A Point Mutation of S Gene from TGEV Isolate TH-98 Followed Construction of Shuttle Plasmid used in *E. coli*/Yeast Expression System

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Abstract: Using primer-specific PCR and multiple-step subcloning, a point mutation of S gene of transmissible gastroenteritis virus (TGEV), TH-98 isolate was performed, resulting in the replacement of a potential pre-termination codon sequence (TTTTTATA) of the S gene by DNA sequence TTTTTACA. The deduced amino acid of S gene is the same as original one due to utilizing nucleic acid silence mutation. The mutated S gene was inserted into *EcoR* I and *Pst* I sites of pCR3.1 vector. Subsequently, the resulting recombinant was digested with *EcoR* I and *Not* I and subcloned into the same sites of *Pichia pastoris* yeast expression vector pPIC3.5, which might be expressed in yeast cells. [The Journal of American Science. 2006;2(1):83-88].

Keywords: TGEV; S gene; reconstruction; shuttle plasmid; construction

Introduction

Swine meat is the main protein source for most Chinese people, and China is one of the countries of the high swine meat production. Therefore, it is very obvious that swine industry owns important portion in Chinese economy. Transmissible gastroenteritis virus (TGEV), a porcine coronavirus belongs to the family of coronaviridae, and is a causative agent for transmissible gastroenteritis (TGE) (1). TGE is associated with high morbidity in susceptible animals of all ages and with high mortality in suckling piglets (2). TGEV is composed of four major structural proteins, the spike (S), the integral membrane (M) glycoprotein, a small membrane protein (sM), and the nucleocapsid (N) protein. In which the spike (S) protein encoded by S gene, about 4.3 kb in length, is the major target of neutralizing antibodies (3), and usually is employed to prepare effective vaccines and other functional studies. The initial aim of our study is to develop genetic subunit vaccine against TGEV by using the S gene of TGEV isolate TH-98 and yeast expression system. However, we found there is a potential pre-termination codon sequence in the S gene cloned in our laboratory, which might influence future expression in some host cells including yeast (4). Therefore, we made a point mutation by PCR and subcloning recombination in order to eliminate the pre-termination codon sequence. The modified S gene was subjected to further subcloning to establish a shuttle plasmid containing the S gene that may be induced to express the S protein in yeast cells. This study produced the useful material for future preparation of TGEV vaccine.

Materials and methods

Vector, host cells, tool enzymes and primers

TA cloning vector (pMD-18T), DH5α competent cells, restriction enzymes, T_4 DNA ligase were purchased from TaKaRa Biotechnology Company. TA cloning vector pCR3.1 and yeast expression vector pPIC3.5 were products of Invitrogen Company. Recombinant pUC-S was constructed as previously described (1). Primers, PS1: 5'-GGTAAGAATTCGTTAACACACC–3', PR1N: 5'-AAAAGTACTAAAGAAATTGTAACCATTAATGTA–3', PS2N: 5'-CAATTTCTTTAGTACTTTTCC-3', PR3: 5'-GGTGTTGTTGTCCAATGTG-3' and PS3: 5'-TACAGTGAGTGACTCGAGCT-3' were used (underlined parts are recognized by *Sca* I, framed part is the induced mutation, which results in the nucleotide T in template was replaced with C in subsequent PCR amplification, but the encoded amino acid is not changed within the correct open reading frame).

PCR amplicfication

Based on the S gene sequence (GenBank accession number: AF494337), an anti-sense primer, PR1N and a sense primer PS2N correspond to nucleotides 1323 and 1337 of S region of TGEV were designed respectively. By the use of the silence mutation of codon, a point mutation was introduced in primer PR1N. Two PCR reactions were performed with primers PS1, PR1N and PS2N, PR3. The temperature profile of each of the 25 cycles comprised at 94℃ for 1.0 min at 51℃ for 1.0 min and at 72℃ for 1.5 min. There was a final extension time of 10 min at 72℃. Two PCR products named SNa and SNb, of 1.3 kb and 1.0 kb, were obtained respectively. The products are identical to the 5' end about half sequence of S gene with the exception of the point mutation.

Strategy of subclonal recombination

SNa was ligated with pMD-18T vector, transformed into DH5α cells and a negative recombinant named TSNa was selected in LB agar plate containing appropriate antibiotic. TSNa was incompletely digested with *Sca* I and *Kpn* I, and a linearized fragment containing the TA vector and SNa, about 3.9 kb, was purified with gel purification kit (Qiagen). The fragment was ligated with SNb fragment digested with the same enzymes. A positive recombinant name TSNab was selected as above described. TSNa. PUC-S was digested with *EcoR* I and *Kpn* I in order to produce a fragment, pUC-Sb containing pUC18 vector and the 3' end half of S gene (1). TSNab was also digested with the same enzymes to give the 5' end half of S gene named SNab. PUC-Sb and SNab were ligated and a recombinant containing the complete S gene was produced that named pUC-SN.

Identification of recombinant

According to the S gene sequence and the physical map of pUC vector, the recombinant pUC-SN was verified with restriction enzymes (RE) and nested PCR using primer PS3 and PR3.

Construction of the S gene–containing shuttle plasmid used in *E.coli*/Yeast cells

Recombinant pUC-SN was digested with *EcoR* I and *Pst* I, and the full-length S gene, about 4.3 kb, was subcloned into the same sites of vector pCR3.1. A positive recombinant named SNPCR3.1 was identified with RE. SNPCR3.1 was then digested with *EcoR* I and *Not* I, and the resulting fragment containing the S gene was subcloned the same sites of a yeast expression vector pPIC3.5. The recombinant designated as SNPIC3.5 was identified with RE, PCR and sequencing.

Results

PCR amplicfication of fragment of interest

Using specific primers, two PCR reactions were performed. Two PCR products named SNa and SNb, of 1.3 kb and 1.0 kb, were obtained respectively. The products are identical with the 5' end about half of S gene

sequence (Figure 1).

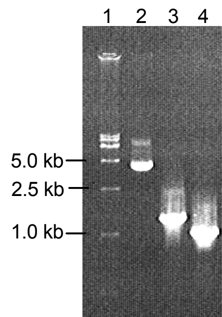

Fig.1. PCR amplification of fragment of interest

Lane 1: DNA Marker (DL15, 000, TaKaRa)

Lane 2: PCR control.

Lane 3: PCR product, SNa, of about 1.3 kb.

Lane 4: PCR product, SNb, of about 1.0 kb.

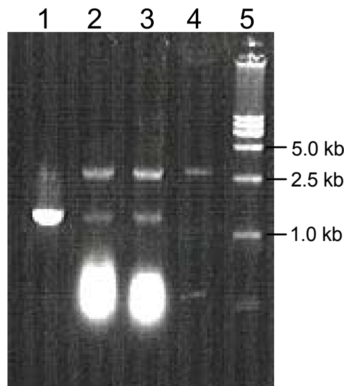

Fig.2. Identification of TSNa with RE

Lane 1: PCR product, SNa, of about 1.3 kb.

Lane 2 and 3: TSNa digested with *EcoR* I,

of about 1.3 kb and 2.7 kb repectively.

Lane 4: Linearized TA vector, of about 2.7 kb.

Lane 5: DNA marker.

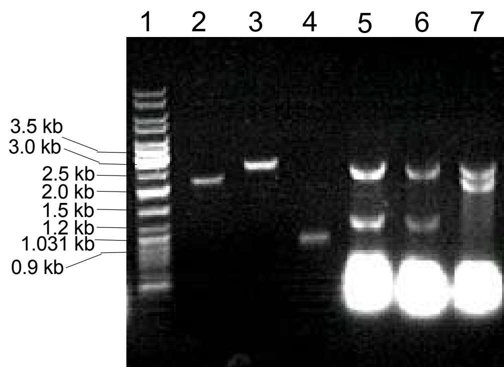

Fig. 3. Identification of recombinant TSNab with RE

Lane 1: DNA Ladder

Lane 2: SNab digested withEcoRI and KpnI，of about 2.3 kb.

Lane 3: Linearized T/A vector, of about 2.7 kb.

Lane 4: SNb digested with ScaI and KpnI, of about 1.0 kb.

Lane 5 and 6: TSNa digested with EcoRI, of about 1.3 kb and 2.7 kb respectively.

Lane 7: TSNab digested with EcoR I, of about 2.3 kb and 2.7kb repectively.

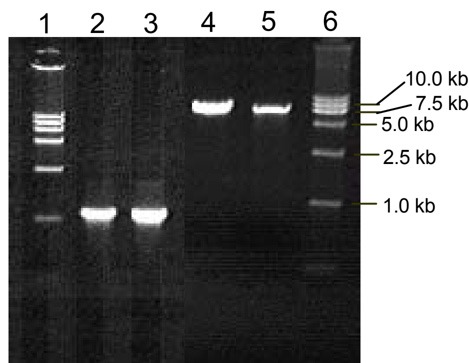

Fig. 4. Verification of pUC-SN by nested PCR and with RE

Lane 1: DNA maker used for evaluating samples in lane 2 and 3.

Lane 2: Identification of pUC-SN by nested PCR,

and the resulting PCR product is about 1.2 kb.

Lane 3: PCR positive control, of about1.2 kb.

Lane 4: Plasmid pUC-SN digested with *EcoR* I, of about 7.0 kb.

Lane 5: Plasmid pUC-S digested with *EcoR* I, of about 7.0 kb.

Lane 6: DNA maker used for evaluating samples in lane 4 and 5.

Point mutation of S gene

As described in materials and methods, a recombinant named TSNa was produced via inserting SNa into T/A cloning vector and identified with RE (Figure 2). A linearized fragment containing the TA vector and SNa, of about 3.9 kb, was purified from TSNa, and ligated with SNb digested with *Sca* I and *Kpn* I. The resulting fragment named TSNab was identified with RE as expected (Figure 3). PUC-S with *EcoR* I and *Kpn* I in order to produce a fragment, pUC-Sb containing pUC vector and the 3' end half of S gene was digested from pUC-S and ligated with the 5' end half of S gene named SNab derived from TSNab. A resulting recombinant, pUC-SN containing full-length S gene was verified with RE and by PCR (Figure 4).

Construction of the S gene–containing shuttle plasmid used in *Ecoli*/Yeast cells

The full-length S gene was obtained from pUC-SN with *EcoR* I and *Pst* I, and subcloned into the same sites of vector pCR3.1. The positive recombinant, SNPCR3.1 was identified with RE as expected (Figure 5). SNPCR3.1 was then digested with *EcoR* I and *Not* I, and the resulting fragment containing the S gene was subcloned the same sites of yeast expression vector pPIC3.5. The recombinant designated as SNPIC3.5 was identified with RE, PCR (Figure 5 and 6) and sequencing. The sequencing report verified there are no nucleotide insertion and deletion, and the artificial mutation (the nucleotide T was replaced by C, see materials and methods) was successful (data not shown).

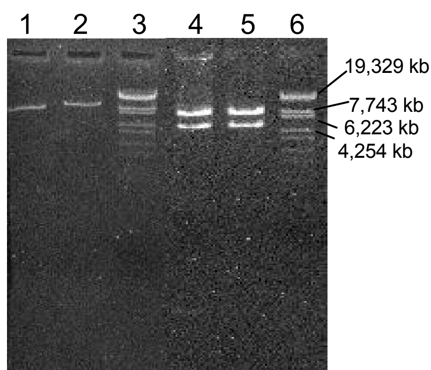

Fig.5: Identification of SNPCR3.1 and SNPIC3.5 with RE

Lane 1: Vector pPIC 3.5 digested with *EcoR* I, of about 7.8 kb.

Lane 2: SNPCR3.1 digested with *EcoR* I, of about 9.4 kb.

Lane 3 and 6: DNA marker (λ-EcoT14 Ⅰ digest, TaKaRa).

Lane 4 and 5: SNPIC3.5 digested with *EcoR* I and *Not* I, of about 4.3 kb and 7.8 kb respectively.

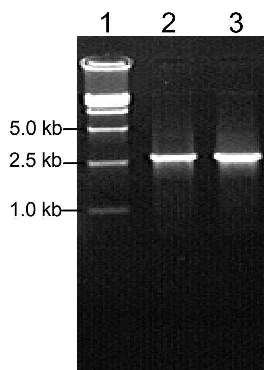

Fig.6: Identification of SNPCR3.1 and SNPIC3.5 by PCR

Lane 1: DNA marker.

Lane 2: Identification of SNPCR3.1 by PCR, of about 1.2 kb.

Lane 3: Identification of SNPIC3.5 by PCR, of about 1.2 kb.

Discussion

At present, the major prevention of TGE is vaccination with inactivated or attenuated vaccines in China. However, these vaccines pose potential disadvantages, such as the recovery of virulence, the need for adjuvant and high cost etc. With the progress of molecular techniques, the genetic subunit vaccines may be one of possible alternatives to conventional vaccines. The S gene of TGEV is widely used for genetic and vaccinological studies due to its important biological functions including inducing the neutralizing antibody. The S protein is a high glycosylation protein with a gross molecular weight (about 180-220 kDa), therefore, a suitable foreign gene expression system is needed for initiating an efficient expression. The S gene expressed in baculovirus/insect cell expression system has been reported (5, 6). We have also constructed a plasmid containing full-length S gene and expressed a soluble S protein of TGEV in this system (7 and unpublished data). However, this system seems a little expensive in terms of the production cost. The S protein has been also expressed by using adenovirus vector and transgenic plant (8, 9, 10, 11). However, no further application information is available.

The *Pichia pastoris* yeast expression system is one of the most successful host expression systems. As a unicellular eukaryote, yeast can potentially produce soluble, correctly folded recombinant proteins that have undergone all the post-translational modifications that are essential for their function (12). The system is freedom from endotoxins and oncogenes. Yeast cells are easier to culture and manipulate genetically than mammalian cells and can be grown to high cell densities. Many foreign proteins have been successful expressed in this system (for a review, see ref.13).

We intended to express the S gene using this system. However, we found there is a potential pre-termination sequence (TTTTTATA), locating about nucleotide 1300 in S gene, which might inhibit or decrease S protein expression in some eukaryotic host systems. In order to facilitate further expression, we mutated one nucleotide by site-specific primer, resulting in a new sequence (TTTTTACA). In this process, some details were important. First, by using TA cloning vector, the PCR product can be directly ligated and transformed, facilitating subsequent subcloning. Second, as there are *Sca* I sites in the sequence encoding antibiotic select maker of pUC18 vector and our designed primer, an incomplete digestion was inevitably adopted to obtain partial S gene and complete vector (see materials and methods). The usage concentration and digestion time of enzyme are key factors for incomplete restriction enzyme reaction. Third, in subsequent multiple-step subcloning, the sticky ligation has facilitated the positive recombination ratio. The shuttle vector, pPIC3.5 can be inserted foreign genes, and transformed in *E. coli*. The resulting recombinant can be transformed into yeast and recombined with the chromosome of yeast. Although there are no suitable RE sites for the S gene insertion, the problem was settled by utilizing TA cloning vector pCR3.1 and subcloning.

In summary, we eliminated a potential pre-termination codon sequence in the S gene of TGEV by site-specific mutation and DNA recombination. The mutated S gene was subcloned into the shuttle vector used in *E.coli*/yeast systems. The resulting recombinant can be used to express S protein in yeast cells in the future.

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