

Cloning and expression of tetanus toxin C fragment (Fc) in prokaryotic vector for constructing recombinant protein based vaccine for tetanus

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Summary

Tetanus is a disease caused by tetanus toxin, a potent inhibitor for the release of inhibitory neurotransmitter in the central nervous system that causes spastic paralysis. Fragment C (52 kD) of this toxin is responsible for binding to the neuronal membrane. For this reason, and also its non toxigenic and immunogenic nature, this fragment might be ideal for new vaccine development. Presently, with respect to the incidence of disease in neonates and animals and the side effects of toxoid vaccine, designing a more effective and efficient vaccine for prevention of this disease is crucial. A segment of *Clostridium tetani* DNA corresponding to C fragment of tetanus toxin was amplified using polymerase chain reaction. This fragment was cloned into expression vector pMalc2x, under the control of the *lac* promoter. Expression of this plasmid in *Escherichia coli* was confirmed by western blotting. In this study, the vector had a strong promoter to allow high level expression of C fragment. Based on our results it appears that this recombinant plasmid may be suitable for the production and development of recombinant vaccine and also has many other applications, such as construction diagnostic kits, production hyperimmune antiserum for serotherapy and as a vehicle for drug delivery to CNS.

Key words: *Clostridium tetani*, Recombinant protein, Vaccine, Fc, pMALc2x

Introduction

Tetanus is now a rare disease in the developed world. However, it remains an important cause of death worldwide (Bruggemann *et al.*, 2003; Blencowe *et al.*, 2010). Fortunately, this disease has been controlled by immunization with tetanus toxoid; nevertheless, according to the World Health Organization cases still occur each year, mainly of neonatal tetanus (Anderson *et al.*, 1996; Blencowe *et al.*, 2010). Tetanus disease is caused by *Clostridium tetani*, an obligate anaerobic spore-forming bacterium that is present in soil, dust and intestinal tracts of animals (Carles *et al.*, 2001; Blencowe *et al.*, 2010)

Tetanus toxin (TeNT) is a member of the

clostridial neurotoxins family, produced by *C. tetani*. TeNT is a potent neurotoxin that inhibits the release of inhibitory neurotransmitter. The toxin is synthesized, as a single 150 kD polypeptide chain. It is cleaved by clostridial protease to yield a heavy chain (100 kD) and a light chain (50 kD) linked by one disulfide bond. Tetanus toxin is known to bind certain gangliosides present on tissues of neural origin; these gangliosides are considered to be potentially relevant eukaryotic cell receptors (Herreros *et al.*, 2000). The heavy chain can be cleaved into two fragments by the papain protease, which corresponds to its C-terminal part (Hc fragment). The Fc fragment is responsible for TeNT binding to the cell membrane (Fitzsimmons *et al.*,

2000; Venom, 2005). Although the N-terminal of the Hc-TeNT is similar in structure to many lectins, deletion mutagenesis studies suggest that the C-terminal is essential for cell and ganglioside binding activity. Fragment C has been demonstrated to retain the ganglioside-binding activity of intact tetanus toxin. Several studies proved that fragment C retains these activities even after intoxication (Qazi *et al.*, 2006). At present tetanus toxoid is used for vaccination. Toxoid has some disadvantages such as irritation at injection site, variable immunity and the risk of toxicity. Therefore, new approaches for vaccine production, such as DNA vaccine and recombinant proteins are investigated. The aim of this study was to construct a prokaryotic vector that expresses Fc fragment of tetanus toxin for future application in vaccine development and diagnostic purposes. This construction can be used as a DNA vaccine for vaccination of animals, especially for horses that are at high risk. It seems that application of this fragment alone or with molecular adjuvants can promote more antibody production and provide more immunity with fewer side effects for animals. Hence, the sequence corresponding to fragment C was amplified using the polymerase chain reaction, ligated into an expression vector, and then colonized in *Escherichia coli* and its protein expression assessed by western blotting.

Materials and Methods

Fc gene amplification

The sequence of Fc was extracted from NCBI and then specific primers were designed with Oligo analyzer program. The sites for restriction endonuclease enzymes on the primers and plasmid pMalc2 were found using Bio edit software and considered in primer designing. The sequence of the designed primers was as follows:

F: 5'-GCCGGGATCCAAAAATCTGGATTGTGGGT-3'

R: 5'-GCCGGTCTGACTTAATCATTTGTCCATCCTC-3'

The *Clostridium tetani* was prepared from the culture collection of the Veterinary Medicine Faculty of Shahid Chamran

University and then cultured in thioglycolate broth and incubated in an anaerobic jar. After 48 h incubation DNA extraction was performed using high pure plasmid isolation kit (Roche Co. USA).

PCR was performed using the high-fidelity thermostable DNA polymerase from *Pyrococcus furiosus*, *pfu* (Fermentas, USA), which possesses 3'→5' exonuclease proof reading activity. Fc was synthesized by using specific primers. The polymerase chain reaction was performed in the following condition, 1u *pfu* DNA polymerase, 10 mM dNTPs, 50 pmol of each forward and reverse primers, MgSO₄ 2.5 mM, 5 µl of 10 × Pfu Buffer and 3 µl template DNA in the final volume 50 µl. The PCR reaction was done in the thermocycler (Corbet Research, Australia) using the following program: primary denaturation at 95°C for 2 min, then 35 repeated cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 4 min and final extension at 72°C for 10 min. The PCR product was visualized by electrophoresis in 1% agarose gel containing ethidium bromide (50 µg/ml) and compared with 1 kbp DNA ladder (Fermentas, USA).

The PCR products were purified by gel extraction kit (Qia quick gel extraction kit, USA) and the purified products were stored at -20°C until use.

Fc gene cloning

A pMalc2x vector (New England Biolabs) was used in this study. This vector produces Maltose binding protein (MBP) that has been located at the beginning of the expressed gene and can be used for recombinant protein purification. For vector amplification this vector was transformed to *E. coli* DH5α. *Escherichia coli* DH5α (CinnaGen, Iran) was cultured in LB broth with ampicillin and incubated overnight at 37°C. The plasmid vector was extracted and purified by High pure plasmid isolation kit (Roche Co. USA). In order to introduce the amplified gene into pMalc2 vector it is necessary that plasmid is turned to linear conformation. For this purpose the vector and amplified gene were digested by *Bam*HI and *Sal*I restriction enzymes (Fermentas, USA). In order to avoid star activity, the

digestion of the vector and Fc gene were done separately with a special buffer for each enzyme. The first and second digestions were performed by *Bam*HI and *Sal*I at 37°C for 90 min, respectively and purified with gel extraction kit. Digestion was confirmed by agarose gel electrophoresis and the digested products were purified by gel extraction kit.

For ligation, the concentrations of plasmid and amplified gene were approximately determined by comparing with the DNA marker and the ligation was done by using T4 ligase for 1 h at 37°C and then overnight at 4°C. After this incubation time the reaction was incubated at 65°C for 10 min in order to enzymes inactivation. The vector containing the Fc gene was used for transformation. The transformation was done by one step transformation method called Chung method in the following manner: first, *E. coli* DH5 α was cultured in LB broth and incubated overnight at 37°C. Then the suspension of overnight culture was added (1:100) to the uncultured LB broth and incubated at 37°C with moderate shaking until its OD₆₀₀ absorbance reached 0.3-0.4. 100 μ l of this bacterial culture was mixed equally with cold TSS 2x solution (Transformation and Storage Solution, Polyethylene glycol 20 W/V, DMSO 10 V/V, LB with 50 mM MgCl₂, pH = 6.5). One hundred μ l of these competent cells were added slowly to 1 μ l (100 μ g) plasmid on ice and incubated for 30 min at 4°C. Then 900 μ l of LB broth containing 20 mM glucose (Merck, Germany) was added to the above solution and then incubated 15 min at room temperature and 45 min at 37°C with moderate shaking. One hundred μ l of this suspension was transferred to LB agar containing 100 mg/ml ampicillin (Jaber Ebne Hayyan, Iran) and incubated at 37°C for 24 h.

In order to confirm the gene cloning and transformation, 10 colonies formed on the LB agar containing ampicillin (Jaber Ebne Hayyan, Iran) were selected and cultured on the LB agar. The presence of plasmid was investigated in these colonies as follows: a few colonies were cultured in 1 ml LB broth/ampicillin. Three μ l of this bacterial suspension was mixed with 17 μ l double distilled water, boiled for 10 min and then

placed on ice for 5 min. Finally, this suspension was centrifuged at 13000 \times g for 5 min and supernatant was harvested and used for PCR reaction. The PCR was conducted as mentioned above and the presence of the gene was confirmed by visualization of the PCR product on the agarose gel electrophoresis. Furthermore, the plasmid was digested with restriction endonucleases and the digested product was evaluated by agarose gel electrophoresis.

Recombinant Fc expression

Expression of the Fc was evaluated by SDS/PAGE and Western blotting. The IPTG (Sigma, USA) was added to transformed cells growing in mild-logarithmic phase, at 2, 4 and 6 h intervals to obtain the best time for protein expression. Then, the cells were harvested by centrifugation, mixed with an equal volume of sample buffer, and finally were electrophoresed on 10% SDS/PAGE at 100 V for 4 h. The fractionated proteins were transferred to a nitrocellulose membrane by electroblotting at 60 V for 4 h. The nitrocellulose membrane was placed overnight in 5% skim milk (Merck, Germany) solution (Ghaffari *et al.*, 2010). Then it was washed 3 times with PBS-Tween and submerged for 1 h in polyclonal human anti-Tetanus Ig solution (1:50 in 2% skim milk/PBS-Tween), and washed as previously mentioned. In the next step the membrane was inserted into peroxidase conjugated anti-human Ig solution (1:500 in 2% skim milk/PBS-Tween) and remained for 1 h. The membrane was washed again and the peroxidase substrate solution (4-Chloro 1-Naphtol, H₂O₂, Methanol) was added to the membrane. The color change on the membrane was considered as positive.

Results

One single band of 1356 bp was produced as a result of PCR reaction with the primers that have been designed. Figure 1 shows the PCR product of the Fc gene.

To confirm the insertion of the amplified Fc into the plasmid, plasmid digestion and also PCR was done on the suspected colonies. The result of plasmid digestion and PCR suggests that gene cloning has been

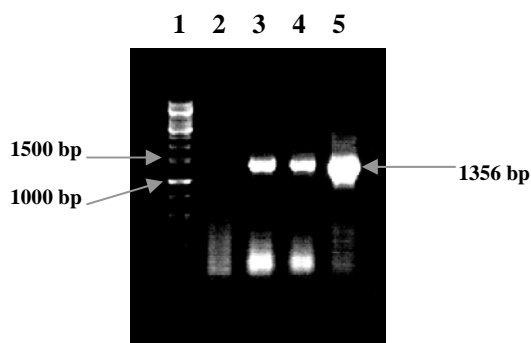


Fig. 1: The PCR product of Fc gene. 1: 1 kbp DNA ladder, 2: Negative control, 3, 4 and 5: 1356 bp product of Fc gene

done successfully.

After confirming the gene cloning and transformation, the transformed colonies were subjected to IPTG for induction of protein expression (52 kD). The results showed that induction with IPTG can cause protein expression, and more incubation time after induction causes more protein expression. The result was shown in Fig. 2.

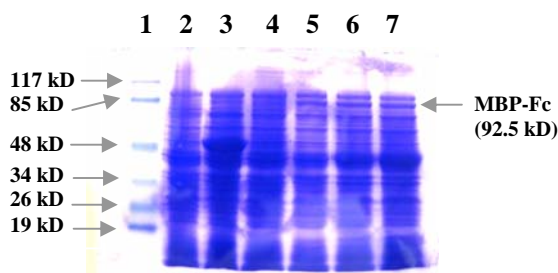


Fig. 2: Induction of protein expression in *E. coli* containing pMALc2x-Fc construction. 1: Molecular weight marker, 2: Plasmid without Fc gene prior to IPTG induction, 3: Plasmid without Fc gene after induction with IPTG, 4: Transformed colony with pMALc2x-Fc prior to IPTG induction, and 5-7: Colonies with pMALc2x-Fc after IPTG induction

Western blot analysis was used for confirming Fc expression. The result showed that Fc has been produced and expressed in transformed *E. coli* DH5 α and specifically reacted with anti-tetanus Ig. Fig. 3 shows the result of western-blot analysis.

Discussion

Tetanus is one of the fatal diseases that occurs in human and animals and due to spore formation of the causative agent, its eradication is impossible. Prevention is very

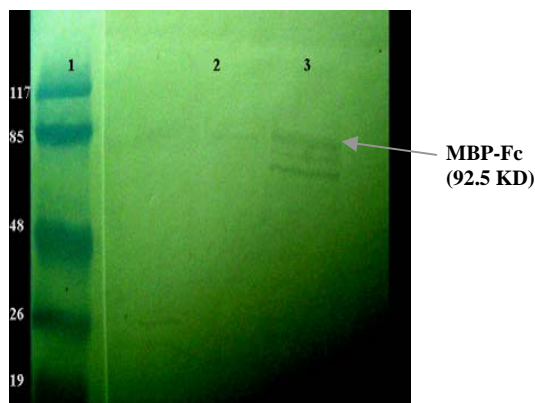


Fig. 3: Western blot analysis. 1: Weight marker, 2: *E. coli* without Fc, and 3: Transformed *E. coli* expressing Fc protein

important in controlling this disease. At present most of the researches have focused on the toxin inactivation and immunization with toxoid. Although this approach has been useful in disease control, cases of tetanus are reported annually around the world. Researches have been done on the application of subunit and or recombinant vaccines (Medaglini *et al.*, 2001; Varfolomeeva *et al.*, 2003). It seems that these vaccines are safer and give greater protection against tetanus. One of the candidate antigens for this purpose is the Fc portion of TeNT that is responsible for binding to the neural cell membrane and hence antibodies against this portion can block TeNT attachment and the disease. Varfolomeeva *et al.* (2003) showed that this fragment has many advantages and enables us to design new vaccine development.

Till now tetanus toxoid has been used as a vaccine for tetanus prevention in Iran. The aim of this study was to produce recombinant TeNT/Fc in the prokaryotic system and to develop new approaches for tetanus vaccine production in Iran. We are aware that this is the first attempt for recombinant tetanus vaccine production in Iran. Such recombinant protein and/or DNA vaccine can be used with greater safety and fewer side effects for controlling tetanus in animals, especially in horses.

In the present study new and different primers that have sites for BamH 1 and SalI restriction enzymes have been used. The pMALc2x was used as a vector for gene expression and protein production. This vector has a strong promoter and so its

protein expression will be high compared to the other vectors. The rate of gene cloning into this vector and subsequently transformation was high and noticeable, which enables work with this vector to be done more easily than with the other vectors. The results of SDS/PAGE and western blotting showed that the transformed *E. coli* DH5 α expresses Fc considerably and after IPTG induction the amount of protein expression has increased because of the presence of lac promoter in pMalc2x. In western blott analysis with specific anti-tetanus Ig there was a band that is the site of Fc on the nitrocellulose that reacted with the antibody.

Varfolomeeva *et al.* (2003) have searched for new tetanus vaccine development based on Hc and its hybrids with other proteins. They reported suitable immunity and protection after immunization with this fragment.

Medaglini *et al.* (2001) represented immunization with recombinant *Streptococcus gordonii* expressing tetanus toxin fragment C can protect mice from lethal challenge.

Anderson *et al.* (1996) have used the Fc DNA for immunization of mice. The results showed that immunization with pcDNA/tetC can protect mice from challenge with tetanus toxin.

Kim *et al.* (2003) have introduced tetanus toxin C-fragment as a universal carrier protein for conjugate vaccines. Saikh *et al.* (1998) have studied the usefulness of DNA based vaccines for protection against secreted bacterial toxins. Their results showed that polypeptide vaccine based on Fc protein, in comparison to plasmid DNA, gives better protection against tetanus, which may be due to different antigen presentation pathways.

The result of this study showed that tetanus toxin C fragment was successfully amplified, cloned and expressed in the prokaryotic system. This recombinant protein was specifically and considerably reacted with a specific anti-tetanus antibody. This is the first time that Fc has been cloned into the pMalc2x vector and expressed in *E. coli* in Iran. The presence of lac promoter in this vector increases the protein expression and under IPTG induction this will be

enhanced considerably. In addition, the presence of maltose in recombinant expressed protein will facilitate its purification. Thus, pMalc2x can be a useful vector for recombinant protein vaccine production. The application of this vector as a DNA vaccine may enhance the antibody level and subsequently the protection against challenge with *C. tetani*. This DNA vaccine can be used especially for animals such as horses, in which irritation and inflammation at the injection site can be a limitation for the use of toxoid, and their activities can be affected by toxoid vaccine. Beyond vaccination and immunization, this recombinant Fc protein can be used for specific drug delivery toward neural cells in the case of neurologic researches or even for neurologic disease treatments. Its applicability as a drug vehicle and as fusion protein with other antigens is an interesting subject.

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