Taxonomy of Botulinum Neurotoxin-Forming Clostridia and their Neurotoxins

In this guide

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1. Report on Botulinum Neurotoxin-Producing Clostridia
2. Taxonomy of Botulinum Neurotoxin-Forming Clostridia and their Neurotoxins
3. Detection
4. Epidemiology
5. Occurrence, Growth and Survival
6. Risk Assessment
7. Recommendations
8. References

The name *Clostridium botulinum* has been used for bacteria that form botulinum neurotoxin. For many decades it has been recognised that *C. botulinum* is not a distinct species but a collection of diverse clostridia that share the common property of forming botulinum neurotoxin[7]. Genes potentially encoding a botulinum neurotoxin have also recently been detected outside of the genus *Clostridium*.

Since 1992 scientific developments, notably whole genome sequencing (WGS), have provided a better understanding of the taxonomy and diversity of botulinum neurotoxin-forming clostridia and their neurotoxins. Information from WGS is valuable when selecting strains for challenge test experiments and process validation, is a valuable resource for improved pathogen detection and discrimination, is vital for tracing outbreaks and is central to studies of pathogen biology and evolution for *C. botulinum* and its neurotoxin genes.

This new information and understanding indicates it is unlikely that properties of botulinum neurotoxin-forming clostridia and their neurotoxins, or the risk of human foodborne botulism that they present, have changed in recent decades (although changes in food processing may impact on risk).

The diverse collection of clostridia that form botulinum neurotoxin can be separated into six genomically and phenotypically distinct groups (Table 1). These six groups are sufficiently distinct for each to be considered a separate bacterial species. Two of these groups, proteolytic *C. botulinum* (including *C. sporogenes*) and non-proteolytic *C. botulinum,* are strongly associated with human foodborne botulism, with the other groups either weakly associated or not associated. Each group includes highly related strains that do and do not form botulinum neurotoxin. There has been a greater interest in sequencing strains that form botulinum neurotoxin rather than non-toxic strains. Thus, for each group, the fraction of sequenced toxic strains is likely an overestimate of the fraction of toxic strains found in the environment.

WGS has provided details of the taxonomy, population structure and diversity within each group, and evidence of horizontal transfer of botulinum neurotoxin genes between distantly related clostridia. The gene encoding the botulinum neurotoxin is located on the chromosome or a plasmid or other mobile genetic element.

Different naming conventions are currently used for botulinum neurotoxin-forming clostridia, and the present situation is dynamic. In this report the framework in Table 1 is used for clarity. Further details of the association with human foodborne botulism are given in Chapter 4 of this report.

Table 1

Six genomically and phenotypically distinct groups of botulinum neurotoxin-forming clostridia

Name of botulinum neurotoxin-forming clostridia used in this report	Association of botulinum neurotoxin-forming clostridia with human foodborne botulism	Serotypes of botulinum neurotoxin formed
Proteolytic *C. botulinum/C. sporogenes*	Strong	A, B, F, X
Non-proteolytic *C. botulinum*	Strong	B, E, F
C. botulinum Group III	Very weak	C, D
C. argentinense	No	G
C. butyricum	Weak	E
C. baratii	Weak	F

2.1 Properties of the botulinum neurotoxins

Eight antigenically distinct botulinum neurotoxin serotypes (A-G, X) have been identified in bacterial strains within six distinct clostridial groups (Table 1). The eight botulinum neurotoxin serotypes are separated into more than 40 botulinum neurotoxin sub-types[5]. Most sub-types have been identified through derivation of their amino acid sequence from the gene sequence and they differ by at least 2.6% in amino acid sequence from any other sub-type. Various hybrid neurotoxins have also been described. Human foodborne botulism is most frequently associated with botulinum neurotoxin serotypes A, B and E. While there is evidence that the botulinum neurotoxin serotypes show minor differences in potency, all botulinum neurotoxins must be considered to represent a major hazard.

2.2 Taxonomy of proteolytic *C. botulinum*/*C. sporogenes* group

The proteolytic *C. botulinum*/*C. sporogenes* group is frequently associated with human foodborne botulism. It comprises highly proteolytic mesophilic bacteria that form spores of high thermal resistance. WGS has separated this group into two major lineages that could be recognised as separate species[8]. Both lineages contain strains responsible for foodborne botulism, and include toxic and non-toxic strains. The first lineage contains strains of proteolytic *C. botulinum*, with most strains forming one or more botulinum neurotoxins of types A, B, and/or F (and in one case of infant botulism type X). These strains are strongly associated with foodborne botulism. The second lineage contains strains of *C. sporogenes*, with 20 out of 104 strains analysed in a recent study forming botulinum neurotoxin type B, and the remaining 84 strains non-toxic. The strains that form botulinum neurotoxin type B were distributed amongst closely related non-toxic strains, rather than clustering together. The toxic strains of *C. sporogenes* have been occasionally associated with foodborne botulism (including one case in the UK).

Recent findings include the recognition that the high thermal resistance strain PA3679 is a non-toxic strain of proteolytic *C. botulinum* rather than a non-toxic strain of *C. sporogenes*, and that some strains that have caused human botulism (foodborne, infant and wound) and form type B neurotoxin belong to *C. sporogenes* rather than to proteolytic *C. botulinum*[8].

The identification of distinct lineages/clusters within the proteolytic *C. botulinum*/*C. sporogenes* group, and further genotypic and phenotypic characterisation of strains within these lineages/clusters has the potential to contribute to improved risk assessments. For example, strains within the proteolytic *C. botulinum* lineage seem to present a greater human foodborne botulism risk than strains within the *C. sporogenes* lineage, since (i) a greater proportion of strains form botulinum neurotoxin, and (ii) a greater fraction of strains has been associated with foodborne botulism. However, currently nothing prohibits other circumstances (e.g. alternative approaches to food processing) that might favour toxic strains in the *C. sporogenes* lineage.

Detailed information on the genotype and particularly the phenotype of botulinum neurotoxin-forming strains in the *C. sporogenes* lineage is presently limited. It is not known how closely the phenotype of neurotoxin-forming strains of proteolytic

C. botulinum is followed compared to that of non-toxic strains of *C. sporogenes*. Further clarity on the homogeneity of the proteolytic *C. botulinum*/*C. sporogenes* group will be of great value in considering the human foodborne botulism risk, and in particular whether it may be appropriate to disaggregate their risks. Currently it is pragmatic to consider neurotoxigenic *C. sporogenes* together with proteolytic *C. botulinum*.

2.3 Taxonomy of non-proteolytic *C. botulinum* group

The non-proteolytic *C. botulinum* group is frequently associated with human foodborne botulism. It comprises psychrotrophic, weakly proteolytic bacteria that form spores of moderate thermal resistance. WGS has separated this group into two major lineages[9]. Both lineages contain strains responsible for human foodborne botulism. The first lineage includes most strains that form type E neurotoxin and also some non-toxic strains. These strains are frequently isolated from fish and the arctic/subarctic environment (including marine and freshwater). The second lineage is dominated by strains that form type B neurotoxin and also contains some strains that form type E or type F toxin and non-toxic strains. The strains are often isolated from European terrestrial environments (including pigs) and marine/freshwater environments.

Foodborne botulism outbreaks associated with strains within the type E toxin lineage frequently involve chilled fish products, while outbreaks associated with strains within the type B (dominant) toxin lineage often involve chilled meat products. This may reflect genotypic/phenotypic characteristics and/or the geographical location in which the strains are found. Preliminary data indicate that spores of strains in the type B (dominant) lineage may have higher thermal resistance than spores of strains within the type E toxin lineage[10]. The identification of distinct lineages within the non-proteolytic *C. botulinum* group, and the genotypic and phenotypic characterisation of neurotoxin-forming strains within these lineages will be of great value in evaluating the human foodborne botulism risk, and in particular whether it may be appropriate to consider risks from the two groups independently.

2.4 Taxonomy of *C. botulinum* Group III and *C. argentinense*

C. botulinum Group III has been only very weakly associated with human foodborne botulism (details of the associations are included in Chapter 4 of this report) and there is no evidence that it presents a new or increased risk. Strains within *C. botulinum* Group III are frequently associated with botulism in animals and birds. *Clostridium argentinense* has not been associated with human foodborne botulism. WGS has been used to describe strain diversity within these two clostridial groups, and strains possessing and lacking genes encoding botulinum neurotoxin appear closely related.

2.5 Taxonomy of *C. butyricum* group

The majority of strains of *C. butyricum* are non-toxic, but a fraction form botulinum neurotoxin type E and have been associated with human foodborne botulism on a few occasions. The WGS of a limited number of *C. butyricum* strains (67 strains including eleven toxic strains) are available in public databases, and although this dataset is very limited there is an indication that the toxic strains may form discrete lineages rather than being widely distributed amongst non-toxic strains[11]. A detailed understanding of the homogeneity of the *C. butyricum* group will contribute to evaluating the human foodborne botulism risk presented by neurotoxigenic strains of *C. butyricum*.

2.6 Taxonomy of *C. baratii* group

The majority of strains of *C. baratii* are non-toxic, but a minority form botulinum neurotoxin type F and have been associated with human foodborne botulism on a few occasions. The WGS of only 16 strains of *C. baratii* (nine non-toxic strains and seven toxic strains) are presently available; including four toxic strains associated with a single outbreak of foodborne botulism. However, initial indications from this very limited dataset suggest that botulinum neurotoxin strains of *C. baratii* could be distributed amongst non-toxic strains[11–13]. A thorough understanding of the genotypic and phenotypic homogeneity of the *C. baratii* group will contribute to evaluating the human foodborne botulism risk.

2.7 Non-clostridial strains that contain potential botulinum neurotoxin genes

Non-clostridial bacterial strains have not been associated with human foodborne botulism. Recent data mining of published WGS has led to the discovery of genes

in non-clostridial bacteria with partial homology to genes that encode botulinum neurotoxins. These include *Enterococcus faecium* from a cow[14], *Weisella oryzae* from fermented rice[15] and *Chryseobacterium piperi* from sediment[16]. The putative neurotoxin sequences differed from those of the neurotoxin serotypes presently described in clostridia. Recombinant DNA methods indicate a potential for biological activity, although it is not yet established whether biologically active botulinum neurotoxins are formed by these bacteria. It is presently unclear whether further non-clostridial strains contain undiscovered potential botulinum neurotoxin-encoding genes, and whether these strains could form botulinum neurotoxin, and potentially cause botulism. As some of these bacteria are from genera that are used as probiotics in humans and animals, a watching brief should be maintained on any future developments regarding neurotoxin gene carriage and expression.

2.8 Conclusion

The understanding of the taxonomy and diversity of botulinum neurotoxin-forming clostridia and their neurotoxins has improved over the last few decades and this progression is likely to continue. New information indicates that it is unlikely that the properties of botulinum neurotoxin-forming clostridia and their neurotoxins have evolved significantly since 1992. A detailed understanding of the genotypic and phenotypic homogeneity of the various groups of botulinum neurotoxin-forming clostridia will contribute to assessment of the human foodborne botulism risk that is associated with botulinum neurotoxin-forming strains within these groups.

Non-clostridial strains with potential botulinum neurotoxin-encoding genes have not been shown to form botulinum neurotoxin, and have not been associated with foodborne botulism, and based on present evidence should not be considered as food safety hazards. However, it is prudent to maintain a close watch on scientific reports that identify botulinum neurotoxin type gene sequences in non-clostridia and to develop clearly identified protocols for responding, rapidly, to any significant new information.