DISCUSSION

In humans, botulism occurs in three forms food-borne, wound, and infant botulism. Food-borne botulism was mainly caused by intake of food those containing preformed botulinum toxins. Current industrial food processing and packaging were designed to kill *C. botulinum* spores but most of the food borne botulism cases is reported from the home made foods such as meats, sausages, fermented fish, seal as well as whale meat. Apart from canned foods, other packed foods such as foodstuffs, yogurt, garlic oil and foil-wrapped baked potatoes will provide the congenial atmosphere for the growth of *C. botulinum*, for example baked foodstuffs that have been left at room temperature or in a warm oven overnight may cause botulism. During baking process anoxic environment will be created which will reduce the bacterial density of other aerobic competing microorganisms (Cardoso et al., 2004; McLauchlin et al., 2006; Brola et al., 2013; Momose et al., 2014; Mazuet et al., 2015). Wound botulism appeared when deep wound is contaminated with *C. botulinum* spores beneath the wound anaerobic environment prevails, these spores convert into vegetative cells and and produce botulinum toxin. Wound botulism is rare illness except in injecting drug abusers, where it is caused by contaminated needles or drugs (De Rosa et al., 2015). It is common among people who insert “black tar heroin” into the subcutaneous tissues (Abavare and Abavare, 2012; Gouveia et al., 2012; McDonald et al., 2013; Tiedeken et al., 2014; De Rosa et al., 2015). Infant botulism was mainly reported in children within 3 to 6 months old. In this type, *C. botulinum* spores are swallowed and will be germinate in the large intestinal tract and produces toxin. In Infants intestine,
micro flora are not well developed so they are unable to compete with clostridial species (Wells and Wilkins, 1996; Arnon et al., 2006; Elad, 2006; Risko, 2006; Vander Vorst et al., 2006). Honey has been linked with a few cases of infant botulism, but spores can also be found in a lot of other sources including dust (Godart et al., 2014). Botulinum spores from the environment can be ingested by children above 3 years and adults without hurt; these spores easily pass through the intestines without germinating (Swaan et al., 2010). Nevertheless, there are rare cases of intestinal colonization botulism in people who have distorted intestinal conditions from gastrointestinal surgery and intensive antibiotic therapy or abnormalities such as achlorhydria (Ramroop et al., 2012; Derman et al., 2014; Kobayashi et al., 2014; Pifko et al., 2014; Ringe et al., 2014; Rosow and Strober, 2015; Sabatini et al., 2016).

The rising threat of bioterrorism and continued materialization of new infectious diseases has driven a major resurgence in biomedical research efforts to build up improved treatments, diagnostics and vaccines, as well as augment the fundamental understanding of the host immune response to infectious agents (Vigil et al., 2010; McCusker and Kelley, 2013; Mifsud et al., 2014). The accessibility of multiple mass spectrometry platforms joint by multidimensional separation technologies and microbial genomic databases provides an extraordinary opportunity to identify the probable potential candidate vaccine molecule (Haag et al., 2001; Purcell and Gorman, 2004; Zhao et al., 2013; Gnanandarajah et al., 2014). The rising area of immunoproteomics as applied to the expansion of new vaccine targets has been also summarized by many researchers (Millon et al., 2014; Wang et al., 2014; Belousov et al., 2015; Liu et al., 2015; Agallou et al., 2016; Pellon et al., 2016). These powerful techniques can identify potential new protein targets; nevertheless, translating these proteomic discoveries to counter-bioterrorism requires collaborative research efforts across multiple basic science and clinical disciplines. The main challenge for subunit-based vaccines is the recognition of the molecules that elicit protective humoral immune responses (Bernstein et al., 1988; Plante et al., 2001; McLain et al., 2012). At the beginning, protective antigen identification has been carried out by fractionating microbial total cell extracts and culture supernatants by classical biochemical procedures and find out those fractions keeping specific components that cross-react with antisera from convalescent patients. These components are then examined proper
in vitro as well as in vivo protection models to select the ones that finally reach human trials. This approach for vaccine candidate assortment has often proved to be lengthy and time-consuming and relies on the hypothesis (not always true) those protective antigens is the ones that induce immune responses by natural infections. Because every subunit-based vaccines eliciting protective antibody responses fall in the category of secreted and highly expressed, surface-exposed proteins, a trustworthy experimental method able to selectively identifying these two categories of antigens would significantly reduce the number of molecules to be tested in protection assays and eventually speed up the whole vaccine discovery process (Doro et al., 2009; Braconi et al., 2011). The aim of the present study was to elucidate the extracellular as well as whole-cell proteome and identify the predominant immunogenic proteins of C. botulinum type B for the development of potential vaccine candidate / diagnostic markers against botulism. In the case of infant botulism spores enter into the body and multiply in large intestine and produces botulinum neurotoxins which eventually cause the flaccid paralysis. While in wound botulism, wound is contaminated with spores then spores germinate and converted into vegetative cells. These cells ultimately produce botulinum toxin which circulate in blood stream and cause flaccid paralysis. Here the objective was to identify the potential immunogenic molecules from C. botulinum type B which can block the bacterial colonization in the body, molecules which can clear the bacterial infection. 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. Extracellular proteins and surface proteins of human pathogens are of special interest; in particular, these proteins play a role in the initial phase of pathogenesis when they come into direct contact with host tissues. If pathogenesis effectively can be inhibited at the initial phase, infection can be halted (Singh et al., 2012). Extracellular proteins and surface proteins can induce protective immunity and also elicit an immune response will be of particular interest for the development of vaccine or diagnostic markers (Stalhammar-Carlemalm et al., 1999; Nayak et al., 2010). In the present study, We have recently proposed a novel proteomics-based approach for the characterization secretory as well as whole-cell proteins of C. botulinum type B. For secretome study, we collected the supernatant from five days old culture of C. botulinum type B, precipitated the soup with 10% tricholoroacetic acid and collect the
secretory proteins by centrifugation. Similarly for the whole-cell proteome preparation, the *C. botulinum* type B culture pretreated with lysozyme followed by sonication. After sonication, the mixture was centrifuged and collected the supernatants. These supernatants was precipitated with pre chilled 100 % acetone. Both secretome as well as whole cell proteins were subjected proteome analysis by two dimensional gel electrophoresis. To find out the immunogenic proteins, the immunoblotting assay were carried out using secretory proteins as well as whole-cell protein antisera raised in BALB/c mice.

The short list the differentiated expressed proteins, *C. botulinum* type B cultures were grown in both TPGY and CMM media. Optimized the 2DE of secretome and whole-cell proteome of *C. botulinum* type B. Most of the secretome and whole-cell proteins were well separated in the narrow \( pH \) range 4-7 and 4.7 to 5.9 strips. In this study we have successfully elucidated the predominantly expressed proteins of the secretome and whole-cell proteome of *C. botulinum* type B grown in TPGY and CMM media. The results reveals that most of the secretome and whole-cell proteins of *C. botulinum* type B was over expressed in TPGY media when compared with CMM media. Among the predominantly expressed secretome proteins in both the media (Table 2) most of the proteins involved in carbohydrate, amino acid, fat and protein metabolism as well as transport functions followed by protease activity, metal binding and different cellular synthesis. Where as in the case of *C. tyrobutyricum* secretome elevated levels acetaldehyde/alcohol dehydrogenase, 3-hydroxybutyryl-CoA dehydrogenase, thiolase, acetyl-CoA acetyltransferase, acrotonase, chaperone proteins and glycerol-3-phosphate dehydrogenase was observed (Ma et al., 2015). The results of the present investigations are correlating with earlier publications and most of the proteins identified in the secretome of Clostridia and few other pathogens have been analysed for their potential as a vaccine / diagnostic markers. Few hits we found are clostripain (Shimizu et al., 2002), Thic C (Martinez-Gomez and Downs, 2008), peptidase T (Strauch et al., 1983), Glycerol dehydrogenase (Monniot et al., 2012), glyceraldehyde-3-phosphate dehydrogenase and acetyl-CoA acetyltransferase (Sengupta et al., 2010; Prasad et al., 2013).

From the secretome proteins, 13.7% accounts for carbohydrate metabolism and its transport followed by 11.76% proteins involved in amino acid metabolism and 13.7%
for lipid protease activity. Similar trends were also observed in B. anthracis secretome in which 17.8% proteins are involved energy metabolism, 10.9% for protein synthesis, 8.7% cellular structure and 13% proteins functions are unknown (Walz et al., 2007). In whole-cell proteome of TPGY grown C. botulinum type B (Table 3) majority of the proteins were involved in energy production and conversion, Carbohydrate transport and metabolism, amino acid transport and metabolism followed by protein transport and metabolism, lipid transport and metabolism, stress response, cell motility, protease activity, receptor binding, cell morphogenesis. Similarly, when bacteria were grown in CMM medium (table 4), most of the protein of C. botulinum were involved in amino acid transport and metabolism followed by carbohydrate transport and metabolism, protein transport and metabolism, lipid transport and metabolism and remaining were involved in nucleotide transport and metabolism, stress response, protease activity, receptor binding and 8% proteins were of unknown functions.

Most of the C. botulinum type B secretome as well as whole-cell proteins were obtained to be located at the cytoplasmic region and did not contain signal sequence which predicted by using Signal P. Most of the enzymes involved in metabolism and energy production were present in the C. botulinum secretome as well as whole-cell proteome. Similar results was also observed in Shigella flexneri (Liao et al., 2003).

Although metabolic proteins are known to play key functions in energy production which are necessary for survival, it has also been considered that they play a role in virulence. The in silico tools such as Signal P and PSORTb facilitated the prediction for cellular localization and the presence of signal peptide of C. botulinum type B secretome proteins. Signal P analysis indicated that only seven proteins were truly secretory proteins they are as follows; hypothetical protein CLOSPO_00563, bacterial Ig-like domain (group 3), clostripain, glycosyl hydrolase, protease inhibitor Kazal-type, thermolysinmetallopeptidase and cell surface protein whereas four proteins were predicted to be extracellular using PSORT. These proteins could have been secreted via the type III secretion system or the two-partner secretion pathway or auto transporters (Lory, 1992). In general, phospholipases such as phospholipid / glycerol acetyl transferase are key virulence factors, contributing to bacterial survival or dissemination without causing tissue destruction. Perhaps the most intriguing aspect
of phospholipases as virulence factors is their potential to interfere with cellular signaling cascades and to modulate the host immune response (Schmiel and Miller, 1999). In the inflammatory environment, these phospholipids-degrading enzymes are further released by the bacterium, like a bacterial defense, when the host responds to microbial invasion. This enzyme has also been suggested to be involved in lipid biogenesis during cell growth and therefore may play an important role in maintaining the cellular integrity of bacterial cells during multiplication in the host (Zhang and Rock, 2008). The ATP-dependent metalloprotease was predicted to be located on the cytoplasmic membrane. TMHMM (De Rosa et al., 2015) predicted two transmembrane helices and therefore this could be a membrane translocated protein. However, the protease ATP-binding predicted as a cytoplasmic heat-shock protein did not have any transmembrane helices or signal peptide suggesting that this was not a secretory protein. ATP-dependent metalloprotease and protease ATP-binding proteins are known to be involved in posttranslational modification protein turnover and chaperones-like protein. Metalloproteases are being considered as efficacious candidates for vaccine development. It was demonstrated that immunizations of mice using a conserved zone metalloprotease peptide decreased the severity of *B. cepacia* infection (Corbett et al., 2003).

Similarly, *C. botulinum* type B whole cell proteins were grouped into different cellular functions and shown in Figure 9. The greater part of the whole cell proteins (TPGY) were involved in energy production and conversion (22% Carbohydrate transport and metabolism), amino acid (21%), protein (14%), lipid (7%) transport and metabolism, stress response (7%), cell motility (5%), protease activity (4%), receptor binding (2%), cell morphogenesis (2%) and remaining 11% were unknown proteins. Similarly, when bacteria were grown in CMM medium, most of the protein of *C. botulinum* were involved in amino acid transport and metabolism (30.5%) followed by carbohydrate transport and metabolism (19.4%), protein transport and metabolism (13.8%) and lipid transport and metabolism (5.5%) remaining were involved in nucleotide transport and metabolism (5.6%), stress response (8.3%), protease activity (5.5%), receptor binding (2.7%) and 8 % proteins were of unknown functions. The role of different category function predictions of *C. botulinum* type B were mentioned in Table 3. Identified proteins were also analyzed using various
bioinformatics software tools, such as Signal P, PROSITE, Secretome P, TMHMM, PSORT and GRAVY score for predicting protein secretion, localization and calculated grand average of hydropathy of extracellular proteins. PSORTb predicted 3-cell wall-associated protein (5.3%) such as hypothetical protein CLOSPO_00563, putative cell surface protein, cell surface protein, only 3-oxoacyl-(acyl carrier protein) synthase II cytoplasmic membrane protein (1.7%), 50-cytoplasmic proteins (89%) and subunit of pyruvate flavodoxin oxido reductase have unknown localization. Putative cell Surface Protein, cell surface protein and hypothetical protein CLOSPO_00563 were predicted by Signal P to be secreted in the classical Sec pathway, which is characterized by the presence of a signal peptide and these proteins also possess the cleavage site for signal peptidase I (SpI) and none proteins had signal peptide II (SpII). The Grand average of hydropathy value for protein sequences were calculated by GRAVY score. In addition, more than 50% proteins from the C. botulinum whole cell have metabolic functions were predicted by UniProt bioinformatic tool.

The C. botulinum type B cultures were grown in TPGY and CMM media. The secretome proteins were collected from both media and antibody was generated in mice against the respective proteins. The immunoblotting assay reveals that the following predominant immunogenic proteins were identified against the sera raised by TPGY secretome proteins are as follows: 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, ATP-dependent clp protease proteolytic subunit, phosphoglycerate kinase, FlaA, flagellin, secreted protease and molecular chaperone GroEL. Similarly 10 predominant immunogenic proteins were identified against the antibody raised by the secretome proteins are as follow: 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. Table 7 clearly indicates that five common immunogenic proteins identified in both TPGY and CMM sera and are as
follows; ornithine carbamoyl transferase, flagellin proteins, secreted protease, molecular chaperone GroEL and hypothetical protein. In whole-cell proteome, 13 immunogenic proteins such as electron transfer flavoprotein, beta subunit/ FixA family protein, peptidil-prolyl isomerase, flaA, flagellin, acetyl-CoA acetyltransferase, ornithine carbamoyltransferase, molecular chaperone GroEL, acetate kinase, hypothetical protein CBO2470, aspartate aminotransferase, conserved hypothetical protein, hypothetical protein CLOSPO_00563 and putative cell Surface were revealed as immunogenic proteins when TPGY grown C. botulinum type B whole-cell proteins probed with whole cell proteins antisera (TPGY). Seven immunogenic proteins such as phosphopyruvate hydratase, peptidase T, pyruvate phosphate dikinase, Ruberythrin, D-prolinereductase, PrdAproprotein, molecular chaperone GroEL and molecular chaperone DnaK were revealed as immunogenic when CMM medium grown C. botulinum type B whole-cell proteins probed with whole cell proteins antisera (CMM). These finding indicates that there are significant differences in the type and number of immunogenic proteins detected using the different antisera preparations and the largest numbers were detected using anti-TPGY expressed proteins sera. Similarly 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). Where as 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 carbamoyltransferase were reported as immunogenic proteins. Mice immunized with recombinant ornithine carbamoyltransferase protein extended the death time of mice challenged with C. perfringens (Sengupta et al., 2010). There has long been documented that flagella is one of the most effectual immunologic stimulator of the immune system. Flagellin is the more predominant immunogenic proteins in secretome and whole-cell proteome of C. botulinum type B. According to researchers, the use of flagellin as a vaccine applicant, part of vaccine or adjuvant has been widely considered. Bacterial flagellin has been considered for malaria vaccine formulations to the development of effective human vaccines. Flagellin intrinsic activity was mediated through TLR5 therefore can be genetically manipulated in a variety of ways for research purpose. In other words, advances in the field of innate immunity have disclosed the successful cellular and molecular mechanisms
Discussion

concerned in pathogen-associated molecular patterns. 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 (Dwyer and Mackay, 1972; Verma et al., 1995; Vanegas et al., 1997). Recombinant flagellin comprises unfamiliar epitopes caused protective immune responses in the absence of any extra adjuvant, flagellin has effects on T cells proliferation and cytokine responses (Bachmann et al., 2006). The induction of potent cell-mediated immunity will be essential and may require the enhancement cytotoxic T cell (CTL) activity which kills host cells infected with intracellular pathogens. 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. Hypothetical protein CLOSPO_00563, cell surface protein and putative cell surface protein are the novel immunogenic proteins mainly present in cell wall predicted by PSORTb. The potential of these candidate molecule to be used as a diagnostic marker as well as vaccine molecule against C. botulinum need be established. Cell wall exposed antigens in Gram-positive bacteria reveal the power for the rapid discovery of new vaccine (McCarty and Morse, 1964; Buschmann and Henrich, 1979). The chaperone protein, DnaKR3, also known as HSP70, belongs to the hsp-70 family. DnaK has been associated with adhesin functionality and to be expressed at significant levels on the surface of Mycobacterium tuberculosis with a propensity for being released into the surrounding environment (Hickey et al., 2009). The surface protein of DnaK has also been shown in other Gram-negative bacteria, including Legionella pneumophila (Garduno et al., 1998a; Garduno et al., 1998b) and Haemophilus influenza (Zhai et al., 2012). As a surface-exposed, immuno-accessible molecule, the chaperone protein DnaK has been identified as the immunodominant antigen of Mycobacterium bovis (Beltran et al., 2011). However, its precise contribution to RA pathogenesis, either direct or by a chaperone-like activity, remains to be elucidated. Both Chaperones DnaK and GroEL are immunogenic proteins in Burkholderia pseudomallei (Woo et al., 2001; Amemiya et al., 2007) Labeorohita (Kumar et al., 2014), Salmonella typhi (Panchanathan et al., 1998), Brucella abortus ( Al Dahouk et al., 2006; Lee et al., 2015), Bacillus anthracis (Chitlaru and Shaffer, 2009), Mycobacterium tuberculosis (Li et al., 2010). In the present study GroEL protein was found highly immunogenic in both secretome as well as whole
cell proteome were grown in both TPGY and CMM media (Table 5-10) while the Dnak protein was found immunogenic in whole cell proteome of *C. botulinum* type B grown in CMM media. The earlier observations were collaborated with the present investigation.

Flagellin is the structural constituent of flagellar filament in a variety of locomotive bacteria. It is the ligand for Toll-Like Receptor 5 (TLR5) of host cells. TLR inspiration by various pathogen-linked molecular patterns leads to activation of innate and adaptive immune responses as a result; TLR ligands are considered attractive adjuvant candidates in vaccine development and showed the highly potent mucosal adjuvant activity of a *Vibrio vulnificus* major flagellin (FlaB). Using an intranasal immunization in mouse model, they observed that when flagellin and tetanus toxoid (TT) both injected, induced considerably enhanced TT-specific IgA responses in both mucosal as well as systemic compartments and IgG responses only in the systemic compartment. The mice injected with TT plus FlaB was entirely protected from systemic challenge from 200 minimum lethal dose of tetanus toxin. Radio labeled FlaB gave into the nasal cavity reached the cervical lymph nodes as well as systemic circulation. Fla B bound with human TLR5 expressed on cultured epithelial cells and as a result induced NF-B and interleukin-8 activation. Intranasally given Fla B co-localized with CD11c as patches in dendritic cells which increases the TLR5-expressing cells in cervical lymph nodes. These results suggested that flagellin would act as an effective mucosal adjuvant enhancing the protective immune responses by TLR5 activation (Lee et al., 2006). In the present study Flagellin protein was found highly immunogenic in both secretome as well as whole cell proteome of *C. botulinum* type B was grown in both TPGY and CMM media.

Elongation factor EF-Tu and EF-Ts represent the typical examples of commonly conserved antigenic proteins. For example EF-Tu, a very plentiful protein, has been shown to be extremely immunogenic in *Anaplasma marginale* (Lopez et al., 2005), *Borrelia hrmsii* (Lopez et al., 2009). In addition, it has been reported that cell surface-associated EF-Tu of many distinct bacteria show multiple activities binding to various mammalian proteins including plasminogen and factor H (*Pseudomonas aeruginosa*) (Kunert et al., 2007). Due to these binding activities, EF-Tu has been identified as an surface immunogenenin *Staphylococcus aureus* (Vytvytska et al.,
2002), and it has potential to be a development of vaccine candidate due to its ability to induce high-level host immune responses. In the present study EF-Tu and EF-Ts proteins were pre dominant proteins in whole cell proteome of *C. botulinum* type B when it was grown in TPGY media (Table 3).

ppiC-Type peptidyl-prolyl *cis-trans* isomerase is a periplasmic enzyme that catalyzes the *cis-trans* isomerization of peptide bonds and act as a molecular chaperone in outer membrane protein (OMP) biogenesis (Zhai et al., 2012). A recent study of the bacterial pathogen *Bartonella henselae* suggested that ppiC-type peptidyl-prolyl cis-trans isomerase elicited an immune response that was solely attributed to a *Bartonella* spp. infection (Eberhardt et al., 2009). In the present study ppiC-Type peptidyl-prolyl *cis-trans* isomerase protein was found highly immunogenic in whole cell proteome of *C. botulinum* type B when it was grown in TPGY media (Table 9).

Peptidase T is a zinc metalloprotease and an amino tripeptidase which catalyzes removal of the N-terminal amino acid residue from tripeptides (Strauch et al., 1983). PepT consists of two domains: a catalytic domain with an active site which contain 2-metal ions, and a smaller domain that is formed by a long insertion into the catalytic domain. It is the first report that identifies pepT as a bacterial antigen. Although the role of this protein in the virulence of RA is unclear, but pepT represents an important RA immunogen (Dyson et al., 1988). In the present study, peptidase T protein was found highly immunogenic in secretome of *C. botulinum* type B when it was grown in TPGY media (Table 5) and also in whole cell proteome of *C. botulinum* type B when it was grown in CMM media (Table 10).

Acetate kinase, an enzyme that is common in bacteria, is responsible for the phosphorylation of acetate. It inhibit growth of *Escherichia coli* by the two inhibitors of acetate kinase, suggesting that the main role of acetate kinase could be a bacteriostatics target (Liu et al., 2014). In the present study, acetate kinase protein was found highly immunogenic in whole cell proteome of *C. botulinum* type B when it was grown in TPGY media (Table 9).

Serine hydroxymethyl transferase is reported as the immunogen in whole-cell protein fraction of *S. aureus* (Brady et al., 2006). Ruberythrins are non-haem iron proteins that have been implicated in oxidative stress protection in anaerobic bacteria and
archaea this is the novel immunogenic proteins identified in \textit{C. botulinum} type B whole-cell fraction (Table 10). Hypothetical protein CBO2470, hypothetical protein CLOSPO\_00563 \textit{(Clostridium sporogenes)} and electron transfer flavoprotein beta subunit/FixA family protein were the novel immunogenic proteins of \textit{C. botulinum} type B which were found in whole-cell proteome fraction. The secretary proteins of \textit{C. botulinum} type B did not show the cross reactivity with other \textit{C. botulinum} type A, E, F secretary proteins antisera at higher dilution (1:30,000) but showed the cross reactivity at lower dilution (1:1000) with the following proteins such as putative cell surface protein, secreted protease, molecular chaperone DnaK and butyrate kinase proteins of \textit{C. botulinum} type B. Similarly hypothetical protein CLOSPO\_00563, aminopeptidase 1, glycosyl hydrolase, family 18 and flaA proteins of \textit{C. botulinum} type B cross reacted with the antisera of secretary proteins of \textit{C. botulinum} type E at the 1: 1000 dilution. When whole-cell proteins of \textit{C. botulinum} type B cross reacted with the antisera of \textit{C. botulinum} type A, E and F at the 1: 30,000 dilution only two proteins were cross reacted with antisera of \textit{C. botulinum} type A. These were molecular chaperone GroEL and Glycine / sarcosine / betaine reductase, component B, subunits alpha and beta but when whole-cell proteins of \textit{C. botulinum} type B probed with the antisera of \textit{C. botulinum} type A, E and F at the 1: 1000 dilution, Conserved hypothetical protein \textit{[Clostridium botulinum Bf]}, Hypothetical protein CBO2470, R-phenyllactate dehydratase small subunit and hypothetical protein CBO2471 were cross reacted with the anti sera of \textit{C. botulinum} type A. similarly Molecular chaperone DnaK, molecular chaperone GroEL and glycine / sarcosine / betaine reductase, component B, subunits alpha and beta proteins were cross reacted with antisera of \textit{C. botulinum} type E and these three proteins conserved hypothetical protein \textit{[Clostridium botulinum Bf]}, hypothetical protein CBO2470 and R-phenyllactate dehydratase small subunit were cross reacted with antisera of \textit{C. botulinum} type F. These cross reacted proteins may be potential vaccine candidate against the botulism. The capability of proteins to display more than one function is called protein moonlighting proteins, and astonishingly, very much conserved proteins concerned in metabolic regulation or the cell stress response have a variety of extra biological actions which are concerned in bacterial virulence. Multiple roles displayed by a range of bacterial proteins, such as molecular chaperones, ornithine transcarbamylase and enolase the role that such moonlighting action plays in the
Discussion

virulence uniqueness of a number of significant human pathogens, including *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Helicobacter pylori*, *Staphylococcus aureus* and *Mycobacterium tuberculosis* (Jeffery, 2003; Gancedo and Flores, 2008; Gancedo et al., 2014). Some of the *C. botulinum* extracellular as well as whole-cell proteins identified here showed metabolic functions that would place them in the cytoplasm. There is growing evidence that such “housekeeping” enzymes have a role in the pathogenesis of/ or immunity to, other infections (Cole et al., 2005; Severin et al., 2007). In addition, some cytoplasmic proteins have been shown to in fact moonlight on the bacterial cell surface or to have more than one role in an organism. For example, both GAPDH and enolase are moonlighting proteins involved in plasmin binding when present on the cell surfaces of some Gram-positive bacteria (Henderson and Martin, 2011). These proteins are involved in cell lysis. However, few such predictably cytoplasmic proteins reported in this study have also been detected on the bacterial surface or in extracellular proteome and in some of the studies; cell lysis was excluded as a reason for this observation by strong experimental evidence. For example, acetyl-CoA acetyltransferase was identified in the extracellular fractions of *Mycobacterium tuberculosis* (Mattow et al., 2003) and *Burkholderia cenocepacia* (Mattow et al., 2003), and triosephosphate isomerase was found in the secretome of *Bacillus anthracis* (Lamonica et al., 2005), *M. tuberculosis* (Connor et al., 2011) and *Streptococcus suis* (Wang et al., 2011), while glyceraldehyde-3-phosphate dehydrogenase was secreted by *Streptococcus pneumoniae* (Vazquez-Zamorano et al., 2014) and *Staphylococcus aureus* (Antikainen et al., 2007). In one other study of *B. anthracis* extracellular proteome observed several predictably cytoplasmic proteins in the extracellular culture supernatant of the bacterium and based on their experimental evidence, cell lysis was excluded as the major contributor to the protein accumulation in the exoproteome. In spite of a growing list of cytoplasmic proteins identified on the bacterial surface or secreted out, the mechanism of their export to the cell exterior remains unclear. Internal signal sequences, posttranslational acylation, or an association with an extracellular protein are hypothesized as possible means (Antelmann et al. 2005).

Currently there is no licensed vaccine for human or animal use to protect against botulism caused by botulinum toxin. Treatment of botulism cases in the USA usually
consists of administration of equine antitoxin antisera and supportive care. The equine antitoxins include a licensed bivalent and monovalent antitoxin that contains neutralizing antibodies against BoNT types A/B and E, respectively and an investigational heptavalent (ABCDEFG) antitoxin. The heptavalent botulinum antitoxin (HBAT, Cangene Corporation) is available through a CDC-sponsored IND protocol. Following expiration of the bivalent and monovalent products in March 2010, HBAT became the only botulinum antitoxin available in the USA for naturally occurring non infant botulism (Hart et al., 2012). Cases of infant botulism are treated with the recently licensed BabyBIG which is derived from the blood of human donors vaccinated with a pentavalent (ABCDE) toxoid vaccine (Dhaked et al., 2010). But due to high cost, people are unable to afford. Subunit vaccines based on selected antigens and/or their respective genes are recognized as the safest type of antibacterial vaccine (Jagusztyn-Krynicka et al., 2009). The bottleneck in the development of effective subunit vaccines is the choice of the antigens (Bill, 2015). A proper candidate for immunization must possess a wide range of different properties, such as extra cytoplasmic localization, abundant presence in the cell, immunogenicity (i.e., ability to stimulate the immune system), and conservation among different pathogen serotypes/genotypes. Further, a candidate vaccine antigen has to be expressed in vivo during infection, when the pathogen is present in the host organism. The surface exposed antigens have been long emphasized as important vaccine candidates since they are involved in the first line of bacterium-host interactions.

In the present study, ornithine transcarbamylase (OTC) and molecular chaperon GroEL was an abundant immunogenic protein found in secretome as well as whole-cell fraction of the bacterium. The PSORTb analysis reveals that, both proteins were the cytoplasmic proteins whereas in the case of C. perfringens as well as C. thermocellum it was reported, the OTC was present in the cell surface of C. perfringens and membrane in C. thermocellum (Sengupta et al., 2010; Peng et al., 2011).

Recombinant OTC protein elicited a predominantly Th2 as well as Th1 type immune response, as revealed by the high titers of anti-rOTC IgG1 and IgG2b followed by IgG3 antibodies and secretion of IFN-γ and TNF-α. To determine the efficiency of rOTC protein in eliciting protection, the mice were infected with 100, 10 and 1 LD₅₀
of *C. botulinum* type B via intraperitoneally route after the 30 day of the post immunization. In the rOTC immunized mice the onset of death was inconsequentially delayed in 100 LD\(_{50}\) but in 10 and 1 LD\(_{50}\) showed 100 percent protection.

Further, the immunized mice were found to be protected from developing botulism disease after intraperitoneal inoculation with a lethal dose of *C. botulinum* type B suggesting that rOTC protein may be promising vaccine candidate for the prevention of botulism infection. Recombinant OTC was identified as an immunogenic surface protein of this bacterium and was upregulated in TPGY grown cells. In another study, rOTC was isolated as a putative adhesin from a surface molecule preparation of *Staphylococcus epidermidis* and was shown to be protective in active immunization challenge experiments for the pathogen (Singh et al., 2010). The homolog of this protein has also been identified as an immunogenic protein in outer surface protein preparations of *Streptococcus agalactiae* (Hughes et al., 2002) and *Streptococcus pyogenes* (Freiberg et al., 2014). Taken together, these findings make rOTC a putative vaccine candidate against *C. botulinum* infection.

In the present study, the antibody raised against rGroEL protein was showed IgG1 response the results directed towards Th2 type response. In the case of rGroEL protein, significantly high expression levels of IFN-\(\gamma\), IL-2, IL-5, GMCSF and TNF-\(\alpha\) 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. To determine the efficiency of rGroEL proteins in eliciting protection, the mice were infected with 100, 10 and 1 LD\(_{50}\) of *C. botulinum* type B via intraperitoneally route after the 30 day of the post immunization. In the rGroEL immunized mice the onset of death was inconsequentially delayed in 100 LD\(_{50}\) but in 10 and 1 LD\(_{50}\) showed 100 percent protection.

Further, the immunized mice were found to be protected from developing botulism disease after intraperitoneal inoculation with a lethal dose of *C. botulinum* type B suggesting that rGroEL proteins may be a promising vaccine candidate for the prevention of botulism infection. Recombinant GroEL protein was identified as an immunogenic surface protein of this bacterium and was upregulated in TPGY as well as CMM grown cells.
According to Paliwal et al., heat shock proteins (Hsps) have been reported to be more expressed antigens for the host immune response to a variety of pathogens and thus, have most potential for use in vaccination. Researcher evaluated the immunogenicity and protective efficacy of recombinant GroEL protein of Salmonella enterica serovar typhi against fatal infection by S. typhi Ty2 in mice model with or without adjuvants. Titers Anti GroEL-IgG were significantly higher in mice immunized with either rGroEL-alone or in miscellaneous with alum / Complete Freund's adjuvant (CFA) as compared to the control. Study of antibody isotypes revealed that predominance of Th2 type immune response in rGroEL + alum immunized animals as revealed by elevated IgG1/IgG2a ratio. While, immunization of animals with rGroEL + CFA or GroEL-alone shifted the immune response toward Th1 type immune response. Mice immunized with rGroEL with or without adjuvants, showed a significant increment in lymphocyte proliferation and cytokine levels. Then animals immunized with recombinant GroEL + CFA or rGroEL-alone showed higher IFN-γ and IL-2 levels compared to alum group, indicating Th1 response whereas IL-4 levels (Th2 response) were found to be significantly higher in alum group as compared to other 2-immunized groups. Immunization of mice with rGroEL-alone, rGroEL + alum and GroEL + CFA showed 70, 50 and 80% protection, respectively, against deadly challenge by S. typhi in mice. The differences among a variety of groups in the percentage protection were credited to the differences in the immune responses raised by respective immunizations. This study showed molecular chaperon GroEL an ideal candidate molecule to make a recombinant protein based vaccine in opposition to human typhoid (Paliwal et al., 2008).

According to Chittradevi et al., Shigella species cause harsh bacillary dysentery in humans and are linked with high morbidity and mortality. The incursion plasmid antigen (IpaB) protein, which is preserved across all Shigella spp., causes macrophage cell death and is requisite to invade host cells. Researcher evaluated the immunogenicity and protective efficacy of the recombinant domain region of IpaB (rIpaB) of S. flexneri. rIpaB was injected either alone or was injected with the rGroEL protein from S. typhi as an adjuvant in a mouse model via intranasal immunization. The 37 kDa IpaB domain region of S. flexneri was PCR amplified from an invasion plasmid, cloned then expressed in BL21 (DE3) cells and purified.
Immunization with the rIpaB domain alone stimulated humoral and cell-mediated immune responses both. In addition, high antibody (IgG, IgA) and T-cell responses were induced when the rIpaB injected with rGroEL. Antibody isotyping study showed higher IgG1 and IgG2a antibody titers and increased level of interferon-gamma (IFN-γ) secretion in the co-administered group. Alone Immunization of mice with the rIpaB protected 60%–70% of the mice from deadly challenge by S. flexneri, S. boydii and S. sonnei, while co-administration with rGroEL augmented the protective efficacy to 80%–85%. Histopathological studies also revealed a significant reduction in lung infection in the co-immunized mice group compared with rIpaB domain alone mice immunized group. This study revealed that the co-administration of both improves immune responses in mice and enhances protective efficacy against Shigella infection (Chitradevi et al., 2015).

According to Huang et al. Clostridium difficile is an endo-spore forming anaerobic bacterium which is accountable for diarrhea and colitis, after treatment with antibiotics. Clinical symptoms are mainly due to two toxins, TcdA and TcdB. However, the primarily step of pathogenesis is the colonization process. C. difficile surface proteins act as the vaccine candidates in the hamster model to prevent intestinal colonization. This vaccination induced an incomplete protection of hamsters against death after C. difficile challenge. A proteomic analysis of animal sera revealed to identify the immunogenic proteins which may be responsible for the protection. The role of GroEL proteins were elucidated in hamsters, the GroEL immunized group delayed the C. difficile colonization. Subsequent to intranasal immunization with the rGroEL protein, they pragmatic a lower C. difficile intestinal colonization in the immunized group as compared to the control group (Huang et al., 2015).

According to Sinha and Bhatnagar, heat shock proteins (Hsps) HSP60 and HSP70 family are extremely conserved and essential to everyone living organisms. Hsps are highly expressed immunogenic proteins in several microbial infections and have been discovered for their vaccine potential. Researcher investigated the immunogenicity and protective efficacy of GroEL and DnaK against B. anthracis in mice model. Both Hsps were establish to be highly immunogenic with mixed antibody response (both IgG1 and IgG2a), representative encouragement of both humoral as well as cell-mediated immunity. Cytokine profile also confirmed highly T-cell response with
increase in lymphocyte proliferation. Immunization with recombinant GroEL conferred 100% protection in mice against *B. anthracis* whereas DnaK couldn’t provide protection (Sinha and Bhatnagar, 2010).

Khan et al. evaluated the immunogenicity and protective efficacy of GroEL (hsp60) of *Streptococcus pneumoniae*, by expressing full length GroEL in BL21 (DE3) cells. The results revealed a significant increment in antibody titre and lymphocyte proliferation in animals immunized with rGroEL as compared with control. Further, there was a considerable increase in interleukin-2 (IL-2) and IL-4 construction in lymphocytes isolated from immunized mice as compared with control. To determine the efficiency of GroEL in eliciting protection, the mice were infected with the fatal dose of *S. pneumoniae* A66 type 3 (capsular strain) via intranasally after the seventh day of the post immunization. In the rGroEL immunized mice the onset of death was inconsequentially delayed and all the mice died by the seventh day post infection (Khan et al., 2009).

Botulism is a fatal disease caused by taking the preformed neurotoxin secreted from the anaerobic endospore-forming bacteria *C. botulinum*. Botulinum neurotoxins are the extremely poisonous toxins known in nature and have been a anxiety in the food industry for a long time. Consequently, rapid identification of BoNTs using molecular as well as biochemical techniques are components in the establishment of synchronized laboratory response systems and is the main focus of current research and development. Sensitive and rapid detection of botulinum neurotoxins is necessary for the detection of contaminated food with toxin, as well as for response of potential bioterrorist threats. Presently, the most trustworthy method for detection of botulinum neurotoxin is mouse bioassay, this assay takes time, sensitive, expensive have limited high throughput and also requires sacrificing the animals. Jain et al. was developed an alternative laboratory based immunoassay which can be utilized to detect suspected human botulism cases as well as botulinum neurotoxins in food and clinical samples. Several researchers have used a powerful promoter of phage T7 and physiological manage to improve the level of expression of a synthetic BoNT/B LC protein as well as codon optimization which reduced protein expression connected with rare codons and elevated AT base composition. The use of a powerful promoter and optimized synthetic BoNT/B LC gene resulted in a 50% elevated yield of protein expression
and/or recovery than in the past reported (Jain et al., 2011). But till now there is no field based detection system is available to detect *C. botulinum* as well as botulinum toxin from clinical as well as environmental samples.

In the present study we developed the sandwich enzyme-linked immune sorbent assay (ELISA) method for reliable detection *C. botulinum* type B from clinical as well as environmental samples. For which, we screened surface associated pre dominant immunogenic proteins of *C. botulinum* type B by immunoproteomics approach. Ornithine transcarbamylase gene was amplified using *C. botulinum* type B whole genome as a template was ligated in pET28a+ expression vector and subsequently transformed in BL21 (DE3) *E. coli* host cell. Cloning of otc gene was confirmed by colony PCR. The recombinant OTC protein was purified by Ni-NTA affinity column chromatography and developed the polyclonal antibodies in mice and rabbit against recombinant OTC protein. Here, we used the *C. botulinum* type B as an antigen and got high specificity polyclonal antibodies and developed the sandwich ELISA assay to identify *C. botulinum* type B. The developed ELISA could able to detect as low as 125 CFU/ml of *C. botulinum* type B (Figure 55). It can be also used to detect the potential source of contamination in outbreak situations of botulism. To check the cross reactivity, *C. botulinum* types A, E and F were used as the antigen. Polyclonal antibody raised in mice and rabbit against recombinant OTC protein bound with *C. botulinum* type A and not bind with *C. botulinum* types E and F. The present study results suggested that antibodies against the rOTC can be used to detect *C. botulinum* types A and B.