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Recent understanding of botulinum toxin and its main detection methods

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Abstract. The Botulinum neurotoxin (BoNT) has the title of the most potent poison known to man. It causes a serious condition known as botulism. Guidelines and regulations for the industrial processing of food have made foodborne botulism rarer, but the use of the toxin in pharmaceutical industries and its potential as a bioweapon make detection methods of the toxin a necessary thing. Currently, the Mouse Lethality Bioassay (MLB) is still considered the golden standard of botulinum toxin detection even though it has several limitations. There had subsequently been the development of several alternatives to the MLB, including immunoassays, endopeptidase-based assays, and more. This paper gives an overview of the mechanisms behind botulinum toxins, current methods of detection, and the methods' advantages and disadvantages.

Keywords: Botulinum Neurotoxin, Toxin Mechanism, Detection Methods, Overview.

1. Introduction

Botulism is a disease that, however rare, might easily become lethal without proper treatment. Between 1920 and 2014, 197 outbreaks of the disease have been reported worldwide, with a median of 3 cases per outbreak [1]. Without treatment, the mortality rate of botulism is ~50% [2].

Because of its toxicity, the botulinum toxin is classified as a Category A bioterrorism agent by the Centers for Disease Control and Prevention (CDC) [3]. However, the botulinum toxin has also been found to be useful in clinical applications. With controlled doses, the toxin can treat spasms, tic disorders, wrinkles, and more [4]. Thus, methods of detecting the dose of botulinum toxins become important both for monitoring health and for the pharmaceutical industry. In this paper, the mechanism of the botulinum toxin is briefly introduced. Then, the Mouse Lethality Bioassays, Immunoassays, Cell-based assays, Endopeptidase-based assays, and Nucleic acid-based assays will be introduced and discussed.

2. Botulinum toxin detection mechanism

The botulinum toxins interfere with acetylcholine release in the Peripheral Nervous System (PNS) by cleaving proteins in the Soluble N-ethylmaleimide-sensitive factor Activating protein Receptor (SNARE) complex [5, 6]. The SNARE complex is a group of proteins that cooperate to facilitate membrane fusion, especially at nerve cells' endings to release neurotransmitters [5]. By destroying proteins in the complex, the toxins effectively render the neurotransmitter-release system useless and induce paralysis [6, 7].

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It is estimated that a few nanograms of the toxin per kilogram of body weight can be deadly [8]. The toxin is composed of a heavy chain and a light chain, bound together by a disulfide bond; the heavy chain binds to receptors on the membrane of the neuron and causes the toxin to be taken into the cell, and once intracellular, the chains are split and the light chain would cleave various proteins in the SNARE complex, depending on which type of toxin it is [7].

There are various methods of detection for the botulinum toxin, some targeting the botulinum toxin's effects on living cells/organisms (like the MLB and cell-based assays), others on the function and reactivity of the toxins (like the endopeptidase assays and immunoassays), and still others on the genetic code that directs the production of the toxins (nucleic acid-based assays). Below is a summary of the main types of assays used in botulinum toxin along with their strengths and weaknesses.

3. Current methods of detection

3.1. MLB and related functional methods

The Mouse Lethality Bioassay (MLB) works by injecting a group of mice with (pretreated and likely diluted) samples suspected to contain the botulinum toxin (or things that are known to contain the toxin, but the dose is needed) [9]. With the symptoms the mice develop, one can check if the samples contain the botulinum toxin, and with the mortality rate in the mice and how much the sample has been diluted, one can estimate how potent the botulinum toxin in the sample is [9, 10].

The MLB is considered the golden standard of botulinum toxin detection. First, it is robust and reliable. The major advantage MLB holds above other assays is that it can detect all types of the botulinum toxin, whereas other methods like ELISA and Endopeptidase-based assays must rely on very specific data on the structure of each toxin it detects [10]. The MLB also allows the biological activities of the toxins to be studied, which is very important in the field of research [10].

However, there are several problems with this method of detection. First, the botulinum toxin usually kills by causing respiratory failure, which causes the painful deaths of the animals [11]. Recently, the scientific community has become more and more concerned about the welfare of animals. To address this, the principle of the 3Rs was put forward. The 3Rs stand for Replacement of animals in testing, Reduction of the use of animals, and Refinement of procedures so that animals suffer less in experiments [12]. The MLB, meanwhile, is dependent on using a substantial number of animals to see how many of them develop symptoms and die. While there is a variation of MLB in which samples are injected under the skin and local muscle paralysis is the endpoint of the test, this variation cannot definitively show that it's the botulinum toxin that's causing muscle paralysis [7]. Moreover, the bioassay is expensive and time-consuming to run, needing sophisticated faculties to maintain all the animals; it might also take days for the botulinum toxin to take effect, which makes the test much less useful in urgent clinical diagnosis [11]. Still, MLB is considered by many as the golden standard of botulinum detection because of its relatively superior sensitivity and accuracy in the history of its use along with the fact that this method is firmly established in scientific research. To replace MLB, several methods have been developed to at least reduce the use or pain of lab mice in testing for the toxin.

3.2. ELISA

Immunoassays are biosensors in which an analyte reacts with an antibody (that is usually bound to a surface), and with the help of a label (which can be an enzyme, fluorescent molecule, or a radioisotope), the amount of analyte is determined [13]. Known for their high specificity and sensitivity, numerous immunoassays have been tried for the testing of botulinum toxins. Among them, the enzyme-linked immunosorbent assay (ELISA) has been used the most.

The ELISA bioassay generally works as follows: first, well plates (usually 96-well plates) are coated with either an antigen or an antibody. Then, after incubation, the plates are washed. In the presence of the analyte (and, in some forms, a secondary antibody), the antigen/antibody will be bound.

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A solution that can react with the substrate is added, and depending on the presence or absence of the analyte, the solution may or may not induce a signal (usually in the form of a color change) [14, 15].

Some of the immunoassays have shown comparable, sometimes even better, accuracies than MLB [3]. In terms of cost, ELISA requires fewer instruments and specialized workers [16]. The speed of the ELISA test is also a great advantage, as only a few hours are needed for results to come out. Thus, it is useful for preliminary diagnostic tests for botulism [7].

However, the ELISA detection method has shown poor performance when with some types of food samples. Thus, according to the FDA, its results must be confirmed by MLB [7]. ELISA also cannot differentiate between the active toxin and its inactive form. Thus, its usefulness is limited in scenarios in which only the active toxin requires quantification [3].

3.3. Cell-based assays

Cell-based assays are something designed to simulate what happens in an MLB, except it does not involve an entire organism. Rather, cells, usually derived from existing cell lines (but can also be taken out of living organisms), are used [17]. There are many proposed endpoints to this assay, including when neurotransmitter release is inhibited (usually measured by attaching radioactive markers on neurotransmitters and detecting the radioactivity that was released during neurotransmitter release) or when SNARE protein fragments are detected (with techniques like Western Blot and ELISA) [18].

Cell-based assays allow the interaction with living cells of the toxins to be studied and can be conducted on a large scale. For this reason, it can be used to replace the MLB in research that involves studying the interaction between living cells and botulinum toxins [10]. However, it is also difficult to maintain cell lines and control many aspects of the assay (like the amount of medium added) [18]. Moreover, many immortalized cell lines (from which cells are derived easier than other methods because they can be maintained more easily) have cancerous origins, which can affect the way they respond to the toxins [10]. Because of its similarity in mechanism to MLB, these assays also take many days to run, and since maintaining cells outside of an organism requires constant conditions, skilled personnel and specialized equipment are inevitably needed [10].

3.4. Endopeptidase reaction-based assays

Endopeptidase reaction-based assays are based on the endopeptidase reactions of the botulinum toxins themselves. Endopeptidase reactions are reactions in which one protein (called a peptidase) cuts another protein at specific places in the middle of the protein [19]. The botulinum toxin light chains are all endopeptidases because they cleave SNARE proteins [10]. The assays work as follows: peptides that the toxins are known to cleave (or mimic the proteins that they cleave) are used as the substrate of the assay, and in the presence of the toxin, some of the substrate will be cleaved. Then, the cleaved substrate can be detected, most commonly with Mass Spectrometry (assay name: Endopep-MS) or ELISA (Endopep-ELISA) [20, 21].

These biosensors are selective and can distinguish between many different serotypes of the toxin. Some papers have also suggested that Endopep-MS has great potential to replace MLB as the golden standard of botulinum detection, being more selective, more sensitive, and more ethical; moreover, the time needed to conduct a test with Endopep-MS is shorter than one with MLB [20]. However, research is necessary for determining what proteins different types of botulinum toxins interact with and what products they leave behind, which makes this assay unreliable in detecting novel types of the toxin [10]. For this reason, this assay cannot replace the MLB, which can detect any type of botulinum toxin, including novel ones.

3.5. Nucleic acid-based

The nucleic acid-based detection methods that have been put forth are based on the detection of the genes that produce the botulinum toxin in a sample. With the help of the polymerase chain reaction (PCR), the genes that code for toxin production can be amplified and detected [22].

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On the premise that the samples are naturally contaminated, this method yields relatively accurate results. However, PCR needs sophisticated equipment and skilled personnel to perform. But perhaps this is an advantage, for as COVID swept throughout the globe, many labs have adapted to conduct PCR on large scales [23]. Still, the biggest limitation to this method is that it doesn't detect the toxin itself and thus will be useless in cases where only the toxin or only the genes are present. As such, it is not able to challenge the MLB in terms of superiority.

4. Conclusion and discussion

There has been a lot of progress from having the MLB as the only method to detect the botulinum toxin, which is as recent as a few decades ago [24]. But, the MLB is still the only golden standard of botulinum toxin detection because of its flexibility and the amount of information it can provide. However, even the MLB is far from perfect. Thus, it becomes important to carefully weigh the priorities in choosing a detection method for botulinum toxin detection.

This paper gave an overview of the main methods for botulinum toxin detection as of 2023. It summed up the main advantages and disadvantages of each general method. In summary, speed, cost, how hard the test is to run, specificity, and sensitivity are the five main factors that make the sensors vary in usefulness.

An ideal botulinum toxin detector would be quick, cheap, and easy to run while giving relatively sensitive results that can distinguish between active/inactive toxins and the type of toxin present. Unfortunately, such a sensor does not exist. To gather complete data, several tests must be run. Thus, future research is needed to improve the abilities of the sensors to provide more information or to reduce the cost and speed of tests while maintaining sensitivity. This paper may be useful for future research into novel methods of botulinum toxin detection.

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