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Historical and current perspectives on *Clostridium botulinum* diversity

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Abstract

For nearly one hundred years, researchers have attempted to categorize botulinum neurotoxin-producing clostridia and the toxins that they produce according to biochemical characterizations, serological comparisons, and genetic analyses. Throughout this period the bacteria and their toxins have defied such attempts at categorization. Below is a description of both historic and current *Clostridium botulinum* strain and neurotoxin information that illustrates how each new finding has significantly added to the knowledge of the botulinum neurotoxin-containing clostridia and their diversity.
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1. Introduction

The original hypothesis was that botulism was caused by a toxin produced by a single anaerobic spore-forming organism. This bacterium was originally named *Bacillus botulinus* and was later changed to *Clostridium botulinum* when the aerobic *Bacillus* genus was separated from the anaerobic *Clostridium* genus [142]. Almost from the beginning, physiological, biochemical and serological studies indicated an underlying diversity within the botulinum neurotoxin-producing (BoNT-producing) clostridia and their toxins. The original two isolates available for study, the European Van Ermengem and Landmann strains [133,85], differed greatly in growth requirements and biochemical reactions [86]. From these early studies we have been able to hypothesize that the Van Ermengem strain was nonproteolytic, most likely a BoNT/B-producing strain, while the Landmann strain was a proteolytic BoNT/A-producing strain.

Botulism related to contaminated sausages and other meats had been a recognized health hazard in Europe for generations, but it appears its recognition in the United States may have been linked to the widespread acceptance of home and commercial canning methods for food preservation in the early 1900s. An additional difference between European and US strains was recognized among the botulinum neurotoxins due to toxin potency, or toxicity. This difference was especially notable during the "olive scare" of 1919–1920, where four separate botulism outbreaks occurred due to commercially canned ripe olives [4,5,7,114,6]. The mortality rate with these cases was greater than 60%, compared to typical mortalities of approximately 35% or less in Europe. Differences in implicated foodstuffs, such as meats (in Europe) versus vegetables and fruits (in the US), were also noted [27].

2. Botulinum neurotoxin-producing clostridial strains

In 1910–1919, serological methods were introduced for categorizing the toxins or bacteria. These methods were based on the abilities of antisera produced using one toxin or bacterial isolate to neutralize toxins or to agglutinate bacteria of

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different isolates. The earliest toxin neutralization methods resulted in differentiation of two toxin types, A and B [86,16], while the agglutination methods separated bacterial strains into seven agglutination groups [111]. Agglutination Groups I–III were composed mainly of BoNT/A-producing bacterial strains and Groups IV–VII were associated with BoNT/B-producing strains. Groups I, II, IV, V, and VI contained multiple members, but Groups III and VII contained single members whose antisera was "ultra-specific" for that strain. Several type A Group I strains cross-agglutinated type B Group V strains, and vice versa, indicating that the same bacterial strains could produce toxins of different types, or possibly multiple toxins, and illustrating from the outset that *C. botulinum* strains were diverse and difficult to categorize.

Despite these diversity issues, the *C. botulinum* species continued to be defined on the basis of a single characteristic, the production of botulinum neurotoxin. Adding confusion to the nomenclature was the presence of *C. botulinum* strains that no longer produce neurotoxin, which is a condition that is particularly found in strains that have been repeatedly subcultured in the laboratory [55].

In 1947 a simple, rapid method for discriminating BoNT-producing *C. botulinum* strains in mixed cultures was introduced based on differential reactions of colonies on egg yolk agar [92]. Two reactions can be discerned – the lipase reaction which results in an oily sheen on the colonies, and the lecithinase reaction, which causes an opaque white ring in the media below the colonies. *C. botulinum* organisms could be distinguished by their positive lipase and negative lecithinase characteristics. However, as different BoNT-producing clostridia with variable lipase/lecithinase reactions were identified, it became clear that these reactions cannot be depended on alone for the identification of *C. botulinum* strains (Table 1).

Using a combination of biochemical characteristics, it was possible to separate *C. botulinum* strains into four metabolic/biochemical Groups [118,58]. Molecular analyses of *C. botulinum* strains have established that these four Groups represent distinct species, and that these species include nontoxic as well as neurotoxic members [24,67,126]. Additional BoNT-producing clostridial strains (*Clostridium baratii* and *Clostridium butyricum*) have been identified and characterized [56,93]. Notably, BoNT-producing clostridial strains of *C. baratii* and *C. butyricum* have never been classified as *C. botulinum*, most likely due to fact that these species were already well established. There is also a report of two non-neurotoxigenic *Clostridium subterminale* strains with *bont/B* genes containing a premature stop codon that prevents the expression of BoNT/B, known as silent B genes [43]. It is now evident that multiple clostridial species may produce BoNTs, and the characterization of BoNT-producing clostridia as a single species is erroneous. To a large extent, researchers indirectly acknowledge the presence of multiple species of BoNT-producing clostridia by using the metabolic Group designations in most contemporary publications. Nonetheless, no alternative nomenclature for this group of organisms has been accepted.

3. Botulinum neurotoxins

As noted above, one of the earliest ways to categorize BoNT-producing strains was by serological methods, to include neutralization of toxins using homologous antisera. Initial serological neutralizations were able to clearly distinguish organisms that produce type A from type B toxins [86,16], and this became a hallmark method for toxin differentiation. In 1922, some toxic *C. botulinum* strains were isolated whose toxins were not neutralized by either of the standard anti-A or anti-B antitoxins. Antisera from one of these strains were used to neutralize other similar isolates, which were designated as type C toxin. However, with type C, there were immediate problems in identification. The original type C strains were isolated by Dr. Ida Bengtson and Dr. Robert Graham in 1922 [12] from larvae of the green fly and contents of chicken crops in affected birds. That same year, Dr. Seddon isolated a very similar organism from a fatal case of "midland cattle disease" in Australia [113], which he designated *Bacillus parabotulinus*. Isolates from these cases were sent to the laboratory of Dr. K. F. Meyer, in San Francisco, where the question was asked "are the type C and Seddon strains serologically and toxicologically alike?" The surprising answer was – yes and no. Results from metabolic and biochemical studies indicated that the bacterial strains could

Table 1
Lipase and lecithinase reactions of neurotoxigenic clostridial strains. References are noted in brackets.

Clostridia	Lipase	Lecithinase	Toxins produced	Nontoxic members [67,72]
Group I (*C. botulinum*)	+	–	A, B, F; Ab, Ba, Bf	*C. sporogenes*
Group II (*C. botulinum*)	+	–	B, E, F	*C. taeniosporum*[a]
Group III (*C. botulinum*)	+	+/–	C, C/D, D, D/C	*C. novyi*
Group IV (*C. argentinense*)	–	–	G	*C. argentinense*, *C. subterminale*, *C. hastiforme*
(*C. baratii*)	–	+	F	*C. baratii*
(*C. butyricum*)	–	–	E	*C. butyricum*

[a] The nontoxic members of each group exhibit the same lipase/lecithinase reactions as the neurotoxigenic bacteria, with the exception of *C. taeniosporum*, for which there is no information.

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