SUMMARY

The neurotoxins produced by Clostridia species are among the most potent toxins of known so far to humans. Because of their extreme toxicity, botulinum neurotoxins were considered as a category ‘A’ agent of biological weapons of mass destruction. Botulinum neurotoxin has been developed as a biological weapon by many countries, including Japan, Germany, the United States, Russia and Iraq. Botulism is a neuroparalytic disease, most commonly caused by food borne ingestion of preformed neurotoxin types A, B, and E, and is often fatal if untreated. In 1817, the German neurologist Justinus Kerner had provided a detailed clinical description of botulism. The bacterial agent and toxicological mechanism of action responsible for botulism were described by Emile van Ermengem in 1895. Other classical bacteria, like C. butricum and C. baratii can also produce the toxin leading to signs and symptoms of botulism. Though rare the illness is potentially fatal and can masquerade as other illnesses making diagnosis difficult. Physicians need to familiarize themselves with the disease as prompt recognition and early treatment can considerably curtail the fatal outcome in the affected and prevent additional cases in the unaffected patients. New diagnostic therapeutic and preventive modalities to tackle the disease have come into focus botulinum toxin, generally considered a potent poison, is successfully being used for treatment of various neuromuscular disorders representing one of the most dramatic role reversals of modern. Centre for Disease Control and Prevention has been classified the human botulism into four categories: food-borne, wound, infant, and other. Food-borne botulism is caused by ingestion of food or drink containing
preformed botulinum toxin. Often this occurs when canned foods that are contaminated with *C. botulinum* spores are not effectively sterilized. It could also result from intentional contamination toxin to food supply. It is the most common cause of botulism in Europe. Wound botulism is the result of profound wounds or abscesses contaminated with *C. botulinum* spores that provide anaerobic conditions for spore germination then spore germinate and converted into vegetative cells further these cells multiply and produce botulinum toxins interior of the wound, which is then absorbed into the bloodstream. Wound botulism has become more widespread in recent years among intravenous drug users in western America. According to CDC report, Infant botulism is the main form of botulism in the United States. It is the result of eating food contaminated with *C. botulinum* spores, then spores reached into gastrointestinal tract where it germinate and colonize and produce botulinum toxins in the large intestine then toxin absorbed into blood stream. It primarily affects infants (younger than 12 months of age) because *C. botulinum* is easily colonized in infant due to normal bowel florae that could compete with *C. botulinum* have not been fully established. Honey is the greater risk of infant botulism because it contains *C. botulinum* spores so it should be avoided for children.

BoNT is an extremely potent substance that is accountable for the disease botulism. Although botulism is a rare disease in humans and animals, the mortality rate is high without proper treatment. Since of its extreme lethality and potency, BoNT can also be used as a biological weapon, such weapon is used by bioterrorists to create severe civic disruption economic hammering and social anxiety. The hazard of bioterrorism has stimulated rehabilitated efforts to generate vaccines and therapies against BoNTs.

The value of the neurotoxin as a therapeutic drug, vaccines have been and will continue to be an significant line of defense for those who labor with the toxin (at-risk workers) and a pick population of the military, law enforcement, and first responders. The first vaccine used to protect against botulinum neurotoxin was a chemically detoxified extract from *C. botulinum*. A Pentavalent botulinum toxoid (PBT) vaccine in examine today is administered under an Investigational New Drug (IND) application held by the CDC. Recombinant subunit vaccines are in development and a bivalent H(c) vaccine (rBV A/B (Pichia pastoris)) is presently being evaluated in a phase II clinical trial.
All above mentioned vaccines are concerned with botulinum toxin neutralization. But till now there is no vaccine is available against *C. botulinum* which is the primary causal agent of infant and wound botulism. Antibiotics should not be used routinely to treat infant botulism and wound botulism should only be used to treat secondary infections (urinary tract infections, pneumonia and otitis media), because their use may result in the lysis of intraintestinal *C. botulinum* with discharge of additional botulinum toxin. Vaccination is an alternative and safe strategy to prevent primary infection of *C. botulinum* that ultimately produces botulinum toxin result botulism. If primary infection is blocked in this case, either colonization or bacterial clearance, there will be no botulinum toxin production in surrounding environment as a result botulism will not occur. To the best of our knowledge so far there is no licensed vaccine available commercially for botulism. Similarly there is no rapid detection system available to detect botulism. To develop the new generation vaccine as well as diagnostic system against many pathogens, the immunoproteomics approach remains the choice of the research.

Immuno proteomic approach is one of the best tool among available to the study the host pathogen interaction. Researchers used combination of two dimensional gel electrophoresis and immuno blotting with sera from infected animals or human patients with mass spectrometry to find out the immunogenic candidate molecules. Similar approach has been widely used for the discovery of new biomarkers for vaccine development in cancer as well as infectious diseases. The secretary proteins / surface proteins play important roles in the pathogenesis of bacterial infection represent the inter-phase of the bacterium–host interaction. In any pathogens the secretary / surface proteins are exposed to the host immune system and are therefore the primary antigen targets of host immune response. The secretary proteins / surface proteins are important for the development of diagnostics and passive immunotherapies. Reports are available for some Gram positive bacteria using secretory proteins, vegetative cell surface proteins and spore surface proteins can elicit a humoral immune response in the course of bacterial infections but no such report are available with respect to *C. botulinum*. Therefore, in the present study we were selected to identify the predominant immunogenic proteins against the sera of secretome and
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whole-cell proteomic of the *C. botulinum* type *B*. In this study, first time we report the immunoproteomics approach to elucidate the secretome as well as whole-cell proteome of *C. botulinum* type *B* and identified the predominant immunogenic proteins further validated for their potential to be used as a diagnostic marker and vaccine potential against *C. botulinum*. As such there is no commercial detection system available in the market to detect botulism; even laboratory expertise is also not available in the country. It is due to the non availability of standard cultures as well as extreme toxicity of the organism and non availability of prophylaxis agent also.

Towards this end, the sub-objectives of the present investigation were as follows: (1) Isolation and identification of *C. botulinum* type *B*. (2) Elucidation of secretome and whole-cell proteome of *C. botulinum* type *B*. (3) Generation of polyclonal antibodies against secretome, whole-cell proteome and live spores of *C. botulinum* type *B*. (4) Identification of immunogenic proteins of secretome and whole-cell proteome of *C. botulinum* type *B*. (5) Validation of selected candidate molecules for their potential vaccine as well as diagnostic application against *C. botulinum* type *B*. (6) Immune responses against the selected candidate molecule in mouse.

To complete this study, first of all different types of environmental samples such as sediments and decayed fishes from the coastal areas were collected and subjected to the selective enrichment in tryptone peptone glucose yeast extract (TPGY) broth and cooked meat medium (CMM) in serum vials and incubated anaerobically at 37°C for 2 to 3 days. At the end of incubation, 1.5 ml of samples from both the media were withdrawn in duplicate into 2ml micro centrifuge tubes and used for ethanol and heat treatment. The sample after their respective treatment were pour-plated on egg yolk agar plates and incubated at 37°C for 48 to 72 hours in an anaerobic workstation. Morphologically distinct well isolated colonies from egg yolk agar plate were inoculated into TPGY broth and also streaked on egg yolk plate with *C. botulinum* supplements (cycloserine, sulphonmethoxazole and trimethoprim) and incubated anaerobically at 37°C for 24 to 48 hours. The pure cultures were used for the biochemical studies. All the media were prepared anaerobically by the standard methods using gassing minifold and serum vials. The complete battery of biochemical test such as lipase, lecithinase, catalase and proteolytic test were carried out for all the test isolates for the identification of *C. botulinum* type *B*. To identify the positive
isolates of *C. botulinum* type B mouse bioassay test was used. Mouse bioassay is the gold standard for detection of *C. botulinum* toxin. The suspected isolate which belongs to *C. botulinum* were grown in TPYG broth for 5-7 days at room temperature. After seven days, the culture supernatant were separated and subjected to 0.2 mµ ultrafiltration. Approximately 500µl of the culture supernatant were intraperitoneally injected to mice and observed the typical botulism symptoms and death for a minimum of 72 hours. All mouse bioassay positive isolates were subjected to the molecular detection of serotype type B of *C. botulinum* and it was further confirmed by nucleotide sequencing.

For the secretome study of the *C. botulinum* type B, it was inoculated in TPYG and CMM media and anaerobically grown at 37°C for 5 days. After five days culture were centrifuged at 10,000 rpm for 15 minutes and supernatant was collected and precipitated by tri chloro acetic acid. Precipitation was centrifuged at 10,000 rpm for 20 minutes and pellet was collected and air dried. Air dried pellet was dissolved in protein solubilisation buffer for further studies. Similarly for whole cell proteome study, *C. botulinum* type B culture was inoculated in TPYG and CMM media and anaerobically grown at 37°C for 24 hours. Cells were harvested and washed with phosphate buffer saline, pH 7.4. The cells were resuspended in the lysis buffer supplemented with protease inhibitor. Cell lysis was performed by sonication and the un-disrupted cells were removed by centrifugation (10000 × g; 20 minutes; 4°C) and supernatant was collected for the 2D proteomic analysis. Proteins samples (secretome & whole cell proteome) were purified using 2D-cleanup kit and the protein pellet was dissolved in protein solubilization buffer and quantified the protein concentrations by Bradford method. Optimized protein concentration (300 µg for 7 cm and 500 µg for 11 cm strips) was resuspended in sample rehydration buffer (8 M urea, 2% w/v CHAPS, 15 mM DTT and 0.5% v/v IPG buffer pH 3–10). The IPG strips (4-7 to 4.7-5.9) were rehydrated overnight and then the strips were focused. After focusing, the strips were incubated for 10 min, in 10 ml of equilibrium buffer I (6 M urea, 30% w/v glycerol, 2% w/v SDS and 1% w/v DTT in 50 mM Tris/HCl buffer, pH 8.8) followed by equilibrium buffer II (6 M urea, 30% w/v glycerol, 2% w/v SDS and 4% w/v iodoacetamide in 50 mM Tris/HCl buffer, pH 8.8). After the equilibration steps the strips were transferred to 12% SDS-PAGE for the second dimension. Protein spots were visualized by staining with Coomassie Brilliant Blue G-250. Gel images were
captured by densitometer. All the protein spots were picked and processed and subjected to mass spectrometry for the identification of the pre dominant proteins. Both the secretory and whole cell proteins of *C. botulinum* type B separately mixed with Freund's complete adjuvant and Freund's incomplete adjuvant and were injected intraperitonealy to BALB/c mice. After 7 days of post immunization blood was collected through a capillary and serum was checked for antibody titer by indirect ELISA. Antibody endpoint titers of secretory and whole cell proteins in mice were 1:128000.

The secretory and whole cell proteins were subjected to two dimensional proteomic analysis and the proteins were transferred to the nitrocellulose membrane and developed the western blot with the raised antibody against the respective protein to screen the immunogenic proteins. Twenty seven and 20 pre dominant immunogenic proteins were identified in secretome and whole cell proteome respectively. Out of 27-proteins of secretome of *C. botulinum* type B, 17 proteins were immunogenic in *C. botulinum* type B grown in TPGY media. These were Thiamine biosynthesis protein ThiC, Hypothetical protein CLOSPO_00563, Butyrate kinase, Aspartate/ornithine carbamoyltransferase family protein, Glycosyl hydrolase, family 18, Ornithine carbamoyltransferase, Flagellar hook-associated protein 2, ECF subfamily RNA polymerase sigma factor, Hypothetical protein RTM1035_02410, Peptidase T, M24 family metallopeptidase, ATP-dependent Clp protease proteolytic subunit, Phosphoglycerate kinase, FlaA, flagellin, secreted protease and molecular chaperone GroEL and 10 proteins, Hypothetical protein CLOSPO_00563, Molecular chaperone GroEL, Putative cell surface protein, Clostripain, Ornithine carbamoyltransferase, Glycerol dehydrogenase, Phenylalanyl-tRNA synthetase, alpha subunit, FlaA, flagellin and secreted protease were scrutinized as immunogenic in CMM medium. Similarly in whole cell proteome, out of 20 immunogenic proteins, 13 proteins were found out as immunogenic proteins in TPGY grown *C. botulinum* type B. These were Electron transfer flavoprotein, beta subunit/FixA family, Peptilid-prolyl isomerase, Molecular chaperone GroEL, FlaA, Flagellin, Acetyl-CoA acetyltransferase, Ornithine carbamoyltransferase, acetate kinase, Hypothetical protein CBO2470, Aspartate aminotransferase, Conserved hypothetical protein, Hypothetical protein CLOSPO_00563 and Putative cell Surface Protein and 7 proteins, phosphopyruvate hydratase, peptidase T, pyruvate phosphate dikinase, Ruberythrin, D-proline
reductase, PrdA proprotein, molecular chaperone GroEL and molecular chaperone DnaK were scrutinized as immunogenic proteins in CMM grown *C. botulinum* type B.

Similarly whole cell proteins 2DE- gels probed with the mice sera generated against live spores of *C. botulinum* type B and there are following proteins were found as immunogenic, these are hypothetical protein HMPREF9332_00280, flaA, fructose-1,6-bisphosphate aldolase, class II, hypothetical protein CBO2471, flagellar hook protein flgE, 2-ketoisovalerate ferredoxin reductase, 3-oxoacyl-(acyl carrier protein) synthase II and acetate kinase.

Further selected immunogenic proteins were validated to check the immunogenicity as well as protective efficacy. To check the immunogenicity and protective efficacy against the *C. botulinum* type B, two immunogenic proteins were selected; these are ornithine transcarbamylase (OTC) and molecular chaperon GroEL. Primers were designed for the selected candidate molecule of *C. botulinum* type B and these primers were synthesized commercially. Total DNA were extracted from *C. botulinum* type B, the full-length / fragment of the immunogenic protein gene were PCR amplified using specific primers and purified by gel extraction method and were cloned into the pET28a+ vector according to the manufacturer’s instructions. The ligated products were transformed into *E. coli* BL21 (DE3) cells. Plasmids were isolated from 10 randomly selected clones and were tested for the presence of the insert by size determination on an agarose gel (1.5%) and PCR amplification of the target gene. The expression of recombinant proteins (rOTC & rGroEL) were induced for 4 h at 37°C in 500 ml LB medium cultures containing 0.25 mM isopropyl--D-thiogalactopyranoside (IPTG). The over expressed recombinant protein were purified by Ni-NTA affinity chromatography in native condition using imidazole buffer. Immunogenicity of recombinant proteins OTC and GroEL were evaluated in 5–8 week old female BALB/c mice and rabbit. The animals (mice) were taken in 9 groups for each recombinant protein immunization and every group contained 6 mice (6 mice /group). All the animal groups were immunized via intra peritoneal (IP) route. Negative control group (first group)–only PBS injected group, 2 to 9 groups were rOTC (2-5 groups) and rGroEL (6-9 groups) proteins immunized groups. Recombinant proteins immunization schedule was carried out in four doses (prime
dose, 14, 21 and 28 days) of different concentration of recombinant proteins (10, 20, 30 and 50 µg) made in 0.35% alum in sterile phosphate buffer saline, PBS. The animals groups were used for antibody isotyping, lymphocyte proliferation, cytokine profiling, protection study and histopathological studies. Similarly polyclonal antibodies against recombinant proteins OTC and GroEL were also raised in rabbit and immunization schedule was carried out in four doses (prime dose, 14, 21 and 28 days) of different concentration of recombinant proteins (50, 100, 150 and 250 µg) made in 0.35% alum in sterile PBS. Immune response to recombinant proteins was studied in BALB/c mice as well as rabbit by peritoneal route. To evaluate the antibody titres raised in BALB/c female mice and rabbit against r OTC and r GroEL proteins, total antibodies in serum were measured by indirect ELISA after seven days of post immunization. The cut-off value for the assays was calculated as the mean OD (+2 SD) from sera of control group assayed at 1:1000 dilution. The endpoint antibody titers were intended as reciprocal of the uppermost serum dilution giving an OD more than the cut-off value (0.02 OD). The antibody endpoint titers of rOTC and r GroEL in mice and rabbit were 1: 2, 56,000.

For protection study, the immunized animals with recombinant proteins (OTC and GroEL) were challenged with 1 LD<sub>50</sub>, 10 LD<sub>50</sub>, 100 LD<sub>50</sub> of live C. botulinum type B including control group. Survivals of the animals were monitored for 45 days post challenge. There was no protection observed in control group. Immunized mice which were challenged with 1 LD<sub>50</sub> (1.9 x 10<sup>3</sup> CFU/ml) and 10 LD<sub>50</sub> (1.9 x 10<sup>4</sup> CFU/ml) of C. botulinum type B showed 100% protection and mice which were challenged with 100 LD<sub>50</sub> (1.9 x 10<sup>5</sup> CFU/ml) of C. botulinum type B significantly increased the death time of mice (approximately 16 hrs) compared with control group.

Antibody response against rOTC and r GroEL were analyzed for different antibody isotypes. IgG1 and IgG2 are considered to be the marker of Th2 and Th1 response respectively. The antibody raised against rOTC was purified under native condition showed IgG1 and IgG2b response, the results showed mixed response biased towards Th2 as well as Th1-type. The antibody raised against r GroEL was purified under native conditions showed IgG1 response significantly higher than the IgG2 response the results showed Th2 type response. The recombinant vaccines were also elicited high levels of serum IgA, and IgM which may be important in providing mucosal
immunity to impart protection in the anogenital tract and complement activation respectively.

Splenocytes from immunized mice were prepared 30 days after the last dose immunization of rOTC and rGroEL proteins to investigate the cellular immune response. Splenocytes from only vaccinated mice had significant high proliferative response to rOTC and rGroEL (stimulated concentration of 2, 5, 10 µg/ml) compared to PBS immunized mice. Concanavalin A mitogen was able to induce spleen cell proliferation in all culture nonspecifically.

Cytokine profiles of rOTC and rGroEL anti-sera were determined by estimating the levels of GMCSF, IL-2, IL-4, and IL-10, IFN-γ, IL-5, IL-1B and TNF-α. In the case of rOTC, significantly high level secretion of IL-1B, TNF-α, and IFN-γ were noticed in antisera in comparison to PBS immunized mice serum but IL-2, GM-CSF, IL-10 were observed significantly low level of increment compare to control mice serum. No significant difference was noticed in the expression levels of IL-5. In the case of rGroEL, significantly high levels of secretion of IFN-γ, IL-2, IL-5, GMCSF and TNF-α were noticed in antisera compared with PBS given mice serum but IL-10 and IL-1B were observed significantly low level of increment compare to control mice serum.

After 30 days post immunization with recombinant proteins, 8h, 30h, 5 and 20 days after challenge with 10 LD$_{50}$ of C. botulinum type B, the lung, brain, liver, kidney, muscles and spleen of the immunized groups including control group as well as treated group with C. botulinum type B were isolated, fixed and prepared for staining. Normal mice that were neither immunized with rOTC and rGroEL vaccines or PBS nor infected with C. botulinum type B were used as naive controls.

Histopatological studies were carried out of visceral organs of recombinant OTC immunized mice. Brain, kidney, spleen, liver, lung and skeletal muscles sections from control mice showed normal histoarchitecture but when these organs collected from treated as well as immunized mice after 8 to 30 hours post exposures with C. botulinum type B mild to severe changes were occurred. These changes were as follow: in brain section changes were occurred in cortex and hippocampal region, in kidney section changes were seen in cortex and medulla, in liver section changes were found in liver histology, in lung sections changes were seen in alveolar, parenchymal...
and bronchioalveolar regions and some mild changes such as degeneration of arrangement of myofibrils were found in skeletal muscles. These changes were recovered after 5 as well as 25 days post exposure with *C. botulinum* type B in immunized group.

Similarly histopathological studied was done for the rGroEL protein immunized mice. The cortex region of control mice brain showing various types of normal neuronal cells arranged in various layers. Brain of immunised and bacteria administered mice, sacrificed at 8hr showing normal brain architecture. Brain of immunized mice challenged with bacteria (10 LD$_{50}$), sacrificed at 30 hr showing necrotic zones in cortical region. Brain of immunized mice challenged with bacteria 5 and 25 days post exposure showing normal arrangement of neurons. The cortical region of control mice kidney showed normal glomerulus, renal tubules and renal parenchyma. Immunized and bacteria exposed kidney showed congestion in blood vessels. Control mice liver showed normal cord pattern of hepatic lobules and hepatocytes with central canal. Liver of immunized mice followed by bacteria infection showed degeneration of hepatocytes, congestion and hemorrhage. Bacteria 5 and 25 days post exposure showed degenerative hepatocytes and congestion in blood vessels. Control and immunized bacteria treated mice lung showed normal alveolar pattern with normal alveolar septa, air duct, alveoli and bronchioles with intact epithelium. Control and immunized bacteria treated mice skeletal muscles showed normal arrangement of myofibres with no visible adverse effect. Control mice spleen showed normal histo architecture of germinal centre, red pulp and white pulp.

To develop an Immunodetection system against *C. botulinum* type B, a sandwich ELISA assay was used. For this assay, the polyclonal antibodies were generated separately in mice as well as rabbits against recombinant OTC protein using alum as an adjuvant via intraperitoneal route. Antibodies raised in rabbit were used as capturing and the antibodies raised in mice were used as revealing antibody. The results represent that the developed antibody against recombinant OTC protein was capable to detect *C. botulinum* type B approximately 125 cfu/ml.
The cross reactivity studies of the generated antibodies against rOTC was carried out with *C. botulinum* type A, E and F using sandwich ELISA. Overnight grown cultures *C. botulinum* type A, E and F were centrifuged and washed thrice with PBS and used for the cross reactivity studies. The results suggested that generated polyclonal antibodies against rOTC bound with *C. botulinum* type A as well as *C. botulinum* type B.