ORIGINAL RESEARCH PAPER

Immunoproteomic analysis of *Clostridium botulinum* type B secretome for identification of immunogenic proteins against botulism

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Abstract

Objectives To identify immunogenic proteins of *C. botulinum* type B secretome by immunoproteomic analysis.

Results In the present study, an attempt was made to elucidate the vaccine candidates/diagnostic molecules against botulism using immuno proteomic approach. *C. botulinum* type B secretome was elucidated when it was grown in TPGY as well as CMM media. Predominant 51 proteins were identified in both the media using 2-DE and mass spectrometry analysis. 2D gels (CMM & TPGY) were probed with respected proteins mice antiserum and obtained 17 and 10 immunogenic proteins in TPGY as well as CMM media respectively. Hypothetical protein CLOSPO_00563, ornithine carbamoyl transferase, FlaA, molecular chaperone GroEL and secreted protease proteins were found as the common immuno dominant proteins in both media. Polyclonal Antibodies raised against *C. botulinum* types A and E showed cross-reactivity with secretome *C. botulinum* type B at the lowest dilution (1:1000) but did not show cross reactivity with highest dilution (1:30,000) with *C. botulinum* type B secretome. Polyclonal antibodies against *C. botulinum* type F secretome did not show cross reactivity with *C. botulinum* type B secretome.

Conclusions Identified immunogenic proteins can be used as vaccine candidates and diagnostic markers for the infant and wound botulism but common immunogenic proteins may be the best vaccine candidate molecule for development of vaccine as well as diagnostic system against the infant and wound botulism.

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Introduction

Clostridium botulinum is a Gram positive, obligate anaerobic and endospore forming bacterium that produces botulinum neurotoxins (Maikanov et al. 2019). It is the most potent neurotoxin so far known to

humans hence it is categorized as a bio-warfare category 'A' agent. It has been divided into seven serotypes designated (A–G) which have similar structure but differ antigenically. Generally, serotypes A, B, E are associated with human clinical cases but rarely serotype F. Among the seven serotypes, A and B are the most poisonous serotypes. It causes neuroparalytic disease in humans and in animals called botulism (Lanci et al. 2019).There are three types of botulism; infant botulism, wound botulism and food borne botulism. Infant botulism is caused by swallowing of botulinum spores present in the environment. These spores are converted into vegetative cells followed by germination which then multiply and colonize in the large intestine leading to secretion of botulinum neurotoxin (BoNT) in situ (Galvis 2019). Similarly when the wounds are exposed to the environment, the *C. botulinum* spores enter into the wound followed by multiplication of the bacteria and produce the toxin. The toxin gets absorbed into the bloodstream and causes the wound botulism (Kuehn 2019). Both infant and wound botulism are caused by primary infection followed by secondary intoxication. But food borne botulism is caused by the intake of preformed BoNT containing food (Forss et al. 2012; Palma et al. 2019). The neurotoxin is absorbed and carried by the bloodstream to neuromuscular junctions where it blocks the release of acetylcholine and causes flaccid paralysis. So far there is no licensed vaccine commercially available for botulism. Similarly there is no rapid detection system available to diagnose botulism. The Centre for Disease Control and Prevention (CDC) used to provide the pentavalent toxoid as an investigational vaccine for select risk groups but it has been discontinued since 2011 due to the decrease in the antibody titer of certain serotypes as well as increasing side effects after the booster (Notice of CDC's discontinuation of investigational pentavalent (ABCDE) botulinum toxoid vaccine for workers at risk for occupational exposure to botulinum toxins 2011). Several studies have been attempted for the development of recombinant protein based subunit vaccine using different domains of the heavy chain of different serotype (Przedpelski et al. 2018). Few studies have been directed towards the use of DNA based vaccines for different serotypes (Kim et al. 2019). Both the studies have revealed that, the binding domains are immunogenic and provide neutralization against the toxins but the risk of using the full domain

of the toxin, reactivity of the recombinant proteins with other human proteins and other ethical concerns using the active toxin part for immunization. Immuno proteomic approach is one of the best tools available to study the host–pathogen interactions (McClean 2012). Researchers used combination of 2DE and immuno blotting with sera from infected animals and mass spectrometry to find out the immunogenic candidates (Pedersen et al. 2005). Similar approach has been widely used for the discovery of new biomarkers for vaccine development in cancer as well as infectious diseases (Forgber 2009; Martinez-Lopez 2008; Mendum 2009; Pitarch et al. 2006; Pitarch and Gil 2011). The secretory proteins play important roles in the pathogenesis of bacterial infection, represent the interphase of the bacterium–host interaction (Tsai-Tien Tseng 2009). In any pathogens the secretory proteins are exposed to the host immune system and are therefore the primary antigen targets of host immune response (Fulton and Twine 2013). Numerous novel secretory proteins produced by different bacteria such as *Helicobacter pylori* (Bernardini et al. 2007), *Pseudomonas aeruginosa* (Nouwens 2002) and *Staphylococcus aureus* (Ziebandt 2001) have been identified in secretome by using two-dimensional gel electrophoresis and mass spectrometry approach to develop the vaccine candidates as well as diagnostic system. Moreover the secretory immunogenic molecules are important for the development of diagnostics and passive immunotherapy (Vanitha Mariappan 2009). Identification of the secretory immunogenic proteins would be further step towards understanding the humoral immune response during *C. botulinum* infection. The aim of the present study was to identify and characterize immunogenic proteins from *C. botulinum* type B secretome by a combination of 2DE and immunoblotting assay using antisera raised in mice against the secretory proteins. Some of these immunogenic proteins have potential to be used as novel vaccine candidates or may be useful for enhancing the protective efficacy of a protective-antigen-based vaccine. However, information regarding the secretome profile as well as immunogenic proteins of *C. botulinum* is not available in literature. The novel immunogenic proteins may be developed as alternative antigens for further study of botulism vaccine and diagnostics.

Materials and methods

Bacterial strains, growth and culture condition

Indian isolates of *C. botulinum* type A SP08 (DRDE Accession No. DB120CLBO8), type B isolate SP11 (DB123CLB11), type E isolate SP01 (DB113CLBO1) and type F isolate SP04 (DB116CLBO4) were retrieved from the DRDE repository and further confirmed by polymerase chain reaction (PCR) using standard primers which were specific for BoNT/A, B, E and F (Lindstrom et al. 2001). Pure culture colonies were inoculated in serum vials containing 40 ml pre-sterilized, de-aerated TPGY and CMM media (Hi Media, India). TPGY media is being used for enrichment and CMM media for maintenance the *C. botulinum* type B culture. These cultures were incubated at 37 °C in an incubator shaker at 200 rpm for 5 days.

Preparation of extracellular proteins

Cultures were centrifuged at $8000 \times g$ for 30 min at 4 °C and collected the supernatant then it was filtered through 0.22 μm filters (Millipore, USA) to remove the suspended vegetative bacterial cells. Culture filtrates were concentrated using 10 kDa cutoff membranes (Millipore, USA) and precipitated using 10% Ice-cold trichloroacetic acid (Sigma USA) after that incubated on ice for 3 h. Precipitate were centrifuged at $8000 \times g$ for 30 min at 4 °C and washed the pellet three times with cold acetone and left the room for air dried (Hirose et al. 2000). Further, the pellets were resuspended in protein solubilization buffer (8 M urea, 2% CHAPS, and 2% carrier ampholytes pH 4–7) (Deatherage Kaiser et al. 2018). The resultant proteins were cleaned using protein clean up kit (Bio-Rad, USA). Protein concentration was estimated by Bradford method (SIGMA, USA) using Bovine serum albumin as a standard protein. Proteins was stored at − 80 °C for further use.

Two-dimensional gel electrophoresis

Secretary proteins 500 μg was added into rehydration buffer (8 M urea, 4% CHAPS, 0.002% bromophenol blue) and applied onto IPG strips (pH 4–7 and pH 4 0.7–5.9, 11 cm). Similarly, 300 μg of protein was loaded onto the 7 cm IPG strips (pH 4–7 and 4.7–5.9).

Rehydration process was performed for 16 h at room temperature and isoelectric focusing (IEF system, Bio-Rad, USA) procedure was carried out using following voltage profile: 200 V constant for 30 min, a gradient from 2 V to 3500 V for 1.5 h, and 3,500 V constant for 1 h 5 min (total, 6500 V/h) (Sharma et al. 2018). After focusing, IPG strips were equilibrated with DTT (50 mM Tris–HCl [pH 8.8], 6 M urea, 30% glycerol, 2% SDS and 0.002% bromophenol blue, 100 mg DTT) and iodoacetamide (50 mM Tris–HCl [pH 8.8], 6 M urea, 30% glycerol, 2% SDS and 0.002% bromophenol blue, 250 mg iodoacetamide) buffers. Strips were then transferred onto 12% SDS–PAGE for the second-dimension resolution. The gel was stained with Bio-Safe colloidal Coomassie Blue G-250 (Bio-Rad, USA) and destained with autoclaved triple distill water and imaged using a GS-800 Densitometer (BioRad) employing PDQuest 7.1 software (BioRad).

MALDI TOF-TOF analysis

Protein spots were excised from 2D gels and 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% acetonitrile and 0.1% trifluoroacetic acid [TFA]) thoroughly by pipetting for spectral scanning. This mixture (1 μl) 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). MS mass spectra had been noted in the reflector positive mode using a laser (wavelength 355 nm) operated at a 200 Hz recurrence rate and at 2 kV accelerated voltage. The MS/MS mass spectra had been developed using data dependent acquisition method. In this method, 20 strongest precursors selected between 850 and 4000 Da and filtered through a signal-to-noise ratio more than 20 from one MS scan. Precursor ions had been selected by timed ion selector (TIS). Fragmentation had been done through collision induced dissociation (CID) method by air (collision gas) at 1 kV energy as well as recharge pressure threshold of 1.5e-006. MS and MS–

MS spectra were obtained by accumulation of at least 1200 and 1600 laser shots, respectively. MS and MS/MS data were analyzed and peak list were generated using the 4000 Series Explorer Software v. 3.5 (Applied Biosystem, USA). A peak intensity filter was used with no more than 50 peaks per 200 Da in the setting parameter of MASCOT search after acquisition. MS/MS peaks were selected based on a signal-to-noise ratio greater than 10 over a mass range of 60–20 Da below the precursor mass. MS and MS/MS data had been analyzed using Protein Pilot version 4.0 (Applied Biosystem) the MASCOT 2.0 search engine (Matrix Science, London). The peak list had been examined against 3,239,079 entries for *Firmicutes* at non-redundant protein sequence database of NCBI. Trypsin digestion with one missed cleavage, oxidation of methionine, carbamidomethylation of cystein and the peptide mass tolerance of 50 ppm for precursor ion and mass tolerance of $\pm$ 0.6 Da for fragment ion with $+$ 1 charge state were search parameters. MS/MS was successfully identified proteins, MASCOT score more than 60 was accepted as significant (p-value < 0.05).

Bioinformatics analysis

Proteins function had been predicted by UniProt Knowledge base (Swiss-Prot and TrEMBL). Signal peptide had been predicted by Signal P software in Gram-positive bacteria. (network http://www.cbs.dtu.dk/services/SignalP). Transmembrane helices numbers had been explored by TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Prediction of lipoproteins and signal peptides had been analyzed by Lipo P 1.0 (http://www.cbs.dtu.dk/services/LipoP/). Grand average of hydropathy had been calculated by gravy calculator (http://www.gravy-calculator.de/). Sub-cellular localization of protein had been predicted by PSORT b version 2.0 (http://www.psort.org/psortb2.

Immunization and generation of polyclonal antibody

The Animal experiments had been approved by the Institutional Animal Ethical Committee (IAEC) of the Defence Research & Development Establishment (DRDE), Gwalior, India. Polyclonal antibodies had been generated in BALB/c female mice via intra-peritoneal route against secretory proteins of *C. botulinum* type B, which were expressed in TPGY and CMM media. Active immunization (0, 14, 21, 28 days) schedule had been performed 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. Similarly polyclonal antibodies had been generated against *C. botulinum* type A/E and F.

Indirect ELISA

Indirect Enzyme-linked immunosorbent assay (ELISA) was performed to check the antibodies titre in mice against extracellular proteins of *C. botulinum* type B. Briefly, the 96 well ELISA plates were coated with 5 μg/ml extracellular proteins (CMM and TPGY medium) and incubated the plates overnight at 4 °C. Then plate was washed three 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) followed by three times washing with phosphate buffer saline (PBS). Plates were blocked with 3% bovine serum albumin (BSA) at 37 °C for 1 h. Plates were washed as mentioned previously followed by addition of 100 μl per well, two fold diluted primary antibody from 1:1000 to 20, 48,000 (mice sera against extracellular proteins expressed in TPGY and CMM media). Similarly the preimmunized serum incubated at 37 °C for 1 h. Then plate was washed three times with PBST and three times with PBS. After washing, 100 μl per well of secondary antibody rabbit anti-mouse IgG-HRP (Dako, Denmark) 1:2000 dilution was added and incubated at 37 °C for 1 h. Then the plate was washed as described previously. Finally the antigen and antibody interactions was 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). Similarly ELISA assay was done for secretory proteins antiserum against *C. botulinum* type A/E and F.

Western blot analysis

2-DE gels were transferred onto PVDF membrane in semi-dry condition using transfer apparatus (Bio-Rad, USA) at 100 V for 1 h at 4 °C in tris glycine transfer

buffer (25 mM Tris, 192 mM glycine and 10% methanol). The membranes were blocked with 5% skimmed milk and incubated at 4 °C overnight. Further membranes were washed three times with PBST followed by PBS twice with 10 min interval. Membranes were probed with polyclonal antibodies (1:30,000 dilutions) generated in mice against the extracellular proteins expressed in TYGY and CMM media. Unimmunized mice serum (1:1000) was used as negative control for immunoblotting. The membranes were washed as washed previously and incubated for 1 h with 1:30,000 dilution Anti-Mouse IgG (Fc specific)–Peroxidase antibody. After washing with PBST, the membrane was developed by DAB substrate (SIGMA, USA). Similarly 2D-gel of type B secretory proteins were probed with other *C. botulinum* types A, E, F secretory proteins anti serum at dilutions of 1:30,000 and 1:1000.

Results and discussion

Identification of predominantly expressed protein spots in TPGY and CMM media

Proteomics has been become a technique for identification of proteins involved during infection and invasion by the pathogens (Das et al. 2019). Bacterial secreted proteins perform key role in the interaction between the bacterial cell and the host environment (Siegrist et al. 2015). Thus secretome of a pathogen are hypothesized to comprise the components of effective vaccines. Secretome exploration facilitates identification of novel vaccine antigen and overall helps in progress of discovery of vaccine (McManus 2020). In the present work, predominantly expressed protein spots were successfully identified by mass spectrometry. Most of the proteins were over expressed in TPGY medium compared to CMM medium. The list of predominantly expressed proteins in both TPGY and CMM media are being shown in Fig. 1 and tabulated in Table 1. Glyceraldehyde-3-phosphate dehydrogenase, type I, chlorohydrolase/aminohydrolase, molecular chaperone GroEL, hypothetical protein CLOSPO_00563, dehydrogenase, FMN-dependent, trios phosphate isomerase, E-cinnamoyl-CoA:R-phenyl lactate CoA transferase large subunit, thiamine biosynthesis protein ThiC, amino peptidase 1, putative cell surface protein, acetyl-CoA acetyltransferase, carbamate kinase, secreted protease, clostripain, molecular chaperone DnaK, enolase, ornithine carbamoyltransferase, butyrate kinase, aspartate/ornithine carbamoyltransferase family protein, thermolysin metallopeptidase, glycosyl
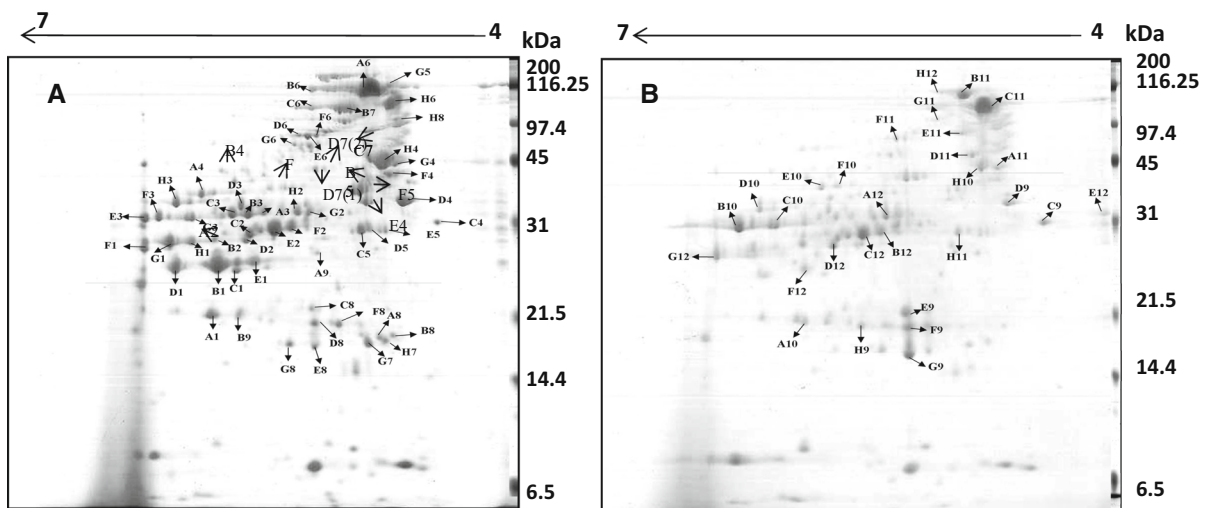
Fig. 1 Two-dimensional map of Bio-Safe Coomassie G-250-stained gel of secretome proteins of *C. botulinum* type B Indian isolate SP11 grown in TPGY (**a**) and CMM medium (**b**). Proteins were separated in the first dimension by a pH 4 to 7 immobilized pH gradient gel (length, 11 cm) and then in the second dimension by a 12% SDS-PAGE. The positions of molecular weight standards are indicated on the right. Protein spots were identified by MALDI-TOF–MS and database searches are indicated by the spot numbers given in Table 1

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Table 1 Proteins from *C. botulinum* type B Indian isolate SP11 secretome as determined by 2-DE using (4–7 narrow range pH strips) and mass spectrometry

S.no	spot ID	Name of the protein	Accession No	Thr.Mr(kDa)	Exp.Mr (kDa)	Isoelectric point(thr.)	Isoelectric point(exp.)	MS-Score	Peptide similarity
1	A2	glyceraldehyde-3-phosphate dehydrogenase, type I [Clostridium botulinum A str. ATCC 3502]	gi|148,378,229	36.39	36.369	6	6.04	125	3
2	A4	chlorohydrolase/aminohydrolase [Clostridium botulinum A str. ATCC 3502]	gi|148,380,839	49.29	49.255	5.8	5.84	743	21
3	A6	hypothetical protein CLOSPO_00563 [Clostridium sporogenes ATCC 15579]	gi|187,777,018	134.68	134.589	5	5.02	567	18
4	A8	bacterial Ig-like domain (group 3) [Lactobacillus crispatus 214–1]	gi|293,380,216	55.8	55.761	9.8	9.81	57	10
5	A9	dehydrogenase, FMN-dependent [Clostridium botulinum A str. ATCC 3502]	gi|148,381,070	35.89	35.868	5.1	5.16	313	10
6	A10	triosephosphate isomerase [Clostridium botulinum H04402 065]	gi|322,804,496	27.3	27.278	5.7	5.76	323	12
7	A12	E-cinnamoyl-CoA:R-phenyllactate CoA transferase large subunit [Clostridium botulinum A str. ATCC 3502]	gi|148,381,235	46.42	46.385	5.3	5.32	917	21
8	B3	thiamine biosynthesis protein ThiC [Clostridium botulinum B1 str. Okra]	gi|170,756,154	48.88	48.841	5.8	5.86	621	4
9	B4	aminopeptidase 1 [Clostridium botulinum A str. ATCC 3502]	gi|148,380,560	57.84	52.632	6	6.11	105	5
10	B5	molecular chaperone GroEL [Clostridium botulinum A str. ATCC 3502]	gi|148,381,241	57.93	57.887	4.7	4.79	88	15
11	B7	putative cell surface protein [Clostridium botulinum A3 str]	gi|170,758,444	146.18	150.882	4.9	4.94	128	8
12	B10	acetyl-CoA acetyltransferase [Clostridium botulinum A str. ATCC 19397]	gi|153,934,044	41.77	41.743	6.1	6.12	685	18
13	C1	carbamate kinase [Clostridium botulinum A str. ATCC 3502]	gi|148,380,549	33.6	33.578	5.6	5.62	126	6
14	C2	secreted protease [Clostridium botulinum A str. ATCC 3502]	gi|148,379,418	65.16	65.113	8.2	8.24	955	15
15	C4	clostripain [Clostridium botulinum A str. ATCC 3502]Best Protein Description	gi|148,379,881	59.67	59.63	4.8	4.88	267	7
16	C5	molecular chaperone DnaK [Clostridium botulinum A str. ATCC 3502]	gi|148,380,911	66.84	66.791	4.8	4.8	1030	18
17	C7	HNH endonuclease [Brucella sp. BO2]	gi|306,840,427	14.23	14.22	9.8	9.87	53	–
18	C9(1)	enolase [Clostridium botulinum NCTC 2916]	gi|168,177,563	46.4	46.363	4.6	4.61	959	–
19	C12	ornithine carbamoyltransferase [Clostridium botulinum A str. ATCC 3502]	gi|148,380,548	37.23	37.207	5.41	5.41	1240	23
20	D2	butyrate kinase [Clostridium botulinum A str. ATCC 3502]	gi|148,381,366	38.97	38.942	5.5	5.53	588	14
21	D3	aspartate/ornithine carbamoyltransferase family protein [Clostridium botulinum B1 str. Okra]	gi|170,754,728	45.36	45.326	5.3	5.36	717	17

Table 1 continued

S.no	spot ID	Name of the protein	Accession No	Thr.Mr(kDa)	Exp.Mr (kDa)	Isoelectric point(thr.)	Isoelectric point(exp.)	MS-Score	Peptide similarity
22	D6	glycosyl hydrolase, family 18 [Clostridium botulinum A str. ATCC 3502]	gi|148,380,791	83.15	83.091	5.6	5.68	1030	23
23	D7(1)	FlaA [Clostridium botulinum]	gi|114,054,817	26.25	26.224	7	7.12	218	3
24	D7(2)	protease inhibitor Kazal-type [Mesorhizobium amorphae CCNWGS0123]	gi|357,026,064	14.79	14.776	8	8.45	60	–
25	D10	serine hydroxymethyltransferase [Clostridium botulinum A str. ATCC 3502]	gi|148,380,550	46.36	46.324	6	6	707	14
26	E4	flagellar hook-associated protein 2 [Clostridium botulinum Ba4 str. 657]	gi|237,796,168	88.91	88.844	5.2	5.25	185	10
27	E6	ECF subfamily RNA polymerase sigma factor [Nitrosomonas europaea ATCC 19718]	gi|30,250,072	19.49	19.475	9.12	9.1	57	–
28	E8	hypothetical protein RTM1035_02410 [Roseovarius sp. TM1035]	gi|149,203,433	70.17	70.112	4.5	4.51	63	–
29	E9	3-hydroxybutyryl-CoA dehydrogenase [Clostridium botulinum A str. ATCC 3502]	gi|148,381,149	30.28	30.256	6.29	6.26	803	12
30	H1	glyceraldehyde-3-phosphate dehydrogenase, type I [Clostridium botulinum A str. ATCC 3502]	gi|148,378,229	36.39	36.369	6.04	6.04	634	-
31	H2	phosphoglycerate kinase [Clostridium botulinum F str. Langeland]	gi|153,941,441	42.98	42.949	5.4	5.44	134	5
32	H4	molecular chaperone GroEL [Clostridium botulinum A str. ATCC 3502]	gi|148,381,241	57.93	57.887	4.79	4.7	1270	–
33	H7	integrase catalytic region [Clostridium phytofermentans ISDg]	gi|160,880,309	35.68	35.65	8.2	9.29	54	–
34	H8	ArgP family transcriptional regulator [Sanguibacter keddieii DSM 10542]	gi|269,795,423	31.97	31.944	5.4	5.41	56	–
35	H9	rubrerythrin [Clostridium botulinum A str. ATCC 3502]	gi|148,379,304	22.37	22.349	5.44	5.44	502	12
36	H11	thermolysin metallopeptidase [Clostridium botulinum B1 str. Okra]	gi|170,754,349	67.17	67.122	6.44	6.44	385	11
37	H12	cell surface protein [Clostridium botulinum A str. ATCC 3502]	gi|148,378,381	150.61	150.505	4.88	4.88	107	6
38	F1	3-hydroxybutyryl-CoA dehydrogenase [Clostridium botulinum NCTC 2916]	gi|168,179,150	30.28	30.268	6.29	6.26	440	9
39	F5	peptidase T [Clostridium botulinum Bf]	gi|168,181,309	45.63	45.595	4.99	4.99	732	16
40	F7	glucose-6-phosphate isomerase [Clostridium botulinum A str. ATCC 3502]	gi|148,381,223	50.48	50.444	5.45	5.45	105	3
41	F8	putative carbamate kinase [Clostridium botulinum]	gi|225,166,838	34.24	34.216	7.74	7.74	57	
42	F10	aminopeptidase 1 [Clostridium botulinum A str. ATCC 3502]	gi|148,380,560	57.84	52.632	5.7	5.7	369	21

Table 1 continued

S.no	spot ID	Name of the protein	Accession No	Thr.Mr(kDa)	Exp.Mr (kDa)	Isoelectric point(thr.)	Isoelectric point(exp.)	MS-Score	Peptide similarity
43	F11	glycosyl hydrolase, family 18 [Clostridium botulinum A str. ATCC 3502]	gi|148,380,791	83.15	83.091	5.68	5.68	676	14
44	F12	flagellin [Clostridium botulinum]	gi|169,950,589	29.8	29.772	8.87	8.87	187	14
45	G2	glycerol dehydrogenase [Clostridium botulinum F str. Langeland]	gi|153,939,851	40.21	40.185	5.11	5.11	406	9
46	G4	phenylalanyl-tRNA synthetase, alpha subunit [Providencia rustigianii DSM 4541]	gi|261,344,012	37	36.968	5.5	5.5	51	
47	G6	M24 family metallopeptidase [Clostridium botulinum F str. Langeland]	gi|153,940,046	67.84	67,794	5.35	5.35	72	6
48	G7	DNA polymerase III subunit alpha [Thermocrinis albus DSM 14484]	gi|289,548,089	131.15	131.047	6.1	6.1	40	
49	G8	ATP-dependent Clp protease proteolytic subunit [Clostridium botulinum A str. ATCC 3502]	gi|148,381,180	21.51	21.489	5.29	5.29	495	12
50	G11	myosin-cross-reactive antigen [Clostridium botulinum A str. ATCC 3502]	gi|148,380,004	67.56	67.51	5.08	5.08	412	13
51	G12	3-hydroxybutyryl-CoA dehydrogenase [Clostridium botulinum A str. ATCC 3502]	gi|148,381,149	30.28	30.256	6.29	6.29	407	9

S.no	sequence coverage	Signal P	Subcellular location	Secretome p	Prosite	TMHMM	Lipo P	GRAVY score b	Function
1	4	No	Cytoplasmic	Yes	D-Erythrose 4-phosphate dehydrogenase (E4PDH)	0	–	−0.139	Carbohydrate transport and metabolism
2	29	No	Cytoplasmic	no	No hit	0	–	−0.268	Unknown function
3	9	Yes	Cell wall	No	No hit	0	–	−0.531	Unknown function
4	–	Yes	Extracellular	yes (0.957834)	No hit	1	SpI	−0.493	Host–cell interaction
5	17	NO	cytoplasmic	no	Lactatedehydrogenase, glycolate oxidase,	0	–	−0.0937	Metal binding
6	34	NO	Cytoplasmic	no	No hit	0	–	−0.214	Carbohydrate transport and metabolism
7	38	NO	Cytoplasmic	no	No hit	0	–	−0.496	Amino acid transport and metabolism
8	6	NO	Cytoplasmic	no	No hit	0	–	−0.414	nucleotide transport and metabolism
9	5	No	Cytoplasmic	no	No hit	0	–	−0.412	Metal binding

Table 1 continued

S.no	sequence coverage	Signal P	Subcellular location	Secretome p	Prosite	TMHMM	LipoP	GRAVY scoreb	Function
10	18	No	Cytoplasmic	No	Escherichia coli groEL protein,Cyanobacterial groEL,	0	–	– 0.094	Posttranslation modification, protein turnover, chaperones
11	–	No	Cell wall	Yes (0.779813)	No hit	0	–	– 0.542	Unknown
12	30	No	Cytoplasmic	No	Thiolases acyl-enzyme intermediate signature,	0	–	– 0.089	Lipid transport and metabolism
13	11	No	unknown	No	No hit	0	SpI	-0.142	Amino acid transport and metabolism
14	16	No	extracellular	Yes (0.921336)	Neutral zinc metallopeptidases, zinc-binding region signature:	0	–	– 0.639	Protease activity
15	8	YES	unknown	Yes (0.900254)	No hit	0	SpI	-0.687	Protease activity
16	22	No	cytoplasmic	yes(0.689319)	Heat shock hsp70 proteins family signature 1,	0	–	– 0.367	Posttranslation modification, protein turnover, chaperones
17	–	No	Cytoplasmic Membrane	No	No hit	0	–		Endonuclease activity
18		NO	cytoplasmic	No	Enolase signature	0	–	– 0.109	Carbohydrate transport and metabolism
19	47	No	cytoplasmic	No	Aspartate and ornithine carbamoyltransferases signature	0	–	– 0.275	Amino acid transport and metabolism
20	25	NO	cytoplasmic	No	Acetate and butyrate kinases family signature 1 and 2	0	–	– 0.22	Lipid metabolism and transport
21	28	No	cytoplasmic	No	No hit	0	–	– 0.429	Amino acid transport and metabolism
22	20	YES	unknown	Yes (0.92417)	No hit	1	–	– 0.708	Carbohydrate binding
23	7	No	cytoplasmic	No	No hit	0	–	– 0.513	Flagellar synthesis and maintenance
24	–	YES	unknown	NO	Prokaryotic membrane lipoprotein lipid attachment site profile, Kazal domain profile	0	SpII	-0.425	Protease activity
25	23	No	cytoplasmic	No	Serine hydroxymethyltransferase pyridoxal-phosphate attachment site:	0	–	– 0.358	Amino acid meta bolism and transport

Table 1 continued

S.no	sequence coverage	Signal P	Subcellular location	Secretome p	Prosite	TMHMM	Lipo P	GRAVY scoreb	Function
26	7	No	Extracellular	yes (0.932446)	No hit	0	–	– 0.558	Flagellar synthesis and maintenance
27	–	No	cytoplasmic	No	No hit	0	–	– 0.118	transcription,
28	–	No	cytoplasmic	No	No hit	0	–	– 0.304	Unknown
29	30	No	cytoplasmic	No	Bacterial 3-hydroxybutyryl-CoA dehydrogenase.Eye lens protein lambda-crystallin	0	–	– 0.037	Lipid metabolism and transport
30	4	No	cytoplasm	No	D-Erythrose 4-phosphate dehydrogenase (E4PDH)	0	–	– 0.139	Carbohydrate metabolism and transport
31	6	NO	cytoplasmic	No	Phosphoglycerate kinase signatur	0	–	– 0.187	Carbohydrate metabolism and transport
32	18	NO	cytoplasmic	No	Escherichia coli groEL protein,Cyanobacterial groEL,	0	–	– 0.094	Posttranslation modification, protein turnover, chaperones
33	–	No	cytoplasmic	yes 0.642398	Integrase catalytic domain profile	0	–	– 0.837	DNA recombination
34	–	No	unknown	No	LysR-type HTH domain profile	0	–	0.02	Transcription
35	43	NO	cytoplasmic	No	Ferritin-like diiron domain profile,Rubredoxin-like domain profile	0	–	– 0.616	Metal binding
36	13	YES	Extracellular	yes 0.918920	Neutral zinc metallopeptidases, zinc-binding region signature	1	–	– 0.675	Protease activity
37	2	yes	Cell wall	yes0.804414	No hit	0	–	– 0.494	Host cell interaction
38	30	no	cytoplasm	No	Bacterial 3-hydroxybutyryl-CoA dehydrogenase, Eye lens protein lambda-crystallin	0	–	– 0.037	Lipid metabolism and transport
39	25	No	cytoplasmic	no	ArgE/dapE/ACY1/CPG2/yscS family signature	0	–	– 0.342	Protease activity
40	4	No	cytoplasmic	no	Glucose-6-phosphate isomerase family profile, Phosphoglucose isomerase signature	0	–	– 0.337	Carbohydrate transport and metabolism
41	–	No	unknown	no	No hit	0	–	– 0.213	Amino acid transport and metabolism
42	5	No	cytoplasmic	no	No hit	0	–	– 0.363	Protease activity
43	13	YES	unknown	No	No hit	0	–	– 0.708	Carbohydrate metabolism and transport

Table 1 continued

S.no	sequence coverage	Signal P	Subcellular location	Secretome p	Prosite	TMHMM	LipoP	GRAVY scoreb	Function
44	35	No	cytoplasmic	no	No hit	0	–	– 0.446	Flagellar synthesis and maintenance
45	18	No	cytoplasmic	no	Iron-containing alcohol dehydrogenases signature 1	0	–	0.075	Glycerol metabolism
46	–	No	cytoplasmic	no	Aminoacyl-transfer RNA synthetases class-II family profile	0	–	– 0.361	Protein translation
47	6	No	CytoplasmicMembrane	no	No hit	0	–	– 0.385	Protease activity
48	–	No	cytoplasmic	no	No hit	0	–	– 0.381	DNA Replication
49	38	No	cytoplasmic	No	Endopeptidase Clp serine active site	0	–	– 0.188	Stress response
50	14	No	cytoplasmic	No	protease inhibitor family signature	1	–	– 0.447	Lipid transport and metabolism
51	30	No	cytoplasmic	No	No hit	0	spI	– 0.037	Lipid transport and metabolism

Spot numbers refer to the numbers on the 2-DE gels shown in Fig. 1a, b

Spot numbers refer to Fig. 1a and b

Protein functions were predicted by UniProt Knowledgebase (Swiss-Prot and TrEMBL)

Presence of signal peptide predicted by SignalP 3.0

Non-classical i.e. non-signal peptide triggered protein secretion by SecretomeP. For each input sequence the server predicts the possibility of non-classical secretion. For bacteria, four scores are generated by the SecretomeP server for each input sequence. The determining score is the 'SecP score', for which a value above 0.5 indicates possible secretion. Values in parenthesis are SecP scores

Number of predicted transmembrane helices in proteins using TMHMM Server v. 2.0

Prediction of lipoproteins and signal peptides using LipoP 1.0

Grand average of hydropathy

Prediction of protein sub cellular localization using PSORT version 2.0

hydrolase, family 18, ATP-dependent Clp protease proteolytic subunit, flagellin and M24 family metallopeptidase were predominant proteins. Elevated levels of these proteins had been also seen in *C. tyrobutyricum* secretome (Ma et al. 2015). Clostripain (Shimizu et al. 2002), ThiC (Martinez-Gomez and Downs 2008), peptidaseT (Strauch et al. 1983), Glycerol dehydrogenase (Monniot et al. 2012), glyceraldehyde-3-phosphate dehydrogenase and acetyl-CoA acetyltransferase (Prasad et al. 2013; Sengupta et al. 2010) predominant proteins had been also identified by other researchers in other microorganism using proteomic approach.

Bioinformatics analysis

Clostridium botulinum type B secretary proteins were grouped into different cellular functions and shown in Fig. 2. Identified secretary proteins are involved in carbohydrate 13.7%, amino acid and lipid 11.76% metabolism, protease activity (13.7%), metal binding,

posttranslational modifications, flagellar synthesis and maintenance (5.88%), host cell interaction and transcription process (3.9%), protein translation, carbohydrate binding, endonuclease activity, stress response, DNA replication and recombination (1.9%) and unknown function (7.8%). Similar trends were also observed in *B. anthracis* secretome in which 17.8% proteins are involved in energy metabolism, 10.9% for protein synthesis, 8.7% cellular structure and 13% proteins functions are unknown (Walz et al. 2007). Hypothetical protein CLOSPO_00563, putative cell surface protein and cell surface protein were predicted as cell wall-associated protein, bacterial Ig-like domain, secreted protease, flagellar hook-associated protein 2 and thermolysin metallopeptidase were identified as extracellular proteins, HNH endonuclease and M24 family metallopeptidase found as cytoplasmic membrane proteins and 35-cytoplasmic proteins and 7-proteins of unknown localization were predicted using PSORTb software. Utmost of the *C. botulinum* type B secretome proteins were situated at
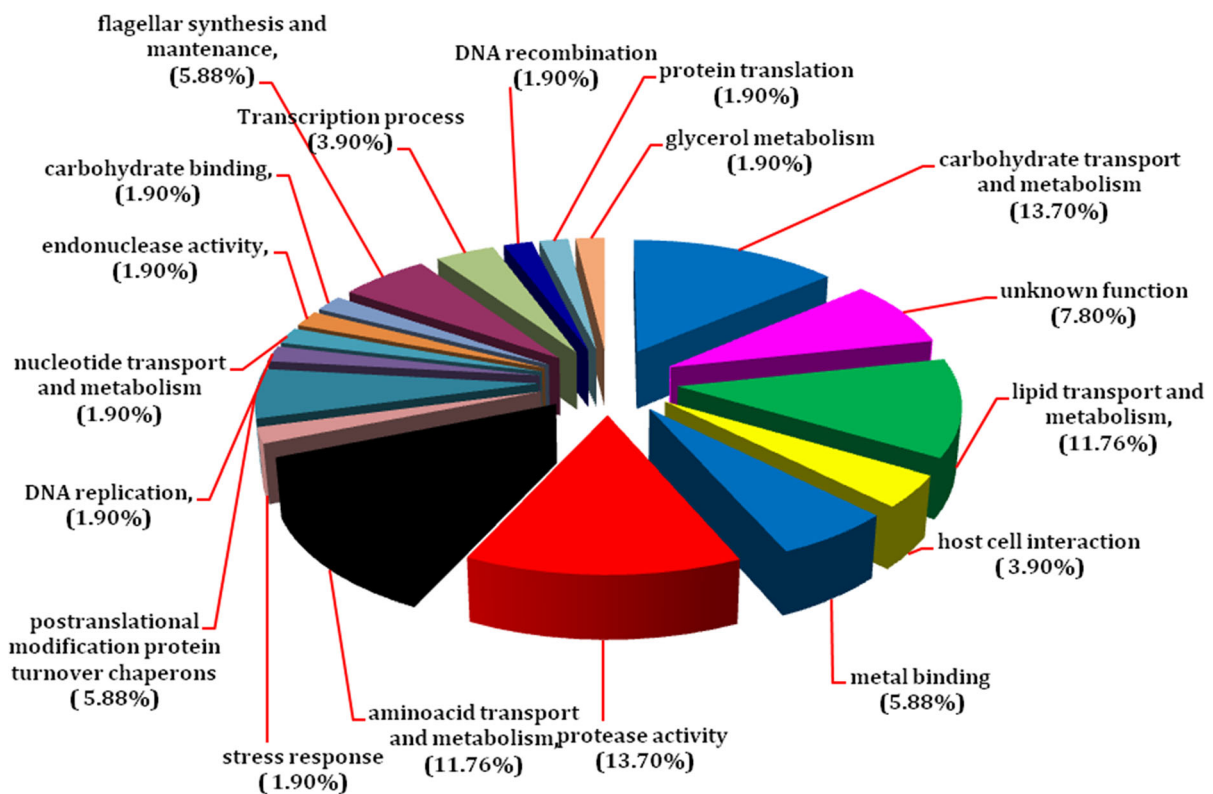

Fig. 2 Graphical representation of the cellular functions of secretome of *C. botulinum* type B Indian isolate SP11: the majority of the proteins were involved in transport and metabolism of carbohydrate, amino acid and lipid, protease activity, post-translational modification and chaperones

the cytoplasmic region and did not contain signal sequence predicted by Signal P. Utmost of the enzymes involved in metabolism and energy production were present in the *C. botulinum* secretome. Similar results were also observed in *Shigella flexneri* (Liao et al. 2003). 15.68% of proteins were predicted by Signal P to be secreted in the classical Sec pathway, which is characterized by the presence of a signal peptide (Shah et al. 2009). But, three proteins were shown to possess the cleavage site for signal peptidase I (SpI) and one protein for signal peptide II (SpII) are being shown in Table 1. Four proteins have transmembrane helices as predicted by TMHMM, representing an extra cytoplasmic but membrane-associated location. Less than 50% of the proteins were predictably secreted by either non-classical pathways as indicated by the presence of a signal peptide (Signal P) or non-signal peptide triggered protein secretion (Dwivedi et al. 2015).

Immunogenic proteins of *C. botulinum* type B

Immunoproteomics is an extension of proteomics, which permits specific elucidation of proteins based on immunoreactivity (Fulton et al. 2019). In procedure of immunoproteomics, 2-D gel blots are probed with antiserum collected from host post infection. This development has bypassed the extensive process of testing immunoreactivity and henceforth vastly enhanced the vaccine discovery by directly permitting the identification of those novel immunogenic proteins which evoke immune system. Secretary proteins of human disease causing agents are of special attention; in particular, these proteins play a role in the preliminary phase of pathogenesis when they arrive direct contact with host tissues (Kennedy 2018). If pathogenesis can be halted efficiently at the preliminary stage, infection can be paused. Secretary proteins can induce defensive immunity and also provoke an immune response which will be of specific attention for the development of vaccine/diagnostic markers. To determine the immunogenic proteins of *C. botulinum* secretome, antiserum of secretary proteins (1:30,000 dilution) were used as primary antibody and serum from unimmunized mice were used as control. Seventeen and ten immunogenic proteins were identified in TPGY and CMM media respectively, results are being shown in Figs. 3, 4 and tabulated in Tables 2, 3. Flagellin, secreted protease, hypothetical protein, ornithine carbamoyl transferase (OCT) and molecular chaperone GroEL were common immunogenic protein and are being tabulated in
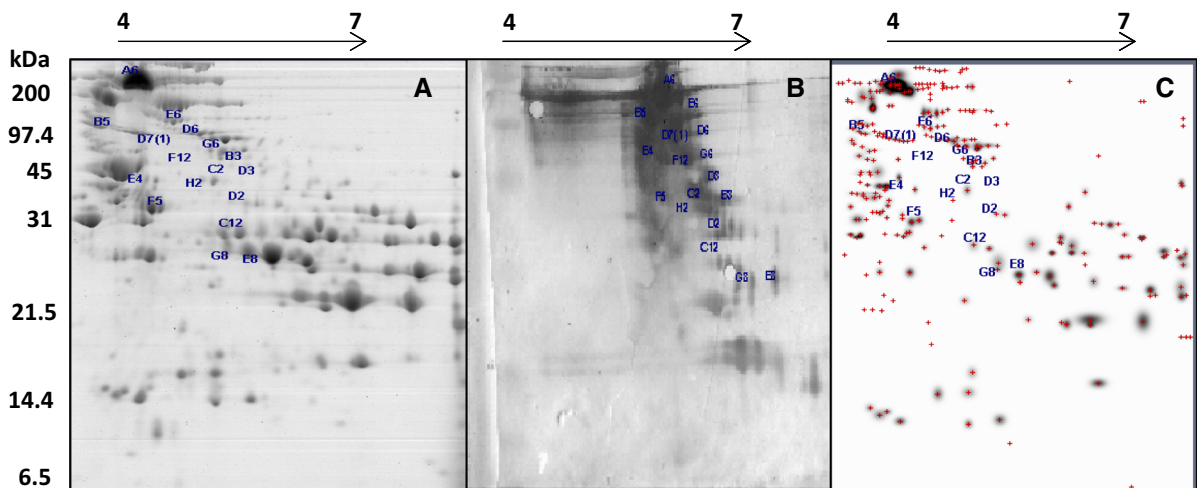

Fig. 3 Immunoblot analysis: antisera of secretory proteins (TPGY) 1/30,000 dilutions binding to secretome proteins of *C.botulinum* type B Indian isolate SP11 grown in TPGY medium. Secretome of *C. botulinum type B* were separated by 2-DE (pH 4–7, length 7 cm) and stained with Bio-safe Coomassie G-250. **a** 2DE-map, **b** Western blot and **c** overlay image showed protein spots which were correspond in both 2-DE as well as immunoblot. The positions of molecular weight standards are indicated on the left. The immunoreactive 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
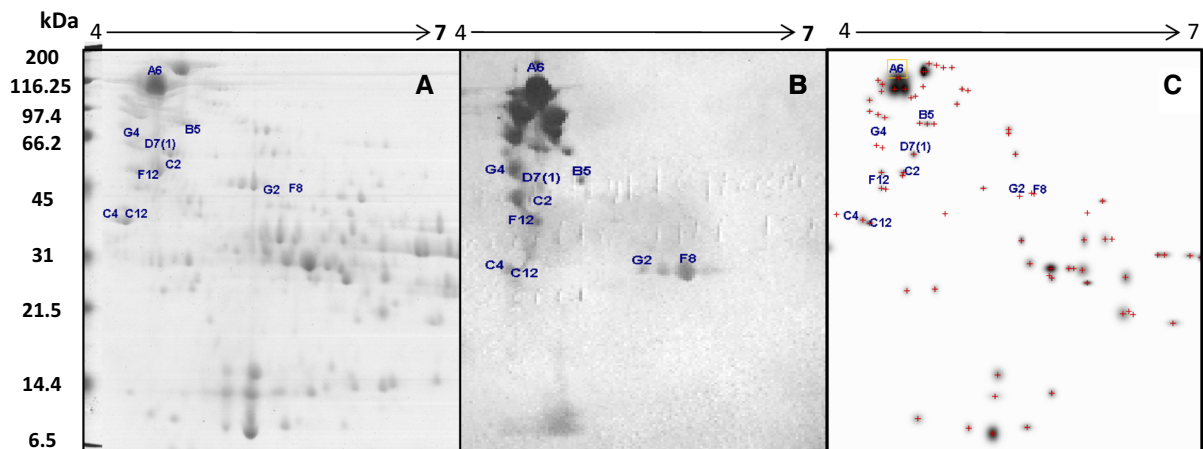
Fig. 4 Immunoblot analysis: antisera of secretory proteins (CMM) 1/30,000 dilutions binding to secretome proteins of *C.botulinum* type B Indian isolate SP11 grown in CMM medium. Secretome of *C. botulinum type B* were separated by 2-DE (pH 4–7, length 7 cm) and stained with Bio-safe Coomassie G-250. **a** 2DE-map, **b** Western blot and **c** overlay image showed protein spots which were correspond in both 2-DE as well as immunoblot. The positions of molecular weight standards are indicated on the left. The immunoreactive protein spots were identified by MALDI-TOF–MS and database searches are indicated by the spot numbers given in Table 3. Three technical replicates were organized in independent experiments; these were very similar, and one of them was revealed

Table 2 Immunogenic proteins from *C. botulinum* type B Indian isolate SP11secretome grown in TPGY media as determined by 2-DE using (4–7 narrow range pH strips) and Mass spectrometry. Spot numbers refer to the numbers on the 2-DE western blot shown in Fig. 4

Spot ID	Extra-cellular Immunogenic proteins in TPGY media
B3	Thiamine biosynthesis protein ThiC [*Clostridium botulinum* B1 str. Okra]
A6	Hypothetical protein CLOSPO_00563 [*Clostridium sporogenes* ATCC 15579]
D2	Butyrate kinase [*Clostridium botulinum* A str. ATCC 3502]
D3	Aspartate/ornithine carbamoyltransferase family protein [*Clostridium botulinum* B1 str. Okra]
D6	Glycosyl hydrolase, family 18 [*Clostridium botulinum* A str. ATCC 3502
C12	Ornithine carbamoyltransferase [*Clostridium botulinum* B1 str. Okra]
E4	Flagellar hook-associated protein 2 [*Clostridium botulinum* Ba4 str. 657]
E6	ECF subfamily RNA polymerase sigma factor [*Nitrosomonas europaea* ATCC 19718]
E8	Hypothetical protein RTM1035_02410 [*Roseovarius sp*. TM1035]
F5	Peptidase T [*Clostridium botulinum* Bf]
G6	M24 family metallopeptidase [*Clostridium botulinum* F str. Langeland]
G8	ATP-dependent Clp protease proteolytic subunit [*Clostridium botulinum* A str. ATCC 3502
H2	Phosphoglycerate kinase [*Clostridium botulinum* F str. Langeland]
D7(1)	FlaA [Clostridium botulinum]
F12	Flagellin [Clostridium botulinum A str. ATCC 3502]
C2	Secreted protease [Clostridium botulinum A str. ATCC 3502]
B5	Molecular chaperone GroEL [Clostridium botulinum A str. ATCC 3502]

Table 4. These proteins may be the best bioproducts for development of vaccine candidates or diagnostic markers against the infant and wound botulism. Cross reactive immunogenic proteins were also identified using secretary protein antisera of *C. botulinum* type A/E & F (supplement Fig. 1) and are being shown in

Table 3 Immunogenic proteins from *C. botulinum type* B Indian isolate SP11secretome grown in CMM media as determined by 2-DE using (4–7 narrow range pH strips) and mass spectrometry

Serial no	Extra- cellular Immunogenic proteins in CMM medium
A6	Hypothetical protein CLOSPO_00563 [Clostridium sporogenes ATCC 15,579
B5	Molecular chaperone GroEL [Clostridium botulinum A str. ATCC 3502]
F8	Putative cell surface protein [Clostridium botulinum A3 str. Loch Maree]
C4	Clostripain [Clostridium botulinum A str. ATCC 3502]Best Protein Description
C12	Ornithine carbamoyltransferase [Clostridium botulinum B1 str. Okra]
G2	Glycerol dehydrogenase [Clostridium botulinum F str. Langeland
G4	Phenylalanyl-tRNA synthetase, alpha subunit [Providencia rustigianii DSM 4541]
D7(1)	FlaA [Clostridium botulinum]
F12	Flagellin [Clostridium botulinum A str. ATCC 3502]
C2	Secreted protease [Clostridium botulinum A str. ATCC 3502]

Table 4 Common Immunogenic proteins from *C.botulinum type* B Indian isolate SP11 secretome grown in TPGY and CMM media

Serial no.	Extra- cellular Immunogenic proteins in TPGY and CMM media
A6	Hypothetical protein CLOSPO_00563 [*Clostridium sporogenes* ATCC 15579]
C12	Ornithine carbamoyltransferase [*Clostridium botulinum* B1 str. Okra]
D7(1)	FlaA [Clostridium botulinum]
F12	Flagellin [Clostridium botulinum A str. ATCC 3502]
C2	secreted protease [Clostridium botulinum A str. ATCC 3502]
B5	Molecular chaperone GroEL [Clostridium botulinum A str. ATCC 3502]

supplementary Figs. 2, 3 and tabulated in Tables 1 and 2. In *C. difficile* GroEL and DnaK were reported as immunogenic proteins and the recombinant GroEL immunized group decreases the intestinal colonization of *C. difficile* (Pechine et al. 2013). GroEL proteins had been also reported to be potent immunogens in a number of infections. Using immunoproteomics, GroEL of *Neospora caninum* has been identified to be immunogenic protein (Shin et al. 2004). Immunization with GroEL of *Bacillus anthracis* provides 100% protection against *Bacillus anthracis* infection in BALB/c mice (Sinha and Bhatnagar 2010). Merakou et al. showed that the flagellin protein enhancing immunity against *P. aeruginosa*. Antibodies against Flagellin protein were more potent in mediating opsonic killing of *P. aeruginosa* and mediating passive immunity in mice. This was attributed to flagellin inducing high titres of antibodies which could neutralise the innate immunity due to TLR5 activation (Merakou et al. 2018). Secreted protease play important roles in bacterial virulence during host infection,

and have been previously proposed as potential vaccine candidates (Wang et al. 2019). OCT was previously suggested as a vaccine candidate to protect against both biofilm-related and acute *Streptococcus suis* infections (Wang et al. 2020). Whereas in the case of *C. perfringens* endo-beta-N-acetylglucosaminidase, SagA protein, phospholipase C, translation elongation factor, acetyl-CoA acetyltransferase, fructose-bisphosphate aldolase and ornithine carbamoyl transferase were reported as immunogenic proteins and the recombinant ornithine carbamoyl transferase extended the death time of mice challenged with *C. perfringens* (Sengupta et al. 2010). Phosphoglycerate kinase was successfully expressed by prokaryotic expression system and the recombinant protein showed favorable immunogenicity in mice (He et al. 2013) Peptidase protein had been known as immunogenic protein in *anopheles culicifacies* or *gambiae* for epitope design using immuno-informatics models (Jakhar et al. 2019). Metallopeptidase was identified as immunogenic proteins in various cancer

including breast cancer (Chantada-Vázquez et al. 2020). Glycosyl hydrolase had been screened as immunogenic proteins in *Clostridium chauvoei* and identified as cell surface associated proteins by immunoproteomic method (Coral 2009). Phenyl alanyl-tRNA synthetase had been identified as immunogenic protein using immunoproteomic analysis of protective response obtained with subunit and commercial vaccine against glasser's disease caused by *haemophilus parasuis* (Li et al. 2017). Immunogenic proteins which identified in different microorganism involved in different disease had also been explore in *C. botulinum* type B secretome these proteins may be used as vaccine candidate as well as diagnostic marker against the botulism. In this work, there were several highly plentiful proteins in the 2-DE gels which did not obtain as immunoreactive. Hence it is rational to recommend that the immunogenic proteins ones may be the noteworthy vaccine candidate as well diagnostic marker against botulism. The protective efficiencies of immunoreactive proteins identified either by alone or in altered combinations remain to be determined in further works.

Conclusions

The present study explored the secretome proteins of *C. botulinum* type B and identified the predominant immunogenic proteins using 2-DE immuno proteomic approach. To the best of our knowledge this is the first study to explore the secretome proteomics as well as identification of immuno dominant proteins of *C. botulinum*. In this study we have identified 17 immuno dominant in TPGY media and 10 proteins in CMM media. Five immunogenic proteins were common in both the media. Apart from that, cross reactive study of proteins had been also carried out to find out the cross reactive proteins in among *C. botulinum* types A, B, E and F. *C. botulinum* types A, B and E secretome showed the cross reactivity but *C. botulinum* type F did not show cross reactivity with the secretome of *C. botulinum* type B. Cross reactive proteins and common immunogenic proteins will be further validated in future study for their potential to be used as a vaccine/diagnostic candidates against infant and wound botulism.

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