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Development of immunodetection system for botulinum neurotoxin type B using synthetic gene based recombinant protein

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***Background & objectives*: Botulinum neurotoxins (A-G) are among most poisonous substances in the world, produced by obligate anaerobic bacteria *Clostridum botulinum*. Among the seven serotypes A, B, E and F are of human importance. In India, the prevalence of *C. botulinum* as well as botulism outbreaks have been reported. Due to its extreme toxicity it has been classified in the Category A of biological warfare agent. So far, there is no commercial detection system available in India to detect botulism. The present study aims to develop an immuno detection system for botulinum neurotoxin serotype B using synthetic gene approach.**

***Methods*: The truncated fragment of the botulinum neurotoxin type B from amino acid 1-450 was synthesized using PCR overlap primers; the constructed gene was cloned in the pQE30UA vector and transformed to *Escherichia coli* SG 13009. The recombinant protein expression was optimized using various concentration of isopropylthiogalactoside (IPTG) induction, further the expression was confirmed by Western blot analysis using anti-His antibody. Recombinant protein was purified under denatured condition using Ni-NTA affinity chromatography. Antibody was generated against the recombinant protein using alum adjuvant in BALB/c mice and tested for cross reactivity with other serotypes of *C. botulinum* as well as closely related clostridia. An ELISA test was developed for the detection of botulinum neurotoxin and the minimum detection limit was also estimated.**

***Results*: The recombinant protein was expressed at maximum yield at 4.3 h of post-induction with 0.5 mM IPTG concentration. The recombinant protein was purified using Ni-NTA affinity chromatography up to the homogeneity level. The polyclonal antibodies were raised in mice with a titre of 1:2048000. The developed antibody was highly specific with a sensitivity of detecting approximately 15 ng/ml of recombinant protein and not showing any cross-reactivity with other serotypes.**

***Interpretation & conclusions*: There is no commercial immunodetection system available in India to detect botulism. The developed detection system is highly specific. It will be useful for growing food industry to detect botulinum neurotoxin in food samples as well as in clinical samples.**

Key words BoNT/B - botulism - ELISA - mousebioassay

Botulism is a neuroparalytic disease caused by obligate anaerobic bacteria *Clostridium botulinum*, which produces seven type of antigenically different toxin (A-G). Botulinum neurotoxins (BoNTs) are released as 150 kDa single polypeptide which are cleaved endogenously or exogenously resulting

into a 100 kDa heavy chain (HC) and 50 kDa light chain (LC) linked through a disulphide bond[1]. The C terminal of HC binds to the nerve membrane and its N terminal facilitate the transport of light chain across the membrane of endosome to cytoplasm. The zinc endopeptidase catalytic domain of the toxin resides in the LC that cleaves one or two out of three SNARE (soluble N-ethylmaleimide factor attachment protein receptor) involved in docking and fusion of acetylcholine containing vesicles to plasma membrane[2,3]. Inactivation of any SNARE protein results in inhibition of acetylcholine secretion at the neurotransmitter junctions resulting in flaccid muscle paralysis[2,4]. BoNT A, C, E cleaves synaptic protein (SNAP-25)[5-7], BoNT C also cleaves syntaxin[8,9] and BoNT B, D, G and F are specific for VAMP/synaptobrevin[10-12]. BoNT is the most toxic substance presently known[13]. Due to its extreme toxicity, relative ease of production, easy transport and need for prolonged intensive care among affected persons BoNT as a bioterror agent can evoke maximum fear in general public[14].

The first food borne outbreak of botulism in India was reported[15] in rural Gujarat resident school. Of the 310 students, 34 developed symptoms, two died and the remaining recovered back to normal after treatments which was mainly caused by *C. butyricum.* In 2004, another food borne botulism case were reported in Ram Maohar Lohia hospital, New Delhi, in which the whole family developed symptoms of botulism within 48 h after eating canned meat products[16]. Similar case was also reported from three year old boy admitted in KG hospital, Coimbatore[17]. Prevalence of proteolytic *C. botulinum* type B from Indian tropical fish was reported in 1990[18]. Occurrence of *C. botulinum* in fresh and cured fish in retail trade in Cochin was also reported with a prevalence around 19 per cent[19]. In fresh retail fish most of the samples were found positive for serotype A, B, and D. Genetic diversity among toxigenic clostridia has also been reported[20]. The spores of type E were isolated from soil of Gwalior[21].

Mouse bioassay is considered as the gold standard for detection of botulism[22]. However, there are several shortcomings associated with mouse bioassay, mice can die non specifically during the process, this test takes 4 days to get the final results and it is intensive, requires animal facility and highly experienced and immunized person to perform the study. Moreover, mouse bioassay is not suitable for routine detection, quantification of samples and cannot meet the extent of real biodefence deployment since a large number of animals is required to get statistically significant results. In addition, there are several ethical issues of using animals for such testing in large number of samples[23]. Several new methods have been evolved to detect BoNTs; among those ELISA has been considered as one of the sensitive, easy and amenable methods to develop a high throughput system. Since there is no indigenous detection system available in the country, there is a need to develop an in-house system to detect botulism in food and clinical samples. The present study was therefore, aimed to construct the synthetic gene of truncated fragment of *C. botulinum* type B toxin gene, clone and express the recombinant protein, raise antibody against the recombinant protein and to develop an immuno detection system for botulinum neurotoxin type B.

Material & Methods

All the work was carried out in the biotechnology division of Defence Research and Development Establishment (DRDE), Gwalior.

Synthetic gene construction and cloning: The catalytic domain of type B botulinum toxin gene sequence position 44853-46192 was selected from the Genbank (Accession no: CP 000940). Primer was designed and commercially synthesized (BiotechDesk, Hyderabad) to construct fragment of synthetic gene of BoNT/B. The synthetic gene fragment of BoNT/B was constructed using PCR by overlapping primers[24]. The catalytic domain was PCR amplified using primers BoNT syn F 5'-TCAGTTACAATAAATAATTTT-3' and BoNT syn R 5'-ATCAACATCAATACATATTCC-3'. All the PCR reactions were carried out for a initial denaturation at 94ºC for 5 min followed by 30 cycles consisting of denaturation at 94ºC for 30 sec, annealing for 60 sec at 48ºC, and extension at 72ºC for 30 sec, then final extension at 72ºC for 6 min. The complete synthetic gene was sequenced to check the mutations. Then the PCR product was purified using 1 per cent low melting point agarose gel electrophoresis and the DNA was extracted using QIAquick gel extraction kit (Qiagen, Germany). The PCR product was cloned to the pQE30UA vector using QIA express UA cloning kit (Qiagen, Germany) according to manufacturer's protocol. The ligated products were transformed into chemically competent *Escherichia coli* SG 13009 (Qiagen, Germany) cells by heat shock method. The transformants were selected on Luria broth (LB) agar plates supplemented with kanamycin (30 µg/ml) and ampicillin (100 µg/ml). Plasmids were extracted from

the clones using QIA prep spin miniprep kit (Qiagen, Germany) following manufacture's protocol. Further, these plasmids were screened for the confirmation of presence of inserts using BoNT/B synthetic gene specific primers mentioned above and also checked inframe using the combination of BoNT/B specific and PQE 30 UA vector specific primer.

Recombinant protein expression and localization: The selected transformant was inoculated into 5 ml LB containing 30 µg/ml kanamycin and 100 µg/ml ampicillin and grown overnight at 37ºC with shaking at 200 rpm. The overnight grown cultures were further inoculated in 20 ml LB broth containing respective antibiotics. The cultures were grown at 37ºC at 200 rpm. Growth was monitored by absorbance measurements (OD_{600}). Approximately at OD_{600} 0.8 to 1.0 the recombinant protein expression was induced with 0.5 mM isopropylthiogalactoside (IPTG) and further growth is resumed for 4.30 h under the same conditions. Prior to IPTG induction, 2 ml aliquots were was taken out aseptically to be used as uninduced control. After induction at every one hour interval 2 ml samples were drawn and cells were harvested by centrifugation at 8000 × *g* for 10 min at 4ºC. Each sample was analyzed by 12 per cent SDS-PAGE using molecular weight marker (Fermentas SM 0671, Canada). Localization of recombinant protein was done by solubilizing the induced pellet in phosphate buffer (50mM NaH_2PO_4, 100mM NaCl, *p*H 8.0). The resuspended pellet was then sonicated on ice and centrifuged at 10,000 x *g* for 10min at 4ºC. The supernatant and pellet both were analyzed by 12 per cent SDS-PAGE.

Preparation of inclusion bodies: The induced cells were harvested at 8,000 × *g* for 10 min and the cell pellet was resuspended in lysis buffer (20 mM Tris, pH 7.5) with a mild nonionic detergent triton X100, at the ratio of 5 ml of the lysis buffer per gram of cell pellet with addition of 5 µg/ml deoxyribonucleaseI and a protease inhibitor cocktail. The suspension was incubated at room temperature for 15 min and centrifuged at 10,000 × *g* for 15 min to pellet the insoluble material. Pellet was resuspended in another 5 ml of lysis buffer and 1 mg/ml lysozyme was added to these suspensions. This mixture was incubated at 25ºC for 15 min. Further the cell suspension was sonicated at 9.9 pulses on/off for 5 min and centrifuged at 12000 × *g* for 30 min at 4ºC. Inclusion body pellet was then washed two times with same lysis buffer and centrifuged at 10,000 × *g* for 10 min at 4ºC. Pellet was resuspended in solubilizing/ equilibration buffer (8M urea, 0.1M NaH_2PO_4 and 0.01M Tris-Cl, *p*H 8.0) and left for overnight and centrifuged at 12000 × *g* for 20 min. The supernatant contains the solubilized inclusion bodies.

Purification of recombinant BoNT/B: Ni-NTA affinity column (Qiagen, Germany) equilibrated with solubilising buffer (8M urea, 0.1M NaH_2PO_4 and 0.01M Tris-Cl, *p*H 8.0) was incubated with cell lysate containing beta-mercaptoethenol (β-ME) to a concentration of 15 mM and triton upto 1 per cent. Column was left for 30 min at room temperature with lysate and in between Ni+-NTA slurry was mixed gently to allow binding. Flow-through was collected. Column was first washed with 10 volumes equilibration buffer and then with 10 volumes of wash buffer (8M urea, 0.1M NaH_2PO_4 and 0.01M Tris-Cl *p*H 6.3) containing triton to a concentration of 2 per cent. Final wash was given with 10 volumes of wash buffer having ethanol to a concentration of 15 per cent. Protein was eluted with elution buffer (8M urea, 0.1M NaH_2PO_4 and 0.01M Tris-Cl *p*H 4.5). Fractions were collected and their purity was checked by SDS-PAGE. Finally urea was removed by dialysis against elution buffer with reducing urea concentration at every hour by 2M. Finally the protein was obtained in Tris buffer of *p*H 8.0 and was concentrated using an Amicon ultra cell with 10 kDa cut-off membrane. The protein concentration was determined using BCA protein assay kit (Sigma, USA).

Western blot analysis: The recombinant BoNT/B is tagged with His6 fusion protein, to confirm the presence of rBoNT/B, Western blot analysis was carried out using anti-His as the primary antibody. The purified recombinant BoNT/B from SDS PAGE was transferred to nitrocellulose membrane. The membrane was blocked overnight at 4ºC in 3 per cent BSA. Membrane was incubated in a 1:1000 dilution of mouse anti-His-monoclonal antibody in blocking buffer with gentle shaking at room temperature for 1 h. After washing with PBST (PBS containing 0.5% Tween-20) three times for 5 min each, the membrane was incubated in a 1:2000 dilution of rabbit anti-mouse IgG horseradish peroxidase (HRP) conjugated as secondary antibody (Dako, Denmark) in blocking buffer, with gentle shaking at room temperature for 1 h. The colorimetric detection was carried out by using 3,3'- diaminobenzidine in PBS containing 8.8 mM H_2O_2 as a substrate, after the membrane was washed with PBST for three times.

Antibody generation: 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. BALB/c mice approximately (16-22g) were selected for immunization, an intra muscular immunization (IM) into claudal thigh muscle with 100ul per mouse as total injection volume. Two sets of five BALB/c mice each were primed on day 0, 14 and 21 with 30 µg of purified recombinant protein per mouse emulsified aluminium hydroxide gel as an adjuvant. Mice were bled through retro-orbital puncture with glass capillary tubes prior to first dose and 7 days after the last dose. Blood was incubated at 37ºC for 30 min. Hyper-immune serum was collected after centrifuging blood samples at 3000 × *g* for 10 min to remove residual blood cells. Serum was stored at -20ºC till further use.

Indirect enzyme-linked immunosorbent assay (ELISA): To determine immunogenicity of recombinant BoNT/B, the presence of serum immunoglobulins specific to recombinant BoNT/B was determined by indirect ELISA. The purified recombinant BoNT/B was diluted to 5 µg/ml in carbonate buffer (0.05M, *p*H 9.6) and used to coat the wells of polystyrene plates (100 µl/well; Nunc-Immuno plate with Maxisorp surface). The plate was incubated overnight at 4ºC and washed three times with PBS-T. Blocking was done with 3 per cent BSA in PBS and incubated for 2 h at 37ºC. Plates washed three times with PBS-T and incubated with specific two-fold dilutions of pre-immune and hyper-immune serum (100 µl/well) in triplicate wells at 37ºC for 1 h. Washed five times with PBS-T, then the plate was incubated at 37ºC for 1 h with HRP-conjugated anti-mouse IgG (1:2000 dilution); (Qiagen, Germany). Washed five times with PBS-T before developing ELISA with 100 µl ortho-phenylenediamine (Sigma, USA) (0.4 mg/ml) in freshly prepared citrate phosphate buffer (0.1M, *p*H 5.0) and H_2O_2 (0.4 µl/ml). The reaction was terminated by addition of 50 µl of 2.5N H_2SO_4/well. The absorbance was read in the Microtiter plate reader (BioTek, USA) at 492 nm and 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 1:1000. The titre of hyper-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 antibody with different serotypes was evaluated by coating 5 µg of recombinant light chain of BoNT/A and E. Also cross-reactivity for other clostridial species was checked by coating the wells with 10 µg of the trichloroacetic acid (TCA) precipitated culture soup of *C. sporogenes* ATCC-11437, *C. perfringens* ATCC-13124, *C. Sordellii* ATCC-9714 (from ATCC, USA), *C.tetani* 49205 (from CRI, Kasauli), C. *butyricum*, and *C. subterminale* (from culture collection Biotechnology Division, DRDE, Gwalior). All the *Clostridium* culture and toxins were handled with utmost safety in BSL-3 biosafety cabinet.

Sandwich enzyme-linked immunosorbent assay (ELISA): To determine the minimum detection limit the raised recombinant BoNT/B specific antibody was diluted to 20 µg/ml in carbonate buffer and was used to coat the polystyrene wells (100 µl/well) in triplicates and incubated overnight at 4ºC. After washing the plate three times with PBST, the remaining sites of absorption were blocked by addition of 200 µl/well 3 per cent BSA (made in PBS) and incubated for 2 h at 37ºC. Then the plate was washed three times with PBST. Purified recombinant BoNT/B (100 µl/well) in two-fold serial dilution (1000 µg to 7.5 ng) was added to the blocked antibody coated plate and incubated for 1 h at 37ºC. The plate was washed three times. Again the plate was incubated with raised BoNT/B specific antibody (100 µl/well) of dilution 20 µg/ml (made in PBS) at 37ºC for 1 h. Washed five times with PBST and further incubated at 37ºC for 1 h with the HRP-conjugated anti-mouse IgG (1:2000). Again washed five times with PBST before developing ELISA. The ELISA was developed in the same way as stated above and the cut-off value for assay was calculated as the mean specific optical density plus three times the standard deviation (SD) of the well having no antigen.

Results

Cloning, expression and purification of recombinant protein: The constructed recombinant BoNT/B synthetic gene was cloned in PQE30UA vector and transformed in *E. coli* SG13009 cells. The recombinant transformant was selected by ampicillin and kanamycin marker. From the selected transformant, plasmid was isolated and checked for the presence of insert using BoNT/B forward and reverse primers. The synthetic gene was sequenced and aligned with the nucleotides of light chain of standard strain of *C. botulinum* type B (CP 000940). There was no ambiguity observed in the synthetic gene, it was perfectly matching with the standard sequence. The recombinant protein expression conditions was optimized the maximum yield of recombinant protein was obtained at OD_{600} 0.8 with 0.5 mM IPTG induction with minimum incubation of 4.30 h after induction. The cells were harvested and localized for the release of recombinant protein and

the results reveal that recombinant protein was present as inclusion bodies. Purification was carried out under denatured conditions and the protein was purified near to homogeneity (Fig. 1). The protein was dialyzed to remove urea and concentrated to 1 mg/ml. The purified recombinant protein was confirmed by Western blot analysis using anti-His antibody (Fig. 2).

Antibody generation: The hyperimmune serum was raised in mice using the recombinant protein of BoNT/B which was purified under denatured conditions up to 98 per cent purity (based on desitometry analysis). The polyclonal antibody was generated and the IgG was purified from the serum and titre was estimated using plate ELISA by coating 5 µg/ml of purified recombinant protein in each well. A titre of 1:2048000 was observed with the cut-off value of >0.127 (OD492 nm) of pre-immunized serum, used as control for assigning the cut-off value. No cross-reactivity was observed when tested with 5 µg of recombinant protein of light chain of BoNT/A and E. Similarly no cross-reactivity was also observed with other closely related clostridia such as *C. butyricum*, *C. subterminale*, *C. sporogenes*, *C. perfringens*, *C. tetani*, and *C. Sordellii.* For these cultures, the culture supernatant was acid precipitated and coated 10 µg of each per well.

Evaluation of detection limit: The recombinant protein was assayed in the concentration ranges from 1 µg/ml to 7.5 ng/ml to determine the limit of detection in the ELISA format (Fig. 3). Recombinant BoNT/B

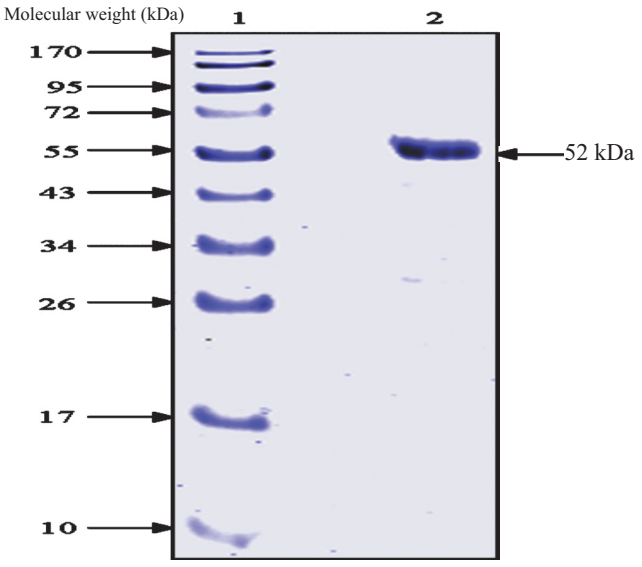

Fig. 1. Affinity purified recombinant protein of BoNT/B. Lane 1: Molecular weight marker; Lane 2: Purified recombinant BoNT/B protein.

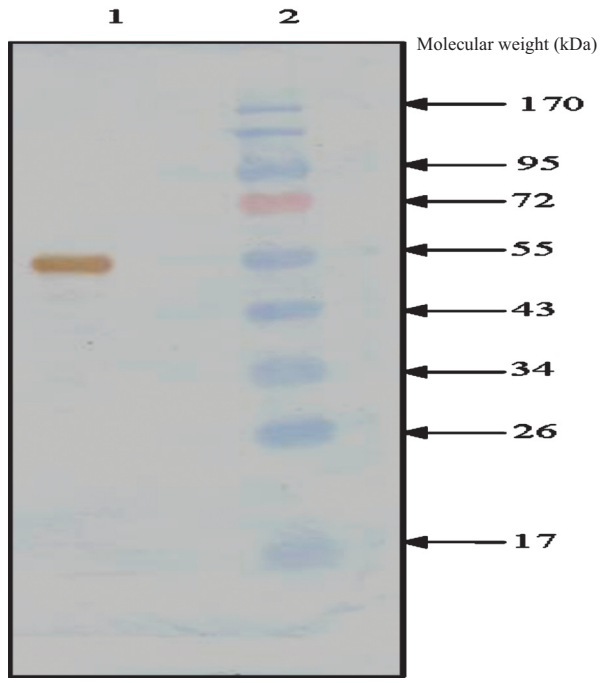

Fig. 2. Western blot of purified recombinat protein of BoNT/B using anti-His antibody dilution of (1:2000). Lane 1: Purified recombinat BoNT/B protein. Lane 2: Molecular weight maker.

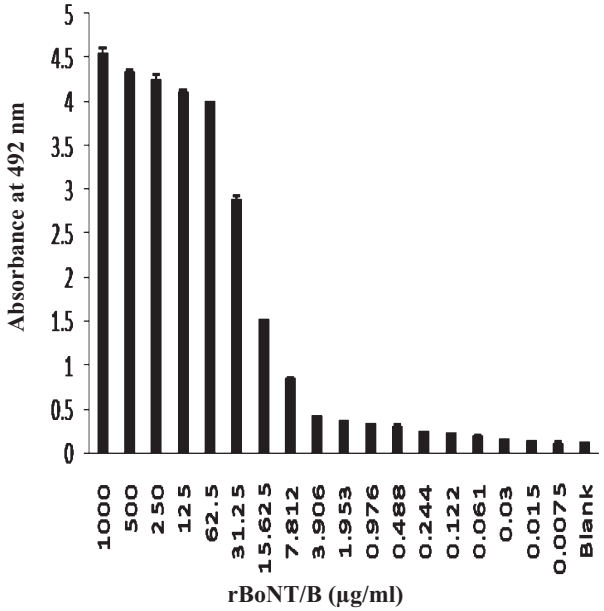

Fig. 3. Minimum detection limit of botulinum neurotoxin type B by sandwich ELISA. OD values represented are means ± SD of triplicate determinations.

specific antibody was used to capture the antigen. Results showed that the recombinant BoNT/B could be detected up to a concentration of approximately 15 ng/ml (mean of triplicate wells). These results were highly

reproducible as when five assays were performed in the single day in separate experiments both accuracy and precision were excellent. There was no deviation from the expected detection limit.

Discussion

Sensitive and rapid detection of botulinum neurotoxins is essential for the detection of poisoned food, as well as for response to potential bioterrorist threats. Currently, the most reliable method for detection of botulinum neurotoxin is mouse bioassay, while this assay is sensitive, it is slow, expensive, has limited high throughput and requires sacrificing the animals. We developed an alternative *in vitro* immuno detection method which can be used to detect suspected botulism cases as well as botulinum neurotoxin in food and clinical samples. Several workers have used a strong promoter of phage T7 and physiological control to improve the level of expression of a synthetic BoNT/B LC as well as codon optimization which limit reduced protein expression associated with rare codons and high AT base composition. The use of a strong promoter and optimized synthetic BoNT/B LC gene resulted in a 50 per cent higher yield of protein expression and/or recovery than previously reported[25-27]. In the present study, truncated fragment of BoNT/B were synthesized by PCR overhang method, cloned in pQE30UA and transformed in *E. coli* SG13009. Expression conditions were optimized, yielded maximum recombinant protein when induced with 0.5 mM IPTG at OD_{600} 0.8 after 4.30 h induction. Similar results were observed when DNA encoding type B *C. botulinum* heavy chain transmembrane binding domain (BoNT/ B HC TBD) was expressed with less non specific proteins at 5 h of induction[28]. Gilsdorf *et al*[29] obtained >12 mg of BoNT/B LC recombinant protein from one litre of culture which was greater than 98 per cent pure based on densitometry analysis of the silver stained gel. Zhou and Singh[28] purified 10 mg of His6-tagged BoNT/B HC TBD from 500 mg of inclusion body from the frozen cell pellet of 1 l culture (4 g bacterial pellet). In our study, >15 mg purified protein was obtained from 1 l culture in 4.3 h.

Mice were injected with purified recombinant BoNT/B with alum as an adjuvant to raise high titre antibody. Similar kind of assay was developed for botulinum like toxins but not for BoNT/B using partially purified and crude toxins and an antibody titre of 1:80000 was achieved using indirect ELISA[30]. In the present study, a high titre antibody of 1:2048000 was obtained. The raised antibody did not show any cross-reactivity with other closely related clostridia as well as with recombinant protein of *C. botulinum* serotype A and E. The capture antibody raised against recombinant BoNT/B could detect rBoNT/B up to a concentration of approximately 15 ng/ml. The developed ELISA systems are highly specific, rapid and can be applied to the testing of a large number of specimens. Various ELISA formats have been developed utilizing fluorescent or chemiluminescent substrates to improve assay sensitivity 10-100 fold but require more specialized equipment[31]. Several rapid, sensitive, and specific tests like real-time PCR, immune PCR, *etc*., have been developed for detection of *C. botulinum* cells as well as the neurotoxin genes. Joshy *et al*[32] described a multiplex PCR for the simultaneous detection of botulinum neurotoxin and perfringens toxin genes. These methods are ultrasensitive in pure culture based studies but when it comes to the food and clinical samples these techniques fail to detect due to the presence of PCR inhibitors. It requires expertise to clean up the samples to get true positive results.

In conclusion, we developed an immunodetection system for botulinum reurotoxin based on the expression of BoNT/B in *E. coli* using an optimized synthetic gene to produce a highly pure, 52 kDa protein. The growth, induction conditions and the recombinant expression were optimized to obtain this protein to raise high titre antibody which will be highly useful for the detection of suspected cases of botulism as well as to detect botulinum neurotoxin in food and clinical samples. In addition, working with large quantities of botulinum neurotoxin poses a health risk. To avoid these problems as well as to obtain high yield of recombinant protein synthetic gene approach was used which will further help to develop a low cost detection system.

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