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RESEARCH ARTICLE

Immunoproteomics: Identification of cross-reactive immunogenic candidate molecules from secretory and whole cell fractions of *C.botulinum* type B

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Abstract

Botulism is the lethal illness caused by botulinum neurotoxins. Botulinum toxin is the most potent poisonous so far known to humans. It is produced by Gram positive; an obligate anaerobic and endospore forming bacteria called *Clostridium botulinum*. There is no detection system / licensed vaccine available commercially for the detection as well as treatment of botulism. The aim of present study was to identify the cross immunoreactive proteins (antigens) of whole cell and secretome of this pathogen. In this study we 0have generated the polyclonal antibody against secretome and whole cell proteins. The two dimensional gel electrophoresis of secretory proteins were probed with the whole cell protein antisera and the whole cell proteins were probed with secretome proteins antisera and identified five cross reactive immunogenic proteins i.e cell surface protein, putative cell surface protein, flagellin, fla-A and hypothetical protein CLOSPO_00563. These identified antigens to be further tested for their efficacy to be used as a potential diagnostic marker / vaccine candidates for botulism.

Introduction

Clostridium botulinum is a gram positive, an obligate anaerobic and endospore forming bacteria produce botulinum neurotoxins (BoNTs). It is the most potent neurotoxin so far known to human (~100 billion times more toxic than cyanide) **(Singh 2000)**. It is categorized as biowarfare agent category 'A'. It can be divided into 8 serotypes designated (A–H) which have similar structure and differ antigenically. Few strains produces dual toxins i.e., Ba, Ab, Bf, and Af (capital letter denotes principal toxin type). Generally the serotypes A, B, E are associated with human clinical cases but rarely serotype F, among the serotypes A and B are the most poisonous. It causes neuroparalytic disease in human and animals called botulism.

Human botulism categorized by CDC into four categories: food-borne, wound, infant, and other. Food borne botulism is caused by ingestion of preformed toxins from contaminated food or drinks. Often this occurs when canned foods that are contaminated with *C. botulinum* spores. The spores converted to vegetative cells, multiply and secrete toxin. It could also result from intentional contamination of food chain supply by spiking of botulinum toxins. 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 toxin inside wound and 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 today. 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 toxin in the large intestine then toxin absorbed into blood stream (**Rosow and Strober 2015)**. It primarily affects infants (younger than 12 months of age) because *C. botulinum* is easily colonized in infants due to the lack of strong gut flora in the intestine. Honey is the greater risk of infant botulism because it contains *C. botulinum* spores so it should be avoided for children. Botulisms are classified as "other" if the patient is not an infant, food and no wounds. It also includes iatrogenic botulism, caused by an unintentional overdose of botulinum toxin (i.e., therapeutic injection) as well as intestinal colonization *C. botulinum* in adults is very infrequent but occurs through a mechanism similar to infant botulism. According to CDC, USA, an average of 145 cases is reported every year, out of this infant botulism occupies higher (65%) followed by wound botulism (20%) and food borne botulism (15%). The majority of wound botulism cases are linked with black-tar heroin injection, especially in California. In 2009 one case of wound botulism was reported in drug user that was caused by *C. botulinum* type B identified by draft genome sequencing **(Fillo et al. 2015)**. In 2011, infant botulism were reported from a 2-month-old boy from Argentina **(De Jong et al. 2015)**. In 2013, four cases of wound botulism were reported in Norway and confirmed in people who injected drugs (PWID) caused by BoNT/ B **(MacDonald et al. 2013)**. In 2013, infant botulism case was reported in Central America where toxin A was identified by polymerase chain reaction and culture from the stools **(Hernandez-de Mezerville et al. 2014)**. In 2015, the European Centre for Disease Prevention and Control (ECDC) and European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) were announced 23 cases of wound botulism in United Kingdom and the Norway and the source was a batch of contaminated heroin.

Detection of botulinum toxin is utmost importance but unfortunately there is no rapid detection system available commercially to detect botulism. Few detection systems have been tested for only serotype A & B. Similarly there is no biomarker has been identified so far against the botulinum toxins.

Deterrence of botulism can be proficiently achieved by vaccination, which generates neutralizing antibodies in opposition to BoNTs. Currently, the most broadly available vaccines for human are pentavalent toxoid (PBT) which is formalin inactivated However, it is very costly and time consuming to construct and is risky during detoxification **(Byrne and Smith 2000)**. DNA vaccines have an attractive approach for construction antigen-specific immunotherapy. DNA vaccines have a number of advantages, together with simplicity of manufacture, purity of product and ease of storage compared to subunit vaccine. But DNA vaccine also has some drawback because DNA vaccines present a risk of potentially disrupting regular cellular processes. This has yet to be revealed as a major concern; however there is a possibility that the entrance of foreign DNA into the body could involve a cell's normal protein expression pathways cause so till now there is no licensed vaccine against the botulism **(Clayton and Middlebrook 2000; Yu et al. 2008)**. In the present study we used the immunoproteomics approach to develop the diagnostic marker / vaccine candidates against the botulism. Now a day Immuno proteomic approach is the most excellent tool among available to the study the host and 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 **(Bunai and Yamane 2005)**. Similar approach has been widely used for the discovery of new biomarkers for vaccine development in cancer as well as other infectious diseases **(Li et al. 2009; Martin et al. 2011)** . 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 **(Dennehy and McClean 2012)**. Numerous novel secretory proteins produced by different bacteria such as *Helicobacter pylori* (Bumann et al. 2002), *Pseudomonas aeruginosa* **(Scott et al. 2013)** and *Staphylococcus aureus* **(Kusch and Engelmann 2014)** have been identified in bacterial culture supernatants by 2DE and mass spectrometry to develop the vaccine molecule as well as biomarker discovery. Similarly, Immunoproteomics approach has been also used to development surface proteins against *Shigella sp* **(Zhu et al. 2010)**, *Bacillus anthracis* **(Mallozzi et al. 2008)**, *S. aureus* **(Carvalhais et al. 2015)**, *C. difficile* **(Wright et al. 2008)** , and *C. perfringens* **(Alam et al. 2009)** using whole cell proteome analysis of these bacteria. Apart from this, the secretary proteins/ surface proteins are important for the development of diagnostics and passive immunotherapy.

Materials and methods

Indian strain of *C. botulinum* type B isolate SP11 (DB123CLB11) was retrieved from the DRDE repository and further confirmed by PCR using standard primers which were specific for BoNT/B as well as nucleotide sequencing. The culture was streaked on egg yolk agar plates and incubated at 37°C in anaerobic work station (Bactron II, Shell Lab, USA) for 24 to 72 hrs supplemented with anaerobic mixed gas (85% N_2 , 5%H_2 and 10% CO_2) . The *C.*

botulinum type B was inoculated in two different serum vials containing 40 ml pre-sterilized, de-aerated TPGY media (Hi Media, India). One serum vial was incubated approximately 16 to 18 hrs (overnight culture) for whole cell proteome study and another serum vial was incubated for five days at 37 °C for secretome study.

Preparation of secretory and whole cell proteins fractions

For secretome fractions, five days old cultures were centrifuged at 10000 X g for 30 min at 4°C and collected the supernatant. Supernatants were kept on ice for 1 h then 10% Ice-cold trichloroacetic acid (Sigma USA) were added gradually and left the 3 hrs on ice for precipitation of proteins **(Lippolis et al. 2013)**. Mixtures were centrifuged at 10,000 X g for 30 min at 4°C and pellets were washed three times with 70% cold acetone and air dried. For the preparation of whole cell protein fractions, Overnight culture was centrifuged at 10000 X g for 10 min at 4 °C and collected the cells pellet and discarded the soup. Cells pellets were washed three times with 1 X PBS then dissolved the pellet in lysis buffer (8 M urea, 4.0% [W/V] CHAPS, and 40 mM DTT and 2.0% [V/V] IPG $p^{H\ 3\text{-}10}$ buffer) and kept on ice for 30 minutes followed by sonication (9.9 pulses on and off for 10 min). Then the samples were centrifuged at 8000 rpm for 30 min at 4°C, collected soup and discarded the cell debris. Three volumes of pre-chilled glacial acetone were added to the sonicated soup and kept at -20°C for overnight for precipitation of proteins. The precipitate was collected by centrifugation at 8000 rpm for 30 min at 4°C and the pellet was air dried. The air dried proteins (secretory and whole cell proteins) were resuspended in protein solubilization buffer (8 M urea, 2.0% [W/V] CHAPS, and 40 Mm DTT and 2.0% [V/V] IPG $p^{H\ 3\text{-}10}$ buffer). Both whole cell proteins and secretory proteins 500 µl each were taken in two different 2 ml eppendorf tubes for desalting using protein clean up kit (Bio-Rad, USA) and finally the protein concentration was estimated by Bradford method (SIGMA, USA) using Bovine serum albumin as a standard protein. The resulting cleaned protein was stored at −80°C or immediately used for 2-DE assay.

Two-dimensional gel electrophoresis of whole cell as well as secretory proteins

A total of 300 µg of protein samples (secretory and whole cell proteins) were added into rehydration buffer (8 M urea, 4% CHAPS, 0.002% bromophenol blue) and applied onto IPG strips (pH 4.7-5.9, 7 cm). Rehydration of strips was done for 16 hr at room temperature and then IPG strips were subjected to isoelectric focusing (IEF system, Bio-Rad, USA). The isoelectric focusing was done at 20°C, 2 mA, and 5 W with the following voltage profile: 200 V constant for 30 min, a gradient from 200 V to 4000 V for 1.5 hr, and 4000 V constant for 1 hr 5 min (total, 6,500 V/hr). After focusing, IPG strips were equilibrated two times for 15 min each in 10 ml equilibration buffer (50mM Tris –HCl [P^H 8.8], 6M urea, 30% glycerol, 2% SDS and 0.002% bromophenol blue) first in 100 mg DTT followed by 250 mg iodoacetamide buffers. Strips were then transferred onto 12% SDS-PAGE for the second-dimension resolution which was carried out in a vertical gel electrophoresis system (Bio-Rad, USA). The gel was stained with Bio-Safe colloidal Coomassie Blue G-250 (Bio-Rad, USA) **(Feist and Hummon 2015).**

Immunization and generation of polyclonal antibody

Experiments on animals were permitted by the Institutional Animal Ethical Committee (IAEC) of the Defence Research & Development Establishment (DRDE), Gwalior, India. Polyclonal antibodies were generated in BALB/c female mice weighing 20 to 30 gm via subcutaneous route against secretory proteins and whole cell proteins of *C. botulinum* type B. Active immunization on a 4-week immunization schedule (0, 14, 21, 28 days) using 10 µg protein with complete Freund's adjuvant followed by three booster doses of 20, 30, 50 µg protein with incomplete Freund's adjuvant.

Indirect ELISA

Indirect Enzyme-linked immunosorbent assay (ELISA) was used to test the antibodies end point titre in mice against secretory proteins and whole cell proteins of *C. botulinum* type B. The ELISA plates were coated with 5µg/ml (secretory and whole cell proteins) and incubated the plate at 4°C for overnight. Then plate was washed five times with PBST (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4 and 0.05% Tween -20, pH *7.4)*. Plates were blocked using 3% bovine serum albumin (BSA made in PBS) at 37°C for 1 hr. Plates were washed as mentioned previously and added 100 µl per well, two fold diluted secretory proteins as well as whole cell proteins sequentially . Similarly the preimmunized sera also added and incubated at 37°C for 1 hr. After washing (4 times with PBST), added 100 µl per well of secondary antibody rabbit anti-mouse IgG-HRP (Dako, Denmark) at 1:2000

dilution and incubated at 37°C for 1 hr. Then the plates were washed as described previously. Finally the antigen and antibody interactions were developed using 2, 2'-azino-bis (3-ethylbenzo-thiazoline-6-sulphonic acid) diammonium salt solution (100 µl/well) containing H_2O_2 and incubated at 37°C for 30 min. Absorbance was measured at 410 nm using an ELISA plate reader (Biotek, USA).

Western blot analysis

Both secretory and whole cell proteins were separated by 2-DE in replicate gels then transferred onto PVDF membrane in semi-dry condition using transfer apparatus (Bio-Rad, USA) at 120 volt for 1 hr at 4°C in Tris–Glycine transfer buffer (25mM Tris, 192 mM glycine and 10% methanol). The membranes were blocked with 5% skimmed milk solution made in PBS and incubated at 4°C for overnight. Membranes were washed three times with PBST with 10 min interval. Secretory proteins transferred membranes probed with polyclonal antibodies (1:20,000) raised in mice against the whole cell proteins and whole cell proteins transferred membranes were probed with polyclonal antibodies (1:20,000) raised in mice against the secretory proteins. Unimmunized mice sera at a conc. of 1:1000 were used as negative control for immunoblotting. Again the membranes were washed three times using PBST then incubated with *Anti-Mouse IgG (Fc specific)–Peroxidase antibody produced in goat* (SIGMA ,USA) with 1:15,000 dilution. After final washing five times with PBST, the membrane was developed with DAB substrate (SIGMA, USA).

Identification of cross reactive immunogenic proteins by Mass spectrometry

Protein spots were excised using fresh surgical blade for the desired protein spot size. At this step, care was taken the spots with adjoining proteins or with skin keratin should not contaminate the actual protein spots. Individual protein spots were washed thrice with proteomic-grade deionized water, destained followed by reduction /alkylation and finally the proteins were digested with trypsin using the Montage In-Gel digestion kit (Millipore, USA) as described by the manufacturer's. The digested protein (0.8 µl) was mixed with 0.8 µl of the matrix solution (5 mg α-cyano-4-hydroxycinnamic acid in 80% [vol/vol] acetonitrile and 0.1% [wt/vol] trifluoroacetic acid [TFA]) thoroughly by pipetting for spectral scanning. This mixture (1µl) was deposited onto the MALDI plate and proteins were identified by mass spectrometry (MS) using a matrix-assisted laser desorption ionization—tandem time-of-flight (MALDI-TOF-TOF) mass spectrometer (Ultraflex III; Bruker Daltonics, Germany). The spectrum was obtained in the mass range of 500 to 4,000 Da followed by calibration using calibration mixture containing angiotensin I, Substance P, adrenocorticotropin (1-17) [ACTH (1-17)], ACTH (18-39) and somatostatin.

Spectra were analyzed with Mascot sequence-matching software from Matrix Science using a non identical protein sequence database based on MSDB in the taxonomy group of *Bacteria*. The following Search parameters were used maximum of one missed cleavage by trypsin, fixed modification of oxidation, charged state of +1, peptide mass tolerance of 50 ppm, and a fragment mass tolerance of ±0.6 Da. Peptide mass fingerprinting (PMF) data for trypsin-digested proteins and MS-MS analysis results for one or more selected peptides were used for the database search as described above **(Sengupta et al. 2010)**.

Results

Immune response against secretory as well as whole cell proteins

To evaluate the antibody titres raised in mice against secretory and whole cell proteins, total antibodies were measured. Sera samples were collected after third boosters. 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. The antibody endpoint titer of secretory and whole cell proteins was 1.28×10^5 which was shown in figure 1.

Identification of cross reactive immunogenic proteins

To determine the cross reactive immunogenic proteins of *C. botulinum* type B, polyclonal antibody was raised in mice against the whole cell proteins and secretory proteins. Antisera (raised against whole cell proteins and

secretory proteins) that elicited high antibody titres with dilution of 1:20,000 at OD 410 nm was used in the Western blot assay and sera from unimmunized mice, OD 410 nm (0.004) were used as control. The secretome 2-DE gels 4.7 to 5.9 IPG strips were probed with whole cell protein antisera and the whole cell proteins 2-DE gels 4.7 to 5.9 IPG strips were probed with secretory protein antisera the results are shown in Figure 2 & 3. Four cross reactive immunogenic proteins i.e cell surface protein , hypothetical protein CLOSPO_00563, FlaA and flagellin were identified when secretory proteins 2DE gel probed with whole cell proteins antisera which was showed in table 1. Similarly four cross reactive immunogenic proteins i.e hypothetical protein CLOSPO_00563 flagellin, FlaA, cell surface protein and putative cell Surface Protein were also identified when whole cell proteins 2DE gel probed with secretory proteins antisera which was showed in table 2. Hypothetical protein CLOSPO_00563, fla-a , flagellin and cell surface protein were identified commonly in both secretory protein antisera and whole cell protein antisera .

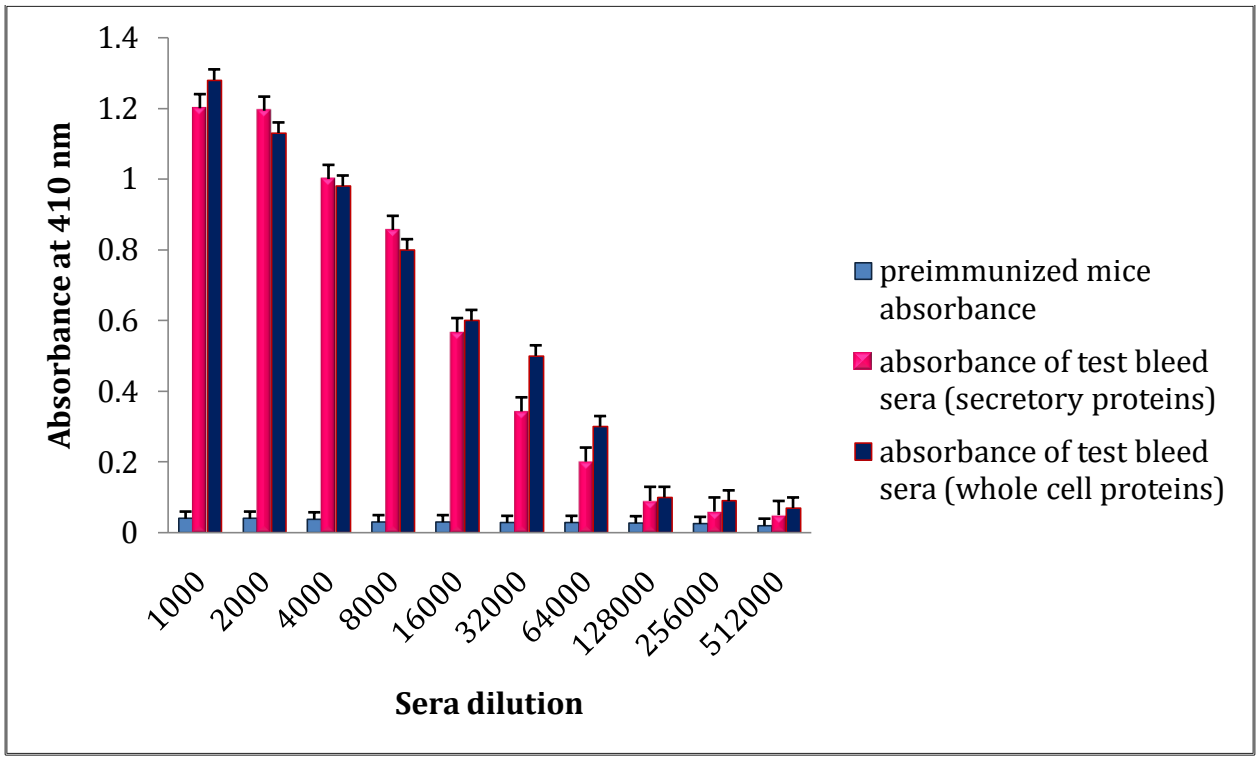

Figure 1. Depicted the Antibody titres against whole cell and secretory protein of *C. botulinum* type B (SP11) grown in TPGY medium. Mice (n = 6 mice per group) were immunized with secretory and whole cell proteins or PBS as the control group. Four weeks after final immunization, antibody titre was measured with indirect ELISA. Results showed that titre against immunization of mice with secretory and whole cell proteins significantly increased compared with control group. Testing was carried out triplicate and values are shown as the mean ± S.D.

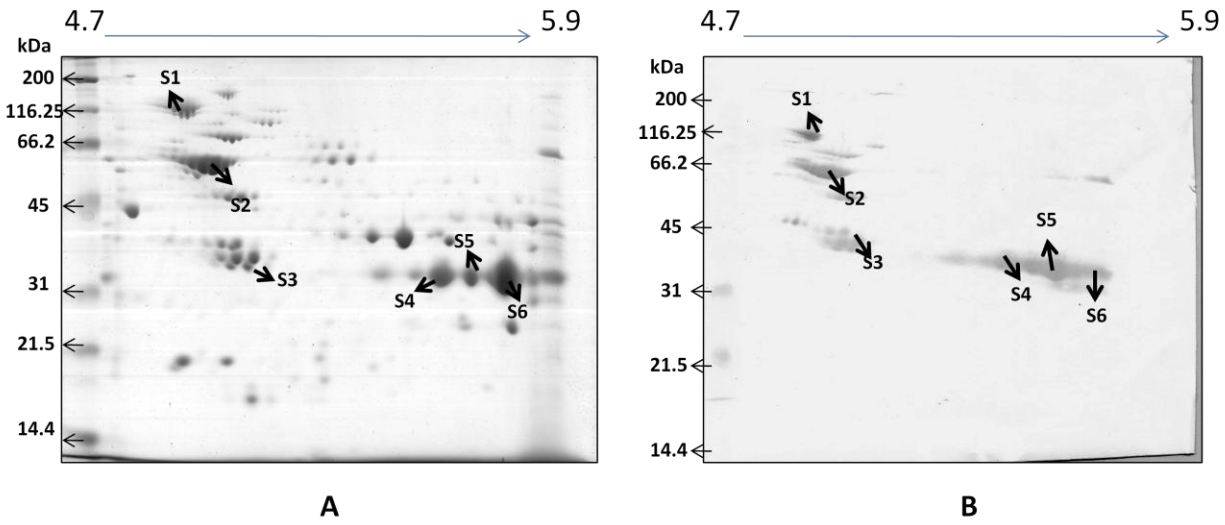

Figure 2. Immunoblot analysis: Antisera of whole cell proteins (TPGY) 1/20,000 dilutions binding to secretome proteins of *C.botulinum* type B Indian isolate SP11 grown in TPGY medium. Secretome of *C.botulinum type B* were separated by 2-DE (pH 4.7-5.9 length 7cm) and stained with Bio-safe Coomassie G-250. (A) 2DE-map (B) Western blot. The positions of molecular weight standards are indicated on the left. The immuno cross reactive protein spots were identified by MALDI-TOF-MS and database searches are indicated by the spot numbers given in Table 1. Three technical replicates were organized in independent experiments; these were very similar, and one of them was revealed

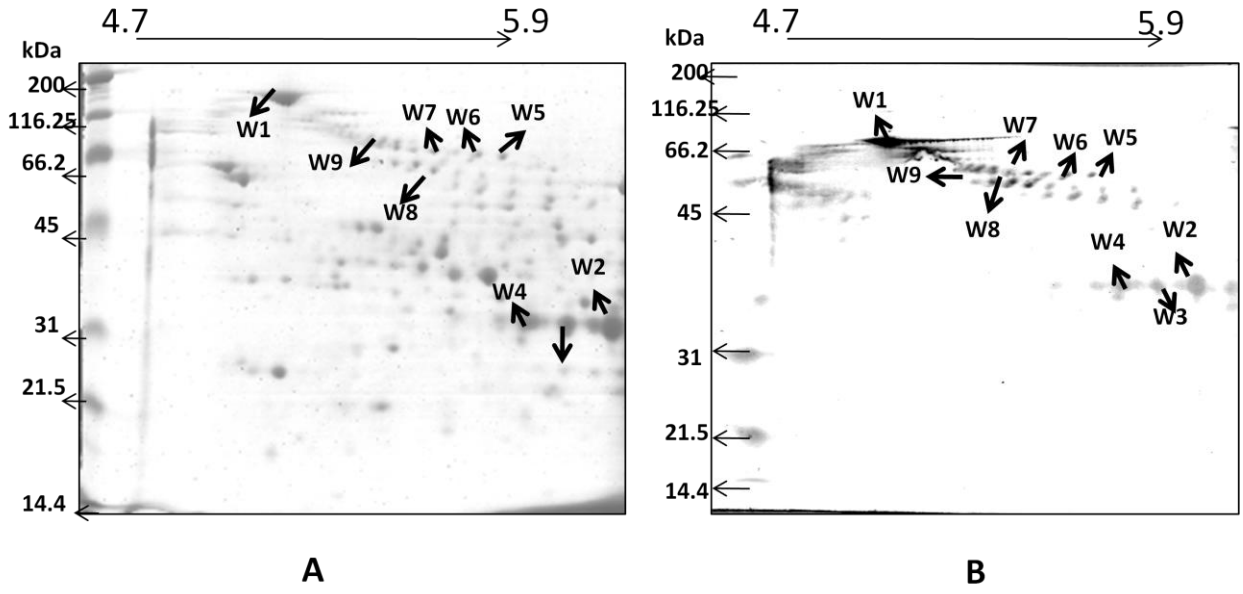

Figure 3. Immunoblot analysis: Antisera of secretory proteins (TPGY) 1/20,000 dilutions binding to whole cell proteins of *C.botulinum* type B Indian isolate SP11 grown in TPGY medium. Whole cell proteome of *C.botulinum type B* were separated by 2-DE (pH 4.7-5.9 length 7cm) and stained with Bio-safe Coomassie G-250. (A) 2DE-map, (B) Western blot. The positions of molecular weight standards are indicated on the left. The immuno cross reactive protein spots were identified by MALDI-TOF-MS and database searches are indicated by the spot numbers given in Table 2. Three technical replicates were organized in independent experiments; these were very similar, and one of them was revealed

Table 1. Cross reactive Proteins (2DE of secretome proteins probed with the antisera of whole cell proteins) of *C. botulinum t*ype B Indian isolate SP11 determined by 2-DE using (4.7 -5.9 narrow range P^H strips) and Mass spectrometry. Spot numbers refer to the numbers on the 2-DE gels shown in Figure 2.

S.No	spot ID	Protein annotation	Accession No	Thr.molar mass(Da)	Expmolar mass (Da)	Isoelectric point(thr.)	Isoelectric point(exp.)	Protein score	Peptide count	sequence coverage	Transmembrane helices	Subcellular location	Secretome p	Prosite	Signal peptide (Signal p/Lipo P)	Grand average of hydropathy value	Cellular Role categories
1	S1	cell surface protein [*Clostridium botulinum A* str. ATCC 3502]	gi|148378381	150610	150505	4.88	4.88	120	6	2	0	Cell wall	Yes	No hit	Yes/spI	-0.494	
2	S2	hypothetical protein CLOSPO_00563 [*Clostridium sporogenes* ATCC 15579]	gi|187777018	134680	134589	5.02	5.02	1021	16	11	0	Cell wall	No	No hit	No/No	-0.531	Unknown
3	S3	hypothetical protein CLOSPO_00563 [*Clostridium sporogenes* ATCC 15579]	gi|187777018	134680	134589	5.02	5.02	1021	16	11	0	Cell wall	No	No hit	No/No	-0.531	Unknown
4	S4	FlaA [*Clostridium botulinum*]	gi|114054801	26250	29270	7.12	7.12	95	8	21	0	cytoplasm	No	No hit	No/No	-0.513	Flagellar synthesis and maintenance
5	S5	flagellin [*Clostridium botulinum* A str. ATCC 3502]	gi|148380688	29800	29798	8.87	7.98	641	14	35	0	cytoplasm	No	No hit	No/No	-0.446	Flagellar synthesis and maintenance
6	S6	FlaA [*Clostridium botulinum*]	gi|114054801	26250	29270	7.12	7.12	219	8	21	0	cytoplasm	No	No hit	No/No	-0.513	Flagellar synthesis and maintenance

Table 2. Cross reactive immunogenic Proteins (2DE of whole cell proteins probed with the antisera of secretory proteins) of *C.botulinum t*ype B Indian isolate SP11 determined by 2-DE using (4.7 -5.9 narrow range P^H strips) and Mass spectrometry. Spot numbers refer to the numbers on the 2-DE gels shown in Figure 3.

S.No	spot ID	Protein annotation	Accession No.	Thr. molar mass(Da)	Exp. molar mass (Da)	Isoelectric point (thr.)	Isoelectric point(exp.)	Proten score	Peptide count	sequence coverage	Transm embrane helices	Sub cellular location	Secretome p	Prosite	Signal peptide (Signal p/LipoP P)	Grand average of hydropathy value	Cellular Role categories
1	W1	hypothetical protein CLOSPO_00563 [*Clostridium sporogenes* ATCC 15579]	gi|187777018	134680	134589	5.02	5.02	1021	16	11	No	No	No	No hit	No/ No	-0.531	Unknown
2	W2	flagellin	gi|148380688	29800	29798	8.87	7.98	641	14	35	No	No	No	No hit	No/No	-0.446	Flagellar synthesis and maintenance
3	W3	flagellin [*Clostridium botulinum* A str. ATCC 3502]	gi|148380688	29800	29798	8.87	7.98	641	14	35	No	No	No	No hit	No/No	-0.446	Flagellar synthesis and maintenance
4	W4	FlaA [*Clostridium botulinum]*	gi|114054801	26250	29270	7.12	7.12	219	8	21	No	No	No	No hit	No/No	-0.513	Flagellar synthesis and maintenance
5	W5	cell surface protein [*Clostridium botulinum A* str. ATCC 3502]	gi|170758444	146.18	150.882	4.9	4.94	128	8	-	No	Cell wall	yes(0.779813)	No hit	No/No	-0.542	Unknown
6	W6	cell surface protein [*Clostridium botulinum A* str. ATCC 3502]	gi|170758444	146.18	150.882	4.9	4.94	128	8	-	No	Cell wall	yes(0.779813)	No hit	No/No	-0.542	Unknown
7	W7	putative cell Surface Protein [*Clostridium botulinum* NCTC 2916	gi|168177721	146180	146072	4.94	4.94	165	10	4	No	Cell wall	yes	No hit	Yes/No	-0.25	unknown
8	W8	cell surface protein [*Clostridium botulinum A* str. ATCC 3502]	gi|170758444	146.18	150.882	4.9	4.94	128	8	-	No	Cell wall	yes(0.779813)	No hit	No/No	-0.542	Unknown
9	W9	putative cell Surface Protein [*Clostridium botulinum* NCTC 2916]	gi|168177721	146180	146072	4.94	4.94	165	10	4	No	Cell wall	yes	No hit	Yes/No	-0.25	unknown

Discussion

The aim of the present study is to identify the cross reactive immunogenic proteins of *C. botulinum type B* secretome and whole cell fractions for the development of potential diagnostic marker / vaccine candidates against botulism. The common cross reactive proteins may be explored for unambiguous detection of botulism irrespective of toxin types. Similarly the same proteins may have the potential to be used as vaccine candidate's molecule against botulism. Now a day's fast food industries are growing across the world and the use of canned foods are increasing heavily. The improper sterilization of the canned foods may leads to the production of preformed toxins in the canned foods leads to food borne botulism. Wound botulism is caused by organisms growing in deep wounds. Infant botulism develops when ingested spores or organisms colonize the large intestine, multiply, and produce toxin. Some intestinal colonization in non infants has also occurred. The clinical aspects of this type of case have been reported. Infant and wound botulism are occurred primary infection followed by secondary intoxication. If primary infection i.e colonization is blocked, toxin will not produce in wound and intestine results infant and wound botulism will not be occurred. In the present study our aim is to mainly identify the cross reactive immunogenic proteins; it can be further explored for their potential to be used as diagnostic marker / vaccine candidates against botulism. To identify the cross reactive immunogenic proteins, we probed the 2DE gels of secretory proteins with the whole cell proteins antisera raised in mice and vice versa. The following cross reactive immunogenic proteins flagellin, fla-a, hypothetical proteins, cell surface protein and putative cell surface protein were identified by western blot and mass spectrometry. These immunogenic proteins may be probable diagnostic marker /vaccine candidate molecules against the botulism. Many researcher have been used these proteins as a vaccine candidate molecules against Gram positive as well as Gram negative bacteria. Several reports are available in literature for flagella proteins. It is one of the most effectual immunological stimulator of the immune system. The use of flagellin as a vaccine candidate or adjuvant has been widely considered. Bacterial flagellin has been considered for malaria vaccine formulations to the development of effective human vaccines **(Bargieri et al. 2011)** . Flagellin intrinsic activity was mediated through TLR5 therefore can be genetically manipulated in a variety of ways for research purpose **(Saha et al. 2007)**. In other words, advances in the field of innate immunity have disclosed the successful cellular and molecular mechanisms concerned in pathogen-associated molecular patterns **(Campodonico et al. 2010)**. Recombinant fla-A induced lymphocyte proliferation, considered that proliferation is a criterion of cell-mediated immunity, thus r-fla-A could promote the cellular immune response **(Delavari et al. 2015)** . Arnon et al showed that recombinant flagellin comprising unfamiliar epitope caused protective immune responses in the absence of any extra adjuvant; flagellin has effects on T cells proliferation and cytokine responses **(Honko and Mizel 2004)** . The induction of potent cell-mediated immunity will be essential and may require the enhanced of cytotoxic T cell (CTL) which kills host cells infected with intracellular pathogens **(Singh and O'Hagan 2002)**. In addition, the innate immune system directs the balance of humoral and cellular mediated responses, and adjuvant can manage the type of acquired immune responses **(Verma et al. 2005)**. Hypothetical protein CLOSPO_00563, cell surface protein and putative cell surface protein are the cell wall associated protein predicted by PSORTb. These are the novel immunogenic proteins may be developed as substitute antigens for further study of *C. botulinum* vaccine and diagnostics. Cell wall exposed antigens in Gram-positive bacteria reveal the power for the rapid discovery of new vaccines **(Cole et al. 2005)**. The above mentioned reports strongly suggested the identified cross reactive immunogenic proteins may have the potential and further to be explored for their efficacy to be used as diagnostic / vaccine candidate molecules against botulism.

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