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Botulinum associated with visceral toxicosis of catfish: Investigation of a viral-vectored

heavy chain subunit vaccine and development of a zebrafish bioassay

By

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Mississippi State, Mississippi

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Botulinum associated with visceral toxicosis of catfish: Investigation of a viral-vectored

heavy chain subunit vaccine and development of a zebrafish bioassay

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Visceral toxicosis of catfish (VTC) is a sporadic, often devastating disease in catfish aquaculture, caused by botulinum neurotoxin serotype/E (BoNT/E). The median lethal dose of BoNT/E for channel catfish (*Ictalurus punctatus*) fingerlings is 13.7 pg/fish. The diagnosis of VTC is suspected if clinical signs and lesions are produced when affected serum is injected into sentinel-catfish and confirmed if this effect is neutralized with BoNT/E antitoxin. However, the assay is complicated in diagnostic cases by small serum samples from affected fish, lack of standardization and availability of small catfish (~10g). Therefore a zebrafish (~0.4g) bioassay for VTC diagnosis was tested and validated. Susceptibility was tested with other serotypes of toxin to help establish zebrafish as a diagnostic model for BoNTs. BoNT/E consist of100kD heavy chain (HC) and 50kD light chain (LC) linked by a disulfide bond. The HC transports the LC into the cytosol of the neuron, where LC (Zn^{2+}-endoprotease) cleaves the SNAP-25 protein thereby blocking the signal transduction at the neuromuscular junction. The HC-based vaccines can induce protective immunity in mammals. To evaluate HC

immunogenicity in catfish, rBoNT/E/HC vaccine produced by USAMRIID was tested; this vaccine did not induce a robust antibody response, but western blot analysis demonstrated specific antibody production in 3 of 11 vaccinated fish. We then developed four channel catfish virus (*Ictaluridherpesvirus* 1, CCV) recombinants expressing synthetic BoNT/E/HC using our established Gateway CCV recombination system to determine if the virus vector could improve the response. Catfish were vaccinated with these recombinants or with a control vector that expressed *Escherichia coli* beta-galactosidase (CCV-lacZ). No significant protective immunity or BoNT/E antibodies were observed but CCV-lacZ induced a strong antibody response. These results suggest that BoNT/E/HC has low immunogenicity in channel catfish and deviates from the high immunogenicity observed in mammals. To develop a protective vaccine for VTC, it will be necessary to enhance the BoNT/E /HC immunogenicity in channel catfish. Development of recombinant animals which are resistant to BoNT/E was explored as another potential way to prevent VTC. However, the attempts to modify SNAP-25 of zebrafish by genome editing using Transcription Activator-Like Effector Nucleases were unsuccessful.

DEDICATION

I dedicate this doctoral dissertation to the loving memory of my father Chatla.
Venkatesham garu for his support and belief in me. I also dedicate this dissertation to my
loving mother, Chatla. Vijaya garu, and my siblings, Chatla. Sridhar, Chatla. Shekher,
and Chatla. Hemalatha for their encouragement, love and support.

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CHAPTER I

INTRODUCTION

1.1   Visceral toxicosis of catfish

In 1998, several catfish farmers observed acute mass mortalities in their Mississippi Delta catfish farms. The mortality rates were higher from mid-February until early May when pond temperatures were between 18 to 22^0C. Mortalities were mainly observed in market sized and brood stock fish [32, 48]. The clinical signs of affected fish included swimming rapidly at the surface for short periods. Post-mortem lesions in affected fish were exophthalmia, ascites, congested spleen and intussusceptions of the intestinal tract and eversion of stomach into the oral cavity [32, 48]. The syndrome was named visceral toxicosis of catfish (VTC) based on lesions and a presumed toxicological etiology. The clinical signs and lesions of VTC were reproduced when sentinel fish were injected with serum of infected fish; however when the serum of infected fish was heated to 85℃ for 5 min prior to the injection, the clinical signs and characteristics were not produced[32]. VTC diagnostic submissions were negative for bacterial or viral pathogens, and algal toxins. Visceral toxicosis was later found to be caused by the botulinum neurotoxin serotype-E (BoNT-E), which was confirmed by serum neutralization in sentinel catfish and endopep mass spectroscopy[32].

Visceral toxicosis of catfish is a sporadic, often devastating disease in catfish aquaculture. From 2003 to 2010, averages of 3.1% of diagnostic fish cases were

confirmed as VTC positive at the MSU-CVM aquatic diagnostic laboratory in Stoneville, MS. Outbreaks of VTC accounted for as much as 37.8% losses on commercial farms in some years[18]. Between 1979 and 1983, outbreaks of botulism occurred in Washington and Oregon which resulted in the loss of $2.3 million steelhead trout, Chinook and coho salmon[58]. The European trout industry also suffered substantial losses due to botulism which was associated with restricted feeding and insufficient removal of dead fish[41]. Although outbreaks of VTC are not regular, when they do occur the impact can be economically devastating for catfish farmers. The high losses of older, market size fish make VTC a severe problem to the catfish industry[32, 48].

1.2   Toxin Production

Clostridium botulinum is a diverse bacterial group, which produces eight types of botulinum neurotoxins A-H. Serotypes H was only recently discovered, hence most of the existing studies were conducted on toxins A-G, which are differentiated based on their immunological differences [5, 19, 37]. Botulinum neurotoxins (BoNT) are synthesized in the bacterial cytosol and because they lack leader sequences cannot be transported from the bacterial cell membrane [42]. The toxins are released after the bacterial cell is lysed. *C. botulinum* produces toxins in the form of multimeric complexes associated with other proteins called progenitor complexes. The progenitor complex contains the BoNT and other components such as hemagglutinin (HA), nontoxic non-HA (NTNH), *orfx* and *botR* components. All these protein genes are linked as clusters. The serotype B, C, D and G are associated with HA+ cluster, and Type E and F are associated with *orfX+* cluster[2]. NTNH are located downstream of these clusters, and BoNT is located upstream of NTNH (figure 1.1).

Figure 1.1 content (gene clusters):

Bont	Strain	Gene cluster
E4	C. butyricum BL5262	IS' orfX3 orfX2 orfX1 p47 ntnha bont/E4 IS'
E3	Alaska E43	IS' orfX3 orfX2 orfX1 p47 ntnha bont/E3 IS'
E1	Beluga	IS' IS6 orfX3 orfX2 orfX1 p47 ntnha bont/E1 IS'
F6	IBCA66-5436 npF	orfX3 IS' orfX2 orfX1 p47 ntnha bont/F6 IS'
A1 of A1(B)	NCTC2916	orfX3 orfX2 orfX1 botR p47 ntnha bont/A1 lycA
F1	Langeland	orfX3 orfX2 orfX1 IS6' botR p47 ntnha bont/F1 lycA
A2	Kyoto-F	orfX3 orfX2 orfX1 IS6' botR p47 ntnha bont/A2 lycA
F2 of Bf	pBf	IS605' lycA orfX3 orfX2 orfX1 IS6' botR p47 ntnha bont/F2 IS3'
A3	Loch Maree	IS605' lycA orfX3 orfX2 orfX1 IS6' botR p47 ntnha bont/A3 IS3'
A4 of Ba4	657 Ba4	lycA orfX3 orfX2 orfX1 IS6' botR p47 ntnha bont/A4
B5 of Ba4	657 Ba4	hypo hypo fla' ha70 ha17 ha33 botR ntnha bont/B5 IS
B1	Okra	hypo hypo fla' ha70 ha17 ha33 botR ntnha bont/B1 IS256'
B5 of Bf	pBf	hypo hypo fla' ha70 ha17 ha33 botR ntnha bont/B5 IS256'
B4 of npB	Eklund 17B	ha70 ha17 ha33 botR ntnha bont/B4 IS256'
(B) of A1(B)	NCTC2916	hypo hypo fla IS3' ha70 ha17 ha33 botR ntnha bont/(B) ISNCY'
A1	Hall	hypo hypo fla IS3' ha70 ha17 ha33 botR ntnha bont/A1 IS3'
A5	H04402 065 IBCA66-5436	ha70 ha17 ha33 botR ntnha bont/A5
C	Stockholm	ha70 ha17 ha33 ntnha bont/C1
D	1873	ha70 ha17 ha33 botR ntnha bont/D
G		ha33 ha70 ha17 botR ntnha bont/G

Figure 1.1   Botulinum neurotoxin gene clusters

The variation in the botulinum neurotoxin gene clusters: from Botulinum neurotoxin by Rummel et.al[2]

The progenitor complex is produced in three different forms: medium (M), large (L) and extra large (LL). All three are in association with non-toxic neurotoxin-associated proteins (NAPs). The M toxin is about 300 kDa and is formed by conjugation of BoNT and NTNH; its sedimentation constant is 12S. The L complex is about 500 kDa and is formed by conjugation of M toxin and HA component; its sedimentation constant is 16S. The LL toxin is about 900 kDa and is assumed to be composed of two L toxins; its

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sedimentation constant is 19S [42, 50]. Botulinum serotype A exists in all three forms (M, L, and LL). Serotypes B, C, and D exist in both the L and M forms. Serotypes E and F exist in only the M complex, and Type G exists in the L complex only[29]. The components of the progenitor complex provide protection and stability to the neurotoxin while they pass through the gastrointestinal (GI) tract in a low pH environment[82]. The activity of the progenitor-protected toxin is 10 to 100 fold higher through the oral route compared to the pure toxin; this shows that the progenitor complex plays a crucial role in food-poisoning by protecting the toxin from the harsh environment of GI tract[20]. BoNT/A and BoNT/B LL and L progenitor protected neurotoxins show resistance to pepsin protease and the acidic environment in the GI tract. NTNH attaches to certain types of carbohydrates in the intestinal wall which helps in the translocation of the toxin across the intestinal mucosal layer [24, 43]. The progenitor complex dissociates in the lymph as the neurotoxin, and the non-toxic components after they pass through the alkaline conditions of the small intestine [36].

1.3   Neurotoxin passage through GI tract and uptake

Ingestion of *C. botulinum* contaminated food is the most commonly form of botulinum intoxication because BoNT can withstand the harsh gastrointestinal (GI) environment. Most proteins are broken down into amino acids and peptides during the digestion process in the GI tract, but the NAPs and other components in the progenitor complex provide the stability to survive the acids of the stomach and intestinal proteases. The GI tract has multiple barriers which can protect the host from harmful external elements, although, the actual mechanism involved in overcoming these barriers by progenitor complex is not fully elucidated. Based on previous research the BoNTs can be

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absorbed through the alimentary canal epithelial barriers, small intestine, stomach and colon, although the upper intestine is considered the most important location for absorption[80]. The BoNT binds to the epithelial cells on the apical surface of the gastrointestinal tract and undergoes a receptor mediated endocyotosis and transcyotosis. It is then carried from the lumen of the gut interstitial fluid where it finally reaches the general lymphatic circulation [89]. The migration of the toxin, via penetration of the epithelial cell barriers to cholinergic nerve terminals, is an essential step for botulinum intoxication.

The primary site for absorption of BoNT is the upper small intestine, but it is also absorbed from the stomach. BoNT binds to the epithelial cells and is transported to the basolateral apical sides of these cells through receptor mediated endocytosis and transcytosis. All forms of the BoNT exist as an un-cleaved or single chain; the di-chain forms with the disulphide bond in the reduced or oxidized states are able to bind and penetrate through the epithelial cells [59]. The BoNT/E heavy chain (HC) or the C-terminal of the HC contains a receptor binding domain, which assists in the transcytotic process. A double receptor model was proposed for BoNTs crossing the intestinal cell monolayer; BoNT/A first binds with GT1b and GD1b gangliosides of the intestinal cells and then with a protein receptor, which mediates the receptor-mediated transcytosis[21]. Evidence suggests that BoNT/A is transported via transcytosis, which is mediated by specific receptors of the intestinal cells, which are different from the BoNT/B receptors [24]. The HC of BoNT/A drives the toxin transport and recognizes the specific receptors GT1b/GD1b and the protein part of the synaptic vesicle glycoprotein 2 (SV2) or immunologically similar SV2 related proteins [24]. This suggests that gangliosides GT1b,

GD1b and SV2 or other immunologically related proteins are important for the transport of the BoNT/A through the intestinal cells. The role of NAPs on epithelial binding is controversial. Some reports suggest that the neurotoxin itself has a binding moiety, and some suggest that NAPs assist in the attachment of the neurotoxin to epithelial cells. Type C progenitor toxin attaches to a high molecular weight glycoprotein like mucin and the surface of HT-29 colon carcinoma cells; this attachment leads to the uptake of the toxin via the clathrin pathway[97]. BoNT acquired by inhalation of the toxin crosses the respiratory system's epithelial layers and finally reaches the peripheral cholinergic nerve endings through the general circulation[67] and BoNT absorbed through the GI and respiratory system reaches the peripheral cholinergic nerve endings through the blood and lymph. All these observations show that different BoNT serotypes use different molecular mechanisms to cross the intestinal barrier of susceptible hosts. .

1.4   Neurotoxin specific binding

The extreme toxicity of BoNTs is due to their efficient delivery of the light chain (Zn^{2+} endopeptidase) into the cytosol of the neuron by their high specificity and efficient uptake by clathrin-coated vesicles [9, 28]. The available data from biochemical assays and studies on gangliosides-lacking mice indicates gangliosides are essential for accumulation of BoNTs on neuronal cell surface. The BoNT/A, E, F and G weakly attach to GD1a, GT1b and BoNT/B, C and D weakly attach to GD1b, GT1b and GQ1b gangliosides. The high abundance of these poly-gangliosides is the first step for BoNT intoxication.

BoNTs are synthesized as 150 kDa single polypeptide proteins subsequently digested by bacterial or host proteases into 100 kDa heavy chain and 50 kDa light chain

connected by a disulphide bond and non-covalent interactions. The 100 kDa HC

efficiently transports the LC into the target, the neuronal cell cytosol. The HC consist of

two 50 kDa domains comprised of N-terminal domains HC_N and C-terminal domain

HC_C. In the seven serotypes (A-G), the structure of these domains is conserved with

differences in amino acid sequences. The HC_C domain contains major receptor

recognition sequences; which can bind to target neuronal surface molecules. Even though

the core structure of the HC_C and HC_N are similar in seven types of BoNTs, the surface

exposed loops of these domains have different structures, which play a major role in

recognizing different gangliosides and target molecules in the substrates. The relatively

low affinity of BoNTs to poly-gangliosides, and the inhibitory effect of BoNTs binding to

neurons after being pre-treated with extracellular proteases suggests a dual receptor

hypothesis[64]. In the dual receptor hypothesis, two sequential binding steps are

necessary: first, low affinity binding to gangliosides with the accumulation of BoNTs;

second, subsequent bindingto protein receptors such as synaptotagmin (syt) or synaptic

vesicle glycoprotein 2 (SV2) isoforms. Synaptotagmin is a calcium sensor which couples

the action of the neurotransmitter release based on calcium influx, and SV2 is associated

with syt-I and may regulate the endocytosis[103]. BoNT/B and G bind to syt-I/syt-II

proteins, and other BoNT serotypes predominately depends on SV2; BoNT/A binds to

SV2C and BoNT/E binds to SV2A and to a lesser extent to SV2B.The available evidence

suggests that BoNT/D and F internalization depends on SV2, but the actual binding

mechanism is not fully elucidated[2, 78].

Once BoNTs bind to their surface receptors; these protein-protein and protein-

lipid interactions trigger the recruitment of a complex cascade of adaptor proteins in the

plasma membrane, which aid in the endocytic process and toxin internalization. One of the early processes is the recruitment of clathrin specific adaptor proteins, which help in the altering of the membrane curvature at the endocytic site by the accumulation of effector proteins. The enrichment of specific lipids and the membrane curvature forms a clathrin basket by a series of complex cascades. The next step is the formation of a clathrin coated vesicle (CCV) and the recruitment of large GTPase which drives the fission of the CCV by hydrolysis.

1.5 Internalization and translocation of neurotoxin

The light chain, zinc containing endopeptidase, blocks the signal transduction between the neuron and muscles at the neuromuscular junction by cleaving soluble N-ethylmaleimide sensitive fusion attachment (SNARE) proteins. The SNARE proteins are present in the cytosol near the plasma membrane, and some are trans-membrane proteins. In order to access these proteins, the LC must be internalized into neuron cytosol. The HC interaction with neuronal cell surface gangliosides and proteins receptors leads to the internalization of BoNT by receptor-mediated endocytosis. Once internalized, the endocytic vesicle containing BoNT will mature into an early endosome where acidification of the vesicle occurs by the H^+-vATPase pump. This acidic environment induces a conformational change in the HC and exposes the hydrophobic segments; these conformational changes will help insert the HC into the endosomal bilayer membrane[2, 27, 30, 49, 84]. Finally, the HC forms a protein-conducting channel which can translocate the LC into the cytosol of the neuron [27, 49]. Here, the heavy chain's N-terminal region plays a critical role in the transfer of the light chain from the endosome to the cell's cytosol. In an acidic environment, the BoNT conformation is changed to an "acid"

structure from a water soluble neutral structure with its surface exposed hydrophobic segments. This conformational change gives the ability to penetrate into the endocytic vesicle lipid bilayer. The acidic environment in endosome does not induce activation of the toxin but it aids in translocation of LC which is proved by studies conducted on acid treated and the non-acid treated LC both LCs blocks exocytosis into the cytosol[46]. In an acidic environment, the HC maintains a stable folded state, but the LC undergoes significant reversible structural rearrangements in which the α-helical structure of the LC changes to a large β-sheet and β-turns structure[49]. The LC will refold to its normal enzymatically active conformation once it reaches the cytosol. The disulfide bridge plays a major role in translocation of the LC into neuronal cytosol. The reduction of the disulphide bridge in the endosome or premature reduction of disulphide bridge after channel formation in the endosome arrests the translocation of LC. The disulphide bridge must be present until the LC reaches the cytosol for productive translocation.

1.6   Light chain or zinc endopeptidase activity

The LC or zinc endopeptidase cleaves one or more SNARE proteins in the neuron's cytosol and inhibits the synaptic exocytosis at neuromuscular junctions (NMJ). The LC is a zinc metalloprotease containing a Zn atom at the center of the active site coordinated by glutamic acid and histadines in a LC conserved motif. The conserved motif of zinc endopeptidase is HEXXH composed of hydrophobic amino acids [10]. Once LC is internalized into neuronal cytosol, the cytosol environment aids LC to refolds its active protease conformation which can cleave one or more SNARE proteins. The SNARE proteins are essential for exocytosis of the synaptic vesicle and signal transduction at the NMJ. Different BoNT serotypes cleave one or more of these proteins:

synaptosomal associated protein of 25kDa (SNAP-25), vesicle associated membrane protein (VAMP), and syntaxin. VAMP is anchored by the hydrophobic carboxyl terminus to a vesicle containing neurotransmitters. The N-terminal region of the VAMP 30 residue is a proline-rich sequence, and the central 70 amino acids of VAMP is predicted to form an α-helical structure with hydrophobic faces centered on the arginine residue while it is attached to other SNAPs (SNAP-25 and syntaxin). SNAP-25 is anchored to the membrane through multiple palmitoylated cysteines at alternatively spaced domains near the middle of the protein. Syntaxin is anchored in the plasma membrane of the neuron cell via a C-terminal hydrophobic stretch of 22 amino acids. The heterotrimeric complex of the VAMP, SNAP-25 and syntaxin is referred to as the core complex. The core complex is formed via a four–helix bundle, two α-helices from SNAP-25, and one α-helix each from VAMP and a syntaxin; these four helixes are parallel to each other. The complex of four helixes brings the presynaptic membrane and synaptic vesicle membrane in proximity, resulting in the merging of the bilayers. This mechanism is referred to as the "Lucine zipper model" of membrane fusion[13]. The helical bundle conformation buries the hydrophobic residue within the core complex. Salt bridges are formed midway along the α-helix by two glutamines from SNAP-25, a glutamine from syntaxin, and an arginine from VAMP. These salt bridges are buried within the core of the helical bundle; the resulting structure is very stable with an unfolding transition over 95ºC *in vitro*.

The proteolysis of one of the SNARE proteins results in blocking the neurotransmitter release, but it does not affect the synthesis of the neurotransmitters. BoNT/A and E cleave the SNAP-25 at different sites within the COOH-terminus; BoNT/B, BoN/D, BoNT/F and BoNT/G cleave the VAMP at different sites. BoNT/C

cleaves both syntaxin and SNAP-25[84]. Evidence suggests that the neurotoxin

recognizes the quaternary structure rather than the primary structure of the substrate

protein at the level of the nine-residue long SNARE motif which is characterized by three

carboxylate residues alternated with hydrophobic and hydrophilic residues[68, 76, 98].

The SNARE proteins are helical and super coil structures in the presence of binding

partners; these structures are stabilized by hydrophobic packaging[13]. A conserved ionic

layer is formed at the center of the SNARE complex by hydrogen bonds and a network of

salt bridges [93]. Different BoNT serotypes cleave one or more SNARE proteins, When

LC cleaves its target SNARE protein the formation of SNARE complex will be blocked.

Site directed mutagenesis results in three negatively charged residues (Asp or Glu) of the

motif of LC; plays a critical role in the recognition of the target by the neurotoxin [68].

Glu224 in the HEXXH motif is critical for BoNT/A activity; it coordinates with water,

which performs a hydrolytic reaction in proteolysis. When, the position of Glu224 is

moved to 225 which results in the complete loss of the enzyme activity [51]. The site-

directed mutagenesis experiments revealed the important role of the catalysis of His

residue and Glu262, which provides a negatively charged carboxylate moiety [72].

Substitution of Glu262 with Asp results in a significant loss of activity which shows the

essential role of the spatial positioning of the carboxyl group of Glu262. Arg362 and

Tyr365 (BoNT/A), which are far from the zinc center, have been shown to be directly

involved in catalysis [10].

The structural analysis and primary sequence of the light chain shows that the

enzymatic mechanism is similar to other metalloproteases, but structural basis on

targeting SNARE proteins, the unusual selectivity and cleavage site selectivity is

remarkable. The scissile bond (a substrate bond cleaved by enzymatic action) for BoNT/A in SNAP-25 is (Gl197-Arg198), which is shifted exactly one position compared to the BoNT/C1 scissile bond (Arg198-Ala199). The light chain efficiently cleaves only long stretches of the SNARE proteins, not the truncated SNARE proteins that are less than 20-30 residues. For the optimal activity of BoNT/A, abundance of 146-202 SNAP-25 proteins are required[13].

SNAP-25 wraps around the light chain and adopts three distinct types of secondary structure after binding to the LC: the N-terminal residue forms an α-helix; the C-terminal residue forms a distorted β-strand, and the residue between N and C terminal are mostly extended. The N-terminal α-helix and C-terminal β-sheets are important for efficient substrate binding and cleavage. The active site of BoNT/A is buried in 20-24Å and covered by the belt region of the translocation domain. The active site of the BoNT/B is 16Å deep and is not covered by the belt region. The neurotoxins are site specific proteases, and they are so potent that a single molecule of toxin is sufficient for stopping the neurotransmitter release from the whole nerve terminal. Except for the blockage of exocytosis, there are no other major changes in the nerve terminal. There are no significant structural changes in the synapse by the action of the toxin.

1.7   Vaccines for BoNT

The BoNTs are considered as "Category A Select Agent and Toxins" (toxins which are potential to pose a severe threat to public health and safety) because of their potential use as a bioterrorist agent. There is a necessity to develop a vaccine or therapies to control BoNT toxicity.   The early BoNT vaccines were formalin treated crude extracts from *C. botulinum*, and these vaccines retained immunogenicity. To make better

vaccines, the toxoid was purified by alum precipitations. This toxoid elicited a good immune response after repeated immunizations. Local and systemic adverse reactions were limited in the first inoculation, but increased in the second inoculation which spurred new vaccine developmental strategies. A pentavelent vaccine (BoNT/A, B, C, D and E) was developed with minimal side effects but the supply of this vaccine is limited because of its impure preparations and difficulty in production [71]. Other therapies for BoNT intoxication include human botulinum immune globulin product (BabyBIG) used for infants and equine based antitoxin treatments. The knowledge of BoNTs action, structure and recent advancements in molecular biology provide new tools and strategies for vaccine production.

Several vaccine studies used different parts of BoNT as the antigen, for example, the recombinant holo toxoid, LC and c-terminal of HC (HC$_C$) and subunits of BoNT. The HC$_C$ domain is the most extensively studied for immunological studies because it binds to the neuronal surface and lacks toxicity. BoNT heavy chain and HC$_C$ are good immunogens to elicit the immune response in mice and rabbit [3, 4, 8, 71, 92, 95].

The objectives of the present study were to:

1. Determine median lethal dose of botulinum E in channel catfish.

2. Evaluate the use of zebrafish as a more standardized bioassay model for diagnosing VTC and for evaluating serum neutralizing activity

3. Establish the zebrafish as model for botulism.

4. Evaluate the following as possible vaccines for VTC

   a. The recombinant BoNT/E/HC (rBoNT/E/HC) vaccine produced by the USAMRIID

b. A codon biased BoNT/E/HC expressed by attenuated channel catfish virus (*Ictalurid herpesvirus* 1)

5. Attempts were made to develop botulinum neurotoxin E resistance zebrafish as long term goal to evaluate development of recombinant animals which are resistant to BoNT/E

CHAPTER II

DETERMINATION OF THE MEDIAN LETHAL DOSE OF BOTULINUM

SEROTYPE E IN CHANNEL CATFISH FINGERLINGS

2.1   Abstract

The median lethal dose of botulinum serotype E in 5.3-g channel catfish *Ictalurus punctatus* fingerlings was determined. Five tanks (five fish/tank) were assigned to each of the following treatment groups: 70, 50, 35, 25, or 15 pg of purified botulinum serotype E. Fish were injected intracoelomically and observed for 96 h. Administration of the toxin resulted in initial hyperactivity followed by erratic swimming, paresis, and death. The cumulative mortality by treatment group was 100% at 70 pg, 96% at 50 pg, 100% at 35 pg, 88% at 25 pg, and 56% at 15 pg. The median lethal dose was calculated as 13.7 pg/fish (equivalent to a 0.81 median lethal dose for mice (*Mus musculus*) using a logistic regression model. All fish were necropsied; lesions included exophthalmia, ascites, splenic congestion, intussusception of the intestines, congested spleen, and blanching of the intestinal tract. The resultant clinical signs and lesions were similar to those noted in the syndrome of visceral toxicosis of catfish. This study indicates that channel catfish are more sensitive to the effects of botulinum serotype E than laboratory mice and the signs

and lesions of visceral toxicosis of catfish were replicated by injecting catfish with the toxin.

2.2   Introduction

Botulinum (BoNT) is a neurotoxin produced by the bacterium *Clostridium botulinum* that causes paralysis in humans and animals by binding to synaptic vesicles at neuromuscular junctions and blocking the release of acetylcholine. In the Mississippi Delta, BoNT serotype E (BoNT/E) was identified in serum of commercially cultured channel catfish *Ictalurus punctatus* afflicted with visceral toxicosis of catfish[32]. Because the mouse bioassay for botulinum was negative on serum samples from these fish, positive results from a catfish neutralization bioassay suggested that catfish were more sensitive to BoNT/E than laboratory mice *Mus musculus*. It is well documented that certain domestic animals are more sensitive to BoNT than laboratory mice [38, 63, 86]. Compared with mice, lactating dairy cattle were experimentally shown to be 12.9 times as sensitive to BoNT serotype C (on a per-kg weight basis;[63]). Previous experiments demonstrate that salmonids are twice as sensitive to BoNT/E as mice when injected intracoelomically (IC)[25]. Using an endopep mass spectrometry (MS) method, the quantity of BoNT/E in sera of VTC-affected channel catfish was estimated as 0.01– 0.5 times the median mouse lethal dose (MLD50)/mL [32]. The findings from the endopep MS assays, coupled with the fact that sentinel catfish died when injected with sera from VTC-afflicted catfish, reinforced the notion that catfish are indeed more sensitive to BoNT/E than mice.

Clinical signs in catfish associated with VTC are erratic swimming, progressive muscular weakness leading to paralysis, lethargy, and death[32]. Postmortem lesions of

VTC include exophthalmia, ascites, splenic congestion, intussusception, and blanching of the intestines. To more accurately define the sensitivity of catfish to BoNT/E, a median lethal dose (LD_{50}) study in channel catfish fingerlings was conducted. In addition, to determine whether the clinical syndrome of VTC could be experimentally reproduced with injected BoNT/E, the clinical signs and lesions in the experimental fingerlings were assessed.

2.3   Materials and methods

This project was implemented under the approval of the Mississippi State University Animal Care and Use Committee and the Institutional Biosafety Committee.

2.3.1   Experimental design

There were five treatment groups with five replicates (individual fish) per group. Treatments were arbitrarily assigned to aquaria according to the quantity of BoNT/E (Metabiologics, Madison, Wisconsin) injected IC into the fish: 70, 50, 35, 25, or 15 pg. The duration of the experimental treatment was 96 h (4 d). On day 1, fish were injected IC with 100 μL of their assigned treatments. Fish were observed three times/d for clinical signs previously reported in fish with BoNT/E intoxication (hyperactivity, lethargy, not swimming, no tail movement, inability to right self in the water column, exophthalmia, distention of the coelomic area, and death;[104]. Fish that died during the study were weighed, necropsied, and examined for gross lesions. After 96 h, surviving fish were euthanized in tricaine methanesulfonate (Western Chemical, Ferndale, Washington), weighed, necropsied, and examined for lesions.

2.3.2 Test fish

We obtained 125 laboratory-reared channel catfish fingerlings, (mean body weight, 5.3 g; SE = 0.89) with no known exposure to *C. botulinum* from the United States Department of Agriculture Agricultural Research Service, Genetics Compound, Stoneville, Mississippi. The fish were arbitrarily counted in groups of five in an undetermined ratio of males and females. Fish were acclimated for 7 d prior to the study, during which time they were fed 36%-protein catfish feed to satiation. Feed was withheld throughout the study beginning the day before BoNT/E injections.

2.3.3 Necropsy methods

All dead fish were removed from aquaria, weighed, and submitted for necropsy. The postmortem examination of the fish included an inspection of the skin, fins, mouth, eyes, gills, and coelomic viscera.

2.3.4 Fish environment and handling

We used 25 water-flow-through glass tanks (60 L) for each study. Nonchlorinated well water was supplied to all tanks, and aeration was achieved utilizing compressed air from air stones. The water temperature was maintained at 25°C (SE = 1.0). Water quality was within acceptable limits for the maintenance of channel catfish fingerlings throughout the studies[96]. The photoperiod was 12 h light and 12 h dark.

2.3.5 Botulinum neurotoxin dilutions

Botulinum was handled within a class 2 biosafety cabinet equipped with HEPA filters. The BoNT/E complex (Metabiologics) was provided at a concentration of 1 mg/mL with specific toxicity (nicked) of 6×10^7 MLD_{50}/mg. We diluted the BoNT/E

with sterile gel phosphate buffer (Fisher Scientific, Fairlawn, New Jersey, and Becton Dickerson, Sparks, Maryland) to yield 10^4 pg/µL of stock BoNT/E. We further diluted 60 µL of 10^4 pg/µL BoNT/E with gel phosphate buffer (pH 6.2) and then incubated it with trypsin (1:250; Difco) at room temperature (about 22–23°C) for 30 min. Following incubation, this solution was further diluted with gel phosphate buffer to yield the following nominal BoNT/E concentrations: 70, 50, 35, 25, and 15 pg/100 µL.

2.3.6 Statistical method

A logit model was used to determine $\log_e LD_{50}$ using PROC LOGISTIC [44], following transformation of the data by taking the natural logs of the dose concentrations. A 95% confidence interval (95% CI) was calculated for the $\log_e LD_{50}$ using methods of Robertson et al[73]. The $\log_e LD_{50}$ and the upper and lower limits were transformed back to the original units by exponentiation.

2.4 Results

2.4.1 Clinical signs

Within 8 h of injection, fish initially exhibited clinical signs of swimming bursts followed by settling on the bottom. Signs progressed to gradual loss of voluntary motor control. The fingerlings attempted to swim, but showed a lack of fin coordination, and with progression, they settled on the bottom displaying loss of equilibrium and righting ability. When stimulated, the fish swam erratically. Signs progressed to total loss of voluntary motor function with increased respiratory rates (evidenced by opercula flaring) and eventual death. Terminally the mouths were opened with abduction of the opercula.

2.4.2   Mortality

The majority of fish from the 70, 50, and 35 pg injection groups were dead by 24 h (Table 2.1). Cumulative mortalities were 100% (25/25fish) at 70 pg, 96% (24/25) at 50 pg, and 100% (25/25) at 35 pg dose. By 72 h all mortalities (22/25 fish; 88%) had occurred in the 25 pg group, and by 48 hours all mortalities (14/25 fish; 56%) had occurred in the 15 pg group. The LD_{50} was calculated as 13.7 (95% CI = 9.9–18.9) pg/fish (equivalent to 0.81 MLD_{50}; Figure 2.1).

Table 2.1   Channel catfish mortality pattern after BoNT/E injections

Dose (pg)	Day 1	Day 2	Day 3	Day 4	Day 5 (euthanized)
70	23	1	1	-	-
50	23	1	0	0	1
35	15	10	-	-	-
25	11	9	2	0	3
15	0	14	0	0	11
Total	72	35	3	0	15

Daily numbers of dead channel catfish fingerlings following injection with BoNT/E at a dose of 70, 50, 35, 25, or 15 pg/fish (*n* = 25 fish/treatment). Survivors of the 96-h challenge were euthanized on day 5.
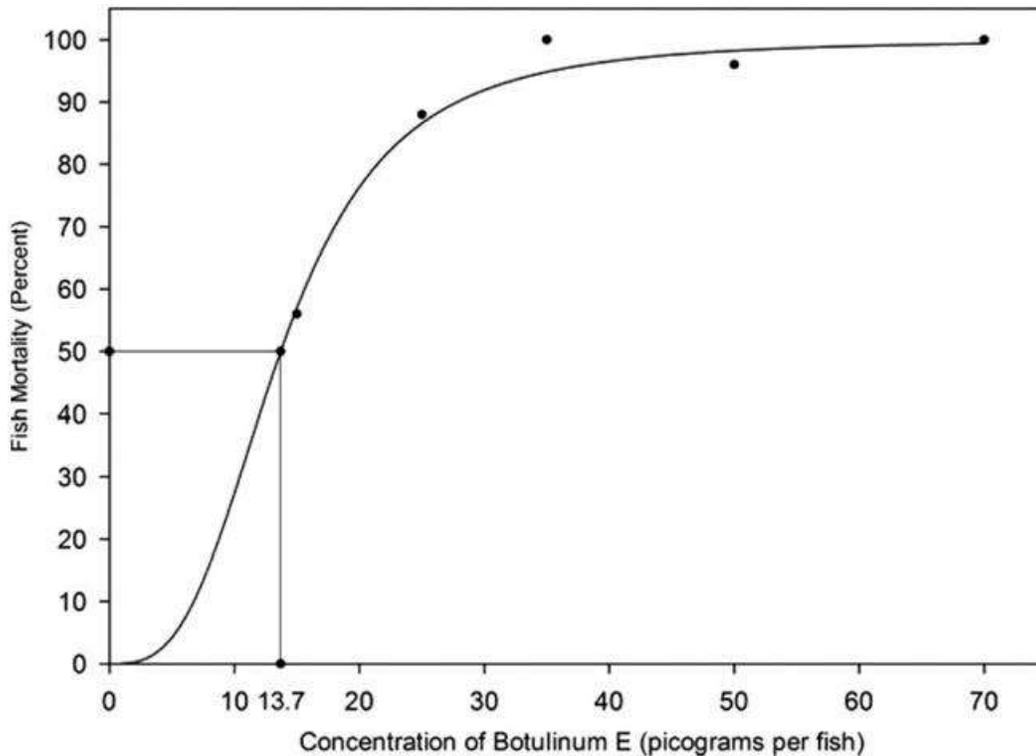
Figure 2.1   BoNT/E LD$_{50}$ Mortality curve for channel catfish

Botulinum E 96-h predicted mortality–dose response curve for channel catfish fingerlings based on parameter estimates from logistic regression analysis. Fish were injected intracoelomically with 100 μL of 70, 50, 35, 25, or 15 pg BoNT/E activated with trypsin (n = 25 fish/treatment). The LD$_{50}$ was calculated as 13.7 pg/fish.

2.4.3   Lesions

The postmortem lesions were similar to those reported in VTC field cases[32].

Postmortem lesions included exophthalmia, ascites, splenic congestion, intussception,

and blanching of the intestines.

2.5   Discussion

This is the first reported experimental replication of the syndrome of VTC using

BoNT/E. Purified BoNT/E produced the same disease (i.e., VTC) pattern observed in

channel catfish naturally infected and in sentinel catfish injected IC with VTC sera.

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Initially, fish were hyperactive with a gradual loss of motor coordination leading to paralysis resulting from blockage of release of acetylcholine. These signs were similar to those reported in other species of BoNT-intoxicated fish; lesions associated with botulism were not reported in other species [22, 91, 104]. However, it is unclear whether pathology was absent or the fish were simply not examined for lesions in those studies. The majority (107/125) of the BoNT/E-injected catfish died within 48 h of injection. Similarly, sentinel catfish fingerlings bioassayed with VTC sera also died within this period[32].

Death from BoNT-intoxication in humans and other mammals is attributed to suffocation resulting from loss of muscular control of the diaphragm [75]. Presumably, loss of muscular control of the branchial pump in its effort to force water over the gills resulted in respiratory failure in catfish.

Bioassays are conducted and standardized with animals of uniform size, and a threshold of BoNT toxin is required to induce clinical signs and lesions[104]. The mouse neutralization assay for botulinum is standardized with female Swiss Webster mice weighing 18–20 g [87]. The channel catfish fingerlings used in this study were of uniform weight (5.3 g, SE = 0.89). Their small size allowed injection of small quantities of BoNT/E for significant bioassay results. Small fish are critical for studies with botulinum and for VTC diagnosis because the U.S. Government limits possession of botulinum to ≤0.5 mg for nonselect agent laboratories (USOFR 2005), and only small quantities of serum are obtained from VTC-afflicted fish. Standardization of catfish bioassays for BoNT/E LD_{50} assays or diagnosis of VTC requires large numbers of small

catfish. Using larger fish would likely require higher doses per fish, necessitating a smaller sample size, which would limit the statistical power of the study.

In the mouse bioassay, when the injected dose is high, mice typically develop signs of botulism within 8 h [87]. At lower doses, mice are affected more slowly; hence, mice are observed for 4 d before a negative result is recorded. Similarly, the high doses of 70, 50, and 35 pg resulted in acute deaths (within 24 h) for the majority of the fingerling catfish. Mortality occurred later with 25-pg and 15-pg doses, the majority of deaths occurring within 48 h.

The LD_{50} BoNT/E for catfish determined from this study was 13.7 pg/fish, which is <1 MLD50. In a previous study, sera from VTC-affected catfish were assayed by endopep MS and (because of the small quantities of the samples) toxin activities were only estimated as 0.01–0.5 MLD_{50}/mL[32]. Although the results of this BoNT/E LD_{50} are not as low as the Gaunt et al. VTC estimates, our study partially explains why the mouse bioassay was negative. In addition, the discrepancy between the quantity of BoNT/E estimated in the Gaunt et al. study and the results of the BoNT LD_{50} (about 0.81 MLD50) could be attributed to deterioration of BoNT/E in VTC serum from affected catfish prior to assaying via endopep MS; BoNT/E is reported to be unstable and ingested toxin is difficult to demonstrate in human serum after 1 week of exposure[12] (J. M. Campbell et al., paper read at the Botulism in Lake Erie workshop, 2002). Sentinel fish bioassays were completed within 21 d after sera were taken from VTC-affected catfish, and the sera were then assayed by endopep MS [32]. To produce its toxic effect, BoNT must bind to its receptor and be taken up by the neuron, and the bound toxin is not free to circulate in blood [38, 63, 86]. Therefore, the quantity of free BoNT in the sera used for the catfish

bioassay and endopep MS assay was likely less than the original blood concentration that killed the affected catfish.

Another explanation for the quantitation difference includes synergistic influences that would lower the toxic dose of BoNT/E in catfish ponds (G. McLaughlin, paper read at the Botulism in Lake Erie workshop, 2002). Potentially toxic blue-green algae were noted in waters with other fish, and BoNT outbreaks and algal toxins were suggested as interacting synergistically with botulinum [38]. Although blue-green algae were found in ponds with VTC affected catfish, the assays were negative for the blue-green algal toxins microcystin, anatoxin *a*, and anatoxin *a* (s)[32].

This LD_{50} assay did not determine the lower limit of a lethal dose of BoNT/E in catfish because mortalities were present in all dose groups. Based on this study, the LD_{50} for purified BoNT/E is estimated at 13.7 pg for fingerling catfish with a mean weight of 5.3 g. This study demonstrated that channel catfish are sensitive to purified BoNT/E, and affected fish had the clinical signs and lesions of VTC, which further reinforces the link between VTC and BoNT/E.

CHAPTER III

ZEBRAFISH (*DANIO RERIO*) BIOASSAY FOR VISCERAL TOXICOSIS OF

CATFISH AND BOTULINUM NEUROTOXIN SEROTYPE E

3.1 Abstract

Visceral toxicosis of catfish (VTC), a sporadic disease of cultured channel catfish (*Ictalurus punctatus*) often with high mortality, is caused by botulinum neurotoxin serotype E (BoNT/E). Presumptive diagnosis of VTC is based on characteristic clinical signs and lesions, and the production of these signs and mortality after sera from affected fish is administered to sentinel catfish. The diagnosis is confirmed if the toxicity is neutralized with BoNT/E antitoxin. Because small catfish are often unavailable, the utility of adult zebrafish (*Danio rerio*) was evaluated in BoNT/E and VTC bioassays. Channel catfish and zebrafish susceptibilities were compared using trypsin-activated BoNT/E in a 96-hr trial by intracoelomically administering 0, 1.87, 3.7, 7.5, 15, or 30 pg of toxin per gram of body weight (g-bw) of fish. All of the zebrafish died at the 7.5 pg/g-bw and higher, while the catfish died at the 15 pg/g-bw dose and higher. To test the bioassay, sera from VTC-affected fish or control sera were intracoelomically injected at a dose of 10 µl per zebrafish and 20 µl/g-bw for channel catfish. At 96 hr post-injection, 78% of the zebrafish and 50% of the catfish receiving VTC sera died, while no control

fish died. When the VTC sera were pre-incubated with BoNT/E antitoxin, they became
nontoxic to zebrafish. Histology of zebrafish injected with either VTC serum or BoNT/E
demonstrated renal necrosis. Normal catfish serum was toxic to larval zebrafish in
immersion exposures, abrogating their utility in VTC bioassays. The results demonstrate
bioassays using adult zebrafish for detecting BoNT/E and VTC are sensitive and
practical.

3.2   Introduction

Visceral toxicosis of catfish (VTC), an important disease in channel catfish
(*Ictalurus punctatus*) aquaculture, is caused by botulinum neurotoxin serotype E
(BoNT/E)[17, 32, 48]. Outbreaks of VTC cause substantial losses to the catfish industry,
accounting for as much as 37.8% of total loss in some years (National Animal Health
Monitoring System: 2011, Catfish 2010 Part III: changes in catfish health and production
practices in the United States, 2002–09. Available at:

http://www.aphis.usda.gov/animal_health/nahms/aquaculture/). Visceral toxicosis of
catfish occurs in the spring and fall when water temperatures are 18–21°C, and is
characterized by a rapid onset with high losses, often occurring in market-sized and brood
stock catfish. Clinical signs commonly observed with VTC include erratic swimming and
progressive muscular weakness leading to paralysis, lethargy, and death[32, 48]. Internal
lesions included chylous or clear fluid (ascites) in the coelomic cavity, intussusception of
the intestinal tract, reticular pattern to the liver, congested spleen, and eversion of the
stomach into the oral cavity[17, 32, 48].

Botulinum neurotoxins are produced by *Clostridium botulinum*, a Gram-positive,
obligate anaerobic, spore-forming, rod-shaped bacterium[37]. Botulinum intoxication

occurs in human beings and various domestic species including cattle, horses, dogs, cats, birds, and cultured fish[22, 101, 102, 104].Toxicosis caused by BoNT/E occurs in aquatic birds after they consume BoNT-laden fish[70].

There are several diagnostic methods to detect botulinum toxin. The assays most commonly used are the mouse bioassay, enzyme-linked immunosorbent assay (ELISA) for botulinum neurotoxins, and polymerase chain reaction (PCR) to detect DNA from the bacterium *C. botulinum*[32, 34]. However these assays are not sensitive enough to reliably detect the low concentrations of BoNT/E present in the serum of VTC-affected fish[32].

A presumptive diagnosis of VTC is made from clinical signs and lesions[48]. A definitive diagnosis is made using a catfish bioassay; small channel catfish fingerlings are injected intracoelomically with serum from affected catfish at a rate of 20 µl/g body weight (g-bw). A positive bioassay results in mortality with characteristic VTC signs and lesions within 96 h[32]. Confirmation requires mixing the test serum with a BoNT/E neutralizing antiserum with a resulting loss of toxicity. The catfish bioassay is a practical answer to an immediate diagnostic challenge. However, it lacks standardization, and many diagnostic laboratories do not have facilities for holding channel catfish. Furthermore, the blood collection yields are often low from moribund or freshly dead catfish that have been submitted as a diagnostic case. Therefore, bioassay fish must be small enough to allow for the 20 µl/g-bw dose in 3–5 replicates. This is complicated by the fact that VTC outbreaks are generally in the fall and early spring when the availability of small channel catfish fingerlings is limited.

In comparison, zebrafish (*Danio rerio*; approximately 0.3–0.4 g) are much smaller than catfish fingerlings (approximately 5–20 g). If zebrafish were sensitive to BoNT/E, they could be used in the bioassay and considerably decrease the amount of VTC-affected catfish sera needed for diagnostic investigations. Additionally, zebrafish adults are relatively uniform in size and have defined genetics, allowing the assays to be standardized. Furthermore, zebrafish are much more economical and convenient to use than channel catfish because they are available year round and use much less tank space. The objective of the current study was to evaluate the zebrafish as a bioassay organism for BoNT/E and VTC by comparing sensitivity of zebrafish and channel catfish and to evaluate a serum neutralization bioassay as a definitive test. Also, because zebrafish larvae are easy to reliably produce, a preliminary study was performed using them in VTC bioassays.

3.3   Materials and methods

3.3.1   Fish maintenance

Zebrafish (Tübingen strain), 0.33 g mean weight, and channel catfish, 20 g mean weight, were produced and housed in the College of Veterinary Medicine, Mississippi State University (CVM-MSU) specific pathogen–free fish hatchery. Production of larvae and husbandry conditions were as previously described[39, 69].Experimentally challenged fish were maintained in 15-liter aerated free-flowing tanks receiving charcoal-filtered dechlorinated municipal water at a rate of 0.5 l/min at 25°C. All production and use of fish were under the oversight and approval of the Mississippi State University Institutional Animal Care and Use Committee and the Institutional Biosafety Committee.

3.3.2   Botulinum neurotoxin

Stock BoNT/E[a] (1 µg/µl concentration with specific toxicity of activated [nicked] BoNT of 6×10^7 mouse lethal dose$_{50}$/mg) was diluted to 10 ng/µl in sterile gel phosphate buffer (2 g/l of gelatin, 4 g/l of Na_2HPO_4; pH 6.2). For challenges, this BoNT/E stock was nicked by digestion with 5% trypsin[b] (1:250; 5 µl of 10 ng/µl BoNT/E, 100 µl of trypsin, and 895 µl of gel phosphate buffer) at 22–23°C for 30 min.

3.3.3   VTC sera

Visceral toxicosis of catfish sera (designated serum 3, 8, 10, and 12) were collected from moribund channel catfish from a confirmed natural VTC outbreak. Control sera were collected from healthy channel catfish, and stored at 4°C.

3.3.4   Bio assay

In the BoNT/E injection trial, 5 catfish and 5 zebrafish were injected with control or each nominal BoNT/E dose. The activated toxin stock (50 pg/µl) was diluted with gel phosphate buffer to working stocks of 1.0, 0.5, 0.25, 0.125, and 0.0625 pg/µl for zebrafish and 3.0, 1.5, 0.75, 0.375, and 0.187 pg/µl for catfish. Zebrafish were intracoelomically (IC) injected with 10 µl and catfish were IC injected with 10 µl/g-bw of the respective working concentrations to provide nominal doses of 30, 15, 7.5, 3.75, and 1.87 pg/g-bw.

In VTC sera bioassays, each serum sample (4 VTC samples and 1 control sample) was injected into 7 zebrafish and 3 channel catfish (numbers of catfish were reduced because of the limited amount of each VTC serum sample). Channel catfish were injected with 20 µl/g-bw, zebrafish were injected with 10 µl per fish of VTC sera or control sera.

Fish were observed 3 times a day for BoNT/E clinical signs: hyperactivity, abnormal swimming pattern, fin in-coordination, lack of tail movement, lethargy, exophthalmia, and mortality[32, 104]. Moribund fish (fish that were unable to swim and settled to the bottom of the tank or were unable to maintain an upright position) were euthanized by immersion in water containing 300 mg/l tricaine methane sulfonate[c] for 10 min past the loss of opercular movement and considered dead at that time point. All dead fish were necropsied and the brain and posterior kidney cultured for bacteria to rule out bacterial infection as a cause of death. This consisted of plating the samples on tryptic soy agar with 5% sheep blood and incubating the cultures aerobically at 22°C. After 96 hr, fish that survived were euthanized as previously described.

3.3.5   Neutralization assay

The VTC serum samples were incubated with monospecific BoNT/E[d] antitoxin (provided at a concentration of 10 international units/ml) at a 4:1 ratio (catfish serum:antitoxin) for 30 min at 22–25°C.[9] The respective no-antibody control samples was produced by incubating the VTC serum with phosphate buffered saline (PBS) at the 4:1 ratio. Five zebrafish were IC injected for each antitoxin-treated serum sample and for each PBS-treated serum sample.

3.3.6   Histology

In the histology experiment, fish were injected with 15 pg/g-bw of BoNT/E, VTC serum, or control serum as described above. The zebrafish coelom is much smaller than that of the catfish, and an IC injection could produce iatrogenic pathology to the viscera. To assess the effect of IC injections, 10 µl of PBS (pH 7.4)-injected zebrafish and

noninjected zebrafish were also processed and examined for histology. At least 5 fish were treated for each of the noninjected, BoNT/E-, control serum, and PBS-injected zebrafish and control serum–injected catfish groups. Smaller numbers ($n = 2$) of catfish were injected with VTC sera because of limited availability. Also, 2 zebrafish injected with serum 3 and 1 injected with serum 10 died before sampling so they were removed from the study.

When fish became moribund, they were euthanized, cultured for bacteria, and processed for histology. Control serum– and PBS-injected fish were sampled at 72 hr postinjection. Zebrafish were fixed in 10% buffered formalin after making an incision the length of the abdomen. The whole body was embedded in paraffin, serially sagittally sectioned, and stained with hematoxylin and eosin. The channel catfish tissues were excised, then fixed and processed similarly.

3.3.7   Exposing zebrafish larvae

Three zebrafish larvae were distributed to each well of 6-well plates containing 2 ml of water per well and 2 replicate wells were evaluated per treatment group. In the initial trial, larvae were exposed to serum from VTC-affected catfish, healthy catfish, or no serum. The dosage was 60 µl serum/2 ml. In the second trial, to assess whether components of catfish serum were toxic, zebrafish larvae were exposed to either 60 or 20 µl of serum/2 ml of either heat-inactivated or non–heat-inactivated serum. Heat inactivation involved incubating the serum at 56°C for 30 min.

Experiments with larvae were initiated on the day of hatch. For each replicate, 3 larvae were placed in a well of a 6-well cell culture plate with 2 ml of water, and a nominal amount of VTC or control serum was added to the well. After 3 hr, the water

was replaced, and the larvae were observed 3 times a day. After 96 hr, surviving larvae were euthanized in tricaine methane sulfonate.[b]

3.4   Results

3.4.1   BoNT/E injections

All zebrafish and catfish that were exposed to the 2 highest dose levels of purified toxin, 30 and 15 pg/g-bw, died (Table 3.1). At the 7.5 pg/g-bw dose, all zebrafish died but no catfish died. These catfish displayed signs compatible with intoxication: erratic swimming, fin in-coordination, followed by resting on the bottom of the tank. No fish exposed to the lower doses or the negative control died. The zebrafish exposed to the 3.75 pg/g-bw dose displayed the same signs of BoNT/E or VTC intoxications seen in the 7.5 pg/g-bw dose catfish. All bacterial cultures taken from moribund zebrafish and catfish showed no growth.

Table 3.1   Catfish and zebrafish BoNT/E comparison studies

Fish	Number of dead fish at the indicated dose of BoNT/E (pg/g)					
	Control	1.87	3.7	7.5	15	30
Zebrafish	0	0	$0^{\dagger}$	5	5	5
Catfish	0	0	0/5	$0^{\dagger}$	5	5

Number of zebrafish and channel catfish fingerlings that died following an injection with purified botulinum neurotoxin serotype E (BoNT/E) activated with trypsin at a dose of 30, 15, 7.5, 3.7, 1.87, or 0pg/gram at 96 hours post inoculation.*
* All treatment groups consisted of five fish.
†Majority of the live fish showed clinical signs of VTC.

3.4.2   VTC sera injections

Mortality was observed in catfish and zebrafish injected with VTC sera (Table 3.2). Overall, the 4 samples of VTC-affected sera killed 78.5% of the zebrafish and 50% of the catfish. There were no mortalities observed in channel catfish or zebrafish that

were injected with control catfish serum. All fish that were injected with VTC sera

displayed lethargy, fin in-coordination, and paralysis, which are all signs typical of VTC.

All bacterial cultures taken from moribund fish showed no growth.

Table 3.2   Catfish and zebrafish VTC sera comparison studies

Fish	No. of fish that died after being injected with indicated serum				
	Control serum	Serum #3	Serum #8	Serum #10	Serum #12
Zebrafish*	0	7	3‡	7	5‡
Catfish†	0	3	0‡	2‡	1‡

Number of fish that died following an intracoelomic injection of visceral toxicosis of
catfish sera or negative control (healthy) catfish serum.
* All treatment groups consisted of 7 fish.
†All treatment groups consisted of 3 fish.
‡Majority of live fish showed typical clinical signs of visceral toxicosis of catfish.

3.4.3   Neutralization assay

All zebrafish injected with VTC sera showed the signs of VTC within 12 hr post-

injection but those injected with VTC sera that were pre-incubated with BoNT/E-specific

antitoxind displayed none of these clinical signs. The VTC-positive sera killed 80% of

the zebrafish, while control sera and VTC sera that were pretreated with BoNT/E-specific

antitoxind caused no mortality (Table 3.3).

Table 3.3   Zebrafish sera neutralization assay

	No of dead fish at indicated control or VTC sera and neutralized sera				
	Control serum	Serum #3	Serum #8	Serum #10	Serum #12
Sera	0	5	5	5	5
Sera + antibody	0	0	0	0	0

Number of zebrafish that died after an intracoelomic injection of visceral toxicosis of
catfish (VTC) sera, negative control (healthy) catfish serum or the respective serum
pretreated with BoNT/E neutralizing antibody*.
* All treatment groups consisted of 5 zebrafish.

3.4.4   Histology

There were varying degrees of histologic changes in zebrafish and channel catfish fingerlings exposed to the VTC serum and purified toxin. The severity of lesions is summarized in Table 3.4. Noninjected control zebrafish demonstrated mildly congested gills, and 1 control fish demonstrated mild cloudy swelling and hydropic degeneration of renal tubular epithelial cells. All other tissues appeared normal. Of 5 zebrafish that were injected with PBS (negative control), all showed a small amount of focal necrosis at the injection site and mild branchial congestion. One of these fish demonstrated mild cloudy swelling of hepatocytes.

Of the 6 zebrafish injected with channel catfish control serum (healthy catfish serum), 4 demonstrated mild hepatopathy that included diffuse hepatic hydropic and ballooning degeneration, distorted architecture, granular cytoplasm, and loss of cellular definition. All 6 displayed mild renal pathology including cloudy swelling and hydropic degeneration of tubular epithelial cells, and varying changes of the Bowman space.

Three of the 10 zebrafish injected with sera from VTC-affected fish died soon after the injection and were not used for histology: 2 injected with serum 3 and 1 injected with serum 10. All remaining VTC serum–injected zebrafish were moribund at 12 h postinjection, and sampled. In zebrafish injected with serum 3, liver tissues demonstrated hydropic and ballooning degeneration, distorted architecture, cloudy swelling, granular cytoplasm, mild focal necrosis, and intercellular edema. Pancreatic tissue demonstrated diffuse necrosis. Kidney tissue demonstrated severe cloudy swelling and hydropic degeneration of tubular epithelial cells, marked diffuse tubular necrosis, necrosis of the interstitial tissue, and intertubular edema. Zebrafish injected with serum 10 demonstrated

hepatic congestion with moderate lesions, but exhibited more severe kidney lesions compared to those injected with serum 3. Kidney lesions included marked congestion, hemorrhage of the interstitial tissue, severe cloudy swelling and hydropic degeneration of tubular epithelial cells, marked diffuse tubular necrosis, necrosis of the interstitial tissue, and intertubular edema. One fish demonstrated increased Bowman space (multifocal), and mild inflammation of the interstitial tissue.

Five zebrafish were injected with 15 pg/g-bw purified BoNT/E and sampled at 12 h postinjection. In all fish, renal tubular epithelia demonstrated hydropic degeneration, karyorrhexis, and necrosis. In 2 fish, hydropic degeneration of hepatocytes was seen.

Of the channel catfish injected with control catfish serum, 1 of 3 demonstrated mild cloudy swelling of hepatocytes; all other tissues appeared normal. Both catfish injected with VTC serum 3 demonstrated moderate hepatic vessel dilation, hepatic perivascular edema, hydropic degeneration, distorted architecture, and cloudy swelling in the hepatocytes. Marked changes in the kidney interstitial tissue compared to the control serum–injected fish included glomerular necrosis, edema, and tubular necrosis. The channel catfish injected with VTC serum 10 demonstrated hepatic vessel dilation and mild hydropic degeneration of hepatocytes. Kidney lesions were mild compared to those of fish injected with serum 3 and consisted of mild glomerular and tubular necrosis. Channel catfish injected with purified BoNT/E demonstrated mild to moderate hypertrophy of renal tubular epithelia and mild leukocytic infiltration of the renal interstitial tissue, congested spleens, and moderate hydropic degeneration of hepatocytes with granular cytoplasm.

Table 3.4   BoNT/E injected zebrafish histology

Fish	Treatment	Tissue	Number of affected fish/number observed
Zebrafish	Non-injected	Gill	5/5 congested
		PKid*	4/5 normal, 1/5 mild renal tubular lesions
		Liver	5/5 normal
	PBS injected	Gill, PKid	5/5 normal
		Liver	4/5 normal, 1/5 mild lesions
	Control serum	Gill	3/6 normal, 3/6 mild lesions
		PKid	6/6 mild lesions
		Liver	2/6 normal, 4/6 mild lesions
	VTC serum 3	PKid	3/3 moderate lesions
		Liver	3/3 severe lesions
	VTC serum 10	PKid	2/4 severe lesions, 2/4 moderate lesions
		Liver	1/4 severe, 2/4 moderate, 1/4 mild lesions
	Purified toxin	PKid	5/5 severe lesions
		Liver	3/5 normal, 2/5 mild lesions
Catfish	Control serum	PKid, Gut	3/3 normal
		Liver	2/3 normal, 1/3 mild lesions
	VTC serum 3	Liver, PKid	2/2 moderate lesions
	VTC serum 10	Liver, PKid.	2/2 mild lesions
	Purified toxin	PKid	3/5Mild lesions, 2/5moderate lesions
		Liver	1/5 normal, 4/5 markedly granular
		Gut	4/5 normal ,1/5 gut vascular dilation and vascular edema
		Spleen	4/5 congested, 2/5 moderate leukocytic infiltration
		Gill, Heart, AKid	5/5 normal

Summary of lesion severity in channel catfish and zebrafish following injection with purified Botulinum Neurotoxin (BoNT/E), sera from channel catfish showing clinical signs of Visceral toxicosis of catfish (VTC), control sera from healthy channel catfish, Phosphate Buffered Saline (PBS), or not injected.
*PKid=posterior kidney, AKid=anterior kidney

3.4.5   Exposing zebrafish larvae

Within 24 h, all larvae exposed to either control catfish serum or VTC serum died, whereas no fish died in the non–serum-exposed wells. Preexposure of the serum to heat (56°C for 30 min) was then evaluated to determine if the toxic component was temperature sensitive. All larvae exposed to 60 or 20 µl serum/2 ml died within 24 hr, whereas all larvae exposed to heat-inactivated serum survived.

3.5   Discussion

The results of the current study support the use of zebrafish as a bioassay organism to detect VTC and BoNT/E intoxication. The zebrafish and channel catfish bioassays using purified BoNT/E were similar in sensitivity. Bioassays using VTC sera demonstrated similar clinical signs in both species and the utility of both species for serotyping using neutralizing antibodies. Because adult zebrafish are readily available throughout the year, and they are relatively uniform in size and much smaller (0.3–0.4 g) than channel catfish, the zebrafish bioassay has more practical utility. Additionally, the defined genetics of inbred zebrafish strains likely reduces genetic-based variation in susceptibility, which should reduce standard error, allow for better between laboratory comparisons, and allow for more precise analytical research. Furthermore, holding facilities and care costs are much lower than that needed for a comparable number of channel catfish or mice[39].

The behavioral signs (hyperactivity, abnormal swimming pattern, fin in-coordination, lack of tail movement, lethargy, exophthalmia, and settling at bottom of the tank) of zebrafish and channel catfish were similar in VTC sera–injected fish and purified BoNT/E-injected fish. All the zebrafish died at 7.5 pg/g and higher doses and showed VTC behavioral signs at 3.7 pg/g. This sensitivity suggests that, with optimization, the zebrafish could be used in titration assays to quantify BoNT/E activity in tissues and serum. The alternative technology, endopep mass spectrometry, is a sensitive, quantitative method for detecting active BoNT/E, but it is expensive and time consuming[32]. The mortality and clinical signs were similar in BoNT/E- and VTC-challenged channel catfish and zebrafish. However, histology of zebrafish and catfish

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injected with control serum suggested some differences. There were mild hepatic and renal changes in the zebrafish that were injected with control serum, whereas changes in the catfish injected with control serum or in zebrafish that were injected with PBS were minimal (focal necrosis was seen at the site of injection but this was thought to be due to mechanical damage that occurred by manually injecting the small zebrafish). Also, larval zebrafish were very sensitive to a heat labile component in the catfish serum in immersion exposures. This suggests that the control adult zebrafish may have been affected by mild serum toxicity. These mild lesion caused by catfish serum should be considered in any future studies that involve cross-species serum injections in fish. Although the current study was not designed for comparative histology, some of the lesions seen in the VTC serum–injected catfish and zebrafish were different from those described in channel catfish with VTC[81]. The current study showed only mild intestinal changes and no marked lymphoid depletion. The differences may be a result of the route and duration of exposure to the toxin.

The preliminary study using zebrafish larvae in immersion exposures was unsuccessful because the serum of channel catfish was toxic to the larvae. The toxic component in the serum was heat labile. One possible explanation for the serum being toxic is heterotypic activation of the complement system. The complement proteins in channel catfish sera may have been activated by zebrafish proteins, and the membrane attack complex or other components from the resulting complement cascade could have killed the larval zebrafish. Cross-species activation of complement has been documented as the cause of hyperacute rejection of xenotransplantations[85] and has been implicated in the acute toxicity of human serum to mice[77]. Botulinum neurotoxins are heat labile,

therefore heat inactivation of the serum is not an option, but zebrafish larvae may be useful as a bioassay for detecting heat-stable toxins in serum samples.

Zebrafish are an attractive and widely used model organism for biomedical studies, genetic research, developmental biology, immunology, and toxicology[15, 17, 23, 55]. Zebrafish are an oviparous cyprinid with year-round fecundity [69].Previously, zebrafish have been used to study enteric septicemia of catfish[69] and the effects of cholera toxin and *Clostridium difficile* toxin[55, 81]. The zebrafish genome is fully sequenced; the availability of various recombinant zebrafish and antibodies to specific markers further aid in research. The zebrafish assay described in the current study is a simple, sensitive method to detect BoNT/E in VTC sera, which can likely be applied to other biological samples. The mouse bioassay is the only method approved by the Centers for Disease Control and Prevention and the most widely accepted definitive diagnostic test for presence of BoNT[11, 70]. However, the mouse bioassay was not sensitive enough to detect the presence of BoNT/E in VTC sera. Endopep mass spectroscopy can detect the low levels of BoNT/E presence in the VTC sera but it is time consuming and expensive. Therefore, the zebrafish bioassay described in the current study is a reliable, practical alternative to rapidly detect BoNT/E in sera from VTC-affected catfish. Furthermore, it is a convenient alternative to the previously established catfish fingerling bioassay[17, 85].

Notes
a. Metabiologics, Madison, WI.
b. 215240, Trypsin 1:250, 100 g; Difco, BD, Franklin Lakes, NJ.
c. MS-222, Tricaine-S; Western Chemical Inc., Ferndale, WA.
d. Catalog no. BS3179, Centers for Disease Control and Prevention, Atlanta, GA.

CHAPTER IV

ZEBRAFISH SENSITIVITY TO BOTULINUM NEUROTOXINS

4.1   Abstract

Botulinum Neurotoxins (BoNT) are among the most potent neurotoxins. The mouse LD_{50} assay is the standard for testing BoNT potency, but is not sensitive enough to detect the ultra low levels of neurotoxin that occur in the sera of affected catfish; zebrafish are important model for many research studies because of their small size, easy maintenance, year-round production, and the molecular techniques available for working with this species of fish. The utility of zebrafish as a model organism for BoNT bioassay was investigated. The96 h median lethal doses of BoNT/A, BoNT/C, BoNT/E and BoNT/F for adult male, Tubingen strain zebrafish (0.3 to 0.4 g weight) at 25°C were 16.31, 125.1,4.7 and 0.61 pg/fish respectively. These findings support the use of the zebrafish-based bioassays for evaluating the presence of BoNT/A, BoNT/E and BoNT/F. The relatively high resistance to BoNT/C may reveal unique functional barriers to the action of this neurotoxin.

4.2   Introduction

Clostridium botulinum is a gram-positive, spore forming, anaerobic, and rod-shaped bacterium that produces extremely potent neurotoxins. Botulinum neurotoxins (BoNTs) are considered high threats for use as bioterrorism or biological warfare agents,

because minute quantities can quickly cause neuro-paralytic illness, which terminates in respiratory failure[79]. Inhalation of the toxin is especially lethal; this may be caused by laboratory exposure or a bioterrorism event[79, 88]. However, *C. botulinum* contamination of food and colonization of wounds and the gastrointestinal tract are the most common causes of botulism toxicity. BoNT is used in medical treatment of muscle hyperactivity, spasticity, blepharospasm, and strabismus, and cosmetology[88].

Botulism isseen in horses, cattle, birds and fish[32, 33, 63, 86, 101, 104], with dairy cattle, horses, and salmonids being more sensitive to BoNT/E than mice[63]. In channel catfish aquaculture, visceral toxicosis of catfish (VTC) is a sporadic disease caused by BoNT/E. Clinical signs of VTC are erratic swimming and progressive muscular weakness leading to paralysis, lethargy, and death. Commonly observed internal lesions include intussusception of the intestinal tract, chylous or clear fluid (ascites) in the coelomic cavity, congested spleen, and eversion of the stomach into the oral cavity. To test the activity or concentration of BoNTs, mouse bioassays are traditionally used. The mouse bioassay failed to detect the BoNT/E in affected sera of catfish afflicted with VTC. The catfish serum neutralization assay detected the presence of BoNT/E in catfish with VTC and was confirmed by endopep mass spectrometry[32].

There are several diagnostic methods available to detect the causative agents of botulism outbreaks, such as polymerase chain reaction (PCR) for *C. botulinum*, enzyme-linked immunosorbentassay (ELISA), and the mouse bioassay. All of these tests failed to detect the BoNTs in catfish sera. Possible reasons include ingestion of only the toxin rather than *C. botulinum* organism, the presence of low toxin levels, and varying sensitivity to BoNTs in different animals[32].

For commercial and diagnostic use, the potency of purified BoNTs must be checked prior to marketing using the traditional mouse LD_{50} assay. It is unknown how many mice are used for this assay, but in 2007 the estimated worldwide number of mice used was 300,000 per year [11]. With the discovery that BoNTs aids in treating muscle hyperactivity, spasticity, blepharospasm, and strabismus, and cosmetic flaws[88], its use increased dramatically in recent years. The present estimation of mouse usage for BoNTs testing is more than 600,000 per year[11]. Additionally, many animals are used to test activity of BoNTs in medical and academic research. The maintenance costs of mice are expensive; and the mouse model does not consistently detect low levels of toxins. Therefore, there is a necessity to develop an alternative, affordable, and sensitive method to detect lower concentrations of BoNTs. Consequently, the zebrafish (*Danio rerio*) was considered as a model in BoNTs research because of its small size and sensitivity to minute amounts of toxin.

The zebrafish is an oviparous cyprinid with high fecundity and exhibits physiological and morphological similarities to other vertebrates. Moreover, the zebrafish is an attractive and widely used model organism for developmental biology, biomedical, immunological, genetic, and toxicology studies[7, 23, 56, 60, 69, 81, 94]. Zebrafish have been used to study the effects of cholera toxin and *Clostridium difficile* toxin[56, 81]. There are many advantages of using the zebrafish as a model organism. The zebrafish genome is fully sequenced; various recombinant zebrafish are available, as are polyclonal and monoclonal antibodies to zebrafish. Additional advantages of using zebrafish in BoNT research are the large offspring numbers produced; faster development compared to other vertebrates, low maintenance, and tiny space requirements for a large number of

animals. Thus, the objective of this research was to establish zebrafish as a model organism to study BoNTs.

The causative agent of VTC is BoNT/E, which cleaves the synaptosomal-associated protein 25 (SNAP-25), one of the SNARE proteins. SNAP-25 is also cleaved by BoNT/A, BoNT/C. In the sediments of VTC outbreak channel catfish ponds, strains of *C. botulinum* expressing BoNT/F were identified in addition to those expressing BoNT/E. The main goal of this study was to estimate the zebrafish LD_{50} for BoNT/A, BoNT/C, BoNT/E and BoNT/F.

4.3   Materials and methods

4.3.1   Experimental design

The LD_{50} experiments in this project were conducted based on guidelines established by the Mississippi State University Animal Care and Use Committee and the Institutional Biosafety Committee. For each experiment we used 5 two-fold serial diluted doses and a control (Phosphate gelatin buffer- 2g of gelatin, 4g of Na_2HPO_4 in 1 L dH20, pH 6.2) dose. In each experiment 10 zebrafish per dose were injected intracoelomically. BoNT was injected in the coelomic cavity posterior to the pelvic girdle with a 35G needle attached to an insulin syringe. All fish were injected with 10µl of an assigned toxin dose or control buffer and observed every 8 hours over a seven day period for clinical signs and lesions of BoNT intoxication including abnormal swimming pattern, lack of tail movement, lethargy, paresis, exophthalmia, settling at the bottom of the tank, fin in-coordination, and erratic swimming. The fish quiescent at the bottom of the tank, upside down, or unable to move fins or tail were considered dead and were euthanized by immersion in charcoal-filtered de-chlorinated municipal water containing 300 mg/L

tricaine methane sulfonate(MS-222,Tricaine-S, Western Chemical, Inc.

http://www.wchemical.com/TRICAINE-S-MS-222-P43.aspx). After 7 days, surviving

fish were euthanized as above. Each experiment was repeated three times to check for

reproducibility.

4.3.2   Zebrafish

All the experimental zebrafish (*Danio rerio*) were obtained from the Mississippi

State University College of Veterinary Medicine (MSU-CVM) fish hatchery. These fish

were propagated and nurtured in a specific pathogen free environment according to

standard operating procedures previously described[39, 69].Adult male zebrafish (mean

bodyweight 0.32±0.045 g) were used for experimental challenges. Experimentally

challenged zebrafish were transferred to15 L aerated tanks receiving charcoal-filtered de-

chlorinated municipal water at a rate of 0.5 L/min at 25 ºC housed in the challenge

rooms.

4.3.3   BoNTs source and handling

One tenth mg (0.1mg) each of purified BoNT/A, BoNT/C, BoNT/E, and BoNT/F

were purchased from Metabiologics, Inc (Metabiologics, Madison, WI;

http://www.metabiologics.com/products.htm). BoNTs were handled in a Class II bio-

safety cabinet equipped with HEPA filters. Each toxin was diluted to a stock

concentration10^4pg/ul with gel phosphate buffer. Each stock was further diluted to a

working stock concentration. BoNT/E was activated (nicked) with 5% trypsin (1:250

Difco, 0.05g in 1ml; 5µl of 10^4 pg/ul BoNT-E, 100µL of 5% trypsin, 895µl of gel

phosphate buffer) at room temperature for 30 minutes prior to injection. The toxin or

activated toxins were further diluted to nominal concentrations with gel phosphate buffer. Based on preliminary studies (data not shown) intracoelomic (IC) doses of 40, 20, 10, 5 and 2.5 pg/fish were assigned for BoNT/A; 1000, 500, 250, 125, and 62.5pg/fish for BoNT/C; doses of 10, 5, 2.5, 1.25 and 0.61 pg/fish for BoNT/E were assigned and for BoNT/F 5, 2.5, 1.25, 0.61 and 0.3 pg/fish were assigned. Confirmation of BoNT/C activity was tested in mice using the same stock of BoNT/C for zebrafish injections.

4.3.4   Statistical methods

Logit model was used to determine the 96 hour post-injection (hpi) LD_{50} of challenged zebrafish, by taking the natural logarithm values of the dose concentration vs. the activity of the toxin using SPSS-18 software for regression analysis. The LD_{50} was calculated at 95% confidence intervals (CI) [45]. After day 4 there were no mortalities in any of the challenges. Based on previous studies, extending the duration of study did not increase the precision of LD_{50} in mice. Therefore our median lethal doses were determined from 96 hour observation periods, but we continued to monitor the fish for clinical signs until day 7.

4.4   Results

4.4.1   BoNT/A 96 h median lethal dose

In all three experiments, 100% mortality was observed at the highest dose (40pg/fish). At 20pg/fish, mortalities were observed in the majority of fish (22 out of 30), but all remaining live fish exhibited typical muscular weakness (paresis) and paralysis associated with botulism. At the doses of 10 and 5pg/fish little mortality was observed, but all live fish exhibited paresis as evinced by their resting in one place on the aquarium

bottom. By end of 6 dpi, the fish were swimming throughout the water column making an apparent recovery from the muscle weakness. At the 2.5pg/fish dose and in the control group, all of the fish survived without showing any clinical signs of BoNT intoxication (Table 4.1). The LD_{50} values were16.31 (95% CI 11.5 to 21.0), 11.56 (95% CI 7.6 to 17.0), and 13.21 (95% CI 9.4to 18.6) pg/fish for the three challenges, respectively (Figure 4.1). The mean 96 h median lethal dose of BoNT/A, based on the three experimentswas13.7 pg/fish (95% CI 11.5 to 21.0).

Table 4.1   Zebrafish response to BoNT/A injections

96 hours	40 pg/fish	20 pg/fish	10 pg/fish	5 pg/fish	2.5 pg/fish	Control
Exp-1	10	8*	None*	None*	None	None
Exp-2	10	6*	5*	None*	None	None
Exp-3	10	8*	2*	1*	None	None

Number of fish that died by 96 h following injection with BoNT/A at a dose of 40, 20, 10, 5 and 2.5 pg/fish and control (phosphate gelatin buffer). Survivors of the 96-hour challenge were euthanized on Day 7 with MS-222, (n=10 fish/dose, N=60/each experiment).
*Denotes that there were live fish in the tanks that showed clinical signs.

Figure 4.1   Zebrafish BoNT/A LD$_{50}$graph

Botulinum Neurotoxin A (BoNT/A) 96-hour LD$_{50}$ concentrations for zebrafish. Fish were injected intracoelomically with 10μL of 40, 20, 10, 5, 2.5pg/fish BoNT/A diluted in gelatin phosphate buffer, (n=10 fish/treatment)

4.4.2   BoNT/C 96 h median lethal dose

In the three experiments, 100% mortality was observed at the highest doses, 1000 and 500 pg/fish. In the remaining doses, a range of 8-10, 1-9, and 0-2 per 10 fish/dose died in the 250, 125, and 62.5 pg/fish doses, respectively (Table 4.2). The calculated LD$_{50}$values were 98.3 (95% CI 57.4 to 123.2), 112.2(95% CI 67.2 to 163.6) and 163.3 pg/fish (95% CI 128.1 to 371) for three repetitions respectively. The mean of combined values was 124.6 pg/fish (95% CI 57.4 to371) (Figure: 4.2). Because this dose was tenfold higher than expected, the median toxic dose of a mouse (100 pg) was intraperitoneally (IP) injected into three Swiss-Webster male mice (18-20 g) and a control mouse was injected with gelatin phosphate buffer to ascertain the potency of the BoNT/C[63]. The BoNT/C injected mice succumbed to the toxin by 24 hpi.

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Table 4.2   Zebrafish response to BoNT/C injections

96-hour	1000 pg/fish	500 pg/fish	250 pg/fish	125 pg/fish	62.5 pg/fish	Control
Exp-1	10	10	10	9*	None	None
Exp-2	10	10	8*	6*	2	None
Exp-3	10	10	10*	1 *	None	None

Number of mortalities following injection with BoNT/C at a dose of 1000, 500, 250, 125, 62.5 pg/fish and control (gelatin phosphate buffer). Survivors of the 96-hour challenge were euthanized on Day 7 with MS-222. (n=10 fish/dose, N=60/each experiment). *Denotes that there were also live fish that showed clinical signs.
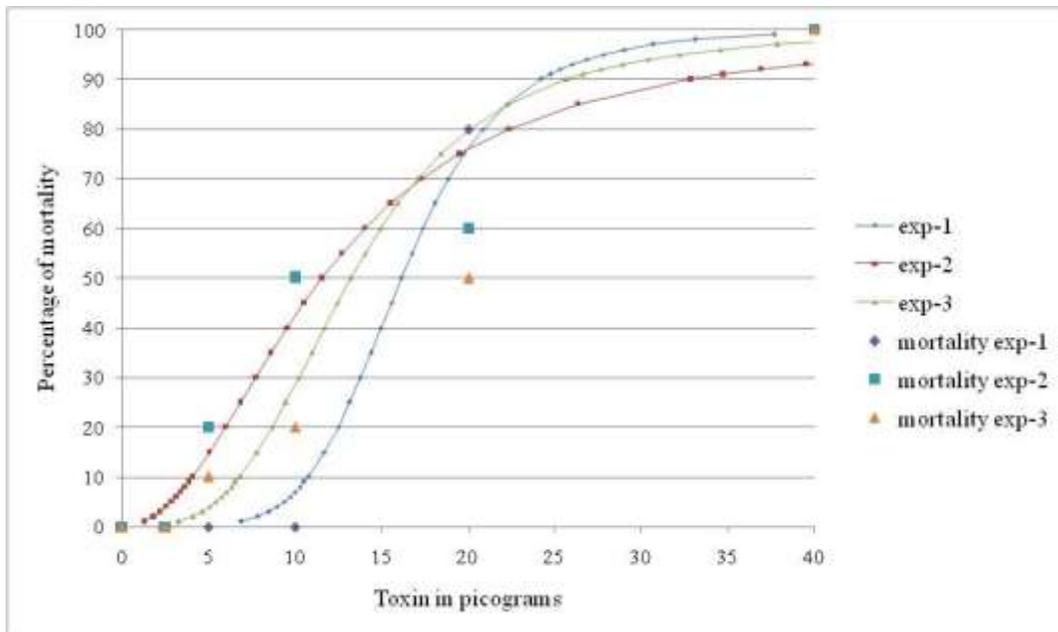

Figure 4.2   Zebrafish BoNT/C LD$_{50}$ graph

Botulinum neurotoxin C (BoNT/C) 96 hour LD$_{50}$ concentrations for zebrafish. Fish were injected intracoelomically with 10μL of 1000, 500, 250, 125, 62.5pg/fish. BoNT/C was diluted in gelatin phosphate buffer (n=10 fish/treatment)

4.4.3   BoNT/E 96 h median lethal dose

In all three experiments, 100% mortalities were observed at the highest dose (10 pg/fish), at 5 pg/fish 60%, 80% and 100% mortalities were observed, respectively in three challenges (Table 4.3). At 2.5 pg/fish, more than 50% of the fish showed clinical signs

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compatible with botulinum intoxication, but little mortality was observed at this dose, and

most of the fish showed reduced paresis signs on day 6 compare to day 1 pi. In the 1.25

and 0.62 pg/fish and control doses, all the fish appeared clinically normal (Table 4.3).

The LD_{50} was calculated as 4.7 (95% CI 1.9 to 6.4), 3.29 (95% CI 2.4 to 4.5), and 3.01

pg/fish (95% CI 2.4 to 6) for the three experiments respectively (Figure 4.3).The median

lethal dose of BoNT/E in zebrafish was 3.7 pg/fish (95% CI 1.9 to 6.4) by intracoelomic

injections.

Table 4.3   Zebrafish response to BoNT/E injections

96-hour	10 pg/fish	5 pg/fish	2.5 pg/fish	1.25 pg/fish	0.62 pg/fish	Control
Exp-1	10	6	None*	None	None	None
Exp-2	10	8	3 *	None	None	None
Exp-3	10	10	2 *	None	None	None

Number of mortalities following injection with BoNT/E at doses of 10, 5, 2.5, 1.25, 0.62
pg/fish and control (gelatin phosphate buffer). Survivors of the 96 hour challenge were
euthanized on day 7 with MS-222. (n=10 fish/dose, N=60/each experiment).
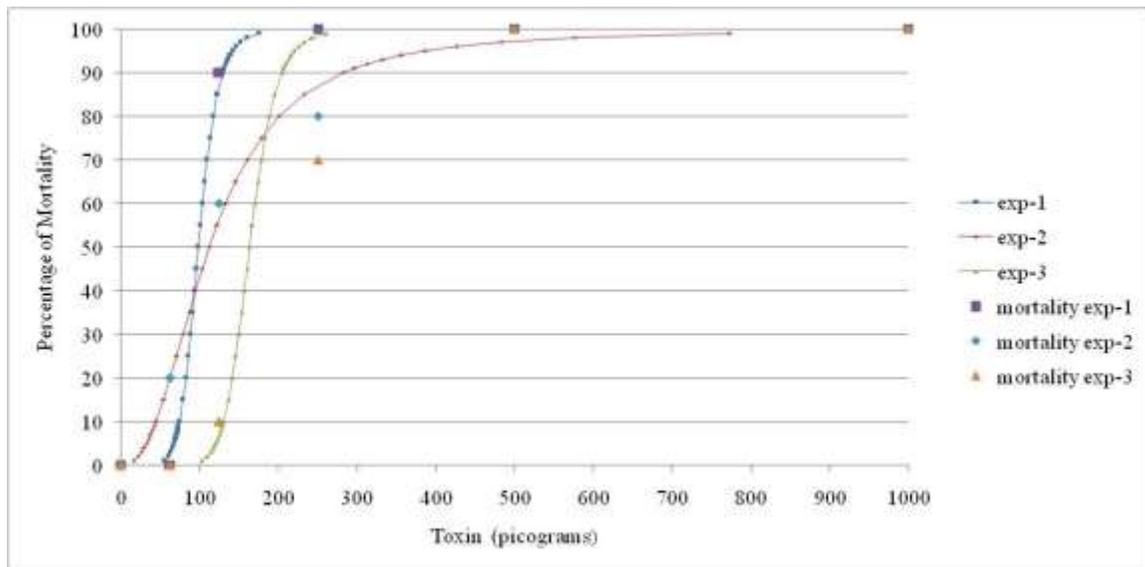*Denotes that there were also live fish that showed clinical signs.

Figure 4.3   Zebrafish BoNT/E LD_{50} graph

Botulinum neurotoxin E (BoNT/E) 96 hour LD_{50} concentrations for zebrafish. Fish were injected intracoelomically with 10μL of 10, 5, 2.5, 1.25, and 0.62pg/fish BoNT/E activated with trypsin in gelatin phosphate buffer (n=10 fish/treatment)

4.4.4   BoNT/F 96 h median lethal dose

Mortalities were observed in all doses (Table 4.4). Fish exhibited clinical signs including paresis within 24 hpi in all doses. By end of day 4, the zebrafish were swimming throughout the water column making an apparent recovery from the effects of BoNT/F. In the three experiments, the BoNT/F LD_{50} was 0.55 (95% CI 0.38 to 0.80), 0.38(95% CI 0.19 to 0.54) and 0.61 (95% CI 0.41 to 0.85) pg/fish. The mean 96 h LD50 of BoNT/F in zebrafish was 0.51pg/fish (Figure: 4.4).

Table 4.4   Zebrafish response to BoNT/F injections

96-hour	5 pg/fish	2.5 pg/fish	1.25 pg/fish	0.61 pg/fish	0.3 pg/fish	Control
Exp-1	10	9*	9*	8*	None*	None

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| Exp-2 | 10 | 10 | 10 | 7* | 4* | None |
| Exp-3 | 10 | 10 | 9* | 5* | 1* | None |

Number of fish dead within 96 h following injection with BoNT/F at a dose of 5, 2.5, 1.25, 0.61, 0.316 pg/fish and control (buffer). Survivors of the 96-hour challenge were euthanized on day 7 with MS-222, (n=10 fish/dose, N=60/each experiment).
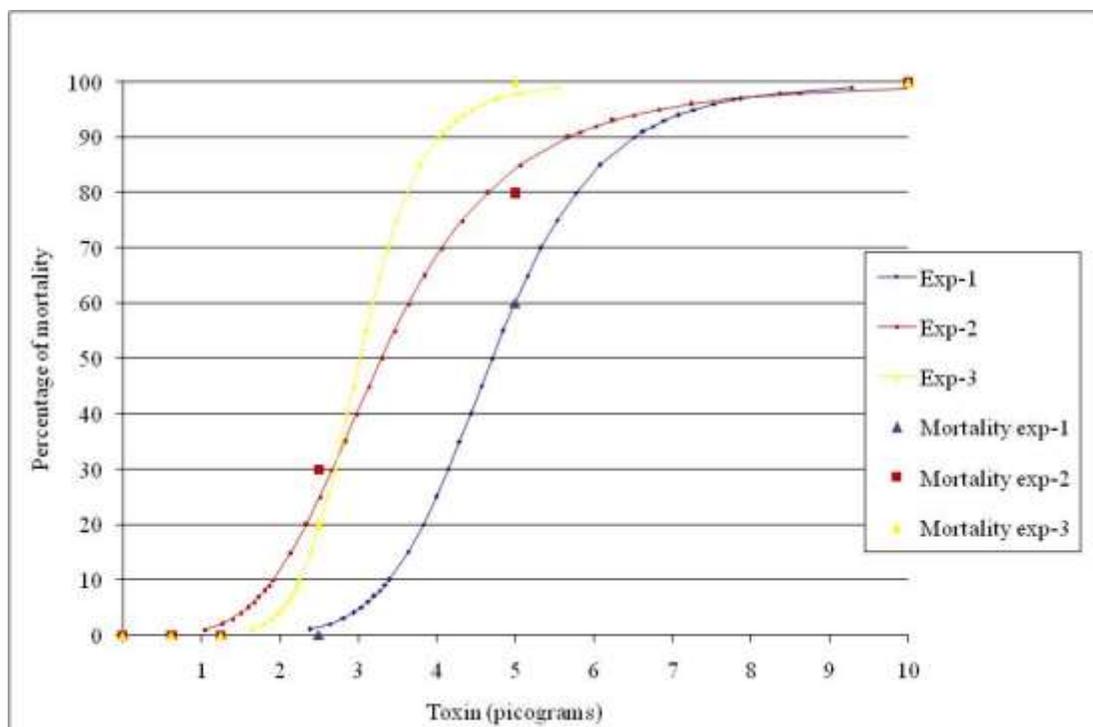* Denotes that there were also live fish that showed clinical signs.
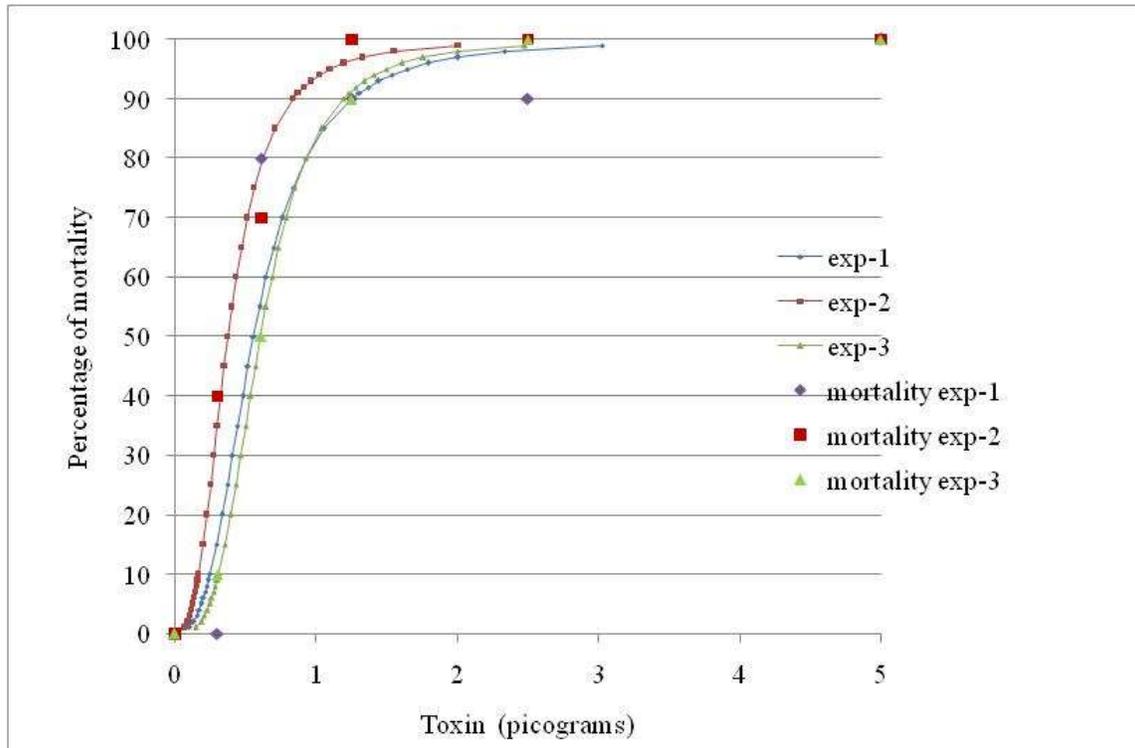
Figure 4.4   Zebrafish BoNT/F LD_{50} graph

Botulinum neurotoxin F (BoNT/F) 96 hour LD_{50} concentrations for zebrafish. Fish were injected intracoelomically with 10µL of 5, 2.5, 1.25, 0.62 and0.3 pg/fish BoNT/F diluted in gelatin phosphate buffer (n=10 fish/treatment)

4.5   Discussion

These study results suggest that the zebrafish response to BoNTs is similar to other vertebrates. We conducted LD_{50} studies in adult zebrafish for four serotypes of BoNTs. The LD_{50} for BoNT/A was 16.31 pg/fish with typical clinical signs at 5pg/fish, BoNT/C LD_{50}125.1 pg/fish with signs at 125 pg/fish, BoNT/E was 4.7 pg/fish with

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typical paralytic signs at 2.5 pg/fish, and BoNT/F LD$_{50}$0.61 pg/fish and signs at 0.31

pg/fish. The zebrafish showed clinical signs as early as 6 to 8 hpi in higher doses and for

lower doses from 24 to 48 hpi. In lower doses the clinical signs were minimal or not

recognizable but the overall swimming activity was lower compared to control fish.

In vertebrates, clinical signs develop after internalization of BoNT into peripheral

nerves. In the cytoplasm of the peripheral nerves, the light chain of the BoNTs acts as a

zinc-dependent endopeptidase and cleaves SNARE proteins, which leads to the blockage

of acetylcholine release at the neuromuscular junction and synapses at postganglionic

sympathetic and postganglionic parasympathetic nerve endings and also at the autonomic

ganglia[40, 54]. Affected animals exhibited clinical signs based on the amount of toxins

internalized; in mice signs were observed within 8 hpi at higher doses, and in lower doses

the response was slower, sometimes longer than 24hpi[32]. Similar results were observed

in zebrafish; at higher doses they showed clinical signs as early as 6 hpi, and by 24 hpi

the majority of mortalities were observed. Zebrafish injected with doses of 2.5pg of

BoNT/E and 5pgof BoNT/A showed clinical signs24 hpi, but after 96 hpi few or no

mortalities were observed. These observations indicate that the internalization of toxins

and the response to the toxin might be similar in zebrafish and other animals.

The persistence of muscle paralysis varies with the serotype of BoNT and

organism intoxicated[16]. Experimental studies showed that, the persistence of BoNT/A

is longer than BoNT/E in humans and rodents[22]. The persistence of BoNT induced

paralysis depends on several factors: the presence of cleaved SNARE proteins blocking

exocytosis, the presence of active BoNT light chains in the cytosol, and also the alteration

or recovery of the presynaptic terminal. Two possible explanations for BoNT/A long

persistence in humans and rodents are SNAP-25^A (SNAP-25 cleaved by BoNT/A) is a more stable product than SNAP-25^E, and the BoNT/A light chain is unusually resistant to polyubiquitination compared to BoNT/E[16]. Muscle paralysis lasts for 4 to 6 months with BoNT/A. It is thought that the recovery of signal transduction during this time achieved by sprouting new nerve terminals at the nerve endings. Studies indicate that the synaptic activity correlate with the growth and elimination of nerve sprouts at neuromuscular junction[16]. Our results in agreement with other studies[2,9,19] suggest that recovery of BoNT intoxication depends on the dose; in lower doses, zebrafish showed progressive recovery with time. On day 7, lower-dose injected fish displayed minimal or no clinical signs of BoNT intoxication with the exception of less activity compared to control fish; the numbers of light chains present in 10 pg of BoNT are approximately 10^{11} which would be distributed in almost all the zebrafish nerve cells. Previous studies have shown that the persistence of BoNT/F was of shorter duration compared to that of BoNT/A in mice[47, 61]. Our experiments showed similar behavior in zebrafish; the fish injected with BoNT/F recovered faster compared to those injected with BoNT/A. The possible explanations for faster recovery might be the turnover rate of vesicle associated membrane protein (VAMP). The cleaved VAMP by BoNT/F (VAMP^F) product might not form SNARE complexes due to truncation. These truncated products might accelerate the turnover rate of VAMP and the formation of new SNARE complexes by newly synthesized VAMP proteins. Another theory involves the possible degradation of BoNT/F LC because of the continuous activity and degradation by the neuronal cytosol cellular proteases. Zebrafish were most resistant to BoNT/C compared to the other BoNT serotypes; the LD$_{50}$ was more variable (98.3 to 163.6 pg/fish with SD of 34.4 pg in three

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experiments) compared to other BoNT serotypes. Previous studies also showed similar results with variability; the oral toxicity of BoNT/C for foxes varies from 10^3 to 10^8 times the mouse lethal dose (MLD), in minks from 10^3 to 10^5, IPMLD[57, 66]. These studies show that BoNT/C toxicity is variable within species and also within individuals.

BoNTs are used in the treatment of muscle hyperactivity and as anti-aging agents in cosmetology products[11, 88]. These products are tested on mice for their potency before being released. The mouse bioassay is a standardized technique to test the acute toxicity of BoNTs, but it failed to detect BoNT/E in channel catfish sera[3]; mortalities were observed when the same sera was injected into catfish fingerlings[32] and zebrafish (data not shown). Because the average weight of adult male zebrafish is consistent, a standardized volume of purified toxin could be used (less than 16 pg of BoNT/A, E and F for fish), whereas in the mouse, the amount of toxin usage much higher (at least 100 pg per mice). By utilizing small bioassay organisms, this allows more laboratories to diagnose and study BoNT related diseases, because the US government limits possession of purified BoNTs to ≤0.5mg for non-select agent laboratories[31].

BoNT/C proteolyzes SNAP-25 and syntaxin proteins of the SNARE complex. In this study, zebrafish were more resistant to BoNT/C compared to other BoNTs. This might be due to the lack of some crucial structural motifs, which aid BoNT/C binding to thesyntaxin and SNAP-25. BoNT/C cleaves syntaxin 1b at Lys252-Ala253, only when they are inserted into the lipid bi-layer[83]. BoNTs tend to recognize their substrate via a double interaction, region A with a neurotoxin binding motif, region B with a peptide bond to be cleaved[76]. There are several syntaxin isoforms expressed based on tissue and cellular distribution, syntaxin 1a and 1b specifically expressed in nervous tissue[83].

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To date, zebrafish syntaxin 1ais not sequenced. Even though the syntaxin 1b sequence is conserved in humans, mouse and cows; the zebrafish syntaxin has ten amino acid differences, five of these differences are present downstream of the cleavage site (amino acid 253 to 288). These differences might reduce or alter the specificity of neurotoxin binding, thereby reducing the action, but further structural studies are needed for conformation. SNAP-25 proteolysis requires1000 fold more BoNT/C light chain compared to BoNT/A, /E for the *in vitro* assay[98].SNAP-25 proteolysis depends on the presence Asn93 to Glu145andIle156 to Met202regions[98]. These regions are conserved in humans, mouse and cow but not in zebrafish. In the region Asn93 to Glu145, zebrafish have several amino acid differences and 2 and 3 gaps in SNAP-25A and SNAP-25B, respectively. Region Ile156 to Met202 also have couple of amino acid differences but no gaps. BoNTs LC can be turned into active proteases only on contact with their cognate substrate, but due to these amino acid differences and gaps, the structure of SNAP-25 motifs might be altered. These differences might be the cause of zebrafish resistance to BoNT/C but further studies are necessary.

Our studies suggested that the clinical signs zebrafish developed after BoNT injections were similar to those reported with VTC and other BoNT intoxicated fish, therefore zebrafish are good models for VTC vaccine development studies. The fact that the development and maturation of the zebrafish immune system is similar to the catfish also facilitates its use to study this catfish disease[69]. The zebrafish life cycle is much shorter than catfish; so using zebrafish has more advantages than catfish. Zebrafish are an attractive and widely used model organism for various toxicology, biomedical, disease model and immunology studies[7, 23, 35, 55, 60, 69, 81, 94]. The availability of

molecular tools and recombinant zebrafish facilitates further research[94]. Having an additional model organism to study BoNT will offer many benefits.

CHAPTER V

EVALUATION OF BOTULINUM NEUROTOXIN-E HEAVY CHAIN EXPRESSING

RECOMBINANT CHANNEL CATFISH VIRUS AS A POTENTIAL VACCINE

FOR VISCERAL TOXICOSIS OF CATFISH

5.1   Abstract

Visceral toxicosis of catfish (VTC) is a sporadic, often devastating disease in catfish aquaculture caused by botulinum neurotoxin serotype/E (BoNT/E). BoNT/E is synthesized by the anaerobic, gram positive bacterium *Clostridium botulinum* as a single 150 kDa polypeptide chain. This product is cleaved by bacterial or host proteases to produce the activated toxin consisting of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) linked by a disulfide bond. The HC binds the cell receptor and helps transport the LC into the cytosol of the neuron, where LC (Zn2+-endoprotease) cleaves SNAP 25, one of the SNARE (soluble N-ethylmaleimide sensitive fusion protein attachment protein receptors) proteins. This cleavage blocks the SNARE complex formation, which is needed for synaptic vesicle exocytosis. The lack of synaptic vesicle exocytosis leads to blockage of signaling molecules transfer which ultimately blocks the signal transfer between neurons and muscles causing paralysis. The HC of botulinum neurotoxin is a non-toxic immunogen which is capable of inducing strong BoNT neutralizing antibody responses in mice and rabbits. To evaluate HC immunogenicity, rBoNT/E/HC vaccine produced by USAMRIID was used to vaccinate channel catfish. This vaccine was unable

to induce a robust antibody response in channel catfish but western blot analysis demonstrated specific antibody production in 3 of 11 vaccinated fish. We then developed four channel catfish virus (*Ictalurid herpesvirus* 1, CCV) recombinants to express BoNT/E/HC to determine if the virus vector could improve the response. We produced the recombinants by inserting synthetic HC genes into our established Gateway CCV recombination system. These recombinants expressed BoNT/E/HC; the protein concentrations and expression patterns were similar in both lactocystein (proteasome inhibitor) treated cell lines and non-treated cell line. The western blots results showed c-terminal of HC expression. The four recombinants differed in promoters and gene sequence. These recombinants were used to vaccinate channel catfish but no significant protective immunity or BoNT/E antibodies presence was observed. In comparison, a control vector that expressed *Escherichia coli* beta-galactosidase induced a strong antibody in the same group of fish. These results suggest that BoNT/E HC has low immunogenicity in channel catfish and deviates from the high immunogenicity previously observed in mouse and rabbit studies. In order to develop a better vaccine, it will be necessary to understand BoNT/E /HC immunogenicity and the channel catfish immune response against HC.

5.2   Introduction

In channel catfish aquaculture, visceral toxicosis of catfish (VTC) is one of the most economically important diseases causing sporadic mortalities in the Mississippi Delta channel catfish ponds. Mortalities mainly appear in brood stock and market sized catfish when pond temperatures are between 18 and22^0C, which causes economic devastation for channel catfish farmers. The first VTC case was documented in 1998;

following this, VTC accounted for 37% of brood stock mortalities in 2003[32, 48].In years 2003 to 2010, an average of 3.1% of diagnostic fish cases were attributed to VTC at the MSU-CVM aquatic diagnostic laboratory in Stoneville, MS. The causative agent of VTC was identified as botulinum neurotoxin type/E (BoNT/E) by the catfish neutralization assay and endopep mass spectroscopy[32].

Botulinum neurotoxins (BoNT) are produced by *Clostridium botulinum*, an anaerobic, spore-forming, rod-shaped, gram-positive bacterium[37]. The BoNTs are divided into seven types based on their immunological difference (type A to G); the molecular mass of each of the seven types is approximately 150 kD [26, 65]. The BoNT consists of a 100 kDa Heavy Chain (HC), which transport the toxin into neurons and a 50 kDa Light Chain (LC), which acts as a metalloprotease (Zn^{2+}-endoprotease) cleaving one of the SNARE proteins in the neuron[52, 90, 99]. The cleaved SNARE proteins cannot form an active SNARE complex which blocks the exocytosis of synaptic vesicles and thereby signal transduction at the neuromuscular junction.

The heavy chain of BoNT/E contains 830 amino acids and is the major portion of the neurotoxin. For over a decade, vaccine research has focused on components of the neurotoxins that could elicit the best protective immune response. Vaccine studies in mammals have experimented with the whole heavy chain, recombinant heavy chain, subunits of the heavy chain, recombinant whole toxin or the C-terminal of heavy chain[3, 4, 8, 71, 92]. All these studies showed that the heavy chain of the BoNT plays an important role in inducing protective immunity. Furthermore, the HC of the neurotoxin is non-toxic because it lacks the endopeptidase activity of the LC.

The goal of this study was to develop a practical and affordable vaccine to protect against VTC losses for the channel catfish producers. This study evaluates the recombinant BoNT/E/HC (rBoNT/E/HC) produced by the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID)and a codon biased BoNT/E/HC expressed by an attenuated recombinant channel catfish virus (*Ictaluridherpesvirus*1) as possible vaccines for VTC.

5.3   Materials and methods

5.3.1   Fish

This project was implemented under the oversight and approval of the Mississippi State University Institutional Animal Care and Use Committee and the Institutional Biosafety Committee. Channel catfish (*Ictalurus punctatus*) for rCCV/HC vaccination were obtained from College of Veterinary Medicine, Mississippi State University (CVM-MSU) specific pathogen free fish hatchery. The fish were arbitrarily assigned to five treatment groups and were maintained in 15-L aerated tanks receiving charcoal-filtered de-chlorinated municipal water at a rate of 0.5 L/min at 30^0C throughout the experiment.

To evaluate the response to rBoNT/E/HC vaccine produced by USAMRIID, channel catfish with no known previous exposure to *C. botulinum* were obtained from the United States Department of Agriculture Agricultural Research Service, Genetics Compound, Stoneville, Mississippi. Twenty three fish were arbitrarily assigned to five treatment groups based on their age and dose of vaccine (Table 5.1).

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5.3.2   Experimental design

Fish were acclimated for 7 d prior to the study. Fish were fed twice a day throughout the experiment except the day before and the day of vaccination and challenge. Fish were observed 3 times a day for mortality, morbidity and clinical signs (inability to swim, settling at the bottom of the tank and fin-in-coordination). Fish that died after the rCCV exposure were necropsied, gross lesions recorded, and the posterior kidney was cultured for bacteria. At the end of the study, surviving fish were anesthetized in tricaine methanesulfonate (Western Chemical, Ferndale, WA, USA), bled to extract serum, and euthanized with an overdose of tricainemethanesulfonate.

5.3.3   Recombinant BoNT/E heavy chain immunization

A total of 23 channel catfish were immunized with rBoNT/E/HC (produced by U.S. Army Medical Research Institute of Infectious Diseases(USAMRIID), kindly provided by Theresa Smith- Integrated Toxicology, USAMRIID, 1425 Porter Street, Fort Detrick, MD. 21702) (Table 5.1). Of 23 fish, 18 fish were around 6 months old and from same spawn; six of these fish were immunized with 1 μg, six fish with 5 μg, and six fish with 10 μg of rBoNT/E/HC. Three adult channel catfish, which were more than 2 years old, were immunized four times with 10 μg of rBoNT/E/HC. Two channel catfish, approximately1- year old were immunized four times with 10 μg rBoNT/E/HC. The fish were immunized 4 times with a 3-week interval between immunizations. In the first immunization the rBoNT/E/HC was mixed with Freund's incomplete adjuvant, and the subsequent 2nd, 3rd and 4th immunizations of rBoNT/E/HC were mixed in saline; ten days after the final immunization the fish were terminally bled, serum was extracted and stored at $^-80^0$C until analysis.

5.3.4 Western blots for recombinant BoNT/E heavy chain immunization

Western blots were performed to analyze for the presence of BoNT/E antibodies in immunized fish sera. The purified BoNT/E toxin (Metabiologics, Madison, WI;http://www.metabiologics.com/products.htm) was mixed with 2x laemmli buffer, heated at 95^0 C for 5 min, cooled on ice, and run on a 8% SDS-PAGE gel for 2 h at 100 V; then the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane at 100 V for 1.5 h. The blot was incubated with 5% non-fat dry milk, 1% bovine serum albumin, and 1% goat serum in tris-buffered saline (blocking buffer) overnight at 4^0C, and washed 5 times with 0.05% tween 20 in tris-buffered saline (TBS). The blot was transferred to Mini-Protein ® II Multiscreen Apparatus containing 20wells; each well was incubated with controls and different sera (1:40 sera in blocking buffer) collected from immunized fish at ambient temperature for 2 h, and washed 5 times with 0.05% tween 20 in TBS. Then the blot was incubated for 1 h with mouse monoclonal antibody 9E1 (against channel catfish Ig) diluted 1:4 in blocking buffer, and washed 5 times with 0.05% tween 20 in TBS. Blots were incubated for 1 h with horseradish peroxidase (HRP) conjugated goat anti-mouse antibodies in blocking buffer (1:50,000 dilution). After HRP incubation blots were washed 5 times with 0.05% tween 20 in TBS and analyzed using amersham ECL prime western blot kit (GE Healthcare Life Science).

5.3.5 BoNT/E antibody analysis by zebrafish neutralization assay

Zebrafish were obtained from College of Veterinary Medicine, Mississippi State University (CVM-MSU) specific pathogen free fish hatchery. The fish were arbitrarily assigned to treatment groups (five fish for each group) and were maintained in 15-L aerated tanks receiving charcoal-filtered de-chlorinated municipal water at a rate of 0.5

L/min at 30 $^{\circ}$C throughout the experiment. Sera extracted from rBoNT/E/HC and rCCV

vaccinated fish were analyzed for the presence of BoNT/E neutralizing Ab. The serum

samples were incubated with activated toxin: 9 μl of sera incubated with 1μl of 15 pg/μl

BoNT/E (7.5pg/μl dose caused 100% mortality in zebrafish previously[18]) at ambient

temperature for 1 h; as a control, the toxin was incubated with phosphate buffered saline

(PBS). Five zebrafish were injected *intracoelomically* (IC) with 10 μl toxin-sera mixture

for each serum sample.

5.3.6   Recombinant channel catfish virus generation

The two synthetic HC genes, one gene codon biased for CCV (HC$_C$) and the other

the natural *C. botulinum* codon usage (HC$_N$) were produced by Biomatik custom gene

synthesis. These synthetic HC DNAs were directionally cloned into two CCV cloning

systems, one which uses the natural thymidine kinase (TK) promoter, and one which uses

the cytomegalovirus immediate early promoter (CMV IE) as previously described[53].

Briefly, the synthetic constructs were directionally cloned into pENTR^{TM}/DTOPO$^{®}$

vector to produce the attL-containing Gateway$^{®}$ entry clone pENTR-HC$_C$ and pENTR-

HC$_N$. Plasmids pENTR-HC(c and n) were used to insert the HC($_C$ and $_N$) into the pTK-

DEST and pCMV-DEST CCV destination vectors by in vitro recombination using the

Gateway$^{®}$ LR Clonase^{TM} II Enzyme Mix according to the manufacturer's instructions

(Invitrogen Inc.) to produce pTK-HC$_C$, pTK-HC$_N$, pCMV-HC$_C$ and pCMV-HC$_N$. In order

to produce the four recombinant cosmids four in vitro reactions were performed. The

proper insertion of the HC in pHC79-395 cosmid was confirmed by PCR fragment length

in 0.7% agarose gel electrophoresis. The HC containing cosmids, PENC and PESΔR

were digested with AscI, NotI and SphI, respectively. The DNA fragments were purified

from 0.7% agarose gels; 1000 μg of each fragment was mixed together and transfected into channel catfish ovary (CCO) cells with Lipofectin® (Invitrogen Inc.) or TurboFect (Thermo Scientific) according to the manufacturer's instructions. The CCO cells were grown to ~80% confluence in a T-25 (5×10^6) flask with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4 mM L-glutamine and 10% fetal calf serum. Two days after transfection, CCV plaques developed. A total of 4 recombinant viruses (rCCV) were created: CCV-TK-HC$_C$, CCV-TK-HC$_N$, CCV-CMV-HC$_C$ and CCV-CMV-HC$_N$. The HC containing CCVs were plaque-purified by end-point dilution assay. The expression of the HC in the rCCV was analyzed by RT-PCR and western blot.

5.3.7 RT-PCR to evaluate BoNT/E/HC in recombinant channel catfish virus

Channel catfish ovary (CCO) cells were grown to ~80% confluence in a T-25 (5×10^6) flask and were washed three times with serum free DMEM supplemented with 4 mM L-glutamine and exposed to 15×10^6 plaque forming units (pfu) of BoNT/E/HC containing rCCV. After 8 h of infection, the cells were washed in ice-cold phosphate saline buffer (PBS); all the cells were collected by scraping the plate in ice-cold phosphate buffer. The cell pellet was washed three times in PBS by centrifugation ($800\times g$, 4^0C, 5 min) and re-suspended in PBS. After three washes the cell pellet was collected; total RNA was extracted using Direct-ZOL^{TM} RNA MiniPrep (ZYMO) kit according to manufacturer's protocol and stored at $^-80^0C$. To synthesize first-strand cDNA from total RNA, SuperScript®III First-Strand Synthesis System for RT-PCR kit (Invitrogen) was used according to the manufacturer's guidelines. For HC amplification, 4 μl of the cDNA synthesis reaction was used with primers 6442U22-CCATGGGGATTCGGAAGTCGAT, 8986L22-CGGGCCCTCTAGATCAACCACT for

HC$_C$ and W6442U22-CCATGGGCATAAGGAAATCAAT, W8986L22-

CGGGCCCTCTAGATCAACCACT for HC$_N$, the PCR product was gel purified from

0.7% agarose and cloned into pCR™4-TOPO® Vector (life technologies). Two plasmids

for each virus were sequenced.

5.3.8 Western blots to analyze BoNT/E/HC expression in rCCV infected cell culture

The channel catfish ovary (CCO) cells were grown to ˜80% confluence in a T-75

flask (1.5×10^7 cells/well) washed three times with serum free DMEM supplemented with

4 mM L-glutamine and 5 μM Lactacystin(20 S proteasome inhibitor to evaluate potential

HC digestion by cellular proteasomes) or without Lactacystin and exposed with 4.5×10^7

pfu of HC containing rCCV. After 6 h of infection, the cells were washed in ice-cold

phosphate saline buffer (PBS); all the cells were collected by scraping the plate in ice-

cold phosphate buffer. The cell pellets were washed three times in PBS by centrifugation

($800 \times g$, 4^0C, 5 min) and re-suspended in PBS. After three washes the cell pellets was

collected and lysed in RIPA buffer (Santa Cruz Biotechnology) according to the

manufactures protocol. The supernatant from lysed cells were collected after the 30 min

agitation and centrifugation steps ($15,000 \times g$ for 30 min at 4^0C) and stored at -80^0C until

analyzed. The supernatants were mixed in 2x laemmli buffer, heated at 95^0 C for 5 min,

cooled on ice, and run on a 8% SDS-PAGE gel for 2 h at 100 V in running buffer (250

mM Tris base, 2 M glycine and 0.1% SDS); the gel was then transferred to a PVDF

membrane at 100 V for 1.5 h in transfer buffer (250 mM tris base, 2 M glycine and 20 %

Methanol). The membrane was blocked overnight with blocking buffer (5% non-fat dry

milk, 1% goat serum and 1% bovine serum albumin in tris-buffered saline (TBS- 50

mMTris, 150mM NaCl PH-7.4), and after washing 5 times with 0.05% tween 20 in TBS, the membrane was incubated for 2 h with sheep anti-BoNT/E/HC in blocking buffer. Again the membrane was washed 5 times with 0.05% tween 20 in TBS and incubated for 1 h with HRP conjugated donkey anti-sheep Ab in blocking buffer. After washing, the proteins were detected by the ECL western blot kit according to the manufacturer's instructions.

5.3.9 Channel catfish exposure to rCCV and BoNT/E challenge

Channel catfish were exposed to all four CCV expressing the BoNT/E/HC, with rCCV/TK- expressing lacZ as a positive control, and media exposed fish were a negative control. For each virus or control, 3 tanks were used, and each tank contained 12 juvenile channel catfish, which were between 8 to 12centimeters in length. All fish were produced from the same spawn and reared indoors under similar conditions. Channel catfish were immersion exposed to respective virus or controls for 30 min with 3×10^6pfu/liter dose. After 3 weeks, the fish were boosted with 6×10^6 pfu/liter dose of respective virus or controls. Vaccinated channel catfish were challenged 10 days after the booster with trypsin-digested BoNT/E (Metabiologics.Inc)at a dose of 30 pg/gram fish as previously described[18].

5.3.10 Dot blots for BoNT/E and CCV antibodies evaluation in vaccinated catfish

Dot blots were performed using individual vaccinated and control fish sera to evaluate the presence of BoNT/E and CCV antibodies. To recognize BoNT/E antibodies purified toxin was used; for each dot 4 μl (10 ng/μl) of purified BoNT/E and similar concentration BoNT/E is mixed with laemmli buffer, heated at 95^0C for 5 min was used

for spotting to analyze polypeptide antibodies. To evaluate CCV specific antibodies CCV lysates of CCV infected CCO cells were used as the spotted antigen and control antigen spots of uninfected CCOs were also added to the blot. To produce the CCV infected cell antigen~80% confluence CCO cells at ~80% confluence in a T-75 flask (1.5×10^7 cells/well)were washed three times with serum free DMEM supplemented with 4 mM L-glutamine and exposed with 4.5×10^7 pfu CCV. After 12 h of infection, the cells were washed in ice-cold phosphate saline buffer (PBS); all the cells were collected by scraping the plate in ice-cold phosphate buffer. The cell pellets were washed three times in PBS by centrifugation ($800 \times g$, 4^0C, 5 min) and re-suspended in 1 ml RIPA lysate buffer and supernatant collected as previously mentioned stored at $^-80^0C$; 4μl/dot of this solution was used for spotting the membrane. The CCO cell lysate was also prepared by using RIPA cell lysate buffer as previously mentioned and stored at $^-80^0C$; 4 μl/dot of this solution was used for spotting membrane. Each dot consisted of 4 μl of BoNT/E, laemmli-BoNT/E, CCV-CCO cell lysate or CCO cell lysate spotted on appropriate grid of nitrocellulose membrane (BIO-RAD) using narrow-mouth tips with a p10 micropipette (Gilson). The membrane was blocked with blocking buffer overnight at 4^0C, and then washed 5 times with 0.05% tween 20 in TBS. The individual fish serum was diluted with blocking buffer in a 1:10 ratio. Two hundred (200) μl of this solution were added to 4 vertical wells (each serum was added to BoNT/E, laemmli-BoNT/E, CCV-CCO cell lysate and CCO cell lysate spots (Fig 5.5). and rBoNT/E/HC vaccinated fish which showed BoNT/E Ab was used as a positive control. The serum with blocking buffer was incubated for 2 h at ambient temperature with minimum agitation and washed 5 times with 0.05% tween 20 in TBS after incubation. Then the membrane was incubated for 1 h

with mouse monoclonal antibody 9E1 (against channel catfish Ig) diluted 1:4 in blocking buffer, and washed 5 times with 0.05% tween 20 in TBS. Blots were incubated for 1 h with horseradish peroxidase (HRP) conjugated goat anti-mouse antibodies in blocking buffer (1:50,000 dilution). After HRP incubation blots were washed 5 times with 0.05% tween 20 in TBS and analyzed using amersham ECL prime western blot kit (GE Healthcare Life Science).

5.3.11   ELISA for β-galactosidase Antibody evaluation in vaccinated catfish

The fish sera were evaluated for presence of anti- β-galactosidase antibodies. Six fish sera from each group: DMEM (control), HC expressing rCCV and rCCV/TK-expressing lacZ sera were arbitrarily chosen to evaluate the presence of antibodies. The 96 well ELISA plates were coated with 200 μl/well of 2.5 μg/ml *E.coli* β-galactosidase (Sigma) in carbonate bicarbonate buffer solution (15 mM Na$_2$CO$_3$, 34.8 mM NaHCO3, pH 9.6) overnight at 4 °C. The plates were washed 5 times with 0.05% tween 20 in PBS and pre-incubated with PBS-blocking buffer (5% non-fat dry milk, 1% bovine serum albumin, and 1% goat serum in PBS) for 2 h at ambient temperature with minimal agitation and washed the plate five times with 0.05% tween 20 in PBS. The serum samples to be tested were diluted 1:10 in PBS-blocking buffer and 200 μl of this solution added to wells, with two replicates per serum sample. The serum was incubated for 2 h at ambient temperature with minimal agitation, and the plate was washed five times with 0.05% tween 20 in PBS after the incubation. Then the wells were incubated with 200 μl of mouse monoclonal antibody 9E1 (anti- channel catfish Ig) diluted 1:4 in blocking buffer for 1 h and the plate was washed five times with 0.05% tween 20 in PBS. The wells were incubated with 100 μl of horseradish peroxidase (HRP) conjugated goat anti-

mouse antibodies (SouthernBiotech) in blocking buffer (1:10,000 dilution) for 1 h.
Afterwards, HRP Ab incubation blots were washed 5 times with 0.05% Tween 20 in
PBS. Finally, 100 μl of 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma-TMB substrate
solution prepared according to the manufacturer's instructions) substrate added to each
well and incubated at room temperature for 20 min and optical densities were measured
at 650 nm using a plate reader (Molecular Devices® ThermoMax Microplate Reader).

5.4   Results

5.4.1   Recombinant BoNT/E heavy chain immunization

The six month old fish vaccinated with the USAMRIID BoNT/E HC sera did not
show any antibodies in western blots and also in zebrafish serum neutralization assay.
One of the ~one year old channel catfish vaccinated with rBoNT/E/HC sera showed
delayed mortality (fish have minimal BoNT/E clinical signs in 24 h post injection and 3/5
were dead by end of the 96 h, in remaining groups fish were dead by 24 h) in the
zebrafish neutralization assay but no antibody was detected in the western blot (Table
5.1). Two of the three older fish vaccinated with rBoNT/E/HC sera showed antibodies in
western blot but serum from these fish did not provide protection or delay mortality in the
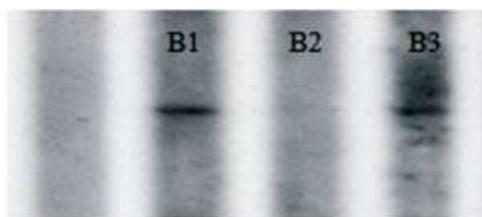zebrafish serum neutralization assay (Fig 5.1)

Figure 5.1   Western blot for rBoNT/E/HC vaccinated catfish

Western blot for analyzing Ab presence in the rBoNT/E/HC vaccinate sera, out of 11 fish two big fish (>2 years) B1 and B3 showed presence anti-BoNT/E antibodies. This serum served as the positive control in further experiments (Figure 5.5).

Table 5.1   rBoNT/E/HC vaccinated catfish

No. of Fish	Age	Dose of BoNT/E/HC	Positive results on western blot	Positive neutralization assay
6	~6 months	1 µg	All Negative	All Negative
6	~6 months	5 µg	All Negative	All Negative
6	~6 months	10 µg	All negative	All Negative
3	>2 years	10 µg	2 positive	All Negative
2	~1 year	10 µg	All negative	1 positive

Results of western blots and zebrafish neutralization assays to assess the immunogenicity of USARMIID rBoNT/E/HC vaccine in channel catfish (*Ictalurus punctatus*). Three different age groups and three different dose groups were tested.

5.4.2   BoNT/E/HC insertion in CCV cosmid

The proper insertion of BoNT/E/HC in the pHC79-395 cosmid was confirmed by the length of PCR fragments in 0.7% agarose gel (Fig 1).
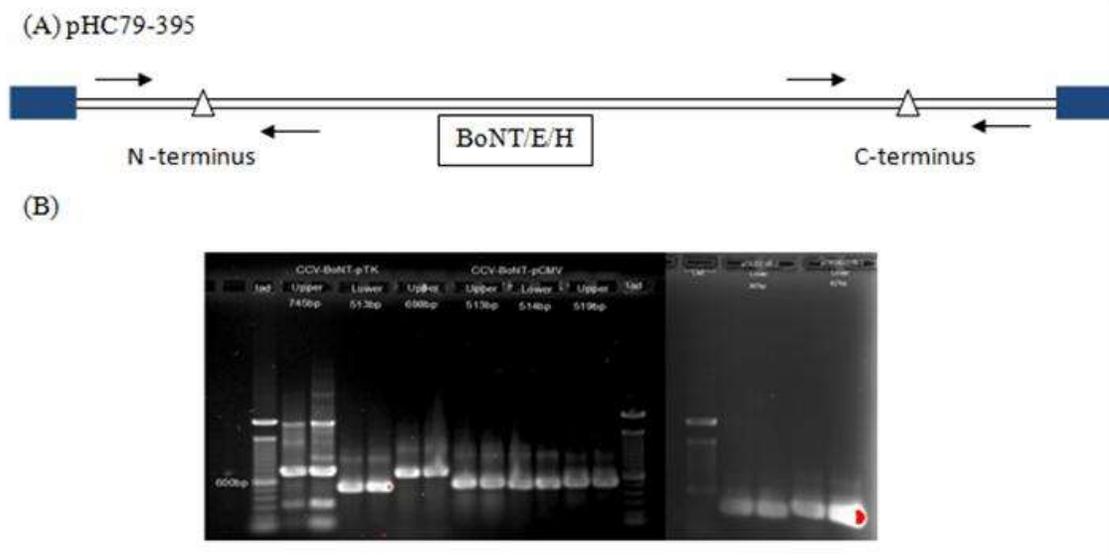
Figure 5.2   Conformation of BoNT/E/HC in pHC79-395 cosmid

(A) The location of primers for PCR evaluation of BoNT/E/HC insertion site in cosmid pHC79-395.(B) Agarose gel (0.7%) electrophoresis of PCR products to analyze the proper insertion of BoNT/E/HC in pTK- HC$_C$ (N-terminal fragment size 745 bp, C-terminal fragment size 513 bp), pTK-HC$_N$ (N- 698 bp, C- 367 bp), pCMV- HC$_C$ (N-513 bp, C- 514 bp) and pCMV- HC$_N$ (N- 519 bp, C- 427 bp)

5.4.3   RT-PCR for BoNT/E/HC expression in CCV

PCR from the cDNA synthesis reaction showed a 2506 bp product in 0.7% agarose gel electrophoresis; which is of the same length as the inserted synthetic BoNT/E/HC. This was further confirmed by sequencing this product, which is a 100% match to the original synthetic BoNT/E/HC.

5.4.4   Western analysis for BoNT/E/HC expression in cell culture

The western analysis showed expression of a 50 kDa BoNT/E/HC product in the CCO cells treated with rCCV expressing codon biased HC. The 50 kDa HC is confirmed as C-terminal BoNT/E/HC protein by specific Ab binding. Similar expression was not observed in the cells infected with natural HC expressing rCCV. The expected HC is

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~100 k Da but only around 50 kDa was shown in westerns (fig 5.3). To determine if

proteasome degradation might be affecting expression BoNT/E/HC, the infected cells

were treated with lactacystin and similar results were obtained in lactacystin treated and
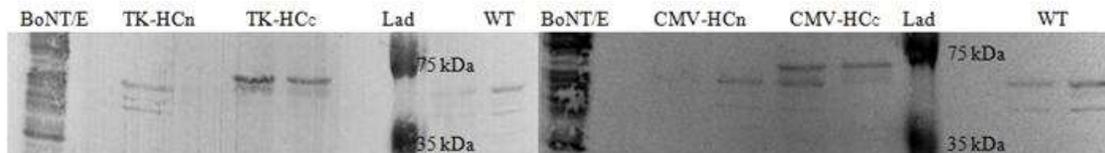
untreated CCO cells.

Figure 5.3   Western blot for BoNT/E/HC expression

Western blot of botulinum E heavy chain (BoNT/E/HC) expression in recombinant
channel catfish virus infected CCO cells. The codon biased HC was expressed in both
promoters and was ~ 50 kD; a similar product was not observed in WT CCV and
attenuated CCV expressing natural C. botulinum HC. HC_C- codon biased BoNT/E/HC;
HC_N: natural *C. botulinum* BoNT/E/HC, TK: thymidine kinase promoter, CMV :
cytomegalovirus immediate early promoter, WT: wild type CCV infected cell lysate,
BoNT/E : BoNT/E positive control and Lad: Ladder

5.4.5   Recombinant BoNT/E/HC CCV (Channel catfish exposure to rCCV and challenge

Channel catfish were vaccinated with the four BoNT/E/HC expressing

recombinant CCV constructs by immersion exposure then challenged with BoNT/E at 28

days post vaccination. Thymidine kinase deleted and *E. coli* lacZ expressing CCV

recombinant was used to vaccinate control groups. The fish in all treatment groups started

showed typical VTC signs such as: abnormal swimming patterns, fin in-coordination,

settling at the bottom of the tank within 12 h. Some live fish in all tanks were resting at

the bottom of the tank but showed no signs of movement, presumably because of paresis

and partial paralysis. When observers approached the tank, the fish swam erratically and

lost balance. There were mortalities within 12 h after BoNT/E injection. There was no

significant protection against mortality in vaccinated fish compared to control fish (Fig 5.4).
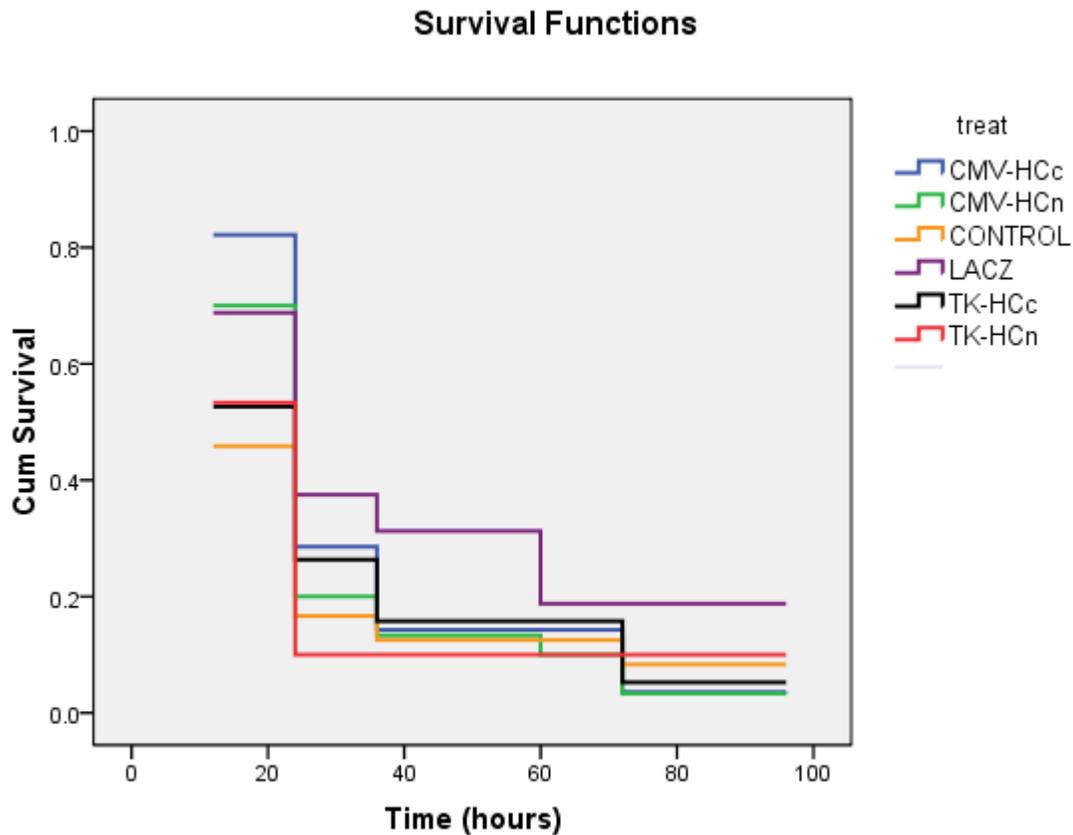
Survival Functions

Figure 5.4   Kaplan-Meier survival curves for recombinant channel catfish virus vaccinated fish

Kaplan-Meier survival curves for recombinant channel catfish virus expressing synthetic botulinum neurotoxin/E heavy chain rCCV/BoNT/E/HC), rCCV expressing *E.Coli* lacZ and control (DMEM) exposed Channel Catfish. CMV-HC$_C$: Cytomegalovirus immediate early promoter (CMV) expressing BoNT/E heavy chain codon biased to CCV (HC$_C$) in rCCV, CMV-HC$_N$: Cytomegalovirus immediate early promoter (CMV) expressing natural BoNT/E heavy chain (HC$_N$) in rCCV, TK-HC$_C$: thymidine kinase (TK) promoter expressing BoNT/E heavy chain codon biased to CCV (HC$_C$) in rCCV, TK-HC$_N$: thymidine kinase (TK) promoter expressing natural BoNT/E heavy chain (HC$_N$) in rCCV, LACZ: thymidine kinase (TK) promoter expressing β-galactosidase in rCCV and CONTROL: fish were exposed to DMEM media only.

5.4.6 Dot blots for BoNT/E antibodies evaluation in vaccinated catfish

The dot blots did not show any BoNT/E Ab presence in BoNT/E or laemmli-BoNT/E spots. All rCCV exposed fish had strong Ab recognition of CCV-CCO lysate and weaker recognition of uninfected CCO cells (fig 4). Control fish showed no recognition of any of the tested antigens. These observations indicate rCCV exposed fish were able to develop Ab against CCV but not to BoNT/E.
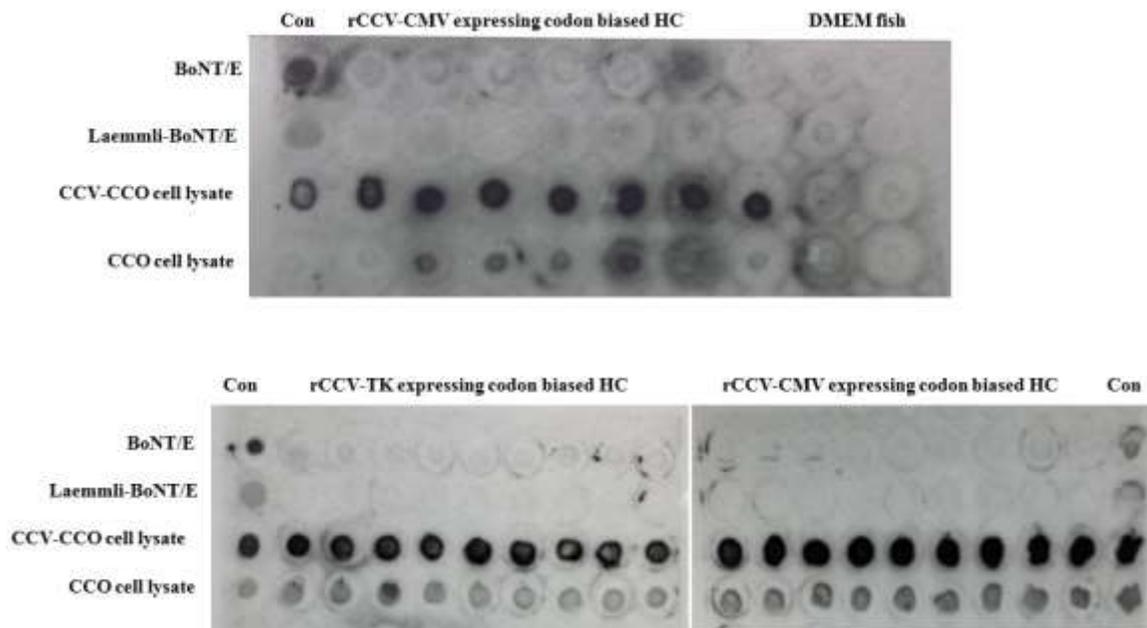
Figure 5.5   Dot blots to analyze anti-BoNT/E antibodies in rCCV vaccinated catfish sera

Dot blot to evaluate the presence of antibodies in vaccinated and control fish sera. Botulinum neurotoxin/E (BoNT/E), laemmli-BoNT/E: BoNT/E mixed in laemmli buffer and heated at 95°C for 5 min (to evaluate polypeptide binding antibodies), CCV-CCO cell lysate: channel catfish virus infected channel catfish ovary (CCO) cell lysate and CCO cell lysate spotted on membrane. Con: Control positive serum from the rBoNT/E/HC vaccination study; rCCV-TK-HC: channel catfish virus expressing BoNT/E/HC with thymidine kinase promoter; rCCV-CMV-HC: channel catfish virus expressing BoNT/E/HC with cytomegalovirus immediate early promoter; DMEM fish: Fish were exposed to DMEM media (Control)

5.4.7   ELISA for β-galactosidase antibody evaluation in vaccinated catfish

In order to evaluate the ability of these channel catfish to respond to expressed foreign antigen we evaluated the anti-β-galactosidase antibodies in the group of channel catfish that were exposed to rCCV-lacZ using β-galactosidase specific ELISA. The CCV-lacZ exposed channel catfish developed a significant anti-body response (OD = 0.659 ± 0.16 (mean±SD)) compared to fish exposed to DMEM media (control) (0.064 ± 0.022) and rCCV expressing BoNT/E/HC (0.085 ± 0.02)   (Figure 5.7)
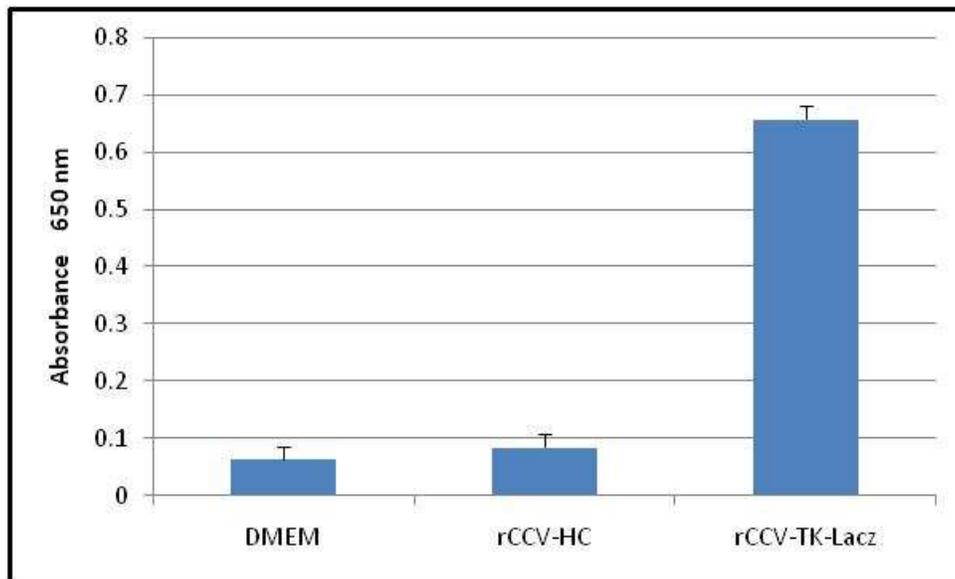
Figure 5.6   ELISA to analyze anti-β-galactosidase antibodies in rCCV vaccinated catfish sera

The average absorbance from six sera samples for each group. DMEM: The channel catfish were exposed to DMEM media (Control), rCCV-HC: The fish were vaccinated with BoNT/HC expressing CCV, rCCV-TK-lacZ: The fish were vaccinated with rCCV expressing lacZ with TK promoter

5.5   Discussion:

The combined information demonstrated weak or no antibody response to the USAMRIID BoNT/E/HC vaccine in channel catfish. Of the 23 channel catfish vaccinated

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with rBoNT/E/HC, only three fish showed an antibody response, and of these, only one demonstrated neutralization activity. Based on these observations; the lack of protection and the lack of anti-toxin Ab could be attributed to no immunogenicity of BoNT/E/HC in fish, and the lacking of the channel catfish's immune system to recognize the BoNT/E/HC.

An efficient immune response depends on several factors of the antigen such as: size, dose, route, foreignness, composition, danger signal, and persistence. A proficient immune response also depends on the host's ability to recognize the antigen by proliferation inducing T cells and antibody presenting B cells, which leads to the production of more antibodies[74]. For a robust immune response, the antigen must be immunogenic and be able to elicit a good immune response against it. Here, we tested the rBoNT/E/HC and C-terminal of BoNT/E/HC expressed by rCCV; none of these proteins were able to induce robust immunity. The lack of immunogenicity of BoNT/E/HC in channel catfish compared to mice and rabbit[3, 4, 8, 71, 95] was perplexing. Antibodies in sera from the two older vaccinated fish that were vaccinated with rBoNT/E/HC recognized the linear BoNT/E peptide, on western blot but failed to neutralize the toxin in zebrafish neutralization assay. Antibodies from the serum of one of the older vaccinated fish failed to recognize linear BoNT/E peptide but they were able to neutralize the BoNT/E toxin in zebrafish serum neutralization assay. One explanation for this impediment involves the processing of the antigen by the host's immune system. The Abs that were produced in the two fish that were able to recognize the linear toxin but unable to neutralize the toxin, so the Abs produced from these fish were possibly derived from the degraded products of rBoNT/E/HC. The Abs that neutralized the toxin might be

derived from the quaternary structure of the toxin. These Abs were unable to recognize linear toxin unlike in the other older catfish. These observations represent the importance of the structure of Ag; in which the rCCV 50 kDa C-terminal of BoNT/E/HC (HCc) was expressed; the structural conformation of this protein was not known. Even though the *E. coli* expressed HCc is highly immunogenic in mouse[8], the rBoNT/E/HC and rCCV expressing HC$_C$ were unable to elicit a robust immune response in channel catfish. Trolletet al,2009 , showed that when plasmids containing HC$_C$ of BoNT/A, BoNT/B and BoNT/E under CMVβ regulation were electro-transferred into the hind legs of mice, the BoNT/E Ab titers were 5.9 fold higher than the titer obtained by electrotransfer of justHC$_C$ of BoNT/E[95]. These results showed that the immunogenicity of HC$_C$varied in the presence of other BoNT HC$_C$ and a danger signal mayenhance antigen recognition. The present study demonstrates the necessity to further analyze the BoNT/E/HC immunogenicity in channel catfish to produce efficient vaccines.

The structure of the antigen plays a crucial role in the immune response; a complex structure tends to give a better immune response compared to simpler protein structures. The western blots using lysates of rCCV infected cells showed only a truncated product of the BoNT/E/HC. This suggests that the BoNT/E/HC might be digested by cellular proteases or were truncated because structural deformities and the cellular proteins could have interfered in the proper conformation of C-terminal BoNT/E/HC. Another possible explanation for the lack of immunogenicity is that the C-terminal domain of BoNT/E/HC expressed in channel catfish might have a simpler protein structure, which could have escaped from the host immune system under the epitope selection pressure in the presence of other CCV proteins. The rBoNT/E/HC was

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expected to produce a better immune response, but despite four immunizations, it was still unable to elicit a robust immune response which questions the ability of BoNT/E/HC to elicit an immune response. Therefore, it is important to evaluate the structural conformation of BoNT/E/HC in channel catfish to understand its ability to elicit immune response in channel catfish.

The channel catfish virus genome has 76 predicted open reading frames (ORF), and the kinetic analysis of CCV polypeptide synthesis revealed three distinct groups of proteins appearing at different time intervals. The expressions of herpes virus genes are coordinately regulated and follow a sequential order of immediate-early (IE), early and late genes. The expression of early and late viral genes depends on the expression of viral regulatory proteins encoded by IE genes act in trans. Based on previous observations of individuals infected with the Epstein –Barr virus (EBV) most of the anti-viral cytotoxic T cell response is directed against the IE protein derived epitopes. This might represent the epitopes generated in IE proteins that have an advantage over the protein epitopes derived from the early and late protein expression. The actual mechanism involved in this anti-viral response is not yet known, but studies suggest that immediate early protein expression leads to earlier antigen processing and presentation of these epitopes is faster as compared to "early" and "late" expressed proteins[1]. In rCCV infection, the CMV promoter expresses HC with the IE genes and the TK expressing HC will be with early genes. There is a possibility of the IE expressed genes having a better immune response, but there is no protection or BoNT/E antibodies in the fish vaccinated with rCCV-CMV expressed HC. Similar results were observed in rCCV-TK expressed HC. The fish were exposed to rCCV with TK expressed lacZ and were able to produce anti-lacz antibodies;

thus time of expression may not be critical for inducing immunity. The combined data suggest that immunogenicity of BoNT/E/HC is low in catfish. In any infection, the immune response tends to focus on only a few epitopes out of the many potential epitopes, this phenomenon is known as immunodominance[1]. The C-terminal of BoNT/E/HC is able to elicit a good immune response in mice, but it failed in channel catfish. The channel catfish challenged with C-terminal of BoNT/E/HC expressing rCCV, failed to produce Ab against BoNT/E/HC, but these fish were able to produce the Ab for CCV proteins and for CCV expressed beta galactosidase. The lack of immune response to the C-terminal of BoNT/E/HC in rCCV infection might be due to the high abundance of co-expressed CCV proteins and their possibility of having immune dominant epitopes compared to C-terminal of the BoNT/E/HC and also the selective pressure of being a better epitope.

In conclusion, the rBoNT/E/HC is unable to generate a robust Ab response, and rCCV expressed C-terminal BoNT/E/HC. Thus, an effective vaccine must use a modified antigen or provide an adjuvant that enhances immune system recognition.

CHAPTER VI

TARGETED MUTAGENESIS TO DEVELOP BOTULINUM NEUROTOXIN E

RESISTANCE ZEBRAFISH USING TALENS

6.1   Abstract

Transcription Activator-Like Effector Nucleases (TALENs) are powerful tools for targeted zebrafish genome editing and also efficiently inactivating genes in zebrafish. Previously researchers were able to modify the zebrafish genome by homology-directed repair using TALENs and single- stranded DNA oligonucleotide at predefined locations. Here, we developed the TALENs that targeted the portion of the SNAP25 gene that encodes *Clostridium botulinum* E recognition region. The TALENS were used in conjunction with an oligonucleotide with modified sequence to develop a recombinant zebrafish that is resistance to botulinum neurotoxin E (BoNT/E). We evaluated these TALENs performance on zebrafish caudal fin cell line from adult zebrafish (SJD) cells. PCR demonstrated the production of recombinant gene. We then used the mRNA of these TALENs and performed the microinjections on one cell stage zebrafish eggs. This study will help us to understand the TALENs performance on handpicked DNA sequence and also shed on the light to development of recombinant animals which are resistant to botulinum neurotoxins

6.2   Introduction

Visceral Toxicosis of Catfish (VTC) is a sporadic and devastating disease in channel catfish aquaculture causing massive mortalities. Botulinum Neurotoxin E (BoNT/E) is the causative agent of VTC in channel catfish[17, 18, 32]. Clostridial neurotoxins (CNT) are A-B type toxins containing two polypeptide chains: B chain helps with internalization of the toxin into neurons, whereas the A chain cleaves one of the SNARE proteins and inhibits the release of the neurotransmitter acetylcholine[13] which blocks the signal transduction at the neuromuscular junction causing paralysis. The substrate recognized by CNT with a double interaction, region 1 is a binding motif, and region 2 contains a peptide bond to be cleaved[83]. The substrate for BoNT/E is SNAP-25, which is cleaved at Arg180-Ile181. Replacement of R180W, I181E and E183I of SNAP-25 completely blocks the proteolysis of BoNT/E, while still maintaining exocytosis *in vitro* in PC12 cells even though the mutant SNAP-25 failed to bind VAMP[100]. If a genetically modified fish was established containing the resistance modification of SNAP-25, this organism would likely be resistant to BoNT/E toxicosis. We planned to test this by using a Transcription Activator-Like Effectors Nucleases (TALEN) based system to modify the zebrafish.

Transcription Activator-Like Effectors Nucleases (TALENs) are engineered to create double stranded breaks in specific locations of chromosomal DNA *in vivo* and *in vitro*. These chromosomal breaks can be repaired by non-homologous end joining (NHEJ) insertions/deletions or homologous recombination, which allows gene replacement or genome editing at specific sites. TALEs are specific DNA binding proteins produced by pathogenic plant bacteria from the genus *Xanthomonas* sp. Upon

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binding to the plant genome, TALEs specifically regulate the plant gene which aids bacterial pathogenesis. TALEs consist of a nuclear localization domain, a central domain of 1.5 to 34.5 tandem repeats, which recognize the DNA sequence and an acidic transcriptional activation domain. Each central domain of the TALE repeat contains an average of 34 highly conserved amino acids; which recognizes a specific nucleotide base pair. Within each repeat, two adjacent amino acids at positions 12 and 13 are hyper-variable and are called repeat variable di-residues (RVD). These RVDs confer the nucleotide base pair specificity in the genome sequence. The most common RVDs His/Asp (HD); Asn/Gly (NG); Asn/Ile (NI); Asn/Asn (NN) recognize cytosine (C), thymine (T), adenine (A), and Guanine (G), respectively. TALEs recognize the DNA sequences by preceding the thymine base pair at the recognition site, and this is crucial in their binding to the genome. Thus, researchers are able to create Transcription Activator-Like Effector Nucleases (TALENs) by fusion of TALEs with non-specific endonuclease FokI. This engineered novel molecular scissor can cleave the specific DNA sequences[62]. The activity of FokI endonuclease depends on two subunits, therefore TALENS are constructed into dimmers each having one subunit; these two subunits fuse at the site of DNA cleavage.

Successful homology-directed repair (HDR) events in predefined locations of the zebrafish genome using Goldy TALENS were reported for insertion of an EcoRV restriction enzyme site in the *ponzr1* locus and insertion of a loxP site in the *crhr2* gene and the*ponzr1* locus using single-stranded DNA (ssDNA) as a template[6]. The goal of our study is to create a HDR using the TALENs system to edit the SNAP-25b genome in zebrafish to obtain a BoNT/E resistant zebrafish. We designed TALENs to target the

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portion of the SNAP-25b gene in zebrafish that encodes the *Clostridium botulinum* E proteolysis region. The TALENS were used in co-injection with ssDNAoligonucleotide with a modified sequence for HDR to create BoNT/E resistant zebrafish.

6.3   Materials and methods

6.3.1   TALENs design

Based on *in vivo* studies, three amino acid replacements were needed to reduce or nullify the SNAP-25 proteolysis by BoNT/E. These 3 amino acids account for a 6 bp change in a span of 12 bp in the SNAP-25b gene. The two suitable pairs of TALENs are chosen by maintaining the cleavage site near the 12bp modification region.

6.3.2   TALEN target sites

The TALEN recognition sites for first pair: left 5'-GGGCAACGAGATCGA-3' and right 5'-TCGACAGGATTATGG-3' with 18 bp spacer "CACCCAGAACCGTCAAA"; the ssDNAoligonucleotide used with this TALEN pair is 5'-CGACATGGGCAACGAGATCGACACCCAGAACCGTCAAATCGAC<u>TGGGAGAT GATC</u>ATGGTGAGATCATGCCGTTGCAATATCACAGAA-3'with the modification site underlined (the original snap-25b base pair in the place of modification was "AGGATTATGGAC"). The 2nd TALEN pair used is left 5'-CGACAGGATTATGGAC-3' and right 5'-TGCAATATCACAGAA-3'with 18bp spacer "ATGGTGAGATCATGCCGT"; the ssDNAoligonucleotide used with this pair is 5'-CCAGAACCGTCAAATCGAC<u>TGGGAGATGATC</u>ATGGTGAGATCATGCCGTTGC AATATCAC-3'.

6.3.3   TALEN construction

The appropriate RVD containing repeats were assembled using the Golden Gate kit (Addgene)[14]. After assembly, the RVDs were cloned into pCS2TAL3-DD (left TALEN) and pCS2TAL3-RR (right TALEN). *In vitro* transcription of TALEN mRNA was synthesizedusing SP6 RNA polymerase system (SP6 mMessage Machine kit, Ambion) according tothe manufacturer's protocol. The mRNA was purified by Direct-Zol^{TM} RNA MiniPrep according to the manufacturer's instruction; the purified mRNA was used for injection.

6.3.4   Treatment of zebrafish cell line with TALENs

Cells of the caudal fin cell line from an adult zebrafish (SJD); were grown in T12.5 cell culture flasks to 80% confluence in DMEM supplemented with 10% fetal calf serum, 4 mM L-Glutamine . Eight flasks were transfected with each set of the TALEN Plasmids (500 ng each) and ssDNA oligonucleotide (1000 ng) or ssDNA oligonucleotide (1000 ng) alone (as a control) using TurboFect Transfection reagent (Thermo Scientific) according to the manufacturer's instructions. After 48 h, the SJD cells were scraped off the plate, and DNA was isolated using the Gentra Puregene cell kit (QIAGEN). After purification, 200 ng of genomic DNA was restriction digested with either HphI or HpyCh4III (New England BioLabs® inc.) at 37°C overnight and 100 ng of the DNA was used as a template for a 25 μl PCR reaction using primers Forward: 5'-CGACTGGGAGATGAT-3' and Reverse 5'- GTGGGATGTGTCATTTCAAGCAC-3' at an annealing temperature of 48^{0}C. The PCR product was gel purified from 0.7% agarose and cloned into pCR™4-TOPO® Vector (Invitrogen™) according to the manufacturer's instructions, and 8 colonies for each transfection were selected; the

plasmids were isolated from these colonies using Miniprep®Plasmid Isolation Kit (INVITROGEN™).These plasmids were sequenced to analyze the HDR events.

6.3.5   TALEN microinjection:

One-cell zebrafish embryos were injected with 40 to 600 pg of TALEN mRNA and ssDNAoligonucleotide(40 to 600 pg - equal to mRNA dose). The caudal fin tissue was biopsied at 2 months post fertilization, and genomic DNA was purified with the Gentra Puregene tissue kit (QIAGEN). To screen for desired recombination events in TALEN injected zebrafish, PCR was performed using primers Forward: 5'-CGACTGGGAGATGAT-3' and Reverse 5'- GTGGGATGTGTCATTTCAAGCAC-3' at an annealing temperature of 48°C.

6.4   Results

6.4.1   Evaluation of TALENs in the SJD cell line

Only one out of 8 colonies from TALEN pair 1 was positive, which produced the HRD product. The remaining 7 colonies did not have any changes in the DNA sequence. Two out of 8 colonies in TALEN pair 2 produced insertions, deletions, and gaps but no homologous recombination; the other 6 sequences did not have changes. The control and ssDNAoligonucleotide sequences also did not have changes from the original DNA sequences.

6.4.2   TALEN microinjection into zebrafish embryos

Approximately 400 zebrafish eggs at the one-cell stage were injected with 75 to 600 pg of mRNA and ssDNAoligo; none of the eggs survived 24 h after injection. Two hundred (200) eggs were injected with 75 to 45 pg mRNA and ssDNAoligo injections, 10

to 50% of eggs survived; with the lower dose containing the most survivors. At the 40 pg

dose, 900 eggs were injected with mRNA and ssDNAoligo and 90% of the fish survived.

A total of 600 zebrafish were screened for homologous recombination, none of them

were positive with desired changes, these fish still carried the wild type SNAP-25.

6.5   Discussion

None of the zebrafish survived when they were injected with≥75 pg mRNA.

Previous studies have shown that 50- 400 pg of mRNA is able to produce HRD in *crhr2*

gene and *ponzr1* locus with minor toxicity[6].In SNAP-25 micro-injections all the larvae

were dead within 6 to 18 h post injection (pi) for dose rates of 75 to 600pg. All the

mortalities were dose dependent; in doses > 200 pg mRNA, 100 % mortality was

observed as early as 6 h. At doses > 100 pg, 90 to 100% mortalities occurred within 12 h

post-injection. In the lower dose of 40pg, there were only 10% mortalities. These results

suggest two possible scenarios: the importance of the role of SNAP-25b in the

development of zebrafish larvae, or the engagement of the cell machinery in translating

the injected mRNA, thus delaying or preventing expression of other important proteins in

development. Eggs injected with 40 pg of mRNA did not show any desired HDR events;

however, this dose might not be sufficient to create any HDR events.

Generally, TALENs targets were optimized using a software program with

defined requirements for proper binding. In SNAP-25b the desired HDR target site is

located at the end of the exon; the intron will start after amino acid 184of SNAP-25b.

There is only a three base pair window between the intron/exon junctions. Because of a

limited window near defined locations, we handpicked several TALENs pairs and

finalized two pairs, which fit best into the binding requirements. To date, there is no

evidence of TALEN activity at the exon ends or near the intron/exon junctions. All the 600 fish screened for HDR events did not have any signs of HDR events, insertions, or deletions. This observation represents targeted genome location also might play the role in occurrence of HDR events using TALENs.

REFERENCES:

1.   Akram A, Inman RD (2012) Immunodominance: a pivotal principle in host response to viral infections. Clin Immunol 143:99-115

2.   Andreas Rummel, Binz T (2013) Botulinum Neurotoxins. Springer Berlin Heidelberg, New York

3.   Baldwin MR, Tepp WH, Pier CL, Bradshaw M, Ho M, Wilson BA, Fritz RB, Johnson EA, Barbieri JT (2005) Characterization of the antibody response to the receptor binding domain of botulinum neurotoxin serotypes A and E. Infection and immunity 73:6998-7005

4.   Baldwin MR, Tepp WH, Przedpelski A, Pier CL, Bradshaw M, Johnson EA, Barbieri JT (2008) Subunit vaccine against the seven serotypes of botulism. Infection and immunity 76:1314-1318

5.   Barash JR, Arnon SS (2014) A novel strain of *Clostridium botulinum* that produces type B and type H botulinum toxins. The Journal of infectious diseases 209:183-191

6.   Bedell VM, Wang Y, Campbell JM, Poshusta TL, Starker CG, Krug RG, 2nd, Tan W, Penheiter SG, Ma AC, Leung AY, Fahrenkrug SC, Carlson DF, Voytas DF, Clark KJ, Essner JJ, Ekker SC (2012) In vivo genome editing using a high-efficiency TALEN system. Nature 491:114-118

7.   Beliaeva NF, Kashirtseva VN, Medvedeva NV, Khudoklinova I, Ipatova OM, Archakov AI (2010) [Zebrafish as a model organism for biomedical studies]. Biomed Khim 56:120-131

8.   Ben David A, Diamant E, Barnea A, Rosen O, Torgeman A, Zichel R (2013) The receptor binding domain of botulinum neurotoxin serotype A (BoNT/A) inhibits BoNT/A and BoNT/E intoxications in vivo. Clinical and vaccine immunology : CVI 20:1266-1273

9.   Bercsenyi K, Giribaldi F, Schiavo G (2013) The elusive compass of clostridial neurotoxins: deciding when and where to go? Current topics in microbiology and immunology 364:91-113

10. Binz T, Bade S, Rummel A, Kollewe A, Alves J (2002) Arg(362) and Tyr(365) of the botulinum neurotoxin type a light chain are involved in transition state stabilization. Biochemistry 41:1717-1723

11. Bitz S (2010) The botulinum neurotoxin LD50 test - problems and solutions. Altex 27:114-116

12. Brashear A, Davis L (2009) Botulinum toxin poisoning. p Botulinum toxin poisoning: diagnosis

13. Brunger AT, Jin R, MA B (2008) Highly specific interactions between botulinum neurotoxins and synaptic vesicle proteins. Cell Mol Life Sci 65:2296-2306

14. Cermak T, Doyle EL, Christian M, Wang L, Zhang Y, Schmidt C, Baller JA, Somia NV, Bogdanove AJ, Voytas DF (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic acids research 39:e82

15. Chakraborty C, Hsu CH, Wen ZH, Lin CS, Agoramoorthy G (2009) Zebrafish: a complete animal model for in vivo drug discovery and development. Current drug metabolism 10:116-124

16. Charles B. Shoemaker, Oyler GA (2013) Botulinum Neurotoxins. Springer Berlin Heidelberg, New York

17. Chatla K, Gaunt PS, Hanson L, Gao DX, Wills R (2012) Determination of the median lethal dose of botulinum serotype E in channel catfish fingerlings. J Aquat Anim Health 24:105-109

18. Chatla K, Gaunt P, Petrie-Hanson L, Hohn C, Ford L, Hanson L (2014) Zebrafish (*Danio rerio*) bioassay for visceral toxicosis of catfish and botulinum neurotoxin serotype E. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 26:240-245

19. Chen Y, Korkeala H, Aarnikunnas J, Lindstrom M (2007) Sequencing the botulinum neurotoxin gene and related genes in *Clostridium botulinum* type E strains reveals orfx3 and a novel type E neurotoxin subtype. Journal of bacteriology 189:8643-8650

20. Cheng LW, Onisko B, Johnson EA, Reader JR, Griffey SM, Larson AE, Tepp WH, Stanker LH, Brandon DL, Carter JM (2008) Effects of purification on the bioavailability of botulinum neurotoxin type A. Toxicology 249:123-129

21. Couesnon A, Pereira Y, Popoff MR (2008) Receptor-mediated transcytosis of botulinum neurotoxin A through intestinal cell monolayers. Cellular microbiology 10:375-387

22.	D.C.Cann, L.Y.Taylor (1982) An outbreak of botulism in rainbow trout, Salmo gairdneri Richardson, farmed in Britain. Journal of Fish Diseases 5:393-399

23.	Dahm R, Geisler R (2006) Learning from small fry: the zebrafish as a genetic model organism for aquaculture fish species. Mar Biotechnol (NY) 8:329-345

24.	Dong M, Yeh F, Tepp WH, Dean C, Johnson EA, Janz R, Chapman ER (2006) SV2 is the protein receptor for botulinum neurotoxin A. Science (New York, NY) 312:592-596

25.	Eklund MW, Poysky FT, Peterson ME, Peck LW, Brunson WD (1984) Type E botulism in salmonids and conditions contributing to outbreaks. Aquaculture 41:293-309

26.	Erbguth FJ (2004) Historical notes on botulism, *Clostridium botulinum*, botulinum toxin, and the idea of the therapeutic use of the toxin. Movement disorders : official journal of the Movement Disorder Society 19 Suppl 8:S2-6

27.	Fischer A, Montal M (2007) Crucial role of the disulfide bridge between botulinum neurotoxin light and heavy chains in protease translocation across membranes. The Journal of biological chemistry 282:29604-29611

28.	Fischer A (2013) Synchronized chaperone function of botulinum neurotoxin domains mediates light chain translocation into neurons. Current topics in microbiology and immunology 364:115-137

29.	Fujita R, Fujinaga Y, Inoue K, Nakajima H, Kumon H, Oguma K (1995) Molecular characterization of two forms of nontoxic-nonhemagglutinin components of *Clostridium botulinum* type A progenitor toxins. FEBS letters 376:41-44

30.	Gambale F, Montal M (1988) Characterization of the channel properties of tetanus toxin in planar lipid bilayers. Biophysical journal 53:771-783

31.	Gaunt P, Chatla K, Hanson L, Gao D, Wills R (2012) Determination of the median lethal dose of botulinum serotype E in channel catfish (Ictalurus punctatus) fingerlings. Journal of Aquatic Animal Health 24:105-109

32.	Gaunt PS, Kalb SR, Barr JR (2007) Detection of botulinum type E toxin in channel catfish with visceral toxicosis syndrome using catfish bioassay and endopep mass spectrometry. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 19:349-354

33.	Gessler F, Hampe K, Bohnel H (2005) Sensitive detection of botulinum neurotoxin types C and D with an immunoaffinity chromatographic column test. Applied and environmental microbiology 71:7897-7903

34.   Getchell RG, W. J. Culligan, M. Kirchgessner, C. A. Sutton, R. N. Casey, Bowser PR (2006) Quantitative polymerase chain reaction assay used to measure the prevalence of *Clostridium botulinum* type E in fish in the lower great lakes. J Aquat Anim Health 18:39-50

35.   Grunwald DJ, Eisen JS (2002) Headwaters of the zebrafish -- emergence of a new model vertebrate. Nature reviews Genetics 3:717-724

36.   Gu S, Rumpel S, Zhou J, Strotmeier J, Bigalke H, Perry K, Shoemaker CB, Rummel A, Jin R (2012) Botulinum neurotoxin is shielded by NTNHA in an interlocked complex. Science (New York, NY) 335:977-981

37.   Hatheway CL (1990) Toxigenic clostridia. Clinical microbiology reviews 3:66-98

38.   Heider LC, McClure JT, Leger ER (2001) Presumptive diagnosis of *Clostridium botulinum* type D intoxication in a herd of feedlot cattle. The Canadian veterinary journal La revue veterinaire canadienne 42:210-212

39.   Hohn C, Petrie-Hanson L (2007) Low-cost aquatic lab animal holding system. Zebrafish 4:117-122

40.   Humeau Y, Doussau F, Grant NJ, Poulain B (2000) How botulinum and tetanus neurotoxins block neurotransmitter release. Biochimie 82:427-446

41.   Huss HH, Eskildsen U (1974) Botulism in farmed trout caused by *Clostridium botulinum* type E; a preliminary report. Nordisk veterinaermedicin 26:733-738

42.   Inoue K, Fujinaga Y, Watanabe T, Ohyama T, Takeshi K, Moriishi K, Nakajima H, Oguma K (1996) Molecular composition of *Clostridium botulinum* type A progenitor toxins. Infection and immunity 64:1589-1594

43.   Inoue K, Fujinaga Y, Honke K, Arimitsu H, Mahmut N, Sakaguchi Y, Ohyama T, Watanabe T, Oguma K (2001) *Clostridium botulinum* type A haemagglutinin-positive progenitor toxin (HA(+)-PTX) binds to oligosaccharides containing Gal beta1-4GlcNAc through one subcomponent of haemagglutinin (HA1). Microbiology (Reading, England) 147:811-819

44.   Institute S (2009) SAS for Windows v9.2. Cary, North Carolina, pp SAS Institute, Inc..

45.   Jacqueline L. Robertson, N. E. Savin, Haiganoush K. Preisler, Russell RM (2007) Multiple explanatory variables body weight. Bioassays with Arthropods, Second Edition. CRC Press, pp 163-178

46.   Jin R, Rummel A, Binz T, Brunger AT (2006) Botulinum neurotoxin B recognizes its protein receptor with high affinity and specificity. Nature 444:1092-1095

47.   Kauffman JA, Way JF, Jr., Siegel LS, Sellin LC (1985) Comparison of the action of types A and F botulinum toxin at the rat neuromuscular junction. Toxicology and applied pharmacology 79:211-217

48.   Khoo LH, Goodwin AE, Wise DJ, Holmes WE, Hanson LA, Steadman JM, McIntyre LM, Gaunt PS (2011) The pathology associated with visceral toxicosis of catfish. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 23:1217-1221

49.   Koriazova LK, Montal M (2003) Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. Nature structural biology 10:13-18

50.   Kubota T, Yonekura N, Hariya Y, Isogai E, Isogai H, Amano K, Fujii N (1998) Gene arrangement in the upstream region of *Clostridium botulinum* type E and *Clostridium butyricum* BL6340 progenitor toxin genes is different from that of other types. FEMS microbiology letters 158:215-221

51.   Kukreja RV, Sharma S, Cai S, Singh BR (2007) Role of two active site Glu residues in the molecular action of botulinum neurotoxin endopeptidase. Biochimica et biophysica acta 1774:213-222

52.   Kukreja RV, Sharma SK, Singh BR (2010) Molecular basis of activation of endopeptidase activity of botulinum neurotoxin type E. Biochemistry 49:2510-2519

53.   Kunec D, van Haren S, Burgess SC, Hanson LA (2009) A Gateway recombination herpesvirus cloning system with negative selection that produces vectorless progeny. Journal of virological methods 155:82-86

54.   Lalli G, Herreros J, Osborne SL, Montecucco C, Rossetto O, Schiavo G (1999) Functional characterisation of tetanus and botulinum neurotoxins binding domains. Journal of cell science 112 (Pt 16):2715-2724

55.   Lanis JM, Barua S, Ballard JD (2010) Variations in TcdB activity and the hypervirulence of emerging strains of *Clostridium difficile*. PLoS Pathog 6:e1001061

56.   Lanis JM, Barua S, Ballard JD (2010) Variations in TcdB activity and the hypervirulence of emerging strains of *Clostridium difficile*. PLoS Pathog 6

57.   Lindstrom M, Nevas M, Kurki J, Sauna-aho R, Latvala-Kiesila A, Polonen I, Korkeala H (2004) Type C botulism due to toxic feed affecting 52,000 farmed foxes and minks in Finland. Journal of clinical microbiology 42:4718-4725

58.   M.W. Eklunda, M.E. Petersona, F.T. Poyskya, L.W. Peck, J.F. Conrad (1982) Botulism in juvenile coho salmon (Oncorhynchus kisutch) in the United States. Aquaculture 27:1-11

59.   Maksymowych AB, Simpson LL (2004) Structural features of the botulinum neurotoxin molecule that govern binding and transcytosis across polarized human intestinal epithelial cells. The Journal of pharmacology and experimental therapeutics 310:633-641

60.   Meeker ND, Trede NS (2008) Immunology and zebrafish: spawning new models of human disease. Developmental and comparative immunology 32:745-757

61.   Mezaki T, Kaji R, Kohara N, Fujii H, Katayama M, Shimizu T, Kimura J, Brin MF (1995) Comparison of therapeutic efficacies of type A and F botulinum toxins for blepharospasm: a double-blind, controlled study. Neurology 45:506-508

62.   Miller JC, Tan S, Qiao G, Barlow KA, Wang J, Xia DF, Meng X, Paschon DE, Leung E, Hinkley SJ, Dulay GP, Hua KL, Ankoudinova I, Cost GJ, Urnov FD, Zhang HS, Holmes MC, Zhang L, Gregory PD, Rebar EJ (2011) A TALE nuclease architecture for efficient genome editing. Nature biotechnology 29:143-148

63.   Moeller RB, Jr., Puschner B, Walker RL, Rocke T, Galey FD, Cullor JS, Ardans AA (2003) Determination of the median toxic dose of type C botulinum toxin in lactating dairy cows. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 15:523-526

64.   Montecucco C (1986) How do tetanus and botulinum toxins bind to neuronal membranes? Trends in biochemical sciences 11:314-317

65.   Montecucco C, Schiavo G (1995) Structure and function of tetanus and botulinum neurotoxins. Quarterly reviews of biophysics 28:423-472

66.   Ohishi I, Sakaguchi G (1980) Oral toxicities of *Clostridium botulinum* type C and D toxins of different molecular sizes. Infection and immunity 28:303-309

67.   Park JB, Simpson LL (2003) Inhalational poisoning by botulinum toxin and inhalation vaccination with its heavy-chain component. Infection and immunity 71:1147-1154

68.   Pellizzari R, Rossetto O, Lozzi L, Giovedi S, Johnson E, Shone CC, Montecucco C (1996) Structural determinants of the specificity for synaptic vesicle-associated membrane protein/synaptobrevin of tetanus and botulinum type B and G neurotoxins. The Journal of biological chemistry 271:20353-20358

69.   Petrie-Hanson L, Romano CL, Mackey RB, Khosravi P, Hohn CM, Boyle CR (2007) Evaluation of zebrafish *Danio rerio* as a model for enteric septicemia of catfish (ESC). J Aquat Anim Health 19:151-158

70.   Piazza TM, Blehert DS, Dunning FM, Berlowski-Zier BM, Zeytin FN, Samuel MD, Tucker WC (2011) *In vitro* detection and quantification of botulinum neurotoxin type e activity in avian blood. Applied and environmental microbiology 77:7815-7822

71.   Pier CL, Tepp WH, Bradshaw M, Johnson EA, Barbieri JT, Baldwin MR (2008) Recombinant holotoxoid vaccine against botulism. Infection and immunity 76:437-442

72.   Rigoni M, Caccin P, Johnson EA, Montecucco C, Rossetto O (2001) Site-directed mutagenesis identifies active-site residues of the light chain of botulinum neurotoxin type A. Biochemical and biophysical research communications 288:1231-1237

73.   Robertson JL, Savin NE, Preisler HK, Russell RM (2007) Multiple explanatory variables: body weight. Bioassays with Arthropods, Second Edition. Taylor & Francis

74.   Rockberg J, Uhlen M (2009) Prediction of antibody response using recombinant human protein fragments as antigen. Protein science : a publication of the Protein Society 18:2346-2355

75.   Roder JD (2004) Biotoxins. In: Plumlee KH (ed) Clinical veterinary toxicology. Mosby, St. Louis, Missouri, pp 98–99

76.   Rossetto O, Schiavo G, Montecucco C, Poulain B, Deloye F, Lozzi L, Shone CC (1994) SNARE motif and neurotoxins. Nature 372:415-416

77.   Rozenfarb E, Eidinger D (1979) Heterotoxicity of human serum. IV. Role of the alternative complement pathway and natural antibody in the lethal toxicity of human serum for mice. International archives of allergy and applied immunology 60:414-426

78.   Rummel A, Hafner K, Mahrhold S, Darashchonak N, Holt M, Jahn R, Beermann S, Karnath T, Bigalke H, Binz T (2009) Botulinum neurotoxins C, E and F bind gangliosides via a conserved binding site prior to stimulation-dependent uptake with botulinum neurotoxin F utilising the three isoforms of SV2 as second receptor. Journal of neurochemistry 110:1942-1954

79.   Rusnak JM, Smith LA (2009) Botulinum neurotoxin vaccines: Past history and recent developments. Human vaccines 5:794-805

80.   Sakaguchi G (1982) *Clostridium botulinum* toxins. Pharmacology & therapeutics 19:165-194

81.   Saslowsky DE, Cho JA, Chinnapen H, Massol RH, Chinnapen DJ, Wagner JS, De Luca HE, Kam W, Paw BH, Lencer WI (2010) Intoxication of zebrafish and mammalian cells by cholera toxin depends on the flotillin/reggie proteins but not Derlin-1 or -2. J Clin Invest 120:4399-4409

82.   Schantz EJ, Johnson EA (1992) Properties and use of botulinum toxin and other microbial neurotoxins in medicine. Microbiological reviews 56:80-99

83.   Schiavo G, Shone CC, Bennett MK, Scheller RH, Montecucco C (1995) Botulinum neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of syntaxins. The Journal of biological chemistry 270:10566-10570

84.   Schiavo G, Matteoli M, Montecucco C (2000) Neurotoxins affecting neuroexocytosis. Physiological reviews 80:717-766

85.   Schilling A, Land W, Pratschke E, Pielsticker K, Brendel W (1976) Dominant role of complement in the hyperacute xenograft rejection reaction. Surgery, gynecology & obstetrics 142:29-32

86.   Schoenbaum MA, Hall SM, Glock RD, Grant K, Jenny AL, Schiefer TJ, Sciglibaglio P, Whitlock RH (2000) An outbreak of type C botulism in 12 horses and a mule. Journal of the American Veterinary Medical Association 217:365-368, 340

87.   Services USDoHaH (1998) Botulism in the United States, 1899-1996 handbook for epidemiologists, clinicians, and laboratory workers, Atlanta, Georgia: CDC

88.   Shukla HD, Sharma SK (2005) *Clostridium botulinum*: a bug with beauty and weapon. Critical reviews in microbiology 31:11-18

89.   Simpson LL, Maksymowych AB, Park JB, Bora RS (2004) The role of the interchain disulfide bond in governing the pharmacological actions of botulinum toxin. The Journal of pharmacology and experimental therapeutics 308:857-864

90.   Singh BR (2006) Botulinum neurotoxin structure, engineering, and novel cellular trafficking and targeting. Neurotoxicity research 9:73-92

91.   Skulberg A, Grande M (1967) Susceptibility of Rainbow Trout Salmo gairdneri Richardson, to *Clostridium botulinum* Toxins. Transactions of the American Fisheries Society 96:67-70

92.   Smith TJ, Lou J, Geren IN, Forsyth CM, Tsai R, Laporte SL, Tepp WH, Bradshaw M, Johnson EA, Smith LA, Marks JD (2005) Sequence variation within botulinum neurotoxin serotypes impacts antibody binding and neutralization. Infection and immunity 73:5450-5457

93.   Sutton RB, Fasshauer D, Jahn R, Brunger AT (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. Nature 395:347-353

94.   Trede NS, Langenau DM, Traver D, Look AT, Zon LI (2004) The use of zebrafish to understand immunity. Immunity 20:367-379

95.   Trollet C, Pereira Y, Burgain A, Litzler E, Mezrahi M, Seguin J, Manich M, Popoff MR, Scherman D, Bigey P (2009) Generation of high-titer neutralizing antibodies against botulinum toxins A, B, and E by DNA electrotransfer. Infection and immunity 77:2221-2229

96.   Tucker C, Hargreaves J (2004) Pond water quality. In: Tucker C, Hargreaves J (eds) Biology and culture of channel catfish, 2nd edition. Elsevier, Amsterdam, pp 215–278

97.   Uotsu N, Nishikawa A, Watanabe T, Ohyama T, Tonozuka T, Sakano Y, Oguma K (2006) Cell internalization and traffic pathway of *Clostridium botulinum* type C neurotoxin in HT-29 cells. Biochimica et biophysica acta 1763:120-128

98.   Vaidyanathan VV, Yoshino K, Jahnz M, Dorries C, Bade S, Nauenburg S, Niemann H, Binz T (1999) Proteolysis of SNAP-25 isoforms by botulinum neurotoxin types A, C, and E: domains and amino acid residues controlling the formation of enzyme-substrate complexes and cleavage. Journal of neurochemistry 72:327-337

99.   Wang HH, Riding S, Lindo P, Singh BR (2010) Endopeptidase activities of botulinum neurotoxin type B complex, holotoxin, and light chain. Applied and environmental microbiology 76:6658-6663

100.   Washbourne P, Bortoletto N, Graham ME, Wilson MC, Burgoyne RD, Montecucco C (1999) Botulinum neurotoxin E-insensitive mutants of SNAP-25 fail to bind VAMP but support exocytosis. Journal of neurochemistry 73:2424-2433

101.   Weber JT, Hibbs RG, Jr., Darwish A, Mishu B, Corwin AL, Rakha M, Hatheway CL, el Sharkawy S, el-Rahim SA, al-Hamd MF, et al. (1993) A massive outbreak of type E botulism associated with traditional salted fish in Cairo. The Journal of infectious diseases 167:451-454

102.   Whitlock RH (2008) Neurotoxigenic closstridia. In: Gyles CL, Prescott JF, Songer JG, Thoen CO (eds) Pathogenesis of Bacterial Infections in Animals. John Wiley & Sons, pp 117-124

103.   Yao J, Nowack A, Kensel-Hammes P, Gardner RG, Bajjalieh SM (2010) Cotrafficking of SV2 and synaptotagmin at the synapse. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:5569-5578

104.   Yule AM, Barker IK, Austin JW, Moccia RD (2006) Toxicity of *Clostridium botulinum* type E neurotoxin to Great Lakes fish: implications for avian botulism. J Wildl Dis 42:479-493