrocellulose paper [8]. Site C is linear and continuous. It is recognized by MAbs in Western blot analysis after treatment of the virus with 2.5% SDS and 5% 2-mercaptoethanol; It is represented by synthetic nonapeptides derived from TGEV spike protein [9]; It is present in recombinant products expressed in bacteria, which do not reconstitute the native spike protein [10]; and it is formed in the absence of glycosylation [8]. In addition, because binding and sequencing studies indicate that site C is not present in the respiratory variants of TGEV, this peptide could be useful to discriminate serum from TGEV or PRCV infected animals. The purified recombinant protein expressed in prokaryotic system in this research, can be recognized specially by polyclonal antibody according to the research of Yang et al [10].

Although, the three recombinant proteins shared the same antigen sites, the quantity of amino acids of the former exceed the latter (the recombinant protein expressed in eucaryotae has 227 amino acids, the recombinant protein expressed in prokaryocyte has 119 amino acids). If this difference has an effect on antigenicity between them or how deeply effect on antigenicity need more experiment to identify.

Recently, some new ways to detection TGEV were developed, such as real-time RT-PCR [11-12], but these ways need expensive equipment and reagent. It seems to unsuitable applied in open country, especially in the third world countries. So we developed the simple ways to detecting antibody induced by TGEV, and the results seem more reliable. The study indicates that the assay reported above is rapid, reliable and sensitive and it has the potential for use as serological method for TGEV diagnosis.

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