ELISA Based Detection of Botulinum Neurotoxin Type 'F' in Red Meat and Canned Fish

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ABSTRACT

Botulinum neurotoxin (BoNT) is the most toxic protein molecule so far known to animals and humans, due to its extreme toxicity, CDC has been listed as category 'A' biological warfare agents. In India, there is no commercial detection system available for the detection of botulism. The present study was aimed to develop an ELISA based detection system for botulinum neurotoxin serotype F in red meat and canned fish. Polyclonal antibodies were generated against the recombinant BoNT/F LC protein and IgG was purified. Sandwich ELISA system was successfully optimised with LOD of ~ 7.8 ng/ml in PBS. Similarly, different concentrations of BoNT/F were spiked in red meat and canned fish and extracted the toxin and the LOD was achieved for red meat ~ 62 ng/ml and for canned fish ~ 31 ng/ml. The developed detection system is highly specific. The developed assay will be useful for the screening of botulinum toxins in a large number of food samples.

Keyword: BoNTs; Warfare agent; Botulism; Detection; Botulinum toxins

1. INTRODUCTION

Botulism is a neurological illness caused by botulinum toxins. BoNT is produced by obligate anaerobic bacterium *Clostridium botulinum*. BoNT is the fatal substances of biological origin so far known to the world. Therefore it was listed as category 'A' Biological warfare agents by the Center for Disease Control and Prevention (CDC)[1]. Out of seven serotypes (A–G), type A, B, E and F are involved in human cases[2]. BoNT is synthesised as 150 kDa protein and it includes 100 kDa heavy chain (Hc) connected with 50 kDa light chain (Lc) through a covalent bond (S-S). The complete BoNT intoxication occurs through receptor binding, followed by BoNT internalisation, translocation of the catalytic domain with the help of heavy chain and finally the cleavage of SNARE proteins by the action of endopeptidase activity of light chain leads to flaccid paralysis[2-4]. *Clostridium botulinum* spores are naturally present in soil, dust, sediments, fresh water, and foods. Favorable conditions such as obligate anaerobic environment, pH and nutrients support the germination of spores into vegetative cells followed by the production of toxins in situ in different canned foods. The ingestion of 0.1 g BoNT contaminated food will be sufficient to cause the intoxication and death[5].

They are three type of botulism: food born botulism, infant botulism and wound botulism. Mostly, the food borne botulism cases are reported due to the consumption of home canned meat and sausages[6,7]. Lack of unhygienic conditions during the course of preparation of home canned foods leads to contamination of *C. botulinum* and the anaerobic conditions of home canned foods facilitate the production of BoNTs.

Inappropriate food preparation processes and the storage temperature conditions are the common reasons for the contamination of BoNT-producing *Clostridial* spores[8-11]. The temperature abuse of the home canned foods while transportation and storage facilitate the production of BoNTs in canned foods[12]. Recent CDC report stated that 53 per cent of botulism cases were identified from outbreaks and 47 per cent of cases were identified through epidemiological investigations[13]. BoNT type A and B are mainly accounted in food borne outbreaks by the consumption of canned meat and pickles made from vegetables of different origin. Similarly BoNT type E is mainly reported by the consumption of fishery products. In 2006, a major food borne botulism outbreak was reported in Thailand, and approximately 163 people were affected due to the consumption of home canned bamboo shoots[14]. Food borne botulism can still be considered as an emerging disease.

The standard mouse bioassay[15] is routinely used for the detection of botulinum toxins in food and clinical samples. This method is very sensitive and specific, but this assay require a huge number of animals. Hence, it is necessary to replace the mouse bioassay with similarly sensitive another assays[16-18].

Continuous research is in progress for development/ improvements in botulinum toxin towards detection systems, food defense and public health preparedness. Due to the lack of availability of the commercial detection system in India, there is an urgent need to develop a system for the diagnosis of botulism. The present study was aimed to detect botulinum neurotoxin serotype 'F' in red meat and canned fish samples.

2. MATERIAL AND METHODS

2.1 Antibody Development and IgG Purification

The recombinant plasmid of BoNT/F LC was sub cultured from the glycerol stock and plated on an LB agar plate containing 30 mg /ml kanamycin, and subsequently inoculated in sterile terrific broth media and grown up to 0.6 OD at 600 nm. Clones were induced with 0.75 mM IPTG and incubated at 21 °C for 6 h for the production of rBoNT/F LC protein. After expression, the recombinant BoNT/F LC protein was successfully extracted using affinity column chromatography (Ni-NTA). The purified rBoNT/F LC protein was used for immunisation in Mice and Rabbit[19].

The IgG was purified from the generated polyclonal antibody against rBoNT/F LC protein. A Antibody Purification Kit (Sigma, USA). Briefly, the hyper immune serum of rBoNT/F LC was clarified and filtered through 0.45 µm filter prior to the purification with Protein A cartridge. Two ml of sera was taken followed by the addition of 4 ml of binding buffer and mixed thoroughly by vortexing. The desalting and Protein A column was regenerated with HEPES buffer and regeneration buffer respectively and further the Protein A cartridge was equilibrated with binding buffer. The serum sample-binding buffer mixture was loaded to the Protein A column (0.5 ml/min). Further, the column was washed with binding buffer. Finally the IgG was eluted using elution buffer (flow rate of 0.5 ml/min). The purity of the IgG was analysed using SDS-PAGE and quantified through Bradford protein assay (Bio-Rad, USA).

2.2 Sample Preparation and Spiking of BoNT/F in Red Meat and Canned Fish

The solid food matrices such as raw meat and canned fish were purchased from a local market and transported to the laboratory in an Ice box. Approximately two gram of raw red meat and canned fish samples were weighed and placed in the Seward closure strainer micro bags, respectively (BA6040). Ten ml of sterile phosphate buffered saline (PBS) was added to the bags. Samples were homogenised for 30 min using Stomacher (Seward, UK). The pH was measured using pH meter (6.9 for red meat and 6.8 for canned fish) and adjusted the pH to 7.0 ± 0.2 using NaOH.

Different concentrations of recombinant BoNT/F LC protein ranging from 1000 to 3.9 ng/ml of was artificially spiked in raw red meat and canned fish samples. These samples were mixed thoroughly by vortexing and further incubated at 37 °C for 30 min. To remove the solid matrices, samples were subjected to centrifugation (7000 rpm for 30 min). After the removal of the solid matrices from the spiked samples, detection of BoNT/F was attempted using S-ELISA and the LOD was calculated[20].

2.3 Sandwich ELISA

Rabbit anti-rBoNT/F LC IgG (2.5 µg/ml) was used in the coating buffer, approximately 100 µl/well in triplicate were coated on the polystyrene 96 well plate and further kept for 12 h at 4 °C. The plate was washed multiple times with PBS and PBST and blocked for 1 h at 37 °C using skimmed milk (5 per cent). Further, the plate was washed as described above.

Different concentration of artificially spiked samples (100 µl/well) were added directly to the plate and kept at 37 °C for 1 h followed by washing. Plate was incubated with mice anti-rBoNT/F LC IgG (2.5 µg/ml) for 1 h at 37 °C and washed as mentioned above. Plate was incubated with HRP-conjugated goat anti-mice IgG at a dilution of 1:10,000 (Sigma, USA) at 37 °C for 1 h. After washing, the plate was finally developed with TMB substrate for 15 to 20 min followed by the addition of 2.5N H_2SO_4 to stop the reaction. OD450 nm was measured using 96 well plate reader (BioTek, USA). The cut-off value for the developed method was calculated using mean specific OD + 3SD of the blank. The concentration of the rBoNT/F LC protein at which the absorbance value was two times the cut-off value (A 450 nm using the well having no antigen) was considered to be the ELISA endpoint (detection limit)[17].

3. RESULTS

3.1 IgG Purification

Since the serum contains some of the nonspecific proteins which may interfere with the sensitivity of the assay. The protein A binds to the Fc portion of IgG antibodies and the nonspecific protein will be removed through washing. The purification results revealed that, elute showed two prominent bands one of 50 kDa of the heavy chain and the other 25 kDa of the light chain and the results are as shown in Fig. 1.
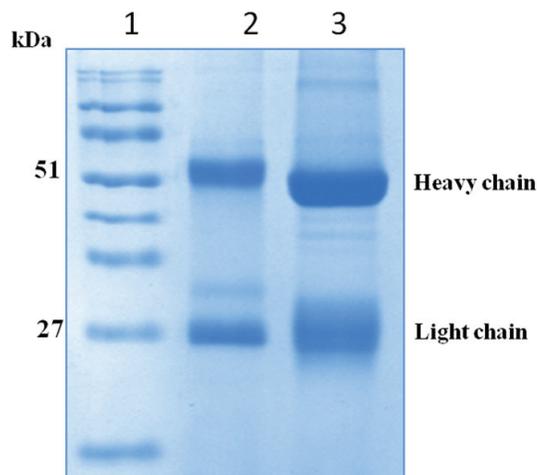

Figure 1. SDS-PAGE profile of purified IgG. Lane 1. MW marker. Lane 2. Purified Mice IgG. Lane 3. Purified Rabbit IgG.

3.2 Detection of BoNT/F in PBS Buffer and Food Matrices

The recombinant BoNT/F LC protein was spiked in PBS, raw meat and canned fish samples. Detection sensitivity of the sandwich ELISA was tested in these matrices using rBoNT/F LC protein in the concentration range from 1000 to 3.9 ng/ml.

Standard graph for BoNT/F was generated using sandwich ELISA in PBS buffer, red meat and canned fish. In the case of PBS, the maximum mean OD of 3.15 was observed at a concentration of 1000 ng/ml. The OD gradually decreased with respect to the concentration of BoNT/F. A 500 ng/ml of
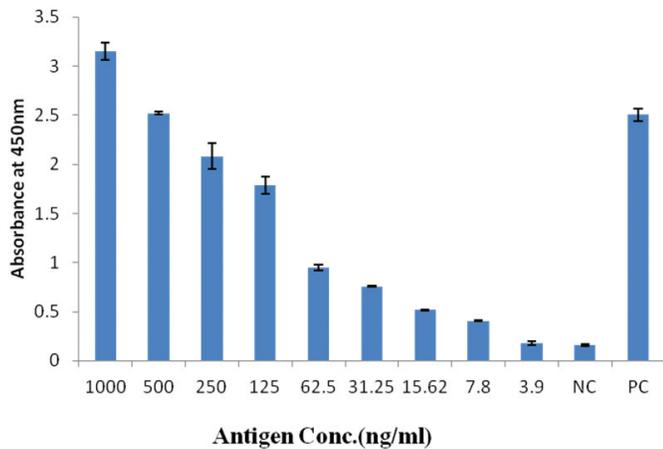
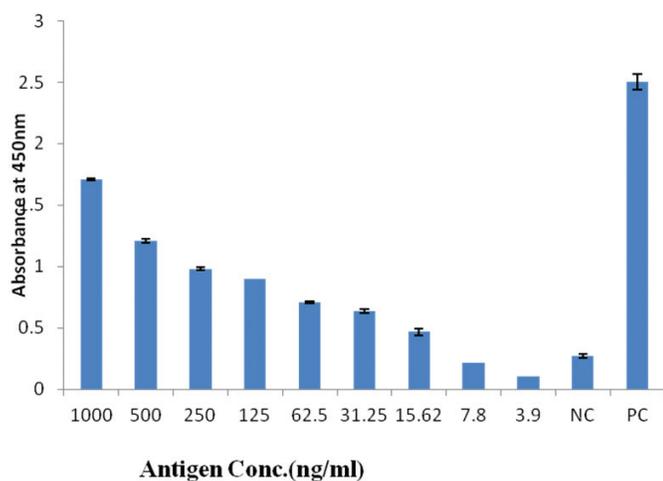**Figure 2. The LOD of rBoNT/ F in PBS buffer. NC: Negative control, PC: Positive control.**

Figure 3. The LOD of BoNT/ F in red meat. NC: Negative control, PC: Positive control.
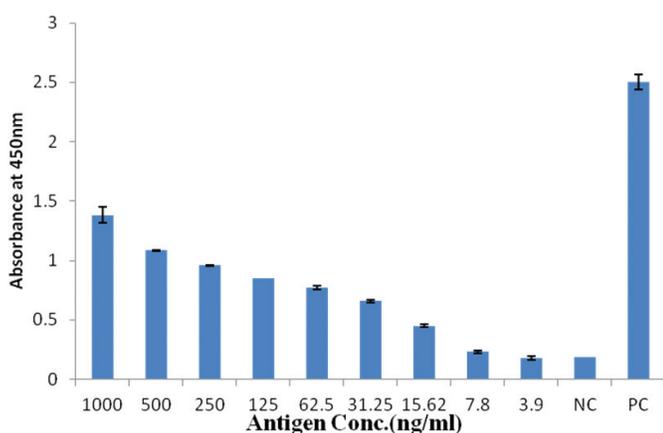

Figure 4. The LOD of BoNT/ F in canned fish. NC: Negative control, PC: Positive control.

rBoNT/F LC protein was used as positive control and PBS was used as negative control. From the standard graph, the limit of detection was ~7.8 ng/ml for PBS as shown in Fig. 2.

In the case of BoNT/F spiked in red meat, the maximum OD of 1.72 was observed at 1000 ng/ml and the OD gradually decreased to 0.10 for 3.9 ng/ml as shown in Fig. 3. The limit of detection was found to be ~62 ng/ml for red meat.

In the canned fish samples, a maximum OD of 1.42 was observed at 1000 ng/ml, which gradually decreased to 0.16 for 3.9 ng/ml as shown in Fig. 4. The limit of detection was ~31 ng/ml for canned fish.

4. DISCUSSION

The BoNTs are produced by *C. botulinum* which cause the disease Botulism. BoNT serotypes (A, B, E, and F) are mainly associated with human botulism[20-21]. BoNTs routinely cause food poisoning in canned foods. Besides, due to the extreme toxicity it's used as BTW agent. LD_{50} of BoNTs reported through different routes is 0.3 ng/kg (intravenous), 1000 ng/ kg (ingestion) and 20 ng/min/m^3 (inhalation)[22-23].

Mouse bioassay is the standard assay routinely used for the detection of BoNTs in food with the sensitivity 10 pg to 20 pg. But this assay has few limitations such as it needs large number of laboratory animals and expertise for handling of animals and toxins, and takes about 96 h for completion. In the present study, a simple and sensitive ELISA was developed for the detection of BoNT/F. Due to the non-availability of the clinical/BoNTs contaminated food samples, attempt were made to spike these toxins in different foods and established their detection limits. The selected food matrices such as red meat and canned fish samples are prone to contamination of *C. botulinum* spores, while the canning of these foods facilitates the growth of *C. botulinum* and produces copious amount of preformed toxins. The developed system was able to detect the BoNT/F ~7.8 ng/ml in PBS, ~ 62 ng/ml in the red meat and ~31 ng/ml in the canned fish. The results of present study clearly indicated that the detection limit varies in different matrices. Hence, determination of detection limit in various foods will help in investigation of the food borne outbreaks. BoNTs was determined in different food matrices with LOD of 5 ng/ml for type A and E and 10 ng/ml type B and F using optical immunoassay[24] Digoxigenin labelled antibodies further increased the limit of detection of different BoNTs in the range of 60 -176 pg/ml[17, 20].

In conclusion, we have developed a highly specific sandwich ELISA based detection system for BoNT/F. This assay will be useful for the screening of botulinum toxins in a large number of food samples as well as biodefence perspectives.

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Contribution in the current study, he designed the study and supervised the experiments. Analysed the data and prepared the manuscript.