INTRODUCTION

_Clostridium botulinum_ is a gram positive, an obligate anaerobic and endospore forming bacteria produce botulinum neurotoxins (BoNTs). It is the most potent neurotoxin so far known to humans (~100 billion times more toxic than cyanide) i.e. it is categorized as biowarfare agents category ‘A’. It is divided into 8 serotypes designated (A–H) which have similar structure and differ antigenically. Few strains that secrete two types of toxins, i.e., Ba, Ab, Bf, and Af (capital letter denotes principal toxin type) (Barash and Arnon, 2004). Generally the serotypes A, B, E are associated with human clinical cases but rarely serotype F, among the serotypes A and B are the most poisonous. It causes neuroparalytic disease in humans and animals called botulism (Sobel, 2005).

BoNT is secreted as a single polypeptide chain which is cleaved endogenously or exogenously and converted into dichain [100 kDa heavy chain (HC) and 50 kDa light chain(LC)] both chain allied by a disulphide bond. HC consist of binding and translocation domain at the carboxy (C) and amino (N) terminal end respectively. HC-binding domain bind with the nerve membrane and its translocation domain facilitate the entry of light chain across the membrane into cytoplasm. Due to cytoplasmic $p^H$ variation the HC dissociates from LC. LC contain zinc endopeptidase catalytic activity that cleaves SNARE-complex (soluble N-ethylmaleimide factor attachment protein receptor) which involved in docking and fusion of acetylcholine containing vesicles to post synaptic membrane which inhibit the liberation of the acetylcholine at the neuromuscular junctions as a result autonomic nervous system will be paralyzed. This
condition is called botulism or flaccid paralysis. BoNT/A, C, E cleaves synaptonemal associated protein-25 (SNAP-25), BoNT C also cleaves syntaxin protein and BoNT B, D, F and G cleave vesicular associated membrane protein /synaptobrevin (Tighe and Schiavo, 2013).

Centre for Disease Control and Prevention has been classified the human botulism into four categories: food-borne, wound, infant, and other. Food-borne botulism is caused by ingestion of food or drink containing preformed botulinum toxin. Often this occurs when canned foods that are contaminated with *C. botulinum* spores are not effectively sterilized. It could also result from intentional contamination toxin to food supply. It is the most common cause of botulism in Europe. A lot of different types of food have been implicated, but the most common ones are home-made preserved foods. The type of toxin varies with location and type of food. For fish and seafood, type E is the most common, while in meats and vegetables types A and B dominate. In turkey 2005, 5 cases were reported as the food borne botulism that was associated with roasted canned mushrooms (Swaan et al., 2010). In Thailand 2006, an outbreak of food borne botulism was reported with 163 people affected, 42 of whom requisite mechanical ventilation. The victims shared a common meal during a religious festival, and consumed home-canned bamboos shoots that were contaminated with *Clostridium botulinum* type A identified by multiplex PCR (Pantukosit, 2007). In Canada (Ontario) from 2003 to 2012, 13 cases of food borne botulism were reported that was associated with home-canned foods (Leclair et al., 2013). In USA 2013, there were 3 outbreaks of food borne botulism were reported (Van Doren et al., 2013). Out of three one case was associated with homemade turshi (associated with 4 probable cases), one with fermented fish, and one with seal oil. All eight patients survived. In Italy 2013, 21 years old patient was hospitalized in the Emergency Unit of the Mauriziano Hospital. After laboratory tests, botulism was confirmed in the patient that was associated with home-preserved food (Anniballi et al., 2015). In Poland 2013, 24 food-borne botulism cases were reported that was associated with commercially canned meat (Czerwinski et al., 2013). In UK 2014, one case of food borne botulism was reported that was associated with hummus. In Ohio 2015, one outbreak of foodborne botulism was reported. In this outbreak 25 cases were found positive for botulism confirmed by laboratory tests (McCarty et al., 2015). This outbreak was caused by consumption of potato salad.
Wound botulism is the result of profound wounds or abscesses contaminated with *C. botulinum* spores that provide anaerobic conditions for spore germination then spore germinate and converted into vegetative cells further these cells multiply and produce botulinum toxins interior of the wound, which is then absorbed into the bloodstream. Wound botulism has become more widespread in recent years among intravenous drug users in western America. Germany in 2010, 16 cases of wound botulism was reported to the health authorities of North Rhine-Westphalia. All patients were injecting drug users and epidemiological investigations suggested contaminated injection drugs as the most probable source of infection (Hope et al., 2012). Italy in 2012, a 41 year old woman was affected by wound botulism after traumatic open fracture. Seventeen days after a traumatic open fracture, a *Clostridium botulinum* wound infection was diagnosed, with self-limiting symptoms. This is the first report of wound botulism in Italy (De Rosa et al., 2015). USA in 2013, The 14 cases of wound botulism was reported. Toxin type A accounted for 11 (79%), toxin type B for 1 (7%), and botulinum toxin type was not determined for 2 (14%). All patients were an injection drug user. In Norway 2013, four cases of wound botulism were confirmed in people who inject drugs (PWID) (MacDonald et al., 2013).

According to CDC report, Infant botulism is the main form of botulism in the United States. It is the result of eating food contaminated with *C. botulinum* spores, then spores reached into gastrointestinal tract where it germinate and colonize and produce botulinum toxins in the large intestine then toxin absorbed into blood stream. It primarily affects infants (younger than 12 months of age) because *C. botulinum* is easily colonized in infant due to normal bowel flora that could compete with *C. botulinum* have not been fully established. Honey is the greater risk of infant botulism because it contains *C. botulinum* spores so it should be avoided for children. In 2006, 26 countries had reported the occurrence of one case of infant botulism among their residents, and the major numbers of cases have been reported, in downward order, by the USA, Argentina, Australia, Canada, Italy, and Japan. Remarkably, large amount countries have not reported infant botulism cases yet. This limited reporting of infant botulism contrasts with the known global occurrence of *C. botulinum* spores in soils and dust, and it suggests that infant botulism could be under recognized, under reported, or both (Vanella de Cuetos et al., 2011). In Argentina from 1982 to 2010, reported 605 laboratory-confirmed cases of infant botulism, the average annual
incidence of infant botulism in Argentina is similar to that of the United States: 2.2 per 100,000 live births in Argentina and 1.9 per 100,000 live births in the United States. All cases of infant botulism registered in Argentina have been caused by BoNT type A, except one case caused by type B. In the United States, BoNT types A and B have been implicated in almost all cases of infant botulism (Vanella de Cuetos et al., 2011). In Italy from 1984 to 2006, 26 cases of infant botulism (and 3 of adult intestinal botulism) were reported. Type A botulism accounted for 4 cases, type B for 17 and type E for 5 (Brook, 2007). In France from 1991 to 2009, 7 cases of infant botulism were reported 1 per year from 2004 to 2008; 2 in 2009 (King et al., 2010). In Canada (Ontario) from 2003 to 2012, 10 cases of infant botulism were reported (Schwartz et al., 2012). In USA 2013, The 135 cases of infant botulism were reported. Out of 135 cases 57, 74, 1 and 3 cases were caused by BoNT/A, B, Bf and F respectively.

Botulism is classified as “other” if the patient is not an infant, has no evidence of ingesting a suspect food, and has no wounds. Consistent with the Council of State and Territorial Epidemiologists position statements, the “other” group includes botulism in which the route of transmission is puzzling. The “other” group also includes iatrogenic botulism, which is caused by an unintentional overdose of botulinum toxin (i.e., therapeutic injection) and adult intestinal colonization botulism, which is very infrequent but occurs through a mechanism similar to infant botulism. In Italy from 1994 to 1995, two cases of adult intestinal colonization botulism were reported and caused by C. butyricum—producing type E which was isolated from affected patient’s stools and confirmed by polymerase chain reaction (Mohanty et al., 2001). In USA from 1981 to 2002, 13 cases were reported as adult intestinal colonization botulism caused by C. botulinum toxin type F (Sobel et al., 2009).

The typical symptoms of botulism include blurred vision, double vision, drooping eyelids, difficulty in swallowing, muscle weakness, slurred speech and dry mouth. Infants with botulism show lethargic, inadequate feeding, constipation, and poor muscle tone and have a weak cry. If these symptoms left untreated result respiratory system failure ultimately leads to death.

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

Ehrlich, Ramon & Von Behring introduced the concept of toxoid based vaccines and they have successfully developed diphtheria and tetanus toxoid. Weinberg and Goy laid the foundation for the development of the first botulinum toxoid vaccine. Weinberg and Goy used Ramon’s toxoiding method for converting a lethal *C. botulinum* neurotoxin mixture into a non-toxic preparation (Botulinum toxoid). The antigenicity of such botulinum toxoids in the beginning was evaluated in experimental animals and then in humans. Crude alum precipitated bivalent AB botulinum toxoid was prepared by the department of defense (USA) during World War II. Moderate to severe local reactions associated with the injection of this toxoid were observed. In 1959, formalin inactivated penta-valent (ABCDE) botulinum toxoids made at Fort Detrick and replaced the bivalent AB toxoid. Initial lots of this vaccine were produced by Parke-Davis in 1957. From 1970 to 1981, more than 1600 individuals received over 6000 doses of the Park Davis penta –valent toxoid vaccine under an Investigational New Drug (IND) application held by the CDC but it is composed of formalin inactivated crude isolates of BoNTs captivated to aluminum phosphate and containing thimerosal as a preservative. However, it is very costly and time-consuming to produce and is hazardous during detoxification later, Centers for Disease Control and Prevention discontinued the pentavalent (ABCDE) botulinum toxoid vaccine. Due to the above small demerits therefore the development of the new generation vaccine-design strategies for the prevention of botulism based on the construct of nontoxic recombinant BoNT proteins is necessary (Rusnak and Smith, 2009).

Later on the synthetic oligonucleotide structure of the Hc domain of *C. botulinum* neurotoxin serotype A (AHc) was developed and a soluble recombinant AHc His-tag fusion was expressed in *E. coli* that conferred protection in mice against challenge with active BoNT/A. To construct a vaccine appropriate for human use, a non-His-tagged rAHc isoform was purified using sequential chromatography with ion-exchange (SP and Q) and hydrophobic-interaction (HIC) resins. The rAHc was
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considered as a subunit vaccine candidate in mouse models of botulism and also reveal that rAHc administered in the presence or absence of adjuvant sheltered mice against a lethal BoNT/A challenge and elicited high anti-rAHc antibodies that were long-lasting, suggesting that this form of rAHc may be fitting for use in humans against botulism (Middlebrook, 2005).

Another recombinant vaccine was also constructed which neutralize all seven serotypes (A-G) of *C. botulinum*. In this approach, HCRs of 7-serotypes of BoNTs (hepta-HCR) were engineered for expression in *Escherichia coli* then each HCR was purified from *E. coli* lysates. Immunization of mice with the *E. coli*-derived 7- BoNT serotypes HCR vaccine elicited an antibody titre to each of the 7- BoNT HCRs and neutralized challenge by 50% lethal doses of each of the 7- BoNT serotypes. A solid-phase analyze showed that the anti-7-serotype HCR sera repressed the binding of HCR serotypes A and B to the Ganglioside GT1b receptor, the first step in BoNT intoxication of neurons. This is the first *E. coli*-derived vaccine that successfully neutralizes each of the 7- BoNT serotypes (Baldwin et al., 2008).

In spite of their safety and effectiveness, there are number of key disadvantages found in subunit vaccinations. Regrettably subunit vaccines are less successful at inducing long lasting immunity against illness. Live vaccines can confer immunity in one or two doses, while subunit vaccines will require to be administered repeatedly over specified periods of time to effectively immunize against a illness. To overcome a few of the drawbacks of the recombinant subunit vaccines, DNA vaccine have been investigated recently.

DNA vaccines have emerged as an striking approach for developing antigen-specific immunotherapy. Compared with subunit vaccines, DNA vaccines have many advantages, including ease of manufacture, purity of product and ease of storage. For the development of DNA vaccine against botulism, researches made Granulocyte-macrophage colony-stimulating factor (GM-CSF) which was a striking adjuvant for a DNA vaccine on account of its capability to recruit antigen-presenting cells to the site of antigen production as well as stimulate the maturation of dendrite cells. This approach evaluated the efficacy of GM-CSF as a plasmid DNA replicon vaccine adjuvants for BoNT/A in mouse model. In BALB/c mice that received the plasmid DNA replicon vaccines made from Semliki Forest virus (SFV) transport the Hc gene
of BoNT/A (AHc), both lympho-proliferative and antibody response specific to AHc were induced, the immunogenicity was improved by co-delivery or co-express of the GM-CSF gene. When AHc and GM-CSF were co-expressed within the SFV based DNA vaccine, the survival rates of anti-AHc antibody titers and immunized mice after challenged with BoNT/A were considerably increased, and further improved by co-immunization with aluminum phosphate adjuvant (Ma et al., 2013).

All above mentioned vaccines are concerned with botulinum toxin neutralization. But till now there is no vaccine is available commercially against C. botulinum which is the primary causal agent of infant and wound botulism. Antibiotics should not be used routinely to treat infant botulism and should only be used to treat secondary infections (urinary tract infections, pneumonia and otitis media), because their use may result in the lysis of intraintestinal C. botulinum with discharge of additional botulinum toxin. Vaccination is an alternative and safe strategy to prevent primary infection of C. botulinum that ultimately produces botulinum toxin result botulism. If primary infection is blocked in this case, either colonization or bacterial clearance, there will be no botulinum toxin production in surrounding environment as a result botulism will not occur. To the best of our knowledge so far there is no licensed vaccine available commercially for botulism. Similarly there is no rapid detection system available to detect botulism. To develop the new generation vaccine against many pathogens, the immunoproteomics approach remains the choice of the research.

Immuno proteomic approach is one of the best tools among available to the study the host pathogen interaction. Researchers used combination of two dimensional gel electrophoresis and immuno blotting with sera from infected animals or human patients with mass spectrometry to find out the immunogenic candidate molecules. Similar approach has been widely used for the discovery of new biomarkers for vaccine development in cancer as well as infectious diseases. The secretary proteins / surface proteins play important roles in the pathogenesis of bacterial infection represent the inter-phase of the bacterium–host interaction. In any pathogens the secretary / surface proteins are exposed to the host immune system and are therefore the primary antigen targets of host immune response. Numerous novel secretory proteins produced by different bacteria such as Helicobacter pylori (Kim et al., 2002), Pseudomonas aeruginosa (Scott et al., 2013) and Staphylococcus aureus
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(Kusch and Engelmann, 2014) have been identified in bacterial culture supernatants by using two-dimensional gel electrophoresis and mass spectrometry approach to develop the candidate vaccine molecule as well as biomarker discovery.

Similarly, Immunoproteomics approach has been also used to development surface proteins against Shigella spp. (Pore et al., 2011), Trichinella spiralis (Robinson and Connolly, 2005), Cronobacter spp. Schistosoma japonicum (Borloo et al., 2013), Bacillus anthracis (Chitlaru et al., 2006), Mycoplasma mycoides (Rebollo Couto et al., 2012), S. aureus (Kusch and Engelmann, 2014), Clostridium difficile (Boetzkes et al., 2012), Clostridium sordelli (Kachman et al., 2010) and Clostridium perfringes (Sengupta et al., 2010) using whole-cell proteome analysis of these bacteria. Apart from this, the secretory proteins / surface proteins are important for the development of diagnostics and passive immunotherapies.

Reports are available for some Gram positive bacteria using secretory proteins, vegetative cell surface proteins and spore surface proteins can elicit a humoral immune response in the course of bacterial infections but no such report are available with respect to C. botulinum. Therefore, in the present study we were selected to identify the predominant immunogenic proteins against the sera of secretome and whole-cell proteomic as well as live spores of the C. botulinum type B. in this study, first time we report the immunoproteomics approach to elucidate the secretome as well as whole-cell proteome of C. botulinum type B and identified the predominant immunogenic proteins further validated for their potential to be used as a diagnostic marker and vaccine potential against C. botulinum.

As such there is no commercial detection system available in the market to detect botulism; even laboratory expertise is also not available in the country. It is due to the non-availability of standard cultures as well as extreme toxicity of the organism and non-availability of prophylaxis agent also. The main objective of present study is elucidation of surface biomarker of C. botulinum as a diagnostic / potential candidate vaccine molecule which will be highly useful for human clinical diagnosis of botulism and it also aimed to make the vaccine for botulism.
To accomplish the main objective, the following sub-objectives were selected and are as follows:

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