ARTICLE

Structure, biological, and paralysis mechanism differences between neurotoxins of *Clostridium botulinum* and *Clostridium tetani*

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ABSTRACT

Clostridium is the genus of spore-forming, mostly anaerobic soil or intestinal, bacteria. The bacteria produce many toxins linked to various conditions in humans and wildlife. *Clostridium botulinum* and *Clostridium tetani* are bacteria that produce neurotoxins that cause paralysis, an uncommon and untreatable disease. While sharing certain common chracteristics, these bacteria exhibit distinct differences in their structures and functions, particularly the impact of their neurotoxic properties. Hence, this study compares the paralysis mechanisms induced by *Clostridium botulinum* (BoNT) and *Clostridium tetani* (TeNT). To differentiate, the researchers used protein sequencing analysis to identify crucial amino acid residues that impact alterations in toxicity, selectivity, and mode of action. Additionally, the researchers utilized protein sequence retrieval and proteomics techniques by accessing the National Centre for Biotechnology Information (NCBI) database. BAP25804 and AAK72964 are the sequences for *Clostridium botulinum* and *Clostridium tetani*, respectively; these sequences have N-terminal extremities containing the entire spectrum of amino acids, making them useful for analysis. Additionally, the investigation demonstrated that both neurotoxins exhibit moderate hydrophilicity, with values of -0.416 (for BoNT) and -0.364 (for TeNT). The analysis of neurotoxins' localization within the cytoplasm has demonstrated that BoNT lacks both cytoplasmic and cell wall activity, in contrast to TeNT. This determination has enabled researchers to figure out these particular manifestations. Thus, it was revealed that BoNT

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Date received: 08 January 2024
Date revised: 05 October 2024
Date accepted: 26 October 2024
DOI: https://doi.org/10.54645/202417SupYHV-52

KEYWORDS

BoNT, Flaccid Paralysis, In-silico, Microbiology, Spastic Paralysis, TeNT

inhibits neuromuscular transmission, whereas TeNT causes excessive acetylcholine release, which causes flaccid and spastic paralysis, respectively. Consequently, using a pool of predicted antigenic peptides, the researchers scrutinize the more significant presence of antigenic sites in TeNT compared to BoNT. This study solely provides an overview of the two *Clostridium* species. Recognizing the distinctiveness of these findings will give more evidence for treating several illnesses, and potential investigators will gain from utilizing the research findings as a cornerstone and point of comparison to go beyond the constraints.

BACKGROUND

Clostridia generate the most toxins among all bacteria and are associated with significant human and wildlife conditions. Among them, botulism and tetanus are diseases caused by neurotoxins produced by *Clostridium botulinum* and *Clostridium tetani*, respectively. Although these bacteria and neurotoxins have many similarities, their structures and functions are distinct, specifically, the effect of their neurotoxin wherein TeNT causes spastic paralysis, while BoNTs cause flaccid paralysis. Botulism is an uncommon but deadly condition produced by a toxin that targets the body's neurons, resulting in difficulty breathing, immobility, and even death (CDC, 2021). There are isolated incidences of *Clostridium botulinum* infection around the world. Since 1973, the Centers for Disease Control and Prevention have monitored botulism cases in the United States. From 2011 to 2015, an average of 162 cases per year was recorded; infant botulism accounted for 71% to 88% of these occurrences, preceded by foodborne, wound, and botulism of questionable etiology.

On the other hand, according to WHO, the average yearly incidence in the United States from 2001 to 2008 was 0.01 per 100,000 people. Children and the elderly are more vulnerable due to weakened immune systems. Tiwari et al. (2021) state that tetanus is a potentially lethal acute condition characterized by overall stiffness and convulsive skeletal muscle spasms. Muscle stiffness typically develops in the jaw (lockjaw) and neck and then progresses. *C. tetani* resides within the soil and can enter the body through a skin pathway. It will produce the tetanus toxin that causes painful muscular contraction. It frequently causes the neck and jaw muscles to lock ("lockjaw") as described by the CDC. The pentavalent vaccine is currently administered globally as a childhood vaccine. However, until now, the spastic paralysis caused by TeNT does not have a cure, and only its symptoms and manifestations are addressed with antibacterial treatment.

In the study of Campbell et al. (2009), they discovered that clinically isolated strains of *C. tetani* were susceptible to penicillin and metronidazole but were resistant to co-trimoxazole. Previously, penicillin was regarded as the predominant course of treatment for tetanus around the world however, according to WHO, metronidazole is currently regarded as the first line of effective medication and is an alternative to penicillin therapy. Tetracycline, macrolides, clindamycin, cephalosporin, and chloramphenicol are all potent anti-tetanus medications. Botulism has a 40% to 50% death risk if left untreated. *C. botulinum*, similar to other *Clostridium* species and anaerobes, is innately resistant to aminoglycosides, sulfamethoxazole, and trimethoprim (Mazuet et al., 2016). Only a couple of crucial strains have been evaluated for antibiotic susceptibility.

This comparative study on the neurotoxins of *C. botulinum* and *C. tetani* has significant medical relevance in understanding the pathogenesis of botulism and tetanus, developing treatments and countermeasures, and exploring therapeutic applications for these potent toxins. Similarities and differences in receptor binding, internalization, and retrograde transport within the neurons can guide the engineering of these toxins for targeted drug delivery to the nervous system.

Other studies could not go in-depth about differentiating the neurotoxins of *Clostridium botulinum* and *Clostridium tetani* in the manifestation of flaccid and spastic paralysis through physiochemical property profiling or using various bioinformatic tools. Meanwhile, this *in-silico* study was able to differentiate both neurotoxins in terms of 1) parameters of manifestation, 2) N-terminal domain, 3) antigenicity and axonal transport, and 4) the significant structure and function of *Clostridium botulinum* and *Clostridium tetani* neurotoxins. Protein analysis has become increasingly important in understanding the molecular mechanisms of bacterial toxins. By comparing the protein sequences of different strains and serotypes, it can identify key amino acid residues responsible for differences in toxicity, specificity, and mode of action. This helps identify new toxins with similar modes of action and understand how these toxins evolve and spread. This information can be used to develop new diagnostic assays, therapeutic agents, and vaccines with the help of the *in silico* method. A method focused on data mining uses relevant information, such as molecular model structure relationships and databases from network analysis tools, including protein analysis.

In silico methods have emerged as a promising approach for differentiating neurotoxins, offering several advantages over traditional in vivo and in vitro methods; this computational method can provide detailed insights into the molecular mechanism of neurotoxicity, enabling the development of therapeutic strategies, offer a cost-effective and time-efficient alternative to traditional toxicity testing, reducing the need for high-cost experiments and lastly, this can enable the integration of genomic and proteomic data to provide a comprehensive understanding of neurotoxicity pathways. However, despite their potential, in silico methods for differentiating neurotoxins are not without limitations; key challenges include the need for high-quality sequences and validation of in silico models, the complex structure of the neurotoxins, which can make it difficult to develop accurate and predictive in-silico model, and lastly, the requirement for expert knowledge and objective skills in interpretation of the model that can limit the applicability.

Definition of Terms:

***Clostridium spp.* -** *Clostridium* is a genus of anaerobic, Gram-positive bacteria that inhabit soils and the intestinal tracts of humans and animals
TeNT - stands for Tetanus neurotoxin, produced by the bacterium *Clostridium tetani*.
BoNT- stands for Botulinum neurotoxins, which are produced by the bacterium *Clostridium botulinum*.
Spastic paralysis – caused by *C. tetani*, a condition characterized by muscle stiffness and uncontrolled muscle contractions
Flaccid paralysis - caused by *C. botulinum,* a condition characterized by muscle weakness and loss of muscle tone
In silico – research is conducted using computer simulations or theoretical models, as opposed to experiments in the laboratory (in vitro) or on living organisms (in vivo).

FASTA Sequence – text-based format used in bioinformatics to represent amino acid (protein) sequences.

METHODOLOGY

Retrieval of Protein Sequence and Proteomics

Through the use of the database of the National Centre for Biotechnology Information (NCBI), the amino acid sequence of the neurotoxins was obtained and confirmed using ClustalOmega (Version 1.2.2) for the arrangement of the sequence with a progressive alignment technique; the purpose of the tool is to identify and compare the relationships between the different sequences. The program's features include multiple sequence alignment, scoring, and visualization to evaluate the quality of alignments. The most similar sequences are aligned first, followed by the least similar sequences until a global alignment is produced. The botulinum neurotoxin gene on a plasmid carrying the full nucleotide sequence of BAP25804 was obtained from a Japanese newborn patient and contained *Clostridium botulinum* type B strain 111. Meanwhile, AAK72964 was obtained from cloning and sequence analysis of the tetanus toxin gene. The BAP25804 for the species *Clostridium botulinum* neurotoxin and AAK72964 for the species *Clostridium tetani* neurotoxin are the elected sequences that have both the N-terminal ends containing the entire stretch of amino acids that can be useful in understanding the primary structure, predicting the secondary and tertiary structures using computational methods, identifying the functional domains, and accurate comparison of homologous proteins across species which enable the identification of conserved regions and evolutionary relationships.

Profiling of Physicochemical Properties

The molecular profile of the neurotoxins was determined using the acquired FASTA sequence, which served as the template for the target protein sequence. The researchers determined the sequences' structural characteristics through the ExPASY Protparam Tool (Expasy 3.0) of the Swiss Bioinformatics Resource Portal. This web-based application allows the computation of various physical and chemical parameters for a given protein sequence, which allows users to paste directly the text format of the amino acid sequence calculating parameters such as molecular weight, theoretical isoelectric point (pI), amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index, and grand average of hydropathicity (GRAVY). The hydropathicity of the sequences was also determined using the ProtParam of the same portal fundamental for predicting protein structure, understanding membrane proteins, annotating protein function, exploring evolutionary relationships, and designing drugs or targeting specific protein regions. A program called SOPMA 2016 Version (Self Optimized Prediction Method from Alignment) is used to predict the secondary structure of proteins; the software from PRABI (2016) is faster than other programs and helps determine the secondary structures. This version of SOPMA was employed to analyze and predict the distribution of alpha-helix, beta-sheet, and random coil elements within protein sequences. The tool was instrumental in determining the secondary structure composition of proteins, providing valuable insights into their structural characteristics and aiding in interpreting experimental data related to protein function and interactions. Analyzing the localization of the sub-cellular environment aids in recognizing how proteins work, and the researchers could determine both the neurotoxin, BoNT, and TeNT's localization using the PSortB (Version 3.0.3). It utilizes a combination of feature predictors and classification systems to analyze protein sequences and determine their likely subcellular locations within organisms. PSORTb has been continuously updated and refined to enhance its accuracy and predictive capabilities. It is a widely used tool in bioinformatics for studying protein sorting and localization in various organisms. The proteins' antigenicity was predicted using Predicted Antigenic Peptides. Besides the localization and antigenicity of the protein, its function was also determined using the DeepGOWeB server (Version 1.0.18), which uses the DeepGOPlus approach to predict protein functions from protein sequences. Deep convolutional neural networks are used to learn sequence features. Sequence similarity-based predictions are combined with predictions.

Modeling and Validation of the Structure

The researchers evaluated the similarity of the proteins through the NCBI Basic Local Alignment Search Tool (BLASTP) Version 2.15.0. The fraction of comparable query and template proteins was determined to be about 40.79%. To comprehend other proteins' structural information, the researchers utilized i-TASSER (Iterative Threading Assembly Refinement) as the prediction server. I-TASSER uses an iterative threading approach to build 3D models of proteins based on structural templates identified from the Protein Data Bank (PDB). Using the SWISS-MODEL workspace QMEAN6 program (2016 Version), the quality of the neurotoxin's structure was evaluated. Through the Chimera software (Version 35.61.1342), the depiction tool PipesandPlanks was used to reduce ribbon and atomic structure and display the N-terminal and C-terminal domains of the neurotoxins. The Pipes and Planks tool in Chimera software is a feature that allows for the visualization of protein structures by representing helices as "pipes" (cylinders) and strands as "planks" (rectangular boxes), with connectors for the intervening coil regions. This tool is part of the UCSF Chimera molecular visualization program, which is widely used in structural biology and bioinformatics for studying protein structures and their interactions.

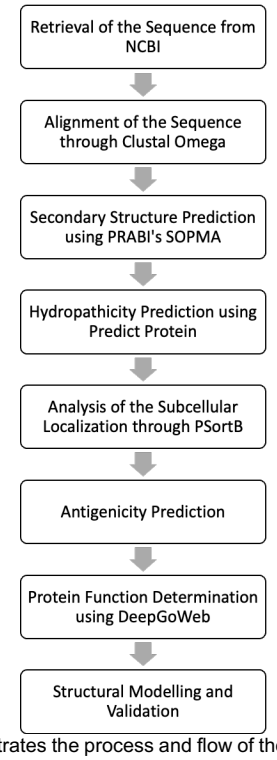


Figure 1: demonstrates the process and flow of the study. The protein sequence of Clostridium botulinum neurotoxin (BAP25804) and Clostridium tetani neurotoxin (AAK72964) are utilized in the study.

RESULTS AND DISCUSSION

SEQUENCE ANALYSIS OF BOTULINUM AND

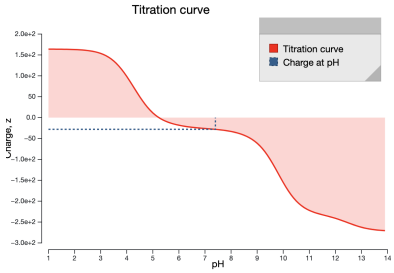
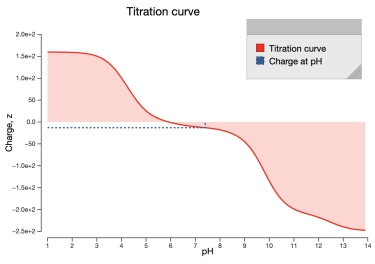
TETANUS NEUROTOXINS

This section summarizes the key aspects of the structural studies on botulinum and tetanus neurotoxins. It highlights their receptor binding and translocation mechanisms, localization in the cytoplasm, aliphatic index N-terminal domain, and antigenicity, which are important for understanding their potent effects and potential therapeutic applications.

Analysis of the Sequence of BoNT and TeNT

The C. botulinum neurotoxin consists of 1291 amino acids, while the C. tetani neurotoxin has 1310 amino acids. The weight of the molecule, number of atoms in totality, net charge, and theoretical pI can be seen in Table 1. To understand how well these neurotoxins interact with water, the researchers measured their hydropathicity. They found that both BoNT and TeNT are moderately hydrophilic, with hydropathicity scores of -0.416 and -0.364, respectively. For context, a standard measure called the GRAVY index indicates that a protein is considered hydrophobic if its score is greater than 1. Using the Kyte and Doolittle hydropathy plot, the researchers confirmed the neurotoxin's moderate hydropathicity (Figs. 2 and 3).

Table 1: Proteomics of the *C. botulinum* and *C. tetani* neurotoxin.

No.	Properties	BoNT (BAP25804)	TeNTs (AAK72964)
1	Number of Amino Acids	1, 291	1,310
2	Molecular Weight	150, 575.14 kDa	150, 317.10 kDa
3	Formula	$C_{6847}H_{10524}N_{1718}O_{2038}S_{33}$	$C_{6795}H_{10570}N_{1734}O_{2042}S_{34}$
4	Total No. of Atoms	21,160	21, 175
5	Theoretical pI	5.22 	5.88 
6	Ext. coefficient	196825	187315
7	Estimated half-life	30 hours (mammalian reticulocytes, in vitro). >20 hours (yeast, in vivo). >10 hours (*E. coli*, in vivo)	1 hour (mammalian reticulocytes, in vitro). 2 min (yeast, in vivo). 2 min (*E. coli*, in vivo).
8	Aliphatic index	90.00	92.05
9	Grand average of hydropathicity (GRAVY)	-0.416 (moderately hydrophilic)	-0.364 (moderately hydrophilic)
10	Localization scores Cytoplasmic Cell Wall Extracellular Final Prediction	 0.00 0.02 9.98 Extracellular	 0.09 0.18 9.72 Extracellular
11	Instability index	32.33 (stable)	32.19 (stable)
12	N-terminal sequence	M (Met)	R (Arg)

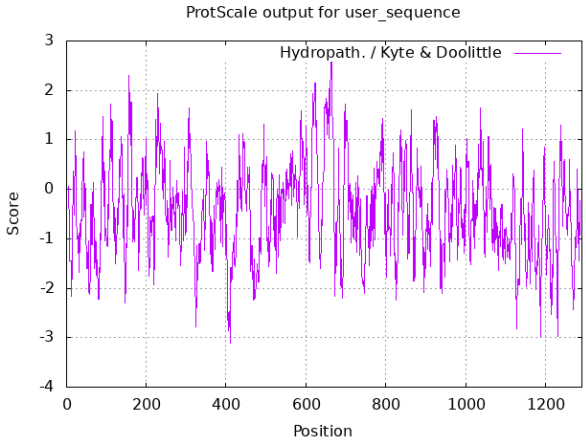


Figure 2: Neurotoxin Hydropathicity-*C. botulinum.* According to the Kyte and Doolittle Hydropathy plot analysis, which assigned negative values to apolar residues, the protein is moderately hydrophilic. (X-axis: location in the protein sequence; Y-axis: hydrophobicity scores)

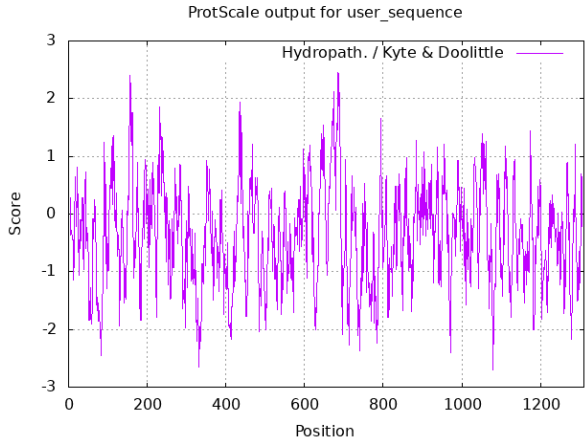


Figure 3: Neurotoxin Hydropathicity-*C. tetani.*

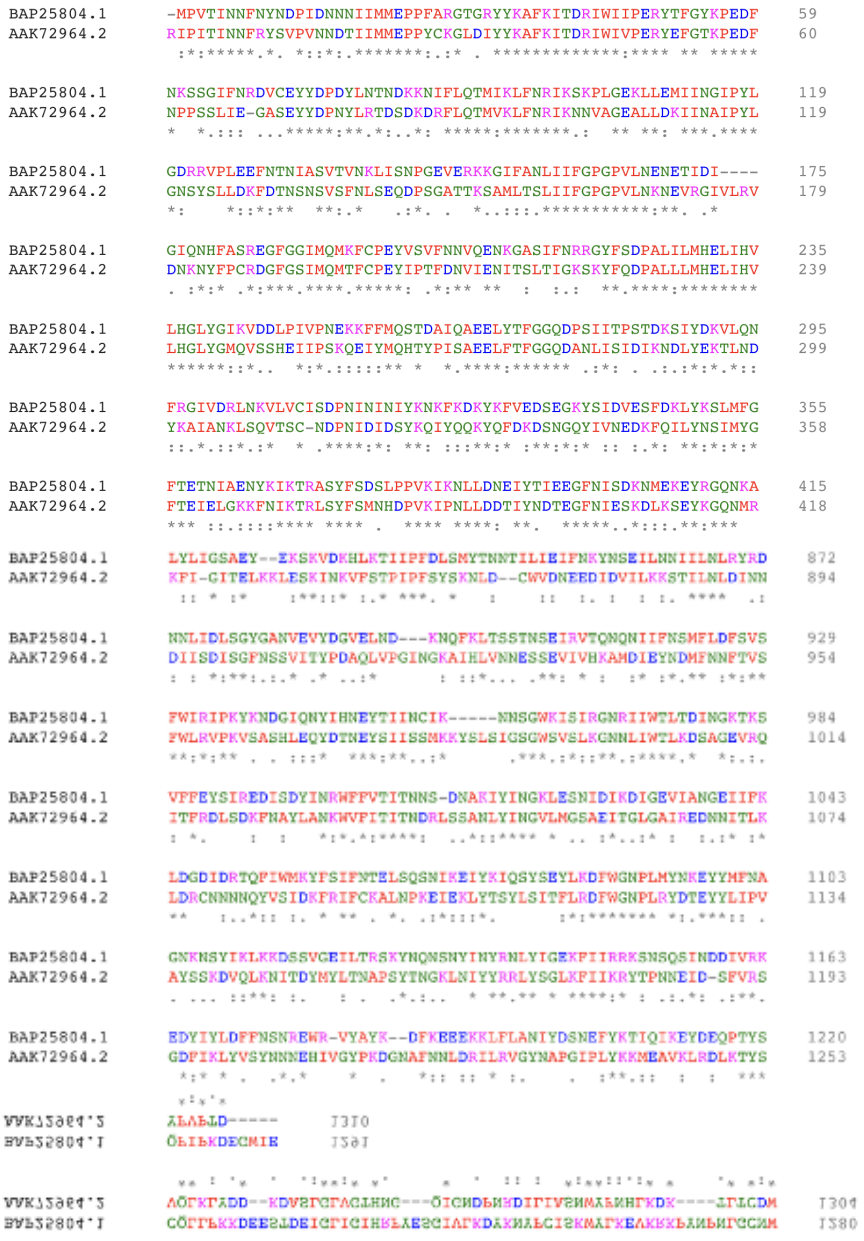


Figure 4: Aligned Sequences of *C. botulinum* neurotoxin (BAP25804) and *C. tetani* neurotoxin (AAK72964) using the Clustal Omega (2023).

Localization in Cytoplasm

The localization scores of the neurotoxins were determined as shown in Table 1 above; compared to TeNT, BoNT has no cytoplasmic and cell wall activity and has slightly more extracellular activity, which may aid in understanding their disease manifestation. The most popular program for predicting signal peptides from amino acid sequences at the moment is called SignalP. Figures 5 and 6 show no signal peptides in the neurotoxin sequences, indicating they are not destined for a secretory route. For the remainder of the translation, proteins lacking a signal peptide remain in the cytosol. These will remain in the cytosol if no other “address labels” exist. But, after translation, they can be delivered to the mitochondria, chloroplasts, peroxisomes, or nucleus if they carry the appropriate labels.

Gram stain is usually utilized to divide bacterial envelopes into one of two groups. Gram-positive bacteria lack the outer membrane and develop a thicker peptidoglycan cell wall. In contrast, a cytoplasmic membrane, a thin peptidoglycan layer, and an outer membrane are seen in Gram-negative bacteria (Depelteau et al., 2019). Takumi and Kawata’s (1969) study discussed that a bacteria’s cell wall has either a cytoplasmic membrane or a cytoplasmic component. Sodium dodecyl sulfonate treatment followed by proteolytic digestion was used to separate and purify the wall peptidoglycan of *Clostridium botulinum* type A. Using the electron micrographs, the cytoplasmic membrane, and other cytoplasmic components were not visibly seen. As seen in Table 1 these results align with BoNT and ToNT localization scores determined by molecular profiling of the ExPASY Protparam Tool.

According to Dong et al. (2019), the absence of cytoplasmic activity in *Clostridium botulinum* showing paralysis compared to *Clostridium tetani* can be explained by the fact that botulinum neurotoxin (BoNT) exclusively targets the release of acetylcholine. In contrast, the tetanus neurotoxin (TeNT) targets inhibiting neuronal activity. As an outcome, botulinum neurotoxin causes a blockage of neuromuscular transmission, resulting in flaccid paralysis with no substantial muscle activation. Tetanus neurotoxin, on the other hand, causes an excessive release of acetylcholine, resulting in spastic paralysis and an increase in muscular activity. To further explain this, botulinum neurotoxin works by cleaving particular proteins needed to expel acetylcholine, a neurotransmitter required for muscular contraction. The nerve cell internalizes botulinum neurotoxin once it attaches to certain receptors on its outermost layer. When it penetrates inside, it breaks down certain proteins needed to release acetylcholine, which stops neuromuscular transmission. The damaged muscles become limp and feeble due to this blockage, which causes paralysis. On the other hand, Tetanus neurotoxin works by cleaving certain proteins responsible for suppressing neuronal activity. Tetanus neurotoxin enters the cell by attaching to certain receptors on the outermost layer of the nerve cells. After entering, it breaks down particular proteins involved in controlling neuronal activity, which causes a high-level secretion of acetylcholine. As an outcome, the damaged muscles experience spastic paralysis, becoming stiff and rigid.

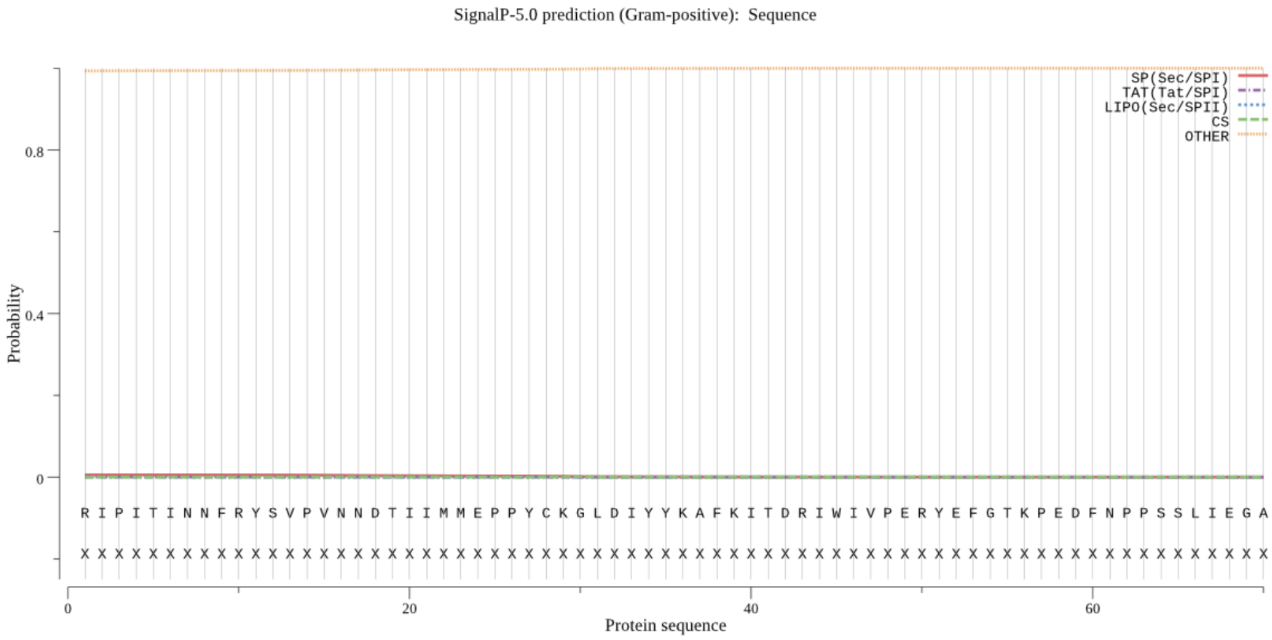


Figure 5: Signal peptide prediction of the *C. botulinum* neurotoxin. The sequence lacks any internal helices or motifs, or signal cleavage sites.

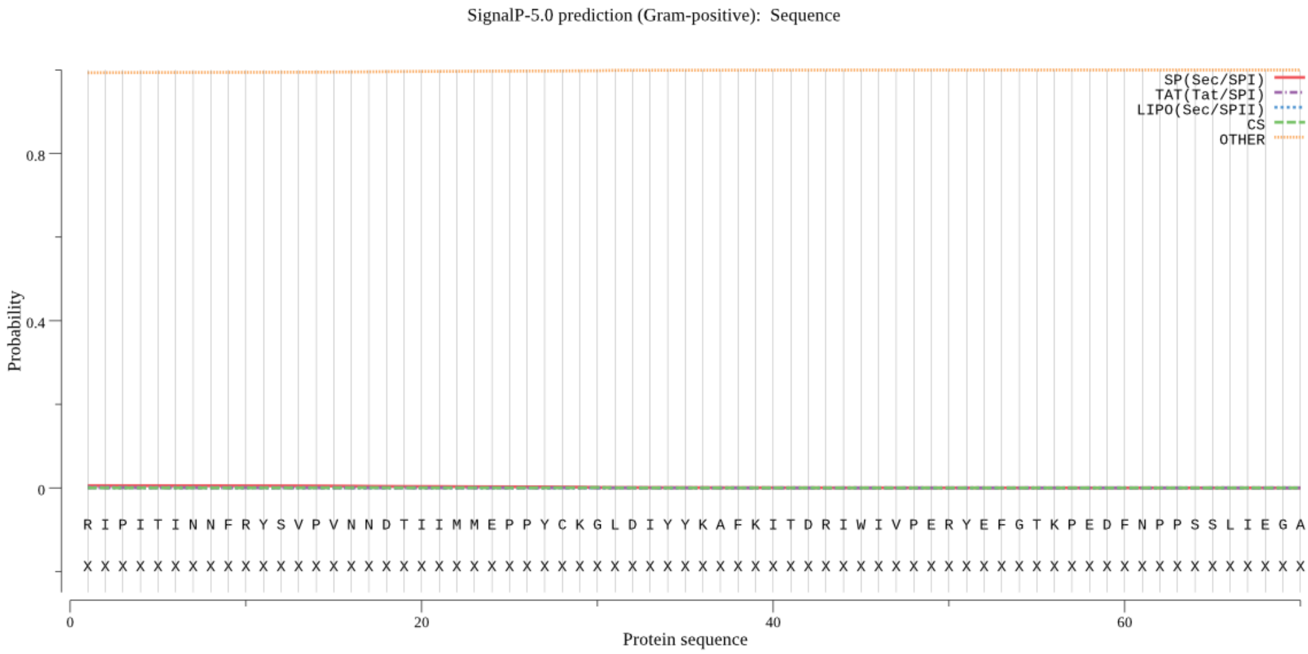


Figure 6: Signal peptide prediction of the *C. tetani* neurotoxin.

Aliphatic Index and Their Relationship to Paralysis

The aliphatic indices of BoNT and TeNT sequences are 90.00 and 92.05, respectively (Table 1). Notably, a high aliphatic index protein is known for its thermal stability across a range of temperatures, as observed with *Clostridium tetani*. Furthermore, the aliphatic index is a determinant of the thermal stability of proteins, while aliphatic amino acids are recognized for their hydrophobic properties.

A protein's aliphatic Index Is the proportional volume filled by aliphatic side chains (alanine, valine, isoleucine, glycine, and leucine). It could contribute to the elevation in globular proteins' 564hermos-stability (Enany, 2014). The present study analyzed putative and conserved protein sequences from *Clostridium botulinum* and *C. tetani*. Furthermore, the aliphatic index is a determinant of the thermal stability of proteins, while aliphatic amino acids are recognized for their hydrophobic properties. As pointed out in the study, although BoNT and TeNT possess comparable functional and structural characteristics, they exhibit distinguished clinical pathology, wherein the hindrance of acetylcholine discharge at the neuromuscular junctions caused by BoNTs leads to flaccid paralysis and the obstruction of glycine or GABA exocytosis in inhibiting interneurons due to TeNT results in spastic paralysis.

N- terminal Domain of BoNT and TeNT

Using the Chimera software, the N-terminal domain of BoNT and TeNT was presented through the PipesandPlanks depiction, which visualizes both neurotoxins' N-terminal and C-terminal sequences. Determining these domains may aid in differentiating where the protein sequence starts, as seen in Table 1 and Figures 7 and 8. The N-terminal of BoNT starts at methionine, commonly known as the "start codon," while the N-terminal of TeNT starts at arginine.

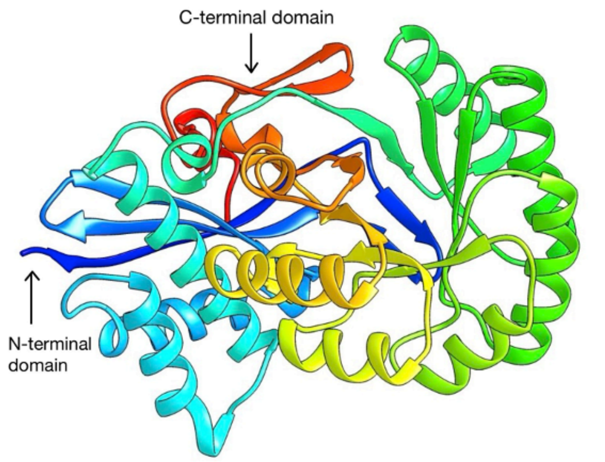


Figure 7: N-terminal domain and C-terminal domain of *C. botulinum* neurotoxin (BoNT). Protein chains are rainbow-colored and coded from blue at the N-terminus to red at the C-terminus.

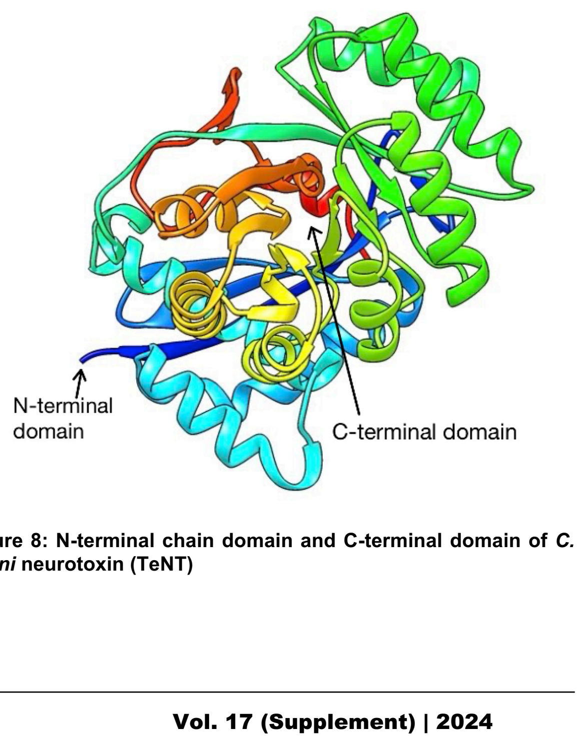


Figure 8: N-terminal chain domain and C-terminal domain of *C. tetani* neurotoxin (TeNT)

In the study of Binz et al. (1990), they were successful in identifying the entire sequence of botulinum neurotoxin A and a portion of the segment of botulinum neurotoxin E. (BoNT/E). Additionally, the researchers differentiated the sequences from those of tetani toxins and partly BoNT/E's amino acids, and they also delineated the start of the Botulinum neurotoxin A gene transcription site. The study reported that Botulinum neurotoxin A's (BoNT/A) actual codon to initiate translation is indeed the ATG codon, which codes for the amino acid methionine. In contrast, the Protparam tool indicated that the N-terminal chain of tetanus neurotoxin is arginine. According to Lee et al. (2022), the amino acid arginine is crucial for neuromuscular function because it regulates the synthesis of nitric oxide (NO) and serves as a substrate for protein arginine methylation. The neurological system that regulates blood flow, neurotransmitter release, and muscle contraction depends on NO as a key signaling molecule. Arginine availability or NOS activity changes may impact NO generation and neuromuscular functionality. Arginine methylation, mediated by protein arginine methyltransferases (PRMTs), is essential for both the development and function of muscles and the release of neurotransmitters.

Antigenicity of BoNT and TeNT

Through the use of Predicted Antigenic Peptides, the researchers found that *C. tetani* neurotoxin (TeNT) has a higher antigenicity as compared to the *C. botulinum* neurotoxin (BoNT) with 51 antigenic determinants and 36 antigenic determinants, respectively (Figures 9 and 10).

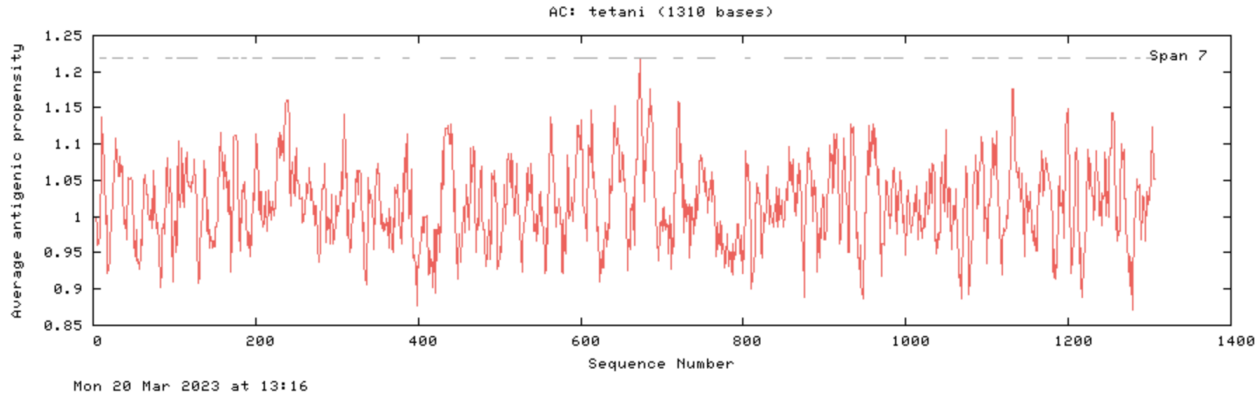


Figure 9: Antigenicity Profile of Tetani Toxin (TeNT). Gray lines represent the 51 antigenic determinants of C. tetani (TeNT).

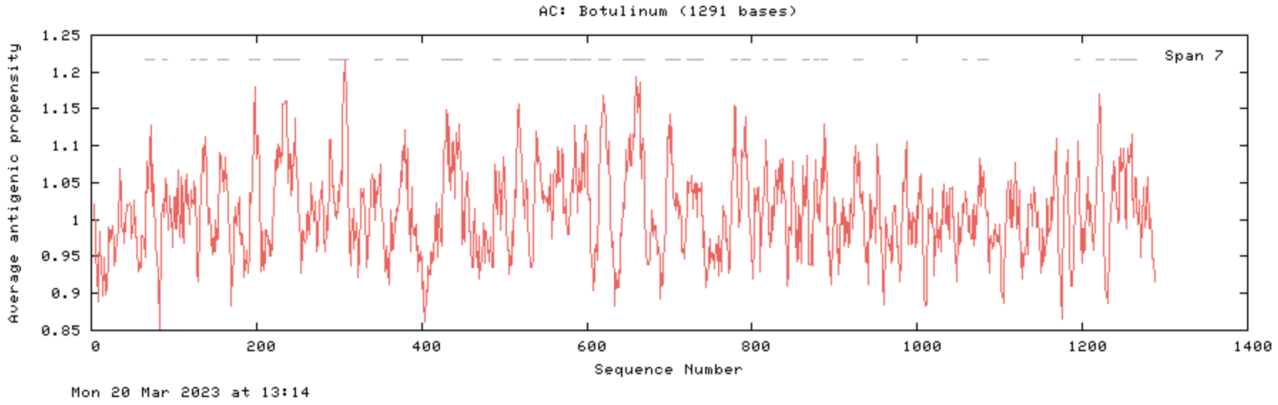


Figure 10: Antigenicity Profile of Botulinum Neurotoxin (BoNT). Gray lines represent the 36 antigenic determinants of C. tetani (TeNT).

The researchers utilized the Predicted Antigenic Peptide tool to compare the higher presence of antigenic sites of the TeNT than BoNT. BoNT has fewer antigenic determinants, consisting of 36 antigenic sites with an average antigenic propensity of 1.0106 (Figure 10; Table 2). Meanwhile, TeNT contains 51 antigenic determinants with 1.0186 average propensity (Figure 9; Table 3). Although TeNT has a lower molecular weight, higher antigenicity may be due to a higher number of amino acids and total atoms. One key distinction is that tetanus is caused solely by intoxication with TeNT. In contrast, botulism can be caused by ingestion of preformed BoNT in food (foodborne botulism) or production of BoNT in the intestine (infant botulism). This means the immune system is more likely to encounter and mount a response against TeNT. This distinct mechanism of action and localization of TeNT may expose more epitopes to the immune system, leading to a more robust antibody response.

According to Mitchison study conducted on mice in 1968, in any event, high protein antigen concentrations are anticipated to have the shortest critical exposure times, and doses in the low zone range are known to cause paralysis slowly. As seen in the predicted antigenic peptides done above, *Clostridium tetani* has a higher antigenicity than *Clostridium botulinum*, which induces flaccid paralysis, wherein the muscle is unable to contract, which causes floppiness and weakness of the muscle. In comparison to spastic paralysis manifested by *C. tetani,* this causes the muscles to tense up and harden. As a result, survivors may feel twitching, spasms, or stiffness of the muscles. Based on the study of Cohen et al. (2017), *Clostridium tetani* and *Clostridium botulinum* synthesize two of the most dangerous neurotoxins known as botulinum neurotoxins (BoNTs) and tetanus neurotoxins (TeNTs). Neurotoxins are the most dangerous toxins because they damage the nervous system. BoNTs are frequently used as medicinal poisons.

Table 2: Antigenic Determinants of Botulinum Neurotoxin.

n	Start Position	Sequence	End Position
1	66	FNRDVCEYYDP	76
2	86	NIFLQTM	92
3	10	GDRRVPL	126
4	132	NIASVTVNK	140
5	154	IFANLIIFGPGP	165
6	192	QMKFCPEYVSVFN	204
7	221	FSDPALILMHELIHVLHGLYGIKVDDLPIVPN	252
8	288	IYDKVLQNFRGIVDRLNKVLVCIS	311
9	344	SFDKLYKSLM	353
10	369	TRASYFSDSLPPVKIK	384
11	425	SKEHLAVYKIQMCKSVRAPGICID	448
12	487	SIDELILDT	495
13	512	TDFNVDVPVYEKQPAIKK	529
14	537	IFQYLYSQTFPLDIRDISLTS	557
15	559	FDDALLFSNKVYSFFSM	575
16	580	TANKVVEAGLFAGWVKQIVDDFVIE	604
17	614	IADISLIVPYIGLAL	628
18	644	EIAGASILLEFIPELLIPVVGAFLLES	670
19	695	DMYGLIVAQWLSTVNTQFY	713
20	721	KALNYQAQALEEIIKYKYNI	740
21	775	INECSVSY	782
22	787	MIPLAVEKLLDF	798
23	813	NKLYLIG	819
24	827	KVDKHLKTIIPFDL	840
25	861	LNNIILNL	868
26	875	LIDLSGY	881
27	883	ANVEVYDG	890
28	923	FLDFSVSFWIRI	934
29	982	TKSVFFE	988
30	1054	IWMKYFS	1060
31	1073	EIYKIQSYSEYLK	1085
32	1191	KKLFLAN	1197
33	1217	PTYSCQLLF	1225

34	1234	EIGLIGIHR	1242
35	1244	YESGIVLKD	1252
36	1254	KNYFCISKWYLKE	1266

Table 3: Antigenic Determinants of Tetanus Neurotoxin.

n	Start Position	Sequence	End Position
1	9	FRYSVPV	15
2	24	EPPYCKGLDIYYKA	37
3	43	RIWIVPE	49
4	62	PPSSLIE	68
5	89	LQTMVKLF	96
6	104	AGEALLDKIINAIPYLGNSYSLLD	127
7	153	AMLTSLIIFGPGPVLN	168
8	172	VRGIVLR	178
9	183	NYFPCRD	189
10	196	QMTFCPEYIPTFD	208
11	221	KSKYFQDPALLLMHELIHVLHGLYGMQVSSHEIIPSK	257
12	260	IYMQHTYPISAEE	272
13	299	DYKAIANKLSQVTS	312
14	320	IDSYKQIYQQKY	331
15	347	KFQILYN	353
16	382	DPVKIPN	388
17	428	DGSGLVSKLIGLCKKIIP	445
18	464	GGELCIK	470
19	508	YSLDKIILDYNLQSKI	523
20	533	VTKGIPYAP	541
21	560	TIYQYLYAQ	568
22	592	KIYSYFPSVISKVNQ	606
23	608	AQGILFLQWVRD	619
24	636	KISDVSTIVPYIGPALNIVKQ	656
25	667	ETTGVVLLLEYIPEITLPVIAALSIAE	693
26	715	KWIEVYKLVKA	725
27	744	RSLEYQVDAIKKIIDYEYKIYS	765
28	802	SRSFLVN	808
29	851	KINKVFSTPIPFSYSKNLDCWV	872

30	877	DIDVILKK	884
31	903	FNSSVITYPDAQLVPG	918
32	921	GKAIHLVN	928
33	931	SSEVIVHK	938
34	950	NFTVSFWLRVPKVSASHLE	968
35	974	EYSIISS	980
36	982	KKYSLSI	988
37	990	SGWSVSL	996
38	1024	FNAYLANKWVFI	1035
39	1043	SSANLYING	1051
40	1083	QYVSIDKFRIFCKA	1096
41	1101	EIEKLYTSYLSITF	1114
42	1127	TEYYLIPVAYSSKDVQL	1143
43	1163	LNIYYRRLYSGLKFIIK	1179
44	1187	IDSFVRS	1193
45	1195	DFIKLYVS	1202
46	1223	DRILRVGY	1230
47	1232	APGIPLYKK	1240
48	1242	EAVKLRDLKTYSVQLKL	1258
49	1264	ASLGLVG	1270
50	1282	RDILIAS	1288
51	1296	KDKTLTCDWYF	1306

STRUCTURAL ANALYSIS OF BOTULINUM AND TETANUS NEUROTOXINS

This section provides the structural analysis of botulinum and tetanus neurotoxins, a crucial area of research that provides valuable insights into the mechanisms of action and potential therapeutic applications of these potent neurotoxins. These toxins, produced by *Clostridium* bacteria, cause the neuroparalytic syndromes of botulism and tetanus, respectively.

Analysis of the Structure of BoNT and TeNT

The secondary structure was determined through the SOPMA software; the researchers derived that *C. tetani* neurotoxin has a higher amount of alpha helix (32.14%) and random coiling (39.54%) compared to *C. botulinum* neurotoxin with 31.53% alpha helix and 38.34% random coiling. The differences in secondary structure elements, specifically alpha helix and random coiling, between *Clostridium botulinum* and *Clostridium tetani* neurotoxins could contribute to the distinct biological effects observed in these toxins. Alpha helices are known for their structural stability and can be crucial in protein-protein interactions; it impacts their ability to interact with specific cellular components or receptors, which plays a crucial role in protein-protein interaction. Both neurotoxins are primarily random coils, as analyzed in the findings. Random coiling region in *C. botulinum* neurotoxins might contribute to their flexibility and adaptability in binding to different targets or environments, which play a role in conformational changes during interaction with host cells. Meanwhile, variations in random coiling patterns in *C. tetani* neurotoxins may affect their ability to penetrate cell membranes, interact with intracellular components, or trigger specific cellular responses, leading to unique biological outcomes. Overall, differences in the distribution and composition of secondary structure elements like alpha helix and random coiling in *C. botulinum* and *C. tetani* neurotoxins could influence their structural stability, binding properties, and interactions with host cells, ultimately contributing to the distinct biological effects observed in these potent neurotoxins.

Although neurotoxins are known to cause different types of paralysis, the lack of molecular-level studies made the researchers seek for the neurotoxin differences. The features of the target-template alignment and the template structure are combined to provide the GMQE (Global Model Quality Estimate). The GMQE is accessible before creating a real model and helps choose the best templates for the current modeling issue. It is considered a valuable tool for assessing the expected accuracy of protein structure models, guiding template selection, and interpreting the reliability of predicted structures in homology modeling and structure prediction pipelines. It is a

key metric used in protein structure prediction to estimate the expected accuracy of a predicted model. It ranges from 0 to 1, with higher scores indicating higher expected quality and reliability of the predicted structure. GMQE scores above 0.5 generally indicate higher quality models, with scores above 0.8 considered very reliable. The TeNT sequence has a GMQE score of 0.85 with 99.85% sequence identity. The BoNT has a GMQE score of 0.86 with 95.66% sequence identity. Neurotoxins with higher sequence identity helped the researchers obtain parameters that helped analyze the differences. Using the i-TASSER, the 3D models of *C. tetani* and *C. botulinum* neurotoxins were predicted (Figs. 11 and 12).

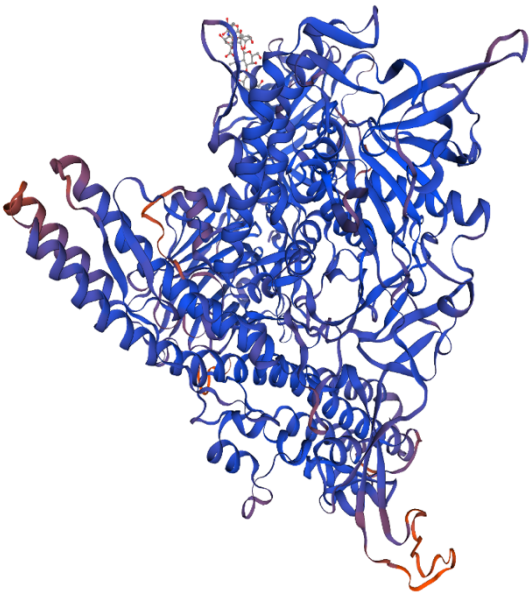


Figure 11: Structure of Tetanus Neurotoxin (TeNT)

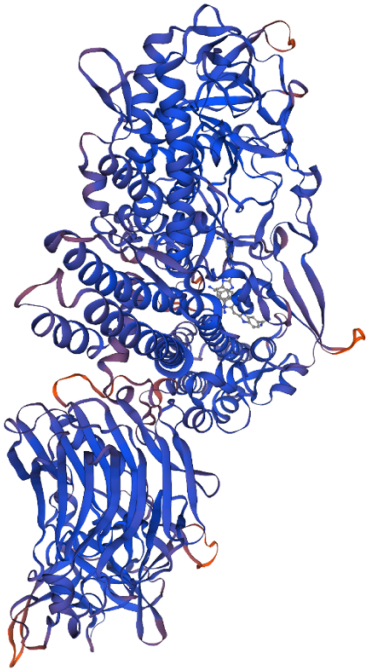


Figure 12: Structure of Botulinum Neurotoxin (BoNT)

FUNCTIONAL ANALYSIS OF BOTULINUM AND TETANUS NEUROTOXINS

Functional studies have revealed that both botulinum and tetanus neurotoxins share a similar mechanism of action despite their distinct clinical symptoms. The use of the DeepGOWeb server, presented the potential drug targets of both botulism and tetanus neurotoxin.

Protein Functions of Botulinum Toxin and Tetanus Toxin

The DeepGOWeb server was used to predict the function based on the sequence. Although both are involved in cellular, molecular, and biological activities, *C. tetani* neurotoxin was found to have a catalytic activity and regulation of biological processes (Tables 5 and 6). Spasticity benefits upper motor neuron syndrome (Reilly et al., 2022). Tetanus neurotoxin induces spastic paralysis by blocking neurotransmitter release from spinal inhibitory interneurons. This action leads to uncontrolled muscle contractions and rigidity, which are characteristic of tetanus. The inhibition of neurotransmitter release by tetanus neurotoxin results in the hyperexcitability regulated by the catalytic activity of motor neurons, leading to sustained muscle contractions regulated by biological processes and spastic paralysis. This mechanism is central to the pathophysiology of tetanus, where sustained muscle spasms and rigidity are characteristic features. As a result, a signal imbalance between the central nervous system (CNS) and muscles manifests as intermittent or recurrent involuntary muscular activation. Compared to *C. botulinum* neurotoxin, *C. tetani* neurotoxin regulates biological processes as its role can be associated with spasms in pulses.

Table 4: Cellular Components of *C. botulinum* and *C. tetanus* Neurotoxins.

Cellular Component	BoNT (BAP25804)	TeNT (AAK72964)
Cellular anatomical entity	0.485	0.586
Intracellular anatomical structure	0.427	0.523
Cytoplasm	0.378	0.463
Organelle	0.374	0.463
Intracellular organelle	0.374	0.461
Membrane	0.333	0.441
Membrane-bound organelle	0.316	0.425
Intracellular membrane-bounded	0.316	0.425

Table 5: Molecular Functions of *C. botulinum* and *C. tetanus* Neurotoxins.

Molecular Function	BoNT	TeNT
Binding	0.488	0.536
Transporter activity	0.474	0.469
Transmembrane transporter activity	0.474	0.467
Protein transporter activity	0.461	0.458
Ion binding	0.386	0.411
Cation binding	0.375	0.401
Metal ion binding	0.374	0.400
Zinc ion binding	0.369	0.395
Transition metal ion binding	0.369	0.395
Catalytic activity	0	0.326

Table 6: Biological Processes of *C. botulinum* and *C. tetanus* Neurotoxins.

Biological Process	BoNT	TeNT
Cellular process	0.851	0.849
Localization	0.589	0.592
Establishment of localization	0.576	0.576
Transport	0.574	0.573
Cellular localization	0.526	0.533
Macromolecule localization	0.497	0.502
Organic substance transport	0.500	0.494
Protein localization	0.488	0.493
Cellular macromolecule localization	0.491	0.493
Nitrogen compound transport	0.490	0.486
Transmembrane transport	0.480	0.473
Establishment of protein localization	0.472	0.472
Protein transport	0.471	0.471
Protein transmembrane transport	0.461	0.458
Biological regulation	0	0.315
Metabolic process	0.305	0.315
Organic substance metabolic process	0.305	0.315
Regulation of biological process	0	0.312

Drug Discovery Recommendation, Change of Function, and Alteration in Molecular Structure

Tuning of Hydrophobic/Hydrophilic Balance

As seen in Table 1, both *C. botulinum* neurotoxin and *C. tetani* neurotoxin have a moderate hydropathicity. Some of its protein structure (Figure 2 and Figure 3) lies >1, indicating its hydrophobicity. The hydrophobicity of *Clostridium botulinum* and *Clostridium tetani* plays a massive role in determining the effects of antimicrobial peptides. In the study of Zhang et al. (2022), they demonstrated that the critical structural factor dictating the efficiency of bacterial death by helical antimicrobial peptides (AMP) is hydrophobicity, not other properties (Figures 13 and 14). The rate of action of AMPs, such as bacterial binding, lipopolysaccharide neutralization, and outer and inner membrane permeabilization, increased with hydrophobicity. In addition to that, the number of carbon atoms has a direct relationship with hydrophobic interaction. The most robust hydrophobic interactions will be found in molecules with the most carbons (Table 7). The intensity of hydrophobic interactions also rises with temperature (LibreText Chemistry, 2023). Recently, antimicrobial polymers (AMP) have been looked up as possible treatments for bacterial infections, having hydrophobic groups that penetrate the bilipid membrane and disrupt it together with cationic groups that enable the polymer to adsorb onto negatively charged bacterial membranes. *C. botulinum* neurotoxins have 181 negatively charged residues from asparagine and glutamine, while *C. tetani* neurotoxins have 151 negatively charged residues. Pham et al. (2021) assessed the antibacterial activity of AMP by adjusting the hydrophobic/hydrophilic balance and overall hydrophobicity, in which they found that hydrophobicity affects biological activity by changing aqueous properties. Suitable adjustments and determining structural characteristics of a hydrophilic group, such as its length, flexibility, and hydrophilicity in which *Clostridium botulinum* and *Clostridium tetani* also fall, may aid in designing antimicrobial peptides.

Table 7: Atomic Composition of the *C. botulinum* Neurotoxin (BoNT) and *C. tetani* Neurotoxin (TeNT).

Atomic Component	*Clostridium botulinum* neurotoxin (BoNT)	*Clostridium tetani* neurotoxin (TeNT)
Carbon	**6847**	**6795**
Hydrogen	10524	10570
Oxygen	2038	2042
Nitrogen	1718	1734
Sulfur	33	34

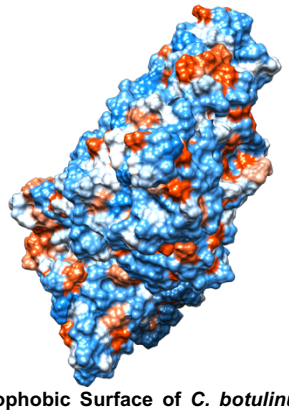


Figure 13: Hydrophobic Surface of *C. botulinum.* Red surfaces represent hydrophobic structures, and the blue surface represents hydrophilic structures.

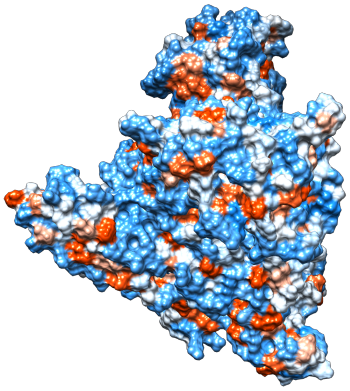


Figure 14: Hydrophobicity Surface of *C. tetani.*

Carbon Dots

Hydrophobicity and heat resistance are due to the carbon atoms present in a structure. A recent discovery on carbon dots (C-dots) as a drug delivery system has been known to improve the solubility and half-life of drugs while reducing the side effects. C-dots have remarkable biocompatibility, size-dependent photoluminescence, excellent photostability, and size tunability in the nanometer range. These carbon nano allotropes have a nanocrystalline graphitic core with excellent sp2 character, virtually isotropic nanoparticles with diameters under 10 nm, and a high oxygen concentration at their surface (Calabrese, 2021). In addition, C-dots can cross the blood-brain barrier, which can target the neurotoxins that cause the paralysis; the blood-brain barrier is vital in keeping toxins and other substances from quickly reaching the brain; creating a mechanism that can cross it to deliver therapeutic and diagnostic substances to the brain is a significant problem. Due to their essential qualities, such as their ultra-small size, low toxicity, high drug-loading capacity, long-term stability, and controlled drug-release capabilities, C-dots have recently gained a lot of attention as a promising technique for drug delivery. To create C-dots (C-dot/Gly) from glycine (Gly), the only starting material, Ruan et al. (2022) assessed the cytotoxicity and cellular absorption in vitro as well as their tissue distribution and imaging properties in vivo. C-dot/Gly has little cytotoxicity, according to in vitro studies on C6 glioma cells, and their uptake is time- and concentration-dependent. Additionally, the in vivo results showed a significant buildup of C-dots in the glioma tissue, which fluoresced more intensely than normal brain tissue, showing that these C-dots can target glioblastoma selectively.

Chemical Modification of Protein

Chemical modification of protein is an approach used to elucidate the biochemical function of proteins. This approach has been incorporated in several metalloenzyme studies that aim to dissect their mechanism and improve enzymatic activity. In addition, more cases use the modification approach, such as enzyme mechanistic studies and drug discovery (Naowarojna et al., 2021).

In pharmaceutical settings, modification of protein therapeutics, including lysine modification, can enhance the stability and circulation half-life to acquire exquisite control of macromolecules (Naowarojna et al., 2021). In the study, it is emphasized that lysine has a high natural abundance. With its nucleophilic primary amine, there can be several biocompatible reactions, making it an attractive choice for chemical modification. Lysine modification is often used in studies where site selection is not prioritized and used when multiple modification sites are needed. Lysine modification with therapeutic antibodies such as trastuzumab can improve the site-selectivity without disturbing the protein of interest's structure and function because it favors the lysine residue with the lowest pK_a. However, in some cases, the lysine with the lowest pK_a is inaccessible. This modification approach can result in homogenous anti-drug antibodies but can be used for further therapeutic development.

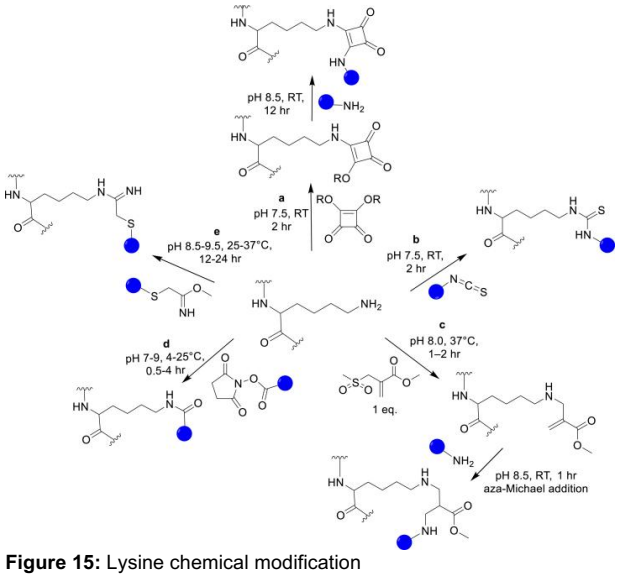


Figure 15: Lysine chemical modification

CONCLUSION AND RECOMMENDATION

In-silico methods and protein sequences have become valuable tools for determining an organism's biological and chemical composition and exhibiting unique characteristics in its molecular structures.

The researchers determined the direct relationship of amino acid numbers with the total amount of atoms, the clinical significance of the aliphatic index that determines their thermostability in terms of the hydrophobic structures despite being hydrophilic, that contributes to the delivery of neurotoxins and inhibiting acetylcholine differently in BoNT covering the lesser amount of acetylcholine in contrast to TeNT which significantly suppresses acetylcholine that creates a burst of reaction associated with its spastic characteristic. The localization in the cytoplasm showed a significant difference between BoNT and TeNT sequences,

where the latter is the only one with catalytic activity and regulated biological processes in the cytoplasm, as determined by its protein function. In line with that, the N-terminal domain of TeNT arginine, in contrast with BoNT's methionine, known as the "start" codon N-terminal domain, also displays regulatory roles in neurotransmitter release. In addition, one of the notable distinctions between BoNT and TeNT is the number of their antigenicity where TeNT has more excellent antigenic peptides, which are found to have shorter critical exposure time compared to BoNT with lower antigenic peptides, explaining its flaccid or slow paralysis.

In the emergence of antimicrobial resistance of these Gram-positive organisms and absence of treatment for both the flaccid and spastic paralysis,this study determined the structure of the neurotoxins, hydrophobic and hydrophilic tuning, and the use of carbon-reducing substances that can cross the blood-brain barrier of small size (nanoparticles); these may aid in drug discovery in future studies, treatment interaction with neurotoxins, and drug delivery in the neurons and alteration of the chemical structure of neurotoxins which may reduce critical symptoms.

The clinical applications derived from the findings on botulinum and tetanus neurotoxins can have significant implications for various medical fields. As presented in the study, utilizing the unique properties of botulinum and tetanus neurotoxins through targeted drug delivery could enhance the efficacy and specificity of treatments for various medical conditions. With this study, an improved understanding of these neurotoxins' molecular structures and mechanisms can aid in developing diagnostic tools for rapid detection and surveillance of botulism and tetanus cases, as the potential to revolutionize medical treatments, enhance patient care, and contribute to advancements in various healthcare fields.

Based on the gaps and limitations identified in the current research on botulinum and tetanus neurotoxins, we recommend future researchers to investigate the specific roles of these neurotoxins in the natural life cycle and environment of *Clostridium* bacteria, search for additional undiscovered botulinum toxin serotypes in the environment, explore the potential therapeutic applications of botulinum neurotoxins beyond their current uses, such as targeting pain processing. Future research should focus on understanding the fundamental biology of these neurotoxins, identifying new variants, elucidating their structures and mechanisms, and exploring their therapeutic potential while ensuring safety. Filling the gaps in our current knowledge could lead to significant breakthroughs in both basic science and clinical applications.

ACKNOWLEDGMENT

This work would not have been possible without our God Almighty, offering endeavors for the wisdom he bestowed upon us, the researchers, to give us strength, patience, and motivation, especially to work under pressure to finish this research. As researchers, we wish to express our sincere appreciation to our Dean, Dr. John Anthony Yason, for his invaluable guidance throughout this research endeavor. Additionally, we are grateful to Far Eastern University-Manila for providing us with this opportunity. Lastly, we extend our thanks to all our friends, particularly the dedicated group whose contributions were integral to the success of this study. "It is not possible to prepare a project or a study without the assistance and encouragement of other people. This one is certainly no exception."

CONFLICT OF INTEREST

The authors declare no conflict of interest, and the information included in this disclosure is accurate and complete.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

MRFL conceptualized, designed, and organized the study. MRFL, MCATP, ZJQ, CSJM, KBL, JJCO, ACMM, ALTP, and LJJP sought the related literature. MRFL and MCATP wrote the manuscript draft, performed the methodology starting from physicochemical profiling alignment up to structural modeling using software. MCATP prepared the tables and figures. MRFL did the final check for the manuscript's cohesiveness and write-up. All authors reviewed and agreed with the final manuscript.

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