ISSN: 2583-8288

Technical Article

Published on: 31.12.2024

Botulinum Neurotoxins and Advancements in Its Diagnosis

Dr. Abhisek Mishra [1*] : M.V.Sc Scholar, Division of Veterinary Medicine
Dr. Ambika Nayak [1]: PhD Scholar, Division of Veterinary Microbiology
Dr. Surya Pratap dhakare[1] : M.V.Sc Scholar, Division of Poultry Science
ICAR- Indian Veterinary Research Institute, Izatnagar, Bareilly, 243122

DOI:10.5281/Vettoday.14578306

Abstract: Botulism is a disease caused by botulinum neurotoxins (BoNTs) that lead to flaccid descending paralysis by disrupting nerve cell communication with muscle cells. Antitoxins can slow disease progression, but complications can be fatal if treatment is delayed. Various Clostridium species produce highly lethal BoNTs that target different substrates, affecting humans and animals differently. The protein structure of BoNTs involves a heavy chain aiding in receptor binding and a light chain that cleaves SNARE proteins. In animals, botulism symptoms include paralysis, vision disturbances, and difficulty swallowing, leading to death from respiratory or cardiac paralysis. Diagnostic methods include mouse bioassays, cell-based assays, endopeptidase mass spectrometry assays, immunoassays, nucleic acid testing, and comparative genomic methods. Biosensors and electrochemical methods have also been developed for detection. Prompt diagnosis is crucial for treatment with antitoxins, and specialized reagents are needed for accurate testing, taking into consideration testing turnaround time for effective public health response.

Keywords: Botulinum neurotoxins (BoNTs), SNARE proteins, MALDI-TOF/MS, FRET, Biosensors

Introduction

Botulism is a rare disease in which botulinum neurotoxins (BoNT) enter nerve cells and prevent communication with muscle cells. This signal disruption at the neuromuscular junction causes flaccid descending paralysis. Although antitoxins are available for treatment, they can only slow the progression of the disease and cannot reverse it. This illness can be fatal if antitoxin is not administered promptly and supportive care is delayed. While most people who develop botulism recover, complications can lead to death. BoNTs are produced by Gram-positive spore-forming anaerobic *Clostridium species* such as *C. botulinum, C. baratii, C. butyricum, and C. argentinense*. These metalloproteases are some of the most lethal substances known. BoNTs exhibit significant immunological and genetic diversity. BoNTs are classified into nine toxinotypes (A, B, C, D, E, F, G, H, or F/A, X) based on their neutralization by specific antisera and actions on various substrates. In addition, each toxinotype is divided into subtypes based on amino acid differences. Currently, 41 subtypes have been identified. Types A, B, E, and F are primarily responsible for human botulism, whereas toxinotypes C and D are associated with animal botulism, which primarily affects birds and cattle. The different BoNTs toxinotypes and subtypes interact with distinct membrane receptors and cleave different intracellular SNARE proteins (soluble N-ethylmaleimide-sensitive factor attachment receptor), VAMP (vesicle associated membrane protein)/synaptobrevin, synaptosomal-associated protein 25 (SNAP-25), and syntaxin at different cleavage sites.

Structure And Mechanism Of Action

The BoNT protein structure is made up of a 100-kDa heavy chain (HC), which aids in nerve cell receptor binding and translocation of the 50-kDa light chain. The LC is then released into the cytosol, where it cleaves the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex proteins required for signal transduction in very specific locations. The cleaved SNAP-25 cannot mediate vesicle fusion with the host cell membrane, preventing the release of the neurotransmitter acetylcholine from axon terminals. This blockage is slowly reversed as the toxin loses activity and the affected cell gradually regenerates the SNARE proteins. The HC and LC are linked by a disulfide bond and surrounded by complexes, allowing BoNT to survive in the environment.

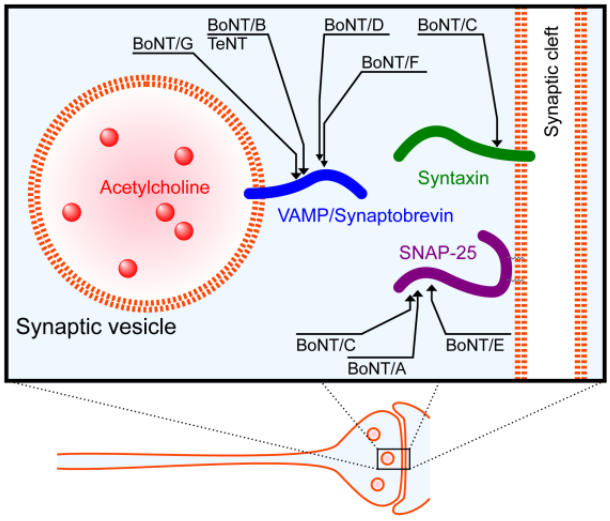

Figure: BoNT molecular targets (source-https://tmedweb.tulane.edu/pharmwiki/doku.php/botox)

Clinical Picture in Animals

Botulism symptoms include progressive motor paralysis, disturbed vision, difficulty chewing and swallowing, and generalized progressive paresis. Death is usually caused by respiratory or cardiac paralysis. The toxin inhibits acetylcholine release at motor endplates (neuromuscular junctions). The passage of impulses down motor nerves and muscle contraction is not hampered. There are no distinctive gross or histologic lesions, and pathologic changes can be attributed to the toxin's general paralytic action, particularly in the respiratory muscles, rather than the toxin's specific effect on any particular organ. Epidemics have occurred in dairy herds, with up to 65% of adult cows developing clinical botulism and dying within 6-72 hours of recumbency. Drooling, decreased tongue tone, dysphagia, inability to urinate, and sternal recumbency that progressed to lateral recumbency just before death were some of the most significant clinical findings. Skin sensation is usually normal, and the limbs' withdrawal reflexes are weak. Clinical signs initially resemble second-stage parturient paresis, but calcium parenteral administration does not improve the cows' condition. The clinical signs reported in horses are very similar, with progressive muscle paresis, recumbency, dysphagia, decreased muscle tone (jaw, tail, and tongue), respiratory distress, and death.

Diagnostic Methods

Due to the specific testing techniques that are currently only available in public health laboratories, botulism testing has historically been carried out there. The majority of BoNT diagnostic testing can be carried out in a biosafety level 2 laboratory by using class II biological safety cabinets, wearing the proper personal protective equipment (PPE), and adhering to the biosafety procedures specified by an institution. A risk assessment must be completed for any testing that uses large amounts of purified toxin or has the potential to produce aerosols or droplets. Additionally, the assays may need to be carried out in a biosafety level 3 laboratory with improved personal protective equipment.

- **The mouse bioassay (MBA):** It has long been the gold standard for botulism laboratory confirmation. Many people favor this approach because it can verify that BoNTs can attach to nerve cells, enter them, and produce symptoms through in vivo proteolytic activity. Despite its sensitivity, this assay can be expensive, labor-intensive, and require specialized staff. It can also take up to four days to confirm test results. Furthermore, when high-throughput testing is needed, this assay is not the best option. Because larger mice may have a higher tolerance for BoNT before developing symptoms, the assay's limited diagnostic age and size restrictions on mice can be problematic. Despite being a reliable test, the MBA can produce confusing results if pathogenic organisms or other interfering substances are present.

Thankfully, BoNT neutralization with a particular mono- or polyvalent antitoxin can rule out false-positive results.

- **Cell based assay**: Since they can demonstrate BoNT intoxication, cell-based assays have been proposed as an alternative to the MBA. These assays are currently the only way to illustrate this process outside of the MBA. In short, samples containing BoNT are added to neuronal cells that have been cultured, and various techniques can be used to measure the evidence of BoNT activity through internalization, receptor binding, and proteolysis. Performance may be impacted by the chosen cell lines, growth conditions, or other elements that can impact cell health, even though these assays may be more sensitive than the MBA. Furthermore, the completion of these tests could take up to three days.
- **Endopeptidase Mass Spectrometry Assays**: For the laboratory confirmation of botulism in clinical and related environmental samples, the endopeptidase-based assay is the main candidate to take the place of the MBA. These assays, in comparison, can be less costly to conduct, yield results in a matter of hours, and—above all—do not necessitate the use of live animals in testing. These assays can detect active BoNT at levels at or below the MBA LOD due to their high specificity and analytical sensitivity. Assays based on endopeptidase that were created by the CDC using high-resolution mass spectrometers have been successfully adapted for use in labs that use mass spectrometers that are frequently used to identify microbiological isolates. An extensive evaluation has been conducted on the detection of BoNT using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF/MS). This technique can be used to confirm active BoNT for serotypes A through G at levels close to or below the MBA LOD in a variety of matrices and specimen types. Each BoNT serotype's antibody-coated beads are added to a sample and then incubated in this Endopep-MS assay. Before any nonspecific proteases are removed from the sample, BoNT will attach to the serotype-specific beads if it is present. The corresponding antibody-coated beads are incubated with peptide substrates that mimic the SNARE complex targets for each BoNT serotype. If toxin is present, these peptides will be cleaved in precise locations, resulting in fragments of a defined mass, because each BoNT serotype has highly specific enzymatic activity. To ascertain whether these cleavage products are present in the sample, the fragmented reaction solutions are spotted onto a MALDI target plate and examined using the MALDI-TOF/MS device. In clinical and environmental samples directly associated with human disease, active BoNT has been successfully detected using the Endopep-MS assay. Analytical sensitivity specifications can be significantly improved by modifying the peptide substrate, according to studies, yielding positive results that are roughly one to two log values below the MBA LOD. Alternatively, research has demonstrated that peptide substrate modifications can also hinder the detection of specific BoNT subtypes, highlighting the significance of carefully assessing assay component modifications. What's interesting is that the Endopep-MS assay has been used to identify botulism outbreaks in livestock using veterinary specimens.
- **Immunoassays:** The detection of BoNT in a variety of clinical and food matrices has been investigated using immunological assays. Many of these assays, like lateral flow assays and enzyme-linked immunosorbent assays (ELISA), are easy to use and can yield results quickly for a variety of samples. Immunoassays are not commonly used for confirmatory testing, despite the fact that they can be effective screening tools for identifying samples that contain BoNT. ELISA and lateral flow assays were previously unable to detect toxin endopeptidase activity; however, a combination of technologies and improved reagents has made this possible.

  While immuno-PCR (iPCR) has been successfully used to detect certain BoNT serotypes with higher specificity and sensitivity than the MBA, its performance in complex matrices and on-site detection capabilities have not been fully assessed. When bound to the BoNT target, nucleic acid reporters are amplified using detector

antibodies in iPCR to generate a signal. In comparison to the performance of traditional ELISA, this signal amplification can result in a sensitivity increase of up to 10^5 times. Even though BoNTs can be detected at very low levels using immunoassay platforms, the presence of inactivated toxin or interfering matrix components could cause false-positive results. The issue of matrix interference remains regardless of the immunological detection technique employed, and the assay's suitability and intended use should be confirmed.

- **Nucleic acid testing:** Because of the genetic diversity among species that produce BoNT, molecular assays are helpful for detecting and differentiating organisms that produce the toxin or screening clinical and related environmental samples for the presence of genes linked to the production of the toxin. PCR-based assays can be very helpful in rapidly and highly sensitively screening primary clinical and environmental samples for the presence of BoNT and related genes, even though these assays do not reveal information on the viability of organisms or their capacity to produce BoNTs. For the qualitative or quantitative identification of BoNT producers or toxin genes, real-time PCR (rtPCR) has been developed. Using automated extraction platforms, these assays can yield vital information in as little as four hours and work with a range of clinical and food matrices. In foodborne outbreaks or bioterrorism incidents, rtPCR would be a helpful screening tool due to its short turnaround time and potential for quick serotype determination. The results could potentially distinguish between different sources of contamination.
- **Comparative genomic methods:** Once botulism has been confirmed, these cases must be investigated to determine the source of contamination and prevent further illness. Several assays were used to compare the genomes of BoNT producers. While some gel-based techniques are still used, sequencing methods are becoming more popular for investigating outbreaks. Pulsed field gel electrophoresis (PFGE) analysis can provide additional information on the relationship between C. botulinum clinical and environmental or food isolates, thereby strengthening epidemiological investigations. Nucleic acid digested by specific restriction enzymes is separated on a gel, and the fingerprints obtained can be compared. National databases can be searched to identify potentially related cases. Traditional multilocus sequence typing (MLST) analysis has also been used as a supplement to initial testing. Sequence data from fragments of seven genes from each isolate are assigned as distinct alleles, and a sequence type is generated. This information can be compared to large databases to find similar isolates.
- **Biosensors and electrochemical methods of detection:** Electrochemical assays have recently been developed to detect the enzymatic activity of BoNT. These techniques allow SNARE complex proteins to be coated onto biosensors. The presence of enzymatically active BoNT is determined by the detectable changes in electrochemical properties that result from the SNARE protein coating being altered by BoNT. By monitoring changes in ion current in nanopores as cleavage products of substrates that mimic SNARE complex regions pass through aerolysin nanopores and are digested, researchers have created a nanopore-based method of detecting BoNT enzymatic activity. After prepared samples are placed on the device, these assays can produce results in a matter of minutes and are sensitive, with some having detection levels in subnanomolar ranges. Nano-biosensors have been used to detect active BoNT. Förster resonance energy transfer (FRET) is made possible by attaching a quencher to serotype-specific substrates and using quantum dots, which fluoresce when excited. In certain situations, no instrumentation is needed to interpret the results of this method, which enables the quick, precise, and quantitative detection of active BoNT at levels at or close to the MBA's LOD. Though additional testing with the remaining serotypes and testing the assay with different foods and clinical matrices would be helpful, this technology has been demonstrated to work for serotypes A, B, and E and may be helpful as a screening assay. Fluorophores and quenchers found in synthetic peptides that

mimic SNARE complex proteins could be used in upcoming FRET-based tests.

Conclusions

By observing clinical signs and symptoms that are consistent with the illness, botulism can be diagnosed. Public health response depends on laboratory confirmation of the clinical case, and effective disease treatment depends on prompt antitoxin administration. Quick identification of BoNT-producing organisms or detection of BoNT is crucial in situations where botulinum antitoxin has not yet been released. Thus, when creating laboratory testing algorithms, testing turnaround time should be taken into account. Serotype-specific antibodies, peptide substrates, or fluorescent reporters are examples of specialized reagents that are necessary for many of the assays that are currently available to confirm a clinical diagnosis of botulism. Since alternative methods have not been fully validated in complex matrices for all serotypes, specialized laboratory equipment or animal testing are currently required to confirm the presence of BoNT. For labs conducting botulism testing, the stability and accessibility of the reagents, consumables, and equipment necessary for such testing are crucial factors.

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