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Molecular medicines for neutralization of *Clostridium botulinum* neurotoxin

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Abstract

Botulism is characterized by symmetrical, descending, flaccid paralysis of motor and autonomic nerves, caused by the spore-forming, obligate anaerobic bacterium *Clostridium botulinum*. Strains of *Clostridium botulinum* are known to produce the most poisonous neurotoxins in mankind. *Clostridium botulinum* produces seven genetically distinct neurotoxins known as BoNT/A, BoNT/B, BoNT/C, BoNT/D, BoNT/E, BoNT/F and BoNT/G.All the serotypes share same structure and molecular weight, but differ in their cellular substrate and target cleavage site. Botulinum toxins work by blocking the release of acetylcholine in four stages, binding, internalization, translocation and inhibition. The food borne botulism, wound botulism, infant botulism and adult botulism are main clinical features. Different molecular techniques like Mouse lethality assay, ELISA, immuno-PCR, chemiluminescent slot blot immunoassay, electrochemiluminenscence, radioimmunoassay, lateral flow immunoassays and End peptidase assay are mostly used to detect the BoNTs. Antitoxins such as BabyBIG, Equine, Mabs and HBAT are used for treatment of BoNTs intravenously or intra- muscularly. At molecular level Peptide Based Inhibitor, Phage display technology and Aptamers are used. A proper delivery system is required to deliver inhibitors to target nerves to reverse the clinical effects. Heavy chain of BoNT has been shown to be the natural, safe and potential delivery system to deliver inhibitory molecules in the affected nerves.

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Introduction

Strains of *Clostridium botulinum* are known to produce the most poisonous neurotoxins to mankind. Even a 30ng (Peck, 2006) oral dosage is enough to kill a person. The dosage amount decreases to 0.09–0.15ng as the route of the entry of toxin changes from oral to intravenous (Kongsaengda *et al.*, 2006). *Clostridium botulinum* produce seven genetically distinct neurotoxins named as Type A, B, C, D, E, F and G. Of them BOTOX C is further divided into Type C1 and C2 (Grend *et al.,* 2012). All the serotypes share same structure and molecular weight but differ in their cellular substrate, target cleavage site, potency, complex protein size and percentage in either nicked or activated form (Aoki and Guyer, 2001). Recently a new *botulinum* toxin named as BoNt type H has also been identified in the IBCA10-7060 strain of *clostridium botulinum* that cannot be neutralized through anti-toxins raised against the BoNts A-G by using standard mouse bioassay.

Neurotoxin

Structure

In the beginning every serotype is made as a continuous polypeptide chain of around 150 kDa. Next a non-toxic polypeptide associates with the toxin polypeptide. The size of the complex increases to almost 900 kDa. In order to become fully functional the polypeptide chain has to be broken down into a heavy and a light chain. The light chain is of 50 kDa, while the heavy chain is of 100 kDa. The heavy chain contains two domains each having a size of approximately 50 kDa. The N-terminal half (Translocation Domain), of the heavy chain is responsible for making ion channels in the cell membrane, while C- terminal half (Ganglioside binding domain),is responsible for binding and internalization of the toxin into neurons (Sakaguchi *et al.*, 1984). Both the chains remain attached to each other with the help of disulfide-bridge (Figure 1). The primary acting site of all the serotypes is the peripheral nerve endings of cholinergic motor nerves (Black and Dolly, 1987; Dolly and Aoki, 2006). Non-toxic polypeptide in the Type C, E, F and Haemagglutinin- negative Type D complexes are almost of the same size as the toxin polypeptide. The Molecular weights of these complexes lie in between 230 – 350 kDa, these complexes are named as M complexes. While the complexes of Type B, A and Haemagglutinin Type D toxins are of greater molecular weights which range from 450-500 kDa. These complexes are known as L Complexes while Type A has also found to make complexes larger up to 900 kDa (Hambleton *et al.*, 1987).

As the pH becomes basic the complexes dissociates into their component proteins but they are reformed when the pH turns to acidic. The non-toxic proteins of the complex have been found to provide protection to the neurotoxin proteins in the gut environment, where the pH may affect the toxicity of the neurotoxins. Due to this protection the chances of the neurotoxin to get entered into blood or lymphatic are raised (Shone, 1987). The light chain of BoNT is also found to have zinc dependent protease activity and target specific group of proteins known as SNARE (NSF (N-Ethylmaleimide-Sensitive Factor) Attachment Receptor). These SNAREs regulate the release of acetyl choline at nerve endings (Schiavo *et al.*, 1992).

Mode of action

Neurotransmitter release from the nerve endings is a complex process involving a number of processes starting from the nerve stimulation followed by the depolarization of the membrane. This activates the Calcium channels in the membrane as a result of which Ca++ ions move inside the cell. The increased the intracellular concentration of calcium stimulates the fusion of synaptic vesicle, consequently releasing the neurotransmitter (Dolly, 2003). The SNAREs are involved in regulating this fusion and are divided into two types depending upon their location (Figure 2). The v-SNARE (Vesicle associated) also known as VAMP (Vesicle Associated Membrane Protein) or Synaptobrevin. The v-SNARE attach to synaptic vesicle via their C-terminal. On the other hand thee t-SNARE (Target Membrane SNARE)arecomposed of two proteins; Syntaxin and SNAP-25 (a 25 kDa Synaptosomal Protein).

As the synaptic vesicle reaches the cell membrane a turnery complex is formed involving two domains of SNAP-25 and one domain from Synaptobrevin andSyntaxin. The complex facilitates the exocytosis by positioning the synaptic vesicle appropriately on the membranes. This is achieved by the binding of soluble N- ethylmaleimide SNAP followed by an ATP dependent enzyme known as N- ethylmaleimide sensitive factor. Then it provides energy to dismantle the SNARE complex and permits the exocytosis (Dolly and Aoki, 2006). Botulinum Toxins work by blocking the release of acetyl choline. The blocking process is divided into four major stages; Binding: Cholinergic neurons have receptors specific to the BoNT on their surface, where BoNT bind through their 100kDaHcDomain in the presence of gangliosides (Montecucco, 1986; Pellizzari *et al.*, 1999). Internalization: After binding the neurotoxin enters the cell through receptor mediated endocytosis and is enclosed into vesicle. The environment of the vesicle is acidic and brings about a conformational change in the structure of neurotoxin (Pellizzari *et al.*, 1999). Translocation: Translocation of light chain into cytosol occurs due to the pH-dependent

conformational change in the structure of neurotoxin molecule (Blaustein *et al.*, 1987; Montecucco *et al.*, 1994). Inhibition of Neurotransmitter Release:

The release of neurotransmitter enclosed in synaptic vesicles is dependent upon SNAR proteins until or unless the SNARE complex does not bring the vesicle and membrane in close proximity, the fusion does not occur. This is where the light chain of BoNt comes into actions as it cleaves the SNARE protein and hence inhibiting the neurotransmission. More precisely the BoNT do not prevent the complexformationrather the complex formed is non-functional, due to this the coupling between Calcium influx and fusion is disrupted. The role of Ca++ ion concentration is crucial to the inhibition as the increase in its concentration is responsible for reversing the effect of BoNT (Sheridan, 1998).

Comparison

Although all the BoNTs more or less work by targeting the SNARE complex, they still exhibit differences among their target protein and target sites even on the same protein (Table1).

Table 1. Comparison of Different Types of BoNTs and their Molecular Target.

Toxin type	Cellular substrate	Target cleavage Site	Cell	Target localization	References
BTX-A	SNAP-25	Near C- terminus Gln197-Arg198	Neuron	Presynaptic Plama Membrane and Other Regions	Schiavo *et al.*, 1993
BTZ-B	VAMP/Synaptobrevin	Gln76-Phe77	Neuron	Synaptic Vesicle	Schiavo *et al.*, 1992
BTX-C	Syntaxin 1A, 1B SNAP-25	Lys-253- Ala-254; Lys252- Ala-253	Neuron Neuron	Presynaptic Plama Membrane and Other Regions	Blasi *et al.*, 1993; Williamson *et al.*,1996.
BTX-D	VAMP/Synaptobrevin	Lys59-Leu- 60; Ala-67-Asp-68 Unknown	Neuron and AllCells AllCells	Synaptic Vesicle	Yamasaki *et al.*, 1994 Yamasaki *et al.*, 1994
	Cellubrevin			Vesicles of Endocytosing/ Recycling System	
BTX-E	SNAP-25	Arg108-Ile-181	Neuron	Presynaptic Plama Membrane and Other Regions	Schiavo *et al.*, 1993
BTX-F	VAMP/Synaptobrevin	Gln58-Lys- 59	Neurons	Vesicles of Endocytosing/ Recycling System	Schiavo *et al.*,1993; Yamasaki *et al.*, 1994
	Cellubrevin	Unknown	All Cells		
BTX-G	VAMP/Synaptobrevin	Ala81- Ala82	Neuron		Yamasaki *et al.*, 1994

Materials and detection techniques used for BoNT produced by Clostridium botulinum

The complete study of different toxins produced by *Clostridium botulinum* strains can be detected by using different techniques. Among major techniques, the most important are PCR (Nakamura *et al.*, 2010), Multiplex PCR assays, Immunochromatography, Bradford, Immunodiffusion assay. For the detailed study of different toxins, first of all toxins producing strains are isolated and then DNA is isolated by using different methods of isolation. Manual process can be used to grow the stains on culture media at different conditions like Bacto^{TM}Brain Heart Infusion at 37°C for 18 hours under anaerobic conditions. The use of centrifuge machine helps in the cell collection of bacterium in the form of pallet. After this the pallet is suspended and cells are ruptured by using lysozymes and the total DNA is extracted through organic method. Then finally DNA is concentrated by using 70% ethanol.
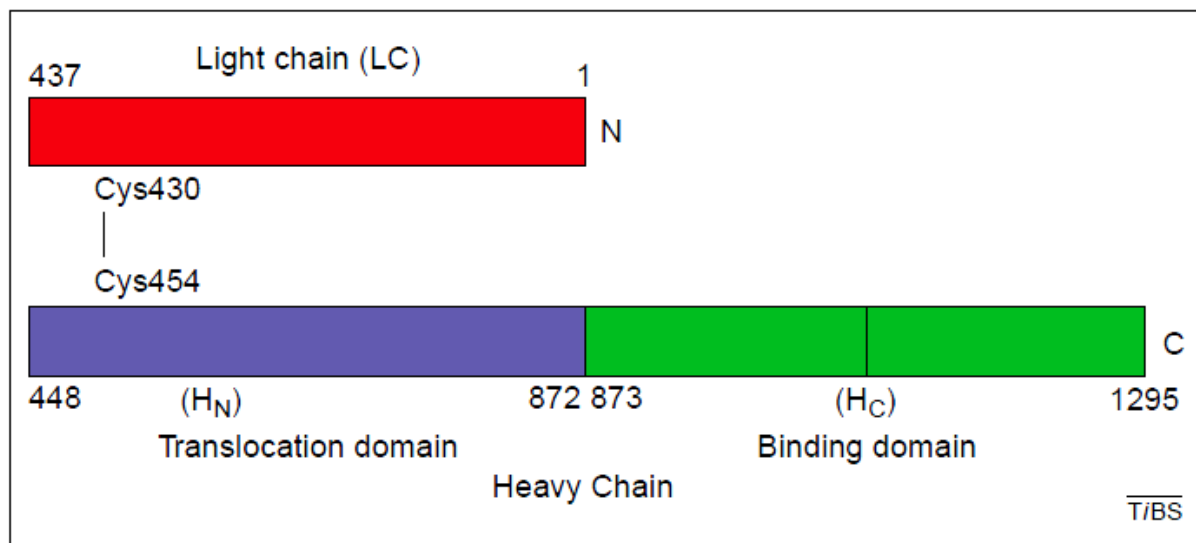
Fig. 1. Structure of BoNT.

The samples for examination can be collected from feces, intestinal contents, gastric juice from patient's stomach (Beaufrere *et al.*, 2016; Tartof *et al.*, 2013). There are different methods which can be used for the identification and characterization toxins produced (Figure 4). Types of samples used for these processes are as follows; Foodborne Botulism: serum, feces, gastric fluid, suspected food. Infant Botulism: feces, intestinal contents, serum, suspected food, environmental samples. Wound Botulism: serum, tissues, wound swab,pus.

Detection of Botulinum Neurotoxin: Mouse Lethality Assay

Mouse lethality assay has been usedfor many decades as standard (Lindström *et al.*, 2001) for the diagnostic purpose of neurotoxins of *Clostridium botulinum* but now there are many rapid assays which can be used and the results can be generated within 20 minutes (Lindström, Keto- Timonen, & Korkeala, 2014). But the sensitivity has beaten the mouse assays. Now the struggle is being done to generate a single assay which would be sensitive and rapid, and it should be for all seven strains of botulinum. Sample feces interfere with the results; fecal proteases degrade toxins which may give the false results.

Antitoxins are used to neutralize the toxin type for its identification so that it may not be degraded (Barash & Arnon, 2014; Solberg *et al.*, 1985). Non-lethal mouse assays has also been used but they do not cause any signs of distress and impaired movement of the animal.

Immunological methods

Immunological assays are simple to use and fast to perform and interpret as comparé to mouse assays (Grenda *et al*, 2014), gel diffusion assay, passive

hemagglutination assay (Grenda *et al.*, 2014). Recently the signal development made the assay almost equally sensitive like that of mouse assays. Due to the unavailability of high quality antibodies causes the major drawback of immunological tests.

The heat is applied in these tests which kill the toxins and these killed toxins cause the main reason of false positive results. The genetic variations of different serotypes of neurotoxins are responsible for false negative results.
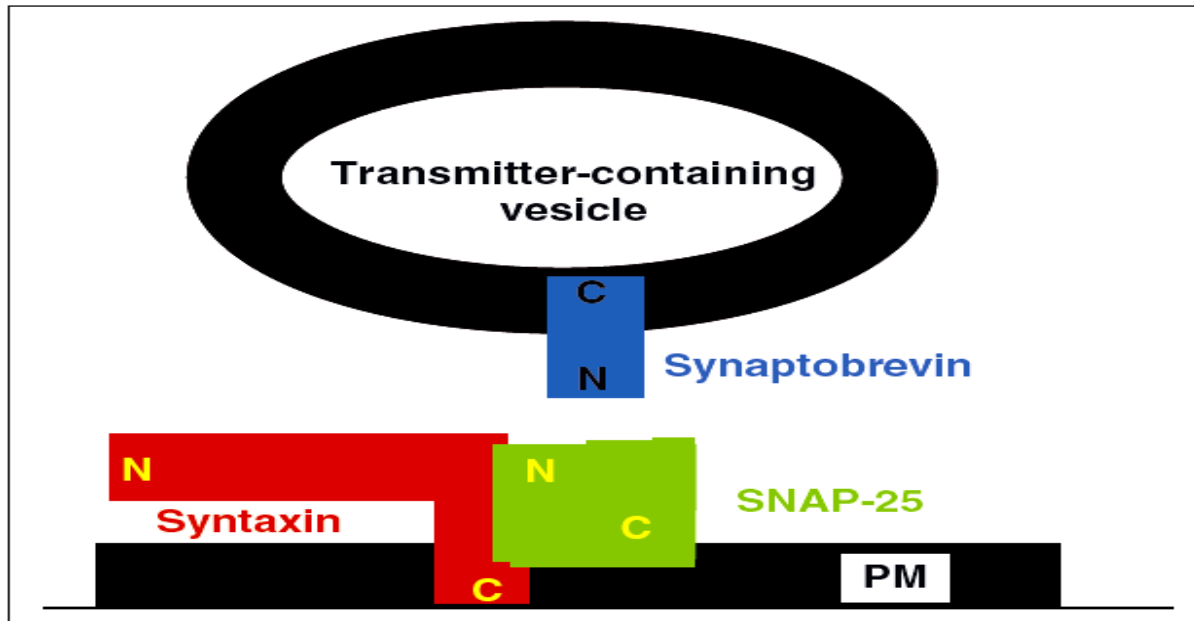
Fig. 2. Components of SNARE Complex.

For the neurotoxin detection ELISA is most commonly used immunological assay. The procedure used is quite simple and easy to use. In this procedure neurotoxins are bind to the solid surface. The surface on which toxin has to bind is usually pre-coated with polyclonal or monoclonal capture antibodies, depending either there are one or more toxins under examination in the reaction plat. Then antitoxin molecule is added which contains enzyme, in most of the cases Horseradish peroxidase or alkaline phosphatase. After complete binding with the toxin, for signal generation substrate is added which combines with enzyme and causes signal generation which can be detected in the reaction center. The sensitivity of ELISA is almost 10 to 100 times lesser than that of mouse assay (Matović, 2013).

Clinical features

Food Borne Botulism: Ingesting performed toxins of *C. botulinum* present in canned food which have survived cooking and canning process cause foodborne botulism. The spores germinate and

reproduce in an anaerobic environment to produce toxin. Incubation time and symptom onset of aerosolized toxin is longer (Mcnally *et al.*, 1994). Out of all syndrome symptoms, the major sign and symptom is toxin induced neurological blockage affecting voluntary and autonomic functions. The severity of symptoms are according to the type of toxin present in food i-e type A toxin has high severity and fatality rate than type B and E toxin (Woodruff *et al.*, 1992). Severity of the symptoms associated with food borne botulism depends upon the time after exposure. Initially 18-36 hours after exposure, Nausea, Vomiting, Abdominal cramps, Constipation, Blurred vision, Dry mouth, Diplopia develop. Followed by onset- 8 days after exposure, Dysphonia, Dysarthria, Dysphagia, Peripheral muscle weakness, Weakness of respiratory muscles, Weakness of upper and lower extremities will develop (Hughes *et al.*, 1981). Severe Cases have also been reported with 8 weeks up to 7 months exposure with symptoms including Respiratory muscle paralysis, Ventilatory failure or Death. In severe cases patient may require

respiratory support commonly needed for 2- 8 weeks (CDC). Recovery require weeks to months as it involves new pre synaptic end plates and neuromuscular junction formation. Supportive care decreased the chances of death by 50-55% from 1950s to date. Wound Botulism: Germination of *C. botulinum* spores within an anaerobic abscessed

wound that allows multiplication of botulinum, production and absorption of its toxins results in wound botulism. Incubation period ranges from 4-14 days (Merson and Dowell, 1971) Symptoms are same as that of food borne botulism expect gastrointestinal one's with fatality rate of approximately 15% (Hatheway,1995).
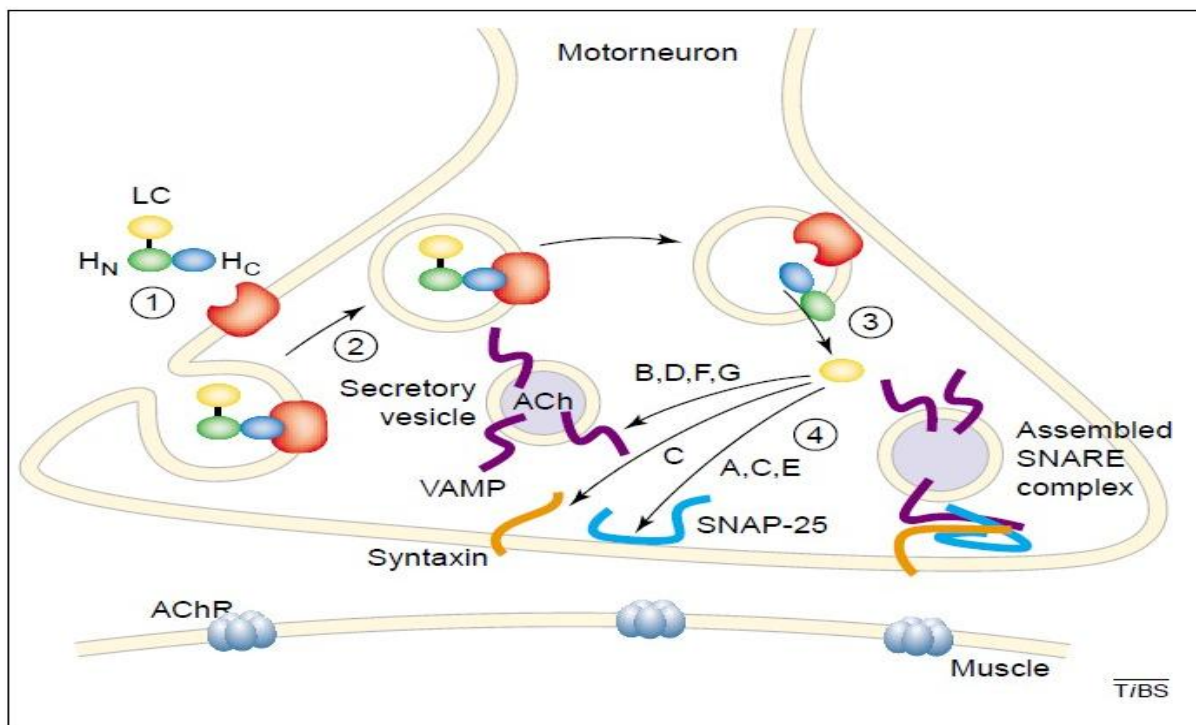

Fig. 3. Mode of Action of BoNT.

Infant Botulism:Infant botulism is caused when spores of *C. botulinum* enter, proliferate and produce toxin in the gastrointestinal tract during the second month after birth. Earliest signs and symptoms include Constipation, Poor feeding, Weak cry, Lethargy, Lack of muscle tone and Floppy head (Wilson *et al.,* 1995) Severity of infant botulism can cause sudden death and recovery takes about weeks or months. 85% of the source of *C. botulinum* spores in infant is unknown the rest is suspected due to the ingestion of honey (Arnon, 1998). Risk factors are not clear (Spika *et al.,* 1989).

Adult Botulism: Adults can also suffer from botulism if *C. botulinum* colonize the intestine and produce toxins like in infant botulism (Griffin *et al.,* 1997). Patient who have undergone any abdominal surgery, have gastrointestinal abnormalities or are treated

with antibiotics (McCroskey and Hatheway, 1988, Chia *et al.,* 1986) are more prone to type A and B *C. botulinum* infection.

Treatment
Contaminated food is removed from the gut by inducing vomiting or by performing enemas whereas infected wound should be surgically treated in order to remove the toxin producing organism. In addition, supportive care i-e IV fluids and breathing support may also be needed during the therapy of all kinds of botulism. Unabsorbed toxin is removed by enemas. Antibiotics are not useful against food borne botulism but wound botulism can be treated with them to some extent when used along with surgery. Toxin strength is increased by magnesium salts, sulfate and citrate. To help manage treatment protocols consultation with a specialistis recommended.
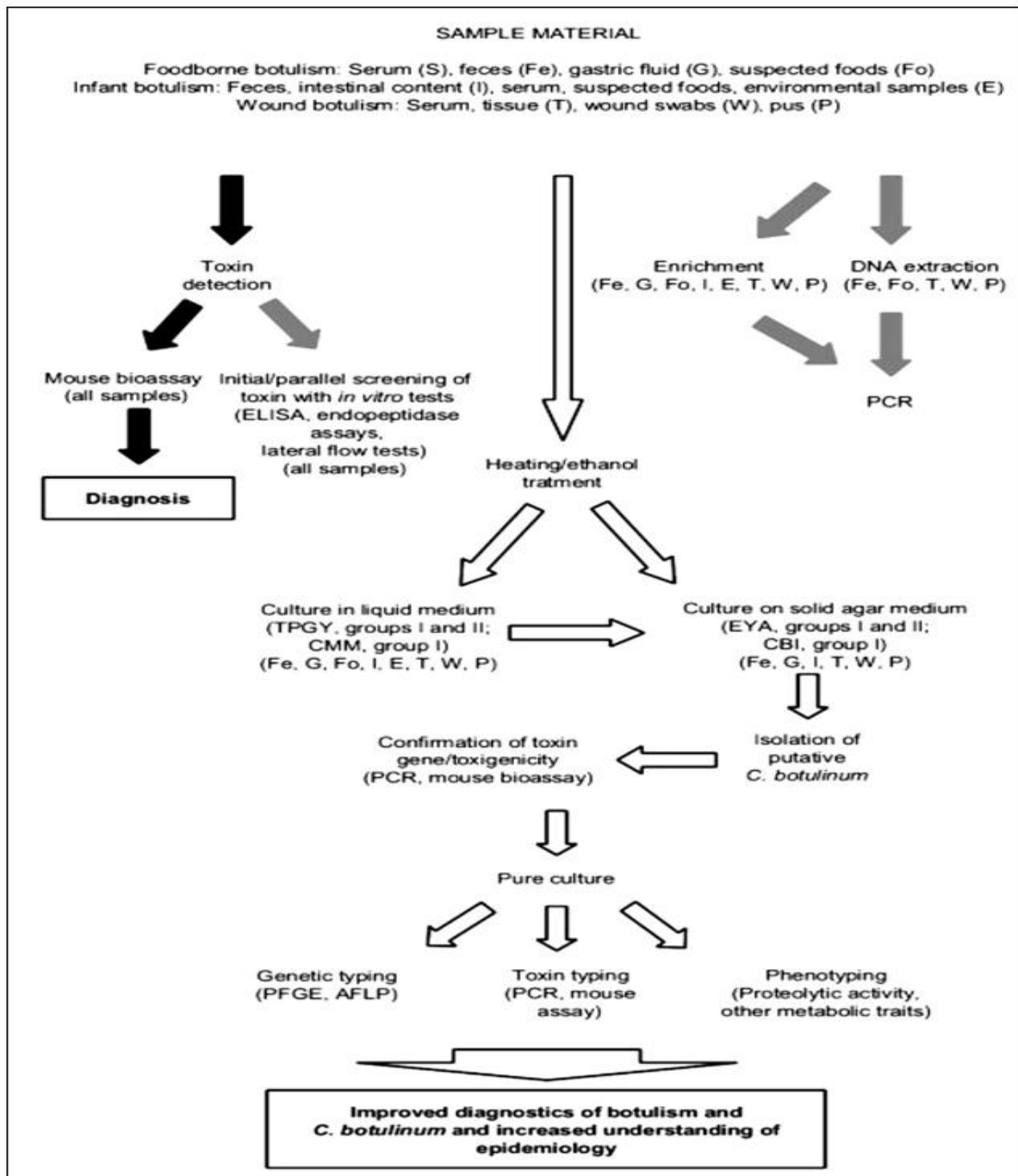
Fig. 4. Laboratory diagnostics of botulism. Black arrows show the standard methods, and the gray arrows are actually responsible for initial screening. But characterization has been shown through white arrows (Feikin *et al.*, 2000; Lindström and Korkeala, 2006).

Antitoxin treatment

BabyBIG: Before the advent of BabyBIG (human immunoglobulin given IV) no antitoxin was used for the treatment *of infant* botulism. BabyBIG is safe and effective but can only be obtained from Public Health department of California, effective against type A, B andC. Equine: This bivalent antitoxin is used nowadays to treat botulism type A and B. it is a

refined antitoxin prepared from horse globulins which are enzymatically digested and modified. 0.4% phenol is used as a preservative (Karashimada, 1997). The exact amount and concentration of antitoxin required to neutralize botulism type A and B is not yet fully documented but one or more vials of equine may be required to counterattack the toxins according to their severity. Precautionary measures are taken

before administering the antitoxin according to the patient's history of having asthma, hay fever or distress due to close proximity of horses. In case of any hypersensitive reaction patients should begiven epinephrine hydrochloride solution (1:1000) immediately.

Dosage and administration

Prevention of Botulism Types A and B

If someone has eaten any suspected food being infected, it is recommended to give a prophylactic dose (1,500-7,500 IU of Type A and 1,100-5,500 IU of Type B given intramuscularly) to the individual. If still any symptoms appear within 12-24 hours, another vial is followed for the treatment (Miller, 1954).

Treatment of botulism types A and B

For best results treatment should be given as early as possible after exposure to infection; Intravenous injection (IV). A dose of 7,500 IU of Type A and 5,500 IU of Type B (one vial diluted with 0.9% saline 1:10dilution) should be given intravenously to neutralize all the toxins present in the fluid.

Intramuscular injection: Same dose is administered intramuscularly to provide a reservoir of antitoxin in the body. Further doses are according to the sign and symptoms monitored in next 2-4 hours. Patients with a history of asthma, allergy or sensitivity of horse serum required great care in administering the antitoxin. Serial dilutions of antitoxins may be administered at 15 mins interval to minimize sensitivity problem.
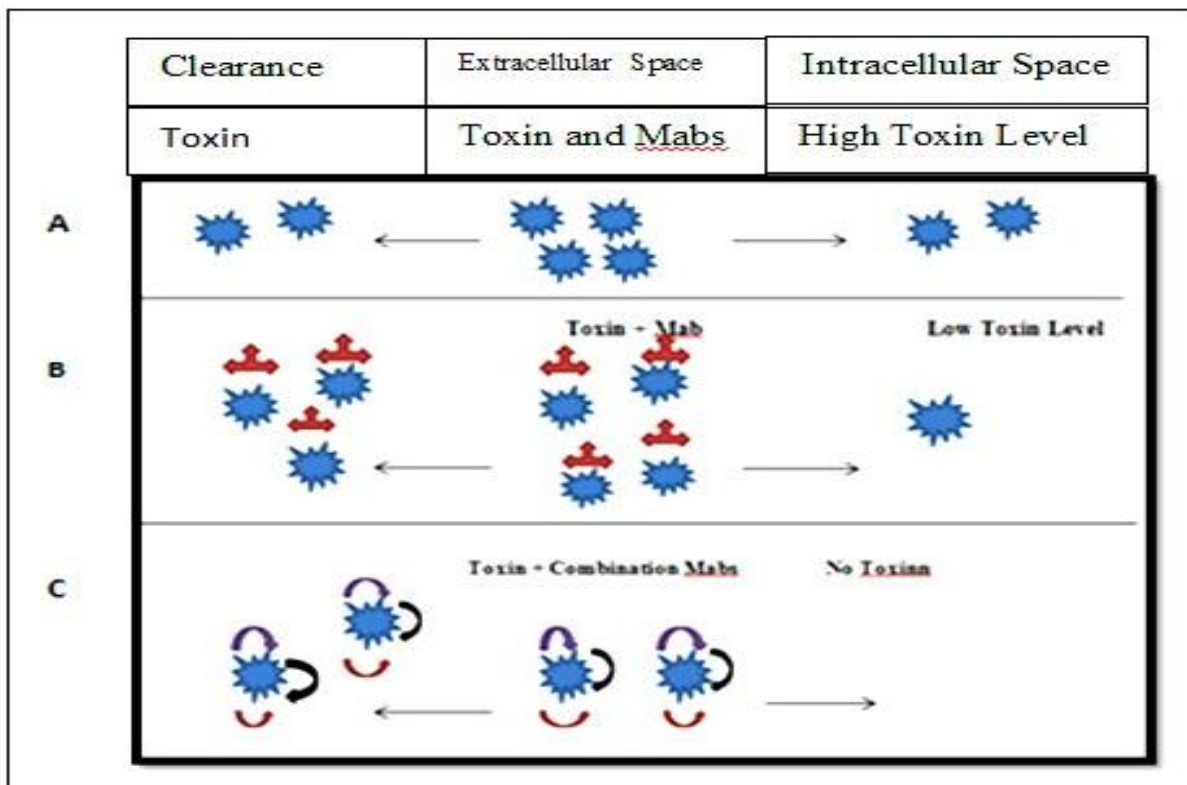
A. Toxin in the absence of MAbs can pass the intracellularbarrier.

B. In the presence of single MAbs low toxin level can pass into intracellularspace.

C. Combinatorial MAbs don't allow the toxin to pass into intracellular space by forming a complex withtoxin.

Fig. 5. Antibody interaction with the toxins.

Mabs

Treatment of Type A, B and E botulism is possible through monoclonal antibody based antitoxins as represented in the Figure 5. Large amount of Mabs

characterized on the basis of epitope and affinity are generated using BoNT/A, B and E immunized human and mice. In-vivo and in-vitro characterization is also taken in account. Combination of 3 Mabs binding

epitopes is more potent than single Mabs in-vivo. Toxin neutralization by Mabs requires an intact Fc receptor (Tomic *et al.*, 2013).

HBAT (Botulism Antitoxin Heptavalent A, B, C, D, E, F,G- equine) US approved 2013

HBAT is an antitoxin against all 7 serotypes of botulism. It contains mixture of fragments of immunoglobulin from equine that neutralize all types of toxins. It can be administered intravenous only (1:10 dilution with 0.9% saline). For adults' one vial at rate of min. 0.5ml/min – 2ml/min max. For pediatrics 20-100% the adult doses at min. rate of 0.01ml/kg/min- 0.03ml/kg/min max. For infants 10% of adult dose at min. rate of 0.01ml/kg/min- 0.03ml/kg/min max. Each single vial contains 4,500 U for type A antitoxin, 3,300 U for type B antitoxin, 3,000 U for type C antitoxin, 600 U for type D antitoxin, 5,100 U for type E antitoxin, 3,000 U for type F antitoxin and 600 U for type G antitoxin. In case of hypersensitivity reaction epinephrine solution is used immediately and patients are monitored for delayed allergic reactions and infusion reactions.

Mechanism of action

HBAT works through passive immunization with polyclonal antibody fragments from equine which are primarily F (ab') and Fab against all serotypes of Botulism neurotoxin.

These circulating antibodies bind to the botulinum toxin and restrict their interaction with ganglioside anchorage site and nerve endings. Then immune system clears these antigen/antibody complexes from the body and thus prevents the toxin internalization into target cells. *Small molecules*

Peptide based inhibitor

Small peptides have been developed based on substrate information as a competitive inhibitor for BoNT. Short peptides of sequence CRATKML have been developed to inhibit BoNT endopeptidase activity on cleavage site EANQRAT, Q and R being the cleavage site (Schmid and Stafford, 2001). Modifications in this basic sequence can be fruitful e.g

replacement of cysteine with 2-mercapto-3-phenylpropionylgenerates Ki peptide of 330nM (Schmid and Stafford, 2002).

Phage display technology: Phage display technology is used to screen potential small peptide inhibitors that target the desired BoNT endopeptidase activity (Zdanovsky *et al.,* 2001). Milimolar to micromolar concentration has proved to have complete inhibition effect on BoNT. Libraries of hinge peptide i-e containing Asp and Glu, zinc chelators His and Cys and scissile-bond amino acide for BoNT/A (Gln and Arg) (Hayden *et al.,* 2003) have inhibition effect on protease activity of BoNT/A. Each library involves structure i-e acetyl-X1-X2-linker-X3-X4-NH2 or X1-X2- linker-X3-NH2.

Pseudopeptides act as competitive inhibitors for synaptobrevin site Gln76-Phe- 77 for BoNT/B (Martin *et al.,* 1999). Amino thiol derivatives is to replace the S1 from the tripeptide inhibitor interacting with cleavage site, that has a strong inhibition effect on BoNT/B. (Anne *et al.*, 2003).SNARE motifs are potential targets for BoNTs (Li and Singh, 1999). VAMP known as V2 have the sequence 62ELDDRADALQ71 has shown to inhibit binding of neurotoxin with SNARE motif (Rosetto *et al.,* 2001, Hayden*et al.,* 2000).

Receptor mimics

BoNT bind to nerve cell by first binding to gangliosides and then receptors (Montecucco *et al.,* 1986, Montecucco *et al.,* 2004). Rreceptor mimics that can bind with gangliosides and receptors (Synaptotagmin for BoNT/A and synaptotagmin II for BoNT/B) can inhibit functional binding activity at 10mM concentration. Sugar mimics and synaptotagmin derived mimics may lead binding inhibition between BoNT and gangliosides (Cai *et al.*, 2005).

Aptamers

Oligonucleotides having high affinity for targets and can be isolated against all protein targets are known as aptamers (Nimjee *et al.,* 2005, Tuerk and Gold,

1990, Pestourie and Duconge, 2005). Aptamers can be used to block the functional protein targets. SELEX, an aptamer screening process includes generation of random and almost 1014-1015 RNA or DNA sequences depending on its target. Extracellular toxins can be neutralized by using aptamer technique as it covers all functional domains of BoNT. It can be used as both therapeutic and prophylactic treatment and can reverse clinical effects when used with proper delivery system.

Delivery system

A proper delivery system is required to deliver inhibitors to targeted nerves to reverse the clinical effects. Heavy chain on BoNT has been shown to be the natural safe and potential delivery system to deliver molecules in affected nerves. (Goodnough *et al.*, 2002; Zdanovskaia *et al.,* 2000).

Inhibition molecules like small peptides or aptamers can be either conjugated or encapsulated with HC liposomes and can be delivered to target site and neutralize the action of toxins. Antibodies can be generated against this HC, thus limiting the use of BoNT delivery system in future.

Prognosis and prevention

Though botulism infection takes weeks and months to recover but with new medical interventions it can be cured completely. Recovery depends on the severity of disease as it can take years for a severe botulism infection to be cured completely as recovery depends on the generation of new proteins of damaged nerve endings. Botulism that is leftuntreatedusedtohaveamortalityrateofabout50%.N owadays, with appropriate and new treatment this rate is reduced to only 3-5%. Early diagnosis plays a vital role in treatment and better prognosis of disease. Botulism can be prevented by reducing the use of canned food, cooking food properly which are suspected to have botulinum spores, avoid using honey for infants for at least 12months, seeking medical help for treating wounds properly, avoiding the potential sources of botulism and using right choice and amount of preservatives. (CDC, 2014).

Conclusion

Reduction in SNARE proteins enhances the binding of toxin with the synapse thus blocking it. Enhancement in the production of SNARE proteins either by analogue molecules or by changes in genes producing SNARE, can reduce the toxin effect. Peptide analogues to target receptors can also inhibit toxin binding. Endopeptidase activity of BoNT is inhibited by CRATKML sequence. Modification in this basic sequence is shown to have potential neutralizing activityagainst BoNT. Competitive inhibitors are also used to inhibit toxin binding with receptors and increasing their concentration can completely inhibit toxin binding. BoNT activates in acidic environment thus, turning the environment basic and inhibiting the HC and LC complex also serves the purpose. As HC of BoNT can induce immune response, so it can be a potential candidate for vaccine synthesis in future.

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