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# Production of recombinant botulism antigens: A review of expression systems

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ABSTRACT

Botulism is a paralytic disease caused by intoxication with neurotoxins produced by *Clostridium botulinum*. Despite their similar mechanism of action, the botulinum neurotoxins (BoNT) are classified in eight serotypes (A to H). As to veterinary medicine, the impact of this disease is essentially economic, since different species of production animals can be affected, especially by BoNT/C and D. In human health, botulism is feared in a possible biological warfare, what would involve mainly the BoNT/A, B, E and F. In both cases, the most effective way to deal with botulism is through prevention, which involves vaccination. However, the current vaccines against this disease have several drawbacks on their process of production and, besides this, can be dangerous to producers since it requires certain level of biosafety. This way, recombinant vaccines have been shown to be a great alternative for the development of vaccines against both animal and human botulism. All BoNTs have a 50-kDa light chain (LC) and a 100-kDa heavy chain (HC). The latter one presents two domains of 50 kDa, called the N-terminal ($H_N$) and C-terminal ($H_C$) halves. Among these regions, the $H_C$ alone seem to confer the proper immune response against intoxication. Since innumerous studies describe the expression of these distinct regions using different systems, strategies, and protocols, it is difficult to define the best option for a viable vaccine production. Thereby, the present review describes the problematic of botulism and discusses the main advances for the viable production of vaccines for both human and veterinary medicine using recombinant antigens.



## 1. Introduction

Botulism is an intoxication caused by potent botulinum neurotoxins (BoNTs) that are secreted during the multiplication and sporulation of *Clostridium botulinum*, a mobile, anaerobic Gram-positive bacilli [1]. *C. botulinum* spores are ubiquitous in the environment, surviving for extended periods under adverse environmental conditions. In appropriate anaerobic conditions, germinating spores produce, grow and secrete BoNTs [2]. Based on the antigenic properties of BoNTs, *C. botulinum* strains are grouped into one of eight serotypes: A, B, C, D, E, F, G and H. The recently isolated serotype H was isolated from a patient with infant botulism, and it secretes a proteolytic toxin that is not neutralized by any of the seven monovalent antitoxins (Anti-A to Anti-G), hence the name of H toxin [3].

*C. botulinum* strains compose four genotypically and phenotypically distinct groups of organisms, designated I to IV [4]. Groups I and II cause botulism in humans, while group III is involved with animal botulism; however, exceptions occur. Group IV, formed by *Clostridium argentinense* [5], is not commonly associated with the occurrence of disease [6]. Group I strains, which are pathogenic for humans, produce the proteolytic BoNT/A, B and F, while Group II strains produce non-proteolytic BoNTs B, E and F. Strains from Group III, which are pathogenic for animals, produce BoNTs C and D. Finally, Group IV strains secrete serotype G neurotoxins, and some Group IV strains were reported to produce two types of toxin. However, strains that are PCR-reactive for both type A and B toxins may generally be considered to be type A toxin-producing strains [7].

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The BoNTs, which are the most poisonous biological substance known, have $LD_{50}$ values in mice ranging from 0.5 to 5 ng/kg, depending on the serotype [8]. The BoNTs are released as progenitor toxin complexes (PTCs), which have distinct molecular compositions. The PTCs are multiprotein complexes composed of a BoNT and several non-toxic neurotoxin-associated proteins (NAPs) that are also called non-toxic-associated proteins [9]. These PTCs cause food poisoning, and BoNT serotype A PTC has $LD_{50}$ values in humans of approximately 0.09–0.15 mg by intravenous administration, 0.7–0.9 mg by inhalation and 70 mg by oral administration, indicating the miniscule amounts of toxin necessary to cause disease in humans [10]. Associated non-toxic proteins (ANTPs) include hemagglutinin (HA) and non-toxic non-hemagglutinin (NTNH), as well as proteins with unknown function, called OrfX.

The genes encoding the BoNTs and ANTPs are clustered in a DNA segment, called the botulinum locus, which is located chromosomally. The gene encoding the NTNH component immediately precedes it. Both genes form an operon located in the 3' region of the botulinum locus that is highly conserved in different species of *C. botulinum*. The BoNTs genes are present in various genetic elements, including phages, plasmids or chromosomes in the different groups of *C. botulinum* and other *Clostridium* species. Plasmids of various sizes and bacteriophages have been found in *C. botulinum* A, B, E, and F. However, these plasmids have not been associated with the toxicity of the strains, but instead with the expression of chromosomal genes [11]. However, in *C. botulinum* C and D, it is clear that BoNT is encoded by bacteriophages [12].

BoNTs are synthesized as inactive 150-kDa single-chain proteins that are activated by proteolytic cleavage to form a disulfide-linked dimer consisting of a 100-kDa heavy chain (HC) and a 50-kDa light chain (LC) [13]. The heavy chain is divided into an amino- ($H_N$) and a carboxy-terminal domain ($H_C$). While the $H_N$ portion is highly homologous among various clostridium neurotoxins, the $H_C$ domain presents more variability [2]. The mechanism of BoNT action can be divided into four stages: binding, internalization, membrane translocation, and proteolysis of specific SNARE proteins. In the first step, the $H_C$ binds on the presynaptic terminal of the neuronic membrane to specific receptors comprising gangliosides (GD1a, GD1b, GT1b, GQ1a, GM1a) and specific proteins including synaptotagmins (Syt-I, Syt-II) and synaptic vesicle associate proteins (SV2A, SV2B, SV2C) [14,15]. After binding, the lateral presynaptic membrane movements trap the neurotoxin inside the array of protein receptors, increasing its interaction with additional binding molecules and making the BoNT activity irreversible. The role of $H_C$ explains the higher rate of protective epitopes in this region when compared with any other part of the molecule. Therefore this domain can induce strong humoral response and consequent protection against the native toxin [16].

The next step, toxin internalization, involves trafficking via vesicles, which is an energy-dependent and temperature-sensitive mechanism that is favored by the high rates of vesicle recycling from the hyperactive nerve terminal. The influx of calcium triggers protein-mediated events that cause synaptic vesicles docked at the presynaptic membrane near the calcium channels to fuse with the membrane and release acetylcholine. The resultant vesicle recycling induces BoNT internalization, which starts a new process of BoNT exocytosis and endocytosis. Once BoNTs are in the lumen of the endosomes, it acidifies and the HC undergoes a conformational change that results in the formation of a HC protein pore in the endosomal membrane. This pore enables the translocation of the LC to the cytosol. Then, it cleaves its cognate SNARE in different places, resulting in the inhibition of acetylcholine release [2]. The lack of this neurotransmitter in the synaptic cleft governs the flaccid paralysis of skeletal muscles. Each serotype cleaves specific peptide bonds in one or more of the SNARE proteins [17].

Different animal species have different susceptibilities to BoNTs and botulism. Equines seem to be sensible to all serotypes [18]. Bovines are susceptible to serotypes C and D with acute and subacute cases, and some description of types A and B intoxication [19]. On the other hand, the ovine disease is commonly chronic [20,21]. Canines are less sensitive and disease occurrence is associated with types A, B and C, but does not occur frequently [22]. In addition, birds are affected by type C and, more rarely, by types A and E, but they can eliminate all serotypes in their excreta [23–25].

Cattle with high nutritional requirements, such as pregnant or lactating females, in inadequate pastures without proper mineral supplementation, especially phosphorus, can develop the habit of eating corpses and its bones [26,27]. Simultaneously, they intake preformed BoNTs on the decomposing carcasses, which lead to large outbreaks resulting in the death of thousands of animals [28–30]. The incubation time and severity of botulism is dependent on the amount toxin ingested and the susceptibility of the animal species involved. In cattle, the disease course can range from hours to a few days, with a mortality rate near 100%. The first clinical signs are difficulty in walking and incoordination of the hind limbs with cranial progression to flaccid paralysis. The animal enters preagonal state, and death, preceded by coma, occurs due to cardiac arrest. Throughout the course of the disease, the physical attributes of the animal remain unchanged. Gross lesions are rare and limited to petechiae in the myocardium as a result of the respiratory distress that precedes death [30–32].

Vaccination with C and D toxoids is still the primary way to control botulism in cattle. In Brazil only, over 150 million vaccine doses with these toxoids are produced annually. Although these immunogens may be effective in preventing disease [1], their quality varies greatly between countries and manufacturers, which consequently results in a large range of immune responses in vaccinated animals [33,34]. BoNT production is one of the most important factors to be considered in the industrial production of botulinum toxoids, because it requires the use of toxigenic strain as well as specific culture media, pH, time and atmosphere suitable for cultivation and incubation [35]. Furthermore, the risk of toxoid handling, the high manufacturing cost and the chemical inactivation of the botulinum toxoid all stimulated research on alternative approaches. To this end, recombinant vaccines have garnered the attention of researchers as an alternative to those made by native toxoids [36]. Preliminary studies with recombinant protein antigens representing one or more of the three domains of the BoNTs were evaluated as potential vaccine candidates. The recombinant protein antigens can be produced in large quantities using expression systems, such as yeast or *Escherichia coli*. These antigens may be purified using conventional chromatography methods or they may not even require this step [37,38]. Recombinant formulations are stable, safe and well tolerated in animal models, as well as target animal species [39–41]. Generally, they induce protective immunity after two to three vaccinations, even when the challenges are generated using large amounts of active neurotoxin. However, these factors were not tested in humans yet [36], being limited to preliminary results in monkeys [42]. The aim of this review is to discuss systems, strategies and protocols to produce recombinant vaccines for controlling botulism.

## 2. Overview of recombinant botulism antigens expressed in *E. coli*

The *E. coli* expression system is a robust way to express heterologous proteins, because its genetic manipulation is simple and well described. Innumerable active proteins have been obtained by this system, which have been useful for many medical applications [43]. The field of immunology has also benefited from the