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Review

Molecular Diversity of BoNT-Producing Clostridia—A Still-Emerging and Challenging Problem

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Abstract: The diversity of BoNT-producing Clostridia is still a worrying problem for specialists who explore the evolutionary and taxonomic diversity of *C. botulinum*. It is also a problem for epidemiologists and laboratory staff conducting investigations into foodborne botulism in humans and animals, because their genetic and phenotypic heterogeneity cause complications in choosing the proper analytical tools and in reliably interpreting results. Botulinum neurotoxins (BoNTs) are produced by several bacterial groups that meet all the criteria of distinct species. Despite this, the historical designation of *C. botulinum* as the one species that produces botulinum toxins is still exploited. New genetic tools such as whole-genome sequencing (WGS) indicate horizontal gene transfer and the occurrence of botulinum gene clusters that are not limited only to *Clostridium* spp., but also to Gram-negative aerobic species. The literature data regarding the mentioned heterogeneity of BoNT-producing Clostridia indicate the requirement to reclassify *C. botulinum* species and other microorganisms able to produce BoNTs or possessing botulinum-like gene clusters. The aim of this study was to present the problem of the diversity of BoNT-producing Clostridia over time and new trends toward obtaining a reliable classification of these microorganisms, based on a complex review of the literature.

Keywords: foodborne pathogens; *Clostridium botulinum*; BoNT-producing clostridia; diversity

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1. Introduction

Theoretically, foodborne botulism has been known since the beginning of mankind. Unconsciously, people have chosen the optimal conditions for *Clostridium botulinum* growth and toxin production during food preservation. Most likely, the symptoms of botulism have been known since ancient times; however, they were not associated with food or feed consumption. A possible example is an annotation of an edict by Leo the VI of Byzantium that appeared in the 10th century, which banned the manufacturing of blood sausages, probably because of the appearance of botulism cases after consumption. Reports of intoxication appeared in the old medical literature, probably misinterpreted because the symptoms of dilated pupils and flaccid paralysis could not be associated, e.g., with atropine intoxication, which was frequently suspected in cases indicated as botulism [1]. The first known recordings of animal botulism were reported in the 18th century and were prepared by Le Vaillant (a French traveller). He noticed fatal symptoms of flaccid paralysis in cattle during his travel through South Africa at the end of the 18th century. He also

recorded the seasonality of the disease, which was observed annually, soon after warm weather had set in, and was particularly prevalent when the grazing was poor and the cattle craved bones [2].

At the beginning of the 19th century, Justinius Kerner conducted thorough research on the symptoms of the mysterious disease. He collected cases of patients with a clinical description of what today is recognised as botulism symptoms and published them in monographs. The mentioned cases were mostly associated with the consumption of sausages. Kerner conducted intensive toxicological research using sausage extracts. For the first time, he used the term 'sausage poison' (botulinum toxin, 'botulus' derived from Latin means 'sausage') [1]. Moreover, he described animal experiments using sausage extracts on birds, cats, rabbits, frogs, flies, locusts, and snails. Kerner was such a passionate scientist that he conducted experiments with the use of sausage extracts on himself. Based on the obtained results, he concluded that the sausage extract interrupted nervous signal transmission. Botulinum toxin and its mechanism of action were described in detail by Kerner; however, the origin of the poison remained unknown up to the end of the 19th century, until it was discovered by Belgian bacteriologist Émile Pierre-Marie van Ermengem, after the funeral of 87-year-old Antoine Creteur. The mentioned funeral took place in 1895 in the small Belgian village of Ellezelles. After the ceremony, 34 people attended a wake at which pickled and smoked ham was served. After the meal, characteristic symptoms occurred among participants such as mydriasis, diplopia, dysphagia, and dysarthria, followed by increasing muscle paralysis. Three of them died and 10 showed severe botulism symptoms. A detailed examination of the ham and an autopsy was ordered and carried out by Van Ermengem. He isolated the microorganism able to produce botulinum toxin and noticed the anaerobic conditions of the growth of the isolated strain. Van Ermengem called this microorganism *Bacillus botulinus* [1]. He also believed that cases of botulism were caused by one specific toxin produced by one strain of bacteria. The correctness of this idea was undermined in 1904 by Landmann. He described another bacterial taxon which caused botulism after the ingestion of contaminated beans. In contrast to the Van Ermengem strain, this was proteolytic [3].

With time, further discoveries were made confirming the serological and taxonomic diversity of *C. botulinum* and the toxins produced by individual strains. In 1910, Leuchs showed the results from examination of the two European strains indicated they had different antigenicities. He observed that treatment with the antitoxin of one did not neutralise the second. In 1919, Georginia Burke, during her work with U.S. *C. botulinum* isolates, made an antigenic distinction between two toxins, and introduced the designation of type A (BoNT/A) and type B (BoNT/B). She initialised the alphabetic designation of toxin types. The mentioned type A toxotype appeared to be similar to that of Landmann's, while type B was similar to the van Ermengem strain. However, unlike with the van Ermengem strain, all the U.S. isolates were proteolytic. This study indicated that different *C. botulinum* strains can produce the same toxin [3].

In the decades that followed Burke's work, the additional diversity was noticed many more times. It was revealed from time to time, on the occasion of discovering new toxinotypes, but also numerous subtypes, of which there are about forty today. The following seven toxic types have been discovered: type C (discovered in 1922), D (1928), E (1937), F (1960), G (1969), H (Hybrid of AF) [4,5], and the recently discovered type X [6].The existence of the mentioned H and X types as separate serotypes is still under discussion by the scientific community. In addition, strains that are capable of producing two toxins (different amounts of them) have also been discovered. A larger amount of toxin is indicated by a capital letter. Commonly, these types of strains are referred to as "bivalent." Among them, Ab, Ba, Af, and Bf have been distinguished, as well as AB strains that are capable of producing the same amount of both toxins. In addition, strains designated as A (B) have also been distinguished. This kind of strain is usually referred to as "A silent B" because their second gene for BoNT/B contains a "stop codon" mutation (thus rendering this gene inactive). Subtypes of toxinotypes A, B, E, and F have also been described. The

mentioned subtypes have been distinguished on the basis of differences in the amino acid sequences of toxins and the nucleotide genes of toxins, surface epitopes, and physiological features [7]. Moreover, the distinction between types C and D is not unequivocal because of the occurrence of a chimeric sequence of C/D or D/C [8,9].

Clostridia capable of producing botulinum toxins form different groups of bacteria that could be considered as separate species. This intricate taxonomic nomenclature is the reason for contradictions and doubts related to the history of individual discoveries. To date, nomenclature problems are the subject of discussion and consideration [3].

2. Diversity of *Clostridium botulinum* Groups I and II

2.1. General Characteristics of Clostridium botulinum Strain Groups I and II

The most common types of botulism reported among humans are mostly caused by strains belonging to two groups within the *C. botulinum* species that produce potent botulinum neurotoxins. Group I and II strains differ in the serotypes of botulinum toxins they produce, which have specific toxicological effects. The use of well-developed methods of molecular biology using gene sequencing techniques and relying on phylogenetic analyses makes it possible to determine the extent of the diversity of genes encoding the production of toxins and the genomic diversity of bacteria of the genus *Clostridium* [10,11].

Clostridium botulinum strains belonging to group I constitute a group of mesophilic bacteria for which the optimal growth temperature is 37 °C. The growth of *C. botulinum* in this group is possible at a pH above 4.6 [12]. The highly proteolytic nature of these strains and the ability to produce heat-resistant spores are the reason for the technological problems in the food production process [13,14]. The detection of toxigenic strains in food can be challenging due to the possible loss of mobile genetic elements which determine their ability to produce botulinum toxin. However, some species, such as *C. sporogenes* can be used in the validation of heat treatment processes. *Clostridium sporogenes* seems to be a very eclectic species. The boundary between some strains considered to be *C. sporogenes* and *C. botulinum* seems to be very thin. Some *C. sporogenes* strains are discussed as being different, undescribed species or even a nontoxic variant of *C. botulinum* group I. *C. sporogenes* PA 3679 could be presented as an example. It has been thoroughly genetically investigated by Lee and Rhiman [15], who revealed that PA 3679 was 100% homologous to the *C. botulinum* strain 62A. Two other studies by Shill et al. [16], and by Butler 3rd et al. [17] investigated genomes of *C. sporogenes* PA 3679 isolates and also several other *C. sporogenes* strains. The most significant difference noticed by the authors was the acquisition of a second *spoVA* operon, *spoVA2*, which is responsible for dipicolinic acid transport into the spore core during sporulation. What is interesting is that *spoVA2* was also found in some *C. botulinum* strains which phylogenetically cluster with PA 3679. The mechanism behind the low heat resistance of spores may be the absence of the *spoVA2* gene. Butler et al. [17] noticed that the *C. sporogenes* strains examined both lack the *spoVA2* locus and are phylogenetically distant within the group I. The authors concluded that *C. sporogenes* could be determined as dispersed *C. botulinum* strains which lack toxin genes. The lack of the ability to produce toxins and the production of heat-resistant spores supports the use of this kind of strain as a surrogates for *C. botulinum*, and could allow the elimination of spores capable of producing toxins without the need to use the target pathogen in food-handling processes [17–19]. Proteolytic *C. botulinum* group I strains can produce botulinum neurotoxin types A, B and F, the genes for which may occur within a plasmid or may be part of a chromosomally integrated genomic island [11,20]. Depending on the number of botulinum toxin genes contained in the genome, a group I strain can form one to three different neurotoxins. The presence of two genes encoding a neurotoxin allows strains to produce one active toxin or two active toxins, one of which is produced in larger quantities [21,22].

In contrast to group I, group II strains are non-proteolytic and capable of producing toxins of types E, B and F, whose genes are located on the chromosome or carried on small plasmids [20,23]. It was assumed that the strains of this group show the presence of single genes encoding neurotoxins, thus forming a single toxin; however, the results of

some studies indicate the possible presence of the non-active, precursor fragments of *bont/b* and *bont/e* genes in *C. botulinum* type F strains belonging to group II [22,24]. *Clostridium botulinum* group II includes saccharolytic bacteria, which are widely distributed in the environment, for which the optimal growth temperature is 25–30 °C [25,26]. Although the spores of strains of this group have less resistance to high temperatures than group I strains, they are capable of surviving heat treatment processes. In addition, the psychrotrophic nature of the strains allows them to grow in refrigerated conditions, which, together with an anaerobic environment and a long period of storage, creates favourable circumstances for the development of spores and the production of toxins, posing a threat to the safety of the food products [27].

Both *Clostridium botulinum* group I and group II strains show significant genetic diversity and the differences that exist between them and the neurotoxin serotypes they produce are an important part of the laboratory diagnosis of botulism outbreaks and the risk assessments of marketed food products (Table 1).

Table 1. General properties of *Clostridium botulinum* groups I and II [21,28–30].

Properties	*Clostridium botulinum* Group I	*Clostridium botulinum* Group II
Proteolysis	+	–
Minimum pH required for growth	4.6	5.0
Optimum growth temperature	37 °C	25–30 °C
Heat-resistance of spores	High	Moderate
Neurotoxins formed	A, B, F	E, B, F
Botulinum neurotoxin gene localization	Chromosome, plasmid	Chromosome, plasmid
Closely related species	*C. sporogenes*, *C. tepidum*	*C. butyricum*, *C. taeniosporium*, *C. beijerinckii*

2.2. Diversity of Botulinum Neurotoxin Genes of Clostridium botulinum Groups I and II

Sequencing of botulinum neurotoxin genes has made it possible to define the subtypes found within each BoNT serotype based on the amino acid sequence variation they exhibit. Strains of *C. botulinum* capable of producing botulinum neurotoxin type A, due to their high potency, are a particularly important etiological agent responsible for the most severe form of botulism occurring among humans [31]. According to the literature, this serotype is the leading cause of botulism cases in the United States, while in Europe it is reported to be the second most common causative agent of food intoxication due to botulism [32,33]. Currently, among *Clostridium botulinum* type A strains, eight subtypes of botulinum neurotoxins (A1–A8) are distinguished, with differences in amino acid sequences ranging from 2.9–15.6%. The BoNT/A serotype is the only one with genes encoding neurotoxins located within a cluster forming two different conformations, one of which contains haemagglutinin (ha^+orfX^-) genes, while the other conformation includes *orfX* (ha^-orfX^+) genes [34]. Genomic sequence studies have shown the possibility of *bont/A* genes within two chromosome sites, one of which is operon *arsC*, in which an *orfX* gene cluster containing *bont/a1*, *bont/a2*, *bont/a3*, *bont/a6*, and *bont/a8* genes may be located, while the other site includes operon *oppB/brnQ,* in which a HA cluster containing the *bont/a1* and *bont*/a5 genes is located. The *bont* genes within the group I strains can also be carried on large plasmids, and *bont/A* is usually present in tandem with *bont/B* gene [35,36]. Significant genetic diversity was observed among the strains forming subtype A1, which is the only subtype that can have genes located within the *ha* or *orfX* clusters. Hill et al. [35], in their study on the possible events responsible for the genetic variation taking place within *C. botulinum* species, pay particular attention to recombination contributing to diversity among the toxin serotypes and the possibility of *bont/a1* in both clusters. This phenomenon is explained by the occurrence of recombination within the *ntnh* gene of serotype B and the *ntnh* gene of serotype A, which had a significant effect on the localisation of the *bont/a1* gene within the haemagglutinin cluster containing the recombinant gene, leading to the formation of a chimaera. The essence of recombination in the creation of genetic diversity

is also manifested in the example of the BoNT/A2 subtype, which was formed following this phenomenon from the BoNT/A1 and BoNT/A3 subtypes [37,38]. Examples of the structure of different botulinum toxin clusters are presented in Figure 1.

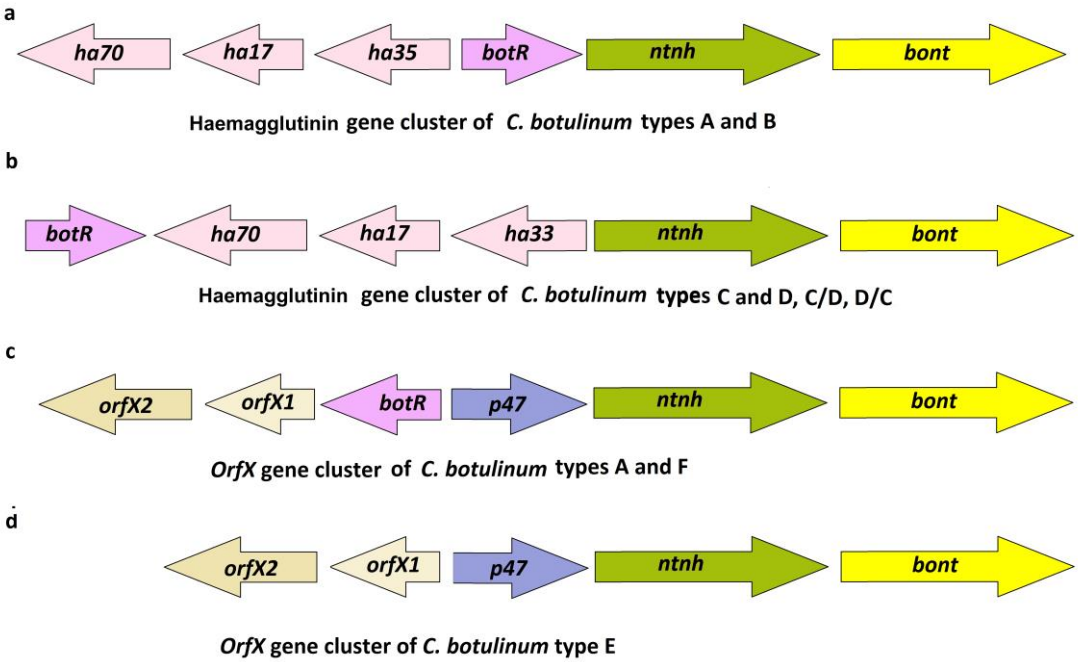

Figure 1. Structure of botulinum genes cluster in various toxin types: (**a**) haemagglutinin gene cluster of *C. botulinum* types A and B; (**b**) haemagglutinin gene cluster of *C. botulinum* types C and D, C/D, D/C; (**c**) *orfX* gene cluster of *C. botulinum* types A and F; (**d**) *orfx* gene cluster of *C. botulinum* type E.

Less genetic diversity was noted in the group of *C. botulinum* type B strains, which is an equally important etiological agent of food poisoning in adults and infants. Within this serotype, eight major subtypes of botulinum neurotoxins (B1–B8) were identified, showing differences in amino acid sequences of 1.6–7.1%. The ability to produce this serotype of botulinum neurotoxins is observed among *C. botulinum* group I, *C. sporogenes* strains, and *C. botulinum* group II strains. The genes encoding the production of all BoNT/B subtypes were organised in a haemagglutinin gene cluster, although depending on the subtype present, it may be located within the chromosome or may be carried by mobile extrachromosomal elements. The plasmid localisation of the *bont*/B genes has been described in multiple publications [39,40] by multiple authors, who, in their studies, indicated the possibility that the genes of each serotype B subtype may be present on plasmids, with genes of non-proteolytic strains associated with only the smallest of them [21,33]. Studies conducted to determine the genetic diversity of *Clostridium botulinum* strains have also enabled the identification of genes of subtype B5 forming bivalent strains, or found in isolates belonging to serotype A, in which the presence of the silent B gene was also detected [41]. Although BoNT/B shows less diversity between subtypes, the serotype manifests significant diversity within specific subtypes that can reach up to 2.1%. For example, this relationship is observed between the BoNT/B2, BoNT/B3 and BoNT/B6 subtypes, with amino acid sequence differences between them ranging from 1.6% to 1.9%. The intra-subtype genetic variability of BoNT/B, observed at a high level, may reflect the higher activity of genetic interactions occurring between strains producing this type of toxin over that observed within the other serotypes [7]. The same BoNT/B subtypes could be produced by *C. botulinum* and *C. sporogenes* (plasmid-borne subtypes BoNT/B1, B2, B6) [42].

The strains producing BoNT/F are much less frequently associated with cases of botulism; however, the literature data provide detailed evidence of the possibility of botulism

outbreaks caused by this type of toxin. Botulism caused by BoNT/F may also be associated with the co-occurrence of *bont/f* genes along with those of other serotypes [43,44].The amino acid sequences of the eight subtypes (F1–F8) vary by 36%, although within the strains representing each subtype, differences in amino acid residues can reach 0.1–1.7% [42,43,45]. Genes of all serotype F toxin subtypes are organised in the orfX cluster, located within the chromosome, or in the case of the *bont/f2* and *bont/f5* genes, located on extra-chromosomal elements. Among the subtypes represented, BoNT/F5 appears to be particularly divergent. Although the heavy chain of this subtype shows very strong similarity to the chain forming the BoNT/F2 subtype, the dissimilarity of BoNT/F5 is evidenced by a specific region located within the light chain, which is the enzymatic fragment of the toxin responsible for the cleavage of VAMP-2 proteins at a site distinct from all other BoNT/F [46–48]. *Clostridium botulinum* type F strains appear to be an ideal example of recombination events leading to the formation of new toxin subtypes. Analogous to the recombination event taking place within the A toxotype, in the case of botulinum neurotoxin type F, a new subtype such as F6 is also formed due to the following recombination between the *bont/f1* gene and the *bont/f2* gene. It is interesting that the created gene *bont/f6* is found within the strains belonging to group II, while the presence of the genes from which it arose is characteristic of proteolytic strains from group I, which indicates the important role of the phenomenon of horizontal gene transfer in the formation of genetic diversity [49,50].

In many regions of the world, the predominant serotype of botulinum neurotoxins associated with fish and seafood is considered to be BoNT/E, for the production of which non-proteolytic *C. botulinum* group II strains are responsible [51].Currently, 12 closely related subtypes of this serotype (E1–E12) have been identified, showing amino acid sequence differences of 0.9–5.9% [27]. The botulinum toxin type E genes are contained in a standard *orfX* gene cluster located in the chromosomal locus or carried by large plasmids [52]. Based on the results of comparative analyses of the gene sequences of each subtype, their close affinity was demonstrated, except for the BoNT/E9 and BoNT/E12 subtypes. BoNT/E9 is a particular standout among the BoNT/E subtypes, as sequence differences between it and other subtypes of this type can reach up to 10.1–11.8% of amino acid residues. Slight differences in amino acid sequences within some subtypes are observed between strains capable of producing *bont/e1*, *bont/e2*, *bont/e4–bont/e6,* and *bont/e11*. Among the E-type neurotoxin-producing strains, another example of the occurrence of recombination ability within the *bont/e6*, *bont/e7*, and *bont/e2* genes leading to the formation of BoNT/E8 is observed [7,51,53]. It should be emphasised that subtypes *bont/e4* and *bont/e5* were found exclusively in strains of neurotoxigenic *C. butyricum* [22].These strains were shown to carry the *bont/e* genes in their chromosomes and to possess a megaplasmid of sizes ranging from 600 to 800 kb. The mentioned subtypes *bont/e4* and *bont/e5* differ from each other by 5.1% in their amino acid composition.

Comparative analyses of the obtained complete genomes of group I and group II strains prove the strong similarity occurring within the group I strains, but the equally strong distance of group II strains from group I. Comparison of the obtained sequences of individual serotypes makes it possible to determine in detail the extent of the differences occurring between the toxinotypes and to distinguish individual subtypes among them [54]. Some of the literature sources indicate that the genetic diversity represented by *C. botulinum* strains of these groups could be associated with the geographic distribution of these microorganisms. Group I strains are presumed to occur in temperate terrestrial climates, while in the case of group II, particularly serotype E, their preference may also apply to the aquatic habitats of the northern hemisphere [25]. However, geographic distribution may result from both natural and man-made global movements of spores into different locations, followed by preferential persistence resulting from differing physiological parameters. It could be stated that the phenomenon of horizontal gene transfer is considered the main source of the formation of new variants of toxins. Gene transfer between strains leading to the formation of new serotypes of botulinum toxins and their subtypes is possible due to the

localisation of a highly differentiated botulinum locus of multiple genetic elements, which for these groups are either chromosomes or mobile extra-chromosomal elements [9,54].

3. Diversity of *Clostridium botulinum* Group III

3.1. General Characteristics of Clostridium botulinum Group III Strains

Group III includes proteolytic and non-proteolytic strains of *C. botulinum* capable of producing toxins of types C, D, CD, and DC. Related species include *C. novyi* type A (22) and *C. haemolyticum*. The optimum growth temperature is 40 °C. Botulism in animals is mainly caused by type C and D toxins and their mosaic variants (BoNT/CD and DC) (Table 2) [9,42,50]. BoNTs produced by group III *C. botulinum* are most commonly responsible for outbreaks of botulism in wild and farmed animals. BoNT type C causes sporadic outbreaks in cattle, but rarely in poultry or wild birds [9,55]. The mosaic strain BoNT/CD is responsible for most outbreaks of avian botulism, while BoNT/DC is the serotype most frequently detected in cattle [10,55]. Gram-positive, spore-forming anaerobes of the species *C. botulinum*, *C. novyi*, and *C. haemolyticum* are pathogens affect animals and humans [56]. Their presence in water reservoirs, soil, and water sediments, among other places, poses a pathogen threat to humans and animals [57,58]. Group III mosaic strains include the chimeric structures of types C and D [59,60]. *C. botulinum* types C and D, but not all of them, produce endotoxin C2 and exotoxin C3, which cause changes in the permeability of blood vessels [60,61]. Considering the higher resistance to toxin type D in birds, this may indicate different binding sites on the BoNT/C and BoNT/D nerve membranes [62–64].

Table 2. General properties of *Clostridium botulinum* group III [60,63,65,66].

Properties	*Clostridium botulinum* Group III
Proteolysis	+/−
Minimum pH required for growth	4.6
Optimum growth temperature	40 °C
Neurotoxins formed	C, D, CD, DC
Botulinum neurotoxin gene localisation	Prophage plasmid
Closely related species	*C. novyi, C. haemolyticum*

3.2. Genetic Diversity of C. botulinum Group III Strains

Extrachromosomal elements play a major role in animal botulism caused by *C. botulinum* group III. *C. novyi* causes gas gangrene, while *C. haemolyticum* causes bacterial haemoglobinuria [3]. These pathogens cause different diseases, but the responsible bacterial strains are related. The genetic relationship between the three species is proven by the similarity of 16S rRNA sequencing, and DNA–DNA hybridisation. The significant genetic similarities revealed by whole-genome sequencing gave rise to a new genospecies, which was designated *C. novyi sensu lato* [65]. The pathogenic features of these species, such as botulinum neurotoxin and *C. novyi* alpha toxin, are associated with a large plasmidome consisting of plasmids and circular prophages, determining the gene transfer in this taxon; however, *C. novyi sensu lato* has not yet been fully characterised [60,61]. Four main lineages (I–IV) have been distinguished, divided into IA and IB. IA and IB consist of *C. botulinum* strains II, III, and IV including *C. novyi* and II *C. haemolyticum*. Lineage IA pathogens produce BoNT/CD, while lineage IB pathogens predominantly produce BoNT/DC. The BoNT/C-producing strains are in lineage II, and the BoNT/D-producing strains belong to lineage IA, IB, and II [60,67]. Genomic analysis of multiple strains of *C. botulinum* group III isolated in Europe indicates high variability within the isolated strains [68]. The genetic components included in the group III *C. botulinum* cluster are genes encoding botulinum neurotoxin (*bont/C*, *bont/CD*, *bont/DC*, and *bont/D*), the haemagglutinin polycistrone (*ha70*, *ha17*, and *ha33*) and *botR*, as well as the non-toxic non-haemagglutinin (*ntnh*). *BotR* is a protein that regulates the expression of a toxin cluster [63] (Figure 1).

Differences in plasmids are used in the genotyping of samples, based on which the genetic diversity of *C. botulinum* strains can be determined. Based on the length and sequence variation of the flagellin (*fliC*) gene, five different *fliC* sequence variants (*fliC-I to V*) are distinguished in *C. botulinum* group III [63,65].

This gene can exist as a single gene or in copies, and is stored on the chromosome. Studies of *fliC* subtypes in samples from Europe suggest that *fliC-I* is the dominant gene in *C. botulinum* type C/D [65,68]. Samples with the *fliC-II*, *-III*, and *-V* genes were rarely detected, while the *fliC-IV* gene was found in D/C-positive samples. The lack of diversity in the flagellin gene of *C. botulinum* proves the clonal spread of C/D strains at different latitudes. The genetic relationship between the *fliC-I*, *-II* and *-III* genes is 87–92%, while *fliC-IV* from *C. botulinum* is less related to the other *fliC*s, and only has a 50% sequence similarity [68]. Moreover, recently, Fillo et al. [65], based on an analysis of Italian *C. botulinum* group III strains, described two new *fliC* variants: *fliC-VI* and *fliC-VII* (they were detected in four strains of serotype C/D), both of which were present in tandem. The presence of different *fliC* types in the same genome could be the result of two different horizontal gene transfer events. It shows that these genes evolved in a common, geographically limited environment but underwent different genetic evolutions. Analysing the review papers on research on *C. botulinum* group III, it can be concluded that this group is less genetically diverse than *C. botulinum* groups I and II. The instability of the prophage carrying the BoNT genes in laboratory cultures of *C. botulinum* strains poses a huge problem in the diagnosis of botulism outbreaks caused by group III. The determination of the *fliC* gene is useful in characterising the genomic diversity of group III *C. botulinum* [65,68].

Taking into account the dynamics of the disease and the difficulty in identifying pathogenic strains occurring in the environment, animal botulism is a serious economic and environmental problem.

4. Other BoNT-Producing Clostridia

The species of *C. botulinum* is historically defined as the group of bacteria able to produce botulinum toxins. Botulinum neurotoxins are produced by at least seven bacterial groups that meet all the criteria of distinct species [3,35,69–71].This definition does not include any phylogenetic relationship. The high diversity of this species is a reason for the division of *C. botulinum* into four physiologically distinct groups. Besides them, there are other species which can produce BoNTs, including some strains of *C. baratii*, which are able to produce BoNT/F (BoNT/F7), certain *C. butyricum* strains, with the ability to produce BoNT/E (BoNT/E4 and E5), and certain *C. sporogenes* strains, which can produce BoNT/B (BoNT/B1, B2, B6) [72–74]. The neurotoxic strains of each group or species are characterised by a high genetic variability. Based on multilocus sequence typing (MLST), several phylogenetic clusters are distinguished within each group [75,76]. Moreover, screening of the *bont* gene sequences in available genomic databases indicates that the presence of these genes is not merely an attribute of Clostridia [77–80].

BoNT homologs other than those of *Clostridium* have been identified in other anaerobes, and not only them. Sequences analogous to botulinum clusters were identified in *Weissella oryzae* (BoNT/I or BoNT/Wo)—i.e., Gram-positive bacteria that cannot produce spores. Similarly, a botulinum-like cluster was identified in *Chryseobacterium piperi* (referred to as BoNT/Cp1), which are Gram-negative bacteria that grow under aerobic conditions. An orfX-like cluster that contained a gene designated BoNT/En was observed in an *Enterococcus faecium* isolate derived from bovines. The mentioned BoNT/En was found to have a 38.7% identity with BoNT/X and was also related (23–25% identity) to the other BoNTs and the tetanus neurotoxin (TeNT). The toxic activity of these genes derived from strains other than Clostridium has not been demonstrated [72,77–80].

The historical designation of *C. botulinum* species is still utilized. Tests based on DNA–DNA hybridisation (DDH) [81] and comparative analysis of 16S rRNA genes [82,83], proved the high genotypic affinity of BoNT-producing Clostridia. Moreover, these tests have also proven that the genotypic and phenotypic groupings for these bacteria supported each

other [69]. The noticed similarity among *C. botulinum* group I strains achieves over a 70% similarity [3]. A close relation of some group I *C. botulinum* was shown to some *C. sporogenes* strains, while the other isolates seemed unrelated to this group. It was also reported that *C. botulinum* group II consisted of closely related strains. The strains of *C. botulinum* group III were noticed to be close relatives to each other and to *C. novyi* and *C. haemolyticum*. The *C. botulinum* group IV is also determined in the literature as a distinct species named *C. argentinense*, and determined to be related to some *C. subterminale* strains. Moreover, it was discovered with the use of 16S rRNA sequencing tools that the toxigenic and nontoxigenic strains within both *C. baratii* and *C. butyricum* were otherwise indistinguishable, which causes a problem in epidemiological investigations of botulism. 16S rRNA gene analysis is still a useful technique in bacterial evolutionary analysis; however, it is limited to a single-gene analysis and because of that, turns out to have less discriminatory power at lower taxonomic levels [3,49,50].

Whole-genome sequencing (WGS) is becoming increasingly common and provides the maximum genetic resolution for phylogenetic and systematic classification. WGS analysis methods can target specific features of the genome and use different evolution tools to generate advanced information on bacterial biology [3,84].

The classification of Clostridial strains able to produce BoNTs as a distinct species is still under discussion [69,71]. Smith et al. [3] suggested the use of the group designation by a Latin binomial nomenclature. These authors suggested the following renaming of known designations: proteolytic *C. botulinum* group I into *Clostridium parabotulinum*; (ii), nonproteolytic group II organisms should be referred to as *C. botulinum*; and (iii) the BoNT/C- and BoNT/D-producing organisms should be redetermined "*C. novyi sensu lato*", because of their documented close relationship with *C. novyi* [60]. The other remaining BoNT-producing species (*C. argentinense*, *C. baratii*, *C. butyricum*, and *C. sporogenes*) retain their current species names [3].The proposed reclassification does not rely solely on the expression of botulinum neurotoxins. The authors further propose that BoNT-producing bacterial strains could be further identified using the toxin type or subtype, such as "*C. parabotulinum* BoNT A1" or "*C. baratii* BoNT F", to distinguish between toxic and nontoxic members [3].

5. Conclusions

The diversity of BoNT-producing Clostridia, as described in this study, is still a worrisome problem for specialists exploring the evolutionary and taxonomic classification of *C. botulinum*. It is also a problem for diagnosticians and laboratory staff conducting epidemiological investigations of botulism cases. The phenotypic heterogeneity of BoNT-producing Clostridia also results in a lack of the selective media for the isolation of all strains able to produce botulinum toxins. Moreover, the occurrence of strains (nontoxigenic) phenotypically similar in all groups (I–IV) makes differentiation possible, mainly by showing the toxigenic ability. The currently unresolved BoNT-producing Clostridia reclassification is still under discussion by microbiologists. New genetic tools such as WGS indicate horizontal gene transfer and the occurrence of botulinum gene clusters which are not limited only to *Clostridium* spp., but also to Gram-negative aerobic species. The diversity of BoNT-producing Clostridia needs further exploration and elaboration with new genetic-based tools for a better understanding and a much more effective epidemiological investigation of botulism cases, in order to assure the safety of humans and animals.

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