Production of *Clostridium botulinum* Type E Antitoxin

Minaei M. E. 1 MSc, Sa’adati M. 1 PhD

1Biological Research Center, Basic Sciences Faculty, Imam Hossein Comprehensive University, Tehran, Iran

Abstract

**Aims:** The Botulinum toxin is known as the most potent toxin. In Iranian population, the type E is prevalent in individuals poisoned by contaminated foods (especially canned fish). Therefore, the purpose of this study was to produce *Clostridium botulinum* type E antitoxin for treatment of food poisoning.

**Methods:** For immunization of lab animals (chicken, mouse and rabbit), first the toxoid was injected with Freund's complete adjuvant either subcutaneously or intramuscularly and in the first to third booster injection, it was injected with Freund's incomplete adjuvant. At the end of injection interval, blood sampling was done and the produced antibody titers were measured using Enzyme-Linked Immune-Sorbent Assay or ELISA method.

**Results:** After each booster injection, a considerable increase in serum antibody titer of lab animals was seen. The titer of *Clostridium botulinum* type E toxin antibodies in the serum of chicken, rabbit and mouse increased to more than 1:3200. After purification of antibody, less than 0.156 micrograms of the purified mouse antibody and less than 0.312 micrograms of rabbit purified antibody were able to detect the toxin.

**Conclusion:** After the injection of toxoid, antibody increased in the body of laboratory animals. Production of type E specific antitoxin based on reaction between antigen and antibody is necessary poisoning treatment.

**Keywords:** antitoxin based on reaction between antigen and antibody (antitoxin with the serological type of the same toxin). In the preliminary studies the antigen quantity was measured using

Introduction

In 1897, Van Ermengen showed that botulism illness is caused by toxin production by an anaerobic bacterium. *Clostridium botulinum* consists of seven different types (A to G), based on the characteristics of the produced toxin. Botulinum toxin at first is synthesized in the form of a single-chain polypeptide with the molecular weight of almost 150kDa, which contains at least one intra-chain disulfide band. This molecule is barely active. When this toxin is broken by proteases, it converts to a two-chain polypeptide having a heavy chain (H) with the approximate weight of 100kDa and a light chain (L) with the approximate weight of 50kDa which is fully active. Among the seven different botulinum toxin serotypes, serotypes A, B and E create disease more commonly in human beings [1, 2, 3, 4].

Preparation of the standard anti toxin was first done for type A, B and C of Clostridium botulinum in the United States by Bengston. He mixed some amount of anti toxin with 100mlDa of toxin and considered a minimum amount of antitoxin, which postponed the death of guinea pigs weighing 250 g for 96 hours, as 0.1unit. Because of the high ability of these anti toxins they were adopted as international standards. The standard preparation of antitoxins of clostridium botulinum type A, B, C, D and E is necessary to estimate the therapeutic ability of antitoxins, and to detect the Clostridium botulinum toxigenic isolates [5, 6, 7].

To identify the Clostridium botulinum bacteria, there are several tests such as "direct observation and microscopic study of sample", "color staining (simple, compound and specific)" , "culture and examination of colonies" , "culture in specific, differentiating media and etc." "Nutritional and growth requirements" , "fermentation capabilities," "the used carbon source", "antibiotic resistance", "injection to sensitive laboratory animals", "phage typing", "bacteriocin typing", etc. Also today, precise and sensitive tests, such as molecular methods are used for detection of *Clostridium botulinum* bacteria. These tests are highly sensitive but are only able to detect bacteria and these methods are not efficient for detection of bacterial toxin.

At the present time, a method that has the highest sensitivity for detection of botulinum toxin and determines the lowest amount of toxin (up to about 5 to 10 pg/ml), is performing experiments on mice in-vivo. To be used as an alternative, should have at least twice the sensitivity on mice and the same specificity [8].

Serological assessment of botulinum toxin in outside-body conditions is done according to the reaction between antigen and antibody (antitoxin with the serological type of the same toxin). In the preliminary studies the antigen quantity was measured using
hemagglutination, Facilitated Hemagglutination Tests (FHT), immune-diffusion and radioimmunoassay. In later studies, specific methods were designed for detection of toxins [9, 10]. "Sandwich ELISA" method is among the best methods of immunoassay. Two antibodies, which are attached to the epitopes without interfering antigens, are needed in this method. For this purpose, two monoclonal antibodies that recognize the distinct sites or purified polyclonal antibodies are used (such as the purified rat and rabbit botulinum antitoxins) [9, 10, 11]. Among the recorded food poisoning cases caused by botulinum in Iran, the most frequent had been due to type E toxin that shows the necessity of producing antitoxin against this type of toxin.

Considering the need for type E anti-toxin in the treatment of food poisoning caused by isolates of Clostridium botulinum type E and detection of toxin in contaminated food, the purpose of this study was to produce antitoxin against serotype E in mice, rabbits and chickens for therapeutic goals.

**Methods**

A) Conversion of toxin to toxoid (detoxification): preparation of anti-toxins against bacterial exotoxins was done by animals' immunization. Initially, toxin was treated by the formaldehyde and lost its toxic properties [12]. For changing toxin into toxoid, formaldehyde 37% (Merck: 3999; Germany) was used. 1mg amount of botulinum toxin type E was solved in one ml PBS with pH of 7. The 37% formalin with the final concentration of 0.2% was added to the toxin and the sample was stored at 32° C. After four days, to ensure the poison deactivation, the amount of 10 micro liters of it were reached up to the volume of one ml by PBS buffer and injected to the peritoneum of two mice (0.5ml for each 18 grams weighing mouse) and the mice were examined up to 4 days. The survival of the mice was considered as the cause of detoxification [13, 14, 15].

B) Deformalization: Since it was possible for formaldehyde to have toxic effects on laboratory animals (rat, rabbit immunization), it is necessary to remove it from the buffer. For this purpose, the sample (Sigma: D 6066 cut off 12kDa; United States) was dialyzed. Dialysis was done before two liters of PBS buffer with pH of 7. Every 12 hours, and for four times, the PBS buffer was replaced (each liter of PBS contained 8g NaCl, 0.2g KCl, 0.29g Na2Hpo4,12H2O and 0.2g of KH2PO4). After dialysis, toxoid protein was determined using Bradford assay method [13, 14, 16].

C) Mixing of toxoid with adjuvant: the amount of 500 micro liters of toxoid was poured into the Eppendorf tube and the same volume of adjuvant was added. Eppendorf tube was shook using the shaker apparatus for 15 minutes and was moved into the syringe and by an interface needle, two syringes were linked. This continued until the mixture became quite thick and its color got milky. In the first injection to rabbits and mice, complete Freund adjuvant (Sigma: F5881; United States) was used and in the booster injections and also in the first injection of chicken, incomplete Freund adjuvant (Sigma: F5506; United States) was used [13, 15, 17].

D) Injection of toxoid to mice, rabbits and chicken: 100 micrograms of toxoid was injected subcutaneously into some points behind the neck of rabbits (New Zealand breed from the Iran Pasteur Institute). Three booster injections were done with a two-week interval. 10 days after each booster injection, in order to evaluate the antibody production, blood sampling was carried out from the rabbits' ears. For injecting of toxoid to the chicken (Leghorn breed from the livestock and poultry company of the Ministry of Agriculture), 50 micrograms of toxoid were mixed with incomplete Freund adjuvant and were injected in some points of chickens' breast muscle. Three booster injections were done and 10 days after each booster injection, blood sampling was done from the blood vessels under their wings. For injecting of toxoid (laboratory white mice from Iran Pasteur Institute), 5 micrograms of toxoid were injected and subcutaneously or intra-peritoneal. Three booster injections were done with a two-week interval. 10 days after each booster injection, in order to evaluate the antibody production and 10 days after each booster injection, blood sampling was done from their eyes [15]. Alongside each set of injections to chickens, mice and rabbits, a control group consisting of two similar laboratory animals was injected by the same amount of toxin and the behavior of laboratory animal was investigated.

E) Determination of antibody titers using indirect ELISA: First, the amount of 20 micrograms of toxin was solved in one ml of carbonate-bicarbonate buffer (pH=9.6), of which the amount of 100µl was poured into the ELISA sink for two hours in 37° C. Then, the content of the sinks were discarded and then were washed using the washing buffer (PBS containing 0.05% Tween20), then were dried. 100 micro liters of blocking buffer (PBS containing 0.05% Tween20 and 1% of bovine serum albumin) was added to sinks and kept for an hour at 37° C. After washing and drying of micro-plate (NUNC; Denmark), 100 micro liters of
antibodies with dilutions of 1:100 to 1:64000 was added to the sinks and kept for an hour at 37° C. After washing and drying of micro-plate, the chicken anti IgG antibody (Sigma: A9046; United States), rabbit (Dako: P0448; Denmark) or mouse (Dako: P0447; Denmark), conjugate was added to peroxidase enzyme with proper dilution by 100 micro liters and were kept for an hour at 37° C. After washing and drying of micro-plate, all sinks were added by 100 micro liters of substrate, which was 6 mg OPD (Sigma: P1526; USA) solved in 10mM of 1 citrate phosphate buffer with pH of 5 plus 5 micro liters of hydrogen peroxide 37% and was placed in the darkness. Finally, each sink was added by the amount of 100 micro liters of one molar sulfuric acid to stop the reaction and was read at the wavelength of 490nm of optical absorbance [13, 18, 19].

F) Purification of antibody type E with protein G column: For purification of antibody presented in the serum of laboratory animal, protein G column was used. Protein G column has high tendency for composing with IgG1 immunoglobulin and is capable of separating the immunoglobulin types of IgG1, IgG2, IgG3 and IgG4 of sera that has higher tendency for composition compared to protein A column. This column is more appropriate for isolation of antibody in the serum of mice and rabbits compared to protein A column [16, 20]. To purify the antibody, first the protein G column should be balanced (with 100mM trace and then with 10mM trace). Equivalent to 0.1 of serum sample volume, 1 M trace was added up to the point that pH equal to 8 and laboratory animal serum’s sample was injected from the top of the column. 15ml of glycine buffer 100mM with pH 3 was added from the top of the column. To collect samples, Eppendorf tube was used and 50 micro liters of 1 molar trace was added to each of them, so that at the collection time the PH would reach from 3 to 8. At the last stage, optical density of each of the tubes was read from the tubes with a wavelength of 280 nm. In order to investigate the process of antibody purification of mice and rabbits’ serum, the SDS-PAGE (12%), determination of protein by Bradford assay method and the indirect ELISA test were used.

**Results**

After the first injection of toxoid to chickens, the titer of antibody against *Clostridium botulinum* toxin type E increased to 1:1600 that the titer reached to 1:3200 or higher after booster injection (Diagram 1).
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Figure 1 - SDS-PAGE gel of the serum passed through protein G column-antibody presented in rabbits and rats serum were well purified and had a specific band with an approximate weight of 150kDa. The purified antibody of rabbit serum was broken in presence of 2ME and part of it showed a band with an approximate weight of 45kDa (Row 1: rabbits serum passed through the column without 2ME; Row 2: mouse serum passed through the column without 2ME; Row 3: protein marker SM 0661Fermentase; Row 4: rabbits serum passed through the column containing 2ME).

After the first injection, the antibody titer increased up to 1:800 in rabbits and reached to 1:3200 and higher after booster injection (Figure 2). The increase of antibody titer in mice was up to 1:1600 dilutions after the first injection, that this titer reached to 1:3200 and higher after booster injection (Diagram 3).

Diagram 4 - Titration of mouse antibody after serum purification

The results of the antibody purification process from the mouse and rabbit serum with electrophoresis and indirect ELISA are shown in Figure 1. Anti-toxin was well purified and reacted well with its toxin. Less than 0.156µg of purified mouse antibody and less than 0.312µg of purified rabbit antibody were able to detect the toxin (diagrams 4 and 5).

Diagram 5 - Titration of rabbit antibody after serum purification

Discussion

In this study, *Clostridium botulinum* type E antitoxin was prepared. Antitoxin is used in identifying the toxigenic *Clostridium botulinum* isolates as well as treating the poisoning cases caused by this toxin. In the identification and diagnosis dimensions, in-vivo experiment on mice to identify the botulinum toxins is yet the most sensitive and the best method. But due to the problems of working with laboratory animals, food and medicine management; therefore, the Center for Disease Control and Prevention has conducted cooperative efforts on developing methods for identifying botulinum toxin in-vitro [21]. So far, different methods have been done based on serological assessment, but ELISA method has had the most usage for food analysis. To improve the sensitivity of measurement, modified ELISA methods have been posed, of which, ELISA sandwich method is the most advanced. This rapid method (less than 6 hours) is highly exclusive and has the same sensitivity as the experiment on laboratory mice. To do this experiment, two antibodies attached to epitope with no interference on the antigens are needed [21]. In this study, purified mouse and rabbit polyclonal antibodies against botulinum toxin type E, which are necessary for sandwich ELISA method, were prepared.

The only available treatment of infection with botulinum toxin is antitoxin use. However, antibody therapy does not cure botulism symptoms, but limits the amount of toxin present in nerve endings; therefore, lessens the severity and duration of the illness. This antitoxin can be used for treatment of the disease [22].
The only way to prevent botulism is vaccination. A pentavalent botulinum toxoid including the botulinum toxins complex of type A, B, C, D and E detoxicated by formalin, has been used for the immunization of laboratory staff since 1961, but no permission has been issued by the U.S. FDA [23]. Except for the United States, in cooperation with three major Japanese laboratories, a tetravalent botulinum toxoid has been made of A, B, E and F serotypes in recent years, for those at risk of being affected by botulism toxicity [24].

In a study in 2008 by Keller on the conversion of botulinum toxin to toxoid through formalin, it was shown that this toxoid can create a desirable protection and this rate depends on the reaction condition during detoxification. Moreover, in-vivo and in-vitro experiments showed that new toxoids have almost same immunogenicity as the main toxin and it seems that they are superior to other botulinum vaccines [11].

The pentavalent toxoid includes botulinum complex of different serotypes and their hem-agglutinin as well as recombinant botulinum (fragment C) as a vaccine, but cannot stimulate the immune response to produce the antibody with cross-diagnosis of major neurotoxin like the new botulinum toxoid [11].

One of the methods of producing toxoid is adding commercial formalin (formaldehyde 37%) to the purified toxin. In a research by J. Bowmer in 1963, for conversion of toxin to toxoid for type A and B, the amount of 0.4%, for type C and D, the amount of 0.3% and for type E, the amount of 0.2% formalin has been respectively added to the obtained toxin. Then this mixture was kept at 37 °C for 3 to 4 weeks. Type E toxin needs less time to change into toxoid. To ensure of toxin intoxication, the obtained toxoid is injected to mice. Then the toxoid is precipitated with 0.5% aluminum phosphate and keeping it at 0 degrees temperature for a night. Types A, B, C and E deposit with 0.5% aluminum phosphate, but 1% aluminum phosphate should be added to type D.

Another study of changing the purified type E toxin into toxoid, has reported that 0.6% formalin is added to it and is kept at 33°C. This formalinated toxoid is injected to mice in order to ensure the lack of toxicity. If the mice stayed alive, the toxoid has converted to toxoid. Then, the toxoid will be absorbed with aluminum phosphate [6, 14, 17].

To prepare toxoid, in another article, 0.4% formalin was added to the purified toxin type E and kept for 2 days at 30°C. Then, complete Freund adjuvant with equal volume has been added and mixed thoroughly. This mixture is appropriate to produce antibody for the first injection to rabbits. For booster injection to rabbits and also for chicken injection, the purified toxin is mixed with Freund incomplete adjuvant. In all these cases, the toxin has been purified incompletely, but antitoxin production against Clostridium botulinum type E, has been reported to be successful [5, 6, 15].

In this study, for preparing toxoid, the Notermans’ method has been followed. After the injection of formalinitated toxin to mice, they did not show any signs of intoxication and survived. Also, the indirect ELISA method was used to determine the antibody titer. ELISA technique had been described by Engvall and Perlman in 1971 that later this method was modified. In 1979, the first experiment to determine the purified Clostridium botulinum toxoid was done by Notermans using ELISA method and conducting experiments on mice. This experience has shown that ELISA technique may be appropriate to determine the Clostridium botulinum toxin type E. In 1982, the ELISA test, in which toxoids type A, B and E had been fixed as antigens at the bottom of microplate could show the antibodies against botulinum toxin in the serum of two patients with neonatal botulism [18, 19, 25, 26]. In this study, to conduct the indirect ELISA test, the microplates’ bottom was covered with toxin and the serum obtained from rabbit, chicken and mice were poured on it. Then the enzyme-labeled antibody was added to each sink. Finally, substrate was added that caused the color reaction in each sink. In each ELISA experiment, the standard antibody was titrated in a column for the control of experiment and the standard antibody was specifically against type E toxin and was of horse type.

Conclusion

The specified antitoxin produced against botulinum toxin type E is used for the diagnosis, prevention and treatment of food poisoning caused by contaminated food.

References