

## Effect of Adjuvants on Antibody Titer of Synthetic Recombinant Light Chain of Botulinum Neurotoxin Type B and its Diagnostic Potential for Botulism

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**Botulism is a neuroparalytic disease caused by *Clostridium botulinum*, which produces seven (A–G) antigenically diverse neurotoxins (BoNTs). BoNTs are the most poisonous substances known to humans, with a median lethal dose (LD<sub>50</sub>) of approximately 1 ng/kg of body weight. Owing to their extreme potency and lethality, they have the potential to be used as a bioterrorism agent. The mouse bioassay is the gold standard for the detection of botulinum neurotoxins; however, it requires at least 3–4 days for completion. Attempts have been made to develop an ELISA-based detection system, which is potentially an easier and more rapid method of botulinum neurotoxin detection. The present study was designed using a synthetic gene approach. The synthetic gene encoding the catalytic domain of BoNT serotype B from amino acids 1–450 was constructed with PCR overlapping primers (BoNT/B LC), cloned in a pQE30 UA vector, and expressed in an *E. coli* M15 host system. Recombinant protein production was optimized at 0.5 mM IPTG final concentration, 4 h post induction, resulting in a maximum yield of recombinant proteins. The immunogenic nature of the recombinant BoNT/B LC protein was evaluated by ELISA. Antibodies were raised in BALB/c mice using various adjuvants. A significant rise in antibody titer ( $p < 0.05$ ) was observed in the Alum group, followed by the Titermax Classic group, Freund's adjuvant, and the Titermax Gold group. These developed high-titer antibodies may prove useful for the detection of botulinum neurotoxins in food and clinical samples.**

**Keywords:** Botulism, BoNT/B LC, synthetic gene, antibody, ELISA, adjuvants

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Botulism is a neuroparalytic disease caused by *Clostridium botulinum*, which produces seven structurally related but antigenically distinct neurotoxins (BoNTs A–G) [19]. Human botulism cases are caused primarily by neurotoxin types A, B, and E [3] and occasionally by type F [4]. The onset of paralytic manifestations can occur from between a few days to over a week after intoxication, but in the majority of cases paralysis occurs within 12–72 h [21]. BoNTs are released as a 150 kDa single polypeptide, which is cleaved endogenously or exogenously resulting in a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) linked through a disulfide bond [32]. The C terminal half of the HC binds to the nerve membrane and the N terminal half facilitates the transport of the light chain across the membrane of the endosome to the cytoplasm. The LC possesses zinc endopeptidase activity, which cleaves the SNARE (soluble N-ethylmaleimide factor attachment protein receptor) proteins involved in the docking and the fusion of acetylcholine-containing vesicles to the plasma membrane [20, 24]. Inactivation of any SNARE protein results in the inhibition of acetylcholine secretion at the neurotransmitter junctions, resulting in flaccid muscle paralysis [20, 29]. BoNTs A, C, and E cleave SNAP-25 [5, 6, 35], BoNT C also cleaves syntaxin [7, 12], and BoNTs B, D, G, and F are specific for VAMP/synaptobrevin [26–28]. BoNT is the most lethal substance presently known [15].

In recent years, incidents of foodborne botulism have been reported all over the world. The efficiency of investigating botulism varies greatly between countries, with the true incidence of foodborne botulism likely to be much higher than what is currently being reported owing to varying levels of expertise. In 2002, two people died from botulism as a result of ingesting commercially tinned pilchards in South Africa [14]. Subsequently, botulism cases were reported after the drinking of pruno in the USA

[36], the consumption of homemade *sume* (condensed yogurt) in Turkey [2], and home-salted unevicerated fish in the USA [33]. In India, a case of foodborne botulism was reported when an entire family developed symptoms of botulism within 48 h after eating canned meat products [1]. In 2006, a severe outbreak in the USA and Canada was associated with commercially chilled carrot juice [31]. In 2009, botulism cases were reported in France after the consumption of commercially vacuum-packed hot smoked white fish [18]. In addition, the botulinum toxin poses a major bioweapon threat because of its extreme potency and lethality, its ease of production and transport, the need for prolonged intensive care among affected persons, and its potential to invoke extreme fear amongst the general public [13]. At present, the treatment for botulinum poisoning is supportive care and the administration of an equine antitoxin. The antidote works by capturing the toxin in the bloodstream, but it cannot neutralize the toxin inside the poisoned cells and so timely intervention with the antidote is critical and requires the rapid and effective diagnosis of botulism.

The development of protocols with recombinant protein always has the benefit of the availability of large amounts of protein without the need to culture *C. botulinum*, which requires a complete anaerobic atmosphere and some very stringent biosafety precautions. Our laboratory has produced recombinant BoNT/B LC as a reagent to study the immunological responses of the catalytic domain of the toxin with different adjuvants. The synthetically constructed BoNT/B LC by the PCR overhang method was cloned in a pQE30 UA vector and the recombinant plasmid was transformed in *E. coli* M15. The recombinant BoNT/B LC was then purified by affinity chromatography and characterized for its purity as well as for its immunogenicity using different adjuvants through the raising of antibodies. The diagnostic potential of the antibody against recombinant BoNT/B LC was also evaluated. Details of the production process of recombinant BoNT/B LC and the effects of the adjuvants on antibody titer are described within this study.

## MATERIALS AND METHODS

### Materials

A QIA miniprep kit, a QIA quick gel extraction kit, Ni-NTA super flow, pQE30 UA expression vector, *E. coli* M15 cells and the anti His antibody were procured from Qiagen (GmbH, Hilden, Germany). Prestained marker, *Taq* polymerase, dNTPs, and PCR buffer were acquired from MBI Fermentas (Canada). Kanamycin, ampicillin, IPTG, aluminum hydroxide gel, Freund's complete/incomplete adjuvant, Titermax Gold and Titermax Classic adjuvant, deoxy ribonuclease I, protease inhibitor cocktail, and a BCA protein assay kit were purchased from Sigma (USA). Nitrocellulose membrane (0.45  $\mu$ M)

was sourced from Millipore (Bangalore, India). Super broth for bacterial culture preparation was obtained from Difco Laboratories (Mumbai, India). Monoclonal antibody for BoNT/B was procured from Biodesign International (USA) and an Immunopure (G) IgG purification kit from Pierce (USA).

### Synthetic Gene Construction and Cloning

The synthetic gene coding the catalytic domain (amino acids 1–450) of the botulinum neurotoxin serotype B (BoNT/B LC) was constructed using the PCR primer overlapping method [10]. Fourteen oligonucleotides of 120 bp length, having 20 bp of overlapping sequence with its partners, were designed. An additional 13 pairs of short 20 bp primers (forward/reverse) were designed to join the larger gene segments. All the designed primers were commercially synthesized (Biotech Desk, Hyderabad, India). Four sequential PCR reactions were performed to link the seven neighboring gene segments, and then these segments were joined into four, then two, then into a complete gene segment with a length of 1,420 base pairs. From this, the catalytic domain of 1,350 base pairs was PCR amplified using primers BoNT syn F 5'-TCAGTTACAATAAATAATTTT-3' and BoNT syn R 5'-ATCAACATCAATACATATTCC-3'. All PCR reactions were carried out with an initial denaturation of 94°C for 5 min, followed by 30 cycles consisting of denaturation at 94°C for 30 s, annealing at 48°C for 60 s, and extension at 72°C for 30 s, and then the final extension at 72°C for 6 min. The synthetic gene was purified using 1% low melting point agarose gel electrophoresis and the PCR products were extracted using a QIA quick gel extraction kit. The PCR product was cloned at the pQE30 UA vector using a QIA express UA cloning kit according to the manufacturer's protocol. The ligated products were transformed into chemically competent *E. coli* M15 cells by the heat shock method. The transformants were selected on Luria–Bertani (LB) agar plates supplemented with kanamycin (30  $\mu$ g/ml) and ampicillin (100  $\mu$ g/ml). Plasmids were extracted from the clones using a QIA miniprep kit following the manufacturer's protocol. These plasmids were then screened for confirmation of inserts using the BoNT/B specific primers mentioned above, with the "in frame" also checked by using a combination of BoNT/B and vector specific primers. The sequence of the synthetic genes was verified by sequencing.

### Recombinant Protein Expression and Localization

The selected transformant was inoculated into 5 ml of Super broth (SB) containing kanamycin (30  $\mu$ g/ml) and ampicillin (100  $\mu$ g/ml), and then grown overnight at 37°C, with 200 rpm of shaking. The culture was further inoculated into 20 ml of SB containing antibiotics and growth was monitored by absorbance measurements. Recombinant protein expression was optimized by inducing the culture with isopropylthiogalactoside (IPTG) with a concentration range of 0.1 mM to 1 M at an approximate culture OD<sub>600</sub> range of 0.5 to 1. Prior to IPTG induction, 2 ml aliquots were taken out aseptically to be used as an uninduced control. After induction, at every one-hourly interval, 2 ml samples were drawn and cells were harvested by centrifugation at 8,000  $\times$ g for 10 min at 4°C. Each sample was processed and the whole cell protein was extracted and analyzed by SDS–PAGE (12%). The localization of recombinant protein was carried out by solubilizing the induced pellet in a phosphate buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 100 mM NaCl, pH 8.0). The resuspended pellet was sonicated on ice, followed

by centrifugation at 10,000  $\times g$  for 20 min at 4°C. Both the supernatant and pellet were then analyzed by SDS–PAGE (12%).

#### Inclusion Bodies Preparation

The positive recombinant transformant of BoNT/B LC was inoculated in 5 ml of SB with antibiotics and grown overnight at 37°C, with 200 rpm. This was subcultured into 1 l of LB broth containing antibiotics and grown until OD<sub>600</sub> ~0.8. The culture was then induced with 0.5 mM IPTG followed by incubation for 4 h. Cells were pelleted at 8,000  $\times g$  for 10 min and resuspended in a cell lysis buffer (20 mM Tris, pH 7.5) containing a mild nonionic detergent (Triton X-100), at a ratio of 5 ml of the lysis buffer per gram of wet cell pellet. Deoxyribonuclease I, at a 5  $\mu g/ml$  final concentration, and a protease inhibitor cocktail at a ratio of 1 ml to 20 g of wet weight of cell pellet was added to the suspension. The suspension was incubated at room temperature for 15 min, followed by centrifugation at 10,000  $\times g$  for 15 min. The pellet was again resuspended in another 5 ml of lysis buffer with Lysozyme (1 mg/ml) and then the mixture was incubated at 25°C for 15 min. The cell suspension was sonicated at 9.9 pulses on/off for 5 min and centrifuged at 12,000  $\times g$  for 30 min at 4°C. The inclusion body pellet was washed twice with the same lysis buffer and centrifuged at 10,000  $\times g$  for 10 min at 4°C. Finally, the pellet was resuspended in a solubilizing/equilibration buffer (8 M urea, 0.1 M NaH<sub>2</sub>PO<sub>4</sub> and 0.01 M Tris-Cl, pH 8.0) overnight, followed by centrifugation at 12,000  $\times g$  for 20 min. The supernatant contained the solubilized inclusion bodies.

#### Purification, Dialysis, and Estimation of Recombinant BoNT/B LC

The recombinant protein was purified under denatured conditions using affinity chromatography. The Ni-NTA column was equilibrated with a solubilizing buffer (8 M urea, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, and 0.01 M Tris-Cl, pH 8.0) and the cell lysate was mixed with  $\beta$ -mercaptoethanol to a concentration of 15 mM and Triton X-100 (1%). The cell lysate and the Ni-NTA slurry were mixed gently to allow for “His”-tagged protein binding with Ni-NTA and incubated at RT for 30 min. Slurry was then poured onto the pre-equilibrated column. The flow-through was collected and the column was washed with 10 volumes of equilibration buffer, followed by 10 volumes of wash buffer (8 M urea, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, and 0.01 M Tris-Cl, pH 6.3) containing Triton X-100 to a final concentration of 2%. Finally, the column was washed with 10 volumes of wash buffer with 15% ethanol and the protein was eluted with an elution buffer (8 M urea, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, and 0.01 M Tris-Cl, pH 4.5). The different fractions of elutes of 1 ml each were collected and their homogeneity checked through SDS–PAGE (12%). Urea was removed by sequential dialysis. For the final step, the protein was concentrated using an Amicon ultracell with a 10 kDa cut-off membrane. The concentrated protein concentration was determined using a BCA protein assay kit.

#### Western Blot Analysis

The purified recombinant BoNT/B LC was separated in SDS–PAGE and then transferred on to a nitrocellulose membrane and blocked with 3% BSA overnight at 4°C. After decanting the blocking buffer, one strip of the membrane was incubated in monoclonal BoNT/B antibody (1:1,000) in blocking buffer with gentle shaking at RT for 1 h to confirm the peptide encoded by the synthetic gene of the 450 amino acids of rBoNT/B. Another strip of the membrane was incubated with an “anti His” monoclonal antibody (1:2,000) to confirm

the presence of the His tag at the N terminal of rBoNT/B LC provided by vector pQE30 UA. After incubation, these strips were washed three times with PBST (PBS containing 0.5% Tween-20) of 5 min each. These strips were again incubated in rabbit anti-mouse IgG horse radish peroxidase (HRP) conjugate (1:2,000) as a secondary antibody in a blocking buffer with gentle shaking at RT for 1 h. Colorimetric detection was carried out by using 3,3'-diaminobenzidine in PBS containing 8.8 mM H<sub>2</sub>O<sub>2</sub> as a substrate.

#### Formulation of Recombinant BoNT/B LC with Adjuvants

Recombinant BoNT/B LC was formulated with aluminum hydroxide gel (Alum), Freund's complete/incomplete adjuvant (FCA/FIA), and Titermax Gold and Titermax Classic adjuvants. In the case of FCA/FIA and Titermax Classic, one part of adjuvant was mixed with one part of antigen (v/v). For Titermax Gold, two parts of adjuvant were mixed with one part of antigen (v/v). Mixing was done in leur-lock syringes until an emulsion was formed. For the aluminum hydroxide gel, the pH was first adjusted to 7.0 using NaOH and then mixed with rBoNT/B LC. The ratio of 61.5% of aluminum hydroxide gel per  $\mu g$  of antigen was used. The aluminum hydroxide gel formulation was incubated for 6 h at 4°C on a rocking platform, and centrifuged at 3,000 rpm for 10 min. The unadsorbed protein in the supernatant was tested through a BCA protein assay kit. More than 90% of recombinant protein was found to have been adsorbed to the aluminum hydroxide gel. The pellet (aluminum hydroxide gel having adsorbed rBoNT/B LC) was resuspended in normal saline and used for the immunization of mice. For a control group, rBoNT/B LC was used for immunization without any adjuvant.

#### Immunogenicity Studies

New Zealand white rabbits (1 kg) and BALB/c female mice (16–22 g), n=5/group, were used for immunization. The animals were maintained and used in accordance with the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals. The study had the approval of the institutional ethics committee of DRDE. For the initial studies, two groups of 5 BALB/c mice were immunized with 100  $\mu l$  of recombinant protein per mouse. One group was immunized intramuscularly through the caudal thigh muscle and the second group was injected intraperitoneally. Mice were injected on days 0, 14, and 21 with 30  $\mu g$  of purified recombinant protein per mouse emulsified in Freund's complete adjuvant (FCA) on day 0 and with Freund's incomplete adjuvant (FIA) on days 14 and 21. In the second set of experiments, five groups of 5 mice each were immunized with rBoNT/B LC through an intramuscular route with FCA/FIA, Titermax gold, Titermax classic, and aluminum hydroxide gel as adjuvants, and one group was immunized with purified rBoNT/B LC without adjuvant with the same immunization schedule as stated above. The first dose in the FCA/FIA group was administered with complete adjuvant and the rest of the doses were given with incomplete adjuvant. Rabbits were immunized intramuscularly with 60  $\mu g$  of purified recombinant protein with the same dosing schedule as stated for mice with alum as an adjuvant. The total injection volume for rabbits was 500  $\mu l$ . In all experiments, the mice were bled through a retro-orbital puncture, and rabbits through an ear vein, prior to the first dose and 7 days after the last dose. The blood was incubated at 37°C for 30 min. Serum was collected after centrifuging the blood samples at 3,000  $\times g$  for 10 min to remove residual blood cells. The serum was stored at –20°C until further use. The IgG antibodies were purified from

respective sera using Pierce, Immunopure (G) columns according to the manufacturer's protocol.

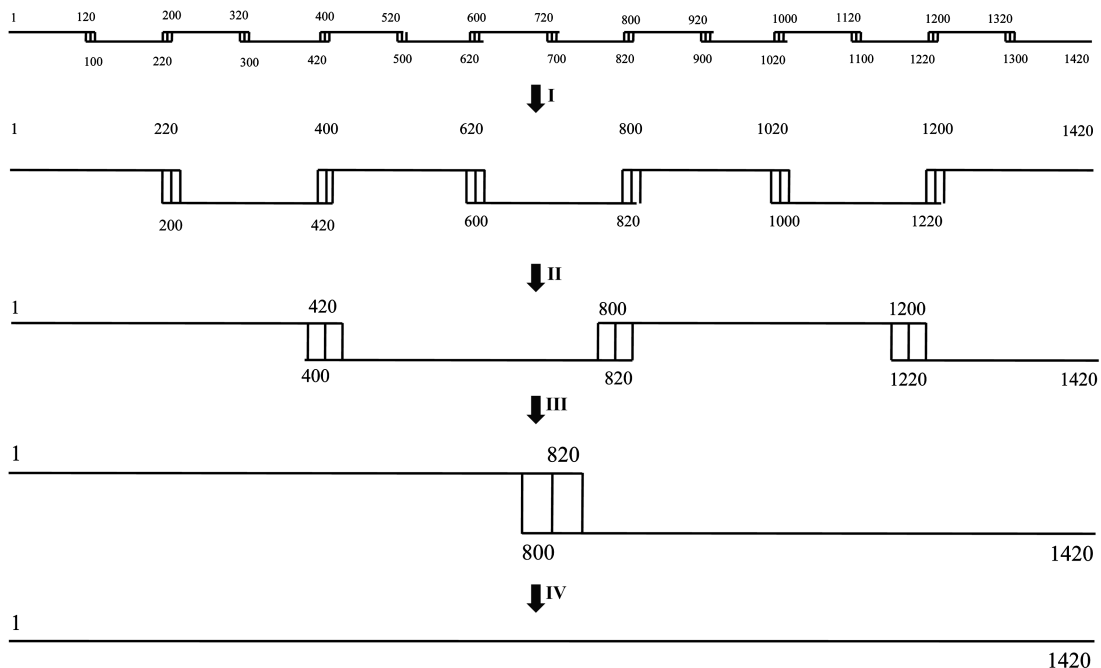
### Indirect ELISA

The presence of serum immunoglobulins specific to BoNT/B LC in mice was determined by indirect ELISA. The purified recombinant BoNT/B LC was diluted to 5 µg/ml in a carbonate buffer (0.05 M, pH 9.6) and used to coat the wells of polystyrene plates (100 µl/well; Nunc-Immuno plate with Maxisorp surface). The plates were incubated overnight at 4°C, and the next morning the plates were washed three times with PBST. The remaining sites of absorption were blocked by the addition of 200 µl/well 3% BSA (made in PBS) for 2 h at 37°C. The plates were washed three times with PBST (PBS/0.05% Tween-20). Sera from each group of immunized animals were 2-fold serially diluted (1:1,000 to 1:4,096,000) and examined in triplicate wells (100 µl/well) of the blocked antigen-coated plates and incubated for 1 h at 37°C. They were then washed five times with PBST and further incubated at 37°C for 1 h with HRP-conjugated anti-mouse IgG (1:2,000). The plate was washed five times with PBST and developed with 100 µl of ortho-phenylenediamine (0.4 mg/ml) in a freshly prepared citrate phosphate buffer (0.1 M, pH 5.0) and H<sub>2</sub>O<sub>2</sub> (0.4 µl/ml). The reaction was terminated by the addition of 50 µl of 2.5 N H<sub>2</sub>SO<sub>4</sub>/well. The presence of serum immunoglobulins specific to BoNT/B LC in rabbits was determined in the same manner as in mice, except the HRP-conjugated antibody used was anti-rabbit IgG. Absorbance was read at 492 nm with a microtiter plate reader. The cut-off value for assay was calculated as the mean specific optical density, plus three times the standard deviation (SD), for pre-immune serum assayed at a dilution of 1:1,000. The titer of immune serum was calculated as the reciprocal

of the highest serum dilution yielding a specific optical density above the cut-off value. The cross-reactivity of raised antibodies with different serotypes was evaluated by coating with 5 µg of a recombinant light chain of BoNT/A and E. The cross-reactivity for other clostridial species was also checked by coating the wells with 10 µg of a trichloroacetic acid (TCA)-precipitated culture supernatant of *C. sporogenes* ATCC-11437, *C. perfringens* ATCC-13124, *C. sordellii* ATCC-9714 (from ATCC), *C. tetani* 49205 (from CRI, Kasauli), *C. butyricum*, and *C. subterminale* (from the culture collection of the Biotechnology Division, DRDE Gwalior). All the *Clostridium* cultures and toxins were handled with the utmost care after wearing gloves and masks in a class III biosafety cabinet.

### Sandwich ELISA

To determine the minimum detection limit, the recombinant BoNT/B specific antibody raised in mice was purified (IgG) and diluted to 20 µg/ml in a carbonate buffer and used to coat the polystyrene wells (100 µl/well) in triplicate batches and then incubated overnight at 4°C. After washing the plates three times with PBST, the remaining sites of absorption were blocked by the addition of 200 µl/well 3% BSA (made in PBS) and incubated for 2 h at 37°C. Then the plates were washed three times with PBST. Purified recombinant BoNT/B (100 µl/well) in 2-fold serial dilution (1,000 µg/ml to 7.5 ng/ml) was added to the blocked antibody-coated plates and incubated for 1 h at 37°C. Then the plates were washed three times and again incubated with a BoNT/B specific antibody raised in a rabbit (100 µl/well) at a 20 µg/ml dilution (in PBS) at 37°C for 1 h. Following this, the plates were washed five times with PBST and further incubated at 37°C for 1 h with HRP-conjugated anti-rabbit IgG (1:2,000) and again washed five times with PBST before the ELISA was developed.



**Fig. 1.** Schematic diagram of synthetic gene construction by PCR.

The synthetic gene (1,420 bp) was constructed from 14 overlapping oligonucleotides. Four sequential PCR fusion reactions were done to link the seven neighboring gene segments, and then join those segments into four, then two, and then one complete gene segment. From this, the catalytic domain (BoNT/B LC) of 1,350 bp was amplified and sequenced.

The ELISA was developed in the same way as stated above, with the cut-off value for assay calculated as the mean specific optical density plus three times the standard deviation (SD) of the well having no antigen.

#### Statistical Analysis

A statistical analysis, to compare the groups, was performed using the *t*-test (Sigma Stat., Jandel Scientific, USA).

## RESULTS

### Synthetic BoNT/B LC Construction and Cloning

Primers were designed to construct the catalytic domain of the botulinum neurotoxin gene type B (BoNT/B LC) and synthesized commercially. Using these primers, the synthetic gene of 1,420 bp was constructed using the polymerase chain reaction (PCR). (The schematic diagram is represented in Fig. 1.) From this, the catalytic domain of 1,350 bp was amplified. The constructed synthetic gene fragment was sequenced and confirmed. The synthetic gene sequence was submitted as GenBank Accession No. HQ116625. After confirmation, the synthetic construct was purified using agarose gel electrophoresis and the purified PCR products were cloned in the pQE30 UA vector. The ligated product was transformed in *E. coli* M15 and the selected transformants were subjected to plasmid profiling for the confirmation of the presence of inserts and their orientation. The vector provides a 6× His tag at the N terminus, which

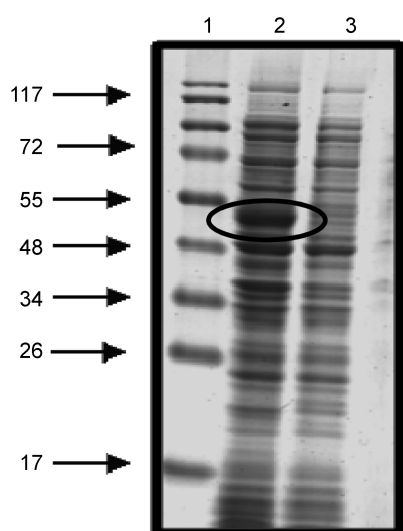
aids in the purification of expressed recombinant proteins by the utilization of metal affinity chromatography.

### Expression of Recombinant BoNT/B LC

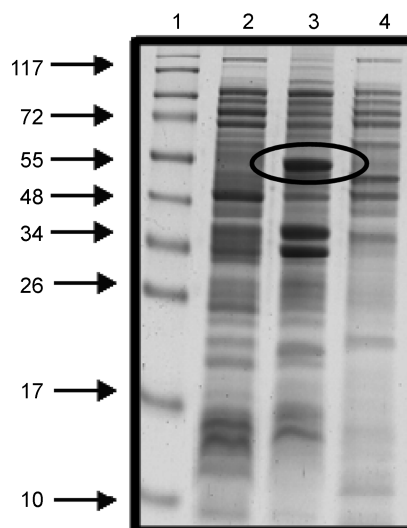
Expression of the His<sub>6</sub>-tagged rBoNT/B LC gene was observed under the control of the IPTG-inducible T5 promoter of M15 cells. The prepared construct encoded 52.8 kDa of rBoNT/B LC protein. Expression conditions were optimized at different IPTG concentrations at various OD<sub>600</sub> values and at different time intervals (data not shown). The maximum yield of the recombinant protein was obtained by inducing the culture with 0.5 mM IPTG at OD<sub>600</sub> 0.8 at the fourth hour of post induction. An SDS-PAGE gel of the recombinant protein expression, with and without IPTG induction, is shown in Fig. 2.

### Purification of His<sub>6</sub>-Tagged Recombinant BoNT/B LC

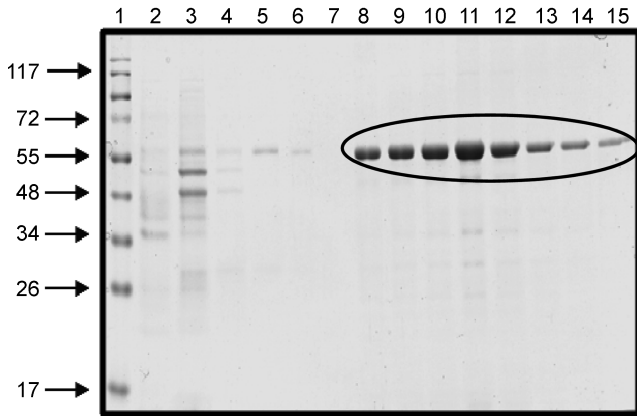
The cells were subjected to localization studies and the results revealed that the recombinant proteins were present as inclusion bodies (Fig. 3). Purification was carried out under denatured conditions. Inclusion bodies were solubilized with 8 M urea and subjected to Ni-NTA affinity chromatography. Triton and ethanol were used as the wash buffers to minimize nonspecific binding. The recombinant protein was purified, as shown in Fig. 4, near to a homogeneity of 98% (based on densitometry analysis). One liter of culture yielded approximately 20 mg of recombinant protein. The



**Fig. 2.** Protein expression analysis of recombinant BoNT/B LC. 12% SDS-PAGE analysis of rBoNT/B LC protein expression. The panel depicts protein molecular mass markers (lane 1), Coomassie-stained polypeptide profiles of *E. coli* lysate induced with IPTG (lane 2) and uninduced lysate (lane 3). The molecular masses of the protein standards are indicated on the left in kilodaltons. A circle within indicates the position of rBoNT/B LC.

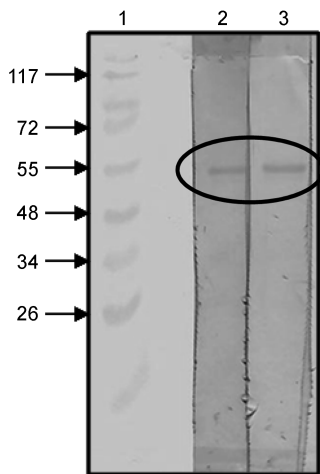


**Fig. 3.** Localization analysis of recombinant BoNT/B LC. 12% SDS-PAGE analysis of recombinant BoNT/B LC protein localization. The panel depicts protein molecular mass markers (lane 1), and Coomassie-stained polypeptide profiles of *E. coli* uninduced lysate (lane 2) sonicated induced pellet (lane 3), and supernatant of sonicated induced pellet. The induced cell culture pellet was sonicated in lysis buffer to localize the protein. The molecular masses of the protein standards are indicated on the left in kilodaltons. A circle within indicates the position of localized rBoNT/B LC in the sonicated pellet.



**Fig. 4.** Purification analysis of recombinant BoNT/B LC. 12% SDS-PAGE analysis of recombinant BoNT/B LC purified protein. The panel depicts protein molecular mass markers (lane 1), Coomassie-stained polypeptide profiles of fraction without inclusion bodies (lane 2), flow-through (lane 3), Wash 1 with equilibration buffer (lane 4), Wash 2 with wash buffer having Triton (lane 5), Wash with wash buffer having ethanol (lane 6), and purified protein elutes (lanes 7–15) in elution buffer. Purification of His-tagged rBoNT/B LC was done by Ni-NTA affinity chromatography. The molecular masses of the protein standards are indicated on the left in kilodaltons. A circle within indicates the position of purified rBoNT/B LC.

protein was subjected to dialysis to remove urea, and finally concentrated to 1 mg/ml. After dialysis, the concentrated recombinant protein was subjected to Western blot analysis to confirm rBoNT/B LC, using BoNT/B specific antibodies, with the results showing a positive reaction with the

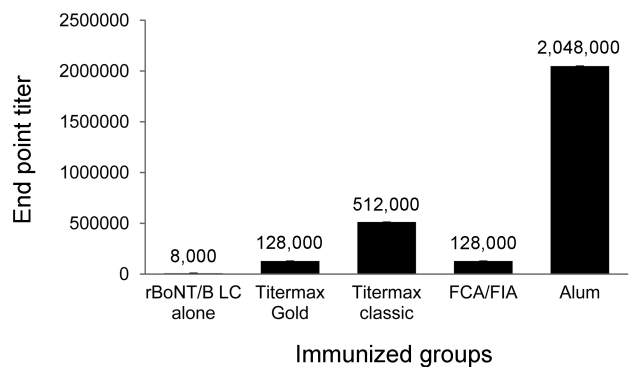


**Fig. 5.** Western blot analysis of BoNT/B LC with anti-His-tag and BoNT/B monoclonal antibody. The purified recombinant BoNT/B LC was run on 12% SDS-PAGE and transferred onto the nitrocellulose (NC) membrane. NC membrane strips were cut. Protein molecular mass markers (strip 1), purified recombinant BoNT/B LC protein developed with monoclonal BoNT/B antibody (strip 2), and purified recombinant BoNT/B LC protein developed with anti-His antibody (strip 3). The molecular masses of the protein standards are indicated on the left in kilodaltons. A circle within indicates the position of purified rBoNT/B LC.

BoNT/B monoclonal antibody, as well as with the His-tagged antibody, illustrated in Fig. 5.

**Antibody Generation and ELISA**

The immune serum was raised in mice and rabbits using the recombinant protein of BoNT/B LC, which had been purified to 98% purity under denatured conditions. One group of mice was injected with the antigen intramuscularly (IM) and another group intraperitoneally (IP). Sera were collected on the 28<sup>th</sup> day and compared through ELISA, with pre-immune sera used as a negative control. Immunogenic response revealed that the IM route is better than the IP route (data not shown). A different set of experiments regarding the effects of different adjuvants on antibody titer was done *via* the IM route in mice. The IgG antibody titer was measured by ELISA in each pooled sera at various dilutions. A significant ( $p < 0.05$ ) value of IgG antibody against recombinant BoNT/B LC was observed in mice of the aluminium hydroxide group (2,048,000), followed by the Titermax Classic group (512,000), the Freund’s group (128,000), the Titermax Gold group (128,000), and the rBoNT alone group (8,000), when compared with their respective group’s pre-immunized sera. The comparison of different adjuvant group’s antibody titer is depicted in Fig. 6. No cross-reactivity was observed when testing with the recombinant light chain of BoNT/A and E. Similarly, other closely related clostridia showed no cross-reactivity (data not shown). The IgG antibody titer in rabbits was 512,000 with alum as the adjuvant. The developed high-titer antibodies raised with different adjuvants were able to detect recombinant protein BoNT/B LC in the range of approximately 15–244 ng/ml and the data are tabulated in Table 1.



**Fig. 6.** Comparative evaluation by ELISA of recombinant BoNT/B LC specific IgG antibody titers in immunized BALB/c mice with different adjuvants. Five groups of animals were formed and each group contained 5 mice. Mean IgG titers of different adjuvants group with respect to their respective pre-immune sera were compared. The cut-off value for assay was calculated as the mean specific optical density plus three times the standard deviation (SD) for pre-immune serum assayed at a dilution of 1:1,000. Here, the graph represents the end point titer values of each group. The groups were compared statistically using the *t*-test. \*= $p < 0.05$ , when compared with pre-immune sera.

**Table 1.** Comparative evaluation by ELISA of minimum detection limit of recombinant BoNT/B LC with sera raised with different adjuvants.

Sera No.	Adjuvants	Detection limit (ng/ml)
1	Alum	15
2	Titermax Gold	61
3	Titermax Classic	122
4	FCA/FIA	244
5	No adjuvant (rBoNT/B LC alone)	312

Minimum detection limit of rBoNT/B LC with sera raised with different adjuvants were compared. The cut-off value for assay was calculated as the mean specific optical density plus three times the standard deviation (SD) for antigen blank.

## DISCUSSION

BoNTs can be used as potential bioterrorism agents and therefore pose a great threat to national security and public health. An outbreak of botulism constitutes a medical emergency that requires the prompt provision of the botulinum antitoxin and often mechanical ventilation. As botulism is a life-threatening disease, a rapid diagnosis is required for successful therapy. The high toxicity of BoNTs is challenging for the detection of BoNTs and the diagnosis of botulism. At present, the only method that can be used with confidence to detect toxins is the toxicity test performed with mice. Apart from that, there are several ethical issues involved in using animals for such testing [9]. Although this test is exquisitely sensitive, with detection limit of 1 mouse 50% lethal dose ( $MLD_{50}$ ), which is equivalent to 10–20 pg of neurotoxin/ml, it has a number of drawbacks. It is time consuming, because it requires time for preliminary screening followed by toxin titration and finally toxin neutralization using specific antibodies for serotype identification. In addition, there are personnel hazards associated with the actual injection of the animals. Consequently, a number of alternative rapid *in vitro* assays, which have a similar sensitivity and reliability when compared with the mouse bioassay, are being developed. The need for a more rapid method for the detection of botulinum toxins, and the requirement for the examination of large numbers of samples, may be satisfied using ELISA.

The ELISA methods are faster to perform than the mouse bioassay and have the added advantage of determining the toxin serotype at the same time. Serotype identification has benefits, as patients who have developed antibody resistance to one serotype may avail of the therapeutic benefits of the botulinum toxin in the future with another serotype. The speed and accuracy of the ELISA methods could result in a faster public health response to suspected botulism cases or incidences. Few assays have been described in the literature for BoNT/B [11, 34], that approach the required sensitivity for low level detection. Electrochemiluminescence

detection offers a relatively better sensitivity than ELISA owing to high luminescent signal-to-noise ratios [23]. However, expensive instrumentation is a major limitation for the further application of the study. Simplicity, rapidity, and the ability to examine large numbers of samples all make ELISA the method of choice for detection where high-titer antibodies are available. Moreover, the ease of operation makes lateral flow assays (LFAs) amenable for large-scale or presumptive test screening and can be effectively used by nontechnical staff for the screening of food samples [30]. In LFAs, high-titer antibodies are also highly desirable as they are economical for mass production.

To the best of our knowledge, no studies have been reported that use synthetic genes of BoNT/B LC for the development of antibodies and then enhance the antibody titer using different adjuvants. In the present study, we attempted to obtain high yields of pure rBoNT/B LC without the need for working with large quantities of botulinum neurotoxins by synthesizing BoNT/B LC *via* the PCR overlapping primer method. Since the expression of recombinant BoNT/B LC was carried out in *E. coli* M15 cells as the heterologous host, the method is easily amenable to an inexpensive scale-up. Previously, a 1,323 bp fragment of a synthetic gene of BoNT/B LC was constructed and cloned into pET 24a+ [16]. Clones were induced at  $OD_{600}$  0.6 by adding IPTG (final concentration 0.1 mM), incubated for 18 h, and the recombinant protein was purified by ion-exchange chromatography, with recovery of the recombinant protein at 4 mg/g of cell paste, whereas the present study yielded higher amounts of recombinant protein, purified by affinity chromatography, with 4 h of post induction and with a final concentration of 0.5 mM IPTG at  $OD_{600}$  0.8. Immunization *via* two routes in mice suggested that the intramuscular route was a little better than the intraperitoneal to raise antibodies. The results clearly indicate that there is a marked difference with different adjuvants on antibody titer. As of 2010, no comprehensive overview and comparative study of adjuvants is available [17] and limited information is available on the mode of action of adjuvants, which leads to their development on an empirical basis. However, the results of this study clearly highlight how the adjuvants affect the antibody titers. Interestingly, the performance of the adjuvants can depend on the antigen, the specificity of the antigenic determinants, the physical nature of the antigen, and the strain of mouse used for raising the antibody [8].

This paper describes the expression of rBoNT/B LC in *E. coli* M15 cells using a synthetic gene to produce a highly pure 52.8 kDa protein and the effects of adjuvants on antibody titer. The marked IgG responses in mice and rabbits immunized with BoNT/B LC support the immunogenic nature of the recombinant botulinum light chain and suggest that these may be used as good immunogens for diagnostic and vaccine studies. The BoNT serotypes exhibit 30–60% identity [22]. However, serotype-specific antisera have been

reported to elicit little or no cross-reactivity [25]. The antibodies used in the assay demonstrated no significant cross-reactivity against the other serotypes B and E synthetic light chain and no cross-reactivity to any other closely related clostridia. In the present study, we were able to detect rBoNT/B LC as low as 15 ng/ml with antibodies raised with alum as the adjuvant, using an ELISA. This is less sensitive than the detection limit of the mouse bioassay, but may be capable of detecting the toxin concentrations that can cause botulism disease symptoms in humans. The detection limit may be further increased by using monoclonal antibodies. However, here our purpose of study was mainly to evaluate the immunogenicity and the effects of adjuvants on antibody titer for synthetic constructs of BoNT/B LC. The present study will provide an insight into the field of antibody development. This simple experiment can be utilized for the raising of high-titer antibodies in an economic fashion, and it is hoped this will further assist in the development of a low-cost detection system.

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