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Research paper

Colonization and Investigation of Vibrio Cholera Recombination Protein in E-Coli

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Abstract

Background and aim: Vibrio cholerae is a gram-negative bacterial pathogen that causes diarrheal disease. One of the most pathogenic factors of V. cholerae is toxin-coregulated pili. This pilus is required as the first factor in the colonization and bacterial persistence in the small intestine. Materials and Methods: In this study, V. cholerae toxin-coregulated pili A (TCPA) gene was amplified using PCR method. The above genes were purified and then expressed by being cloned into the pGEX4T-1 plasmid. Then the recombinant plasmid structure was introduced into the E. coli bacterium. Protein production was carried out by IPTG induction and optimization of culture conditions. The recombinant proteins were purified using Glutathione S-Transferase (GST) Assay Kit and western blot test was then carried out for confirmation of recombinant protein. Protein levels were measured using Bradford protein assay. Results: The results of the present study proved the successful expression of recombinant proteins in E. coli cells. The recombinant protein was purified by affinity chromatography. The reaction pattern between these proteins and their anti-antibodies showed that these proteins have antigenic properties. Conclusion: Since it was proved that these proteins have antigenic properties in this study, they may be used as an appropriate antigen for vaccination of V. cholera.

Keywords: *Polymerase chain reaction, Toxin-coregulated pili A gene, Vibrio Cholera.*

1. Introduction

Cholera is a diarrheal disease caused by a gram-negative bacterium called Vibrio (V.) cholera (1). V. cholerae belongs to the vibrionaceae family and the vibrio genus, which lives as anaerobic in fresh and salty waters (2). This bacterium has the fermentation potential (2, 3), and the most important symptom of this disease is plenty of vomiting and watery diarrhea. The contamination transmission occurs mostly through contaminated food or water. The disease leads to dehydration and electrolyte imbalance (4, 5). There are about 200 serotypes of V. cholera, with only two serotypes of 1o and 139o were known to be associated with the cholera epidemic (6). The V. cholerae genes expression takes place with the inset of changes in environmental stimuli such as salt, PH and temperature. This environmental trasmission occurs with the digestion of contaminated food or water (7, 8) and the bacterium colonizes into the small intestine and needs type IV pilus for this colonization. TCPA is a toxin-tcpco regulated of pilus, which belongs to the B4 group (9). Pili production and the secreted virulence factor, tcpF, facilitate the microcolony formation, and is required for colonization of intestinal epithelial cells (10, 11). The pilus plays a very complex and essential role in the pathogenesis of V. cholerae and the mutated forms of this protein are not capable of creating colonization in the small intestine of humans and mice (12). The TCPA protein is a thin string pilus that generates a specific class on the surface of V. cholerae bacterium. The expression of virulence cassette genes of TCPA is controlled by toxR, which is a membrane protein. When this bacterium is cultured under elevated-toxin expression conditions, this regulation indicates a state of affinity between tcp and toxin positions in the toxR regulon (13). The pilus is observed in the form of a fiber made up of identical polymers of tcpA (14). TcPA is then processed as a leading protein in the cytoplasmic part of the internal membrane of V. cholerae by a protease enzyme called TcpJ (15). The mature tcpA subunits, along with other attachments, transform into mature tcp. It is believed that these proteins have the ability to form a scaffold that acts as a TCPA (8). The TcPA protein is considered as the major subunit of this pilus. This protein can be easily affected by the immune system since it is located on the bacterial surface. The TcP protein is a polymer of repeating units found among the V. cholerae pathogenicity island (VPI) (16, 17). The DNA tcp sequences for the El Tor 1o and 139o species are very similar (18). Therefore, this protein is immune to these species when this serum is formed. The TcPA protein plays an important role in the formation of the bacteriophage receptor and this protein is an appropriate candidate for the development of anti-colonization immunity against cholera due to its arrangement against antibodies. The TCPA protein with molecular weight of about 45.5 kDa is one of the important components of V.cholera. The gene of this protein consists of 598 bp. E. coli 3BL21 (DE) was used to produce this protein in the present study. The production of recombinant TcPA protein warrants further studies on its immunogenicity.

2. Materials and Methods:

The production of recombinant TcPA protein is a fundamental-applied study. The bacteria used in this study included Vibrio cholera Inaba strain prepared from Pasteur Institute of Iran, DH5α E. coli strain and 3E.coli, BL21 prepared from the National Center

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for Genetic Research and Biotechnology. 1pGEX4T Plasmid was used for the cloning and production of TcPA protein. The mentioned plasmid was prepared from the National Center for Genetic Engineering and Biotechnology. Purification of V. cholerae chromosome was carried out using CTAB / NaCl method, in which V. cholerae was first cultured in a nutrient broth medium at 37 ° C. The precipitate obtained from bacterial culture suspension was dissolved in EDTA buffer (TE 1 mM, Tris.HCl 10 mMa , 8 = PH) and bacterial cells were lysed by sodium dodecyl sulphate and Proteinase K. The bacterial chromosome was extracted using CTAB / NaCl solution (CTAB 10%, NaCl 0.7%). Proteins and other cellular components were harvested using a mixture of phenol / chloroform / isoamyl alcohol at ratios of 25:24:21. The obtained DNA was precipitated using alcohol isopropanol and then washed with 70% ethanol. DNA quality was evaluated by 0.8% agarose gel electrophoresis gel in TBE buffer. The amount of DNA purified was obtained by measuring absorbance at wavelengths of 260 and 280nm was. The "forward" and "reverse" primers were designed using the tcpA gene sequence:

Forward: 5'-AGGGATCCATGACATTACTCGAAG-'3

Reverse: 5'-

The forward 3'-primer contains the TGTTACCAAATGC sequence required for identification and cutting of AACTCGAGGC by BamHI enzyme and the reverse primer for identification and cutting by the XhoI enzyme. The replication of tcpA gene was performed using 50µl PCR. The concentration of PCR agents included 500ng / ml of the template DNA, 1 mM of each primer, 3 mM of magnesium ion, 200 mM of each desoxygenucleotide triphosphate, 2.5 unit of Taq DNA Polymerase enzyme and 10 ×PCR. The PCR cycle used was as follows: initial heating at 94 ° C for 5 minutes (one cycle), the second stage of PCR consisting of 30 cycles, each cycle including a denaturation phase (for one minute at 94 ° C), the annealing of primers to the template DNA (for one minute at 64 °C), replication of the target gene (for one minute at 72°C). Finally, the final replication step was carried out at 72 ° C for 5 minutes. The result of electrophoresis was evaluated by being stained with Ethidium Bromide solution and observed with a UV Transilluminator. Purification of the PCR product was carried out using a purification kit prepared by Roche Co. based on its instructions. The tcpA gene was cloned in 1 pGEX4T plasmid as follows: First, the PCR product was cut by BamHI and XhoI restriction enzymes and then entered into the above vector. The above plasmid was also cut with these enzymes. The annealing of the tcpA gene in this plasmid was performed using a T4 DNA ligase enzyme at 16 ° C for 24h. The tcpA 1pGEX4T plasmid was introduced into the susceptible cells of E. coli DH5α and 21BL strains, respectively. To verify the nucleotide sequence of the gene derived from the PCR product, the tcpA pGEX4T1 plasmid structure was sent to Gene Fanavaran Company, Tehran, Iran. In order to produce the TCPA protein, pGEX4T-1-tcpA plasmid was introduced into the E. coli 21BL strain by thermal shock and cultured on a nutrient agar medium containing ampicillin (100 mg / ml). It was then incubated at 37 ° C for 24 h. The colonies grown on this medium were cultured on a nutrient broth medium containing ampicillin (100 mg / ml) for 24 h. In order to induce TCPA protein production, the cultured bacteria (about 500µl) were transferred to the induction medium and placed in a shaker incubator at 24 rpm. After the bacterial count reached the appropriate level (0.6 absorbance), 1 mM IPTG solution was added to the bacterial suspension. Bacterial sedimentation was achieved by centrifugation at 4000 rpm for 8 minutes two and four hours after adding IPTG solution. To test the induction outcome, the bacterial sedimentation was electrophoresed on 12% SDS-PAGE gels. Protein purification was carried out using the G.S.T kit according to the manufacturer's instructions (QIAGEN). The level and quality of the purified protein was measured using the Bradford method and 12%SDS-PAGE gel electrophoresis, respectively. Western Blot method was used to confirm the TCPA protein. The serum of rabbits, rats and cholera serum of the affected individuals were used in the present study (18).

3. Results and Discussions

The chromosomal DNA concentration obtained from V. cholerae bacterium was estimated to be 500 µg / ml. The chromosome was purified from V. cholerae bacterium and used as a template for the replication of the tcpA gene. The fragment size of the PCR- amplified gene was 598 bp as compared to the marker. In order to confirm the gene replication and evaluate the design of primers, a tcpA-containing plasmid was sent to the above company. The results showed that the PCR product had homology with the relevant gene. In this study, TCPA protein was evaluated in different induction media using 3 E. coli, BL21 (DE) (Fig. 1)

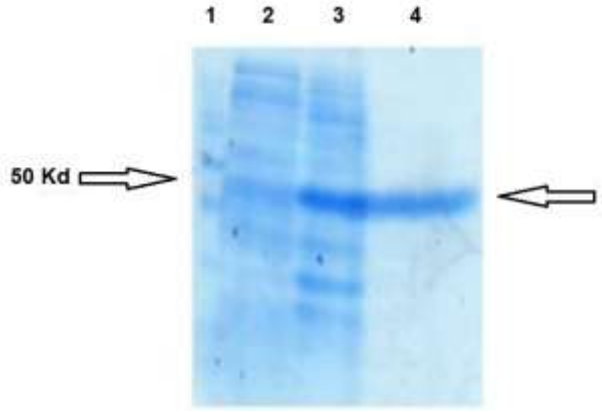


Figure 1:. Protein induction gel. Column # 1: Marker, Column # 2: Protein sample before induction in Nutrient Broth medium with glucose, Column # 4 and # 3: Protein sample after induction in the Nutrient Broth medium with glucose.

The TCPA protein with a molecular weight of 45.5 kDa was purified in a suitable induction medium by G.S.T kit (Fig. 2). To determine the antigenicity of the intended protein and carry out the final confirmation on the recombinant protein, western blot was used (Fig. 3).

The bacterium (3E.coli, BL21 (DE), due to lacking protease enzymes, is able to express the above protein ay high levels. TcPA was then produced using the 1pGEX4T system as one of the most powerful protein expression vector. The 1pGEX4T expression vector has a replication source of 1F and tryptophan promoter and the protein was induced using IPTG solutio .The 1pGEX 4Texpression vector has a specific protein sequence called glutathione S transferase, which adds about 23 kilograms of molecular weight to the protein of interest. The pure molecular weight of the tcpA protein is about 21 kDa. The recombinant TCPA protein appears to be an important antigen for serological diagnosis of cholera.

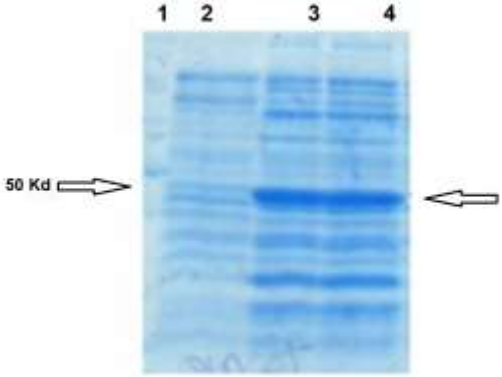


Figure 2:. Protein purification. Column # 1: Marker, Column # 2: Protein sample prior to induction in the nutrient broth medium with glucose, Column # 3: Protein sample after induction in nutrient broth medium with glucose, Position No. 4: Purified KD 50 protein sample

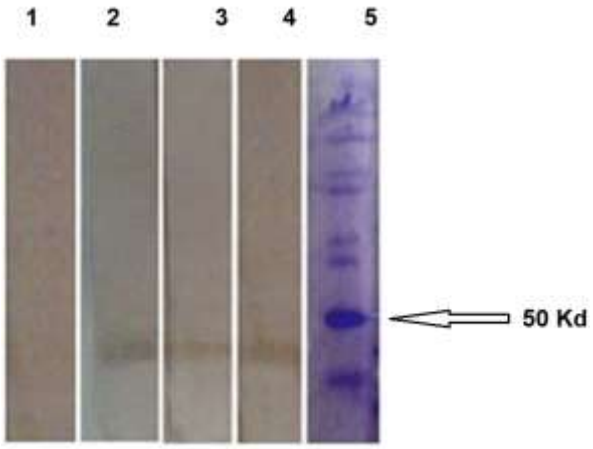


Figure 3: Western blot of recombinant protein tcpA V. cholerae Column 1: Negative control, Column 2: Western blot of recombinant protein with immunized mouse serum, Column 3: Western blot of recombinant protein with immunized rabbit serum, Column 4: Western blot of recombinant protein with immunized human serum, Column 5: Marker

In this study, Toxin-coregulated pili A gene was isolated using PCR. The TcPA protein with a molecular weight of 45 kDa was purified using 1pGEX4T expression vector. Purification of the intended protein was carreid out in induction media containing glucose. The TcPA protein is considered as a large subunit of vibrio cholera toxin coregulated pili A and is very similar to Type IV pili (9). This subunit, after being processing by peptidase (tcpJ) located in the inner membrane, is moved by a series of transport proteins to the periplasmic space, and then reaches the outer membrane through a special channel, and tcpA finally forms a definite peptide at the bacterial surface (19). Of the proteins involved in the formation of a toxin coregulated pili, only a few of them that are not within the cell membrane range can react with antibodies that can play a protective role in such way that TcPB, TcPA, and TcP F proteins can be exposed to antibodies (8). The protective effects of antibodies against tcpA seem to be effective by reducing the amount of V. cholerae colonization. The results of studies on the synthetic model of this protein have shown that dummy peptides induce protective antibodies in some TcPA areas in mice. These antibodies are attached to a high-density tcp fiber (20). In another study, anti-tcp antibodies were produced in rabbits. Administration of this serum in v. cholera-infected mice showed that the only anti-serum that was protective was the high-density anti-15TcP A serum that binds to TcP (5). Taylor et al. showed in a study that any impairment in the TcPA protein production would increase about 50% of the deadly dose of this bacterium (21). In another study, they identified a TcPA protein area that has an effective protective effect on newborn mice and has been proposed as compounds in the killed vaccine. These regions have hydrophilic property in such way that IgA antibody titers can be measured in mucosal responses of jejunal fluid after 7 days (22). The best immune responses studied are humoral immune responses, and general and mucous antibody responses can be induced for protection purposes (25-23). Serologic responses such as complement-dependent vibriocidal antibody responses, vibrio- lipopolysaccharides (VPS) antibody responses, chlorotoxin and tcp responses, colonization factor and the potential antigen increase the antibody secreted cell (ASC), fecal as well as plasma antibodies in patients. The V. cholerae pathogen has the ability to induce antibacterial immune responses and general and mucosal responses in patients (26, 27) in such way that effective vaccines should induce such responses. The immunity against this disease depends on the stimulation of the mucosal immune system and the production of IgG secretion (SIgA) in the lymphoid tissue of the intestine in such way that the antibodies are placed on the mucous membranes of the intestine and memory B cells of the individual and protect them against subsequent attacks (28). Secreted IgA antibodies have been studied against large protective genes in mucosal secretions of patients (13). Harrington et al. confirmed the pathogenicity of the TcP protein. They proved that these bacteria are not able to create colonization in the small intestine of humans and mice in V. chloride strains, the tcpa gene of which was mutated (13). According to the arrangement of the tcp-constituting proteins, and considering the possibility of exposure of these proteins, including TcPA and TcPB, to antibody molecules, we were forced to clone and express the intended protein. The researcher could isolate this protein either synthetically or directly from V. cholerae bacterium in previous studies on tcpA. Considering the importance and necessity of tcpA function in the V. cholerae pathogenesis, attempts were made in the present study to take an effective step in the field of immunogenicity and vaccine design in subsequent studies aim by producing this protein. The attempts were also made in the current study to produce a recombinant protein in order to effectively express this protein at a lower cost. TcPA protein was expressed in E. coli 21BL strain. The molecular weight of the G.S.T fusion protein is about 20 kDa. This protein sequence in the 1pGEX4T plasmid, which was used in the present study to produce the recombinant TcPA protei, was added to the beginning of the protein. So, the weight of the resulting protein is about 40.5 kDa. The TcPA protein was produced under the control of the Trp operator. This protein has been able to maintain its sustainability in the cytoplasmic space of E.coli bacteria through fusion proteins. The binding of these two proteins prevented the destruction of the recombinant TcPA protein by intracellular proteases of E.coli. The aim was to investigate the antigenicity of recombinant TcPA protein and ultimately to produce highly effective vaccines, with due regard to the importance of pathogenicity of the intended protein (TcPA), and to provide an appropriate immunity against colonization of V. cholerae in the intestine.

4. Conclusion

The results of the present study indicate that by employing suitable hosts such as 21E.Coli Bl, which lack protease enzymes, and also by using vectors such as 1pGEX 4T consisting of fusion protein with low molecular weights, the recombinant protein is maintained in the E-coli bacterium and its function remains unchanged . This research showed that, considering the antigenic similarity between the normal and recombinant forms, recombinant proteins can be used to investigate the V. cholera-induced infections and take an effective step in the production and design of anti-cholera vaccine by investigating the immunogenicity.

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