Chapter 1

Botulinum Toxins, Diversity, Mode of Action, Epidemiology of Botulism in France

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Abstract

Botulinum toxins (BoNTs) are the most potent toxins and are responsible for botulism, which is a neurological disease in man and animals. Botulism is characterized by flaccid paralysis and inhibition of secretions. BoNTs are produced by distinct clostridial species including *Clostridium botulinum* that consist in four physiological and genetic groups, atypical strains of *C. baratii* and *C. butyricum*. Recently, nonclostridial bacteria have been found to synthesize BoNTs. The particularity of BoNTs is to associate with nontoxic proteins to form large-size complexes that are resistant to acidic pH and protease degradation of the digestive tract. BoNTs are divided into 10 types based on neutralization by specific antisera and into more than 40 subtypes according to their sequence variations. All BoNTs retain a common core structure and mode of action, which consists in the inhibition of neurotransmitter release, notably acetylcholine. Human botulism occurs in three main forms: foodborne botulism, botulism by intestinal colonization including infant botulism, and wound botulism. In France, type B foodborne botulism is the most prevalent form, resulting from the traditional consumption of pork products such as home-made cured ham. Albeit less frequent, human botulism is still present in France including diverse types and origins.

Keywords: botulism, botulinum toxin, *Clostridium botulinum*, flaccid paralysis

1. Introduction

Botulinum toxins (BoNTs) are the most potent toxins among bacterial, animal, and plant toxins. Indeed, the lethal activity as tested in laboratory animals by determining the lethal dose 50% (LD50) is the lowest compared to that of all other toxins. Because of its extreme lethal potency,

BoNTs are considered as the greatest threat of toxin weapon and are classified as Category A threat agent by the Centers for Disease Control and Prevention Select Agent Program [1]. In the natural conditions, BoNTs are responsible for a neurological disease in man and animals, botulism, which is characterized by flaccid paralysis and inhibition of secretions. Outbreaks of animal botulism are worldwide distributed and cause important economic losses, notably in cattle and farmed birds. Human botulism is much rarer than animal botulism, but it is a severe disease often fatal without treatment. Human botulism is the most severe food poisoning, and botulism surveillance by health and food authorities is performed in most of the countries in order to rapidly identify and withdraw contaminated foods and also to address recommendations to industrials and consumers regarding hygiene and food preservation practices. However, the paralytic effects of BoNTs are used in the treatment of numerous diseases including muscle hyperactivity such as dystonia, strabismus, limb spasticity, sphincter dysfunction, or hypersecretion (hyperhidrosis, hypersialorrhea, and drooling in neurodegenerative diseases), but also in the treatment of pain and in cosmetology. BoNTs are largely used as therapeutic drugs and are one of the drugs that have the most numerous medical indications [2, 3].

Botulism was described in the second part of the eighteenth century and at the beginning of the nineteenth century by Steinbuch (1817) and Kerner (1817–1822). Both described a particular form of foodborne poisoning due to ingestion of a "sausage poison." An increased number of fatal food poisoning cases occurred at the end of eighteenth century in the southwest German region of Wurtenberg due to a decline in hygiene of rural food productions subsequently to Napoleonic war perturbations. The paralytic disease was mainly associated to the consumption of blood sausages and was termed "sausage poisoning." This disease was also known as "Kerner's disease" and the name "botulism" was coined later in the second half of the nineteenth century from the Latin word *botulus* meaning sausage [4]. Interestingly, albeit the nature of this poisonous substance was not known, Kerner envisioned the possibility of using this poison to treat diseases associated with an overactive nervous system, including muscle hyper-contraction and hyper-secretion of body fluids. Then in 1895, Emile Pierre Marie Van Ermengem, a professor of Microbiology at the University of Ghent and who had worked in the laboratory of Robert Koch in Berlin, isolated an anaerobic-sporulating microorganism that he had named *Bacillus botulinus*, from the ham, the intestine, and spleen of one of the victims of a severe outbreak of botulism which occurred in a small Belgian village (Ellezelles). The term *Clostridium* was then used to designate anaerobic spore-forming bacteria in contrast to *Bacillus* which was reserved for aerobic or facultative anaerobic bacteria. Subsequently, the other types of botulism with the identification and characterization of BoNTs and bacterial organism producers were reported [5].

2. Botulinum toxins

2.1. Structure

BoNTs share a common structure. They are synthesized as a precursor protein (about 150 kDa), which is inactive or weakly active. The precursor that does not contain a signal peptide is

released from the bacteria by a yet unknown mechanism. The precursor is proteolytically activated in the extra-bacterial medium either by *Clostridium* proteases or by exogenous proteases such as digestive proteases in the intestinal content. The active neurotoxin consists of a light chain (L, about 50 kDa) and a heavy chain (H, about 100 kDa), which remain linked by a disulfide bridge. The structure of BoNTs shows three distinct domains: L-chain containing α-helices, and β-strands and including the catalytic zinc-binding protease motif (His-Glu-X-X-His), the N-terminal part of the H-chain forming two unusually long and twisted α-helices, and the C-terminal part of the H-chain consisting of two distinct subdomains (H_{CN} and H_{CC}) involved in the recognition of the receptor. While the three domains are arranged in a linear manner in BoNT/A and BoNT/B, both the catalytic domain and the binding domain are on the same side of the translocation domain in BoNT/E. This domain organization in BoNT/E might facilitate a rapid translocation process [6–16].

The overall sequence identity at the amino acid level between BoNTs ranges from 34 to 97%. Several domains are highly conserved which account for the common mode of action of these toxins including the central domains of L chains containing the catalytic site and the N-terminal half of the H-chains that is involved in the translocation of the L-chain into the cytosol. Thus, a similar mechanism of internalization of the intracellular active domain into target cells is shared by all the clostridial neurotoxins. In contrast, the C-terminal half of H-chain, mainly the H_{CC} subdomains, is the most divergent [17–19]. This accounts for the different receptors recognized by the clostridial neurotoxins (see subsequent text).

2.2. Botulinum complexes

BoNTs associate by non-covalent bounds with non-toxic proteins (ANTPs) produced by *C. botulinum* to form large complexes of different sizes (medium M or 12S, large L or 16S, large-large LL or 19S), also known as progenitor toxins (**Figure 1**). Botulinum complexes are synthesized in *in vitro* cultures and in naturally contaminated food or intestinal content. The complexes are stable at acidic pH, but dissociates at alkaline pH (≥pH 7) (reviewed in [20]).

All BoNT complexes contain the non-toxic non-hemagglutinin (NTNH) protein. NTNH is highly conserved. Two main classes of botulinum complexes can be distinguished based on their composition in additional proteins, the botulinum complexes containing hemagglutinins (HAs, including HA33, HA17, and HA70) (HA-BoNT complexes) and those possessing OrfX (including OrfX1, Orfx2, and OrfX3) and P47 proteins (OrfX-BoNT complexes) [17, 20–23]. The composition and structure of HA-BoNT complexes have been extensively investigated, whereas the OrfX-BoNT complexes are still poorly characterized. The stoichiometry can vary according to the strain, culture conditions (culture media, temperature, period of culture, etc.), and the method of complex preparation [20]. *C. botulinum* A produces three types of botulinum complexes M (medium), L (large), and LL (large/large) [24, 25], whereas the other *C. botulinum* types yield only M and L complexes.

The 12S or M complex results from the association of a BoNT molecule together with a NTNH at a 1:1 ratio [26]. L HA-BoNT complexes of *C. botulinum* A, B, and C consist of BoNT/NTNH/

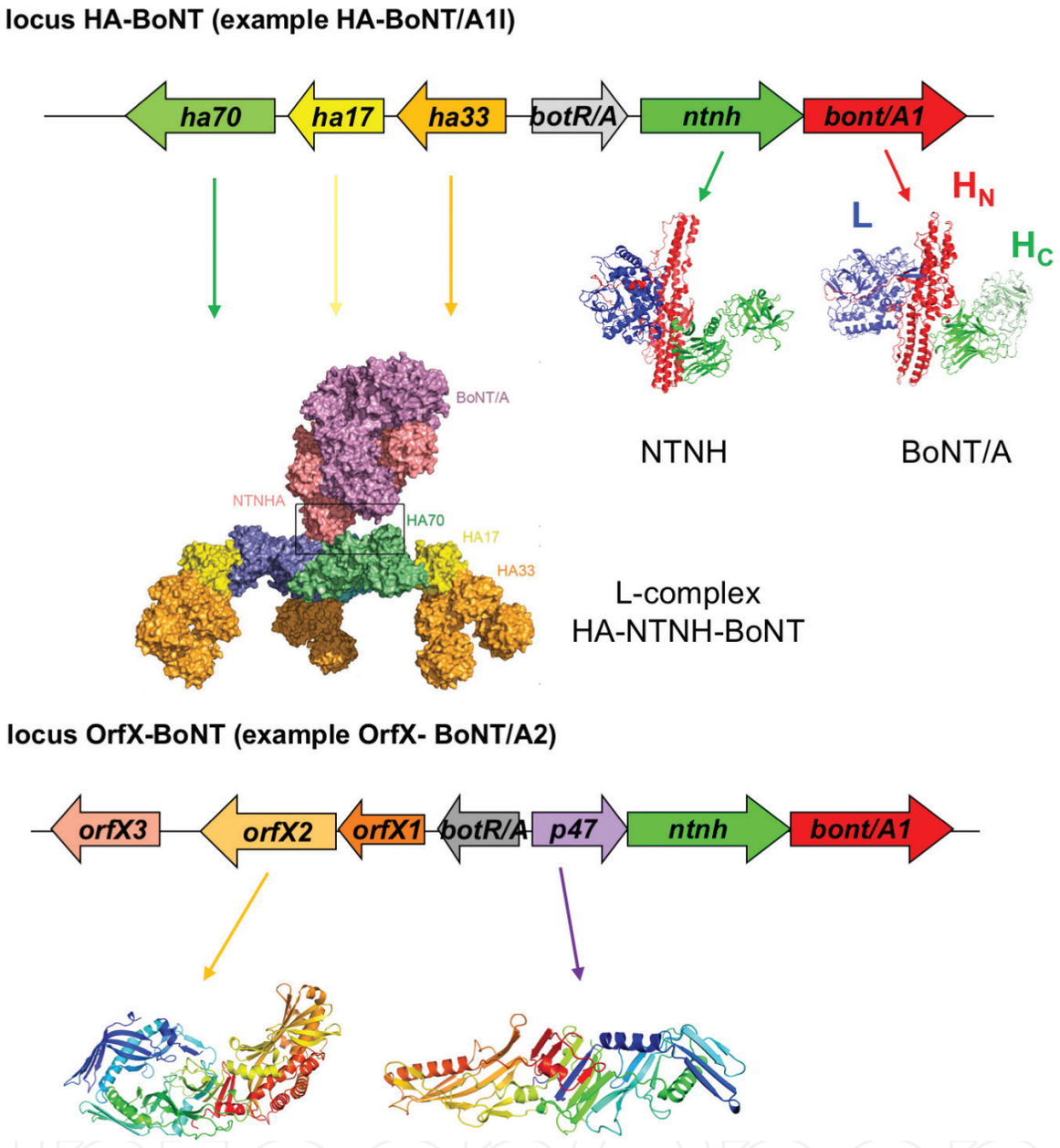

Figure 1. Genetic organization of *ha-bont* and *OrfX-bont* locus and structure of BoNT/A, NTNH, HA-NTNH-BoNT/A complex, OrfX2 and P47. L, BoNT light chain; H_N, N-terminal part of BoNT heavy chain; H_C, C-terminal part of BoNT heavy chain. The structure of OrfX-BoNT complex is not yet known.

HA70/HA17/HA33 in a molar ratio of 1:1:2:2:3 as determined by gel electrophoresis and densitometry [27]. The HA33 are likely to be at the periphery of the complex. Using stain electron microscopy and single particle averaging analysis, a stoichiometry of 1:1:3:3:6 was deduced. L HA-BoNT complexes of *C. botulinum* A or B share a similar ovoid structure with three flexible appendages, whereas the M OrfX-BoNT complex from *C. botulinum* E lacks these arms [25]. Further crystal structure analysis supports the 14 subunit complex of L HA-BoNT/A

[24]. The LL complex produced only by *C. botulinum* A is presumed to be a dimer of the L complex linked by an oligomeric HA33 consisting of four molecules and thus containing two molecules of BoNT/A [21, 22, 28]. However, a refined analysis of LL complex showed a composition of 1 BoNT/A, 1 NTNH/A, 5–6 HA17, 4–5 HA23, 3–4 HA48, and 8–9 HA34 (HA23 and HA48 resulting from HA70 nicking) [29].

The composition and organization of OrfX-BoNT complexes from *C. botulinum* A1, A2, and E is poorly characterized [30]. *C. botulinum* A2, A3, A4, A6, A7, A8, E, and F only produce M complexes devoid of hemagglutinating activity, and *C. argentinense* produces only L complex [22]. *M. botulinum* complex type A2 only contains BoNT/A2 and NTNH, although P47, Orfx2, and OrfX3 are produced in the culture supernatant, but not OrfX1 or in very low amount [31]. OrfX1 has been detected in botulinum complex type E but not type F, whereas neither OrfX2 or P47 has been evidenced in both toxinotypes [32, 33]. The structure of OrfX2 and P47 showed a similarity with TULIP family of proteins which are lipid-binding proteins [34].

NTNHs from the different *C. botulinum* types retain a high identity level (76–83.5%) and are the most conserved proteins among the botulinum complex components [17, 20]. NTNH/A, NTNH/C, and NTNH/D contain a cleavage site within their N-terminus, yielding 15 kDa N-terminal and 115 kDa C-terminal fragments. NTNH/A is split into 13 and 106 kDa fragments by cleavage between Pro144/Phe145 [35]. NTNH/C and NTNH/D are cleaved at Lys127 by a trypsin-like protease with 7–13 amino acids removed from the N-terminus of the 115 kDa fragment that subsequently results in three proteins starting at Leu135, Val139, or Ser141 [36]. NTNH is only cleaved in the 12S (M) complexes from *C. botulinum* types A, C, and D, but not in the L (16S) or LL (19S) complexes. The cleaved NTNH molecules constituted a nicked structure since the two fragments still remain together after NTNH purification [36]. In contrast, NTNH/E and NTNH/F show an identical deletion of 33 residues in the corresponding region of NTNH/A, NTNH/C, and NTNH/D encompassing the cutting site, and NTNH/G possesses a slightly different sequence in this region. It is presumed that the processing and additional sequence of NTNH in *C. botulinum* A, C, and D are responsible for forming 12S-, 16S-, and 19S-sized complexes. The inability of *C. botulinum* E and F to form L complexes may result from the absence of hemagglutinin (HA) or other related proteins that bind to NTNH and from the absence of a putative binding site in NTNH/E and NTNH/F [17, 22].

BoNT and NTNH share a weak amino acid sequence identity (~20%), but both proteins retain a common structure (**Figure 1**). NTNH associates with BoNT by non-covalent bonds in a pH-dependent manner to form an interlocked compact M complex, which is resistant to acidic pH and protease degradation, whereas each protein separately is sensitive to proteolysis [23, 25, 26, 37]. Thereby, NTNH is a non-toxic protein which acts as a chaperone protein to protect BoNT. NTNH does not contain the catalytic HExxH motif, but another zinc-binding motif, KCLIK, at the same position. Indeed, NTNH binds one zinc atom per each molecule but exhibits no proteolytic activity [38]. This strongly supports that all NTNH and BoNT variants derive from a common ancestor gene by duplication and subsequent independent reshuffling.

HA33-35 is the most abundant hemagglutinin component of the HA-BoNT complexes. Type A HA35 binds to oligosaccharides containing galactose-β1-4glucose-*N*-acetyl-D-neuraminic acid (Galβ1-4GlcNAc) [39]. Thereby, hemagglutination induced by L and LL type A botulinum complexes is mainly mediated through HA35 binding to erythrocyte membrane glycolipids and glycoproteins containing Galβ1-4GlcNAc [39, 40]. Similarly, HA33 from types C and D botulinum complexes binds to paragloboside on Galβ1-4GlcNAc and also sialylglycolipids (GM3), as well as sialoglycoproteins (sialosylparagloboside) on the *N*-acetyl-D-neuraminic acid-α2-3-galactose-β1 motif [41]. The importance of HA33-35 in hemagglutination is also supported by monoclonal antibody studies. Type C-specific monoclonal antibodies against HA33 inhibit hemagglutination, contrary to those against HA50 and HA17 [42]. However, type C HA70 and its derivative HA50 recognize sialosylparagloboside and GM3 at the *N*-acetyl-D-neuraminic acid-α2-3-galactose-β1motif in erythrocyte membranes, like the corresponding L botulinum complex. Thus, HA50 could also be involved in hemagglutination [41]. HA35 purified from *C. botulinum* A is predominantly a dimeric, β-sheet protein in aqueous solutions. In *C. botulinum* A, five N-terminal amino acids are removed from HA35, but similar posttranslational modification has not been observed in HA33 from *C. botulinum C*. The significance of HA35 processing on its biological activity is not known [43]. It was first discovered that the 31 C-terminal amino acids, which contain a predicted carbohydrate recognition site, play an essential role in hemagglutination [44]. The structure of type C HA33 shows two β-trefoil domains consisting of a six-stranded, antiparallel β-barrel capped on one side by three β-hairpins. Related β-trefoil structures bind to oligosaccharides and are found in other proteins, including various lectins like the ricin B-chain, cytokines, trypsin inhibitor, xylanase, as well as the C-terminal part of BoNTs. Type A and B HA35 retain a similar structure related to the carbohydrate-binding site of ricin, a plant toxin. It is worth noting that Asp263 and Asn285 of HA35, which are conserved in the lactose-binding site for ricin B chain, are critical for carbohydrate binding [45–47]. HA17 also adopts a β-trefoil fold, whereas HA70 forms a three-bladed propeller-like trimer with a pore located at the center of the trimer [48, 49].

More recently, a novel function has been attributed to HA complexes consisting in the disruption of intercellular junctions between intestinal epithelial cells. HAs recognize E-cadherin, which plays a crucial role in basolateral junction. The interaction of HAs with E-cadherin is species and isoform specific. Thereby, HAs directly bind to the extracellular domain of (epithelial) E-cadherin, but not of (neural) N-cadherin, nor (vascular endothelial) VE-cadherin. Type B HAs specifically bind to human, bovine, and mouse E-cadherin but not to that of rat and chicken [50]. This is consistent with the fact that botulism type B is common in humans and is rarely observed in chickens. Type A BoNT complexes also recognize human E-cadherin, whereas type C BoNT complexes do not [50]. The combination of HAs (HA33, HA17, and HA50/70) organized in complex is required for the optimum binding to E-cadherin, whereas individual HAs do not interact with E-cadherin. HAs assemble in a threefold symmetric hetero-dodecameric structure, and the whole HA complex exhibits the highest affinity to E-cadherin. The minimal HA complex interacting with E-cadherin consists of domain 3 of HA70 (Pro-378-Asn-626), one

molecule of HA17, and two HA33 molecules [51]. HAs bind to the distal extracellular domain (EC1) of E-cadherin near the cadherin trans-dimer interface [50]. Thus, the HA-binding sites to carbohydrates and E-cadherin are functionally and structurally distinct [52].

The structures of OrfX2 and P47 are unrelated to that of HAs and show that they belong to the tubular lipid-binding (TULIP) protein superfamily. Thereby, OrfX1 and OrfX2 have been found to bind to phosphatidylinositol [34]. In contrast to HAs, OrfX proteins and OrfX complexes have not been reported to interact with E-cadherin or to alter the intestinal epithelial barrier. This raises the question whether OrfX complexes are involved in BoNT passage through epithelial barriers. In *C. botulinum* strains type E, F, and some type A, BoNTs form complexes lacking HAs and are responsible for foodborne botulism, which is as severe as the classical type A and B botulism.

2.3. Botulinum toxin gene organization

The BoNT and ANTP genes are clustered in close vicinity in a DNA fragment which is called the botulinum locus. BoNT and ANTP genes are organized in two operons. The operon localized in the 3′ part of the botulinum locus contains *ntnh-bont* which is highly conserved in all *C. botulinum* strains. In *C. botulinum* types E and F and certain *C. botulinum* A strains, this operon contains an additional gene called *p47* encoding a 47-kDa protein (**Figure 1**). The second operon consists of the *ha* or *orfX* genes and is localized upstream of the *ntnh-bont* operon. The *ha* or *orfX* operon is transcribed in opposite orientation to that of the *ntnh-bont* operon and shows more strain variation. In *C. botulinum* B, C, D, and some A strains, the *ha* operon consists of three genes (*ha70*, *ha17*, and *ha33*). The *ha* genes of *C. botulinum* G only comprise *ha*17 and *ha*70. The *ha* genes are missing in the non-hemagglutinating toxinotypes A1, A2, A3, A4, E, and F and an *orfX* operon (*orfX1*, *orfX2*, *orfX3*) instead of *has* lies upstream of the *ntnh-bont* operon [53–55] (**Figure 1**). It is worth noting that a same *bont* gene can be inserted into a HA or a OrfX locus. However, *bont*/*A1* is the only gene which has been found in either of the two types of botulinum locus.

The *bot*R gene encoding for an alternative sigma factor is a positive regulator of the *ntnh-bont* and *ha* operons. *botR* is localized differently according to the *C. botulinum* strains, either between the *ntnh-bont* and *ha* operons or upstream of the *ha* operon. This gene is missing in *C. botulinum* E and toxigenic *C. butyricum*.

Most of *C. botulinum* strains produce only one type of BoNT, and the botulinum locus is present in a single copy on the genome. However, some rare strains synthesize two different BoNTs: BoNT/A-BoNT/B, BoNT/A-BoNT/F, and BoNT/B-BoNT/F producing strains have been isolated. The two neurotoxins are usually produced in different proportions. Thus, in Ba and Bf strains, BoNT/B is produced 10 times more than BoNT/A and BoNT/F. Some clostridial strains contain silent neurotoxin genes. Several *C. botulinum* A strains isolated from foodborne and infant botulism contain a silent *bont*B gene. These strains are noted A(b). These strains contain two distinct botulinum loci. One *C. botulinum* strain has been found to harbor three botulinum loci containing *bontA2*, *bontF4*, and *bontF5* [56].

The botulinum loci are distributed on different genetic elements, including chromosome, plasmid, or phages depending on the species and strain of Clostridia. In *C. argentinense*, *C. botulinum* type B, mainly in subtype B1, bivalent, and non-proteolytic strains, and in some *C. botulinum* A strains, the botulinum loci are located on large plasmid. For example, in the bivalent strain Ba657, the two botulinum loci, locus A and locus B, are harbored by the same plasmid (pCLJ) separated by approximately 97 kbp. Similarly, the neurotoxin genes, *bontB* and *bont/f*, from one Bf strain are located on a same plasmid (pBf), which is very much related to pCLJ. In *C. botulinum* type E and neurotoxigenic *C. butyricum* strains, the *bont/E* loci are mainly on the chromosome. However, in three *C. botulinum* E strains from 36, *bont/E1* is located on a large plasmid. In *C. botulinum* C and D, it has been clearly evidenced that BoNT is encoded by bacteriophages (reviewed in [57]).

The location of botulinum locus within chromosome or plasmid seems to occur not at random but at specific sites. Indeed, in strains from group I or II, whose genome sequencing is available, five specific sites of botulinum locus integration have been identified. *orfX-bont/A2*, *orfX-bont/A1*, and *orfXbont/F* loci are located in the *ars* operon, which contains three to five genes involved in arsenic reduction. *orfX-bont/A1* and *orfX-bont/F*loci share a similar integration site at the 5′ end of the *ars* operon, whereas *orfx-bont/A2* locus is inserted between two copies of *arsC* gene. *ha-bont/A1* and *ha-bont/B* loci, which contain a recombinant *ntnh* gene type A and type B strains, are found in the *oppA/brnQ* operon, encoding for extracellular solute-binding protein and branched chain amino acid transport proteins, respectively. This operon is lacking in non-proteolytic *C. botulinum* type B, *C. botulinum* type E, and *C. butyricum* type E strains. The third integration site is the *rarA* gene in group II and V strains, which contains the *orfX-bont/E* locus in *C. botulinum* type E and *C. butyricum* type E strains. *rarA* encodes a resolvase protein involved in recombination or insertion events of transposons. Interestingly, the botulinum E locus is inserted in the same codon [102] of *rarA* gene in both *C. botulinum* type E and *C. butyricum* type E strains, and the inserted botulinum locus contains an additional intact *rarA* gene [58]. The trivalent strain A2f4f5 contains the *orfX-bont/A2* and *orfX-bont/F4* loci located in the chromosome at the *arsC* and *pulE* (type II secretion system protein E) genes, respectively [56]. In *C. botulinum* F, the *orfX-bont/F6* locus has been found in a new chromosomal integration site *topE* [59].

Two specific sites of botulinum locus location have been identified on plasmids from group I strains, one contains *orfX-bont/A3*, *orfXbontT/A4* from Ba strain, or *orfX-bontF* from Bf strain, and the second harbors the *ha-bont/B* locus from *C. botulinum* B1 strain or bivalent Ba4 or Bf strains. The *ha-bont/B4* locus in nonproteolytic strains is located on a plasmid different from those of group I strains. However, the downstream flanking region of the HA-npB locus contains an IS element, a transposon-associated resolvase, and a site-specific recombinase [58]. It is worth noting that *C. botulinum* plasmids harboring *bont* genes such as pCLJ, pCLL, and pCDC-A3 (related to pCLK) are transferable by conjugation into a group I *C. botulinum* strain [60].

The toxin gene location on the various genetic elements chromosome including mobile genetic elements (plasmid, phage) supports horizontal *bont* gene transfer between *Clostridium* strains and also between clostridia and non-clostridia strains. In addition, insertion sequences or transposases genes have been identified in the flanking regions of most of botulinum loci. These genetic elements are associated to gene mobility and contribute to the extreme plasticity of these BoNT-producing bacteria. It is worth noting that most of the insertion sequences are partially modified, suggesting a very ancient process of gene mobility and subsequent DNA

rearrangement or modification (review in [61–63]. Indeed, the BoNT-producing clostridia strains are heterogeneous and do not form a unique bacterial species. The *C. botulinum* species has been designed on the basis of only one phenotype, the production of a paralytic toxin. However, it appeared that they show variable physiological and biochemical properties and they have been divided into four physiological groups (I–IV). Moreover, it was shown that atypical strains of other *Clostridium* species than *C. botulinum* such as *C. baratii* and *C. butyricum* were able to synthesize a BoNT related to those produced by *C. botulinum*. Genetic analysis including whole genome sequencing confirmed the distinction of the multiple groups and species of BoNT-producing bacteria [64–66]. More recently, *bont* genes have been found in the genome of non-clostridial species (see subsequently and in **Table 1**). Clostridia and other bacteria, which contain *bont* genes, are from the environment, raising intriguing question which

Botulinum toxin type	**BoNT/A**	**BoNT/B**		**BoNT/E**	**BoNT/F**	
Subtypes	A1, A2, A3, A4, A5, A6, A7, A8	B1, B2, B3, B5, B6, B7, B8	B4	E1, E2, E3, E6, E7, E8, E9, E10, E11, E12	F6	F2, F2, F3, F4, F5, F8
Enzymatic substrate (cleavage site)	SNAP25 (QR)	VAMP1, 2, 3 (QF)		SNAP25 (RI)	VAMP1, 2, 3 (QK) F5: VAMP2 (LE)	
Neurotoxin-producing bacteria	*C. botulinum* group I		*C. botulinum* group II			*C. botulinum* group I
Main physiological properties	Proteolytic Lipase Temperature growth: minimum 10–12°C, optimum 30–40°C Highly heat-resistant spores		Non-proteolytic Lipase Growth at low temperature: minimum 2.5–3°C, optimum 25–30°C Moderate heat-resistant spores			Idem group I
Botulism	Human, occasionally animal					

Botulinum toxin type	**BuNT/E**	**BaNT/F**	**BoNT/C**	**BoNT/D**	**BoNT/G**	**BoNT/H**
Subtypes	E4, E5	F7	C/D, D/C		G	H or F/A or H/A
Enzymatic substrate (cleavage site)	SNAP25 (RI)	VAMP2 (QK)	SNAP25 (RA) Syntaxin (KA)	VAMP1, 2, 3 (KL)	VAMP1, 2, 3 (AA)	VAMP1, 2, 3 (LE)
Neurotoxin-producing bacteria	*C. butyricum*	*C. baratii*	*C. botulinum* group III		*C. argentinense* group IV	*C. botulinum* group I
Main physiological properties	Non-proteolytic Glucidolytic	Lecithinase	Non-proteolytic Lipase Temperature growth 37–40°C		No protease No lipase	Group I
Botulism	Human, animal not reported		Animal, very rare in human		No natural case reported	Human

Botulinum toxin type	**BoNT/X**	**BoNT/I or BoNT/Wo**	**BoNT/J or eBoNT/J or BoNT/En**	**Cp1 toxin (BoNT homolog)**	**BoNT/Ba** **BoNT/Bf** **BoNT/Ab** **BoNT/Af** **BoNT/A(B)** **BoNT/A2F4F5**
Subtypes	Bivalent BoNT/B2-BoNT/X				
Enzymatic substrate (cleavage site)	VAMP1, 2, 3, 4, 5 Ypkt6 (RA)	VAMP2 (WW)	VAMP2 (DL) SNAP25, 23 (KD) Syntaxin (MD)	?	
Neurotoxin-producing bacteria	*C. botulinum* strain 111 group I	*Weissella oryzae*	*Enterococcus faecium*	*Chryseobacterium piperi*	Bivalent and trivalent *C. botulinum* strains Group I
Main physiological properties	Group I	Gram-positive bacillus Nonspore-forming Facultative anaerobic	Gram-positive cocci	Gram-negative bacillus Strictly aerobic Non-spore forming	Group I
Botulism	Infant botulism Japan	No natural botulism case reported			Human botulism

Table 1. Botulinum toxin (BoNT) types, subtypes, and their main properties including enzymatic substrates and cleavage sites, as well as the neurotoxin-producing microorganisms with their main physiological properties and involvement in naturally acquired botulism.

are the molecular mechanism and selection pressure of neurotoxin gene transfer and which are the advantages conferred by genes encoding paralytic toxins for higher organisms. It is worth noting that whether *bont* genes can be mobilized in diverse bacteria, their transfer is mainly restricted to *Clostridium* species.

2.4. Botulinum toxin diversity

BoNTs form a family of diverse proteins which share the common property to induce a flaccid paralysis. Historically, it was found that these toxins can be antigenically distinguished. On the basis of neutralization of the biological effects on small rodents with specific antisera, seven BoNT types (A–G) were identified. Each type-specific antitoxin only neutralizes the corresponding BoNT type. The differences in amino acid sequences range from 37.2 to 69.6% [19]. In 2013, a novel eighth BoNT type called H (or F/A or H/A) has been described from a bivalent *C. botulinum* strain isolated from an infant botulism case and producing both BoNT/B2 and

BoNT/H [67, 68]. It was claimed that this novel BoNT type was not neutralized by the already known anti-BoNT sera justifying its assignment to a novel type. More recently, genome analysis showed the presence of a related *bont* sequence in an OrfX locus in *C. botulinum* type B strain 111 which also produces BoNT/B2. BoNT/X retains a low sequence identity with the other BoNT types, and it is not recognized by the antibodies against the previous BoNT types [69]. Moreover, *bont*-related sequences have been identified in non-clostridial bacteria including Gram-positive/Gram-negative, spore-forming/non-spore-forming, anaerobic/aerobic bacteria such as *Weissella oryzae* (BoNT/Wo or BoNT/I) from fermented rice [70], an *Enterococcus faecalis* strain (BoNT/J, or BoNT/En, or eBoNT/J) isolated from a cow [71, 72], and *Chryseobacterium piperi* (Cp1) from sediment [73] (**Table 1**). This suggests a complex and long evolution of *bont* genes, the ancestral source of which still remains mysterious [63, 74, 75].

An increased sequencing of *bont* genes and/or whole genome of individual strains shows that each BoNT type contains variable isoforms based on sequence variations. Therefore, BoNT types are divided into subtypes which were initially defined as displaying at least 2.6% amino acid sequence difference [76]. However, some BoNT subtypes, notably from types B and E, only exhibit 0.9–2.1% amino acid sequence difference, but they were assigned to distinct subtypes according to phylogenetic clade analysis. Among more than 500 BoNT sequences, 41 subtypes have been identified [19] (**Table 1**).

Amino acid sequence variations might impact BoNT biological functions including receptor recognition, the efficiency of entry into cells and persistence, recognition by monoclonal antibodies, and enzymatic activity. For example, BoNT/A2 has been shown to enter more efficiently into neuronal cells than BoNT/A1 and to have a higher affinity for its receptor [77, 78]. BoNT/A2 induces a faster paralysis than BoNT/A1/A4/A5, and BoNT/A3 has a shorter duration of effect [79]. In addition, BoNT/A2 retains a lower immunogenicity [80]. Thus, BoNT/A2 would be a more efficient therapeutic agent than BoNT/A1 [81–83]. BoNT/A8 binds less efficiently to gangliosides embedded into a membrane and has a lower enzymatic activity than BoNT/A1 [84]. BoNT/B1 binds to synaptotagmin 1 and 2 receptors, whereas BoNT/B2 only recognizes synaptotagmin 2 [85]. In contrast to the BoNT/F subtypes which cleave VAMP1 and VAMP2 at QK site, BoNT/F5 uses a distinct cleavage site (LE) [86] (**Table 1**). Monoclonal antibodies against BoNT/B differently recognize the subtypes BoNT/B1 to BoNT/B5 [87]. Similarly, monoclonal antibodies against BoNT/A recognize and/or neutralize the distinct BoNT/A subtypes with variable efficiently [88, 89].

3. Mode of action

BoNTs enter by oral route (foodborne botulism) or are produced directly in the intestine (infant or intestinal botulism) subsequently to a *C. botulinum* intestinal colonization. BoNTs are able to transcytose across the intestinal mucosa (review in [90] or can pass through the paracellular way with the help of HA complexes (review in [91]). After diffusion into the extracellular fluid and blood stream circulation, BoNTs target motoneuron endings.

Each type of BoNT and TeNT recognizes specific receptors on demyelinated terminal nerve endings, mainly through the H_{CC} subdomain. BoNT/A/C/E/F exploit the three isoforms of

the vesicle protein SV2 as specific receptors, while BoNT/B and /G bind to synaptotagmin I or II [92–98]. BoNT/C and BoNT/D interact with gangliosides (GD_{1b}, GT_{1b}) and phosphatidylethanolamine, respectively, by their H_{CC} subdomain [99]. Gangliosides (GD_{1b}, $GT_{1b,}$ and GD_2) and SV2A/B/C also mediate the entry of BoNT/D into neurons, but by a different mechanism than that used by BoNT/A and BoNT/E [100, 101]. The role of H_{CN} subdomain, which may interact with phosphatidylinositol phosphates [102], is still unclear. The co-presence of the *ad hoc* ganglioside(s) and protein receptors likely facilitates the identification of cell subset targeted by BoNTs at very low concentrations encountered in the physiological medium during the disease. At higher concentrations, binding to the protein receptor is likely sufficient for mediating toxin binding. Indeed, the number of cell types affected by these toxins expands with increasing toxin concentrations. Therefore, BoNTs can target numerous neurons but not all, as well as non-neuronal cells at high concentrations, inhibiting the release of various compounds.

Neurotoxin bound to its receptor is internalized by receptor-mediated endocytosis. Acidification of the vesicle lumen triggers a conformational change of the neurotoxin and subsequent translocation of the L chain into the cytosol. In addition, the disulfide bond between the two chains has a crucial role in the translocation process [103–106]. Then, the L chain refolds in the neutral pH of the cytosol. Cytosolic translocation factors such as β-COPI are possibly involved in this mechanism, as it has been found for diphtheria toxin [107–110].

L chains of all clostridial neurotoxins are zinc-metalloproteases that cleave one of the three members of the SNARE proteins. BoNT/B, D, F, and G attack synaptobrevin (or VAMP), BoNT/A and E cleaves SNAP25, and BoNT/C1 cut both SNAP25 and syntaxin. The cleavage sites are different for each neurotoxin. The cleavage of SNARE proteins occurs only when disassembled. Since VAMP, SNAP25, and syntaxin play a major role in the regulated fusion of synaptic vesicles with the plasma membrane at the release sites, their cleavage induces a blockade of the neurotransmitter exocytosis.

SNAP25 cleavage by BoNT/A or BoNT/E alters SNAP25 and synaptotagmin interaction, thus strongly reducing the responsiveness to Ca^{++} of exocytotic machinery [111–114]. Indeed, the removal of the nine C-terminal amino acids of SNAP-25 by BoNT/A deeply disrupts the coupling between Ca^{2+} sensing and the final step in exocytosis [112]. Truncated SNAP-25 can behave as a dominant-negative mutant upon the exocytotic process, suggesting that after BoNT/A treatment, the block of release is due to both functional elimination of SNAP-25 and accumulation of the cleavage product which competitively inhibits exocytosis [115–117]. In contrast, the blockade of exocytosis by BoNT/E is only due to the elimination of functional SNAP-25 and not to the production of competitive antagonists of SNARE complex formation. Indeed, the inhibition of exocytosis by BoNT/E can be rescued by supplementing the C-terminal portion of SNAP-25 removed by the toxin [118–120]. Truncation of SNAP-25 by BoNT/E destabilizes the four-helix bundle of the SNARE complex [118, 119], and SNAP-25 truncated by BoNT/E is not retained by syntaxin [121].

VAMP cleavage abolishes the interaction of VAMP with the adaptor protein AP3 and affects synaptic vesicle recycling *via* early endosomes [122]. The blockade of neuroexocytosis likely results from a disturbance of synaptophysin-1/VAMP2 interaction and of coupling between

detecting Ca^{2+} and synaptic vesicle triggering [112]. Since the synaptic vesicles docked with unproductive complexes cannot fuse or undock, they stay at the fusion sites (with slightly increased numbers), irreversibly plugging the fusion sites that would normally accommodate intact vesicles. This progressively reduces the number of active release sites to which exocytosis can occur. When VAMP is cleaved by BoNT/B or /G, the VAMP portion (~20 amino acids) remaining in the synaptic vesicle membrane does not contain interaction sites for the other SNAREs. Therefore, the synaptic vesicle membrane is no longer linked to a SNARE complex, and fusion with the plasma membrane cannot occur. When VAMP is cleaved by BoNT/D or /F, the C-terminal fragment remaining in the vesicle membrane is long enough to anchor the synaptic vesicle to the SNARE complex, but fusion cannot occur because the SNARE complex cannot transit into the thermally stable four-helix bundle.

BoNT/C cleaves both syntaxin-1 and SNAP-25, but *in vitro* cleavage of SNAP-25 by BoNT/C occurs with a low efficiency (~1000-fold difference) versus cleavage by BoNT/A or /E [123, 124]. This raises the following question: which of the two targets is involved in BoNT/C neuroexocytosis blockade?

Although the physiological properties induced by the cleavage of either VAMP, SNAP25, or syntaxin are not equivalent at the neuromuscular junctions, all the clostridial neurotoxins cause a blockade of the regulated neurotransmission, which varies in intensity and duration according to each neurotoxin type.

4. Epidemiology of botulism in France

4.1. Main clinical forms of human botulism

Several clinical forms of botulism are distinguished according to the mode of acquisition of BoNT and/or neurotoxigenic bacteria. Foodborne botulism occurs after the consumption of food contaminated by *C. botulinum* in which sufficient amount of toxin has been produced. Foods stored for a sufficient period such as home-made canned foods, home-fermented products, or commercial minimally heated and chilled foods are at risk of botulism. Ingestion of preformed BoNT in food is responsible for botulism by intoxication. Foodborne botulism is the main form in adults.

Infant botulism results from the ingestion of *C. botulinum* spores that germinate, multiply, and produce BNT in the infants intestinal content. A low contaminating dose of 10–100 *C. botulinum* spores is sufficient to induce intestinal colonization and production of BoNT in the intestinal tract, since the intestinal microbiota, which has an inhibitory effect on the growth of *C. botulinum* in the digestive tract, might be not sufficiently developed or non-functional in babies under 1 year.

Botulism by intestinal colonization occasionally occurs in adults. Predisposing factors consist in factors that perturb or modify the microbiota composition such as antibiotherapy, intestinal surgery 1 or 2 weeks prior consumption of a food contaminated by *C. botulinum* spores, chronic inflammation, and necrotic lesions of the intestinal mucosa, which might support the intestinal growth of *C. botulinum*.

Wound botulism is caused by *C. botulinum* growth and toxin production in a contaminated wound or a lesion-like tetanus. Wound botulism is much rarer than tetanus. Drug users by injection who handle contaminated materials or drugs are notably at risk of wound botulism.

Inhalational botulism is very rare. A few cases have been reported in laboratory workers preparing concentrated BoNT by continuous centrifugation and in two patients who inhaled cocaine (review in [125]). BoNT dissemination by aerosol has been considered as a possible bioterrorist attack.

Iatrogenic botulism is a recent novel form of botulism which develops subsequently to toxin overdoses for a therapeutic or a cosmetic purpose or to a hematological dissemination of toxin at a therapeutic dose.

4.2. Botulism in France

4.2.1. Foodborne botulism

The first case of human botulism was reported in 1875. The disease was very rare until the second war, since the consumption of canned foods was not traditional in France. This not excludes that the disease was underestimated or misdiagnosed. Only 24 cases were recorded from 1875 to 1936 and eight from 1936 to 1940 [126, 127]. In contrast, in the USA where the first industrial canned foods treated by heating were developed, large outbreaks of botulism occurred from 1899 to 1954, 514 outbreaks, 1350 cases including 861 deaths [127]. However, the incidence of botulism was very high in France during the Second World War. About 500 outbreaks and 1000 cases were estimated between 1940 and 1944 [126]. Food deprivation and poor quality of home-made food preservation were the main factors responsible for this high incidence. Type B botulism predominated, and most of the incriminated foods (93%) were from pork meat, notably cured ham [126].

The incidence of botulism decreased after the Second World War (**Figure 2**). Albeit no systematic recording of botulism cases was performed during this period, only a few outbreaks were identified, mainly in the Anaerobe Laboratory of Pasteur Institute. During the period 1956–1970, a 22.4 annual mean of botulism cases was observed based mainly on the detection of BoNT and/or *C. botulinum* in the incriminated food. Since 1971, the diagnosis of human botulism was improved by the detection of BoNT in patient's serum [128]. Thus, the incidence of botulism increased to an annual mean of 76 cases per year within the 1971–1977 periods. This corresponds to a better survey of human botulism, but possibly also to the introduction of novel foods or modes of food preservation at risk of botulism, such as minimally heated and chilled foods or vacuum-packed chilled foods. Type B was the most frequent type of botulism (96.9% of outbreaks), and home-made or small-scale preparation of ham was the main source of botulism (63.7% of the outbreaks). However, commercial products or restaurant meals were incriminated or suspected in 30 (7.2%) outbreaks and were responsible for six deaths [129].

From 1986, human botulism is subjected to mandatory declaration to health authorities and since 1998 botulism declarations are coordinated by the national organism of disease survey InVS (Institut national de Veille Sanitaire called Sante Publique France since 2016). Since 1980, human botulism decreased, but every year, 10–40 cases are recorded in France. Home-made

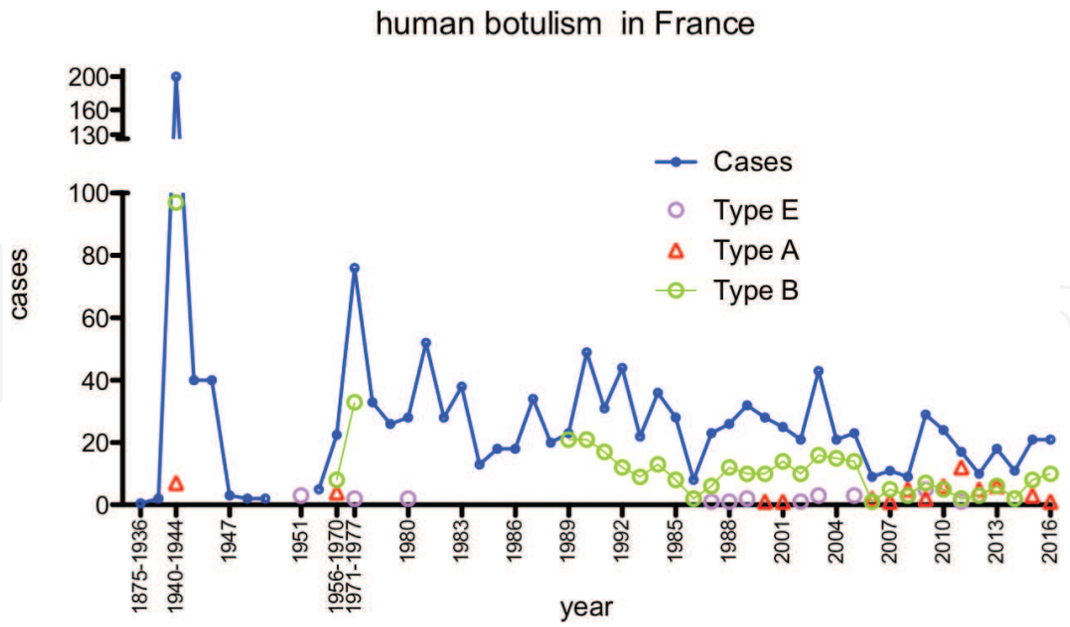

Figure 2. Incidence of human botulism in France, 1875–2016. The numbers indicated in the period ranges 1875–1936, 1940–1944, 1956–1970, and 1971–1977 are the annual mean values. Total cases (blue), type B botulism (green), type A botulism (red), type E botulism (purple), according to [127, 129–135, 147–151]. The two outbreaks of *C. baratii* type F botulism in 2014 and 2015 are not reported in the figure.

preserved foods are less used but remain traditional in certain areas of France. Type B is predominant, and cured ham and pork meat preparations are the main origin of human botulism [130–135]. Pork is often a healthy carrier of *C. botulinum* type B and rarely develops botulism symptoms [136, 137]. Insufficient or inadequate sanitary measures in the preparation of pork meat and absence or insufficient heat treatment are the main risk factors. However, more diverse types and sources of botulism occurred since 2000 (**Figure 2**). Botulism type A, which was extremely rare in France, was more frequent since 1997 notably from canned vegetables [132]. Severe outbreaks of botulism type A occurred in 2008, one from commercial "enchiladas" containing chicken meat, vegetables, and cereal cake, and another one from home-made pumpkin jam [134]. During the period 2010–2012, botulism type A was predominant (23 cases out of 51) and resulted from diverse origins: home-made canned beans, commercial tapenades (olives, dried tomatoes), commercial pasta, and imported home-made eggplant preparation [135]. Only one outbreak of botulism type A (from home-made pheasant pie) was recorded within 2013–2016 [133].

Botulism type E is extremely rare in France. An outbreak of botulism type E occurred in 2009 after the consumption of smoked and vacuum-packed fish which was bought a few days ago in Finland. The fish was from Canada and was processed in Finland [134]. In 2010, an unusual case resulted from a ham contaminated with *C. botulinum* B and a novel *C. botulinum* E subtype (E12) [135, 138]. It was hypothesized that marine salt used for the ham preparation could be the origin of the contamination.

Two atypical outbreaks of botulism type F occurred in 2014 and 2015. Both were *Clostridium baratii* F7 botulism. The first outbreak included two patients, one of which was totally paralyzed and showed a very high level of BoNT/F in the serum (400 mouse lethal doses/ml),

but she recovered after 46 days in intensive care unit. The origin of this outbreak was not determined [139]. The second outbreak concerned three patients who have had their meal at the same restaurant on the same day. A Bolognese sauce prepared 2 days in advance with industrial ground meat was the common food. A sample of the ground meat in the refrigerator of the restaurant was contaminated with *C. baratii* F7 [140, 141].

4.2.2. Infant botulism

Infant botulism is a rare form of botulism in France. Only 15 cases were identified from 2004 to 2016. They resulted from group I *C. botulinum* type A or B and from different subtypes: A1(B), A2, Bf, B2, and B5. All food samples investigated for the origin of contamination were negative. In two outbreaks, an environmental contamination was strongly suspected. In one of them, the baby's home was close to a reconstruction work. *C. botulinum* B was identified in stool sample of the baby and soil samples of the reconstruction work [133]. Another 2-month-old baby developed botulism with several relapses over a period of 4 months. *C. botulinum* A2 was isolated from stool samples all along the course of the disease. The particularity of this strain was its high resistance to penicillins and to metronidazole [142]. It was the first report of an antibiotic-resistant clinical *C. botulinum* strain. The baby's home was at proximity of a thermal power station that intermittently released sprays of vapor and smoke/dust and that was suspected to be the origin of the contamination.

4.2.3. Wound botulism and inhalation botulism

Only one case of wound botulism was identified from 1995 to 2017. In 2008, a patient had an open fracture of the leg abroad and was hospitalized again when back to France for persistent suppuration of the wound. He developed a type B botulism during the course of the second hospitalization [134]. Wound botulism in injection drug users was reported in several European countries and North America, but no such case was reported in France [143, 144]. However, in 2007, two patients who inhaled cocaine developed a botulism type B [145].

4.2.4. Botulism diversity in France

Albeit botulism is a rare disease, human botulism is identified every year in France. Foodborne botulism is the main form of botulism in France. Historically, home-made cured ham or pork products were the main source of type B botulism. During the recent period, home-made preserved foods including ham are no longer commonly used, but human botulism is still present albeit to a lower extent than in the past. Thereby, the origin of botulism is more diverse including imported products, commercial minimally heated foods, or meals at a restaurant. The diversity of BoNT types and subtypes as well as of the BoNT-producing clostridia reflects the diverse origins of human botulism in France [146].

5. Conclusion

BoNTs form a wide diverse family of toxins which target specific neurons, leading to the inhibition of release of neurotransmitters, notably acetylcholine. At least 10 BoNT types and more

than 40 subtypes have been identified. All BoNTs retain a common core structure and mode of action which consists in the inhibition of neurotransmitter release, notably acetylcholine, leading to flaccid paralysis. However, they use distinct pathways and distinct intracellular targets to drive the blockade of neurotransmission. Indeed, the distinct BoNT types recognize different neuronal receptors such as different sets of gangliosides and different membrane proteins (SV2 isoforms, synaptotagmin) and target either one of the three SNARE proteins at distinct cleavage sites. In addition, BoNTs are produced by diverse bacterial species, mainly from the *Clostridium* genus which are environmental bacteria. This raises the questions about the evolution and selection pressure involved in the emergence of so diverse bacterial proteins with unique function on the neurological system of higher eukaryotic organisms. BoNTs are responsible for severe neurological disease in man and animals which are still present in some countries such as in France. However, they also constitute valuable therapeutic tools for the treatment of diverse neurological dysfunctions. The increased number of medical indications of BoNTs contrasts with the high poisonous activity of these toxins. The wide BoNT diversity offers a panel of natural variants which can be adapted to specific applications.

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