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Research Article

Production and Evaluation of a New Recombinant Cloning Vector for the *Clostridium novyi* Type B Vaccine Strain Alpha Toxin Gene

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Abstract

Clostridium novyi is a rod-shaped, gram-positive, obligate anaerobic, motile and spore-forming bacterium, which is widely distributed in the environment. *C. novyi* is subdivided to 4 groups (A to D); based on 4 produced major toxin types (alpha, beta, epsilon and gamma). Alpha (α) toxin is the major toxin of the C. novyi A and B types which causes Black disease, Gas gangrene and Infectious Necrotic Hepatitis. The purpose of the present study was to clone and sequence *C. novyi* vaccine strain alpha toxin gene. The genomic DNA was extracted using phenol-chloroform procedure. Alpha toxin sequence was taken from GenBank and using Oligo software appropriate primers were designed. With the use of PCR and proof reading enzymes, α toxin gene was amplified and ligated into pJET1.2blunt vector. The ligation product was transformed using *E. coli*/TOP10 and the recombinant clones were chosen from on LB-AMP. Finally, recombinant gene containing plasmid was extracted from bacterium host and sequenced. The results of sequencing showed a high identity to those of alpha toxin genes registered in GenBank previously. Considering the novelty of cloning and expression of *Clostridium novyi* alpha toxin gene in *E. coli* in Iran, the results of this study can lead to recombinant vaccine production.

Keywords: *Clostridium novyi Vaccine Strain; Alpha Toxin; Black Disease; Recombination*

Introduction

Clostridium novyi belongs to gram-positive bacteria with a length of 4 to 8 micron and a thick cell wall. This is not the bacteria able to consume the oxygen and lacking Catalase and Peroxidase. As a result, in the presence of oxygen, H_2O_2 concentrations accumulate toxic. Clostridiums divided to three categories according to toxin production or invasion of tissues and proliferation of disease that creates enterotoxin, histotoxic and neurotoxic that *C. novyi* belong to Histotoxic category is named *Clostridium oedematiens* [1].

This, bacterium can cause a wide variety of diseases in man and animals [3]. It divided in three types, known as A, B, and a non-pathogenic type C distinguished by the range of toxins they produce. Sasaki [4] have demonstrated by 16S rDNA sequence analysis demonstrated that *Clostridium haemolyticum* as *Clostridium novyi* type D. *C novyi* is closely related to *Clostridium botulinum* types C and D.

Clostridium novyi type A produces alpha, delta, gamma and epsilon toxins. *Clostridium novyi* type B produces alpha, beta and zeta toxin [5]. Alpha Toxin is lethal and necrotizing that causes edema and also effects on the central nervous system (CNS) [6]. *C. novyi* has a circular

chromosome with 2547720 bp in length and GC content of the 28.9% and AT content of 71.9%. Near to 139 genes has found in this bacteria that have no homologues in the other bacteria [7].

Bormann., *et al*. [8] studied the ways to distinguish the toxin alpha in C. novyi using cell culture systems. They surveyed ten stable cell lines for react to toxin alpha in *C. novyi*. Staedtkea., *et al*. [9] are shown that *Clostridium novyi* non-toxigenic (C. novyi-NT) spores could be used as anti-tumor therapy.

Bettegowda., *et al*. [7] had determined the sequence of *C. novyi*. They found a new replication and 139 genes without any homologue in other bacteria. Guttenberg., *et al*. [10] studied the auto proteolysis processing of this toxin by Inositol hexa phosphate (InsP6) and compared with *Clostridium difficile*.

C. novyi produces a fragment consisting of 6534 nucleotide (alpha-toxin gene, *tcn* alpha) open reading frame which encodes a polypeptide of the N-terminal alpha-toxin (Tcn alpha) sequence [11].

The main purpose of this study was possibility of cloning of *C. novyi* type B vaccine strain alpha toxin gene, in *E. coli* strain Top10 for further studies on recombinant vaccine production using these bacteria.

Material and Methods

Bacterial strains and culture conditions

C. novyi vaccine strain (CN804) and *E. coli* strain Top10 were obtained from Razi Vaccine and Serum Research Institute and were cultured in sterilized liver extraction medium and incubated in anaerobic condition (37°C).

DNA extraction

Whole genomic DNA was extracted and purification carried out after culturing rapidly. The protocol used in this study was Phenol-Chloroform procedure. Quality and quantity of extracted DNA was measured on 1% agarose gel electrophoresis and was visualized using Ethidium bromide.

Primers and PCR procedure

Special primer was used for amplification of fragments of alpha toxin coding gene. Forward primer as 5´-AGA TCT ATG CTT ATA ACA AGA GAA CAA-3´ and reverse primer as 5´-GGA TCC ATC TTT TTC TTT TAT TAT ACT-3´ were published previously [12].

Each PCR reaction was contained 12.5 µl of master mix, 0.5 µl of each primer, 0.5 µL of template DNA, 11 µl of ionized water, in a total volume of 25 µl. DNA amplifications were performed using Master cycler programmed for a preliminary step of 5 min at 95°C, followed 30 cycle by 60 seconds at 94°C, 60 seconds at 52°C and 3 minutes at 72°C, with a final extension of 15 minutes at 72°C.

Ligation and transformation

For cloning process, the linearized pJET1.2/blunt vector (Fermentas) was used as cloning vector. Amplified alpha toxin gene was ligated into pJET1.2/blunt to produce pJETcnα recombinant vector. Then pJETcnα was transformed into *E. coli*/Top10 using electroporation method.

Screening and selection

When transformation was completed, screening of recombinant clones was performed by antibiotic resistance (culture of suspension on LB-amp agar plate and incubation at 37°C) and colony PCR. After overnight growth on selective media, PCR was conducted for some of the colonies with pJETcnα recombinant plasmids. The pJET1.2/blunt universal forward and reverse sequencing primers were used for colony PCR based on what recommended by the manufacturer. On the other hand, for negative control, non-recombinant cloning vector was subjected to the same PCR procedure. Also, the similar colony was cultured on a new plate containing LB-Amp, and later plasmid extraction was carried out. The recombinant pJETcnα was extracted using a dedicated plasmid extraction. Sequencing of nucleotides for purified recombinant vector was carried out.

Results

Genomic DNA extraction of *C. novyi* vaccine strain in the first attempt (Figure 1), and the amplification of alpha toxin gene after optimization (Figure 2) was successful. All extracted DNAs from samples yielded a specific single band PCR product without any nonspecific band.

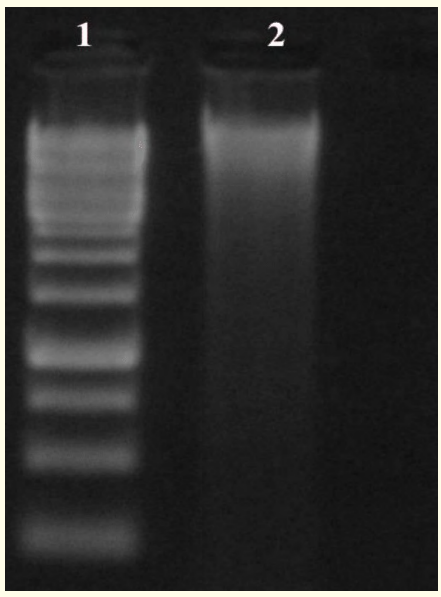

Figure 1: *Genomic DNA extraction of C. novyi vaccine strain. Lane 1; 100bp plus DNA size marker, lane2; Genomic DNA.*

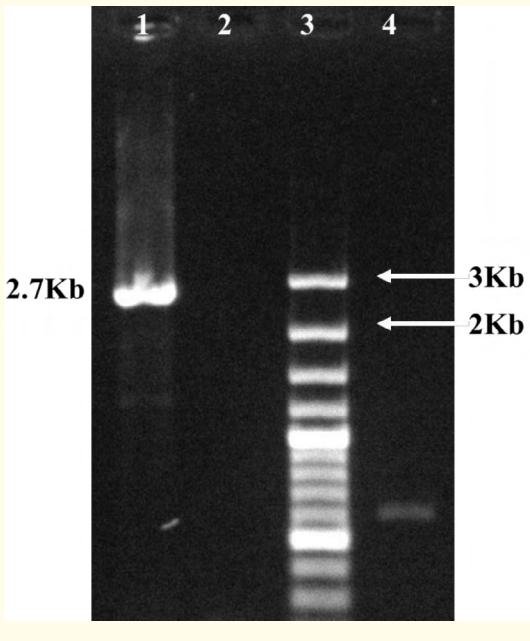

Figure 2: *PCR amplification of alpha toxin gene shown on 1% agarose gel. Lane 1; PCR products (alpha toxin gene), lane 2; negative control, lane 3; 100bp plus DNA size marker, lane 4; positive control.*

After ligation procedure, alpha toxin gene (2700bp) was ligated into the linearized pJET1.2/blunt cloning vector (2974bp) and after production of the pJETcnα recombinant vector (5674bp) and transformation the recombinant *E. coli*/Top10/pJETcnα was produced. Colony PCR of recombinant *E. coli*/Top10/pJETcnα colonies using the pJET1.2/blunt universal forward and reverse sequencing primers, and then plasmid extraction showed the 5674bp pJETcnα recombinant cloning vector (Figure 3).

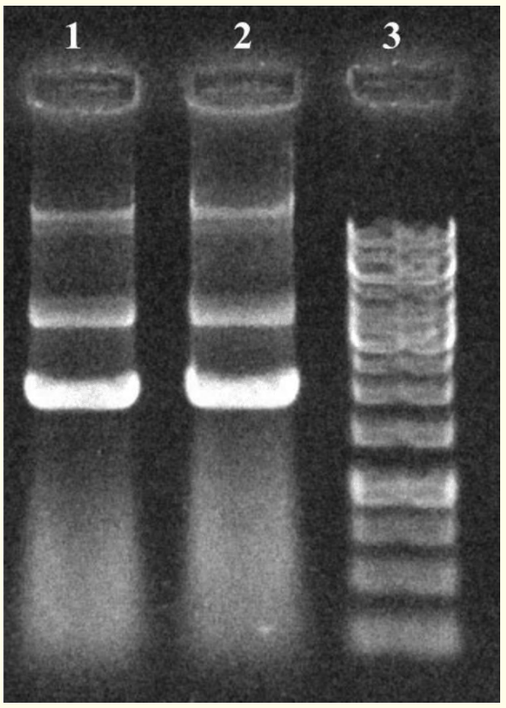

Figure 3: *Plasmid extraction showed the 5674bp pJETcnα recombinant cloning vector. Lanes 1 and 2; pJETcnα recombinant cloning vector, lane 3; 100bp plus DNA size marker.*

Colony PCR of some recombinant colonies using alpha toxin forward and reverse primers confirmed the presence of DNA fragment on 1% agarose gel electrophoresis has shown in figure 4. Extracted plasmid sequencing for showing alpha toxin gene was done by SinaClone Company.

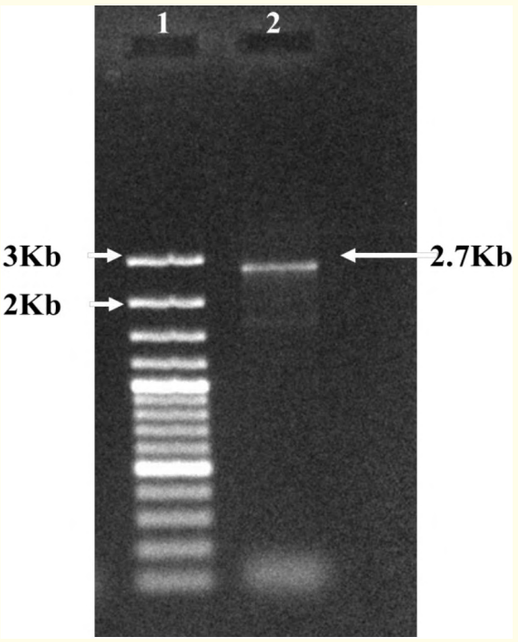

Figure 4: *Colony PCR of E. coli/Top10/pJETcnα recombinant colony using alpha toxin forward and reverse primers. Lane 1; 100bp plus DNA size marker, lane 2; PCR product of colony PCR.*

Discussion

Black disease is an acute and fatal disease of sheep and goats in Iran. Fifty-one strains of *Clostridium oedematiens* types A, B, and D had been isolated and typed from the liver lesions specimen which had been received from different parts of the country. The techniques of isolation and rapid identification by using fluorescent labelled antibodies, typing, sugars fermentation, toxicity and haemolytic activity of the isolated strains had been described [13].

Genetic, biochemical and pharmacological evidence [14] had been shown that three toxins consisting of *C. novyi* Tcn alpha and both toxins A (TcdA) and B (TcdB) of *C. difficile* are belong to one subgroup of large Clostridial cytotoxins (LCT) [11].

Staedtkea., *et al*. [9] are shown that *Clostridium novyi* non-toxigenic (*C. novyi*-NT) spores could be used as anti-tumor therapy, Bettegowda., *et al*. had identified a new type of transposition and 139 genes in the 2.55-Mb genomic sequence of *C. novyi*-NT that do not have homologs in other bacteria. As they had demonstrated the *C. novyi*-NT spores contained mRNA and the spore transcripts are distinct from those in the vegetative forms of the bacterium. Recognizing the full length alpha toxin gene of vaccine strain in our study and comparing to the one that Bettegowda., *et al*. had been showed, can lead to better understanding of this toxin gene structure and functions. So here we are proposing that *Clostridium novyi* vaccine strain (CN804) non-toxigenic (*C. novyi*-NT) spores could be used as anti-tumor therapy.

Bozorgkhoo., *et al*. [15] had used the pJET1.2/blunt cloning vector in their study to cloning of *Clostridium septicum* vaccine strain alpha toxin successfully. The vector pJET1.2/blunt with 2974 bp length has a lethal gene located between nucleotides 371 and 372. When DNA (alpha toxin gene) was inserted into this site, this lethal gene was destroyed, so that each cell that have received recombinant plasmids was capable to reproduce. By using this vector, white and blue colonies are no longer necessary.

In this study that was a part of cloning and expression of Clostridial major toxins gene project in Razi Serum and Research Institute, we had cloned and sequenced the alpha Toxin Gene of *C. novyi* in *E. coli*. We hope that its molecular information can be used in production vaccine against these bacteria.

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