Original Article

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Production and Purification of Polyclonal Antibody against Cholera Toxin

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ABSTRACT

BACKGROUND AND OBJECTIVE: Cholera is a debilitating enteric disease, caused by Vibrio cholerae. Cholera toxin is the most important virulence factor in the pathogenesis of Vibrio cholera. Cholera toxin B subunit (CTxB), which forms a bond between the toxin and eukaryotic cells, has immunogenic features. The purpose of this study was to produce and purify antibodies against CTxB recombinant protein.

METHODS: The CTxB recombinant protein was expressed and purified by Ni-NTA affinity chromatography. In total, ten 5-week-old BALB/C mice were divided into control and test groups. The test group subcutaneously received 10 micrograms of the recombinant protein along with Freund's adjuvant. Antibody titers were measured by ELISA method. The serum of immunized mice, receiving phosphate-buffered saline, was used in ELISA as the control. Immunoglobulin G was purified by the use of affinity column of G protein. The inhibiting effect of antibody against CTxB on toxin was examined using GM1-ELISA method.

FINDINGS: The results of ELISA method showed the binding of recombinant protein to cholera toxin antibody. The amount of purified protein for each liter of the medium was 9 milligrams. ELISA findings showed that after each injection, the amount of antibody in mice was increased. The absorption rate of serum with the dilution of 1:500 was higher than three. According to Bradford assay, the density of purified antibody was 1 mg/ml. In ELISA'S reaction, 156 ng of toxin-binding subunit was identified by the antibody. The binding of toxin to GM1 increased by 70%, using immunized animal serum.

CONCLUSION: The results of this study showed the efficiency of CTxB recombinant protein as an effective immunogen for provoking humoral response against cholera toxin. The antibody against the recombinant B subunit was able to identify toxins and inhibit its binding to GM1 receiver.

KEY WORDS: Vibrio Cholera, B Subunit of Cholera, Recombinant Protein, Polyclonal Antibody, GM1-ELISA.

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Introduction

Vibrio cholera is the bacterium that causes cholera. It mostly emerges in developing countries and leads to high death rates (1,2). Cholera is caused by the strains of Vibrio cholera, which produce cholera toxin. Despite the fact that 200 serotypes of Vibrio cholera have been identified, only two types of serotypes,

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known as O1 and O139, cause pandemic and epidemic cases of cholera (3, 4). As a result of toxin activity, electrolytes and water are released from the body, and consequently, blood plasma volume significantly reduces, leading to the patient's death in a few hours (5). The most important factor among several virulence factors of Vibrio cholera is cholera toxin, which is an exotoxin and the major cause of cholera disease (6). With respect to the considerable antigenic features of cholera toxin, many studies have evaluated the use of this toxin for immunizing the body against cholera (7,8). This toxin is a protein consisting of heterodimeric A subunits (CtxA) with the molecular weight of 27,400 daltons and B-subunits (CTxB) with the molecular weight of 58,000 daltons. The B subunit is composed of 5 parts and 103 amino acids, with an annular arrangement and affinity to GM1 in jejunal epithelial cells (9). The A subunit is proteolytically cut, forming two polypeptide chains known as A1 and A2. It seems that the responsible factor for all biological interactions is cholera toxin A1 subunit (7,10).

The B subunit, which has no toxic features, is responsible for binding the toxin to the existing receivers in the cytoplasmic membrane of host cell. This subunit enables A1 subunit to penetrate into the cell (11). The function of A1 subunit leads to the intercellular increase of adenylatecyclase and the steady increment of annular cyclic adenosine monophosphate (cAMP), which eventually results in the excessive discharge of electrolytes and water into the intestine (8,9). In spite of the immunological features of cholera toxin, which have been highly regarded by scientists, its toxicity limits its application in human vaccination. Instead, given the non-toxic features of B subunit, the usage of CTxB has been widely investigated as a mucosal immunogen in humans (12-14). Consequently, CTxB recombinant protein can have several applications in producing oral vaccines (15, 16). The previous methods of CTxB purification were based on the mass culture of Vibrio cholera, collecting the culture media containing grown bacteria, and finally extracting the toxin.

In these methods, in addition to deficits in pure separation of CTxB, the obtained protein contains some CTxA and other unwanted proteins which limit the usage of this protein (17). In the method of producing recombinant protein, in addition to lowering the risks of working with pathogens, the obtained protein is pure and its production is cost-effective, i.e., we can produce more proteins in a shorter period of time at lower costs (17). In human studies, use of cholera toxoid, conjugated by chemical compounds, did not show an appropriate protective function. This can be one of the possible reasons for the effect of glutaraldehyde on the antigenic features of toxin (3,18). Levin et al. showed that use of toxoid, produced by chemical compounds, does not lead to the production of high titers of anti-toxin antibodies (18). In a study by Clemens et al., it was determined that the use of dead bacteria together with purified toxin-binding subunit is more effective than using the dead bacteria alone (19).

In a study by Dakterzada et al., 1 milligram of CTxB recombinant protein was expressed and purified. The function of recombinant protein was studied using GM1-ELISA method (20). Moreover, in a study by Zeighami et al., the maximum amount of purified recombinant protein was reported to be 480 micrograms in each liter of culture medium (21). Additionally, Yuki et al. investigated the effects of produced antibodies against catalytic toxin-binding subunits on the function of toxin and diarrhea in an animal model. It was shown that the produced antibody was more effective in immunizing the body and preventing diarrhea (22). Also, in a study by Nochi et al., the produced antibody in mice was able to inhibit the binding of cholera toxin to GM1 receivers up to 80% (23). By using the recombinant protein, we can immunize and form polyclonal antibodies. Polyclonal antibodies are a combination of monoclonal antibodies, which are formed against different epitopes and willingly bind to antigen molecules, probably due to the existence of different types of antibodies against antigen epitopes (24). Since these antibodies are formed against epitopes, they are important in confronting microbial agents. In order to be immunized against Vibrio cholera, different anti-toxin and anti-bacterial methods have been introduced (1). In

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this study, immunization and formation of antibodies, based on toxin subunits, were discussed. The CTxB recombinant subunit was suggested as an immunogen, which could be used for the formation of antibodies against toxin. The purpose of this paper was to produce a polyclonal antibody against cholera toxin subunit binding and investigate the prevention of binding between the toxin and receiver in vitro.

Methods

In this study, we used Escherichia coli BL21-DE3 bacteria. For the growth of this bacterium, liquid lysogeny broth (LB) medium and agar were used. The chemical compounds, kits, and molecular markers were provided by Merck, CinnaGen, Kiagen, and Fermentas companies. To purify the recombinant protein, we used affinity chromatography column of nickel-nitrilotriacetic acid (Ni-NTA) by Kiagen Company; to purify the antibody, we used G column by Roche Company. About ten 5-week-old BALB/C mice were divided into control and test groups.

Formation and purification of CTxB protein: After expression, the recombinant protein was purified, using Ni-NTA affinity chromatography (25).

Verification of the purified protein by ELISA method: For verifying the purified protein, we used anti-CTxB antibodies and ELISA method. The purified CTxB protein with serial dilution of 4 mg to 125 ng was stabilized in all wells. Then, these wells were rinsed with phosphate-buffered saline (PBS) three times (PBS containing 0.05% tween 20). At this stage, about 100 microliters of blocking buffer (5% non-fat powdered milk in buffered saline) was poured in all wells and kept at 37 °C for one hour. After cleaning, 1:5000 dilution of Anti-CtxB antibody and 1:2500 dilution of conjugated rabbit antibody were used, respectively. OPD and H_2O_2 substrates were added to the wells and after 15 minutes, by inhibiting the bonding with sulphuric acid, light intensity was determined by ELISA analysis at a wavelength of 495 nm.

Immunization analysis: In order to test immunization in a mouse model, 150 microliters, containing 50 micrograms of purified protein, was reached to a volume of 500 microliters with PBS. Afterwards, by adding the same amount of Freund's adjuvant, the final volume was reached to one milliliter. Five mice were chosen for injection and about 200 microliters of the mixture (containing 10 micrograms of antigen) were subcutaneously injected in mice.

In order to investigate and evaluate the results and prevent fake responses, buffer alone was injected to a group of 5 mice as controls; injections were performed with a 14-day interval. For the sake of immunization analysis, blood was taken from the eyes of the animals (immunized or unimmunized). The blood samples were transferred into sterile micro tubes and after heating for one hour at 37 °C, they were kept at 4 °C overnight. To separate the serum, blood clots were first removed. The serum was centrifuged at 1000 rpm at 4°C for 10 minutes, and the resulting clear yellow liquid was separated to be used in the next steps. In the next stage, the existing polyclonal antibody in the serum was investigated and evaluated by ELISA method.

Purification of polyclonal antibody: Purification of polyclonal antibody in the immunized serum with CTxB recombinant antigen was performed by G column. At first, the column was rinsed with 5 milliliters of Tris 100 mM and Tris 10mM (pH=8) and the buffer was allowed to completely exit the column. Then, Tris 1 M solution was added to 0.1 of serum volume (pH=8) and transferred from the column after vortex; the outlet of the column was collected. Then, the column was rinsed again with 5 milliliters of Tris 100 mM and Tris 10 mM (pH=8) and the outlet of the column was collected. In the next stage, the column was washed with 5 millilitres of 100 mM glycine solution (pH=3) and the outlet of the column was collected. Finally, the collected samples were studies by Bradford assay and SDS-PAGE after density determination. After the purification of antibody and determination of its density, dilution series of the antibody was provided and used in ELISA method.

Evaluation of the activity level of antibody in inhibiting LT toxin binding to GM1 receivers: In order to evaluate the activity of the provided antibody,

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inhibition of the bonding between LT toxin and GM1 receivers was evaluated. At first, 100 ng of GM1 receiver was poured in 100 microliters of coating buffer in microplate wells and kept in an incubator shaker at 37 °C for 2 hours. After evacuating the wells, their content was washed and blocked with 5% powdered milk. Bacterial supernatant containing 50 ng of toxin in serum concentrations, as well as the resulting solution of the control sample, were added to the wells. After washing and drying, the anti-CTxB antibody was added to the wells. Conjugated mouse antibody and OPD substrate were used in the next stages of ELISA method (26,27). By comparing the amount of OD in ELISA reaction in the presence of unimmunized serum, the purified antibody, and the standard diagram of toxin binding to the receptor, the percentage of binding to GM1 was calculated.

Results

Production of the recombinant protein and its verification by ELISA technique: After transferring pET28a/ctxB structure to E. coli BL21-DE3-susceptible cells, induction of gene expression was started, and the recombinant protein was purified using nickel column and denaturation method. By using urea gradient dialysis, 8-molar urea was replaced by PBS buffer. In order to confirm the recombinant protein, ELISA technique was used by applying the anti-CTX antibody. This antibody was able to identify the recombinant protein in gene expression (fig 1). As the results indicated, a small amount of the recombinant protein could also greatly bind to the antibody; in fact, the binding between the antibody and 125 ng of protein was also significant ($p<0.05$).

The evaluation results of anti-CTxB antibody by ELISA method: The immunization of animals was accomplished by injecting the resulting CTxB protein into 5 mice. During the intervals between the injections and two weeks after the last injection, blood samples were obtained from the mice, the serum was taken, and ELISA reaction was performed. After each injection, the amount of antibody production in mice's bodies increased (fig 2).

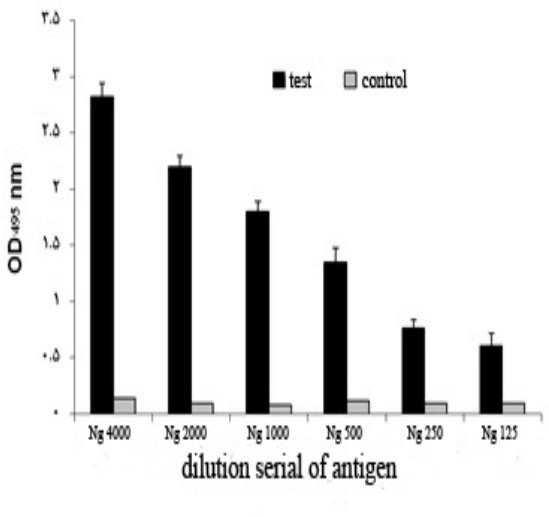


Figure 1. Confirmation of CTxB recombinant protein by ELISA method

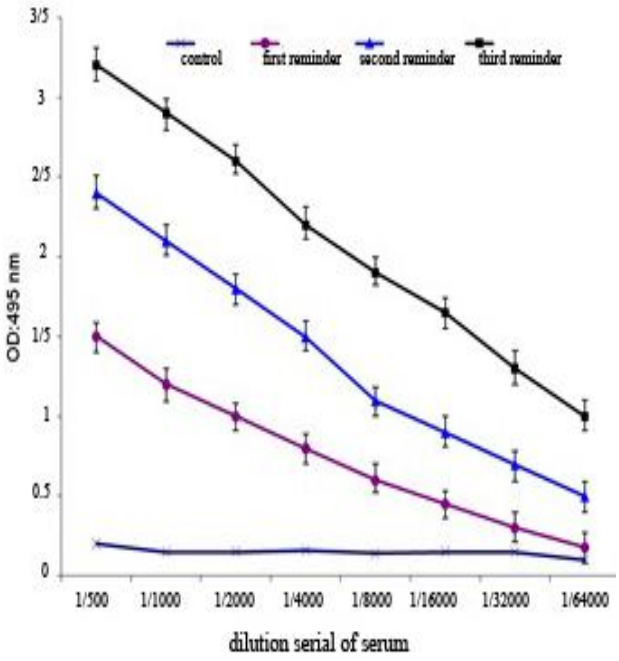


Figure 2. The ELISA results related to the antibody formed by immunization

Purification of polyclonal antibody: By using the column G, immunoglobulin G antibody was purified from the immunized serum of mice (fig 3). The density of the purified antibody from the serum of the third reminder was calculated to be 1µg/µl based on Bradford assay. By using the purified antibody, ELISA results showed that this antibody could perfectly identify the recombinant protein (fig 4).

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Antibody's inhibition of binding between the toxin and GM1 receiver: For evaluating the activity of the produced antibody, the inhibition test of toxin binding to GM1 was performed. By comparing the amount of OD in ELISA reaction in the presence of unimmunized serum, the antibody was purified. By using the standard table of toxin's binding to receptors, the percentage of binding to GM1 was calculated. Accordingly, the produced antibody inhibited the binding of 70% of toxin to GM1 receiver (fig 5).

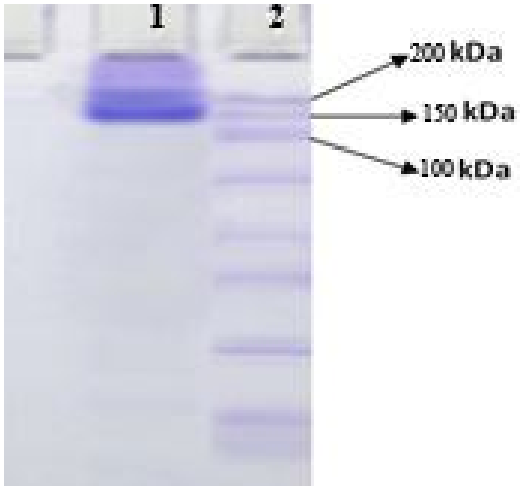


Figure 3. Study of the purified antibody on SDS PAGE gel. Column 1) the purified antibody, Column 2) protein size marker

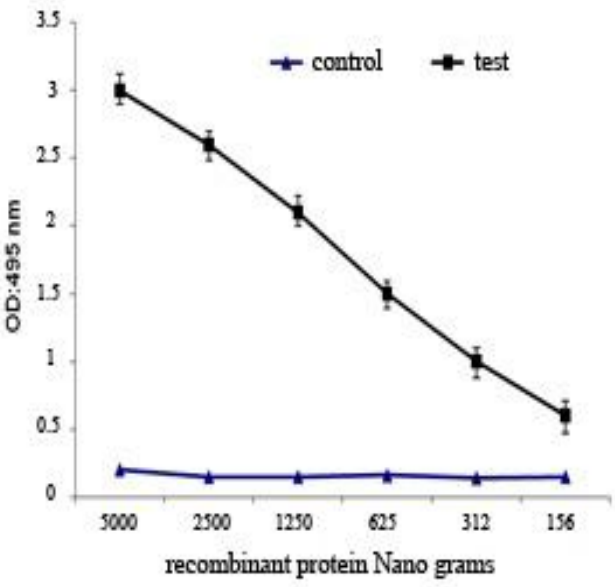


Figure 4. The results of ELISA regarding the purified antibody by CTxB recombinant protein

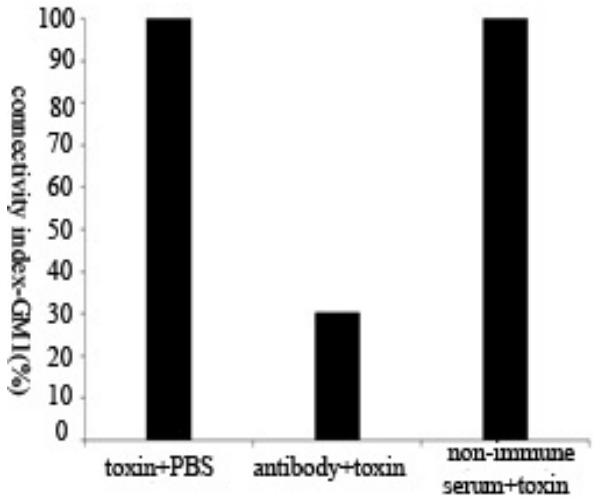


Figure 5. Study of the antibody's activity in the inhibition of binding between the toxin and GM1 receiver

Discussion

In this study, polyclonal antibody was produced against the binding subunit protein of cholera toxin, which was able to inhibit the binding of toxin to the receiver. Cholera toxin proteins and their binding subunits were considered as effective adjuvants. Previous studies have shown that unlike cholera toxin, which is toxic and its usage is limited to adjuvant, CTxB in addition to having immugenetic and adjuvant mucous features, does not have toxic features. In fact, CTxB by binding to antigens can stimulate immune responses through antigen's interactions with cells expressing adenomatous polyposis coli (APC) antigen in digestive and respiratory mucosa. CTxB forms a covalent bond with antigens and then carries them to mucous cells through ganglioside 1. Thus, the adjuvant feature of CTxB was confirmed when it was conjugated alongside with other proteins (17,28).

CTxB molecule plays an important role in the binding of cholera toxin to intestinal epithelial cells and the produced antibody can inhibit the toxic effects (1). With regard to the adjuvant features of this protein and its importance in vaccination, it seems that the expression of this protein as a recombinant protein is

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an appropriate, efficient, and cost-effective method for producing this protein. Moreover, future studies should evaluate the immunizing effects of this protein alone or alongside other immunogenic factors or as edible samples.

In this study, after improvisation and transmission of synthetic gene construct (pET28a/ctxB) to bacteria, the expression and purification of the recombinant protein were performed and confirmative tests were also carried out. The amount of recombinant protein after purification was 9 milligrams in each liter of culture medium. In a study by Zeighami et al., the maximum amount of purified recombinant protein was reported to be 480 micrograms in each liter of culture medium (21). After injecting the protein to lab animals, serum antibody titers, isolated from the blood of the animals, were evaluated by ELISA method, and at the end, the purification of polyclonal antibody against cholera toxin was performed using column G. The reason for using column G in the purification of antibody was to increase the speed and accuracy of purification. Therefore, there was no need to use ammonium sulphate to sequestrate the antibody initially; also, there was no need to use ion exchange chromatography. Since the obtained antibody is pure and it is possible to determine its density, it can also be used in methods of identifying cholera toxin such as ELISA sandwich.

In the current study, toxin binding subunit was used for immunization and the catalytic subunit of toxin was not considered. In a study by Yuki et al., the effects of the produced antibody against catalytic and binding subunits of toxin on the function of toxin and diarrhea were studied in an animal model. The results showed that using binding subunit as an immunogen has a double effect in comparison with the binding subunit after the manifestation of diarrhea symptoms in animals (22). The neutralizing features of the produced antibody showed that the anti-CTB antibody can identify the pentameric and natural structure of B subunit in toxin and inhibit its binding to GM1 receiver. The produced antibody is an immunoglobulin, which could reduce toxin's binding to the receiver by 70%. In a study by Nochi et al., the produced antibody was also able to reduce the binding between cholera toxin and GM1 receiver by 80% (23). Since the method of antigen prescription in this study was different from Nochi's study, the difference in the inhibition of toxin binding cannot be analysed. However, in both studies, the effect of binding subunit antibody of toxin in the inhibition of binding function of cholera toxin was shown.

The binding subunit of cholera toxin has more than 80% amino acid similarity to toxin binding subunit sensitive to enterotoxigenic E. coli (ETEC) heat. Therefore, the results of this study can be compared with the results of studies on LTB. Saliman et al. investigated the effectiveness of an anti-LTB antibody in inhibiting the binding of LT toxin to GM1 receiver. The antibody against LTB was able to inhibit the binding between toxin and receiver by 80% (29). The 10% increase in binding inhibition in comparison with the current study can be related to the amount of used antigen in animal immunization. In the study by Salimian et al., 20 micrograms of antigen was used in each stage of immunization. However, in this study, a lower amount of antigen (about 10 micrograms) was used in each stage of immunization. As mentioned earlier, considering the high amino acid similarity between cholera toxins and toxins sensitive to ETEC heat, polyclonal antibody can be used for the initial identification of toxins sensitive to heat. Nazarian et al. in their study used anti-cholera toxin antibody in order to confirm chimer protein containing ETEC antigens (26).

In another study by Saliman et al., antibody against cholera toxin binding subunits was used to confirm LTB recombinant protein (29). Also, the produced immunogen can be used in immunization studies against ETEC. Walker et al. indicated that using CTxB recombinant protein alongside with five common binding factors in ETEC would lead to immunization against ETEC and its toxin (30). The results of this study showed the efficiency of CTxB recombinant protein as an effective immunogen which stimulates hormoral response against cholera toxin. Also, polyclonal antibody can be used in methods of identifying cholera toxin, based on antibody.

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