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Cedric Woudstra

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Présentée et soutenue publiquement par

Cédric WOULDSTRA

Date 21 Mars 2016

***Clostridium botulinum*, from toxin and flagellin genotyping to Whole Genome Sequencing:**

An insight into the genetic diversity of human and animal botulism associated Clostridia

Directeur de thèse : **Dr Patrick FACH**

Jury

M. Henri-Jean Boulouis , Professor, Veterinary College of Alfort, France	President
M. Patrick Fach , Professor, French agency for food safety, Maisons-Alfort, France	PhD supervisor
M. Frederic Carlin , Research director, National Institute for Agricultural Research, Avignon, France	Reporter
M. Daniel Gillet , Professor, Atomic Energy Commission, Gif sur Yvette, France	Reporter
M. Pierre Wattiau , Professor, Veterinary & Agrochemical Research Centre, Brussels, Belgium	Examiner
M. Hervé Morvan , Veterinary doctor, LaboCEA, Ploufragan, France	Examiner
M. Martin Dorner , Doctor, Robert Koch Institute, Berlin, Germany	Examiner

***Clostridium botulinum*, from toxin and flagellin genotyping
to Whole Genome Sequencing:**

**An insight into the genetic diversity of human and animal
botulism associated Clostridia**

Cédric WOULDSTRA

Anses – Laboratoire de sécurité des aliments - Plateforme IdentityPath

*Thèse de Doctorat, Université Paris Est, Ecole Doctorale ABIES,
spécialité sciences du vivant*

Title: *Clostridium botulinum*, from toxin and flagellin genotyping to Whole Genome Sequencing.

Keywords: *C. botulinum*, detection, typing, *bont*, flagellin, WGS

Abstract: *Clostridium botulinum* is the etiologic agent of botulism, a deadly paralytic disease that can affect both human and animals. Different bacteria, producing neurotoxins type A to G, are responsible for the disease. They are separated into different groups (I to VI) on the basis of their phenotypical and biological characteristics. My PhD work was structured by the different projects I was involved in, which were related to *C. botulinum* detection and typing, like BIOTRACER and AniBioThreat European projects, the French national CBRN program, or the French NRL mandate for avian botulism. The main transversal objective I followed lead me to develop new methods to trace back the origin of *C. botulinum* contamination, in case of a deliberate, accidental or naturally occurring botulism outbreak. I investigated the flagellin genes as potential genetic targets for typing *C. botulinum* Group I-II and III, responsible for human and animal botulism respectively. Flagellin genes *flaA* and *flaB* showed *C. botulinum* Group I and II strains to cluster into 5 major groups and up to 15 subgroups, some being specific for certain geographical areas, and *flaB* being specific to *C. botulinum* type E. Flagellin *fliC* genes of *C. botulinum* Group III showed to cluster into five groups, with *fliC*-I and *fliC*-IV associated to type C/D and D/C respectively, being not discriminative enough to differentiate highly genetically related strains. I also studied the prevalence of mosaic toxin genes in *C. botulinum* Group III in animal botulism, mainly in poultry and bovine. The results brought out the mosaic toxin types C/D and D/C to be predominant in the samples investigated throughout Europe. Finally, I explored the full genome sequences of 14 types C/D and 3 types D/C *C. botulinum* Group III strains, mainly originating from French avian and bovine botulism outbreaks. Analyses of their genome sequences showed them to be closely related to other European strains from Group III. While studying their genetic content, I was able to point out that the extrachromosomal elements of strains type C/D could be used to generate a genetic ID card. Investigation of Crispr typing method showed to be irrelevant for type C/D, due to a deficient Crispr-Cas mechanism, but deserve more investigation for type D/C. The highest level of discrimination was achieved while using SNP core phylogeny, which allowed distinguishing up to the strain level.

Titre: Clostridium botulinum, du génotypage de la toxine, en passant par les flagellines jusqu'au séquençage de génomes.

Mots clés: C. botulinum, détection, typage, bont, flagellin, WGS

Résumé: Le botulisme est une maladie nerveuse, commune à l'homme et aux animaux, due à l'action de la neurotoxine botulique produite par Clostridium botulinum. Il existe 7 types de toxines dénommées A à G. Les bactéries capables de produire cette toxine se différencient en six groupes sur la base de leurs caractéristiques phénotypiques et biologiques. Mon projet de doctorat s'est organisé autour de plusieurs projets de recherche visant à développer des méthodes de détection et de typage du germe et de sa toxine (projets Européens BIOTRACER et AniBioThreat ; projets NRBC-bio ; mandat du LNR botulisme aviaire en France). Lors de mes recherches j'ai concentré mon travail sur le développement de méthodes capables de suivre et remonter à la source d'une contamination, qu'elle soit délibérée, accidentelle ou naturelle. Afin d'y parvenir j'ai investigué les gènes codant pour les flagellines de C. botulinum groupe I à III, responsables du botulisme humain et animal. L'analyse des gènes flaA et flaB a mis en évidence 5 groupes majeurs et 15 sous-groupes, certains étant spécifiques de régions géographiques. FlaB s'est montré spécifique de C. botulinum type E. Les gènes flagellines fliC, spécifiques à C. botulinum du groupe III, se divisent en 5 groupes, avec fliC-I et fliC-IV associés aux types mosaïques C/D et D/C. J'ai étudié la prévalence des souches productrices de toxine de type mosaïques chez les volailles et les bovins. Les résultats montrent que les types C/D et D/C sont majoritaires en Europe. Enfin, j'ai séquencé le génome de 17 souches associées à des cas de botulisme animal en France (14 types C/D et 3 types D/C). Leur analyse montre que ces souches sont très proche génétiquement, entre elles et avec les souches Européennes. Grâce à ces données j'ai mis en évidence un large contenu extra chromosomique dans les souches C/D, qui peut être utilisé pour créer une carte d'identité génétique. D'autre part, l'étude des séquences Crisprs à des fins de typage ne s'est pas avérée suffisamment résolutive, du fait de système Crispr-Cas déficient chez les souches C/D. Enfin, un très haut degré de discrimination a été atteint par typage SNP, qui a permis de distinguer jusqu'à l'origine de chaque souche.

Acknowledgments

This PhD study was carried out at the Laboratory for Food Safety of the French agency for food, environmental and occupational health safety (Anses), in Maisons Alfort, in the IdentityPath platform.

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Patrick Fach, my supervisor: you fought even harder to defend my candidature as a PhD. We have been working a long way since the beginning of the platform, from an empty lab to a fully functional and well-equipped platform. You believed in me and gave me a fantastic opportunity to work as an engineer first, and then as a PhD student. Under your guidance I was able to develop my skills and my scientific mind. I owe you so much; I hope that I gave you back as much input as you gave me.

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My beloved mermaid: your arrival in my life opened my eyes on a totally different field that I'm so happy to explore with you. You gave me an extraordinary motivation to look forward.

If I would have to retain one important thing out of my PhD:

Everything is possible when you work together with others.

Collaborating and sharing make difficult things achievable.

We is my I.

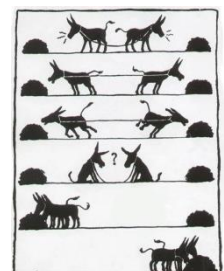


Table of content

Abstract	3
Acknowledgments	5
List of publications	10
Abbreviations	10
List of figures and tables	13
Foreword	15
Introduction	17
Chapter I Botulism and Clostridium botulinum	19
I.1. Botulism, a historical overview	19
I.2. <i>Clostridium botulinum</i> , the organism.....	19
I.3. Botulism, the disease	22
I.3.1. Human botulism	22
I.3.2. Animal botulism	27
I.4. The botulinum neurotoxin	33
I.4.1. BoNT Characteristics	33
I.4.2. Mechanism of action.....	35
I.4.3. BoNT serotypes and subtypes	37
I.4.4. <i>bont</i> gene.....	40
I.4.5. <i>bont</i> genetic locus	43
I.4.6. BoNT uses.....	44
I.5. <i>Clostridium botulinum</i> genome.....	46
I.5.1. Genomic characteristics of <i>C. botulinum</i> Group I	49
I.5.2. Genomic characteristics of <i>C. botulinum</i> Group II	50
I.5.3. Genomic characteristics of <i>C. botulinum</i> Group III	51
I.5.4. Genomic characteristics of <i>C. botulinum</i> Group IV, <i>C. butyricum</i> Group V and <i>C. baratii</i> Group VI.	54
I.5.5. Mobile genetic elements and horizontal gene transfer	57
Chapter II Diagnosis	58
II.1. Clinical symptoms.....	58
II.2. Botulism laboratory confirmation.....	58
II.2.1. Biological detection methods.....	59
II.2.2. Immunological tests	61

II.2.3.	Endopeptidase Assays	63
II.2.4.	DNA-based detection methods	64
	Chapter III Genotyping methods used to characterise <i>C. botulinum</i> diversity.....	68
III.1.	Enzyme restriction characterisation methods: PFGE and AFLP	68
III.2.	Sequence type characterisation.....	70
III.2.1.	Ribotyping.....	70
III.2.2.	MLVA	70
III.2.3.	Rep-PCR	71
III.2.4.	RAPD	71
III.2.5.	MLST	71
III.2.6.	DNA microarrays	72
III.2.7.	Flagellar typing	73
III.2.8.	Whole genome sequencing (WGS).....	75
	Objectives and Results	79
	Chapter IV Aim of the thesis	81
	Chapter V Results obtained during the course of the PhD.....	83
V.1.	<i>C. botulinum</i> Group I and II diversity investigation using flagellin <i>flaA</i> and <i>flaB</i> genes.....	83
V.1.1.	Genetic diversity of the flagellin genes of <i>Clostridium botulinum</i> groups I and II.	84
V.2.	<i>C. botulinum</i> Group III detection and characterization studies	93
V.2.1.	Investigation of <i>C. botulinum</i> Group III <i>bont</i> types throughout Europe.	93
V.2.2.	Characterization of <i>C. botulinum</i> Group III strains using flagellin as genetic target... 103	
V.3.	Investigation of <i>C. botulinum</i> Group III French isolated strains by Next Generation Sequencing (NGS).	117
V.3.1.	Draft genome sequences of 17 French <i>Clostridium botulinum</i> Group III strains.....	118
V.3.2.	Analysis by Whole Genome Sequencing (WGS) of 17 French strains of <i>Clostridium botulinum</i> from Group III.	123
	General Discussion and Conclusion.....	137
	Chapter VI Discussion	139
VI.1.	Genetic signatures for characterizing strains of <i>C. botulinum</i> from Groups I to III.....	139
VI.2.	Whole genome sequencing and SNP genotyping of <i>Clostridium botulinum</i> Group III strains from France	142
VI.3.	Main achievements	145
VI.4.	Next perspectives	146
	Chapter VII General Conclusion	148

***Related publications*151**

***References*.....153**

List of publications

This thesis is based upon the work contained in the following papers:

Article N°1:

Woudstra C, Lambert D, Anniballi F, De Medici D, Austin J, Fach P. Genetic diversity of the flagellin genes of *Clostridium botulinum* groups I and II. Appl Environ Microbiol. 2013 Jul;79(13):3926-32.

Article N°2:

Woudstra C, Skarin H, Anniballi F, Fencia L, Bano L, Drigo I, Koene M, Băyon-Auboyer MH, Buffereau JP, De Medici D, Fach P. Neurotoxin gene profiling of *Clostridium botulinum* types C and D native to different countries within Europe. Appl Environ Microbiol. 2012 May;78(9):3120-7.

Article N°3:

Woudstra C, Le Maréchal C, Souillard R, Bayon-Auboyer MH, Anniballi F, Auricchio B, De Medici D, Bano L, Koene M, Sansonetti MH, Desoutter D, Hansbauer EM, Dorner MB, Dorner BG, Fach P. Molecular gene profiling of *Clostridium botulinum* group III and its detection in naturally contaminated samples originating from various European countries. Appl Environ Microbiol. 2015 Apr;81(7):2495-505.

Article N°4:

Woudstra C, Le Maréchal C, Souillard R, Bayon-Auboyer MH, Mermoud I, Desoutter D, Fach P. Draft Genome Sequences of 17 French *Clostridium botulinum* Group III Strains. Genome Announc. 2015 Oct 1;3(5).

Article N°5:

Woudstra C, Le Maréchal C, Souillard R, Bayon-Auboyer MH, Mermoud I, Desoutter D, Fach P. New Insights into the genetic diversity of *Clostridium botulinum* Group III through extensive genome exploration. Frontiers Microbiol. 2016 May 19; Epub.

Abbreviations

AFLP	Amplified fragment length polymorphism
<i>bont</i>	botulinum neurotoxin gene
BoNT	botulinum neurotoxin protein
<i>C. botulinum</i>	<i>Clostridium botulinum</i>
CDC	Centers for Disease Control and Prevention
CDS	Coding DNA sequence
DGAL	General Directorate for Food Safety
ELISA	Enzyme-linked immunosorbent assay
FlaVR	Flagellin variable region
FliC	Flagellin gene
HA	Hemagglutinin
HC	Heavy chain
IS	Insertion sequence
Kbp	Kilo base pair
kDa	KiloDalton
LC	Light Chain
MBA	Mouse bioassay
MLST	Multi-Locus Sequence Typing
MLVA	Multiple Loci Variable number tandem repeat Analysis
NGS	Next Generation Sequencing
NRC	National Reference Centre
NRL	National reference laboratory
NTNH	non-toxic non-hemagglutinin
ORF	Open reading frame
PCR	Polymerase Chain Reaction
PFGE	Pulse-field gel electrophoresis

RAPD	Randomly amplified polymorphic DNA analysis
Rep-PCR	Repetitive element sequence-based PCR
SNAP	Synaptosomal-associated protein
SNARE	Soluble N-éthylmaleimide-sensitive-factor Attachment protein Receptor
SNP	Single nucleotide polymorphism
SRA	Sequence Read Archive
VAMP	Vesicle-associated membrane protein
VNTR	Variable number of tandem repeat sequences
WGS	Whole genome sequencing

List of figures and tables

Tables

Table 1. Characteristics of the six physiologically and phylogenetically distinct Clostridia producing botulinum neurotoxin.	21
Table 2 : Botulinum toxin and animal species affected.	28
Table 3. BoNT type and subtype produced by the different Clostridia Group species.	38
Table 4. Nucleotide and amino acid identity comparisons of the <i>bont</i> genes or BoNT proteins.	38

Figures

Figure 1. <i>Clostridium botulinum</i> bacteria.	20
Figure 2. Human form of botulism.	24
Figure 3. Flaccid paralysis characteristic of botulism in cow and turkey.	29
Figure 4. The maggot cycle.	30
Figure 5. Botulinum toxin structure.	34
Figure 6. Structure of the precursor toxin complex.	35
Figure 7. Mechanism of action of botulinum toxin.	36
Figure 8. Phylogenetic tree of BoNT toxin sequences.	39
Figure 9. Genetic relatedness of <i>bont</i> type A to G encompassing the mosaic type H-FA.	40
Figure 10. Genetic relatedness of <i>bont</i> subtypes diversity: <i>bont/A1</i> to <i>A8</i> , <i>bont/B1</i> to <i>B8</i> , <i>bont/C</i> , <i>C/D</i> , <i>D</i> , <i>D/C</i> , <i>bont/E1</i> to <i>E12</i> , <i>bont F1</i> to <i>F7</i> , <i>bont/G</i> , <i>bont/H-FA</i>	41
Figure 11. Alignment of <i>C. botulinum</i> Group III <i>bont/C</i> , <i>C/D</i> , <i>D</i> and <i>D/C</i> sequences.	42
Figure 12 : <i>Bont</i> cluster arrangements.	43
Figure 13. Phylogenetic overview of representative <i>C. botulinum</i> genomes.	47
Figure 14. Phylogenetic whole genome comparison of BoNT producing Clostridia and non-BoNT toxigenic related species.	48
Figure 15. Phylogenetic comparison of <i>C. botulinum</i> Group I and <i>C. sporogenes</i> genomes.	50
Figure 16. Phylogenetic comparison of <i>C. botulinum</i> Group II genome sequences.	51
Figure 17. Average whole genome similarity of <i>C. novyi sensu lato</i> genomes.	53
Figure 18. Comparative analysis of <i>C. novyi sensu lato</i> plasmids.	54
Figure 19. 16S RNA phylogenetic relatedness between <i>C. botulinum</i> type G and <i>C. subterminale</i> . .	55
Figure 20. Whole genome phylogenetic relatedness between <i>C. butyricum</i> Group V type E and non-BoNT producing <i>C. butyricum</i>	56

Figure 21. 16S gene phylogenetic relatedness between <i>C. baratii</i> Group VI type F and non-BoNT producing <i>C. baratii</i> .	56
Figure 22. Glove box used for <i>C. botulinum</i> cultivation.	59
Figure 23 : Bead based assay principle.	62
Figure 24. Endopept-MS assay principle.	64
Figure 25. PFGE genotyping of <i>Clostridium botulinum</i> associated with type B infant botulism in Japan.	69
Figure 26. Genomic indexing of 43 strains of Group II <i>C. botulinum</i> .	73
Figure 27. Flagellar arrangement.	74
Figure 28. Circular genome representation of <i>C. botulinum</i> ATCC 3502.	77

Foreword

When I look back at the path that brought me to the PhD level, I would say it was not an easy play. I am from this generation of child who grew with video games in their hands. I remember my parents fighting for me and my siblings to play outside. But for me, a video game was more like reading an interactive book, where you can help the main character to achieve a heroic and spectacular goal, even saving an imaginary world! For a kid like me, with an overrunning imagination, it helped developing skills like patience and perseverance (when you try for the thirty-sixth time to find your way out of a labyrinth without falling into a trap...), curiosity (when you are allowed to explore an entire new world), cleverness (when you have to solve particularly though enigma), thoroughness (when you have to collect plenty of items to upgrade your character), qualities which turned to be fundamental while I was graduating as a student, and then afterwards in my scientific life. After finishing my master degree I tried unsuccessfully three times to become a PhD student. The first time I was not chosen among the candidates, the second time the project did not get the funding, and the third time I tried to apply for a PhD in the U.S. but the faculty cut down the number of foreigner applicants due to a larger number of U.S. student applicants this year. Despite that, I succeeded in integrating the professional world as an Engineer in molecular biology, keeping in mind my personal PhD project, waiting for another opportunity.

It turned out that I was later recruited at Anses, the French agency for food, environmental and occupational health & safety, in the Laboratory for Food Safety, by Patrick Fach, in the IdentityPath platform. The Laboratory for Food Safety is in charge of taking action in case of chemical and biological hazards that can affect food quality and safety. The IdentityPath platform was created to answer public health issues for detection and typing of pathogenic bacteria involved in food or animal safety, by developing molecular tools. When I started to work at the IdentityPath platform, I was in charge of developing the subject linked to the anaerobic bacterium *Clostridium botulinum* (*C. botulinum*). This bacterium produces the deadly botulinum neurotoxin which is responsible for the botulism disease, characterized by a flaccid paralysis. Without proper medical assistance it could lead to a fatal issue due to respiratory failure. The disease concerns both human and animals. The number of human cases per year in France is relatively low (15 to 20, generally associated with consumption of homemade canned food). The number of outbreaks in animal production such as cattle and poultry is higher, around 50 per year, and could concerns thousands of animals. Economical loss in highly valuable animal production (e.g. bovine,

horse) could be catastrophic. Despite a relatively low occurrence, botulism disease is a major health issue because of its medical implication for human health and its economic impact on animals. The only specific treatment for botulism is botulinum antitoxin and supportive care activities, especially mechanical ventilation, for several days to several weeks. Despite its extreme potency (1 g could potentially kill one million people), botulinum toxin finds several therapeutic and cosmetic applications, but could also be misused as a biological warfare weapon or as a bioterrorism threat.

The IdentityPath platform was involved in two European projects and one French program regarding the development of methods to counter bioterrorism threats linked to potential misuse of *C. botulinum*. The BIOTRACER project (FP6-2006-Food-036272, VIth PCRD) which aimed to improve bio-traceability of unintended micro-organisms and their substances in food and feed chains; the ANIBIOTHREAT project (DG Justice, Freedom and Security) which aimed to improve bio-preparedness measures concerning prevention, detection and response to animal bio-terrorism threats; and the French program CEA/NRBC (Convention D7683). The IdentityPath platform is also associated with the National reference laboratory for avian botulism created in 2011 by our colleagues from Anses Ploufragan - Plouzané Laboratory (Avian and Rabbit Epidemiology and Welfare Unit, and Hygiene and Quality of Poultry and Pig Products Unit).

Here are the circumstances where my work on *Clostridium botulinum* took place, which turns out to become my PhD project. My final goals were to investigate different genetic markers to improve detection and typing of *C. botulinum*. Usual detection methods are based on the presence of the toxin (BoNT) or the coding gene (*bont*). To date eight different toxin types are characterized (A to H), which are divided into several subtypes. This genetic diversity does not allow for thorough epidemiological investigation, but it could be completed using other genetic targets. Flagellins, for example, are proteins involved in structural motility which give specific bacterial antigenic properties, which could be used as typing targets. When I started my work on *C. botulinum*, little information was publicly available about genetic diversity in these organism. Therefore, I investigated the potential genetic diversity of flagellins genes *flaA/flaB* and *fliC* in *C. botulinum* responsible for human and animal botulism respectively. I completed my work by investing the full genome diversity of *Clostridium botulinum* responsible for animal botulism, giving the work a deeper genetic dimension. Here are the results I am going to develop in this PhD manuscript.

Introduction

Chapter I

Botulism and *Clostridium botulinum*

I.1. Botulism, a historical overview

Although it is reasonable to hypothesise that botulism is a disease known to man for a long time, it has received a great deal of medical and scientific attention since the end of the 18th century, when poor sanitary measures in rural food production left the door open to foodborne disease (Ting and Freiman, 2004). Knowledge of botulism increased when outbreaks of food poisoning followed the consumption of meat and blood sausages. German physician Justinus Kerner (1786-1862) was the first to study in detail the mysterious “sausage poison”. He made several important observations about the poison: it develops in sour sausages under anaerobic conditions, it interrupts motor signal transmission in the peripheral and autonomic systems, and is lethal in small doses. He also accurately described the neurological symptoms, including vomiting, intestinal spasms, mydriasis, ptosis, dysphagia and respiratory failure. Furthermore, he speculated about the use of small doses of the poison as a therapeutic treatment for hyperexcitability of the nervous system (Erbguth, 2008). However, he was unable to determine and isolate the biological causative agent. Microbiologist Emile Pierre Marie van Ermengem, of the University of Ghent, was later able to isolate the bacterium in ham and the corpses of botulism victims. He grew it, used it for animal experiments, characterised its culture requirements, described its toxin and called it “*Bacillus botulinus*”, after the Latin word *botulus*, meaning sausage. The pathogen was later renamed “*Clostridium botulinum*” (*C. botulinum*) because of its spindle shape (Zhang et al., 2010).

I.2. *Clostridium botulinum*, the organism

C. botulinum is the aetiological agent of botulism. It is a Gram-positive, rod-shaped, anaerobic, spore-forming, motile bacterium belonging to the *Clostridium* genus (Figure 1).



Figure 1. *Clostridium botulinum* bacteria.

Coloured scanning electron micrograph of Clostridium botulinum bacteria (rod-shaped). The rods are 0.3-1.9 μm in width and 1.6-9.4 μm in length (Smith and Hobbs, 1974). Magnification: x13,300. Credit EYE OF SCIENCE/SCIENCE PHOTO LIBRARY.

The taxonomic designation defining *C. botulinum* is its ability to cause botulism in humans or animals by producing botulinum neurotoxin (BoNT). The spores of *C. botulinum* are naturally present in the environment worldwide (particularly in soil and marine sediments). To date, seven types of BoNT—designated A to G, based on their biochemical and serological characteristics—have been described. Recently, a new serotype, H, has been identified but remains to be confirmed (Maslanka et al., 2015). Toxin types A, B, E and F are subdivided into several subtypes (see chapter I.4.1). *C. botulinum* exhibits such cultural and physiological diversity that these bacteria could be considered different species (Peck, 2009), and are thus divided up into different phenotypical Groups (Table 1).

Toxigenic Clostridia	<i>C. botulinum</i> Group I	<i>C. botulinum</i> Group II	<i>C. botulinum</i> Group III	<i>C. argentinense</i> Group IV	<i>C. butyricum</i> Group V	<i>C. baratii</i> Group VI
Neurotoxins produced	A, B, F, H	B, E, F	C, C/D, D, D/C	G	E	F
Non-toxigenic equivalent Clostridia	<i>C. sporogenes</i>	<i>C. beijerinckii</i>	<i>C. novyi sensu lato</i>	<i>C. subterminale</i>		
Proteolytic activity	+	-	-	+	-	-
Lipase activity	+	+	+	-	-	-
Lecithinase activity	-	-	+	-	-	+
Optimum growth temperature	37°C	25°C	40°C	37°C	30 – 37°C	30 – 45°C
Minimum growth temperature	10 – 12°C	2.5°C	15°C		12°C	10 – 15°C
Spore heat resistance	D _{104°C} , 2.22 min	D _{82.2°C} , 2.4 min	D _{104°C} , 0.9 min	D _{104°C} , 1.1 min	D _{100°C} , <0.1 min	

Table 1. Characteristics of the six physiologically and phylogenetically distinct Clostridia producing botulinum neurotoxin.

Where values are not given, they are not readily available in the literature. From Peck, 2009.

Group I and III organisms seem to be mainly of terrestrial origin and are present in temperate climates, whereas Group II strains—and particularly type E—are frequently found in aquatic environments in the northern hemisphere. Group I strains producing BoNT types A, B, F and the new type H toxin, also referred to as mosaic toxin FA (see chapter 1.4.3 for more information on type H-FA (Gonzalez-Escalona et al., 2014a)), are proteolytic and mesophilic, with an optimal growth temperature of 35 - 40°C, and a minimum growth temperature of 10°C (Carter and Peck, 2015). The spores are highly heat-resistant (2.2 min, 104°C) and cause problems in canning and home preservation of vegetables and meat (Lindstrom et al., 2003). Their characteristics are close to those of *C. sporogenes*. Group II strains produce toxin types B, E and F, which are non-proteolytic psychotropic substances with an optimal growth temperature of 30°C, and a minimum growth temperature of 2.5°C (Carter and Peck, 2015). The spores have low heat resistance (2.4 min, 82.2°C) and are of

great concern in processed packaged foods that have extended shelf lives at refrigerated temperatures (Stringer et al., 1997). Due to the lack of endogenous proteolytic enzymes, the toxin requires a trypsinisation step to be fully activated. Group III consists of BoNT types C, D and the mosaic forms C/D and D/C (Oguma et al., 1986) (see chapter 1.4.3 for more information on mosaic toxins C/D and D/C). They are mostly non-proteolytic, and both lipase- and lecithinase-positive. They have an optimal growth temperature of 40°C and a minimum growth temperature of 15°C (Smith and Hobbs, 1974; Lindstrom and Korkeala, 2006). The spores have an intermediate heat resistance (0.9min, 104°C) (Smith and Hobbs, 1974; Lindstrom and Korkeala, 2006). They are considered to be responsible for the majority of botulism outbreaks in animals. Recent genetic studies have shown that *C. botulinum* Group III is genetically related to *C. novyi* and *C. haemolyticum* (Skarin et al., 2011b). Group IV consists of the proteolytic, lipase- and lecithinase-negative type G strain (re-designated *Clostridium argentinense*), which has an optimal growth temperature of 37°C. Its spores have an intermediate heat resistance (1.1 min, 104°C) (Puig de Centorbi et al., 1997; Taylor et al., 2010). Initially isolated from soil samples (Gimenez and Ciccarelli, 1970), type G has not been clearly identified as a source of human botulism (Sonnabend et al., 1981; Taylor et al., 2010). This Group is closely related to *C. subterminale*. Some strains of *C. butyricum* and *C. baratii* are capable of producing BoNT types E and F respectively. They have been associated with botulism cases and assimilated as Group V and Group VI (Augustynowicz et al., 2003). They are both non-proteolytic, lipase-negative mesophilic bacteria with an optimal growth temperature around 37°C. Neurotoxic strains are closely related to non-pathogenic *C. butyricum* and *C. baratii* rather than other *C. botulinum* neurotoxin-producing Clostridia based on their cultural and physiological characteristics.

1.3. Botulism, the disease

1.3.1. Human botulism

BoNTs are extraordinarily potent neurotoxins. The estimated lethal dose for purified crystalline BoNT type A is estimated at 1 ng/kg. The lethal dose for a 70-kg person by the oral route is estimated at 70 µg, by the inhalation route 0.80 to 0.90 µg, and by the intravenous route 0.09 to 0.15 µg (Arnon et al., 2001b).

1.3.1.1. The different forms of human botulism

Human botulism can be divided into three major categories depending on the source of the disease: foodborne botulism, infant/intestinal botulism and wound botulism (Figure 2).

- Foodborne botulism.

Foodborne intoxication occurs following ingestion of food containing preformed neurotoxins after growth of the *C. botulinum* organism. The severity of the disease is related to the quantity ingested and type of toxin, type A being the most effective in humans. It is the most common form of botulism encountered worldwide (in France, from 2010 to 2012 both types A and B were equally reported (Mazuet et al., 2014)). Many different types of food have been implicated, but the most common are home-made preserved foods (Pingeon et al., 2011). Any food allowing anaerobic spores to survive and which is not subsequently heated before consumption could support the growth of the bacteria and production of BoNTs associated with botulism. Almost any type of food that is not very acidic (pH above 4.6) can be concerned, such as canned corn, peppers, green beans, soup, beets, asparagus, mushrooms, ripe olives, spinach, tuna fish, chicken, chicken livers and liver pâté, luncheon meats, ham, sausage, stuffed aubergines, smoked and salted fish or lobster. Inadequate sterilisation allows the spores of *C. botulinum* to germinate and grow without competition, leading to production of BoNT. The food industry recommends a heat treatment equivalent of maintaining the food at 121°C for 3 minutes to fully inactivate spores of *C. botulinum* (Ronald G. Labbé, 2013). If the food containing the toxin is not properly cooked before being consumed (the toxin being inactivated after 3 minutes at 80°C (Losikoff, 1978)), it could lead to a botulism toxico-infection.

- Infant botulism.

Infant botulism is a result of a toxico-infection by ingestion of *C. botulinum* spores which colonise the intestine of children younger than 12 months of age and produce neurotoxins *in situ*. It is often associated with the consumption of honey, or the intake of soil or dust containing *C. botulinum* spores (Abdulla et al., 2012). Such colonisation can only occur since a normal competitive internal microflora has not yet been established. The symptoms include constipation, lethargy, general muscle weakness and a “floppy” head, weak cry, poor sucking ability and difficulty in swallowing. It could be associated with sudden infant death syndrome (Rosow and Strober, 2015). Rarely, *C. botulinum* or a neurotoxin-forming strain of *C. butyricum* can become established in the adult intestine. This can occur if competing bacteria in the normal intestinal microflora are suppressed (e.g. hospitalised patients treated with antibiotic therapy), or as a result of intestinal tract malformation (Fenicia et al., 2007).

- Wound botulism.

Wound botulism results from BoNT production *in vivo* after colonisation of injured tissue by *C. botulinum* spores, which germinate and produce toxin *in situ*. It has been associated with wounds from trauma, surgery, or the injection sites of drug users leaving the door open for *C. botulinum* spores to enter the body (MacDonald et al., 2013; Moreno et al., 2014). The spores germinate in the wound or an abscess, providing the necessary anaerobic conditions for the bacteria's development and production of botulinum toxin.

- Iatrogenic botulism.

Other types of human botulism are extremely rare. Botulism can also occur after the injection of licensed or unlicensed toxin for therapeutic or cosmetic purposes (iatrogenic botulism (Ghasemi et al., 2012)). The inadvertent inhalation of a toxin aerosol by veterinary workers, named inhalational botulism, was reported in 1961 (Holzer, 1962). More data are available on the exposure of animals to toxin aerosols (Park and Simpson, 2003).

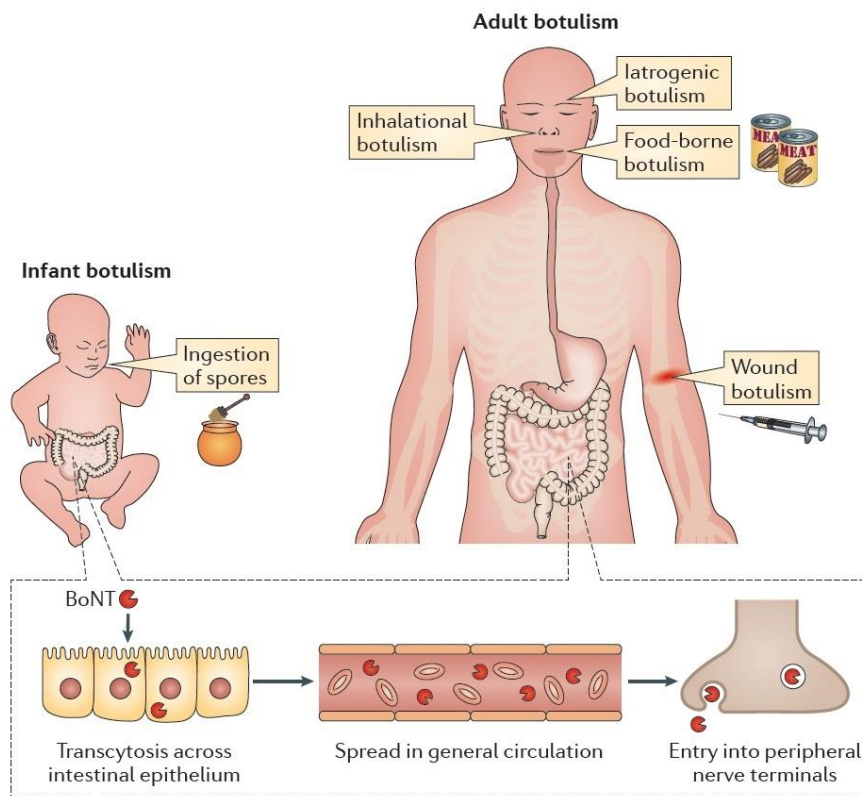


Figure 2. Human form of botulism.

Intoxication due to consumption of food contaminated with botulism toxin is responsible for the majority of human botulism outbreaks. Toxicoinfection occurs in young children by ingestion of C. botulinum spores that will produce the toxin in-vivo. Wound botulism is mainly responsible for drug addict outbreaks. From Rossetto et al., 2014.

1.3.1.2. Symptoms

The first symptoms of botulism can be observed within 2 hours, even if the typical incubation time is 12 to 36 hours and up to 8 days. Typical symptoms are blurred vision, difficulty in swallowing and speaking, paralysis of face muscles, descending bilateral flaccid paralysis and generalised muscle weakness. If not treated, it can lead to fatal respiratory failure and cardiac muscle paralysis (Kolho et al., 2012).

1.3.1.3. Origins

Outbreaks of BoNT types A and B are generally associated with temperate and warm areas, with one type often predominating. Proteolytic *C. botulinum* Group I types A and B are predominantly related to outbreaks in the United States, China, South America and southern European countries, the most frequently implicated foods being vegetables. In Central Europe, meat products are frequently associated with outbreaks of non-proteolytic *C. botulinum* Group II type B strains. More than 90% of the outbreaks are caused by home-prepared/home-preserved foods (Peck, 2009). Type E occurs primarily in northern countries such as Canada, Finland, Japan, Norway, Sweden, Russia, and Alaska in the United States (Macdonald et al., 2011). Botulism due to type F is rare (<1% of cases) and associated with food outbreaks of *C. botulinum* or *C. baratii* strains (Raphael et al., 2010a).

1.3.1.4. Treatment

Rapid diagnosis and the availability of well-coordinated, multidisciplinary supportive care constitute the most effective measures in the treatment of botulism, especially mechanical ventilation. Supportive care is generally necessary for several days up to several weeks until the patient is capable of surviving. The only specific treatment for botulism is botulinum antitoxin. It can stop the progression of paralysis and decrease the time of hospitalisation, but only if administered early; usually within 24 hours of the first symptoms, when the toxin has not yet bound to its target. Equine-derived antitoxin preparations, however, have many side effects (15–25%), including serum sickness and anaphylaxis. Once the toxin reaches the central nervous system and binds to its targets, antitoxin is ineffective, and full recovery takes months (Sobel et al., 2009). The case fatality rate for foodborne botulism is 5–20%, whereas infant botulism and wound botulism have current rates of approximately 15% and 1% respectively (Caya, 2001).

1.3.1.5. Epidemiological data

In the United States, a total of 153 laboratory-confirmed cases of human botulism were reported to the Centres for Disease Control and Prevention (CDC) in 2013. Foodborne

botulism accounted for two of these cases, found to be caused by toxins type A and E— associated with home-canned peaches and fish oil respectively. One hundred and thirty-five infant botulism cases were reported: 57 involved toxin type A, 74 type B, 1 type Bf and 3 type F. Fourteen wound botulism cases were reported, mainly associated with drug users: 11 involved toxin type A, 1 type B and 2 remained undetermined. Finally, two cases of botulism of an unknown or other aetiology were reported: they involved toxins type A and F.

In France, the French National Reference Centre (NRC) for anaerobic bacteria and botulism at the Institut Pasteur, directed by Dr. Michel Popoff in 2016, is involved in identifying anaerobic strains (mainly human related) isolated by hospital laboratories, as well as in the diagnosis and surveillance of botulism. In France, 11 confirmed cases of human botulism, related to four outbreaks, were reported in 2014 to the Pasteur NRC for anaerobic bacteria and botulism. All were foodborne botulism associated with the consumption of commercial pesto sauce or home-prepared ham and green beans. Four cases were related to *C. botulinum* producing toxin type B, three were uncertain between type B and E, two were related to toxin type F produced by *C. baratii*, and finally in two cases the toxin type was undetermined. Recently, three new cases caused by *C. baratii* type F were reported in August 2015 (Tréhard et al., 2016), this being the second episode reported in France caused by this rare pathogen.

1.3.1.6. Examples of human botulism-related events worldwide

Sources: <http://www.promedmail.org/>.

Two cases of infant botulism type E relating to *Clostridium butyricum* in 2010 and 2013 were reported in Dublin, Ireland. The origin of contamination was related to pet turtles (Shelley et al., 2015).

On 2 August 2013, the Chinese dairy producer Fonterra informed eight customers (including the French company Danone) that three batches of whey protein concentrate potentially contaminated with *Clostridium botulinum* had entered the supply chain. The contaminated product was used to produce infant formula and sports drinks. It led to a major recall (over 1,000 tonnes) of the concerned products in China and New Zealand. Danone estimated the economic impact of the recalls to be €350m. Fortunately, no cases of consumers being sick were finally reported. It turned out to be a false alarm as tests later revealed that the bacterium found was *C. sporogenes*, one of the non-toxic surrogate species of *C. botulinum*.

The Norwegian Institute of Public Health (NIPH) and the National Health Service (NHS) in Scotland reported 10 and 15 wound botulism cases respectively linked to contaminated

heroin since the first case recorded in late December 2014. Police are investigating the hypothesis that these cases are the result of a single batch of heroin contaminated with *C. botulinum* spores, but this remains to be confirmed.

In Argentina, the Food Science part of the Rio Negro Ministry of Health reported in March 2015 a case of botulism due to the consumption of marinated octopus.

In the United States, one person died and at least 20 others were hospitalised in April 2015 after an outbreak of botulism following a potluck dinner at a church in central Ohio.

In the republic of Mari El, Russia, type F botulinum toxin was found in industrially canned tomatoes linked to a case of botulism in July 2015.

1.3.2. Animal botulism

Like for human botulism, three kinds of animal botulism may be distinguished: contamination by the ingestion of preformed toxins in feeds, toxico-infection caused by germination of *C. botulinum* spores in the intestinal tract or a wound, and BoNT production *in vivo* (Holzhauer et al., 2009; Souillard et al., 2015).

1.3.2.1. Animal species affected

Animal botulism is a major environmental and economic concern, mainly because of the high mortality rate during outbreaks. Animal botulism mostly affects wild and domesticated animals such as poultry, cattle, horse, fish or animals raised for fur, summarised in Table 2 (Galey, 2001; Lindstrom et al., 2010; Myllykoski et al., 2011; Souillard et al., 2014; Yule et al., 2006). Sensitivity to BoNTs seems to vary widely between different animal species. Cattle botulism is most frequently caused by type D/C toxin and less frequently by type D (BoNT / D/C toxic activity being the highest of all BoNT types (Nakamura et al., 2010)), followed by type C/D, although the rarer type B has also been reported (Souillard et al., 2015b; Yeruham et al., 2003). In poultry, the most common toxin is of type C/D, though a few cases of type C and D/C have also been reported (Souillard et al., 2015). The mosaic type C/D toxin seems to be more lethal to chickens than type C (Takeda et al., 2005). In wild birds, the most commonly isolated toxin is of type C/D and in fish-eating waterfowl, type E (Jang et al., 2014). Type A has also been reported in gulls, however (Ortiz and Smith, 1994). In recent years, there have been large outbreaks caused by type E in the Great Lakes of the United States, with high mortality among fish and birds. These episodes have been well documented and analysed (Chun et al., 2013). Equine botulism is caused by toxins of type B,

C or A (Johnson et al., 2015). Fur farm animals such as foxes, ferrets and mink appear susceptible to type C and C/D toxins, but rare outbreaks due to type A and E toxins have also been reported (Myllykoski et al., 2011). Some animal species are rarely contaminated and seem to be more resistant to botulism (e.g. cats, dogs or carrion-eaters such as lions) (Elad et al., 2004; Greenwood, 1985; Uriarte et al., 2010). Vultures are known to be naturally resistant (Ohishi et al., 1979). Pork seems to be naturally resistant to botulism, although scarce cases of type C have been reported (Raymundo et al., 2012). It could therefore be a host reservoir, at the origin of human foodborne outbreaks (Czerwinski et al., 2015; Myllykoski et al., 2006).

Animal species	Toxin type
Cattle	D/C, D*, C/D, B*
Fish	E
Fish eating birds	E
Fur farm animals [#]	C/D, C, A*, E*
Horse	B, C, A*
Sheep	C, C/D, D, D/C
Pork	C*
Poultry	C/D, C*, D/C*
Wild birds	C/D

Table 2 : Botulinum toxin and animal species affected.

* Rarely reported; [#] fox, ferret, mink; mosaic forms are reported with a slash /.

1.3.2.2. Symptoms

All forms of animal botulism are characterised by a progressive, symmetrical, flaccid paralysis that often starts in the hindquarters with weakness, muscle tremors, stumbling and recumbency (Figure 3). Disturbed vision, difficulty in chewing and swallowing complete the symptoms. Most often, the disease results in death due to respiratory paralysis. Generally, clinical signs appear from 24 hours up to 17 days post-infection. For toxico-infectious forms, the incubation period is usually longer, and an indicative period of 4 to 14 days has been reported. No characteristic gross and histological lesions develop.



Figure 3. Flaccid paralysis characteristic of botulism in cow and turkey.

Credit: Cow picture, Bruckstein Shmuel, DVM and the Center for Food Security and Public Health, with permission (at <http://www.cfsph.iastate.edu/>). Turkey picture, Rozenn Souillard, Ploufragan - Plouzané Laboratory, Hygiene and Quality of Poultry and Pig Products Unit, Ploufragan, France, with permission.

1.3.2.3. Origins

Outbreaks typically occur in environments that contain *C. botulinum* spores, which can germinate in decomposing organic material under the appropriate anaerobic environment, but little is known about the primary substrate in botulism outbreaks. *Clostridium botulinum* is ubiquitously present in the environment in soils, dust, and the marine and fresh water sediments of wetlands, rivers and lakes where spores persist for decades. Contaminated soils and sediments are primary environments for spores and could serve as an initial proliferation area, from which the pathogens may be mobilised by surface waters in heavy rain, or dust carried away by wind (Long and Tauscher, 2006). Whatever the animal species, botulism is often reported to be seasonal. The number of outbreaks increases during summer and autumn, when ambient conditions are near the optimal temperature for bacterial growth (Rocke et al., 1999), but cases are also reported during the rest of the year. Several vertebrates can carry neurotoxicogenic *C. botulinum* as part of their intestinal microbiota (Myllykoski et al., 2006; Yamakawa et al., 1992). Animals dying for other reasons but containing spores of *C. botulinum* in their digestive tract can serve as a substrate for bacterial germination (Anza et al., 2014b). It also appears that *C. botulinum* may be associated with various organisms that are not affected by the toxins, such as algae, plants and invertebrates (Bohnel, 2002). Fish are carriers of *C. botulinum*, but botulism outbreaks in fish populations may lead to death on a large scale (Hannett et al., 2011). These organisms

represent a biotic reservoir for *C. botulinum*, and may become toxic upon anaerobic decomposition. Animals may directly ingest decaying organic matter containing toxins, or may ingest toxins through the consumption of zooplankton or invertebrates such as larvae, that have themselves consumed toxic material. Fly larvae and other invertebrates are unaffected by the toxin, but feeding on toxigenic carcasses may concentrate toxins (Gismervik et al., 2014). The ingestion of a single toxigenic maggot could be lethal. Once established, a botulism outbreak is self-perpetuating. This is described as the wildlife carcass-maggot cycle of avian botulism (Figure 4). Environmental conditions that favour botulism outbreaks in wildlife include warm temperatures (optimum growth temperature between 25°C and 42°C), shallow alkaline waters that contain abundant invertebrate populations, and decomposing carcasses (Soos and Wobeser, 2006).

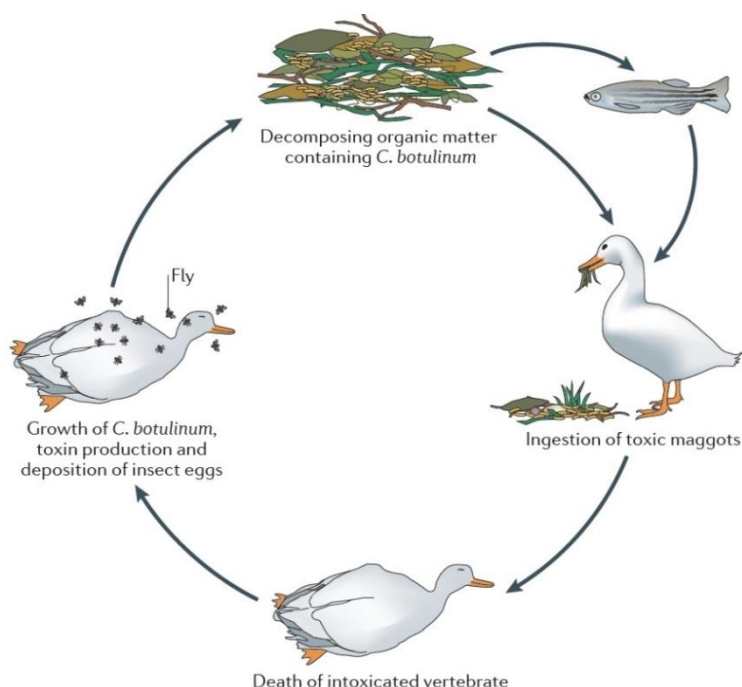


Figure 4. The maggot cycle.

From Rossetto et al., 2014.

Several routes of contamination have been suggested for poultry and cattle farms. The cadavers of small animals (e.g. rodents) could contaminate feed or water, providing the optimal conditions for *C. botulinum* growth and toxin production. Raw material such as grass, hay, rotting vegetation or slaughterhouse waste, as well as decaying carcasses, invertebrates (darkling beetles) and sewage may contain spores of *C. botulinum* and support germination and toxin production (Souillard et al., 2014). There is also some evidence that

exposure to poultry litter for feed or bedding may constitute a risk factor in the occurrence of cattle botulism (Bienvenu et al., 1990; Kennedy and Ball, 2011). Changes in intestinal microbiota due to poor-quality feed, for example, could allow *C. botulinum* to grow in the gastrointestinal tract. Data about asymptomatic carriage of *C. botulinum* Group III in cattle and poultry production are particularly scarce. The few studies available concluded that the organism was absent in healthy animals (Hardy et al., 2013; Seyboldt et al., 2015). Souillard et al. presented a study of 17 commercial poultry farms contaminated by *C. botulinum* after botulism outbreaks between 2011 and 2013 (Souillard et al., 2014). Their findings concur with previous reports that the disease occurs more frequently during the warm season. They evaluated the presence of *C. botulinum* type C/D in 39.5% of the samples that originated from the environment of affected farms. Contamination was also detected after the cleaning and disinfection process, indicating the difficulties of decontaminating poultry houses after an outbreak. To prevent the recurrence of botulism after an outbreak, darkling beetles, drinking water, the ventilation system and soil around the concerned farms have to be considered as potentially contaminated by *C. botulinum*.

1.3.2.4. Treatment

When botulism is suspected, the first critical therapeutic step is to give polyvalent antitoxin to affected animals. As the treatment is expensive and the antitoxin difficult to obtain, only very valuable animals are concerned (e.g. horses). Antitoxin treatment should be initiated as soon as possible because it is effective only against circulating toxin. Antibiotic administration (beta-lactams) has been successfully used to treat the toxicoinfection form of botulism. Other therapies include supportive care (oral water and electrolytes) and reduced physical activity. Vaccination can be considered to be effective in preventing the outbreak from spreading (Anniballi et al., 2013b; Cunha et al., 2014).

1.3.2.5. Human health considerations

Animal botulism could be considered to be a zoonosis as it may be a source of botulism for humans. Botulism has been characterised as a particularly substantial risk to humans in northern climatic regions due to intoxication by poorly preserved food (Austin and Leclair, 2011). Human botulism is mainly due to BoNT types A and B, to a lesser extent type E, and rarely type F. Toxins produced by *C. botulinum* Group III are not related to confirmed human cases. Therefore, only animals that are sensitive to the same toxins as humans could be considered a potential zoonotic threat. For example, several reports of human foodborne type E intoxication were related to contaminated fish (Fach et al., 2002). There has also been

a report of infant botulism caused by toxin type E related to *C. butyricum* where contamination originated from pet turtles (Shelley et al., 2015).

1.3.2.6. Epidemiological data in France

Outbreaks of animal botulism can spread rapidly, leading to the death of hundreds of thousands of animals in just a few days. In European countries, the number of animal botulism outbreaks involving avian species and cattle has been increasing over the last decade. It is still difficult to estimate the real prevalence of the disease as it is not notifiable in all European countries. The reason behind this upsurge is still unknown, but it attracted the attention of the French health services, which created the National Reference Laboratory (NRL) for avian botulism in 2011 to assess the issue. In France, 482 cases of avian botulism were reported between 2000 and 2011. The French epidemiological surveillance network for poultry (RNOEA) reported 89 cases between 2000 and 2006, and 393 cases between 2007 and 2011. Chickens (49% of cases) and turkeys were the most impacted. Toxin type C was the most frequently observed (58% of botulism cases), followed by type D (20%). Mosaic types were not investigated at that time. In 2015, the NRL for avian botulism investigated 33 cases, of which 22 were positively identified as botulism outbreaks. The main toxin detected was type C/D, which accounted for 17 positive cases. One case was shown to contain both types C/D and C, while another contained types C/D and D. Type D/C was detected in one case, and type D in two. It is interesting to note that the dual presence of toxin types was reported. Even though the majority of cases were due to mosaic toxins, some historical C and D types were also detected. There is currently no reference centre or network on a European or international scale regarding animal botulism. Depending on the country, animal botulism may or may not be a notifiable disease. More data related to European countries are available in the report of the “Animal botulism in Europe” workshops held in 2013 in Sweden as part of the European AniBio Threat project (Skarin et al., 2013).

1.3.2.7. Examples of botulism-related events worldwide

Sources: <http://www.promedmail.org/>.

In the United States, water bird mortality was investigated in three areas of northern Lake Michigan from 2010 to 2013. A total of 4,447 dead birds were retrieved. A subset of fresh carcasses was collected throughout each year of the study and tested for botulinum neurotoxin type E (BoNT/E). Sixty-one percent of carcasses (57/94) and 10 of 11 species collected throughout the sampling season tested positive for BoNT/E, suggesting that avian

botulism type E was a major cause of death for both resident and migratory birds at Lake Michigan (Chipault et al., 2015).

In February 2013, more than 200 wild ducks were found dead on two ponds in Nowra, Australia. The Cumberland Livestock Health and Pest Authority discovered the birds had died from botulism, though no information on type was reported.

In December 2013, the New York State Department of Environmental Conservation in the United States reported botulism at the eastern basin of Lake Ontario. Reports indicate that at least 200-300 common loons had been washed ashore along Jefferson and northern Oswego County shorelines. The loon deaths were all attributable to type E botulism.

In April 2014, more than 400 feral chickens in Bodden town on the Cayman Islands died of botulism according to a post-mortem examination carried out by the Department of Agriculture (the type was not reported).

In April 2014, New Zealand's Department of Conservation and Fish and Game of Tauranga City Council retrieved dead or ill ducks affected by type C botulism from Papamoa's Royal Palm Beach Reserve.

In August 2014, the UK's Animal Health and Veterinary Laboratories Agency reported several suspected outbreaks of type C botulism after dead ducks were discovered along the river Weaver in Winsford.

In August 2015, the United States' Department of Natural Resources reported 30 dead Mallard ducks on beaches along the eastern branch of Grand Traverse Bay (Lake Michigan, Michigan, USA). All tested positive for type C botulism.

In November 2015, the United States' State Department of Environmental Conservation investigated the deaths of wildlife caused by type E botulism along the shores of Lake Ontario in Oswego, Jefferson and Wayne counties.

I.4. The botulinum neurotoxin

I.4.1. BoNT Characteristics

BoNTs are zinc endopeptidases from 1,251 (BoNT/E) to 1,296 (BoNT/A) amino acids long, produced as single-chain proteins of 150 kDa polypeptides, which are released after bacterial lysis then cleaved by proteolytic nicking by bacterial or tissue proteases to give the active toxin (Montecucco and Schiavo, 1995). The active di-chain molecule consists of a light chain (LC) of 50 kDa and a heavy chain (HC) of 100 kDa that are linked by a disulphide bond (Figure 10).

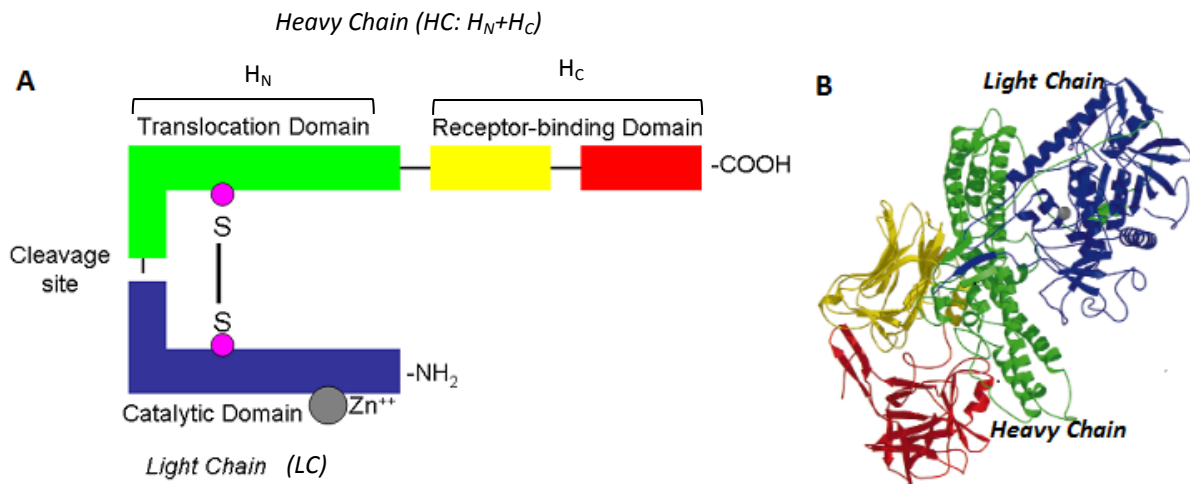


Figure 5. Botulinum toxin structure.

A) Schematic representation of BoNT; B) Crystal structure of BoNT/A. The catalytic domain (light chain) is coloured in blue. The translocation domain (heavy chain) is coloured in green, N-terminal and the C-terminal receptor binding domains are coloured in yellow and red respectively. The catalytic zinc is represented as a ball in grey. The colour code is same for Figures 5A and 5B. From Lacy et al., 1998.

The C-terminal domain of the HC (H_C) is responsible for binding to the target receptor of the neuronal cell membrane and internalising the toxin molecules by endocytosis. The N-terminal domain of the HC (H_N) is responsible for translocating the toxin from the endosomal vesicle into the neural cell, allowing the LC — which is the zinc-dependent endopeptidase catalytic domain — to transfer into the cytosol where it will reach its target. When produced, the toxins are released together with other non-toxic components with or without hemagglutinin activity (NTNH, ha17, ha33, ha70). They form the so-called precursor complex of 300 to 900 kDa. Its role is not yet fully understood, but it probably protects BoNTs from enzymes or environmental conditions (notably pH and temperature) when released in the environment and after ingestion, and facilitates absorption into the blood stream (Figure 6) (Benefield et al., 2013; Lee et al., 2013). The mechanism of transport across the intestinal epithelium involves the toxin complex binding to receptors on the mucosal surface of gut epithelial cells, followed by transcytosis and delivery of the toxin in the same conformation to the basolateral side (Ahsan et al., 2005; Lam et al., 2015b; Maksymowych and Simpson, 2004).

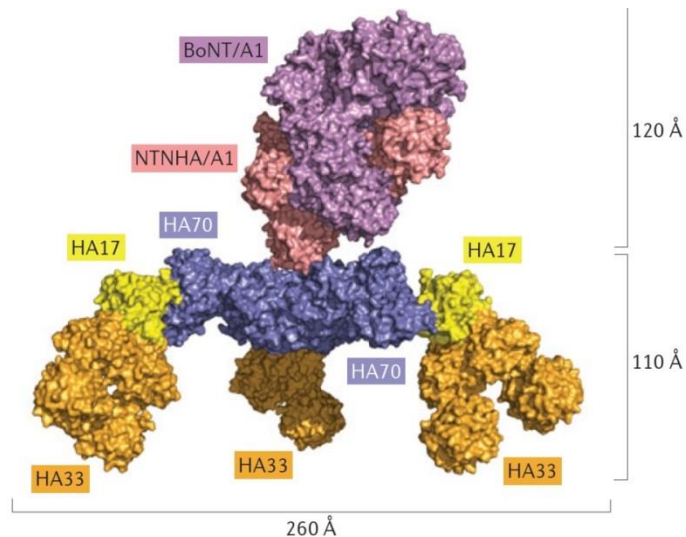


Figure 6. Structure of the precursor toxin complex.

Non-toxic non-hemagglutinin (NTNH) forms a heterodimer with BoNT. NTNH BoNT-like structure allows protection of BoNT toxin with a molecular hand-shake. Hemagglutinin proteins (HA17, HA33 and HA70) form a three spider-like legs structure linked to the NTNH-BoNT complex. From Lee et al., 2013.

I.4.2. Mechanism of action

All botulism neurotoxins act by blocking the release of acetylcholine at the neuromuscular junction, thus preventing transmission of neurotransmitters, inhibiting muscle contraction and leading to flaccid paralysis. When the unaffected motor neuron is depolarised through the neuronal signal, acetylcholine is released from the cytosol into the synaptic cleft between the neuronal cell and the muscle, allowing the normal transmission of information and the muscle to contract (Figure 7A). Acetylcholine is released by a transport protein chain named the “Soluble N-ethylmaleimide-sensitive-factor Attachment protein Receptor” (SNARE) complex. When BoNT reaches its target tissue—the motor neuron—the heavy chain receptor-binding domains (H_C and H_N) of the BoNT bind to glycoprotein receptor structures specifically found on cholinergic nerve terminals and is internalised (Rossetto et al., 2014). The extreme toxicity of BoNTs is predominantly due to their specific docking to cholinergic synapses through recognition of two host receptors, a polysialo-ganglioside (synaptotagmin) and, in the majority of cases, a synaptic vesicle protein (Lam et al., 2015a). After internalisation, the light chain of the BoNT is released into the cytosol and specifically binds to its targets. These are SNAP-25 (synaptosomal-associated protein of 25 kDa), synaptobrevin (or VAMP—vesicle-associated membrane protein), and syntaxin. They are collectively referred to as SNAREs and form a synaptic fusion complex which mediates the

fusion of the neurotransmitter vesicle to its target membrane. The enzymatic action of BoNT results in SNARE degradation thus preventing neurotransmitter vesicle fusion and the release of acetylcholine, stopping muscle contraction and causing paralysis (Poulain et al., 2009). SNAP-25 is specifically cleaved by BoNT/A and BoNT/E. Synaptobrevin is cleaved by BoNT/B, BoNT/D, BoNT/F, BoNT/G and by BoNT/H-FA at different peptide positions. BoNT/C can cleave both syntaxin and SNAP-25 (Figure 7B). The inhibition of acetylcholine exocytosis by BoNT is terminated by restoration of the SNARE protein complex turnover or the internal degradation of the neurotoxin. It has been estimated that symptoms and even death could occur by ingestion of as little as 30 to 100 ng of toxin (the oral lethal dose for humans being estimated at 1 µg/kg (Arnon et al., 2001)).

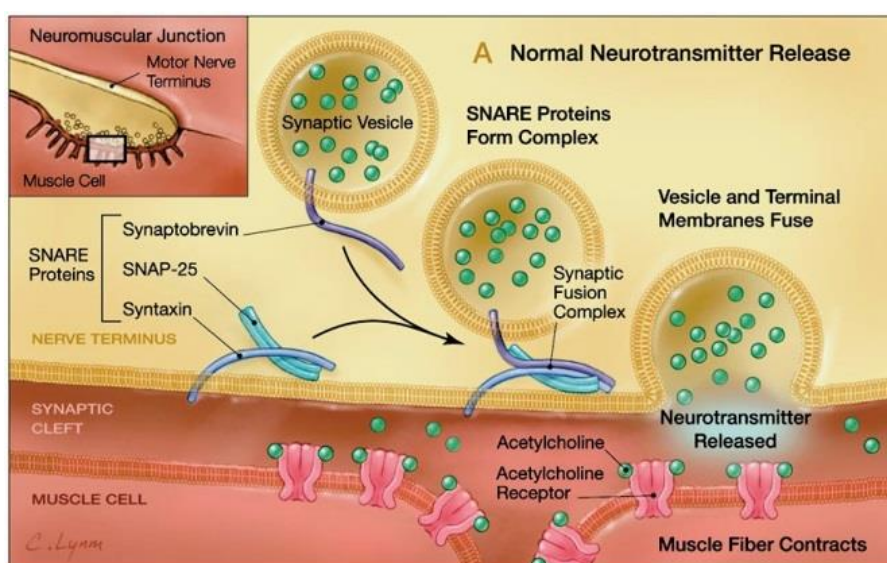
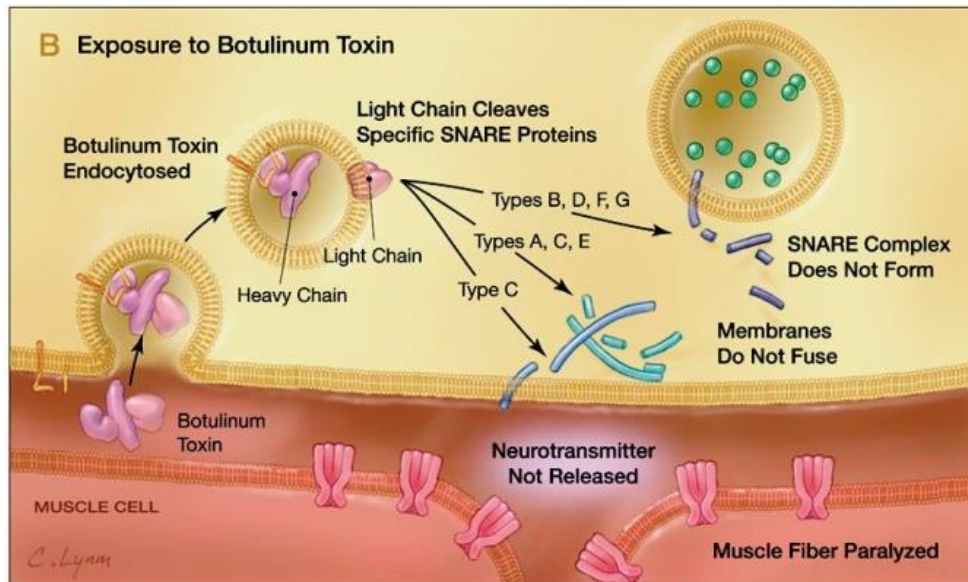


Figure 7. Mechanism of action of botulinum toxin.

A. Release of acetylcholine at the neuromuscular junction is mediated by the assembly of a synaptic fusion complex that allows the membrane of the synaptic vesicle containing acetylcholine to fuse with the neuronal cell membrane. The synaptic fusion complex is a set of SNARE proteins, which include synaptobrevin, SNAP-25, and syntaxin. After membrane fusion, acetylcholine is released into the synaptic cleft and then bound by receptors on the muscle cell.



B. Botulinum toxin binds to the neuronal cell membrane at the nerve terminus and enters the neuron by endocytosis. The light chain of botulinum toxin cleaves specific sites on the SNARE proteins, preventing complete assembly of the synaptic fusion complex and thereby blocking acetylcholine release. Botulinum toxins types B, D, F, and G cleave synaptobrevin; types A, C, and E cleave SNAP-25; type C also cleaves syntaxin. Without acetylcholine release, the muscle is unable to contract.

SNARE, Soluble N-éthylmaleimide-sensitive-factor Attachment protein Receptor; SNAP-25, synaptosomal-associated protein of 25 kDa. From Arnon et al., 2001.

1.4.3. BoNT serotypes and subtypes

Botulinum neurotoxins are the most poisonous substances discovered so far, even more dangerous than ricin or cyanide. Six phylogenetically distinct Clostridia produce botulinum neurotoxin serotypes A to G, including the new questionable type H. All BoNT neurotoxins share the same biological structure, which is similar to that of the tetanus toxin (Popoff and Bouvet, 2013). BoNT serotypes are defined by antibody neutralisation, as antibodies that neutralise BoNT/A do not neutralise other BoNT serotypes. The amino acids of serotypes A to G differ by up to 70%. The study of Maslanka et al. reported that toxin type H was neutralised by antitoxin type A, and that it contains a hybrid genetic structure containing regions similar to bont/F5 for the LC (>80%) and to bont/A1 for the HC_c (84%) (Maslanka et al., 2015). Therefore, toxin type H should not be considered a new toxin type, but a mosaic type and should be named F/A, in keeping with the previously characterised types C/D and

D/C. Each toxin serotype is categorised according to amino acid sequences into various subtypes, of which 41 have been identified so far. Subtypes can differ by 2.6% to 31.6% at amino acid level (Smith et al., 2005) (Tables 3 and 4). These differences can affect the binding and neutralisation potency by monoclonal and polyclonal antibodies also in addition to the affinity and catalytic efficiency of BoNTs with respect to their substrate (Popoff and Bouvet, 2013).

Clostridial species	Type	Subtype	Localisation
Proteolytic <i>C. botulinum</i> Group I	A; B; F; H-F/A	A1; A2; A3; A4; A5; A6; A7; A8; B1; B2; B3; B5; B6; B7; B8; F1; F2; F3; F4; F5; H-F/A	Chromosome and plasmid
Non-proteolytic <i>C. botulinum</i> Group II	B; E; F	B4; E1; E2; E3; E6; E7; E8; E9; E10; E11; E12; F6	Chromosome and plasmid
<i>C. botulinum</i> Group III	C; D	C; D; C/D; D/C	Bacteriophage
<i>C. argentinense</i> Group IV	G	G	Plasmid
<i>C. butyricum</i> Group V	E	E4; E5	Chromosome
<i>C. baratii</i> Group VI	F	F7	Chromosome

Table 3. BoNT type and subtype produced by the different Clostridia Group species.

Adapted from Rossetto et al., 2014.

	Nucleotide identity %								
	A1	B1	C	D	C/D	D/C	E3	F1	G
A1	-----	60.2%	55.3%	57.8%	58.8%	59.0%	60.7%	62.1%	59.6%
B1	37.5%	-----	56.6%	58.0%	61.3%	60.8%	59.6%	59.5%	71.8%
C	30.4%	30.8%	-----	68.3%	86.2%	65.4%	56.7%	56.8%	56.3%
D	31.2%	32.6%	51.4%	-----	65.2%	85.9%	57.7%	57.8%	57.8%
C/D	31.9%	34.8%	78.6%	44.1%	-----	64.6%	58.6%	60.4%	60.2%
D/C	32.8%	35.6%	44.1%	78.5%	43.1%	-----	59.0%	61.3%	59.9%
E3	37.7%	35.9%	30.9%	31.6%	32.5%	34.1%	-----	75.5%	59.0%
F1	38.7%	37.4%	30.9%	32.7%	34.4%	37.7%	62.8%	-----	59.0%
G	38.0%	57.1%	32.5%	34.0%	33.3%	33.9%	36.4%	36.8%	-----

Amino acid identity %

Table 4. Nucleotide and amino acid identity comparisons of the *bont* genes or BoNT proteins.

Nucleotide identities are indicated upward; amino acid identities are downward. Accession numbers: A1 strain Hall AF488749; B1 strain Okra CP000940; C strain Stockholm D90210; D strain 1873 AB012112; C/D strain BKT015925 CP002411; D/C strain OFD05 AB461915; E3 non proteolytic strain AlaskaE43 EF028403; F1 strain Langeland X70821; G strain 113/30-NCFB3012 X74162. From Hill and Smith, 2013.

BoNT serotype A is distantly related to other neurotoxin types. BoNT/C, D, C/D and D/C are closely related to each other, as are serotypes B to G and E to F. Newly discovered serotype BoNT/H (Barash and Arnon, 2014; Dover et al., 2014) is half way between BoNT/F and BoNT/A so may be considered a mosaic form of types F and A (Figure 8).

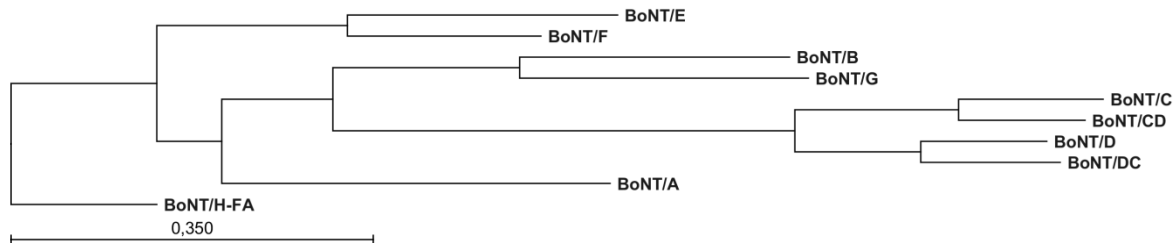


Figure 8. Phylogenetic tree of BoNT toxin sequences.

The phylogenetic tree of *C. botulinum* BoNT toxin was generated using consensus amino acid sequences of the 41 BoNT subtypes. The scale represents the branch length and is given in terms of expected numbers of substitutions per site. Elaborated by C. Woudstra from publicly available sequences using neighbour joining method in CLC® Genomic Workbench 7.5.1.

Several strains produce multiple toxins. Bivalent *C. botulinum* strains, each producing two toxin types, have been reported (Ab, Ba, Af, and Bf, where capitalised letters refer to the predominant toxin type). In such strains, the two neurotoxins are usually produced in different proportions. Thus, in Ba and Bf strains, BoNT/B is produced ten times more than BoNT/A or BoNT/F respectively (Henderson et al., 1997; Hutson et al., 1996). Several *C. botulinum* strains producing type A toxin have been found to also encode a silent *bont/B* gene that does not produce active toxin protein due to mutation or truncation. They are denoted A(B) (Rossetto et al., 2014). Silent *bont/B* has also been evidenced in non-toxigenic *C. subterminale* strains (Franciosa et al., 1994). Furthermore, a *C. botulinum* strain that expresses three BoNTs (chromosomally encoded BoNT/A2 and BoNT/F4 genes and a plasmid-borne BoNT/F5 gene) has recently been described (Dover et al., 2013). *C. botulinum* Group III produces types C, D, C/D or D/C (Sakaguchi et al., 2015). BoNT/CD and BoNT/DC are mosaic toxins resulting from genetic recombination between *bont* genes of type C and type D. BoNT/CD consists of the N-terminal part of serotype C and the C-terminal domain of

serotype D, whereas BoNT/DC comprises the N-terminal domain of serotype D and the C-terminal part of serotype C (Moriishi et al., 1996b). *C. botulinum* strain IBCA10-7060, isolated from infant botulism, was recently reported to produce a novel BoNT serotype, designated BoNT/H. This is the first new toxin type to be discovered in the past 40 years, i.e. since the discovery of type G. A genetic comparison with known *bont* sequences suggests that it is a hybrid-like structure containing regions of BoNT/A1 and BoNT/F5, thus being renamed mosaic BoNT/FA (Maslanka et al., 2015).

1.4.4. *bont* gene

C. botulinum is defined by its ability to produce BoNTs. The *bont* gene coding for the toxin is approximately 3.8 Kbp long. It contains sequences similar to collagenase enzymes which encode a HExxH domain (Doxey et al., 2008), suggesting that they evolved from a common ancestor. *Bont* sequences of each *C. botulinum* toxin types A to H show sequence variations leading to new subtypes. A new subtype is defined on the basis of a difference of at least 2.6% in the amino acid sequence of the BoNT protein. All BoNT serotypes share the same biological activity but little genetic identity; nucleotide differences among the toxin gene serotypes A-H range from 24.5 to 44.7% (Hill and Smith, 2013) (Figure 9).

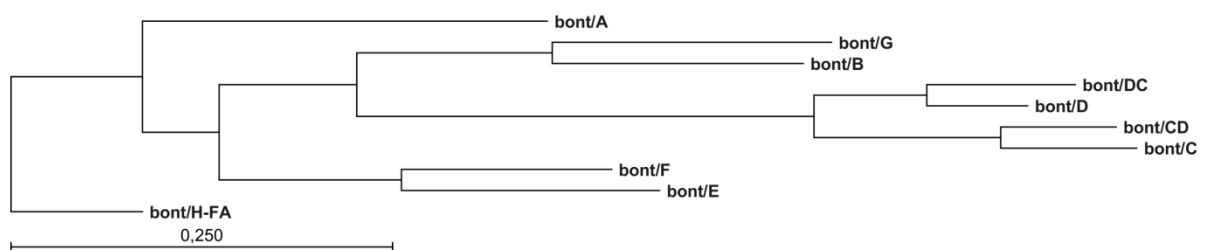


Figure 9. Genetic relatedness of *bont* type A to G encompassing the mosaic type H-FA.

*The phylogenetic tree of the genetic relatedness of *C. botulinum* bont genes was generated using consensus sequences of the 41 bont subtypes. The scale represents the branch length and is given in terms of expected numbers of substitutions per site. Elaborated by C. Woudstra from publicly available sequences using CLC® Genomic Workbench 7.5.1.*

To date, there are 41 BoNT subtypes: BoNT/A1 to A8, B1 to B8 encompassing bivalent B and non-proteolytic B, C, C/D, D, D/C, E1 to E12, F1 to F7, G and the new type, H-FA (Figure 10).

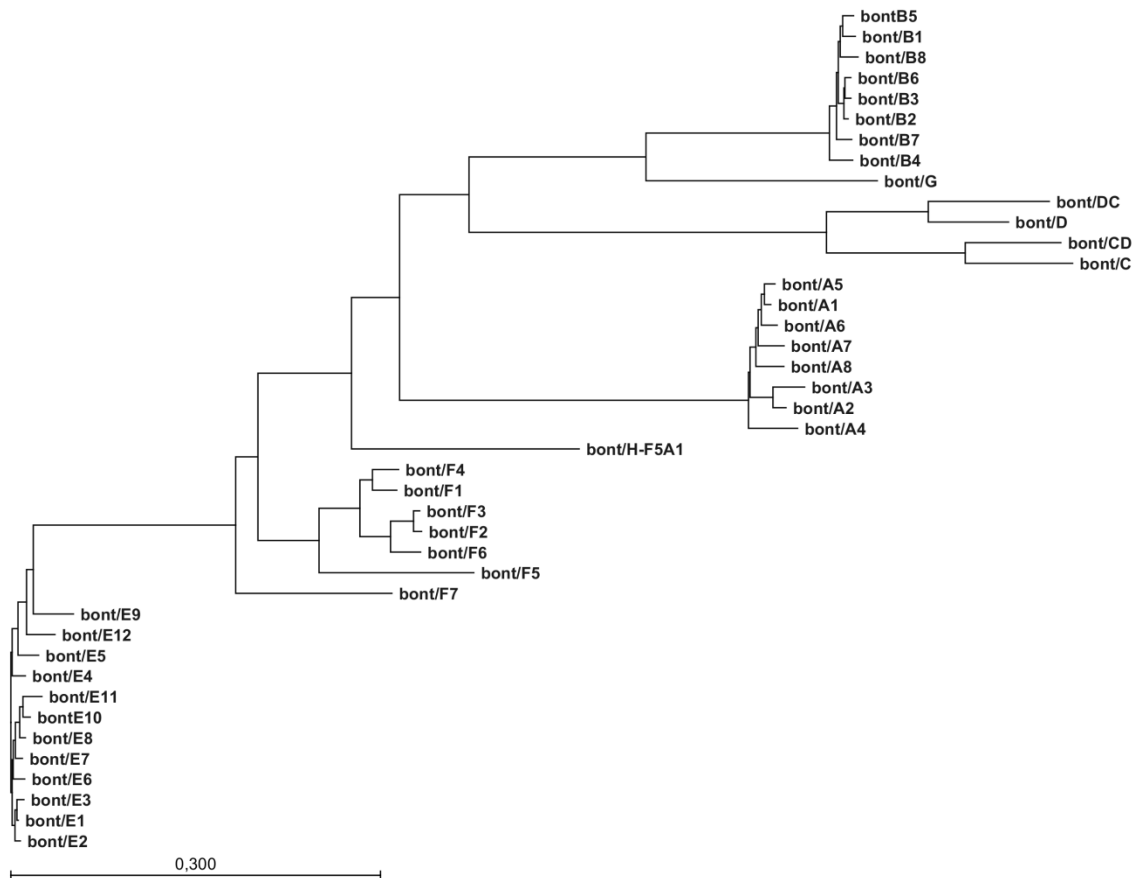


Figure 10. Genetic relatedness of *bont* subtypes diversity: *bont*/A1 to A8, *bont*/B1 to B8, *bont*/C, C/D, D, D/C, *bont*/E1 to E12, *bont* F1 to F7, *bont*/G, *bont*/H-FA.

C. botulinum bont subtypes origins are indicated with accession number in bracket; *bont* origin from *C. baratii*, *C. butyricum* and *C. argentinense* are specified. *Bont* subtype A1 strain ATCC3502 (accession number NC009495); A2 Kyoto (NC012563); A3 Loch Maree (NC010520); A4 657Ba (NC012658); A5 H04402 065 (NC017299); A6 CDC41370 (FJ981696); A7 2008-148 (JQ954969); A8 Chemnitz (KM233166); B1 Okra (AB232927); B2 Prevot59 (EF033128); B3 CDC795 (EF028400); B4 non-proteolytic ATCC17844 (EF028394); B5 bivalent CDC593 (AF300466); B6 Okayama2011 (AB665558); B7 NCTC3807 (JN120760); B8 Maehongson2010 (JQ964806); C Stockholm (AP008983); CD Eklund (ABDQ01000029); D 1873 (AB012112); DC OFD05 (AB461915); E1 NCTC11219 (X62683); E2 CDC5247 (EF028404); E3 INGR16-02E1 (KF719371); E4 *butyricum* ATCC43755 (X62088); E5 *butyricum* KZ1890 (AB037711); E6 K36 (AM695759); E7 IBCA97-0192 (JN695729); E8 E134 (JN695730); E9 CDC66177 (JX424534); E10 FWKR11E1 (KF861887); E11 SW280E (KF861877); E12 84-10 (KM370319); F1 Langeland (GU213203); F2 CDC4013 (GU213209); F3 CDC54086 (GU213218); F4 CDC54078 (GU213214); F5 54074 (GU213211); F6 non-proteolytic VPI7943 (GU213228); F7 *baratii* CDC59837 (GU213231); G *argentinense* 113-30_NCFB3012 (X74162); H-FA IBCA10-7060 (JSCF01000006). The scale represents the branch length and is given in terms of expected numbers of substitutions per site. Elaborated by C. Woudstra from publicly available sequences using CLC® Genomic Workbench 7.5.1.

Bont genes from type A1 to A8 have 92–95% nucleotide identity corresponding to 84–90% amino acid identity (Kull et al., 2015). Type B genes differ from 2 to 4% at nucleotide level and 3–6% at amino acid level (Wangroongsarb et al., 2014). Mosaic genes C/D and D/C can be distinguished from classical type C and D strains in that the first letter designates catalytic activity and the second, receptor binding activity (Figure 11) (Nakamura et al., 2010; Takeda et al., 2005).

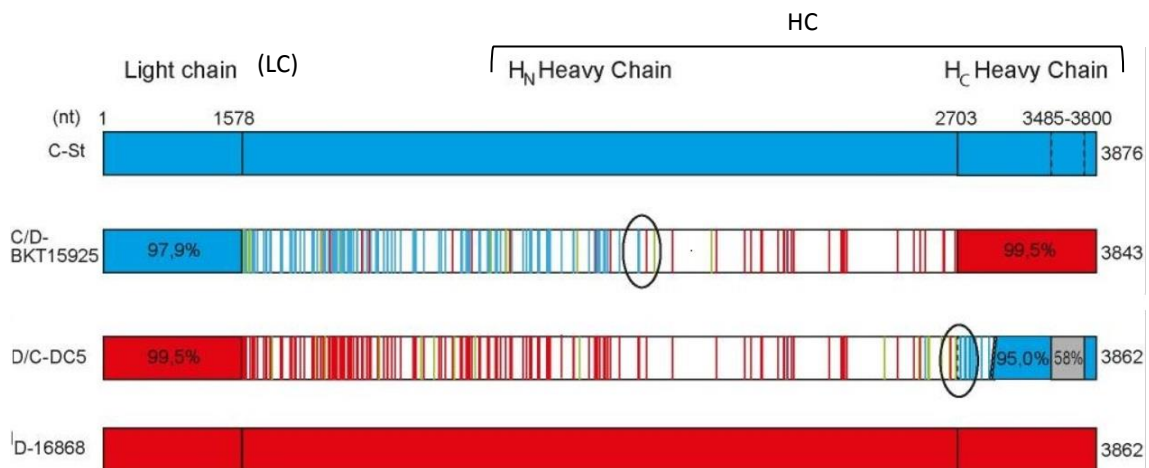


Figure 11. Alignment of *C. botulinum* Group III *bont*/C, C/D, D and D/C sequences.

Chimeric sequences are aligned against non-chimeric sequences from type C strain C-Stockholm and type D strain 16868. The chimeric sequences are from type C/D strain BKT015925, and type D/C strain DC5. In the H_N domain, nucleotides that are conserved are not coloured, whereas unique nucleotides are marked in green, nucleotides corresponding to the C-sequence are marked in blue, and nucleotides corresponding to the D-sequence are in red. The light chain and the H_C domain are not represented according to scale and they are coloured as the closest related sequence. Estimated recombination sites are encircled. From Skarin and Segerman, 2014.

For BoNT/C and C/D, two-thirds of the molecules are highly similar (96.9–97.7% identical in their light chain (LC) and 92.6–93.6% in their H_N-domain), while the H_C domains are less well conserved (>40%). Similarly, BoNT/D and D/C have very similar LC and H_N domains (97.8–98.2% identical in their LC and 94.8–95.5% in their H_N-domain), and less conservation in the H_C domains (>37%). Conversely, the H_C domains of BoNT/D and C/D are markedly homologous (>91%), while the H_C domains of BoNT/C and D/C are less well conserved (74%

overall) (Skarin and Segerman, 2014). *Bont/E* sequences are divided into 12 subtypes; the ten subtypes from *C. botulinum* share 99% nucleotide identity and 97–99% amino acid identity, while the two *bont/E* subtypes from *C. butyricum* strains (E4 and E5) have 97–98% nucleotide and 95–96% amino acid identities (Mazuet et al., 2015b; Weedmark et al., 2014). Nucleotide sequences of *bont/F* are even more different (25%) and are clustered into seven subtypes. *bont/F* sequences from *C. botulinum* type F (Group I *bont/F*1 to F5; Group II *bont/F*6) form a different cluster to those from *C. baratii* (*bont/F*7) (Guo and Chen, 2015; Raphael et al., 2010a). The small number of type G strains available does not allow neurotoxin gene diversity to be evaluated in a significant manner.

I.4.5. *bont* genetic locus

Bont belongs to a genetic cluster containing a non-toxic non-haemagglutinin (NTNH) gene and several other haemagglutinin components (HAs) or OrfX proteins of unknown function, along with regulatory proteins (Sharma et al., 2003). The NTNH gene is always located on the 5' side of the *bont* gene; they are transcribed in the same direction and constitute a transcriptional operon. A second transcriptional unit is made of the HAs (ha17, ha33 and ha70) or OrfX (orfX1, orfX2, orfX3) genes that are transcribed in the opposite direction from the NTNH/*bont* operon. The botulinum neurotoxin cluster is made up of the NTNH/*bont* operon together with either the “HA cluster” or the “OrfX cluster”. The botulinum locus is then denoted by the presence or absence of the HA and OrfX clusters (HA^{+/-} versus OrfX^{+/-}) (Figure 12).

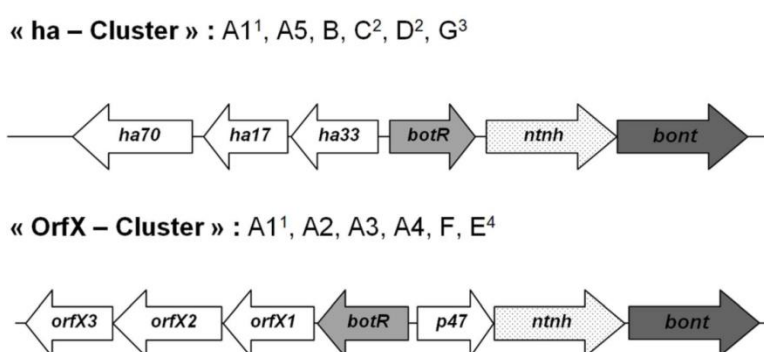


Figure 12 : *Bont* cluster arrangements.

The “ha cluster” appears to be associated with type A1, A5, B, C, D and G *bont* genes, and the “orf-X cluster” with type A1, A2, A3, A4, F and E *bont* genes. ¹ Type A1 *bont* gene has also been found associated with orf-X cluster in single toxin strains and in all bivalent strains. ²The *botR* gene arrive ahead of *ha* genes in all toxinotype C and D strains. ³The *Ha33* gene is absent in all toxinotype G strains. ⁴The *botR* gene is absent in all toxinotype E strains. HA, hemagglutinin; ORF, open reading frame; NTNH, non-toxic non-hemagglutinin. BOTR is a regulatory gene identified in the HA cluster. From Vanhomwegen et al., 2013.

Between *bont*/NTNH and HA-OrfX transcriptional units lies a gene encoding a sigma 70 factor (botR) involved in the positive regulation of the neurotoxin gene (Dupuy and Matamouros, 2006). It is absent in *C. botulinum* and *C. butyricum* type E, here it is replaced by a p47 gene encoding a 47kDa protein of an unknown function located in the OrfX cluster. The HA operon is associated with *bont*/B, C, D, and G, whereas the OrfX operon is linked to *bont*/A, E and F (Jacobson et al., 2008). *Bont* genes are present on different genetic elements, including phages, plasmid or chromosomes (see Table 3). Proteolytic and non-proteolytic *C. botulinum* Group I and II harbour the neurotoxin genes either on the chromosome or a plasmid (Carter and Peck, 2015). In *C. botulinum* Groups III and IV, the neurotoxin genes are respectively located on a bacteriophage and plasmid (Bhandari et al., 1997; Skarin et al., 2011). Genomic analysis of the *C. botulinum* Group III bacteriophage genome encoding type C, C/D, D and D/C neurotoxins revealed that this phage is a circular plasmid prophage which does not integrate in the host chromosome. Toxin is produced during the lysogenic state (Sakaguchi et al., 2005). In *C. butyricum* Group V, the *bont*/E locus is located on a plasmid, whereas in *C. baratii* Group VI, *bont*/F is chromosomal.

I.4.6. BoNT uses

The potential misuse of botulinum toxin has resulted in very strict legislation worldwide on the commercial or scientific exchange of any biological material from *C. botulinum*. In the United States, the Centre for Disease Control and Prevention (CDC) maintains a well-established surveillance system for human botulism based on clinician reporting (Shapiro et al., 1997). The French Agency for Medicines and Health Products Safety, ANSM, is in charge of delivering special authorisations for possession and use of living organisms, toxin, or any genetic material from *C. botulinum*, which is on the French “MOT” list of highly pathogenic microorganisms.

I.4.6.1. Military use

BoNT was first developed as a biological weapon over 80 years ago by military regimes against their opponents because of its lethality. It can be aerosolised or used to contaminate food. The Soviet Union and Iraq have produced large amounts of BoNT, and Iraq loaded toxin into missiles and bombs (Roffey et al., 2002). Nevertheless, BoNT is still a poor choice as a biological weapon or a weapon of mass destruction because it is rapidly degraded in the

environment and becomes non-lethal minutes after release; it is rapidly inactivated by standard water sanitation protocols; it does not penetrate the skin; it is not transmissible from person to person, and finally, antitoxin treatment is available.

1.4.6.2. Bioterrorism

Biological threats have been the centre of much attention since the Biological Weapons Convention (Lebeda, 1997). Because of their extreme potency (a single gram of crystalline toxin could potentially kill one million people), BoNTs are classified as one of the highest risk threat agents for bioterrorism (Arnon et al., 2001). *Clostridium* BoNTs have been included in the Australia Group (AG) list of biological agents. The Centres for Disease Control and Prevention (CDC) in the United States also consider BoNTs as category A agents that could pose a risk to national security (other agents/diseases in this category include anthrax; plague; smallpox; tularaemia, and viral haemorrhagic fevers (Rambhia et al., 2011)). The deliberate release of aerosolised BoNT (the lethal dose for a 70-kg person being 0.8 – 0.9 µg by inhalation) or contamination of the feed or food chain (the lethal dose for a 70-kg person being 70 µg via this route) for the purpose of bioterrorism is the subject of concern (Wein and Liu, 2005; Woudstra et al., 2013b). In the 1990s, the Aum Shinrikyo cult released aerosolised toxin in Japan, but fortunately unsuccessfully. Other terrorist groups, such as Hezbollah, have bought and sold counterfeit BoNT to raise cash. The Al Qaeda movement also tried to obtain BoNTs for terrorist purposes, but they failed due to rudimentary laboratory equipment and limited access to lethal strains (Katona, 2012).

1.4.6.3. Therapeutic and cosmetic use

Despite its high toxicity, Botulinum neurotoxin type A (BoNT/A) is approved for therapeutic use for many neurological disorders, including dystonia and spasticity, and non-neurological troubles such as achalasia or hyperhidrosis as well as muscular contraction disorders (Persaud et al., 2013). Its mode of action is well understood, which has in turn led to increasing indications (e.g. pain, gastrointestinal and urological disorders) for which the toxin can reduce disturbing symptoms (Arbizu and Rodriguez, 2015). The application is generally safe, with few unwanted reactions. At worst, BoNT-treated patients may develop neutralising antibodies. More recently, BoNT/B serotype has been approved as a substitute for patients who do not respond to BoNT/A (Pickett and Perrow, 2011). However, because BoNT/B is less potent in humans, higher doses have to be administered, leading to only transient success in the treatment. Commercial botulinum toxin BoNT/A called “Botox” (first

licensed in 1989 by Allergen) is also available for cosmetic use. Botulinum toxin type A injections are one of the most popular cosmetic procedures for diminishing the appearance of facial lines caused by habitual facial muscle contractions. The “muscle relaxant” effect lasts about three to four months and can be repeated when needed (Yeilding and Fezza, 2015).

I.5. *Clostridium botulinum* genome

One hundred and seven genome sequences (21 complete genomes, 86 draft genomes) of *C. botulinum* strains are currently available at the National Centre for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/genome/> accessed on the 1st of September 2015). These include 58 genome sequences (14 complete and 44 draft) of *C. botulinum* strains from Group I, 14 from Group II (five complete and nine draft) and 31 from Group III (one complete, *C. botulinum* BKT015925 type C/D, and 30 draft genome sequences – two for type C, 20 for type C/D, three for type D, and five for type D/C). One draft genome of Group IV *C. argentinense*, two draft genomes of Group V *C. butyricum* and one complete genome of Group VI *C. baratii* Sullivan are also available. The genomes consist of a circular DNA chromosome ranging in size from 3.66 to 4.15 Mbp for *C. botulinum* Groups I and II. Group III displays a smaller genome (3.2 Mbp). The *C. argentinense* Group IV genome is 4.4 Mbp long, and the *C. butyricum* Group V 4.51 Mbp, while the *C. baratii* Group VI is 3.15 Mbp long. Most strains also possess plasmids or prophages which vary in size from 16 Kbp to 270 Kbp. The GC content (27–28%) is similar to that of most Clostridial species. The comparison of neurotoxin-producing Clostridia genome sequences has shown that their genomes are organised differently; they form distinct phylogenetic clusters which each represent one of the different physiological Groups (Figure 13). This indicates that each *C. botulinum* Group is genomically heterogeneous and could be assimilated to entirely different species (Hill et al., 2007). Areas of divergence include the presence of plasmid, the flagellar glycosylation island and prophages.

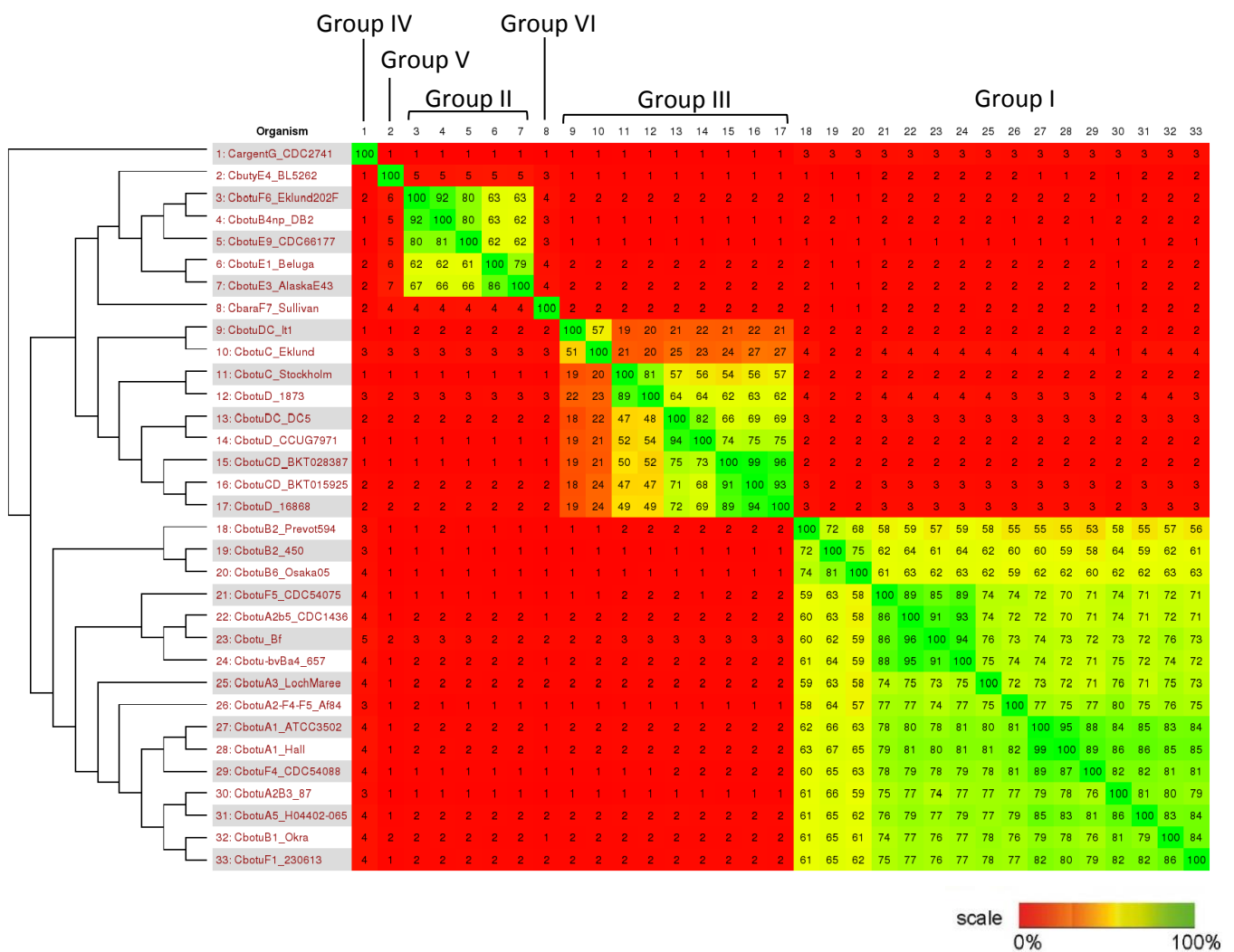


Figure 13. Phylogenetic overview of representative *C. botulinum* genomes.

A subset of 33 *C. botulinum* genomes on the 107 currently available in public database, representing Group I to VI, was analysed using Gegenees 2.1 software (www.gegenees.org) and Splitstree 4 (www.splitstree.org). Both heat-plots of the similarity matrices and trees were created using the same data. The heat-plot is based on a fragmented alignment using BLASTN made with settings 200/100. A dendrogram was produced in SplitsTree 4 (using neighbor joining method) made from a Nexus file exported from Gegenees. Elaborated by C. Woudstra from publicly available sequences.

C. botulinum is a taxonomic designation for many different anaerobic spore-forming rod-shaped bacteria that have the common property of producing BoNTs. Within the Clostridia genus, several species are genetically and physiologically closely related to *C. botulinum* but do not produce the neurotoxin. An analysis of phylogenetic relationships based on genome sequencing demonstrated that *C. sporogenes* is part of the proteolytic Group I *C. botulinum* lineage. Non-proteolytic saccharolytic Group II forms a distinct group separate from other

saccharolytic Clostridia and is phylogenetically far removed from Group I. *C. novyi* and *C. haemolyticum* are related to Group III, and *C. subterminale* to Group IV type G (Suen et al., 1988a, see also figure 19), in agreement with the four phenotypical criteria divisions. *C. butyricum* and *C. baratii* BoNTs producing Group V and VI respectively are related to their own non toxigenic species sequences (Figure 14) (Poehlein et al., 2015).

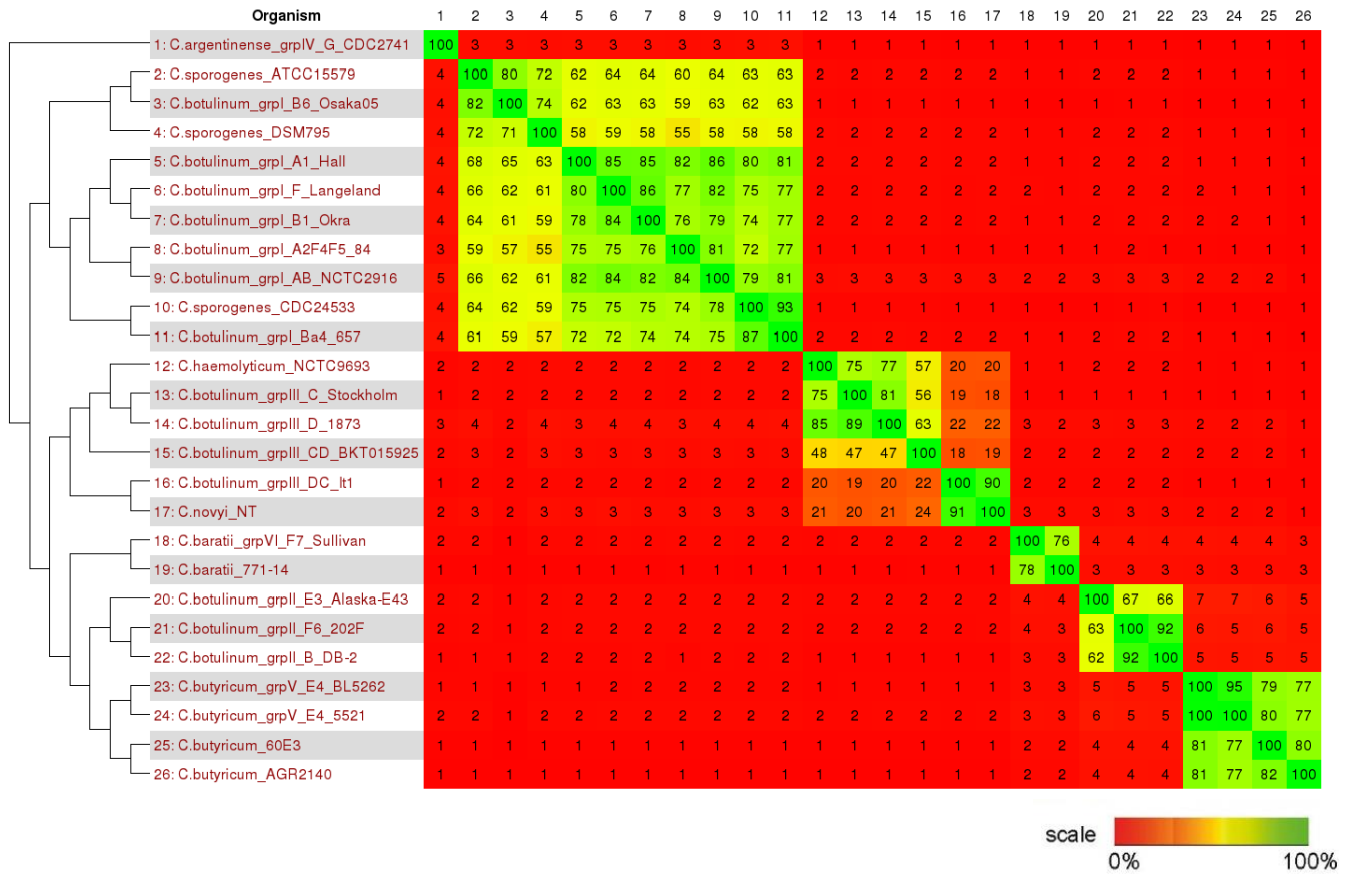


Figure 14. Phylogenetic whole genome comparison of BoNT producing Clostridia and non-BoNT toxigenic related species.

A subset of BoNT producing Clostridia genomes together with non BoNT producing Clostridia were analyzed using Gegenees 2.1 software (www.gegenees.org) and Splitstree 4 (www.splitstree.org). Both heat-plots of the similarity matrices and trees were created using the same data. Elaborated by C. Woudstra from publicly available sequences.

I.5.1. Genomic characteristics of *C. botulinum* Group I

Physiological (proteolytic) Group I includes *C. botulinum* strains producing toxins of serotypes A, B or F and is mainly associated with human cases. The BoNT gene could be located either on the bacterial chromosome or a plasmid. The non-toxic species *C. sporogenes* is so close to proteolytic Group I *C. botulinum* that it shares nearly identical metabolic properties, including chromosome organisation (Hill and Smith, 2013). It has been used for the last 70 years as *C. botulinum* surrogate in thermal processing control for the food industry. Whole genome analysis based on the genome of *C. botulinum* type A1 Hall (ATCC3502) demonstrates the close genetic relationship between strains of proteolytic *C. botulinum* and *C. sporogenes*, and possession of a relatively stable genome (Carter et al., 2009). Phylogenetic analyses of *C. botulinum* Group I and *C. sporogenes* whole genomes showed them to clearly separate into two discrete clades with 93% average nucleotide identity (Weigand et al., 2015), supporting their distinction as two separate species. The work of Weigand et al. showed some BoNT type B strains such as Osaka05 and Prevot94 to be more closely related to the *C. sporogenes* clade than to the *C. botulinum* cluster. As the BoNT B toxin gene is frequently carried by plasmids (Franciosa et al., 2009), one explanation could be that these strains are *C. sporogenes* having acquired the BoNT toxin gene via plasmid horizontal transfer. Similarly, the presence of *C. sporogenes-like* strains in the *C. botulinum* clade suggest the possibility that these strains were true *C. botulinum* that underwent BoNT B toxin plasmid loss and were falsely characterised as *C. sporogenes* (Figure 15). It suggests that some toxin gene variants may be highly mobile within these two groups of organisms and that some strains classified as *C. sporogenes* could be reclassified as *C. botulinum* having lost their toxin gene, and vice versa. This work constitutes a key milestone on the way to understanding the diversity of human-related *C. botulinum* Group I strains.

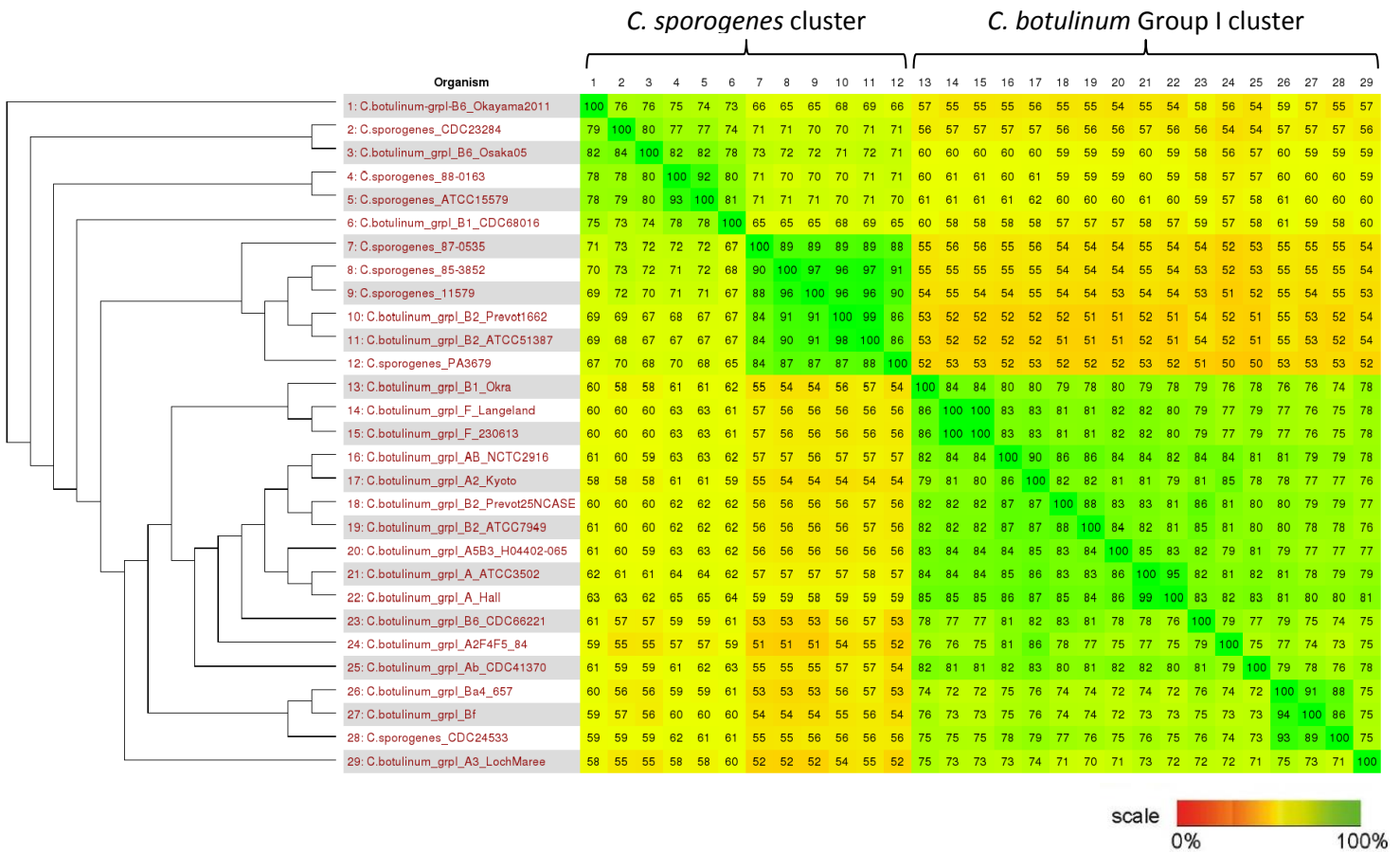


Figure 15. Phylogenetic comparison of *C. botulinum* Group I and *C. sporogenes* genomes.

A subset of *C. botulinum* Group I versus *C. sporogenes* genomes were analyzed using Gegenees 2.1 software (www.gegenees.org) and Splitstree 4 (www.splitstree.org). Both heat-plots of the similarity matrices and trees were created using the same data. Adapted from Weigand et al., 2015.

I.5.2. Genomic characteristics of *C. botulinum* Group II

Physiological Group II (non-proteolytic) *C. botulinum* consists of strains producing toxins of serotype B, E or F responsible for human botulism. It is distinct from other *C. botulinum* Groups. Although Group II strains are closely related, the strains can be grouped into two distinct subpopulations based on their genetic diversity: the Eklund17B cluster encompassing type B and type F strains, and recently a type E9 strain (Raphael et al., 2012); and the AlaskaE43 cluster formed of only type E strains (Hill et al., 2007; Raphael et al., 2012) (Figure 16). No relationship was observed between the clusters and geographical origin, environment, food type or isolation date (Stringer et al., 2013). Recently, a large genomic

study based on the genetic analysis of 163 Group II isolates and their SNP profiles confirmed this clustering distinction (Weedmark et al., 2015). Interestingly, the cluster containing the Eklund17B strain produces only type B4, E9 and F6 toxin types, located on a plasmid. It has been reported that the neurotoxin gene located on a plasmid for some strains of Group II type B4 could be spontaneously lost in both the environment and in laboratory conditions, leading to non-toxigenic strains which have not yet been given a specific name (Carter et al., 2014; Stringer et al., 2013). The presence of non-toxigenic isolates belonging to the Eklund17B cluster could thus be explained by their loss of the plasmid containing the botulinum neurotoxin locus.

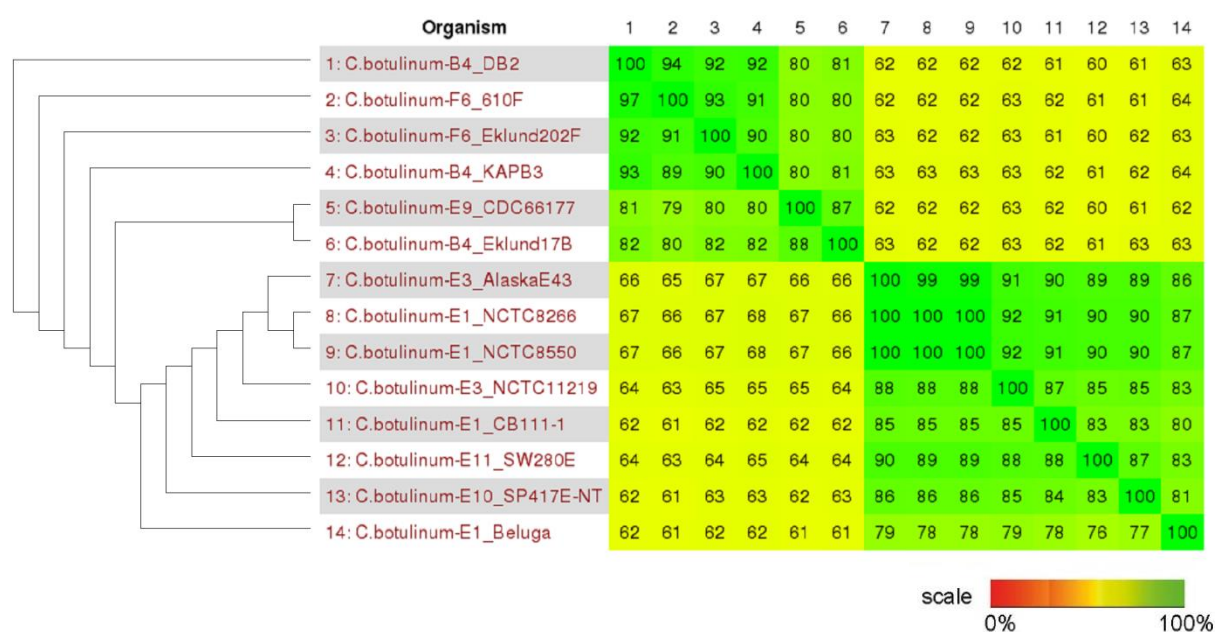


Figure 16. Phylogenetic comparison of *C. botulinum* Group II genome sequences.

Fourteen *C. botulinum* Group II genomes were analyzed using Gegenees 2.1 software (www.gegenees.org) and Splitstree 4 (www.splitstree.org). Both heat-plots of the similarity matrices and trees were created using the same data. Elaborated by C. Woudstra from publicly available sequences.

I.5.3. Genomic characteristics of *C. botulinum* Group III

Group III produces BoNT of serotype C or D (and their mosaic forms C/D and D/C). The *bont* genes are located on a large prophage of 200 Kbp. They are associated with avian and non-human mammalian botulism. Group III produces two additional toxins named C2 and C3. The C2 gene is located on a large plasmid, whereas the C3 gene is located on the BoNT prophage. C2 is a binary toxin composed of a binding component (C2-II) and an enzymatic

component (C2-I) which disorganise the actin cytoskeleton through ADP-ribosylation of actin, making exposed mammalian cells to become round and leading to the accumulation of fluid in exposed organs (Reuner et al., 1987). C3 is an exoenzyme that acts on Rho GTP-binding proteins by ADP-ribosylation (Moriishi et al., 1991). Their role in animal botulism has revealed their enterotoxic properties (Ohishi, 1983). Most virulence genes are carried on plasmids or phages, which can be exchanged by horizontal transfer; this leads to the inter-conversion from toxigenic to non-toxigenic strains and vice versa (Eklund et al., 1974; Iida et al., 1974). The genome sequences of *C. botulinum* Group III strains are very different from those of Group I and II strains, but are closely related to that of *C. novyi* and *C. haemolyticum* (Skarin and Segerman, 2014). Their close relatedness led the scientific community to encompass them as a new genus named *Clostridium novyi sensu lato* (Skarin and Segerman, 2011). *C. botulinum* Group III is responsible for animal botulism, *C. novyi* for gas gangrene and black leg disease (Oakley et al., 1947), and *C. haemolyticum* for bacillary haemoglobinuria (Hauer et al., 2004). Although causing different diseases, these strains are clearly genetically related, as previously shown (Skarin and Segerman, 2011). A genomic comparison of strains representing *C. novyi sensu lato* species revealed four separate lineages (I to IV), with lineage I subdivided into Ia and Ib given their close genetic content (Figure 17). Lineage I contains only BoNT-producing strains, and subgroup Ia seems to be predominant in the light of available genome sequences. Lineage II consists of all three species and contains the oldest isolates. The few strains investigated and found to be part of lineage III may indicate that it could be a less common lineage. Lineage IV has evolved towards *C. novyi* type A strains, but also contains one *C. botulinum* strain (Figure 17).

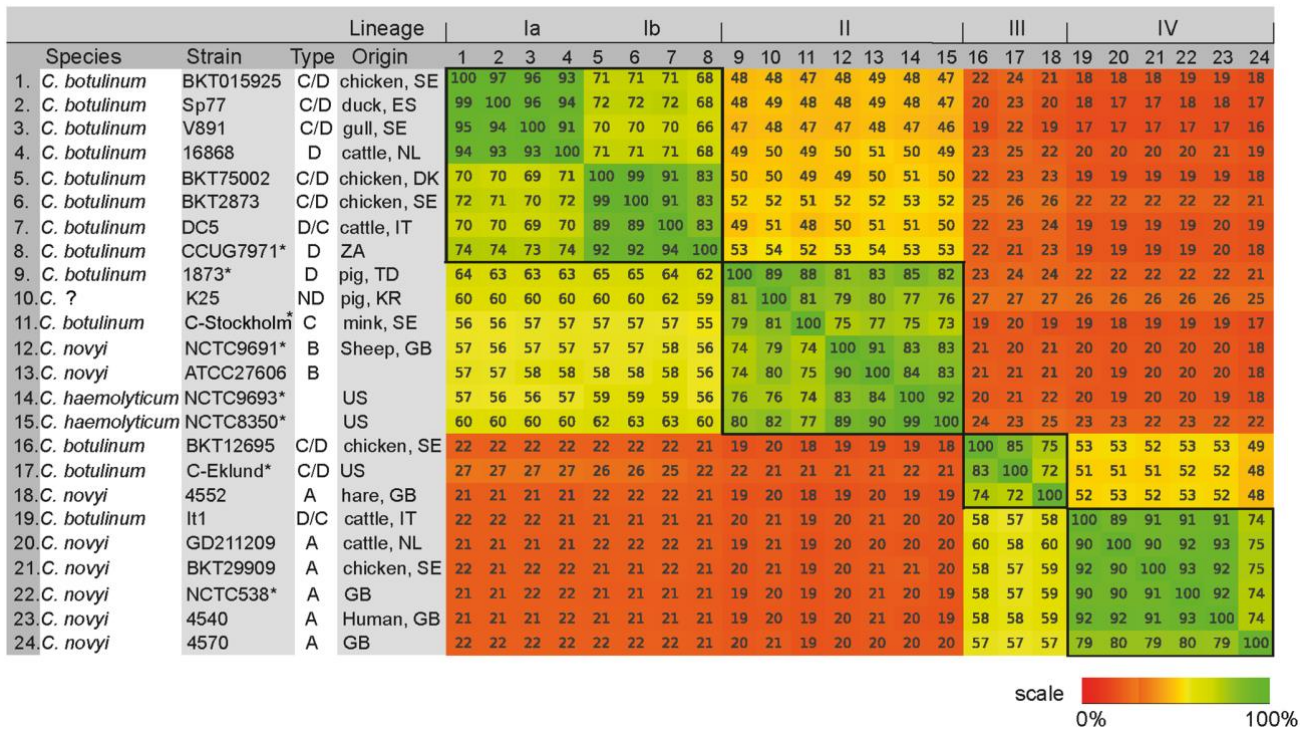


Figure 17. Average whole genome similarity of *C. novyi sensu lato* genomes.

A similarity matrix based on normalized average BLASTN scores for fragmented comparisons covering the whole genomes (200 bp fragment size) illustrated by a heat-plot. Four lineages were identified (I–IV) and are framed with black squares. The geographical origins of the strains are listed with two-digit country codes. Strains isolated more than 50 years ago are marked with a star. From Skarin and Segerman, 2014.

Another interesting property of the *C. botulinum* Group III genome is the presence of several insertion sequence (IS) elements located at different positions in closely related strains. This indicates that the genome is influenced by high transposon activity (Skarin and Segerman, 2011). Group III genomes also differ from other *C. botulinum* groups by a much higher number of plasmids. The study of Skarin et al. based on Whole Genome Sequence (WGS) analysis of 24 *C. novyi sensu lato* strains revealed 61 plasmids organised in 13 plasmid groups (Figure 18), which are actually phage-like plasmids (due to the presence of phage gene functions).

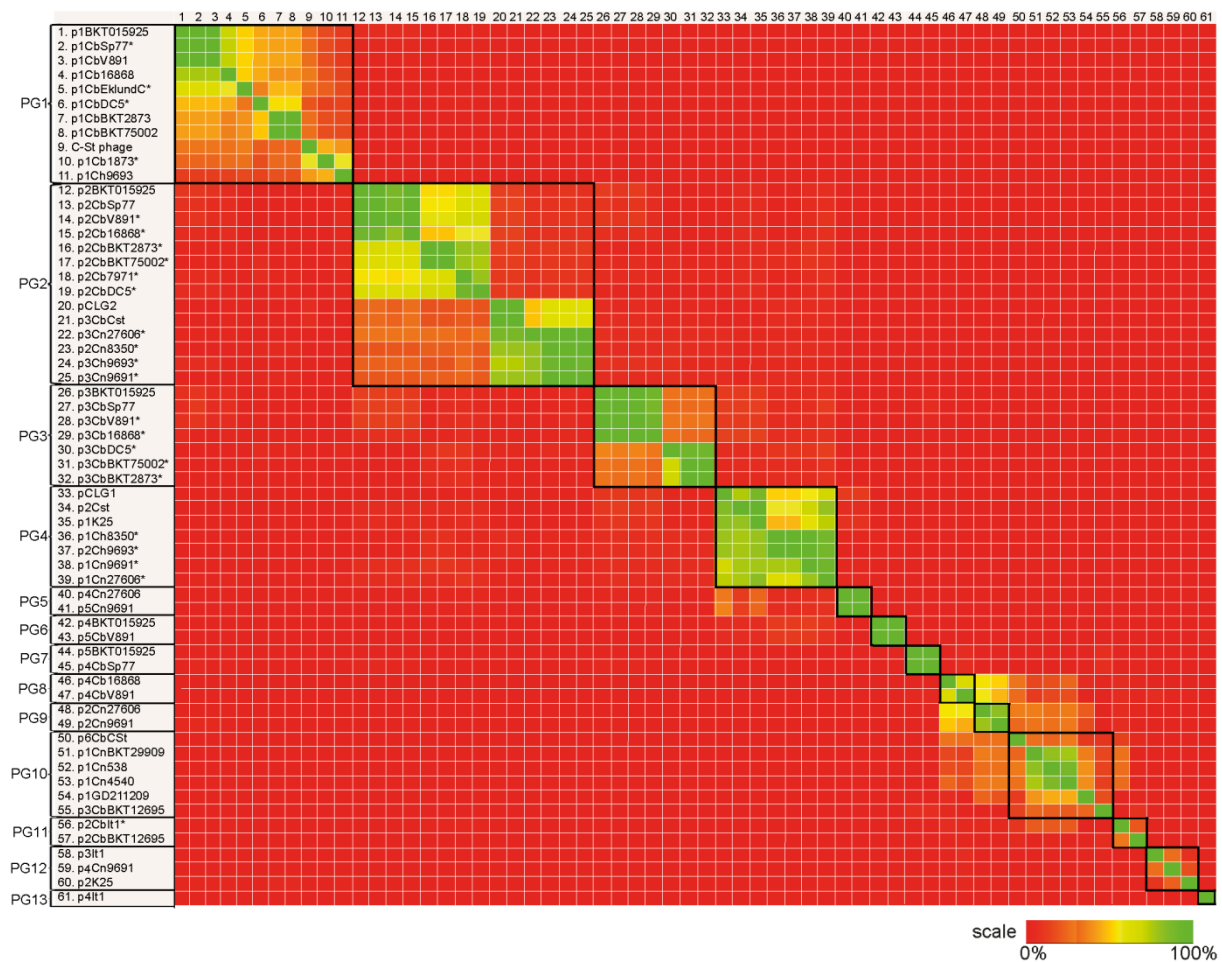


Figure 18. Comparative analysis of *C. novyi sensu lato* plasmids.

Heat-plot of the relative amount of shared genetic material between plasmids (40% normalized BLASTN score threshold). Thirteen plasmid groups (PG1–PG13), framed by black squares, were identified from these results and from analysis of plasmid replication and partitioning genes. Some of the plasmids in lineage I strains (PG1–PG3) shared several IS elements, and this resulted in an apparent increase in the background level of shared genetic content between otherwise dissimilar plasmids within this lineage. Uncompleted plasmids are marked with a star. From Skarin and Segerman, 2014.

I.5.4. Genomic characteristics of *C. botulinum* Group IV, *C. butyricum* Group V and *C. baratii* Group VI.

Within Group IV Clostridia, all *C. botulinum* type G strains (renamed *C. argentinense* (Ciccarelli et al., 1977)) are closely related to each other and represent a single genomic species based on multilocus enzyme electrophoresis and 16S RNA analysis (Figure 19) (Altwegg and Hatheway, 1988). Distantly related to other *C. botulinum* Groups, *C.*

argentinense BoNT G is closer to *C. subterminale*, which could be assimilated as its non-toxigenic phenotypical counterpart with sequence homologies up to 70% relatedness (Suen et al., 1988a).

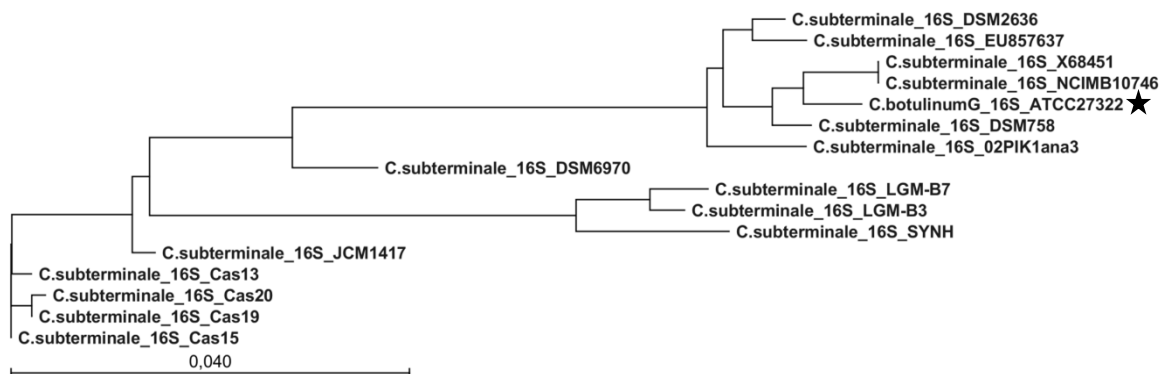


Figure 19. 16S RNA phylogenetic relatedness between *C. botulinum* type G and *C. subterminale*.

C. botulinum type G is marked with a star. The scale represents the branch length and is given in terms of expected numbers of substitutions per site. Elaborated by C. Woudstra from publicly available sequences using CLC® Genomic Workbench 7.5.1.

DNA-DNA pairing studies have shown that BoNT-synthesising strains of *C. baratii* and *C. butyricum* are genetically closely related to non-toxigenic reference strains (including type strains) of their respective species (Suen et al., 1988b). These toxigenic strains are genetically remote from Group I-IV *C. botulinum* strains. *C. butyricum* Group V whole genome sequencing showed the species to be closely genetically related (Figure 20).

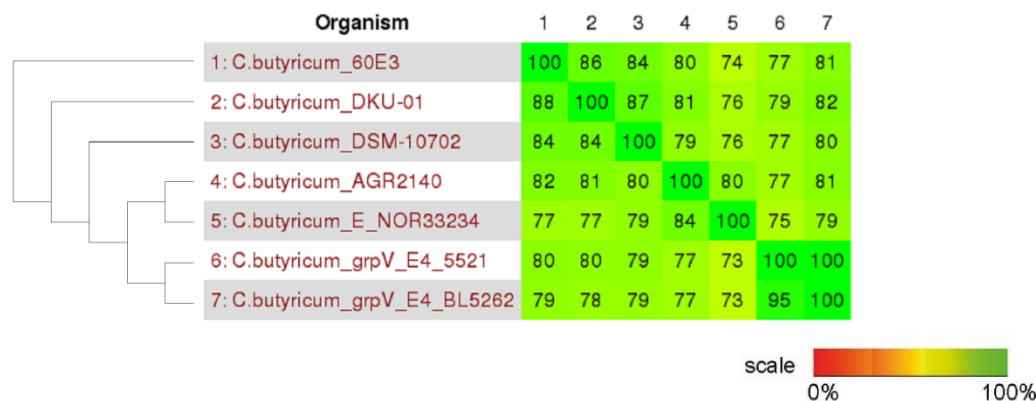


Figure 20. Whole genome phylogenetic relatedness between *C. butyricum* Group V type E and non-BoNT producing *C. butyricum*.

C. butyricum Group V producing BoNT E4 (strains 5521 and BL5262) genomes were compared to *C. butyricum* non-BoNT toxigenic using Gegenees 2.1 software (www.gegenees.org) and Splitstree 4 (www.splitstree.org). Both heat-plots of the similarity matrices and trees were created using the same data. Elaborated by C. Woudstra from publicly available sequences.

16S ribosomal RNA sequencing has also shown that BoNT-synthesising strains of *C. baratii* Group VI are genealogically indistinguishable on the 16S level from non-toxigenic strains of their respective species (Hutson et al., 1993) (Figure 21).

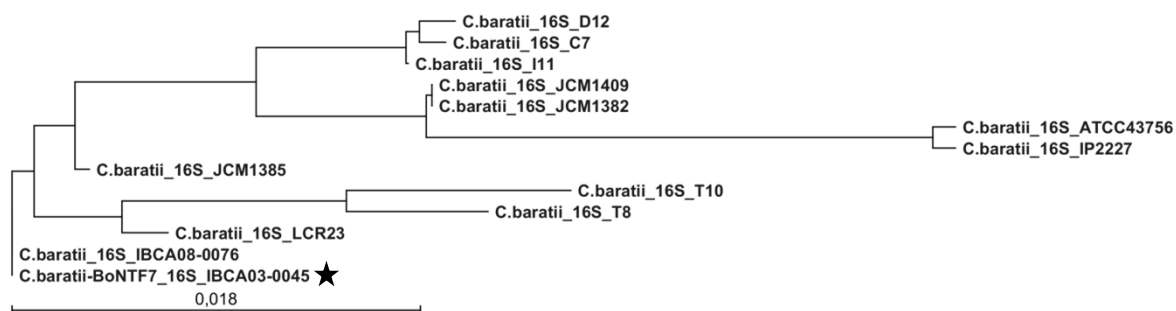


Figure 21. 16S gene phylogenetic relatedness between *C. baratii* Group VI type F and non-BoNT producing *C. baratii*.

C. baratii type F7 is marked with a star. The scale represents the branch length and is given in terms of expected numbers of substitutions per site. Elaborated by C. Woudstra from publicly available sequences using CLC® Genomic Workbench 7.5.1.

I.5.5. Mobile genetic elements and horizontal gene transfer

Microbial communities evolve and adapt to different environments by the transmission of genetic material from parent bacterial cells to newly divided cells. Different mechanisms may be used to promote the genetic diversity necessary to adapt to environmental changes. Horizontal gene transfer, which refers to the transfer of genetic material from bacteria to bacteria without a reproduction mechanism, is one way of promoting genetic diversity. This mechanism is usually mediated by mobile genetic elements. In microbial communities, the latter consist of viruses, plasmids and associated elements (insertion sequences, transposons and integrons) that are either self-transmissible or that use mobile plasmids and viruses as vehicles for their dissemination (Sobecky and Hazen, 2009). Clostridia comprise a heterogeneous group of environmental bacteria containing 15 pathogenic species (e.g. *Clostridium tetani*, *Clostridium perfringens* or *Clostridium botulinum*) producing lethal toxins (tetanus toxin TeTx, epsilon toxin, botulinum toxin BoNT (Popoff and Bouvet, 2013)). A comparison of the *C. botulinum* sequence, the location of neurotoxin genes on different genetic elements (plasmid, phage or chromosome) and the wide genetic diversity between the physiological Groups all suggest that the different toxin types have evolved separately in different bacterial species (Hill et al., 2007). The location of the *bont* locus within the chromosome or plasmid appears to occur not at random but at specific sites. In strains from Group I or II, whose genome sequences are available, three specific sites of *bont* locus integration have been identified: the *ars* operon, which contains three to five genes involved in arsenic reduction; the *oppA/brnQ* operon encoding for extracellular solute binding proteins and branched chain amino acid transport proteins respectively; and the *rarA* gene which encodes a resolvase protein involved in recombination or transposon insertion events. The presence of insertion sequences (IS) flanking the *bont* locus supports a horizontal gene transfer by IS elements and/or transposons (Hill et al., 2009). Genes of the *bont* locus of different types of neurotoxigenic bacteria appear to have been rearranged, probably by homologous recombination. This is supported by the identification of atypical strains carrying mosaic genes C/D and D/C containing elements derived from *C. botulinum* C and D (Moriishi et al., 1996a), or mosaic NTNH genes from *C. botulinum* type A and C (Kubota et al., 1996). The *bont* locus has a complex genetic diversity that has undergone recombination, insertion and horizontal gene transfer events among various strains of Clostridium species. This is what has led to the multiplicity of toxin types and subtype diversity observed today.

Chapter II

Diagnosis

II.1. Clinical symptoms

Clinical manifestations and patient history are the first indications that should guide investigations towards botulism. The typical clinical symptoms of all forms of human botulism include cranial muscle paralysis, such as double vision and dilated pupils, slurred speech, dry mouth, difficulty in swallowing and speaking, and facial paralysis. Limb and respiratory dysfunctions become apparent when the disease progresses. A demonstration of *C. botulinum* neurotoxin in the patient's serum or gastrointestinal tract, coupled with its demonstration in suspect foodstuff, constitute the "gold standard" for diagnosing foodborne botulism (Brola et al., 2013). Infant botulism is diagnosed by demonstration of BoNTs in the patient's serum or gastrointestinal tract contents, as well as by faecal cultures of *C. botulinum* organisms (Rosow and Strober, 2015). Wound botulism is ideally diagnosed by the demonstration of the neurotoxin in the wound or serum, as well as a positive culture of *C. botulinum* organisms (Caya, 2001).

Diagnosis of animal botulism is at first based on clinical signs that are indicative but not specific. A presumptive diagnosis is based on the combination of signs observed in sick animals, the duration of the outbreak, post-mortem findings and by ruling out other differential diagnoses (Anniballi et al., 2013b).

The definitive confirmation for human and animal botulism diagnosis requires detection and identification of the neurotoxin type and, if possible, isolation of the pathogenic strain involved in the outbreak after an enrichment step.

II.2. Botulism laboratory confirmation

As botulism is a life-threatening condition, an early and accurate diagnosis, with determination of the serotype, is critical in order to administer the appropriate antitoxin for a successful therapy. First based on clinical symptoms, botulism has to be confirmed by laboratory analysis. Demonstration of BoNTs and of BoNT-producing Clostridia in serum and samples collected during the outbreak are the best complementary analyses to confirm the

clinical diagnosis. The extreme potency of BoNT demands strict requirements to ensure the safety of laboratory workers. Despite the potency of its neurotoxin, *C. botulinum* is a non-invasive and non-contagious bacterium, which is why it has been graded as a class II pathogen. An appropriate biosafety level 2 containment facility and trained personnel are a minimum requirement for dealing with *C. botulinum*. An anaerobic chamber (Figure 22) filled with nitrogen, hydrogen and carbon dioxide (80-10-10%) could be used to provide the adequate anaerobic conditions for cultivating samples suspected to contain *C. botulinum*.



Figure 22. Glove box used for *C. botulinum* cultivation.

Credit: SVA, AnibioThreat project.

II.2.1. Biological detection methods

Botulism is diagnosed by testing for the presence of the neurotoxin. Serum and gastrointestinal contents are used to detect the presence of active BoNTs. Different biological methods are available for toxin detection. The mouse bioassay (MBA) is still considered the “gold standard” method for BoNT detection and identification. The presence of a BoNT in suspected samples is tested by analysing serum, gastrointestinal content, organs, wound, food, feed, faeces or samples from the environment close to the outbreak. Mice are injected intraperitoneally with each suspected sample and monitored for symptoms of botulism up to four days, though they generally appear within 6 to 24 hours. In mice, typical symptoms are ruffled fur, hind limb paralysis, a contracted abdomen (wasp

shape), and laboured breathing. The toxin type is identified by monitoring mice protected with neutralising antibodies. The mice treated with antibodies that protect them against the type of toxin present in the sample material survive, whereas the others die (Wheeler et al., 2009). BoNT activity is generally described in mouse lethal dose LD₅₀ units, which is defined as the quantity of toxin required to kill 50% of the population of injected mice (5 to 10 pg is considered the LD₅₀ for a mouse) (Torii et al., 2010). The mouse bioassay is specific, sensitive and could be used with different kinds of sample material. Although the mouse test is considered the most sensitive test, a false negative result can occur if the toxin in samples is already degraded or if serum is sampled when the toxin is no longer circulating in the bloodstream at the time of sampling. The period of time during which the toxin is detectable in blood before being internalised at the neuronal junction is actually quite short. Furthermore, only a few laboratories worldwide, such as certain NRLs, are fully equipped to perform the assay. Another drawback of the MBA is the partial cross-reactivity of mosaic toxin types. For example, BoNT C/D can be neutralised by both type C and type D antitoxin, or sometimes only by type D antitoxin (Moriishi et al., 1996a). The MBA method is laborious, time-consuming and expensive. It furthermore requires animal facilities and presents ethical issues.

To reduce the number of animals used, alternative *in vivo* assays have been developed such as subcutaneous injection, resulting in muscle paralysis avoiding animal death (Sesardic et al., 1996), or the *ex vivo* hemidiaphragm assay, which uses electrical stimulation measurements of an isolated phrenic nerve from a rat or mouse (Rasetti-Escargueil et al., 2011). The specificity and sensitivity are comparable to conventional mouse lethality assays, but *in fine*, the methods still require animal use.

Recently, the U.S. Food and Drug Administration approved a BOTOX[®] Cell-Based Potency Assay (CBPA) elaborated by Allergan Inc. to replace the mouse bioassay (<http://agn.client.shareholder.com/releasedetail.cfm?ReleaseID=587234>). The assay requires incubation of the cells with BoNTs for a defined time period, followed by removal of the toxin and a quantitative endpoint for determining toxin activity by Western blot or ELISA. Cell-based assays measure fully functional BoNT receptor binding, translocation and enzymatic activity in a single assay (Pellett, 2013) which exceeds the sensitivity of the mouse bioassay and offers a sensitive model for the testing of fully functional BoNTs.

The development of an alternative *in vitro* analytical method comparable to the MBA has been a challenge for the scientific community in the last decade. The objective was both to develop a substitute to the MBA method that could offer very high detection sensitivity and specificity with respect to the active toxin, and to develop rapid assays for screening purposes in the event of botulinum toxin contamination of food supplies.

II.2.2. Immunological tests

II.2.2.1. ELISA

Immunoassays using specific antibodies to detect targeted toxins are routinely performed. The most common immunoassay format is the conventional enzyme-linked immunosorbent assay (ELISA). Samples presumed to contain the toxin are added to toxin-specific polyclonal or monoclonal antibodies. The toxin-antibody complexes are then revealed using a second toxin-specific antibody linked to a specific enzyme (phosphatase or peroxydase) producing a light signal through enzymatic cleavage of a chromogenic substrate. Several ELISA detection methods have been developed as diagnostic tools to detect botulinum neurotoxins easily and quickly (Brooks et al., 2011; Stanker et al., 2008; Stanker et al., 2013). With the development of signal amplification, the method is as sensitive as the MBA. Sharma et al. developed a sensitive and specific ELISA to detect *C. botulinum* neurotoxins A, B, E and F. The assay uses toxin type-specific polyclonal antibodies to capture the toxins and digoxigenin (DIG)-labelled toxin type-specific polyclonal antibodies as secondary antibodies. This detection method requires adding a chromogenic substrate and anti-DIG antibody conjugated with horseradish peroxidase. The test proved sensitive up to 60 pg/ml for BoNT/A as well as specific, a variety of food samples having been successfully tested (Sharma et al., 2006). More recently, Zhang et al. developed a specific and sensitive simultaneous detection assay for BoNT serotypes A, B, C, D, E and F. Sensitivities were up to 0.2 pg/ml for BoNT/E, and several clinical and food matrices (serum and milk) were used to assess the assay (Zhang et al., 2012).

II.2.2.2. Immuno-PCR assay

The ELISA antibody method could be coupled to a variety of amplification systems to improve limit of detection sensitivity, such as Polymerase Chain Reaction (PCR) instead of using an enzyme, or a fluorophore for direct detection coupled to the secondary antibody. DNA-labelled antibodies are used for amplification by PCR methods. Fach et al. developed

and evaluated a specific and highly sensitive PCR-ELISA method to detect *C. botulinum* types A, B, E and F by testing fish and sediment samples originating from northern France. The investigated samples showed a significant occurrence of *C. botulinum* (14.5%) (Fach et al., 2002). Mason et al. described an adapted immunoassay for BoNT/A toxin detection using liposomes harbouring ganglioside receptors and containing encapsulated DNA as reporters. After immobilisation of the targeted toxin by a capture antibody, the ganglioside receptor on the liposome surfaces bind to the toxin. Real-time PCR can detect a signal when the liposomes rupture and the DNA reporters are released. Assays developed for cholera and BoNT/A claim to be several orders of magnitude more sensitive than current ELISA detection methods (Mason et al., 2006).

II.2.2.3. *Bead-based assay*

Bead-based technologies can use specific targets to identify pathogenic microorganisms. The assay uses colour-coded polystyrene or superparamagnetic beads conjugated with protein-specific capture antibodies. Beads recognising different target proteins are mixed together and incubated with the sample. Bound target proteins are subsequently detected using biotinylated detection antibodies and streptavidin-phycoerythrin (PE) conjugate. Beads are read on a dual-laser flow-based detection instrument such as the Luminex[®], or Bio-Rad[®] Bio-Plex[®] analyser (Figure 23). One laser classifies the bead and determines which protein is being detected. The second laser determines the magnitude of the signal, which is in direct proportion to the amount of protein bound. BoNT/A and BoNT/B (up to 21ng/ml) were detected in multiplex with other toxins (e.g. cholera toxin, ricin and staphylococcal enterotoxin B (SEB)) using different colour-coded beads in spiked food samples (Garber et al., 2010; Pauly et al., 2009).

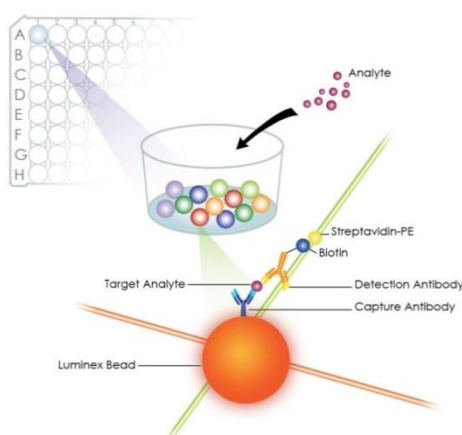


Figure 23 : Bead based assay principle.

Source <https://www.rndsystems.com>.

II.2.2.4. Lateral Flow Tests

Lateral flow tests (such as a pregnancy test) are immunochromatographic assays that rely on the target being captured by a specific antibody, revealed by a second antibody interaction typically on a nitrocellulose strip resulting in a visual (colour) change. Basically, a liquid sample is applied on one end of the strip and then migrates towards the opposite end via capillary action. It goes through a first line of fixed antibody conjugated with a reporter such as dye or gold nanoparticles, and then migrates along the strip through the second antibody control line, finally being stopped in the detection zone by the capture antibody, resulting in a final colouration. Lateral flow tests are inexpensive, easy to use, generate a visual read-out with no equipment needed and are very fast (15 min). Lateral flow assays for qualitative detection of BoNTs in various foods have been described, offering limits of detection ranging between 5 and 50 ng/ml (Ching et al., 2012; Sharma et al., 2005).

However, these immunological detection methods are limited due to the difficulty in obtaining high-quality antibodies. Genetic variation within toxin serotypes may also result in reduced test affinity, causing false negative results. Furthermore, they are not able to differentiate between active and inactivated toxins that may cause false positive results.

II.2.3. Endopeptidase Assays

Endopeptidase assays offer a new approach based on monitoring the zinc protease cleavage activity of BoNT on an artificial specific substrate containing the same cleavage sites as the natural VAMP or SNAP-25 (synaptic protein SNARE). It uses the specificity of the unique peptide products to overcome immunoassay issues. Detection of peptide products was first revealed by anti-SNARE antibodies (Poulain et al., 1993). The detection limits of this type of assay in optimised reaction buffer ranged from 200 pg/ml to 40 fg/ml for BoNT/A (Jones et al., 2008). To overcome the inherent problem of sample matrix interference and to improve sensitivity, developments focused on coupling the methods with an immunoseparation step (Poras et al., 2009). Functional enzymatic activity receptor-binding assays using brain synaptosomes have also been developed. Toxin types A, B and F are captured by brain synaptosomes, incubated with the appropriate substrate, then detected using specific antibodies (Evans 2009). More recent developments have used mass spectrometry to detect the unique peptide products (Endopep-MS) (Boyer et al., 2005). Each BoNT serotype having

a unique cleavage site on a unique peptide, the mass-specific peptide products detected differentiate active BoNT serotypes. Peptide substrates specific to each serotype are incubated with BoNTs, then serotype-specific product peptides are detected by matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) (Figure 24). Mass spectrometry (MS) is an instrumental analysis technique that measures the mass-to-charge ratio of ions, making it possible to calculate molecular masses. In combination with different fragmentation techniques, it is possible to use MS to measure the mass of different parts of a molecule, giving fingerprint-like results that can be used for identification purposes (Barr et al., 2005; Tevell Aberg et al., 2013). This method has proven to be highly sensitive and specific. These functional approaches detect active toxin and discriminate between serotypes in a single experiment.

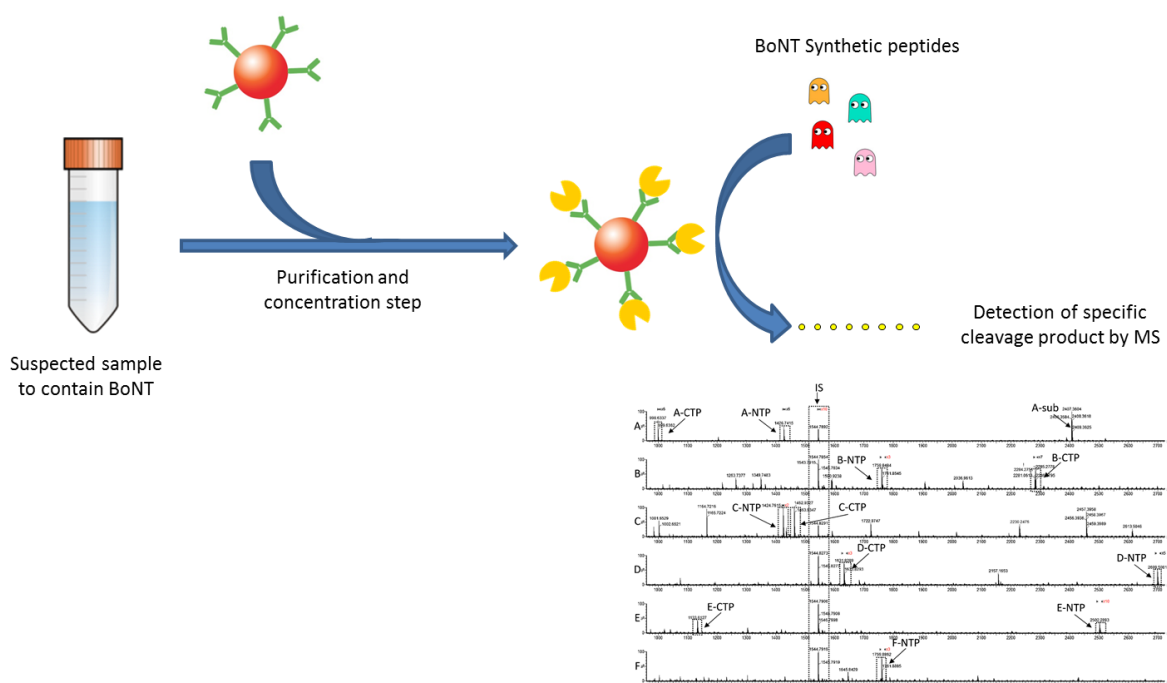


Figure 24. Endopept-MS assay principle.

Adapted from Wang et al., 2015.

II.2.4. DNA-based detection methods

In parallel to neurotoxin detection, a diagnostic alternative is to detect BoNT-producing bacteria using molecular biology methods. Molecular techniques do not require the use of laboratory animals, making them a suitable tool for screening bacterial colonies, pure liquid cultures and sample enrichments for the presence of *C. botulinum*. Despite the fact that the bacteria's presence does not necessarily indicate toxin production, when taken together

with specific botulism symptoms, it is sufficient evidence to confirm a botulism diagnosis (Anniballi et al., 2013b).

II.2.4.1. Sample treatment

The number of *C. botulinum* organisms in naturally contaminated samples is often very low (10 to 1,000 spores/kg) and because of their ability to form resistant spores, it can be difficult to detect the target organism directly from sample materials. Anaerobic enrichment is thus a prerequisite for germinating spores and increasing the concentration of vegetative cells in the sample. Careful consideration of enrichment temperature is important when different *C. botulinum* Groups are suspected to be involved. Proteolytic *C. botulinum* Group I will grow at 35 to 37°C, while non proteolytic Group II, for example, will grow at 26 to 30°C. The duration of the enrichment step is also an important issue to allow the bacteria to multiply enough to be detected. It should always be determined for each sample material and enrichment medium. If the enrichment process is too short, the competitive flora may overtake *C. botulinum*, and if the incubation time is too long, it may result in lysis or sporulation of *C. botulinum* cells. The optimal incubation time ranges from 2 to 5 days (Lindstrom and Korkeala, 2006).

II.2.4.2. DNA extraction process

The quality and quantity of extracted DNA are critical for *Clostridium botulinum* DNA-based detection methods. An ideal extraction method has to optimise DNA yield, minimise DNA degradation, eliminate inhibitory substances (e.g. protein, fat content and salts), allow extraction from multiple sample materials and be efficient in terms of cost, time, labour and supplies. Auricchio et al. compared four DNA extraction procedures: Chelex® ion exchange resin, which acts by removing metal ions from the reaction, thus inactivating nuclease enzymes that could degrade DNA; Phenol-Chloroform-Isoamyl alcohol, which is based on the chemical properties of DNA; the NucliSENS® automatic magnetic extraction kit and finally, the DNeasy® Blood & Tissue kit which uses silica-based DNA purification spin columns. The integrity, purity and amount of amplifiable DNA were evaluated. The results show that the DNeasy® Blood & Tissue kit is the best extraction method, providing the most pure, intact and amplifiable DNA. However, the less expensive Chelex® 100 matrix also appears to be suitable for PCR-based methods intended for laboratory diagnosis of suspected outbreaks of botulism (Auricchio et al., 2013).

II.2.4.3. PCR-based detection methods

Bonts are distinct genes located on a specific genetic locus situated either on the chromosome, a plasmid, or a phage (see Table 3) (Peck et al., 2011). This genetic locus is specific to *C. botulinum* and needed to produce the toxin. It thus constitutes an ideal genetic target for *C. botulinum* detection using DNA-based methods. DNA amplification using polymerase chain reaction (PCR) has been widely used in many laboratories. *Bont* PCRs to detect botulism of all toxin types have been developed (Fach et al., 1995; Franciosa et al., 1996). The sensitivity of the method could be increased using a two-step (nested) PCR that uses two primer sets targeting the same gene in different positions in subsequent reactions (Fach et al., 1996; Jang et al., 2015; Shin et al., 2007). For both PCR and nested PCR, a post-PCR gel migration step is required to visualise the amplified products. Real-time PCR has therefore been a real step forward. By monitoring amplification of the target gene after each PCR cycle, it enables rapid interpretation and quantification of the target sequences, reflecting the level of contamination by the organism. Real-time PCRs have been developed for all *bont* types (Johnson et al., 2014; Johnson et al., 2012; Lindberg et al., 2010; Satterfield et al., 2010; Takahashi et al., 2010; Vidal et al., 2011). The introduction of fluorogenic probes increases specificity and allows the simultaneous detection of different targets in the same assay by multiplexing them (Anniballi et al., 2012; Anniballi et al., 2013a; Fach et al., 2009). Using DNA intercalate chemistry, it is also possible to calculate the melting temperature of the amplified target DNAs, and to differentiate between specific and unspecific PCR products (Anniballi et al., 2012). *Bont* genes are the preferential targets for botulism detection as they confirm the presence of BoNT-producing bacteria. The NTNH gene is also a good candidate for molecular detection as the gene is always present in the botulism genetic locus (Raphael and Andreadis, 2007). The use of real-time PCR methods to confirm botulism has been successfully validated in international proficiency tests (Fenicia et al., 2011; Woudstra et al., 2013a), reaching limits of detection up to ten genome equivalents (Kirchner et al., 2010). Evidence of a toxin gene does not mean that the toxin is being actively produced. Another approach consists in detecting the expression of *bont* genes using reverse transcriptase real-time PCR (Lovenklev et al., 2004; Shin et al., 2006), but as it is time-consuming to obtain high-quality RNA, this method is mainly used for research. However, detecting mRNA related to BoNT expression is not necessarily associated with the production of a functional neurotoxin, so positive detection results using these methods merely indicate the presence of the organisms. Furthermore, as environmental and feed samples may harbour PCR

inhibitors, internal amplification controls as well as an internal positive control should be implemented for routine diagnostic PCR to check for potential inhibition by the sample material or contamination (Fencia et al., 2011).

Chapter III

Genotyping methods used to characterise *C. botulinum* diversity

Epidemics of foodborne diseases must be monitored and understood to implement appropriate prevention methods. *C. botulinum* genotyping enables investigations of botulism cases to evaluate similarities between strains for epidemiological studies and forensic discrimination. The genetic characterisation of *C. botulinum* neurotoxin-producing strains is challenging because of the great diversity not only of toxin types and subtypes, but also of their host genetic background, each phenotypical group being considered a different species.

III.1. Enzyme restriction characterisation methods: PFGE and AFLP

Pulsed-field gel electrophoresis (PFGE) is a powerful genetic typing method for foodborne pathogenic bacteria. The method is based on the electrophoresis of genomic DNA digested with rare-cutting restriction enzymes, yielding a distinctive fingerprint pattern for each bacterial strain. These fingerprints consist of 5 to 15 fragments ranging from 10 to 1,000 Kbp in length (Luquez et al., 2015). PFGE has been used for both diagnostic and biodiversity studies of *C. botulinum* (Lindstrom et al., 2004; Nevas et al., 2005). The analysis of 55 Group I type A, B, AB, and F organisms revealed marked similarity among types AB and B, and among type F organisms. For *C. botulinum* Group II, PFGE proved to be highly discriminative, revealing broad genetic diversity among strains isolated from aquatic environments (Hielm et al., 1998). The genotyping by PFGE of *C. botulinum* Group I type B, responsible for infant botulism in Japan, revealed genetic diversity among subtypes B1, B2, and Osaka05 (Figure 25). The diversity within subtype B2 strains was greater than that within B1 strains (Umeda et al., 2009). Anza et al. studied the genetic relatedness of avian *C. botulinum* strains originating from Spain and Sweden. The method revealed four highly similar pulsotypes, two of which contained strains from both countries. These results support a clonal spreading of the mosaic *C. botulinum* type C/D throughout Europe (Anza et al., 2014a).

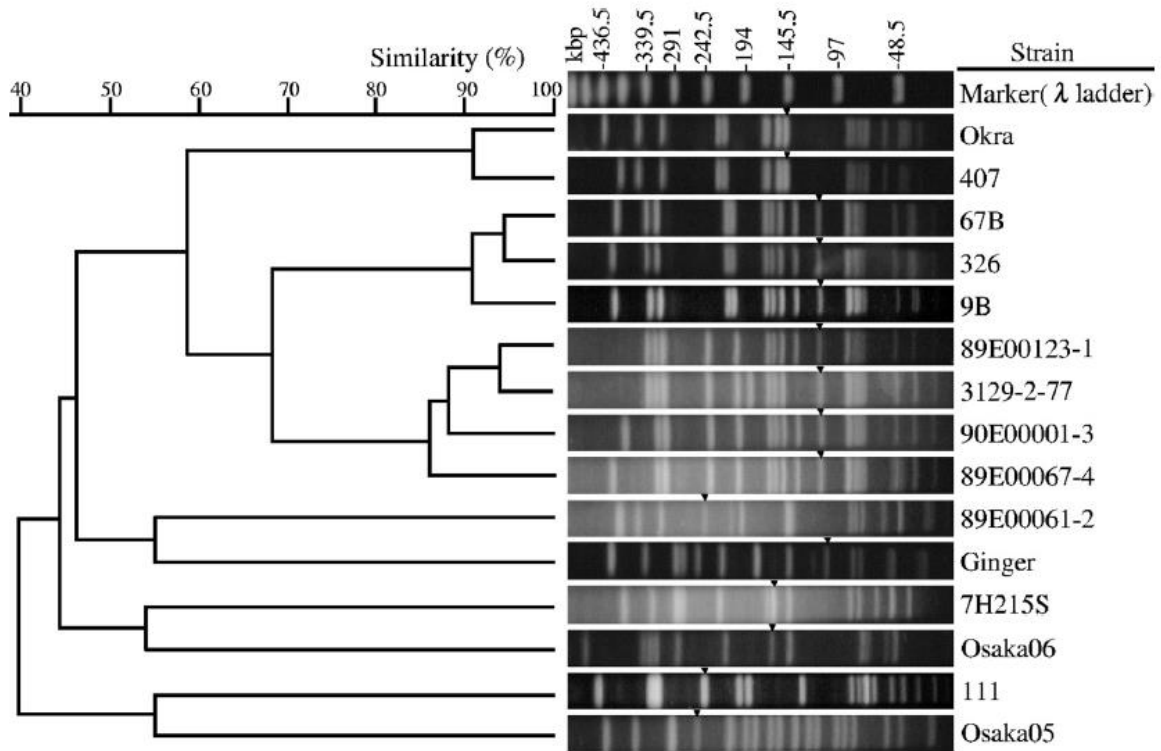


Figure 25. PFGE genotyping of *Clostridium botulinum* associated with type B infant botulism in Japan.

The dendrogram and PFGE patterns of SmaI-digested DNA from 15 C. botulinum type B strains. Arrowheads indicate the DNA fragment containing the bont/B gene detected by Southern blot hybridization. From Umeda et al., 2009.

Amplified fragment length polymorphism (AFLP) analysis provides an alternative technique with which to examine and compare *C. botulinum* strains. AFLP is based on the digestion of genomic DNA using two restriction enzymes, followed by ligation of restriction site-specific adapters and amplification of a subset of fragments by PCR (Vos et al., 1995). An AFLP analysis of 33 Group I and 37 Group II *C. botulinum* strains with *HindIII* enzyme and *HpyCH4IV* revealed that the method is fast, highly reproducible and discriminative. The method clearly differentiated between *C. botulinum* Groups I and II, identifying a total of 42 AFLP types. The method proved to be an excellent typing tool for *C. botulinum* phylogenetic analysis (Keto-Timonen et al., 2005).

PFGE and AFLP have proven to be useful typing methods for epidemiological studies and are excellent tools for investigating relatedness between strains isolated from patients and food. Although reproducible and discriminative, such analyses are laborious and could take up to several days to complete. Yet DNA degradation patterns have been observed from time to

time using a standard PFGE protocol. An empirical solution was to add urea to the migration buffer.

III.2. Sequence type characterisation

Advances in molecular biology in the last 20 years have led to the emergence of a new era in sequencing. The rapid technological advances in sequencing platforms and chemistry have made it possible to fill in the publicly available databases with an amazing amount of sequence data, first with specific gene sequences, then with complete or incomplete whole genome sequences. This scientific knowledge constitutes the cornerstone for molecular epidemiology and phylogeny studies.

III.2.1. Ribotyping

The conservative ribosomal genes (e.g. 16S RNA gene) have been intensively studied as targets for phylogenetic analysis of *Clostridium* species (Kalia et al., 2011). Based on the genetic analysis of ribosomal 16S RNA gene sequences, comparisons have shown that *C. botulinum* strains form four distinct clusters which correspond to phenotypical Groups I to IV (Collins and East, 1997; Hutson et al., 1994), confirming that these groups belong to distinct phylogenetic lineages, which does not tie in with *bont* genetic relatedness (Bhushan et al., 2015).

III.2.2. MLVA

Multiple Locus Variable number tandem repeat Analysis (MLVA) is a method based on amplification of a variable number of tandem repeat sequences (VNTR) using PCR. A VNTR is a short nucleotide sequence organised as a tandem repeat in the genome. Generally, the tandem repeat regions are enumerated by the size of the PCR amplicon for each locus. The resulting information is a code which can be easily compared to a reference database. It is used as a fingerprinting method to trace back to the origins of a particular strain in an outbreak situation. A 15-locus MLVA scheme used to investigate 89 Group I strains identified 86 distinct genotypes, revealing highly diverse genetic content in Group I *C. botulinum*, making it possible to clearly differentiate strains involved in different outbreaks (Fillo et al., 2011). A study of *C. botulinum* Group I A(B) strains involved in a food outbreak in the U.S. and having the same PFGE profile, showed the MLVA method to be partially but sufficiently discriminatory to differentiate such strains (Raphael et al., 2014).

III.2.3. Rep-PCR

Repetitive element sequence-based PCR (Rep-PCR) uses conservative repetitive extragenic elements present in bacterial genomes as targets for PCR, with single or multiple consensus primers (Versalovic et al., 1991). The number and size of amplification products define a species-specific fingerprint. A Rep-PCR protocol established for *C. botulinum* allowed Group II type B and E toxin-producing strains to be differentiated up to strain level, whereas the Group II type F toxin-producing strains and all Group I strains were differentiated only up to toxin type level (Hyytia et al., 1999). A different amplification fragment pattern was obtained for Groups I and II, suggesting the possibility of using this method to determine the physiological group of *Clostridium botulinum* strains involved in an outbreak situation. However, because differentiation up to strain level is limited, the method's applicability in epidemiological and clinical research also appears limited.

III.2.4. RAPD

Randomly amplified polymorphic DNA analysis (RAPD) is based on PCR using randomly annealing universal primers under low-stringency conditions. Due to random annealing of unspecific primers, the method lacks sufficient reproducibility and is thus not well-suited for bacterial strain comparison. Few studies have evaluated the use of this method for *C. botulinum* typing. Nevertheless, Hannett et al. applied it in order to study a type E animal botulism outbreak in wildlife affecting a wide range of bird and fish species. Positive toxin type E isolates were typed by RAPD and revealed 12 different RAPD profiles and multiple subtypes. They furthermore demonstrated that multiple genetically distinct strains of *C. botulinum* type E present in the sediments of Lake Erie were involved in the studied outbreak (Hannett et al., 2011).

III.2.5. MLST

Multi-Locus Sequence Typing (MLST) is a method used to determine the degree of evolutionary relatedness using the sequence variation of housekeeping genes to differentiate bacterial strains. Partial sequencing of the *adk*, *atpH*, *gyrB*, *proC*, *rpoD* and *spo0A* genes from 51 various *C. botulinum/sporogenes* isolates resulted in 37 different sequence types, showing a consistent but slightly lower resolution of Group I strains compared to MLVA (Olsen et al., 2014). An analysis of 41 *C. botulinum* serotype E strains clustered the strains into several clades according to AFLP analysis (Macdonald et al., 2011).

III.2.6. DNA microarrays

Microarrays have been developed to identify specific *C. botulinum* Groups or BoNT serotypes and to provide information about both toxin gene clusters and other genes of interest (Raphael, 2012). Strains are differentiated on the basis of the presence or absence of specific genes using specifically-designed oligonucleotides to probe the genome of interest. DNA microarray data can be used to check for genetic diversity among multiple strains. A microarray assay was performed by Raphael et al. (Raphael et al., 2010b) using 62 different sequences based on known variable regions in the genome of proteolytic *C. botulinum* type A ATCC 3502. The assay was successfully used to differentiate *C. botulinum* type A strains. In another study, a Group II subtyping microarray demonstrated that the genomic background of a *C. botulinum* type E strain isolated in Argentina was more similar to Group II type B strains than to other type E strains (Raphael et al., 2012). Vanhomwegen et al. evaluated performance of the PathogenID v2.0 resequencing microarray for the simultaneous detection and characterisation of BoNT-producing Clostridia. This approach led to the successful identification and typing of DNA extracts from a representative panel of reference *C. botulinum* strains of the different toxin types and subtypes, as well as neurotoxin-producing *C. botulinum* strains in a naturally contaminated food sample (Vanhomwegen et al., 2013). In a study by Stringer et al., the genome of Group II strain *C. botulinum* Eklund 17B was used to construct a whole genome DNA microarray to compare the relatedness of 43 strains of Group II *C. botulinum* (14 type B, 24 type E and 5 type F strains) isolated from a variety of foods and environmental sources from different locations over a long period of time. The clustering patterns obtained using the microarray data were consistent with other typing methods (e.g. AFLP) previously used to classify Group II *C. botulinum* strains (Figure 26). Nonetheless, microarrays are developed based on available genetic sequence information, so unique genetic variations could be encountered yet remain undetected.

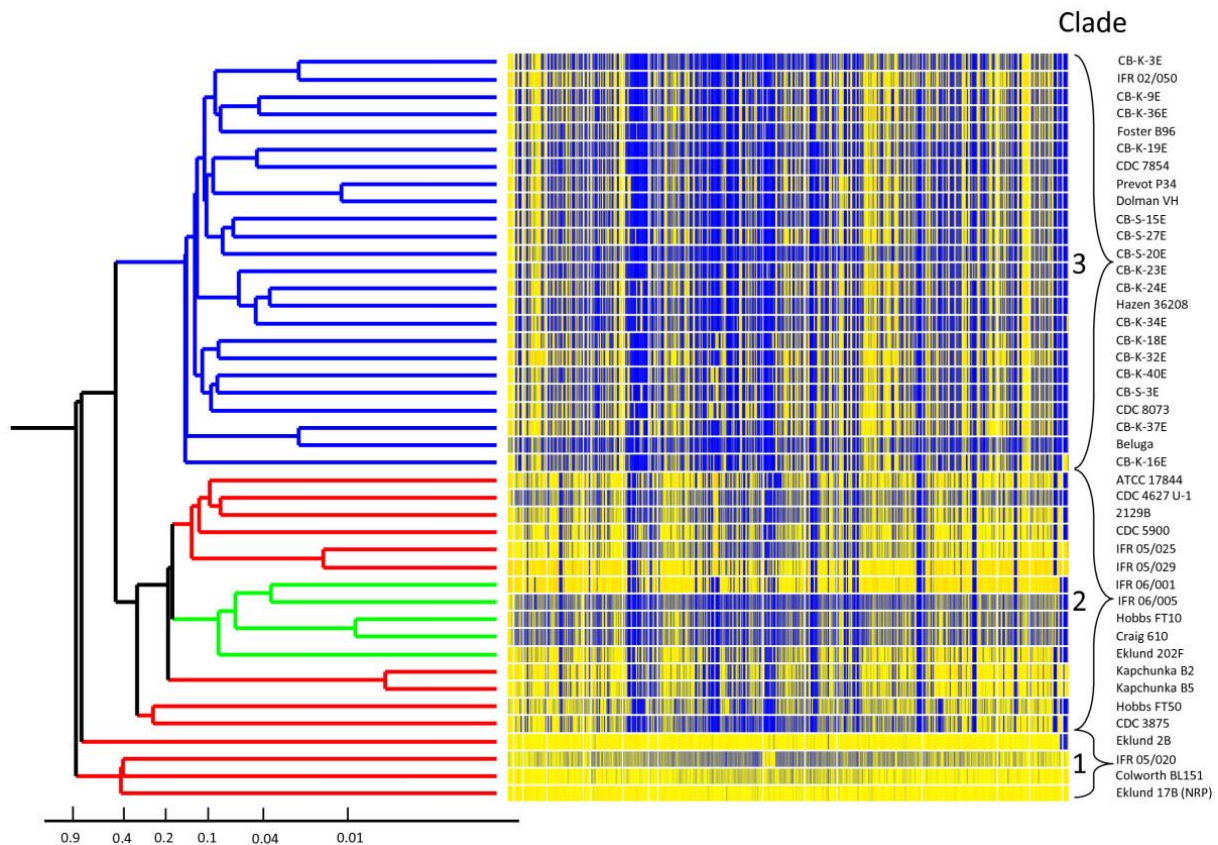


Figure 26. Genomic indexing of 43 strains of Group II *C. botulinum*.

An average linkage hierarchical clustering dendrogram of the *C. botulinum* strains was created in GeneSpring version 7.0 from microarray data using the Pearson coefficient correlation. Each row of the heatmap represents one strain and consists of a series of vertical bars which represent the CDS content of *C. botulinum* Eklund 17B. The colour of each bar indicates the ratio of the signal from the test strain over that of the index strain. Where the signal from DNA binding is equal for both the test and control strains the bar is coloured yellow and where there is lower binding by the test strain the bar is blue. Where there is no probe the bar is white. The hierarchical clustering dendrogram has been coloured according to neurotoxin type: red = type B, blue = type E and green = type F. Strains were divided into three clusters using a distance of 0.5. From Stringer et al., 2013.

III.2.7. Flagellar typing

The flagellum is the organelle responsible for motility in the majority of bacterial species. It plays a role in the ability of bacteria to adapt to their unique biological niches. Flagella from a wide range of bacteria have been shown to be important in colonisation and as virulence factors; they are also critical to biofilm formation in many species (Haiko and Westerlund-

Wikstrom, 2013). Flagella may be arranged differently depending on bacterial species (Figure 27).

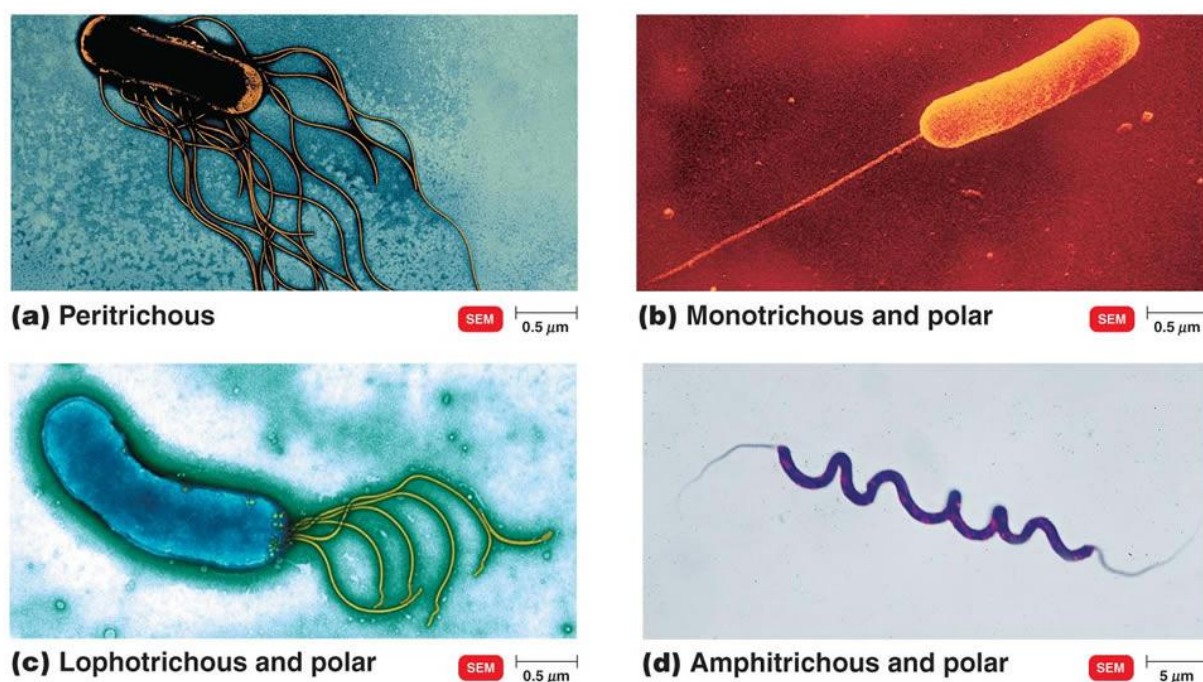


Figure 27. Flagellar arrangement.

A single monotrichous flagellum, multiple lophotrichous flagella, a single amphitrichous flagellum, or peritrichous flagella projecting in all directions on each of the two opposite ends. Credit: Pearson Education, 2010.

The basic structure of a bacterial flagellum can be divided into three parts: the basal body, the hook and the flagellar filament. The flagellar filament consists of repeating subunits of the flagellin protein (FliC). Flagellin proteins can be divided into three distinct subdomains: the N and C terminal ends, which are conserved within a given species, and a variable region in between (Macnab, 2004). The N- and C-terminal parts are responsible for secretion and polymerisation of the flagella. The central region, which may vary considerably among bacterial species in both amino acid sequence and size, constitutes the surface-exposed variable domain of the flagellin which is antigenically diverse. This forms the basis for a wide range of typing methods for strain identification (Ayala Cde et al., 2012; Winstanley and Morgan, 1997). The flagella of gram-negative bacteria have been extensively studied, but less is known about the flagella of Gram-positive bacteria. Some studies have investigated the flagella in *Clostridium* species (e.g. *Clostridium chauvoei* (Kojima et al., 1999; Kojima et

al., 2000)). The phylogenetic relationship between several *Clostridium* species has also been analysed (Sasaki et al., 2002). Pheno- and genotypical variability of the *Clostridium difficile* flagellin gene (*fliC*) and its correlation with serogroups in isolates from different origins has been studied. PCR amplification of flagellin genes combined with RFLP analyses have revealed nine groups (Tasteyre et al., 2000). More recently, flagellin diversity in *Clostridium botulinum* has also been studied to improve genotyping of Group I and II strains (Paul et al., 2007). The investigation of *fliC* sequences in Group I showed the flagellin variable region (flaVR) sequence diversity to be 39% over a region of 400 bp, providing a sufficient level for strain-to-strain discrimination. FlaVR sequences within Group II were less diverse, with a maximum of only 8% differences at nucleotide level, demonstrating that other methods are preferable for the phylogenetic analysis of Group II strains. It has also been found that the majority of strains produce several to multiple peritrichous flagella with the standard characteristics of other well-characterised flagella (e.g. glycan modifications) (Twine et al., 2008). Another study of genetic diversity focusing on the flagellar glycosylation island (FGI) in *C. botulinum* Group I showed that it does not match that of the *bont* locus. This indicates an independent evolution of the loci of flagellin and *bont* genes in proteolytic *C. botulinum* strains (Carter et al., 2009; Sebahia et al., 2007).

III.2.8. Whole genome sequencing (WGS)

Complete *C. botulinum* genomes are especially valuable for strain comparisons using various bioinformatic analysis methods. The finished genomes can be used as reference sequences. New whole genome sequences can then be assembled and compared against the reference sequences in order to identify insertions, deletions, synteny and recombination events. The availability of high-quality finished genomes is crucial for accurate mapping when short reads produced by the current genomic sequencing platforms are used. However, the road from genomic DNA to sequences of such quality is long and full of ambushes. First, pure strains have to be isolated from complex sample materials. This is particularly difficult for *C. botulinum* Group III because the BoNT gene cluster is located on a phage which could be easily lost during cultivation (Oguma, 1976). Second, the target strain has to be cultured to obtain sufficient genomic material before the DNA is extracted using suitable methods for WGS (e.g. DNA has to be eluted in molecular grade water without DNase, or without EDTA which could inhibit the sequencing process). Next, DNA is submitted to the library

preparation procedure, which consists of size-specific DNA fragmentation and tagging (specific adapter sequences are added to identify each DNA fragment) in order to be useable by the different sequencing platforms, each sequencing technology requiring a different DNA library preparation (Mayo et al., 2014). Sequencing platform manufacturers have made convenient, user-friendly kits available for library preparation which allow the user to prepare and run the sequencing reaction in less than two working days (Baym et al., 2015). The sequencing reaction itself can take several days. Sequencing outputs are raw reads, consisting of millions of short sequences of lengths ranging from 50 bp to 300 bp for Illumina® based flow-cells, for example. These short sequences have to be processed by dedicated bioinformatic tools (e.g. the free “Galaxy” bioinformatic platform (Goecks et al., 2010), licensed software “CLC® genomic workbench” or “BioNumerics®”) to be meaningful. These tools are used to check the run’s quality, remove (trim) the adapter sequences from each read, *de novo* assemble the reads together to obtain the complete genome sequence, or map the reads to a reference genome if one is available. The coding sequences are then annotated using several software programs such as PROKKA (Seemann, 2014) available on the Galaxy web platform, “RAST” (Aziz et al., 2008) or the automatic annotation pipeline of the NCBI (<http://www.ncbi.nlm.gov/genomes/static/Pipeline.html>).

A huge amount of information can be extracted from a genome sequence. It is possible to check for plasmids, for specific properties such as antibiotic resistance, toxin production, metabolic pathways, or look for the presence or absence of specific genetic sequences. The first released genome sequence of *C. botulinum* was the Hall A ATCC 3502 type A1 strain (Figure 28) (Sebahia et al., 2007).

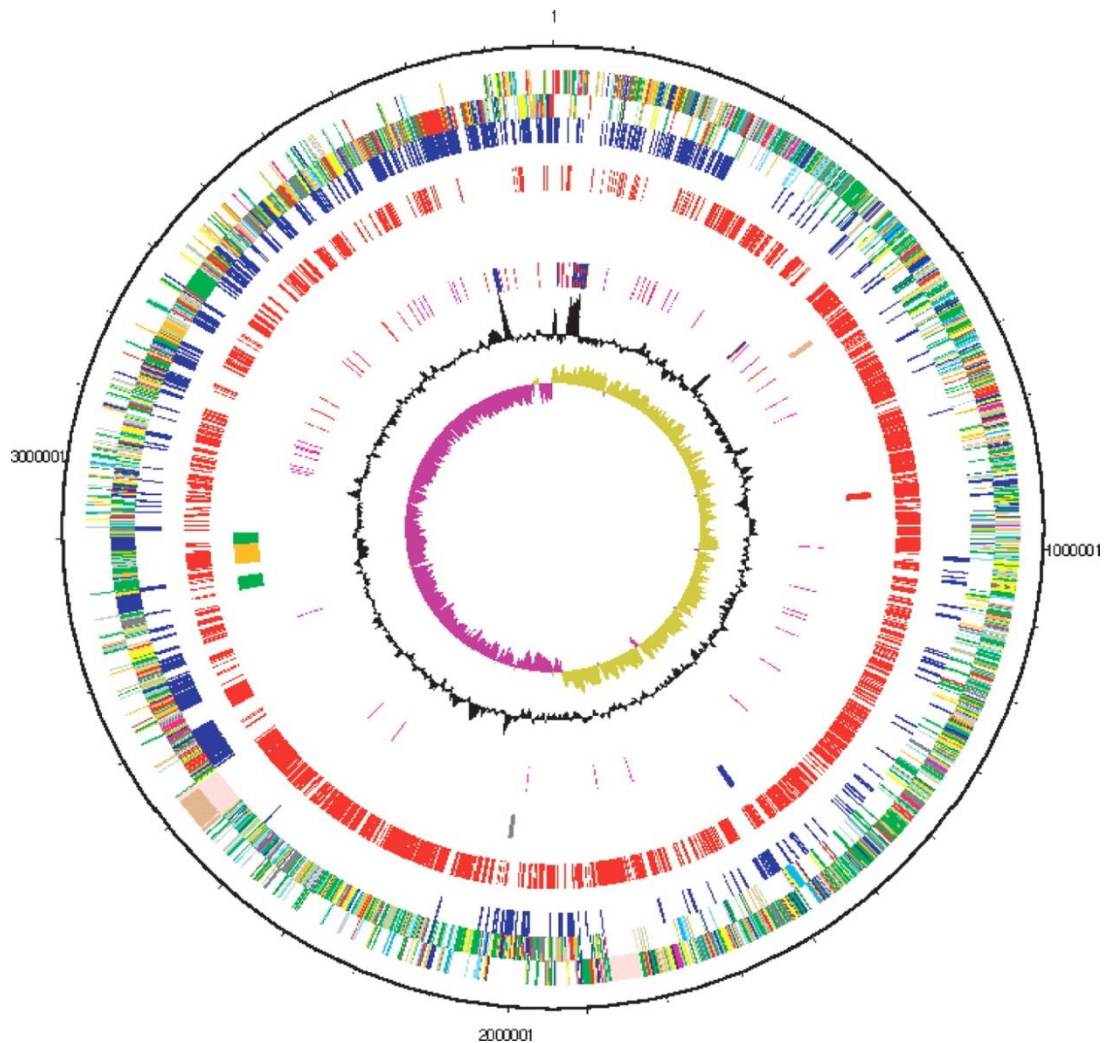


Figure 28. Circular genome representation of *C. botulinum* ATCC 3502.

*The circles represent (from the outside in): (1 and 2) All CDS (transcribed clockwise and anticlockwise); (dark blue) pathogenicity/adaptation; (black) energy metabolism; (red) information transfer; (dark green) surface-associated; (cyan) degradation of large molecules; (magenta) degradation of small molecules; (yellow) central/intermediary metabolism; (pale green) unknown; (pale blue) regulators; (orange) conserved hypothetical; (brown) pseudogenes; (pink) phage and IS elements; (gray) miscellaneous. (3) (Blue) CDSs shared with sequenced Clostridia. (4) (Red) *C. botulinum*-unique CDSs relative to the sequenced Clostridia. (5) Virulence factors; (brown) streptolysin; (red) neurotoxins; (blue) metalloproteases; (black) type IV pilus; (green) flagellar and chemotaxis operons; (orange) flagellar glycosylation island. (6) RNA genes; (blue) rRNAs; (red) tRNAs; (purple) stable RNAs. (7) G+C content (plotted using a 10-kb window). (8) GC deviation plotted using a 10-kb window; (khaki) values >1; (purple) values <1. From Sebahia et al., 2007.*

Since this first release, many more genome sequences have been added to public databases (Basavanna et al., 2013; Clauwers et al., 2015; Fillo et al., 2015; Gonzalez-Escalona et al., 2014a; Mazuet et al., 2015a; Sakaguchi et al., 2014). Some are complete genome sequences, but most are incomplete scaffolds or contigs, or even only raw reads available in the Sequence Read Archive (SRA, www.ncbi.nlm.nih.gov/sra). With this information it is possible to search for the botulinum neurotoxin cluster, check its organisation, genetic content (HA / OrfX locus arrangement or location), identify insertions, deletions, synteny and recombination events. It is also possible to perform *in silico* MLST (McCallum et al., 2015). With the release of more and more genomic sequences, it is gradually becoming possible to make whole genome comparisons using software such as “*Gegenees*” (Agren et al., 2012). This program was first developed to investigate the genetic relatedness of *Bacillus anthracis*. Since then, it has also been used to compare whole genome sequences of *C. botulinum* Group III strains, emphasising its genetic diversity and its relatedness with *C. novyi* and *C. haemolyticum* (Skarin and Segerman, 2014). Finally, it has become possible to analyse genetic diversity up to a single nucleotide polymorphism (SNP) (Candela et al., 2015). The analysis of *C. botulinum* Group I whole genome SNP sequences (Gonzalez-Escalona et al., 2014b) proved useful in differentiating strains harbouring identical toxin gene subtypes that are undistinguishable by PFGE and MLST. It revealed that SNPs could be used to further divide Group I into five different lineages, reinforcing the findings of previous studies on the genomic plasticity of *C. botulinum*.

With the expansion of sequencing capabilities and the associated drop in cost, it is expected that whole genome sequencing will increasingly replace conventional genetic typing methods currently used to characterise strains. At the same time, the knowledge gained will provide greater insights into physiological processes within BoNT-producing Clostridia.

Objectives and Results

Chapter IV

Aim of the thesis

The PhD research presented in this dissertation aimed to investigate diagnostic, typing genetic markers and genomic diversity, for the characterization and surveillance of the bacterium *Clostridium botulinum* responsible for human and animal botulism.

The context in which this work took place was linked to the different projects I participated in, which were about developing new detection and typing methods for the characterization of the bacteria responsible for botulism disease. *C. botulinum* Group I and II responsible for human botulism, despite a low prevalence, are of great concern for public health. In 2013, the story case of Danone milk products suspected to contain such organism, sold by its supplier Fonterra in China, had a huge economic impact. Finally, it turned out fortunately to be a false alert. Group III, which is responsible for animal botulism, concerns livestock farming where animals could either be destined to food production or breeding. With presence of other animals in the farm surrounding, the contamination could even extend to other sensitive species (e.g. cattle botulism due to exposure of contaminated poultry litter). The subject has gained much attention after 2007, when the number of animal botulism outbreaks recorded a significant rise. It is a major concern for livestock farmer as the entire flock of birds, or herd of cattle could die in the outbreak, which could even be recurrent once the farm has been contaminated.

To address such issues, my PhD focused on:

- Developing typing methods for *C. botulinum* Group I and II responsible for human botulism.
- Developing detection and typing methods for *C. botulinum* Group III responsible for animal botulism.
- Investigating the genomic diversity within *C. botulinum* Group III responsible for animal botulism in France.

The work of this thesis was part of the European collaboration project AnibioThreat which started in October 2010, encompassing several institutes from all over Europe (in Italy, the Istituto Superiore di Sanità - ISS, and the Istituto Zooprofilattico Sperimentale delle Venezie - IZSVE; in the Netherlands the Central Veterinary Institute - CVI; in Sweden the National

Veterinary Institute - SVA). We collaborated also with the Health Canada institute, the French NRBC program (Nuclear, Radiological, Biological and Chemical threats) from CEA (Atomic Energy and Alternative Energies Commission), the French National Reference Laboratory of avian botulism, the National Laboratory of New Caledonia and the Robert Koch institute - RKI in Germany. The methods developed in this project were based on real time PCR and Next Generation Sequencing (NGS), using genetic material gathered from Europe, Canada and New Caledonia.

The first objective, which was to develop typing methods for *C. botulinum* Group I and II, was realized together with the Health Canada institute (Dr. John W. Austin and Dr. Dominic Lambert from the botulism reference service for Canada). Their research activities mainly focus on foodborne and infant botulism together with growth and ecology of Clostridia in foods. We assessed the flagellin genes *flaA* and *flaB* as potential targets for epidemiological purposes. The results are presented in article N°1.

The second part of this PhD work was to develop detection and typing methods for *C. botulinum* Group III. The study of *C. botulinum* Group III constitutes the main goal of this PhD work. In Paper N°2 I developed specific genetic targets allowing the detection and distinction of *bont* genes of types C, C/D, D and D/C, mainly responsible for animal botulism in poultry and cattle farms. Paper N°3 deals with the investigation of flagellin gene *fliC* as a suitable target for typing and epidemiological purposes to characterize *C. botulinum* Group III. This work was supported by the Directorate General for Food Safety (DGAL) from the French Ministry of Agriculture, and in collaboration with several institutes throughout Europe.

Finally, the last part of my work is dealing with whole genome sequencing of *C. botulinum* Group III type C/D and D/C French isolated strains. It's covered by paper N°4 and N°5 which present the analyses of *C. botulinum* group III genome sequences produced. This work was part of an effort of the French national reference laboratory for avian botulism, the LABOCEA from Brittany, and the national laboratory of New Caledonia.

Chapter V

Results obtained during the course of the PhD

V.1. *C. botulinum* Group I and II diversity investigation using flagellin *flaA* and *flaB* genes

C. botulinum Group I and II are responsible for synthesis of BoNT associated with human cases of foodborne botulism. Because of their severity, the toxins were particularly well studied. Strains of *C. botulinum* are traditionally identified by their BoNT type and subtype. However, identification of an additional target for typing would improve differentiation of strains and allow more thorough epidemiological investigations.

The flagellar genetic locus is known to be one of the hot spots of genetic diversity in *C. botulinum* (Hill et al., 2007). A previous work of Paul et al. showed that flagellin proteins of *C. botulinum* Group I and II are diverse. They used the flagellin variable region of *flaA1/A2* genes for single locus sequence typing of 80 environmental and clinical strains of *C. botulinum* Group I and II (Paul et al., 2007).

Based on this assumption, we investigated the flagellin genetic locus of 62 *C. botulinum* Canadian references strains from Paul et al. work and a collection of 210 strains isolated in European countries. We found out that the genetic diversity of *flaA* genes among *C. botulinum* strains clustered into 5 major groups and up to 15 subgroups, some being specific for certain geographical areas. Also *C. botulinum* type E showed to harbour a specific flagellin gene named *flaB*. Therefore, profiling of *C. botulinum* Group I and II using the flagellin variable region was carried out. Gene's *flaA* and *flaB* turn to be promising targets for molecular surveillance and epidemiology.

The results of this study were published in 2013 in the scientific journal *Applied and Environmental Microbiology* (Article n°1).

Article n°1:

Woudstra C, Lambert D, Anniballi F, De Medici D, Austin J, Fach P. Genetic diversity of the flagellin genes of *Clostridium botulinum* groups I and II. *Appl Environ Microbiol.* 2013 Jul;79(13):3926-32.

Article n°1

Genetic diversity of the flagellin genes of *Clostridium botulinum* groups I and II.

Woudstra C, Lambert D, Anniballi F, De Medici D, Austin J, Fach P.

Applied and Environmental Microbiology

2013 Jul;79(13):3926-32

Genetic Diversity of the Flagellin Genes of *Clostridium botulinum* Groups I and II

Cedric Woudstra,^a Dominic Lambert,^b Fabrizio Anniballi,^c Dario De Medici,^c John Austin,^b Patrick Fach^a

ANSES (French Agency for Food, Environmental and Occupational Health Safety), Food Safety Laboratory, Maisons-Alfort, France^a; Botulism Reference Service for Canada, Bureau of Microbial Hazards, Health Products and Food Branch, Health Canada, Ottawa, Ontario, Canada^b; Istituto Superiore di Sanità, Department of Veterinary Public Health and Food Safety, National Reference Centre for Botulism, Rome, Italy^c

Botulinum neurotoxins (BoNTs) are produced by phenotypically and genetically different *Clostridium* species, including *Clostridium botulinum* and some strains of *Clostridium baratii* (serotype F) and *Clostridium butyricum* (serotype E). BoNT-producing clostridia responsible for human botulism encompass strains of group I (secreting proteases, producing toxin serotype A, B, or F, and growing optimally at 37°C) and group II (nonproteolytic, producing toxin serotype E, B, or F, and growing optimally at 30°C). Here we report the development of real-time PCR assays for genotyping *C. botulinum* strains of groups I and II based on flaVR (variable region sequence of *flaA*) sequences and the *flaB* gene. Real-time PCR typing of regions flaVR1 to flaVR10 and *flaB* was optimized and validated with 62 historical and Canadian *C. botulinum* strains that had been previously typed. Analysis of 210 isolates of European origin allowed the identification of four new *C. botulinum* flaVR types (flaVR11 to flaVR14) and one new flaVR type specific to *C. butyricum* type E (flaVR15). The genetic diversity of the flaVR among *C. botulinum* strains investigated in the present study reveals the clustering of flaVR types into 5 major subgroups. Subgroups 1, 3, and 4 contain proteolytic *Clostridium botulinum*, subgroup 2 is made up of nonproteolytic *C. botulinum* only, and subgroup 5 is specific to *C. butyricum* type E. The genetic variability of the flagellin genes carried by *C. botulinum* and the possible association of flaVR types with certain geographical areas make gene profiling of flaVR and *flaB* promising in molecular surveillance and epidemiology of *C. botulinum*.

Clostridium botulinum is the etiological agent of botulism, a disease characterized by a descending symmetrical flaccid paralysis. Food-borne botulism develops after consumption of contaminated foods containing botulinum neurotoxin (BoNT) (1, 2). Nonetheless, there are other forms of botulism in which *C. botulinum* produces toxin *in vivo*, including infant botulism, adult intestinal colonization botulism, and wound botulism.

C. botulinum is a taxonomic designation for Gram-positive, spore-forming, anaerobic bacteria that encompasses most organisms that produce BoNT but that are otherwise unrelated genetically. Physiological traits, biochemical tests, and toxin serotyping based on the mouse bioassay are used to divide strains among major groups. Group I strains secrete proteases, produce toxin serotypes A, B, or F, and grow optimally at 37°C, whereas group II strains are nonproteolytic, produce toxin serotype E, B, or F, and grow optimally at 30°C. While these two groups are associated with botulism in humans, animals are affected by toxin serotypes C and D, and mosaic forms of these two serotypes (produced by group III strains). Other clostridial species can produce BoNT, i.e., *C. baratii* (serotype F), *C. butyricum* (serotype E), and *C. argentinense* (serotype G; formerly known as *C. botulinum* group IV).

Although physiological traits, biochemical tests, and toxin serotyping are still used to characterize *C. botulinum* strains, this information does not possess the discrimination required for source attribution and epidemiological investigations. Other methods were developed to this end, e.g., randomly amplified polymorphic DNA analysis, amplified rRNA gene restriction analysis, pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism, single locus and multilocus sequence typing, and multilocus variable-number tandem repeat analysis (MLVA) (3–7). Real-time PCR-based approaches for identifica-

tion of neurotoxin genes have also been developed (8–13). Real-time PCR allows rapid, simple detection and typing. This approach can be expanded by inclusion of new target sequences to make typing methods more discriminatory.

Flagellin subunits are composed of conserved C- and N-terminus domains bracketing a variable domain (14). During assembly, monomers bury their termini toward the central channel of the filament, leaving the variable domains exposed to the surface. Flagellin genes and their products have been used in genotypic and phenotypic typing methods for food-borne pathogens (15). Utilizing flagellin variable region (flaVR) sequences, Paul et al. (5) classified 80 historical and Canadian *C. botulinum* strains into 10 flaVR types and identified *flaB*, a putatively type E-specific flagellin gene. Here, we present the development of real-time PCR assays for typing *C. botulinum* groups I and II using flaVR sequences and *flaB*. Specific identification of *bont/A*, *bont/B*, *bont/E*, and *bont/F* by previously developed PCR assays (8) was used as a control. A PCR assay targeting *fldB* was employed as a marker for proteolytic clostridia (16). The assays were first tested using a panel of 62 *C. botulinum* strains as reference and characterized as described by Paul et al. (5) before being extended to 210 isolates of European origin. We report the finding of four new *C. botulinum* flaVR types and one new flaVR type specific to *C. butyricum* type E.

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Address correspondence to Patrick Fach, patrick.fach@anses.fr.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. botulinum* strains used as references ($n = 62$) were stored on Microbank beads (Pro-Lab Diagnostics, Richmond Hill, Canada) at -86°C and archived at the Botulism Reference Service for Canada. Spores were inoculated onto McCleung-Toabe 1.5% agar (Difco, Tucker, GA) with 5% egg yolk and 0.5% yeast extract (MTEYE) and allowed to grow for approximately 72 h at 35°C (group I) or at room temperature (group II) in an atmosphere of 10% H_2 , 10% CO_2 and 80% N_2 . Single colonies were transferred into SPGY broth containing (wt/vol) 5% special peptone (Oxoid Inc., Basingstoke, United Kingdom), 0.5% peptone, 2% yeast extract, 0.4% glucose (Difco), and 0.1% sodium thioglycolate (Sigma-Aldrich, St. Louis, MO) adjusted to pH 7.2 using HCl. Broth cultures were grown for 24 h at 35°C (group I) or 48 h at room temperature (group II). *C. botulinum* strains native to Europe ($n = 210$) investigated in this study were cultivated in TPGY medium under anaerobic conditions as described previously (10). Ten *C. botulinum* strains of types C, C-D, D, D-C, and G were included in the panel of *C. botulinum* strains. Eighteen strains of other clostridial species were used as non-BoNT-producing negative controls (*C. butyricum*, *C. baratii*, *C. beijerinckii*, *C. bifementans*, *C. chauvoei*, *C. difficile*, *C. mangenotii*, *C. oedematiens*, *C. perfringens* type A, C, D, and E, *C. septicum*, *C. sordellii*, *C. sporogenes*, *C. subterminalis*, and *C. tetani*). Eighteen strains of other bacterial species were also analyzed as non-*Clostridium* negative controls (*B. cereus*, *B. thuringiensis*, *Citrobacter* sp., *Escherichia coli*, *Hafnia alvei*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Proteus* sp., *Pseudomonas* sp., *Salmonella enterica* serovar Virchow, *S. enterica* serovar Hadar, *S. enterica* serovar Enteritidis, *S. enterica* serovar Infantis, *S. enterica* serovar Typhimurium, *Shigella* sp., *Staphylococcus aureus*, *Streptococcus faecalis*, and *Yersinia enterocolitica*).

Genomic DNA isolation. The DNA of the 62 *C. botulinum* reference strains encompassing the original 10 flaVR types was isolated as described previously (17) or using a QIAamp minikit (Qiagen, Hilden, Germany). The DNA of the 210 *C. botulinum* strains native to Italy, France, Germany, and the Netherlands was extracted using the following protocols and apparatus: phenol-chloroform extraction (18), a DNeasy blood and tissue kit (Qiagen, Hilden, Germany), Chelex 100 (Bio-Rad Life Science Research, Hercules, CA), and a MicroLab Starlet (Hamilton, NV) automatic system employing a MegMax total nucleic acid isolation kit (Ambion, Austin, TX), according to the manufacturer's instructions for Gram-positive bacteria. DNA samples were stored at -20°C until high-throughput real-time PCR analysis.

High-throughput real-time PCR. A LightCycler 1536 (Roche, Meylan, France) was used to perform high-throughput real-time PCR amplifications. For the PCR setup of the LightCycler 1536 multiwell plates, a Bravo liquid dispenser automat (Agilent Technologies, Massy, France) equipped with a chiller, and a PlateLoc thermal microplate sealer (Agilent Technologies) was used. The PCR mixtures contained 1 μl DNA sample and 1 μl master mix containing 1 \times RealTime ready DNA probe master mix (Roche), a 300 nM concentration of each primer, and a 300 nM concentration of each probe. Amplifications were performed using HEX-labeled TaqMan probes. The following thermal profile was used for PCR: 95°C for 1 min followed by 30 cycles of 95°C for 0 s and 55°C for 30 s. Primers and probes used for PCR amplifications are listed in Table 1. Oligonucleotides used for determining the flaVR type were derived from the *flaA* (accession numbers DQ844946 to DQ845031) and *flaB* (DQ658239) sequences. Oligonucleotides targeting *fldB* were designed to differentiate group I and group II *C. botulinum* strains (16). Specific oligonucleotides for typing *bontA*, *bontB*, *bontE*, and *bontF* were described previously (8). Primers and probes were purchased from Sigma-Aldrich (St. Quentin Fallavier, France) and Eurofins MWG Operon (Courtaboeuf, France).

Flagellin DNA locus sequencing. Double-stranded DNA sequencing of flagellin genes was performed according to a previously published method (5) by Eurofins MWG Operon (Courtaboeuf, France). Primers used for DNA sequencing of *flaA* and *flaB* are listed in Table 1.

TABLE 1 Primers and probes

Primer or probe ^a	Sequence (5' → 3') ^b	Amplicon size (bp)
FlaVR-1-F FlaVR-1-R FlaVR-1-P	CGCTGCTAATGTAACAAA GCTCTAATCTGTTTTGGTTA CTGAAATTAATAGCATTATCTATTGTCGCT	101
FlaVR-2-F FlaVR-2-R FlaVR-2-P	CTGCTGCTAATATAACAAA GTCTAATCTGTTTTGGTTA AGCAACAATAGATTACAGCTATTAATTACAG	102
FlaVR-3-F FlaVR-3-R FlaVR-3-P	AAGACCAAGTTATGGAGTTA ATTCCTGATATTGACTTAGC CCTTTTCCACTTCTATATTTATACTTCC	106
FlaVR-4-F FlaVR-4-R FlaVR-4-P	GCTGCTTCAATATCAGGC TCTAATCTGTTTTGATTGCTCC ACTGTATCAGCAGAAAGGCCAAGCT	98
FlaVR-5-F FlaVR-5-R FlaVR-5-P	AATAGGAGCAAATAGTGGA CTTCCTATTGTAACITTTATCAC CAACACCTAATGCAGCAACATC	96
FlaVR-6-F FlaVR-6-R FlaVR-6-P	GAAGCAGACTTAACCTTAGATA ATCAGCAACACTTACACTT TGCTCTGAACACTCATGCTTCC	87
FlaVR-7-F FlaVR-7-R FlaVR-7-P	GATACTGCATTAACCTTAGAAG GTAGCATCTGCATTAGTTC TGGATTCAACAGCATTAAACAATTACAG	101
FlaVR-8-F FlaVR-8-R FlaVR-8-P	GATTTAGCGCAAAACATT CAGTACTAAGTGAACCTCT TGCTGACATATCTCCAATGTGTAATGT	114
FlaVR-9-F FlaVR-9-R FlaVR-9-P	AATGAGAGAAGTACTGTGAC CAGTAATTTCTGTTTTATAGCTTC AGGAACTAACGATGATCAACCTTACTGCA	80
FlaVR-10-F FlaVR-10-R FlaVR-10-P	AAGAACTCACAAAGATTCC ATTTCTGTTTTATAGCGGC TCTGTTAGTGCTCTCTGCTGT	153
FlaVR-11-F FlaVR-11-R FlaVR-11-P	GTCTGCAAAAAGTATGATG CTTTTACTCCACTTCTATTC CAAGCTATTAAGTAACTATAGGAGCT	126
FlaVR-12-F FlaVR-12-R FlaVR-12-P	GAAATATGGGAATCAACACTAC TTCAGAAATGCTAGTCCAG AGCTTAATTTTCCATAGCTTTCCAGA	110
FlaVR-13-F FlaVR-13-R FlaVR-13-P	ACAACAAATGTAAGTGTTC gTTTTGGAAAGCACCAAG AGCTTTAGATGTTGCATTATCAGCAACAC	114
FlaVR-14-F FlaVR-14-R FlaVR-14-P	AAGCATAACTTCAACAAGCG GTTTTGGAAAGCACCAAG ACGCTGATTATCAACAACACTTACGC	124
FlaVR-15-F FlaVR-15-R FlaVR-15-P	CAGCAGGTCTATGTATATCGG GTTGAGCTTCTATAGTTTGAAC ATCCATCTTGAGTATTCGTGCTTGCTT	112
FlaB-F FlaB-R FlaB-P	ggAAACAGAGCTGGAGATG gGAAGCTTGRCTAATCCCTC TCTGAGATTGCAAGACCTGCTGC	81
FldB-F FldB-R FldB-P	CTAGTATCATATTGTGCTTCTGAG GGAAGAAGATTAGAAAAGGAGAC AATTTCTTGGGTCTGCTTATCCCC	98
Sequencing primers		
FlaB_seq-F1	gCAGTTAATTCAGGAAAGAGC	
FlaB_seq-F2	AGGGAAATGCAGATGTTAAAG	
FlaB_seq-F3	ACTGCAGTAGCTAATGTAGATG	
FlaB_seq-F4	TGTTGAAAATGCATTAGATGTAAG	
FlaB_seq-R1	TGAAGAACTCCTTGTGGTTG	
FlaB_seq-R2	gCTTACATCTAATGCATTTTCAAC	
FlaB_seq-R3	TCATCTACATTAGCTACTGCAG	
FlaB_seq-R4	GCITTAACATCTGCATTTCC	
FlaA_butyrr-seq-F1	gTAATATATCATCATTAGCGAGTTG	
FlaA_butyrr-seq-F2	ACTAATACCAATGATGATAGAAAGC	
FlaA_butyrr-seq-R1	gACTAATTTTAAACATCACTCCGC	
FlaA_butyrr-seq-R2	CTTGATGAAATTCATCACAATTGCC	

^a F, forward (primer); R, reverse (primer); P, probe.

^b All probes were labeled with 6-HEX and BHQ1 (Black Hole Quencher). Lowercase indicates nucleotides not present in the original gene sequence added to increase primers annealing temperature.

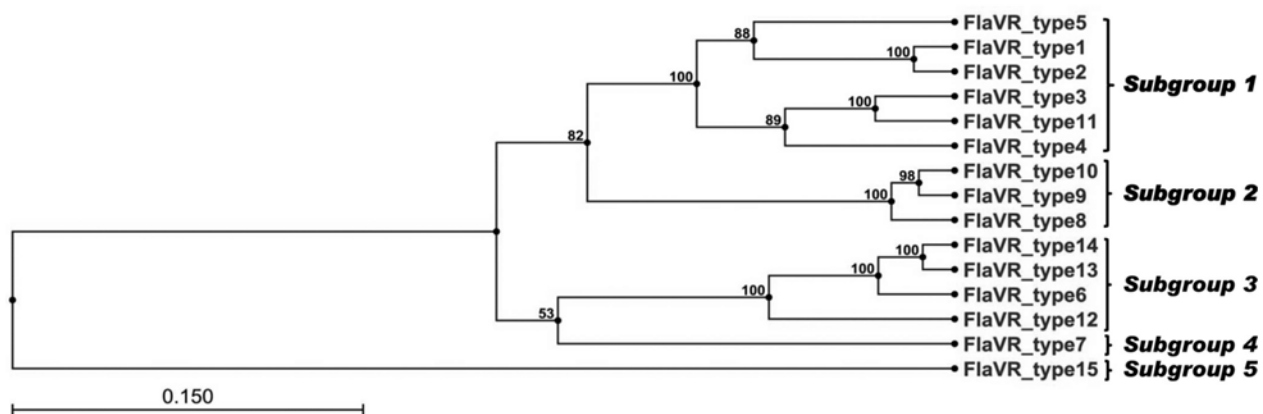


FIG 1 Clustering of flaVR types.

Phylogenetic analysis. Phylogenetic analysis of the flagellin genes is shown in Fig. 1. The dendrogram comparing the flagellin gene sequences used was obtained using the unweighted pair group method with arithmetic averages (UPGMA) with default settings.

Nucleotide sequence accession numbers. New sequences of *C. botulinum* flagellin genes were deposited in GenBank with accession numbers KC235357 to KC235394 and KC407595 to KC407603.

RESULTS

Molecular characterization of reference *C. botulinum* strains. A panel of 62 strains of *C. botulinum* was characterized for their flagellin genes as previously reported (5). These strains were also characterized by high-throughput real-time PCR for flaVR1 to flaVR10, *flaB*, *bont/A*, *bont/B*, *bont/E*, *bont/F*, and *fldB* (Table 2). The expected results of *bont* subtypes and detection of the proteolytic strain-specific gene *fldB* were confirmed, with the exception of 3 strains previously serotyped as type A. The latter were identified by PCR as type AB and found to carry a silent BoNT/B gene. The flaVR1 to flaVR10 genotype determined by real-time PCR corroborated the results obtained by sequencing of *flaA* and previous flaVR classification (5). Likewise, *C. botulinum* type E strain Bennett was shown to carry both flaVR8 and *flaB* sequences as previously reported (5). Interestingly, *flaB* was observed not only in 3 other flaVR8 strains but also in 15 flaVR10 strains. The *flaB* sequences detected in isolates positive for flaVR8 were sequenced (GenBank accession numbers KC407601 to KC407603). It is noteworthy that all *flaB*-positive strains were reported as group II *C. botulinum* type E and that *C. botulinum* type B strains belonging to the group II tested all negative for *flaB*.

Molecular characterization of 210 *C. botulinum* strains isolated in European countries. A collection of 210 *C. botulinum* strains native to Italy ($n = 196$), Germany ($n = 7$), France ($n = 5$), and the Netherlands ($n = 3$) was typed, using high-throughput real-time PCR, based on flaVR1 to flaVR10 sequences in *flaA* and *bont* type. The same approach was used to determine the presence of *flaB* and *fldB*. Among the 210 strains investigated in this study, 167 were subtyped based on flaVR sequences as described previously (Table 3). Four *C. botulinum* type E strains derived from France, Germany, and the Netherlands tested positive for *flaB*. Of the 210 isolates, 43 were not typeable based on flaVR and *flaB*, despite having their BoNT type determined by real-time PCR. Double-strand sequencing of *flaA* of 38 of these isolates revealed

new flaVR types, which we named flaVR11 to flaVR14 (Table 4). Six *Clostridium butyricum* type E strains failed to be sequenced (5). Based on the sequence of *flaA* of *C. butyricum* type E strains BL5262 and 5521, new sequencing primers and a flaVR15 real-time PCR assay were designed. The 6 strains gave a specific positive signal for flaVR15 and were successfully sequenced (GenBank accession numbers KC407595 to KC407600).

The flaVR, *flaB*, and *fldB* real-time PCR assays were evaluated for their specificity. Ten *C. botulinum* strains types C, C-D, D, D-C, and G, 18 nonbotulinum clostridia, and 18 nonclostridium bacteria tested negative for flaVR and *flaB* (data not shown). *fldB* was detected only in *Clostridium sporogenes* ATCC 3584. This finding is consistent with the draft genome sequence of *C. sporogenes* ATCC 15579.

Phylogenetic analysis of flaVR1 to flaVR15 types. The dendrogram shown in Fig. 1 gives an illustration of the genetic diversity of the flaVR among *C. botulinum* strains investigated in the present study. It reveals the clustering of flaVR types into 5 major subgroups: flaVR1 to flaVR5 and flaVR11 belong to subgroup 1; flaVR8 to flaVR10 are classified in subgroup 2; flaVR6, and flaVR12 to flaVR14 belong to subgroup 3; flaVR7 is part of subgroup 4; and flaVR15 is reported as subgroup 5.

DISCUSSION

Flagellar structure and genetics of Gram-positive bacteria, in particular *Clostridium* species, have not been investigated as extensively as those of Gram-negative bacteria. Paul et al. (5) reported the first study showing the genetic diversity of the flagellin genes of *C. botulinum* strains belonging to groups I and II. Their work opened the door for the development of new genotyping methods based on the sequence of the variable region of *flaA*.

In the present study, we developed real-time PCR genotyping assays for flaVR and *flaB* sequences for better discrimination of *C. botulinum* strains derived from groups I and II. Real-time PCR typing of flaVR1 to flaVR10 regions and *flaB* was optimized and validated on 62 historical and Canadian strains of *C. botulinum* that have been previously typed (5). These PCR assays were highly specific for *C. botulinum* strains belonging to groups I and II. *C. botulinum* strains from groups III and IV, other *Clostridium* species and non-*Clostridium* bacteria all tested negative. Our results are in accordance with a recent study (19) showing that *C. botuli-*

TABLE 2 Molecular characterization of 62 reference strains

Strain	Origin ^a	BoNT serotype (15)	<i>bont</i> type (5) ^b	flaVR type (15)	PCR flaVR type (this study)	<i>Clostridium botulinum</i> group	<i>flaB</i> (this study)
62A	NA	A	A	1	1	I	+
A6	NA	A	A	1	1	I	+
CK2-A	Feces	A	A	1	1	I	+
FE0101AJ0	Feces	A	A	1	1	I	+
GA0101AJ0	Gastric liquid	A	A	1	1	I	+
NG0107ASA	Gastric liquid	A	A	1	1	I	+
F9801-A	Feces	A	A	1	1	I	+
FE0205A1AK	Feces	A	A	1	1	I	+
INWB2202A2	Seal intestine	A	A	1	1	I	+
PC0101AJ0	Pork	A	A	1	1	I	+
SO300A1	Soil	A	A	1	1	I	+
1344-1-77	Feces	B	B	1	1	I	+
1366-1-77	Honey	B	B	1	1	I	+
368B	Feces	B	B	1	1	I	+
426B	Honey	B	B	1	1	I	+
427-2-76	Honey	B	B	1	1	I	+
920A-2-76	Feces	B	B	1	1	I	+
GA0108BEC	Gastric liquid	B	B	1	1	I	+
FE9504ACG	Feces	AB	A, B	1	1	I	+
FE0207AMB	Feces	A	A, B	1	1	I	+
F9604-A	Feces	A	A	2	2	I	+
FE9508BPD	Feces	B	B	2	2	I	+
FE9508BRB	Feces	B	B	2	2	I	+
PA9508B	Pate	B	B	2	2	I	+
FE0303A1YO	Feces	A	A, B	3	3	I	+
FE9909ACS-Alberta	Feces	A	A, B	3	3	I	+
NCTC2916	Canned corn	AB	A, B	3	3	I	+
Langeland	Liver paste	F	F	3	3	I	+
FE9904BMT	Feces	B	B	4	4	I	+
17A	NA	A	A	5	5	I	+
1B1-B	Feces	B	B	6	6	I	+
MRB	Mushrooms	B	B	6	6	I	+
EN0509BLP	Dust	B	B	7	7	I	+
FE0507BLP	Feces	B	B	7	7	I	+
Bennett	Gastric liquid	E	E	8	8 and <i>flaB</i>	II	-
SO329E1	Shoreline soil	E	E	8	8 and <i>flaB</i> ^c	II	-
SP455/456E2	Coastal rock	E	E	8	8 and <i>flaB</i> ^d	II	-
SOKR 38E2	Shoreline soil	E	E	8	8 and <i>flaB</i> ^e	II	-
17B	Marine sediment	B	B	9	9	II	-
19501F	Marine sediment	F	F	9	9	II	-
70F	Marine sediment	F	F	9	9	II	-
205F	Marine sediment	F	F	9	9	II	-
KAP-B-3	Kapchunka	B	B	9	9	II	-
KAP-B-8	Kapchunka	B	B	9	9	II	-
190F	Marine sediment	F	F	9	9	II	-
II60-15B	Feces	B	B	9	9	II	-
610F	Salmon	F	F	9	9	II	-
Gordon	Clinical sample	E	E	10	10 and <i>flaB</i>	II	-
F9508EPB	Feces	E	E	10	10 and <i>flaB</i>	II	-
FE0005EJT	Feces	E	E	10	10 and <i>flaB</i>	II	-
FE9507EEA	Feces	E	E	10	10 and <i>flaB</i>	II	-
FE9709EBB	Feces	E	E	10	10 and <i>flaB</i>	II	-
FE9909ERG	Feces	E	E	10	10 and <i>flaB</i>	II	-
GA9709EHS	Gastric liquid	E	E	10	10 and <i>flaB</i>	II	-
GA9709EJA	Gastric liquid	E	E	10	10 and <i>flaB</i>	II	-
MU0005EJT	Muktuk	E	E	10	10 and <i>flaB</i>	II	-
GA9709ENS	Gastric liquid	E	E	10	10 and <i>flaB</i>	II	-
MI9507E	Misraq	E	E	10	10 and <i>flaB</i>	II	-
SO326E1	Shoreline soil	E	E	10	10 and <i>flaB</i>	II	-
SOKR-23E1	River sediment	E	E	10	10 and <i>flaB</i>	II	-
SOKR-29E1	Shoreline soil	E	E	10	10 and <i>flaB</i>	II	-
FE9709ELB	Feces	E	E	10	10 and <i>flaB</i>	II	-

^a NA, not available.^b Shading indicates the silent B gene.^c GenBank accession number KC407601.^d GenBank accession number KC407602.^e GenBank accession number KC407603.

num type C strains carry flagellin gene sequences that are significantly different from those of *C. botulinum* types A, B, and E. The flaVR types of *C. botulinum* types C and D and their mosaic variants C/D and D/C remain to be investigated, and this would con-

stitute another study focusing on the characterization of *C. botulinum* strains involved in animal botulism. *flaB*, first identified in *C. botulinum* type E strain Bennett (5), was detected in all *C. botulinum* type E isolates investigated. The sequence of *flaB* in *C.*

TABLE 3 Search for flaVR1 to flaVR10 and *flaB* in *C. botulinum* strains isolated in Europe

Strain(s) ^a	Origin ^b	BoNT serotype (15)	<i>bont</i> type (5)	flaVR type (this study)	<i>flaB</i> (this study)
IP64 ^c , IP910 ^c , IP969 ^c , BfR7272 ^d , RIVM2 ^e	NA	A	A	1	+
ISS29, ISS103, ISS186, ISS260	Feces	A	A	1	+
ISS345	Canned spinach	A	A	1	+
ISS30	Sauerkraut	A	A	2	+
ISS117, ISS137, ISS138	Feces	A	A	3	+
ISS280, ISS289, ISS292, ISS300, ISS301, ISS303, ISS312, ISS313, ISS317, ISS318, ISS326, ISS327, ISS328, ISS329, ISS330, ISS348	Honey	A	A	3	+
ISS334, ISS391, ISS420, ISS451	Feces	A	A	3	+
ISS452	Mushroom in oil	A	A	3	+
ISS22	Mushroom in oil	A	A	5	+
ISS104	Feces	A	A	5	+
ISS126	Ribollita toscana	A	A	5	+
ISS127	Honey	A	A	5	+
ISS129	Feces	A	A	5	+
ISS130	Feces	A2	A	5	+
ISS270	Feces	A	A	5	+
ISS362, ISS363	Honey	A	A	5	+
ISS364	Homogenized meat	A	A	5	+
ISS365, ISS390, ISS392	Feces	A	A	5	+
ISS395	Foal gastric content	A	A	5	+
ISS464	Sea salt	A	A	5	+
ISS31, ISS264, ISS380, ISS382, ISS413	Feces	B	B	1	+
ISS268	Canned tuna	B	B	3	+
ISS173, ISS269, ISS277, ISS304, ISS305, ISS344, ISS361, ISS376, ISS385, ISS393, ISS403, ISS404, ISS414, ISS421, ISS441, ISS442	Feces	B	B	3	+
ISS311	Turnip tops	B	B	3	+
ISS347	Mushrooms	B	B	3	+
ISS368	Creamed truffle	B	B	3	+
ISS406, ISS407, ISS408, ISS409, ISS410, ISS411	Truffle in oil	B	B	3	+
ISS415	Canned chicory in oil	B	B	3	+
ISS463	Sea salt	B	B	3	+
BfR1944 ^d	NA	B	B	4	+
ISS64	Peppers in oil	B	B	4	+
ISS67, ISS200, ISS266	Mushroom in oil	B	B	4	+
ISS95	Pesto	B	B	4	+
ISS211	Food	B	B	4	+
ISS238	Olives	B	B	4	+
ISS275	Tissue	B	B	4	+
ISS156, ISS212, ISS213, ISS214	Sea salt	B	B	4	+
ISS343, ISS397	Canned tuna in oil	B	B	4	+
ISS90, ISS131, ISS133, ISS134, ISS136, ISS140, ISS167, ISS199, ISS201, ISS202, ISS203, ISS204, ISS210, ISS235, ISS236, ISS265, ISS272, ISS322, ISS402, ISS416	Feces	B	B	4	+
ISS257, ISS331, ISS274	Feces	B2	B	4	+
ISS276, ISS314, ISS338, ISS341	Honey	B2	B	4	+
BfR89 ^d	NA	B	B	5	+
ISS142	Olives	B	B	5	+
ISS223, ISS224, ISS225, ISS226, ISS227, ISS228, ISS229, ISS230, ISS231	Sea salt	B	B	5	+
ISS261	Honey	B	B	5	+
ISS32, ISS70, ISS135, ISS172, ISS208, ISS307, ISS308, ISS309, ISS346, ISS384, ISS389, ISS418, ISS419, ISS443, ISS444	Feces	B	B	5	+
ISS267	Feces	B2	B	5	+
IPBL10 ^c , BfR7273 ^d , RIVM3 ^e	NA	B	B	6	+
ISS386	Feces	AB	A, B	1	+
ISS89	Salami	A2B3	A, B	5	+
ISS87C	Feces	A2B3	A, B	5	+
RIVM5 ^e	NA	E	E	8 and <i>flaB</i>	-
IPHV2 ^c , BfR1718 ^d , BfR8550 ^d	NA	E	E	<i>flaB</i>	-
BfR1956 ^d	NA	F	F	3	+
ISS356, ISS357	Feces	F	F	3	+
ISS358	Asparagus	F	F	3	+

^a The ISS prefix indicates Italian origin.

^b NA, not available.

^c French origin.

^d German origin.

^e Dutch origin.

botulinum type E strains of flaVR type 8 has been determined and showed high sequence similarity to the *flaB* sequence of strain Bennett (99.71 to 99.92% identity; 99.43 to 99.64% similarity).

We investigated the flaVR genetic diversity in 210 group I and II strains of *C. botulinum* native to European countries (mainly of

Italian origin). The flaVR type of 167 isolates fell into the various flaVR types previously determined in the panel of 62 *C. botulinum* reference strains. However, 43 isolates remained unclassified (mostly *C. botulinum* type B group I and type E group II). Sequencing the flagellin gene of these strains revealed four new

TABLE 4 Search for flaVR11 to flaVR15 and *fldB* in *C. botulinum* strains isolated in Europe

Strains (GenBank accession no.) ^a	Origin	BoNT serotype (15)	<i>bont</i> type (5)	flaVR type (this study)	<i>fldB</i> (this study)
ISS234 (KC407595), ISS237 (KC235358), ISS430 (KC235359), ISS431 (KC235360), ISS432 (KC235361), ISS445 (KC235362)	Feces	B	B	11	+
ISS446 (KC235363)	Canned spinach	B	B	11	+
ISS447 (KC235364)	Mushroom in oil	B	B	11	+
ISS250 (KC235365), ISS253 (KC235367), ISS428 (KC235370), ISS429 (KC235371)	Feces	B	B	12	+
ISS251 (KC235366)	Canned chickpeas	B2	B	12	+
ISS254 (KC235368)	Canned vegetables in oil	B	B	12	+
ISS427 (KC235369)	Peppers in oil	B	B	12	+
ISS271 (KC235372)	Tofu	B2	B	13	+
ISS434 (KC235373)	Feces	B	B	13	+
ISS396 (KC235375)	Ileum	AB	A, B	13	+
ISS128 (KC235376)	Infant formula	B	B	14	+
ISS178 (KC235377), ISS181 (KC235378), ISS239 (KC235379), ISS241 (KC235381)	Feces	B	B	14	+
ISS240 (KC235380)	Meat in aspic	B	B	14	+
ISS242 (KC235382)	Canned beans	B	B	14	+
ISS243 (KC235383)	Beans	B	B	14	+
ISS244 (KC235384)	Mushroom in oil	B	B	14	+
ISS245 (KC235385)	Canned beans	B	B	14	+
ISS248 (KC235385, KC235388)	Canned beans and carrots	B	B	14	+
ISS246 (KC235386), ISS247 (KC235387), ISS249 (KC235389)	Peppers in oil	B	B	14	+
ISS252 (KC235390)	Canned chickpea	B2	B	14	+
ISS256 (KC235391)	Feces	B2	B	14	+
ISS259 (KC235392), ISS401 (KC235393), ISS422 (KC235394)	Feces	B	B	14	+
ATCC 43755 ^b (KC407599), ATCC 43181 ^b (KC407600)	Feces	E4	E	15	–
ISS109 ^b (KC407595), ISS145 ^b (KC407596), ISS146 ^b (KC407597)	Feces	E	E	15	–
ISS184 ^b (KC407598)	Soil	E	E	15	–

^a The ISS prefix indicates Italian origin.

^b *Clostridium butyricum*.

flaVR types for *C. botulinum* strains belonging to group I. Likewise, a new real-time PCR assay targeting *flaA* of *C. butyricum* type E was designed.

Molecular typing of flaVR sequences showed that 75.1% of historical and Canadian strains previously typed by Paul et al. (5) harbor flaVR1, flaVR9, and flaVR10, whereas 75.3% of the European strains carry flaVR1, flaVR3, flaVR4, and flaVR5. Interestingly, 20.8% of the European isolates display new flaVR types. Neither flaVR7, flaVR9, nor flaVR10 was recorded among isolates of European origin. However, it should be noted that these isolates were mostly (95.3%) group I *C. botulinum* strains, in contrast to the historical and Canadian strain collection, in which *C. botulinum* groups I and II were almost equally represented. These results suggest that flaVR distribution may vary with the geographical origin; further investigations are necessary to confirm this hypothesis. No significant association between the BoNT type and the flaVR determination was observed. Conversely, *flaB* was highly related to *C. botulinum* type E. Analysis of the genetic diversity of flaVR and *flaB* resulted in the clustering of *C. botulinum* strains into 5 major subgroups. Subgroups 1, 3, and 4 contain proteolytic *Clostridium botulinum*, subgroup 2 is made up of non-proteolytic *C. botulinum* only, and subgroup 5 is specific to *C. butyricum*. The genetic variability of the flagellin genes carried by *C. botulinum* and the possible association of flaVR types with certain geographical area make gene profiling of flaVR and *flaB* promising in molecular surveillance and epidemiology of *C. botulinum*.

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Main results:

- Analysis of 62 historical and Canadian *C. botulinum* group I and II strains, and 210 isolates from European origin allowed the identification of 15 flagellin groups, associated with *flaA* and *flaB* genes.
- Real time PCR genotyping assays targeting the internal variable region of the flagellin genes were developed for better discrimination of *C. botulinum* strains groups I and II.
- Molecular typing of *flaVR* sequences showed that 75.1% of historical and Canadian strains harbour *flaVR1*, *flaVR9*, and *flaVR10*, whereas 75.3% of the European strains carry *flaVR1*, *flaVR3*, *flaVR4*, and *flaVR5* flagellin types.
- 20.8% of the European isolates display new *flaVR* types.

Main conclusions:

- Results suggest that *flaVR* distribution may vary with the geographical origin; further investigations are necessary to confirm this hypothesis.
- No significant association between the BoNT type and the *flaVR* determination was observed except for *flaB* which showed to be specific to *C. botulinum* toxin producing type E.
- Genetic diversity of *flaVR* and *flaB* resulted in the clustering of *C. botulinum* strains into 5 major subgroups. Subgroups 1, 3, and 4 contain proteolytic *C. botulinum*, subgroup 2 is made up of non-proteolytic *C. botulinum*, and subgroup 5 is specific to *C. butyricum*.
- The genetic variability of the flagellin genes carried by *C. botulinum* and the possible association of *flaVR* types with certain geographical area make gene profiling of *flaVR* and *flaB* promising in molecular surveillance and epidemiology.

Perspectives:

C. botulinum type C strains carry flagellin gene sequences that are significantly different from those of *C. botulinum* group I and II. The *flaVR* types of *C. botulinum* types C and D and their mosaic variants C/D and D/C remain to be investigated, and this would constitute another study focusing on the characterization of *C. botulinum* strains involved in animal botulism.

V.2. *C. botulinum* Group III detection and characterization studies

C. botulinum Group III is mainly responsible for animal botulism cases in poultry and cattle farms. As botulinum toxins types C, D, C/D and D/C are harmless for human, this *Clostridium* species received less scientific interest during the last decades, until the work of the Japanese team of Dr. S. Kozaki. In 2005 they were able to solve the problem of cross neutralization reaction with type C and type D antitoxin on isolates from cases of avian botulism (Takeda et al., 2005). They found out that all avian botulism-related isolates and specimens they tested possessed the gene for a mosaic form C/D of the neurotoxin, comprising one third of type C, the catalytic part, and two third of type D, the receptor part. Later on they also characterized the counterpart D/C mosaic toxin in bovine isolates (Nakamura et al., 2010). Those data were the starting point of my work on *C. botulinum* Group III, as part of the European project AnibioThreat.

V.2.1. Investigation of *C. botulinum* Group III *bont* types throughout Europe.

At the time were I started to work on *C. botulinum* Group III, little was known regarding the epidemiology of the disease, and the factors behind the outbreaks. Also the prevalence of mosaic strains in Europe remained unclear, mostly due to the absence of methods that were able to discriminate non-mosaic from mosaic *C. botulinum* types. My objectives were to develop detection and typing methods to investigate *C. botulinum* Group III strains involved in animal botulism outbreaks through different European countries. Based on the work of Takeda et al. and Nakamura et al., I developed a molecular detection tool based on real time PCR to differentiate between *bont* genes type C, D and their mosaic C/D and D/C. Thanks to the several collaborating European institutes throughout Europe, I was able to test 56 isolated strains and 292 naturally contaminated samples from animal botulism events reported from different locations in Europe. The data obtained during the study indicated that mosaic types C/D and D/C are predominant in sample material gathered in the investigated regions. The results of this study were published in 2012 in the scientific journal *Applied and Environmental Microbiology* (Article n°2).

Article n°2:

Woudstra C, Skarin H, Anniballi F, Fenicia L, Bano L, Drigo I, Koene M, Båyon-Auboyer MH, Buffereau JP, De Medici D, Fach P. Neurotoxin gene profiling of *Clostridium botulinum* types C and D native to different countries within Europe. **Appl Environ Microbiol. 2012** May;78(9):3120-7.

Article n°2

Neurotoxin gene profiling of *Clostridium botulinum* types C and D native to different countries within Europe.

Woudstra C, Skarin H, Anniballi F, Fenicia L, Bano L, Drigo I, Koene M, Bâyon-Auboyer MH, Buffereau JP, De Medici D, Fach P.

Applied and Environmental Microbiology

2012 May;78(9):3120-7

Neurotoxin Gene Profiling of *Clostridium botulinum* Types C and D Native to Different Countries within Europe

Cedric Woudstra,^a Hanna Skarin,^b Fabrizio Anniballi,^c Lucia Fenicia,^c Luca Bano,^d Ilenia Drigo,^d Miriam Koene,^e Marie-Hélène Bâyon-Auboyer,^f Jean-Philippe Buffereau,^f Dario De Medici,^c and Patrick Fach^a

ANSES (French Agency for Food, Environmental and Occupational Health Safety), Food Safety Laboratory, Maisons-Alfort, France^a; National Veterinary Institute, Department of Bacteriology, Uppsala, Sweden^b; Istituto Superiore di Sanità Laboratorio Alimenti, Rome, Italy^c; Istituto Zooprofilattico Sperimentale delle Venezie, Laboratorio di Treviso, Treviso, Italy^d; Central Veterinary Institute of Wageningen, Lelystad, Netherlands^e; and Analysis and Development Laboratory 22, Ploufragan, France^f

Clostridium botulinum types C and D, as well as their mosaic variants C-D and D-C, are associated with avian and mammalian botulism. This study reports on the development of low-density macroarrays based on the GeneDisc cyclor platform (Pall-Gene-Disc Technologies) applied to the simultaneous detection of the *C. botulinum* subtypes C, C-D, D, and D-C. The limit of detection of the PCR assays was 38 fg of total DNA, corresponding to 15 genome copies. Artificially contaminated samples of cecum showed a limit of detection below 50 spores/g. The tests were performed with a large variety of bacterial strains, including *C. botulinum* types C ($n = 12$), C-D ($n = 29$), D ($n = 5$), and D-C ($n = 10$), other botulinum neurotoxin (BoNT)-producing *Clostridium* strains ($n = 20$), non-BoNT-producing clostridia ($n = 20$), and other bacterial species ($n = 23$), and showed a high specificity. These PCR assays were compared to previously published real-time PCRs for the detection of *C. botulinum* in 292 samples collected from cases of botulism events in four European regions. The majority of the samples originated from wild birds ($n = 108$), poultry ($n = 60$), and bovines ($n = 56$). Among the 292 samples, 144 were positive for either the *bont*/C-D or the *bont*/D-C gene by using the GeneDisc arrays. The reliability of the results tallied to 97.94%. Interestingly, only BoNT mosaics, types C-D and D-C, were found in naturally contaminated samples whatever their animal origin and their geographical location. Further investigations should now be performed in order to check that mosaic types dominate in Europe and that acquisition of mosaic types helps in survival or adaptation to particular niche.

Botulism is a severe flaccid paralytic disease caused by seven different neuroparalytic toxin subtypes (A to G) (20). These botulinum neurotoxins (BoNTs) are produced by anaerobic Gram-positive bacteria species such as *Clostridium botulinum* (BoNT A to F), *C. baratii* (BoNT F), *C. butyricum* (BoNT E), and *C. argentinense* (BoNT G) (23). All BoNT subtypes act at the neuromuscular junction, blocking the release of acetylcholine interfering within the exocytose mechanism and thus leading to a flaccid paralysis (26, 41). BoNT types A, B, E, and, more rarely, F are mainly responsible for human botulism, whereas toxin types C and D are involved in animal botulism worldwide (40). Regarding animal botulism, the most common sources of contamination are poultry litter and contaminated ground or carcasses (22, 38). The disease is common in wild and domestic animals. It occurs as sporadic cases and also massive outbreaks all over the world (5, 33, 38, 50).

In Korea, five outbreaks of botulism in water birds were reported over a 5-year period from 2004 to 2008. In October 2008, an outbreak of avian type C botulism affected approximately 2,000 wild water birds in the Namdong flood control basin, Incheon, South Korea (47, 52). In Europe, several cases of animal botulism were reported in the recent years. In Sweden from 2000 to 2004, more than 10,000 seabirds, primarily Herring Gulls, died from type C botulism in the Blekinge archipelago in southeastern Sweden (37). From 2003 to 2009, 168 cattle and 19 sheep botulism incidents were reported in England and Wales, with the notable emergence of *C. botulinum* type D, suggesting a change in the source or epidemiology of botulism in the United Kingdom (39). Finland also reported their first case of a bovine type C botulism outbreak in 2008 (35).

In intensively farmed animals, animal botulism is responsible for high mortality (32, 46). In order to take appropriate measures, veterinarians need to quickly identify the nature of the disease. Strains producing interserotype recombinant toxins, primarily the C-D and D-C mosaic subtypes, have been reported (34, 36). Furthermore, since toxic activity is not equivalent between the different botulinum neurotoxins, the quick typing of botulism toxins is of major importance (36, 43).

Botulism diagnosis in animals is based on specific clinical symptoms such as abnormal posture of the head, weakness, loss of tongue tone, dilated pupils, and flaccid paralysis (5, 6). A laboratory confirmation is also required to determine the BoNT serotype. The reference method is currently the mouse lethality bioassay (standard mouse bioassay), followed by seroneutralization (8, 21). However, the mouse bioassay has major drawbacks: it is time-consuming and expensive, and there are ethical considerations associated with the use of animals (6, 7). For the seroneutralization test, there is a commercially available equine antiserum for types C and D, provided by the NIBSC (Health Protection Agency, United Kingdom), but only a few laboratories are able to perform routinely this analysis. The seroneutralization test is used to dem-

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Address correspondence to Patrick Fach, patrick.fach@anses.fr.

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TABLE 1 Primers and probes

Primer or probe ^a	Sequence (5'–3') ^b	Target <i>bont</i> types	Position ^c	Amplicon size (bp)
C-I_F	TCCTGGGAATAACAATACTC	C, C-D	348–367	135
C-I_R	CTAGGTCCAGTTATTATAACAC		485–464	
C-I_P	[ROX]AACCAGTTGTTACCTTGTCTAGTTT[BHQ2]		441–416	
C-II_F	GGGTCAAATTTATCTCG	C, C-D	1233–1250	135
C-II_R	AGCTCTCTACAATCTAATG		1367–1349	
C-II_P	[ROX]ATCCAGCATTAAGAAAAGTCAATCCT[BHQ2]		1253–1278	
C-III_F	TCAGCTTAATCCAATATTTC	C, D-C	2697–2717	77
C-III_R	GGGTTACTATAACTTTACCTC		2773–2753	
C-III_P	[ROX]CCCTGAACTACCTAATTTAAAGTCAAA[BHQ2]		2745–2719	
D-C_F	GACTGATTTAGTTCCTACTAG	D-C	3561–3580	82
D-C_R	GCATGGTTGTATTATAAACG		3642–3623	
D-C_P	[ROX]ACGTATCTCATCCATTGGTTGATC[BHQ2]		3612–3589	
D-I_F	TCCAGTAATAGCTTTAATGC	D, D-C	666–685	141
D-I_R	TCCTCAAATTTGACGTTG		806–789	
D-I_P	[ROX]AAATCCCTCGCTAAGTTGTGGAC[BHQ2]		771–749	
D-II_F	GAAGCATTAGTTATAGCAATAAG	D, D-C	1696–1719	116
D-II_R	TCCTCAACTACTTCATTTCG		1811–1793	
D-II_P	[ROX]AGAATAAACCTGCTGAACACCTTT[BHQ2]		1783–1759	
D-III_F	ATGGCAATATAGAATGGA	D, C-D	2949–2966	138
D-III_R	ACCCCATATATTATTAGTTATAG		3086–3063	
D-III_P	[ROX]ATCCTGTATGACTTAATGATTCACT[BHQ2]		3038–3014	

^a Suffix: F, forward (primer); R, reverse (primer); P, probe.

^b ROX, 6-carboxy-x-rhodamine; BHQ, Black Hole Quencher.

^c GenBank accession numbers: *bont*/C, X62389, strain C-Stockholm; *bont*/C-D, AB200360, strain 003-9; *bont*/D, AB012112, strain 1873; and *bont*/D-C, AB461915, strain OFD-05.

onstrate the presence of BoNT in clinical samples and to identify the toxin type, but a negative result does not exclude the possibility of botulism since the toxin can be rapidly degraded (51). Thus, recent research has focused on the development of rapid, specific, and reliable alternative biological techniques for the identification of BoNT-producing clostridia (6, 31). PCR-based assays have been recently developed for various bacterial diagnostic methods. While not solving the shortcoming of the detection of bacteria instead of toxin, PCR-based assays have the advantage of being rapid, easy to perform, and highly specific. A large number of studies have focused on the detection of *C. botulinum bont*/A, *bont*/B, *bont*/E, and *bont*/F genes, which are responsible for toxin production leading to human botulism (1–4, 11, 13, 14, 17, 18, 30, 45). Several studies have also reported on the detection of type C (*bont*/C) and type D (*bont*/D) genes by conventional PCR (9, 15, 19, 24, 42, 48, 49), while only a few such genes have been detected by real-time PCR (25, 28, 29). Real-time PCR technique presents the advantages of being highly specific and sensitive with no need for a post-PCR detection assay, in contrast to conventional PCR. In a recent study, we described the advantage of using macroarrays such as the GeneDisc array from Pall-GeneDisc Technologies (Bruz, France) for the screening of *C. botulinum* types A, B, E, and F in food samples (13). In the present study, two GeneDisc arrays were developed for the simultaneous detection of *C. botulinum* types C and D and mosaic types C-D and D-C for veterinary and epizootic study purposes. These PCR assays have been optimized to differentiate mosaic C-D and D-C strains from parental C and D strains, whereas no other methodologies are currently able to differentiate these two subtypes. The GeneDisc arrays were evaluated for their specificity and sensitivity and used to investigate the neurotoxin gene profile (molecular toxinotype) of neurotoxin-producing clostridia involved in animal botulism across Europe.

MATERIALS AND METHODS

Primers and probes. The primers and probes used in the present study are listed in Table 1. These primers and probes were designed by alignment of the neurotoxin gene sequences *bont*/A to *bont*/G from *C. botulinum*, *C. baratii*, and *C. butyricum* available in the National Centre for Biotechnology Information GenBank (<http://www.ncbi.nih.gov/GenBank/>) using the multi-alignment program CLUSTAL W (<http://align.genome.jp/>). The specificity of the oligonucleotides for toxin gene-specific identification was checked by *in silico* analysis against the published sequences from the GenBank database using Basic Local Alignment Search Tool (BLAST) algorithm (<http://www.ncbi.nih.gov/BLAST>). Primer pairs and probes were designed using the *bont*/C, *bont*/C-D, *bont*/D, and *bont*/D-C gene sequences available (the accession numbers for *bont*/C and *bont*/C-D are AB061780, AB200358, AB200359, AP008983, D90210, X53751, X62389, X66433, X71126, X72793, AB037166*, AB200360*, AB200361*, AB200362*, AB200363*, AB200364*, ABDQ01000029*, AY251553*, D49440*, FN436021*, and FN436022*; the accession numbers for *bont*/D and *bont*/D-C are AB012112, S49407, X54254, AB037920*, AB461914*, AB461915*, AB461916*, AB461917*, AB461918*, AB461919*, AB461920*, AB461921*, D38442*, and EF378947*). Accession numbers labeled with stars indicated sequences of the mosaic types. All probes were 5'-labeled with 6-carboxy-x-rhodamine (ROX) and 3'-labeled with Black Hole Quencher (BHQ-2). All primers and probes were purchased from Sigma-Aldrich (St. Quentin-Fallavier, France).

Design of GeneDisc arrays. Two GeneDisc arrays were designed. The *C. botulinum* type C and D GeneDisc array (GD1 C&D) contains the primer and probe sets C-I, C-II, D-I, and D-II designed in the gene sequences encoding for the light chains (i.e., the N-terminal portion) of BoNT/C and BoNT/D, respectively. These oligonucleotides allowed the detection of *C. botulinum* type C and D and mosaic type C-D and D-C *bont* genes, respectively, but without the possibility to differentiate non-mosaic and mosaic types. GD1 C&D was designed for the simultaneous examination of six different samples, each being tested for *bont*/C- and *bont*/D-specific gene targets, together with negative and internal amplifi-

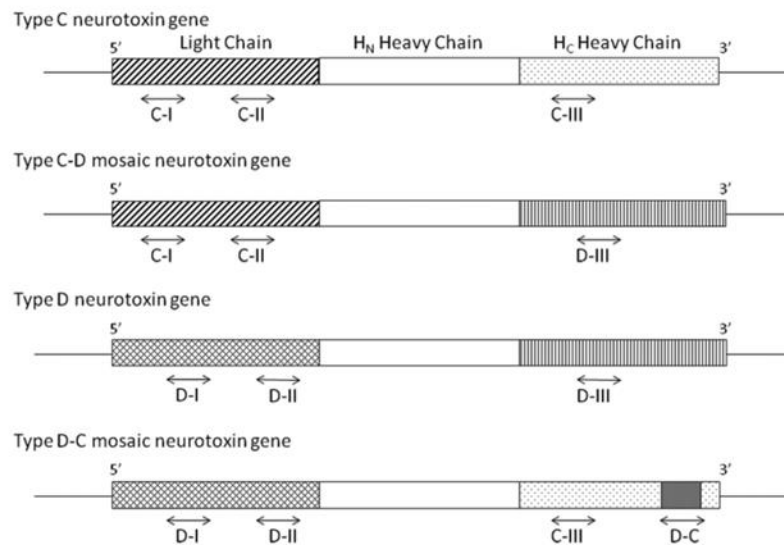


FIG 1 Position of oligonucleotides. Highly homologous domains are represented with the same pattern. GD1 C&D contains the C-I, C-II, D-I, and D-II oligonucleotides, together with the IAC and the negative control. This allows the detection of N-term part of the neurotoxin genes and also allows the detection of but not distinction between nonmosaic and mosaic genes types. GD2 C,D&mosaic contains the C-II, C-III, D-C, D-II, and D-III oligonucleotides, together with the IAC and the negative control. It targets both the N-terminal and C-terminal parts of the neurotoxin genes, allowing detection and also distinction between nonmosaic and mosaic gene types.

cation controls. Each sector had the following settings: microwell 1, negative PCR control; microwell 2, *bont/C-I*; microwell 3, *bont/C-II*; microwell 4, *bont/D-I*; microwell 5, *bont/D-II*; and microwell 6, PCR internal amplification control (IAC). For a type C-positive result to be valid, positive signals were required for C-I and C-II, together with the IAC, and negative results for D-I, D-II, and the negative control. For a type D-positive result to be valid, positive signals were required for D-I and D-II, together with the IAC, and negative results for C-I, C-II, and the negative control. Schematic locations of the various primers and probes used in the assays are shown in Fig. 1.

The *C. botulinum* type C and D and mosaic GeneDisc array (GD2 C,D&mosaic) contained the primer and probe sets C-II (targeting the light chain of BoNT/C), C-III (targeting the heavy chain of BoNT/C), D-C (targeting specifically the mosaic D-C), D-II (targeting the light chain of BoNT/D), and D-III (targeting the heavy chain of BoNT/D). These oligonucleotides were designed to target both the N-terminal and the C-terminal parts of the *bont/C* and *bont/D* genes permitting the detection and specific identification of nonmosaic types and mosaic types. GD2 C,D&mosaic used the following settings: microwell 1, *bont/C-II*; microwell 2, *bont/C-III*; microwell 3, *bont/D-C*; microwell 4, *bont/D-II*; microwell 5, *bont/D-III*; and microwell 6, negative PCR control together with the PCR internal amplification control. To validate a *C. botulinum* type C-positive result, we considered the positive signals for C-II and C-III, together with the IAC, and negative results for D-C, D-II, D-III, and the negative control. To validate a *C. botulinum* type D-positive result, we validated the positive signals for D-II and D-III, together with the IAC, and negative results for C-II, C-III, D-C, and the negative control. To validate a *C. botulinum* mosaic type C-D-positive result, we looked at the positive signals for C-II and D-III, together with the IAC, and negative results for C-III, D-C, D-II, and the negative control. To validate a *C. botulinum* type D-C-positive result, we considered the positive signals for C-III, D-C, and D-II, together with the IAC, and negative results for C-II, D-III, and the negative control. D-C was specifically designed to detect only *C. botulinum* mosaic type D-C. Schematic locations of the various primers and probes used in the assays are shown in Fig. 1.

GeneDisc spotting and manufacturing were performed by Pall GeneDisc Technologies. The negative PCR control and internal amplification

control are part of the GeneDisc technology. The GeneDisc is preloaded with desiccated PCR primers and fluorescent TaqMan probes labeled with the reporter dye 6-carboxy-x-rhodamine (ROX; 575 to 602 nm). Primers are incorporated at final concentrations of 600 nM and probe is incorporated at final concentrations of 400 nM. The primers and probes incorporated in the GeneDisc arrays were previously validated on a LightCycler 480 (Roche Diagnostics, Meylan, France), showing that they could be easily transferred and adapted to the GeneDisc technology (data not shown).

To confirm the results obtained with the two GeneDisc arrays, all strains and naturally contaminated samples were tested on the LightCycler 480 using previously published conventional real-time PCR systems (25, 28). The real-time PCR conditions were identical to those previously published, and we used the same DNA sample concentrations as those used in the GeneDisc assays.

Bacterial strains and growth. The selected strains used in the present study are listed in Table 2. The specificity was evaluated with 12 *C. botulinum* type C (strain C-Stockholm as the *bont/C* reference), 29 mosaic type C-D (strain 07-BKT002873 as the *bont/C-D* reference), five type D (strain 1873 as the *bont/D* reference), and 10 mosaic type D-C (strain 4456/11 as the *bont/D-C* reference) strains. A total of 20 BoNT-producing *Clostridium* strains (BoNT/A, BoNT/B, BoNT/Ab, BoNT/E, and BoNT/F) were evaluated as negative controls. All BoNT-producing *Clostridium* strains were toxinotyped by a reference mouse bioassay according to the previously described method (7); the tests were performed in accordance with European Directive 86/609&EEC on the protection of animals used for experimental and other scientific purposes. Briefly, 2 ml of the enrichment culture (48 h) were centrifuged and 1 ml of the culture supernatant was incubated with 200 μ g of trypsin/ml for 20 min at room temperature. A volume of 0.5 ml of 10-fold serial dilutions was then injected intraperitoneally into Swiss male mice (two mice per sample), and the mice were monitored for the characteristic symptoms of botulism (labored breathing, pinching of the waist, and paralysis) for up to 4 days. Botulinum toxins were confirmed, and types were identified by using a seroneutralization test on mice with specific botulinum antitoxins for types C and D (Istituto Superiore di Sanità, Rome, Italy).

Twenty strains of other clostridial species were used as non-BoNT-

TABLE 2 Specificity of GeneDisc arrays

Strain	No. of strains	GeneDisc and PCR specificity ^a			Reference PCR ^b
		GD1 C&D	GD2 C,D&mosaic		
<i>Clostridium botulinum</i>					
Type C	12	C	C	C	
Type C-D	29	C	C-D	C	
Type D	5	D	D	D	
Type D-C	10	D	D-C	D	
Type A	7	–	–	–	
Type B	4	–	–	–	
Type Ab	2	–	–	–	
Type E	3	–	–	–	
Toxic <i>Clostridium butyricum</i> type E	2	–	–	–	
<i>Clostridium botulinum</i> type F	1	–	–	–	
Toxic <i>Clostridium baratii</i> type F	1	–	–	–	
<i>Clostridium botulinum</i> type G	1	–	–	–	
Non-BoNT-producing clostridia ^c	20	–	–	–	
Non- <i>Clostridium</i> bacteria ^d	23	–	–	–	

^a –, no amplification.

^b Performed as described previously (25, 28).

^c Non-BoNT-producing *Clostridium* strains tested: *C. butyricum*, *C. baratii*, *C. beijerinckii*, *C. bifementans*, *C. chauvoei*, *C. difficile*, *C. manganotii*, *C. edematiens*, *C. perfringens* type A, *C. perfringens* type E, *C. perfringens* type C, *C. perfringens* type D, *C. septicum*, *C. sordellii*, *C. sporogenes*, *C. sporogenes*, *C. subterminale*, and *C. tetani*.

^d Non-*Clostridium* strains tested: *Bacillus anthracis*, *B. cereus*, *B. thuringiensis*, *Citrobacter* sp., *Escherichia coli*, *Hafnia alvei*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Proteus* sp., *Pseudomonas* sp., *Salmonella enterica* serovar Virchow, *S. enterica* serovar Hadar, *S. enterica* serovar Enteritidis, *S. enterica* serovar Infantis, *S. enterica* serovar Typhimurium, *Shigella* sp., *Staphylococcus aureus*, *Streptococcus faecalis*, and *Yersinia enterocolitica*.

producing negative controls. Twenty-three strains of other bacterial species were also analyzed as non-*Clostridium* negative controls. The *C. botulinum* strains were grown overnight in Trypticase-peptone-glucose-yeast extract (TPGY) broth at 30°C under anaerobic conditions, in brain heart infusion medium (Difco, Paris, France), or in broth-fortified

cooked meat medium (27). Nonclostridial strains were grown in brain heart infusion medium at 37°C for 24 h.

DNA extraction. After culturing, 1 ml of the cell cultures was DNA extracted using different protocols, such as a phenol-chloroform method (16), a DNeasy blood and tissue kit (Qiagen, Hilden, Germany), Chelex 100 (Bio-Rad Life Science Research, Hercules, CA), and the Automatic MicroLab Starlet System (Hamilton, Nevada), employing a MegaMax total nucleic acid isolation kit (Ambion, Austin, TX), according to manufacturers' instructions for Gram-positive bacteria. DNA was stored at –20°C until analysis.

DNAs from *C. botulinum* types C (C-Stockholm), C-D (07-BKT002873), D (NCTC 1873), and D-C (OFD-05) were used to determine the limit of detection (LOD) of the PCR assays. Purified DNAs were quantified prior to serial dilution by using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The LOD was determined using serial 10-fold dilutions of genomic DNA over a range of 6 orders of magnitude (Table 3). Each dilution was tested in triplicate. The PCR efficiency was assessed for each primer-probe combination with a serial dilution in accordance with the correlation coefficient (R^2). LOD was the lowest amount of *C. botulinum* in a test sample that was reproducibly detected in three experiment sets. It was expressed as the genomic copy number after converting the total genomic DNA from each strain.

Spiking experiments. Spiking of chicken cecum samples with a known concentration of botulinum spores was performed to evaluate the performance of the enrichment protocol and to determine the capability of detecting a low number of spores. Spore solutions from *C. botulinum* type C (strain Stockholm), type C-D (strain 07-V891), or type D (strain CIP-105256) were prepared and titrated by the five-tube most-probable-number (MPN) method as previously described (10, 29). Samples of chicken cecum (1 g) that previously tested negative for the presence of *C. botulinum* were inoculated with 50 and 100 spores of each strain. The first spore dilution for which no visible growth was recorded was also included in the experiment (<1 spore/g). Spiked samples were enriched and incubated under anaerobic conditions in prerduced TPGY medium for 48 h at 30°C. A volume of 1 ml was then subjected to DNA extraction using a DNeasy blood and tissue kit (Qiagen) and analyzed with GeneDisc arrays GD1 and GD2 (Table 4). All experiments were performed in triplicate.

TABLE 3 Limit of detection of GeneDisc arrays

Purified genomic DNA type (strain)	GeneDisc array data					
	Primers and probes	LOD (fg) for DNA ^a	Genome equivalents ^b	R^2 ^c	Slope ^d	Efficiency (%) ^e
<i>C. botulinum</i> type C (C-Stockholm)	C-I	66	21	0.9928	–3.11	109.48
	C-II	66	21	0.9962	–3.01	114.74
	C-III	66	21	0.9969	–3.09	110.66
<i>C. botulinum</i> type D (1873)	D-I	38	15	0.9990	–3.46	94.40
	D-II	38	15	0.9981	–3.33	99.76
	D-III	38	15	0.9984	–3.43	95.64
<i>C. botulinum</i> type C-D (07-BKT002873)	C-I	70	22	0.9973	–3.36	98.58
	C-II	70	22	0.9933	–3.27	102.36
	D-III	70	22	0.9925	–3.17	106.52
<i>C. botulinum</i> type D-C (OFD-05)	D-I	103	40	0.9984	–3.44	95.33
	D-II	103	40	0.9989	–3.35	98.75
	C-III	103	40	0.9997	–3.34	99.39
	D-C	103	40	0.9941	–3.41	96.61

^a LOD, limit of detection.

^b Genome equivalents were calculated based on the masses of the genomes of *C. botulinum* types C (2,961,186 bp) and D (2,379,404 bp) and on the assumption that the average mass of a base pair is 650 Da.

^c R^2 , regression correlation coefficient.

^d The slope was calculated based on logarithm dilution plotted against their corresponding threshold cycles (C_T).

^e The efficiency was calculated based on the following formula: $E = -1 + 10^{-1/\text{slope}}$.

TABLE 4 Use of GeneDisc arrays for testing spiked cecum samples^a

Toxin type (strain)	Estimated no. of spores/g	GD1 C&D		GD2 C,D&mosaic	
		C_T	SD	C_T	SD
C (C-Stockholm)	<1	35.00	0.61	34.62	1.03
	50	21.90	4.48	21.65	4.34
	100	18.85	1.92	18.14	2.08
C-D (07-V891)	<1	34.95	1.21	33.08	0.55
	50	25.09	1.16	24.78	1.22
	100	27.46	2.50	27.60	1.58
D (CIP-105256)	<1	35.16	2.00	34.78	2.25
	50	30.99	0.79	31.16	0.67
	100	23.23	1.00	23.59	0.60

^a Cecum samples were inoculated with the estimated number of spores as determined by MPN and tested with the GeneDisc arrays GD1 and GD2. All spiking experiments were performed in triplicate. SD here indicates the standard deviation of the mean C_T values for the whole experiments.

Naturally contaminated samples. A total of 292 naturally contaminated samples collected across Europe during animal botulism events reported in the last few years were investigated (see Table S1 in the supplemental material). Of these, 120 naturally contaminated samples were collected by Analysis and Development Laboratory 22 (Brittany, France) during animal botulism epizootic events identified in France in 2009. An additional 112 naturally contaminated samples were collected by the Istituto Superiore di Sanità (Rome, Italy) during botulism outbreaks recorded from 2006 to 2009. Another 36 naturally contaminated samples were collected by the Istituto Zooprofilattico Sperimentale delle Venezie (Treviso, Italy) during botulism outbreaks reported in 2010. Lastly, 24 naturally contaminated samples were collected in 2011 by the Central Veterinary Institute (Lelystad, Netherlands). Each sample (1 g) was 10-fold diluted (wt/vol) and then incubated in anaerobic conditions in pre-reduced TPGY medium. After 48 h of incubation at 30°C, 1-ml aliquots of the enrichment broths were collected and centrifuged at 9,000 × *g* for 5 min. The supernatant was discarded, and the cell pellet was subjected to DNA extraction using either a DNeasy blood and tissue kit or Chelex 100 (Bio-Rad) according to the manufacturer's instructions.

RESULTS

Specificity study. Specificity was evaluated on genomic DNA extracted from strains of *C. botulinum* type C ($n = 12$), strains of mosaic type C-D ($n = 29$), strains of type D ($n = 5$), and strains of mosaic type D-C ($n = 10$). The results obtained with the GeneDisc arrays GD1 and GD2 on these strains were consistent with conventional real-time PCR assays used as reference (Table 2). The 20 strains of BoNT-producing clostridia expressing types A, B, E, F, and G toxin tested negative with GeneDisc arrays GD1 and GD2. No amplification was observed with the GeneDisc arrays when 20 non-BoNT-producing clostridial and 23 nonclostridial bacterial DNAs were used as a template (Table 2). The PCR assays included negative and internal inhibition controls to exclude PCR inhibition by the sample material: all samples yielded positive results for the internal amplification control and negative results for the negative control. Molecular genotyping of *bont*/C and *bont*/D genes using the GeneDisc arrays perfectly matched the results of the conventional real-time PCR used as reference (Table 2) and those of the standard mouse bioassay (data not shown).

Sensitivity study. Purified genomic DNA of *C. botulinum* types C (strain C-Stockholm), C-D (strain 07-BKT002873), D

(strain NCTC 1873), and D-C (strain OFD-05) were used as references for determining the LODs of the GeneDisc arrays. The DNA concentrations were converted to genomic copy number, assuming that the size of each strain's genome is equal to those of the sequenced *C. botulinum* types C (2.9 Mbp) and D (2.4 Mbp) strains. The LOD was determined using triplicate serial dilutions of each DNA strain and ranged from 15 to 40 genome copies using the BoNT GeneDisc arrays with PCR efficiency ranging from 94.40 to 114.74% (Table 3). All real-time PCR assays of the GeneDisc arrays showed a strong linear correlation ($R^2 > 0.99$) between the threshold cycle (C_T) values and the template concentration over a range of 6 orders of magnitude. The slopes ranged from -3.46 to -3.01 , revealing high PCR efficiencies (Table 3).

Spiking experiments. Enrichment broths of chicken cecum samples (1 g) spiked with *C. botulinum* spores, with spiking levels ranging from 0 to 100 spores, were tested with GeneDisc arrays GD1 and GD2 for the presence of *C. botulinum* types C, C-D, D, and D-C. The GeneDisc arrays yielded specific positive signals with the various toxin type-specific detection systems of the BoNT GeneDisc arrays. The results were consistent with the toxin types, as determined with conventional approaches (data not shown), and both GeneDisc arrays were able to detect the *bont* genes in all spiked cecum samples tested with a limit of detection below 1 to 50 spores/g (Table 4).

Molecular toxinotyping of neurotoxin-producing clostridia in naturally contaminated samples gathered in Europe. Naturally contaminated samples ($n = 292$) from animal botulism events reported in different European regions in the last few years were analyzed for the presence of *C. botulinum*. Most of the samples ($n = 181$) were tested in the mouse lethality bioassay, and all were tested for their neurotoxin gene profile (molecular toxinotyping) using conventional real-time PCR systems as reference methods (25, 28) and the GeneDisc arrays GD1 C&D and GD2 C,D&mosaic. The results are summarized in Table S1 in the supplemental material. All samples gave positive results for the internal amplification control, showing that the samples carried no significant levels of PCR inhibitors. Of 292 samples, 144 were tested positive for either the *bont*/C-D or the *bont*/D-C gene by using the GeneDisc arrays. Of these them, 110 were type C-D, 24 were type D-C, and 9 were detected as both types C-D and D-C. The data obtained with the GD1 and the GD2 arrays correlated perfectly and showed no discordant results: the agreement between the GD arrays and previously published real-time PCR systems used as a reference reached 97.94%. Only 4 of the samples detected as type C with the conventional real-time PCR test ($C_T = 36.49$ to 38.08) were negative in the GeneDisc arrays, 1 sample detected as type D ($C_T = 38.18$) was negative with the GeneDisc arrays, and 1 sample was detected as type D-C ($C_T = 34.42$) with the GeneDisc arrays but was negative with the reference PCR systems, among a total of 292 samples.

DISCUSSION

Animal botulism is a worldwide problem that causes large economic losses since it affects cattle and other livestock (39). It has become an emerging and serious problem in poultry flocks and waterfowl in Sweden for the last few years (37) and in other European regions (35). Little is known regarding the epidemiology of the disease, and the factors behind the outbreaks are not well identified. The prevalence of mosaic strains in Europe has also remained unclear, mostly due to the absence of methods able to

discriminate nonmosaic from mosaic *C. botulinum* types. In the present study two GeneDisc arrays (GD1 C&D and GD2 C,D&mosaic) based on real-time PCR were developed for detecting the neurotoxin genes carried by *C. botulinum* types C and D and the corresponding mosaic types C-D and D-C. Identification of the mosaic types represents an improvement in regard to the current PCR approaches since it could be used for epidemiological purposes and for *bont/C* and *bont/D* gene profiling. The aim of the present study was to assess both GeneDisc arrays in an investigation of animal botulism events occurring in different geographic regions within Europe and to gain further insight regarding the neurotoxin gene profiles (molecular toxinotype) of these neurotoxin-producing clostridia.

The LOD of the GeneDisc arrays was determined using serial dilutions of clostridial DNA as a template. The lowest LOD was observed for *C. botulinum* type D (15 genome copies), and the highest LOD was reported for *C. botulinum* type D-C (40 genome copies). Type C and D *bont* genes are carried by circular plasmid prophages that express unstable lysogeny in the bacteria (12, 44), which can partly explain the observed differences regarding the LOD values. The high efficiency and coefficient of correlation characterizing the various PCR assays presented here emphasize that the primers and probes used in these assays are highly sensitive for the detection of *C. botulinum bont/C*, *bont/C-D*, *bont/D*, and *bont/D-C* genes.

The GeneDisc arrays GD1 and GD2 were found specific for detecting *bont/C*, *bont/C-D*, *bont/D*, and *bont/D-C* genes of BoNT-producing clostridia since no cross-reaction was observed with other BoNT-producing clostridia and other bacterial species. The identification of the *bont* type by the PCR assays correlated very well with the BoNT toxinotyping mouse bioassay. GD1 was able to detect *bont/C*, *bont/C-D*, *bont/D*, and *bont/D-C* genes but did not differentiate between nonmosaic and mosaic types. GD2 was designed to confirm the GD1 results and to differentiate between nonmosaic and mosaic *C. botulinum* subtypes. All results obtained with GD2 were concordant with the GD1 results, offering also the possibility of differentiation between nonmosaic and mosaic types. No cross-reaction was observed with other clostridia and other bacterial species that are frequently isolated from environmental samples. These results confirmed that both GeneDisc arrays GD1 and GD2 were suitable for the rapid and specific molecular characterization of BoNT/C- and BoNT/D-producing clostridial strains.

Data obtained with both GeneDisc arrays GD1 and GD2 showed 97.94% agreement with the real-time PCR tests used as references (25, 28). Since the GeneDisc arrays contained an internal amplification control, the discrepancies observed between the two methods were not attributed to PCR inhibition but were most probably due to uncertainty of detection in case of very low levels of contaminants. The C_T values of these discrepancies (between 36.49 and 38.18) strengthened this hypothesis. Moreover, the GeneDisc arrays GD1 and GD2 provided a double-detection system per target. Positive results were only valid when both targets were amplified, thus increasing the specificity of the system.

The LOD of the complete method, including enrichment and analysis by the GeneDisc arrays, was also evaluated by testing the recovery of known quantities of inoculated spores of *C. botulinum* types C, C-D, and D in spiked cecum samples. The LOD for artificially contaminated cecum samples was found to be <50 spores/g irrespective of the strains used for spiking. Positive re-

sults, although with high C_T values, were also reported for the samples inoculated with <1 spore/g. This can be explained by the fact that the concentrations were based on the MPN method, which counted only viable cells. However, smaller numbers of dead cells were probably also present in these samples.

In order to test the applicability of the GD1 C&D and GD2 C,D&mosaic arrays for the investigation of botulism outbreaks across Europe, 292 naturally contaminated samples from various origins (bovine, coot, coypus, dog, egret, environment, feed, gallus, goose, guinea fowl, heron, herring gull, horse, maggot, mallard, mink, moorhen, partridge, peewit gull, pheasant, pigeon, pochard, poultry, rabbit, rat, raw material, swan, turkey, and wild duck) were tested. Samples gathered from France, Italy, and the Netherlands were tested blindly with GD1 and GD2, and the data were compared to former published real-time PCR tests used as reference methods during the present study (25, 28). Of the 292 samples tested, 144 were determined to be positive, and only samples collected from horses, pigeons, and raw material were determined to be negative. The data have shown that D-C types were highly represented among the bovine isolates, whereas C-D types were highly represented among isolates from several avian species. Interestingly, all positive samples were recorded as mosaic types C-D or D-C irrespective of the nature of the samples and the regions where they were collected. Further investigations in other regions across Europe should be performed in order to consolidate the current data and study the prevalence of mosaic types C-D and D-C. It may be hypothesized that acquisition of mosaic types helps in survival or adaptation to a particular niche. This might result in mosaic types being found more frequently than usually expected. Despite considerable efforts to isolate strains from the positive samples, no strain could be isolated during the present study. Isolation of *C. botulinum* types C and D is really challenging, probably because the neurotoxin genes are mediated by bacteriophages and hence easily lost through laboratory experiments. In the absence of sequences of the entire toxin genes from strains derived from the samples investigated here, the hypothesis of clonal spreading of the mosaic gene types in Europe cannot be supported. However, our data support that mosaic gene types are common in Europe. The mosaic types are probably underdiagnosed since most of the PCR assays described in the literature failed to distinguish mosaic neurotoxin gene types from nonmosaic types C and D. These types might also possibly be missed due to incomplete serological toxinotyping in diagnostic laboratories.

In contrast to many other diagnostic tests, the results obtained with the GeneDisc arrays GD1 and GD2 allowed clear discrimination between nonmosaic and mosaic types. The results were generated without the need for specifically trained personnel, and the assays were performed in a very short time, providing data in only 2 days after receiving the sample. The total assay time included the enrichment step (48 h), the DNA extraction (30 min), and the PCR detection (<1 h). The method is not a substitute for a standard mouse bioassay, but it can be used to reduce the number of animal tests, shorten the time to result, and decrease the cost of analysis. The GeneDisc arrays GD1 and GD2 described here provide a reliable alternative detection tool for the routine diagnostics detection and molecular typing of the neurotoxin genes of *C. botulinum* types C and D and their mosaic C-D and D-C variants. Such GeneDisc arrays offer important tools to veterinary laboratories, allowing epidemiologists to better investigate the neurotoxin gene profiles (molecular toxinotype) of neurotoxin-produc-

ing clostridia associated with animal botulism across Europe. The data obtained here indicate that mosaic types C-D and D-C are predominant in the samples gathered in the regions investigated.

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Main results:

- Specific real time PCR detection assays were developed to detect and characterize *C. botulinum* group III type C, D, C/D and D/C; they were implemented into two GeneDisc arrays.
- The assays developed show 98% of concordance with previously published methods.
- 292 samples were tested, originating from animal botulism cases (mainly avian and bovine origin), from France, Italy and The Netherlands.
- 144 samples were positively detected for mosaic types C/D and D/C.
- Data shown that D/C types were highly represented among the bovine isolates, whereas C/D types were highly represented among isolates from several avian species.
- Some samples were detected for both type C/D and D/C.

Main conclusions:

- Our study show all positive samples recorded to be mosaic types C/D or D/C, irrespectively of the nature of the samples and the regions where they were collected.
- The samples dually detected for mosaic types indicate not only one *C. botulinum* group III strain could be responsible for a botulism outbreak.
- The GeneDisc arrays developed are commercially available and could be used as a routine method for diagnostic laboratory.

Perspectives:

The absence of detection of *C. botulinum* type C and D could mean they are currently less frequent, but it remains to be confirmed. The use of the GeneDisc arrays as a routine diagnostic method in veterinary laboratory would help in confirming this hypothesis. Further investigations in other regions across Europe should be performed in order to consolidate the current data and study the prevalence of mosaic types C/D and D/C.

V.2.2. Characterization of *C. botulinum* Group III strains using flagellin as genetic target.

As a result of the AnibioThreat project, *C. botulinum* Group III scientific interest increased. Dr. H. Skarin from the National Veterinary institute of Sweden (SVA) was in charge to investigate the genomic diversity of *C. botulinum* Group III strains responsible for animal botulism. Together with Dr. I. Anza from the Spanish Instituto de Investigación en Recursos Cinegéticos (IREC), they reported a potential clonal spread of *C. botulinum* mosaic type C/D through Europe using PFGE typing method (Anza et al., 2014a). Despite the high resolution of the PFGE method, in some cases it was not discriminatory enough to distinguish highly genetically related strains (Raphael et al., 2014).

Based on the previous work related to the study of *C. botulinum* Group I and II flagellin genes (Article N°1), I investigated the flagellin *fliC* gene as a potential molecular target to assess the genetic diversity of *C. botulinum* Group III. Flagellin genetic sequences of 68 *C. botulinum* isolated strains were investigated and clustered into five groups. Investigation of *fliC* types in 560 samples, with various European origins, showed that *fliC*-I was predominant and found exclusively in samples contaminated by *C. botulinum* type C/D. *fliC*-II was rarely detected, no sample was recorded to be positive for *fliC*-III or *fliC*-V, and only *C. botulinum* type D/C samples tested positive for *fliC*-IV. The lack of genetic diversity of the *C. botulinum* Group III flagellin gene compared to *C. botulinum* Groups I and II supports the clonal spread hypothesis.

The results of this study were published in 2015 in the scientific journal *Applied and Environmental Microbiology* (Article n°3).

Article n°3:

Woudstra C, Le Maréchal C, Souillard R, Bayon-Auboyer MH, Anniballi F, Auricchio B, De Medici D, Bano L, Koene M, Sansonetti MH, Desoutter D, Hansbauer EM, Dorner MB, Dorner BG, Fach P. Molecular gene profiling of *Clostridium botulinum* group III and its detection in naturally contaminated samples originating from various European countries. **Appl Environ Microbiol. 2015 Apr;81(7):2495-505.**

Article n°3

Molecular gene profiling of *Clostridium botulinum* group III and its detection in naturally contaminated samples originating from various European countries.

Woudstra C, Le Maréchal C, Souillard R, Bayon-Auboyer MH, Anniballi F, Auricchio B, De Medici D, Bano L, Koene M, Sansonetti MH, Desoutter D, Hansbauer EM, Dorner MB, Dorner BG, Fach P.

Applied and Environmental Microbiology

2015 Apr;81(7):2495-505

Molecular Gene Profiling of *Clostridium botulinum* Group III and Its Detection in Naturally Contaminated Samples Originating from Various European Countries

Cedric Woudstra,^a Caroline Le Maréchal,^{b,c} Rozenn Souillard,^{b,c} Marie-Hélène Bayon-Auboyer,^d Fabrizio Anniballi,^e Bruna Auricchio,^e Dario De Medici,^e Luca Bano,^f Miriam Koene,^g Marie-Hélène Sansonetti,^h Denise Desoutter,^h Eva-Maria Hansbauer,ⁱ Martin B. Dorner,^j Brigitte G. Dorner,^j Patrick Fach^a

Ansès (French Agency for Food, Environmental and Occupational Health and Safety), Food Safety Laboratory, Maisons-Alfort, France^a; Ansès, Ploufragan-Plouzané Laboratory, Hygiene and Quality of Poultry and Pig Products Unit, Ploufragan, France^b; Université Européenne de Bretagne, Rennes, France^c; LABOCEA, Ploufragan, France^d; Istituto Superiore di Sanità (ISS) Department of Veterinary Public Health and Food Safety, National Reference Centre for Botulism, Rome, Italy^e; Istituto Zooprofilattico Sperimentale delle Venezie (IZSve), Laboratorio di Treviso, Treviso, Italy^f; Central Veterinary Institute (CVI) of Wageningen, UR, Lelystad, The Netherlands^g; LNC, Veterinary Diagnostic Laboratory, Paita, New Caledonia^h; Robert Koch-Institut (RKI) Centre for Biological Threats and Special Pathogens, Biological Toxins, Consultant Laboratory for *Clostridium botulinum*, Berlin, Germanyⁱ

We report the development of real-time PCR assays for genotyping *Clostridium botulinum* group III targeting the newly defined *C. novyi sensu lato* group; the nontoxic nonhemagglutinin (NTNH)-encoding gene *ntnh*; the botulinum neurotoxin (BoNT)-encoding genes *bont/C*, *bont/C/D*, *bont/D*, and *bont/D/C*; and the flagellin (*fliC*) gene. The genetic diversity of *fliC* among *C. botulinum* group III strains resulted in the definition of five major subgroups named *fliC*-I to *fliC*-V. Investigation of *fliC* subtypes in 560 samples, with various European origins, showed that *fliC*-I was predominant and found exclusively in samples contaminated by *C. botulinum* type C/D, *fliC*-II was rarely detected, no sample was recorded as *fliC*-III or *fliC*-V, and only *C. botulinum* type D/C samples tested positive for *fliC*-IV. The lack of genetic diversity of the flagellin gene of *C. botulinum* type C/D would support a clonal spread of type C/D strains in different geographical areas. *fliC*-I to *fliC*-III are genetically related (87% to 92% sequence identity), whereas *fliC*-IV from *C. botulinum* type D/C is more genetically distant from the other *fliC* types (with only 50% sequence identity). These findings suggest *fliC*-I to *fliC*-III have evolved in a common environment and support a different genetic evolution for *fliC*-IV. A combination of the *C. novyi sensu lato*, *ntnh*, *bont*, and *fliC* PCR assays developed in this study allowed better characterization of *C. botulinum* group III and showed the group to be less genetically diverse than *C. botulinum* groups I and II, supporting a slow genetic evolution of the strains belonging to *C. botulinum* group III.

Botulism is a severe flaccid-paralytic disease that can affect both humans and animals. The disease symptoms are caused by botulinum neurotoxins (BoNTs), typically produced by *Clostridium botulinum*, a Gram-positive bacterium. The species *C. botulinum* can be divided into four groups (I to IV) based on physiological and genomic traits (1). *C. botulinum* group I encompasses proteolytic strains producing toxin serotype A, B, F, or H, whereas group II strains are nonproteolytic and produce toxin serotype B, E, or F. Other clostridial species can produce BoNT, i.e., *C. baratii* (serotype F), *C. butyricum* (serotype E), and *C. argentinense* (serotype G; formerly known as *C. botulinum* group IV). Groups I and II are associated with botulism in humans. In contrast, most cases of animal botulism are caused by group III *C. botulinum* strains that produce type C and D toxins or a chimeric fusion of C and D termed C/D or D/C toxin (2). Animal botulism is considered an emerging disease in Europe, notably in poultry production (3). Although physiological traits, biochemical tests, and toxin serotyping are still used to characterize *C. botulinum* strains, this information does not possess the discrimination required for source attribution and epidemiological investigations. To perform such analysis, it is essential to investigate the strains at the genetic level using methods such as randomly amplified polymorphic DNA (RAPD) analysis, amplified rRNA gene restriction analysis, pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), single-locus and multilocus sequence typing, multilocus variable-number tandem-repeat analysis (MLVA), or real-time PCR (4–8). Despite the large range of

technical methods available, *C. botulinum* group III, responsible for animal botulism, has not been as intensively studied as strains responsible for human botulism. However, this has recently started to change with the publication of scientific developments of great interest, such as the discovery and characterization of the mosaic toxin types C/D and D/C (2), development of molecular tools for rapid detection (9), and full-genome sequencing (10, 11), which gave the scientific community invaluable new insights. These developments revealed the genetic relatedness between *C. botulinum* group III, *C. novyi*, and *C. haemolyticum* to be so close that the new genospecies name *Clostridium novyi sensu lato* was proposed (10). The availability of whole-genome data provides genetic information to perform epidemiological investigations.

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Address correspondence to Patrick Fach, patrick.fach@anses.fr.

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To date, only a few studies have been published in regard to the epidemiological knowledge of animal botulism (3, 12), warranting further investigation of the topic. Flagellin gene detection assays have been used in molecular epidemiology for different *Clostridium* species (13). Previous studies have shown that considerable variation occurs between species in both the length and the sequence of the central region of the clostridial flagellin genes (14).

Our objective was to develop real-time PCR assays to investigate the genetic diversity of *C. botulinum* group III among a large number of strains and samples collected during animal botulism outbreaks from all over Europe. The assays developed encompass a *C. novyi sensu lato* detection system to correlate the sample tested with the *C. novyi sensu lato* group (*C. novyi*, *C. haemolyticum*, and *C. botulinum* group III), *bont/C*, *bont/C/D*, *bont/D*, and *bont/D/C* PCR typing of botulinum toxin genes; an *ntnh_grpIII* assay specifically targeting the nontoxic nonhemagglutinin (*ntnh*) gene present within the neurotoxin gene cluster of *C. botulinum* group III; and *fliC*-based PCR assays for genotyping the flagellin genes. The PCR-based assays developed in this study enable a rapid and deeper genetic characterization of *C. botulinum* group III strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The panel of *C. botulinum* group III strains ($n = 112$) investigated was composed of 12 *C. botulinum* type C (strain C-Stockholm as the *bont/C* reference), 73 mosaic type C/D (strain 08-BKT015925 as the *bont/C/D* reference), 5 type D (strain 16868 as the *bont/D* reference), and 22 mosaic type D/C (strain 16564 as the *bont/D/C* reference) strains (Table 1). In addition, a total of 91 BoNT-producing *Clostridium* strains (BoNT/A, BoNT/B, BoNT/Ab, BoNT/E, and BoNT/F) were tested as negative controls. All BoNT-producing *Clostridium* strains were toxinotyped using the reference mouse bioassay, as described previously (15). Twenty non-BoNT-producing strains of other clostridial species were used as negative controls: *C. butyricum*; *C. baratii*; *C. beijerinckii*; *C. bifermentans*; *C. chauvoei*; *C. difficile*; *C. mangenotii*; *C. novyi*; *C. haemolyticum*; *C. edematiens*; *C. perfringens* types A, C, D, and E; *C. septicum*; *C. sordellii*; *C. spirogenes*; *C. sporogenes*; *C. subterminale*; and *C. tetani*. Eighteen strains of other bacterial species were also analyzed as non-*Clostridium* negative controls: *Bacillus cereus*, *Bacillus thuringiensis*, *Citrobacter* sp., *Escherichia coli*, *Hafnia alvei*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Proteus* sp., *Pseudomonas* sp., *Salmonella enterica* serovar Virchow, *S. enterica* serovar Hadar, *S. enterica* serovar Enteritidis, *S. enterica* serovar Infantis, *S. enterica* serovar Typhimurium, *Shigella* sp., *Staphylococcus aureus*, *Streptococcus faecalis*, and *Yersinia enterocolitica*. Clostridial strains were grown overnight in Trypticase-peptone-glucose-yeast extract (TPGY) broth at 37°C under anaerobic conditions, in brain heart infusion medium (Difco, Paris, France), or in broth-fortified cooked-meat medium (16). Nonclostridial strains were grown in brain heart infusion medium at 37°C for 24 h.

Enrichment of group III *C. botulinum* from naturally contaminated samples. A total of 560 clinical (intestinal contents and organs) and environmental (insects, droppings, feces, feed, litter, manure, soil, farm swabs, udder, and water) samples with various origins (bovine, chicken, duck, goose, guinea fowl, mink, poultry, and turkey), collected across Europe during animal botulism events reported between 2006 and 2014, were investigated. These samples were previously PCR screened for *bont* genes (9). Of these, 427 samples were collected by the National Reference Laboratory for Avian Botulism and the LABOCEA laboratory (Brittany, France) between 2009 and 2013. Sixty-four samples were collected by the Istituto Superiore di Sanita (Rome, Italy) and by the Istituto Zooprofilattico Sperimentale delle Venezie (Treviso, Italy) during botulism outbreaks recorded from 2006 to 2010. In addition, 31 samples were collected in 2011 by the Central Veterinary Institute (Lelystad, The Netherlands),

30 samples were collected in 2013 and 2014 by the National Laboratory of New Caledonia, and 8 samples were collected by the Robert Koch-Institut (RKI) (Berlin, Germany). Each sample (1 g) was 10-fold (wt/vol) diluted in prerduced TPGY medium and incubated for 48 h at 37°C under anaerobic conditions. After incubation, a 1-ml aliquot of the enrichment broth was collected and centrifuged at $9,000 \times g$ for 5 min. The supernatant was discarded, and the cell pellet was subjected to DNA extraction.

Genomic DNA isolation. DNA was extracted using one of the following protocols according to the manufacturer's instructions for Gram-positive bacteria: phenol-chloroform extraction (17), the DNeasy blood and tissue kit (Qiagen, Hilden, Germany), the QIAamp DNA minikit (Qiagen, Hilden, Germany), or Chelex 100 (Bio-Rad Life Science Research, Hercules, CA). DNA samples were stored at -20°C until high-throughput real-time PCR analysis.

Sequencing. Double-stranded-DNA sequencing of the flagellin genes was performed according to a previously published method (4) by Eurofins MWG Operon (Courtaboeuf, France). The primers used for flagellin gene sequencing were as follows: forward, ATGATTATTAATCACAATA TGAACGC, and reverse, TTAATAATTGAAGTACACCTTGTGG. The NTNH-encoding gene was amplified and sequenced as previously described (18).

fliC protein analysis. Alignment of the translated consensus *fliC*-I to *fliC*-V protein sequences (Fig. 1) was obtained using CLC Genomics Workbench software version 6.6.2 (Qiagen, Aarhus, Denmark).

High-throughput real-time PCR. The LightCycler 1536 (Roche, Meylan, France) was used to perform high-throughput real-time PCR amplifications as previously described (14). Briefly, the PCR was performed in a 2- μl final volume. Primers and TaqMan probes (6-carboxy-fluorescein [FAM] and 6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein succinimidyl ester [HEX] labeled; listed in Table 2) were used at 300 nM final concentration for the PCR. The following thermal profile was used: 95°C for 1 min, followed by 40 cycles of 95°C for 1 s, 50°C for 15 s, and 55°C for 30 s. The oligonucleotides used for determining the flagellin type were derived from the aligned flagellin gene sequences (for accession numbers, see "Nucleotide sequence accession numbers" below). The *ntnh_grpIII* PCR assay targeting the NTNH-encoding gene was used as a control to detect *C. botulinum* group III strains. *C. botulinum* *bont* type C, C/D, D, and D/C PCR assays were described previously (9) and were used as *C. botulinum* group III positive controls. DNA from *C. botulinum* type B (strain ISS-244), together with previously published primers and probe (19) targeting the *bont/B* gene, was used as an internal control to check for PCR inhibition. All primers and probes developed in this study were designed using Beacon Designer Software (version 7.91) and purchased from Eurofins MWG Operon (Courtaboeuf, France).

Validation study. The validation study was carried out using a panel of 72 *C. botulinum* group III (Table 1), 91 *C. botulinum* group I and group II, 20 non-BoNT-producing *Clostridium*, and 18 non-*Clostridium* strains (see "Bacterial strains and growth conditions" above). As reference strains, C-Stockholm (for *bont/C* and *fliC*-II), 08-BKT015925 (for *bont/C/D* and *fliC*-I), Eklund (for *fliC*-III), 1873 (for *bont/D* and *fliC*-II), 1585-19-11 (for *bont/D/C* and *fliC*-IV), and RKI-7 (for *fliC*-V) were chosen. According to the ISO 16140:2011 norm, the following performance parameters were evaluated: selectivity (inclusivity and exclusivity), linearity, limit of detection, relative sensitivity (SE), relative specificity (SP), and relative accuracy (AC). Linearity and limit of detection testing was performed with three replicates of each serial dilution of DNA extracted from the reference strains. The amplification efficiencies (E) for the different targets were calculated according to the following equation: $E = 10^{(-1/\text{slope})} - 1$.

Statistical analysis. The frequencies of *fliC*-I to *fliC*-V genes were calculated in *bont*-positive samples (see Table 5). The frequencies of all genetic markers were calculated according to sample origins (see Table 6). Fisher's test was used for calculation. As a significance level, α was set to 0.05. All P values less than or equal to α were considered statistically significant.

TABLE 1 *C. botulinum* group III strains

Strain	Origin	Assay result					GenBank accession no. ^f	
		SMB ^a	<i>bont</i> type ^{b,g}	<i>C. novyi</i> <i>sensu lato</i> ^{c,g}	Ntnh group III ^{d,g}	<i>fliC</i> type ^{e,g}	Flagellin	NTNH
Reference strains								
Stockholm	Mink	C	C	+	+	II	KM496352	AP008983
08BKT015925	Poultry	C	C/D	+	+	I	NC_015425	CP002411
Eklund	Unknown	C	C/D	+	+	III	ABDQ00000000.1	ABDQ00000000.1
1873	Unknown	D	D	+	+	II	NZ_ACSJ01000007	AB012112
D-4947	Unknown	D	D	+	+	V	KM496392	
1585/19/11	Milch cow ruminal content	D	D/C	+	+	IV	KM496395	KM496453
Group III <i>Clostridium botulinum</i> strains used for validation study								
Bal64	Unknown	C	C	+	+	II	KM496350	KM496418
4165	Unknown	C	C	+	+	II	KM496345	KM496412
1663	Unknown	C	C	+	+	II	KM496344	KM496411
850131	Unknown	C	C	+	+	II	KM496348	KM496416
468	Horse	C	C	+	+	II	KM496343	X72793
CKIII	Unknown	C	C	+	+	II	KM496351	KM496419
15586	Unknown	C	C	+	+	II	KM496346	KM496414
870505	Unknown	C	C	+	+	II	KM496349	KM496417
75965	Unknown	C	C	+	+	II	KM496347	KM496415
RKI-1	Unknown	C	C	+	+	II	KM496353	
RKI-2	Unknown	C	C	+	+	II	KM496354	
V891	Unknown	C	C/D	+	+	I	AESC01000016	
NRCB1	Duck liver	C	C/D	+	+	I	KM496380	KM496445
NRCB2	Duck intestinal tract	C	C/D	+	+	I	KM496381	KM496446
NRCB3	Seagull liver	C	C/D	+	+	I	KM496382	KM496447
92962XIV	Unknown	C	C/D	+	+	I	KM496376	KM496441
80671III	Unknown	C	C/D	+	+	I	KM496374	KM496440
481290	Unknown	C	C/D	+	+	I	KM496378	KM496443
13451II	Unknown	C	C/D	+	+	I	KM496367	KM496435
4622	Unknown	C	C/D	+	+	I	KM496360	KM496427
81290	Unknown	C	C/D	+	+	I	KM496375	
31354	Unknown	C	C/D	+	+	I	KM496369	KM496436
32150	Unknown	C	C/D	+	+	I	KM496370	KM496437
24992-1	Unknown	C	C/D	+	+	I	KM496368	
32670	Unknown	C	C/D	+	+	I	KM496371	KM496438
97371	Mink feed	C	C/D	+	+	I	KM496377	KM496442
37393	Unknown	C	C/D	+	+	I	KM496372	
2286-3	Fox	C	C/D	+	+	I	KM496358	KM496424
38997	Unknown	C	C/D	+	+	I	KM496373	KM496439
12792	Chicken	C	C/D	+	+	I	KM496366	KM496434
621125	Chicken intestine	C	C/D	+	+	I	KM496379	KM496444
09/03/5231	Duck intestine	C	C/D	+	+	I	KM496355	
4456/11	Duck intestine	C	C/D	+	+	I	KM496359	KM496426
5391/09	Duck intestine	C	C/D	+	+	I	KM496363	KM496430
5017/2/11	Duck intestine	C	C/D	+	+	I	KM496362	KM496429
4732/2/10	Duck intestine	C	C/D	+	+	I	KM496361	KM496428
12LNR1	Duck	+	C/D	+	+	I	KM496356	KM496421
5674/10	Duck intestine	C	C/D	+	+	I	KM496364	
6136/A/12	Gull intestine	C	C/D	+	+	I	KM496365	KM496432
12LNR10	Turkey spleen	+	C/D	+	+	I	KM496357	KM496422
RKI-3	Unknown	C/D	C/D	+	+	I	KM496383	
RKI-4	Unknown	C/D	C/D	+	+	I	KM496384	
RKI-5	Unknown	C/D	C/D	+	+	I	KM496385	
RKI-6	Unknown	C/D	C/D	+	+	I	KM496386	
RKI-16	Unknown	C/D	C/D	+	+	I	KM496387	
RKI-20	Unknown	C/D	C/D	+	+	I	KM496388	
RKI-12	Unknown	C/D	-	+	-	I	KM496406	
RKI-13	Unknown	C/D	-	+	-	I	KM496407	

(Continued on following page)

TABLE 1 (Continued)

Strain	Origin	Assay result					GenBank accession no. ^f	
		SMB ^d	<i>bont</i> type ^{b,g}	<i>C. novyi</i> <i>sensu lato</i> ^{c,g}	Ntnh group III ^{d,g}	<i>fliC</i> type ^{e,g}	Flagellin	NTNH
RKI-18	Unknown	C/D	–	+	–	I	KM496408	
RKI-21	Unknown	C/D	–	+	–	I	KM496409	
RKI-23	Unknown	C/D	–	+	–	I	KM496410	
2639-2	Unknown	D	D	+	+	I	KM496389	KM496448
16868	Unknown	D	D	+	+	I	KM496390	KM496450
16983	Unknown	D	D	+	+	I	KM496391	
16564	Unknown	D	D/C	+	+	IV	KM496399	KM496449
564424	Cow intestinal content	D	D/C	+	+	IV	KM496402	KM496459
86469	Vaccine strain	D	D/C	+	+	IV	KM496401	KM496458
18128	Unknown	D	D/C	+	+	IV	KM496400	KM496457
4456/11	Milch cow feces	D	D/C	+	+	IV	KM496397	KM496455
1585/18/11	Milch cow ruminal content	D	D/C	+	+	IV	KM496394	KM496452
SP28	Milch cow intestine	D	D/C	+	+	IV	KM496405	KM496460
360	Milch cow abomasum	D	D/C	+	+	IV	KM496393	KM496451
3859/5/11	Milch cow intestine	D	D/C	+	+	IV	KM496396	KM496454
7574/5/12	Milch cow intestine	D	D/C	+	+	IV	KM496398	KM496456
RKI-8	Unknown	D/C	D/C	+	+	IV	KM496403	
RKI-9	Unknown	D/C	D/C	+	+	IV	KM496404	
Additional group III <i>Clostridium</i>								
<i>botulinum</i>								
10/02/5009	Duck intestine	C	C/D	+	+	I		KM496420
13LNR1	Turkey cloacal swab	+	C/D	+	+	I		KM496423
2659/1/10	Chicken intestine	C	C/D	+	+	I		KM496425
OFD05	Bovine ruminal content	D	D/C	+	+	IV		AB745668
7296	Unknown	D	D/C	+	+	IV		
7357	Unknown	D	D/C	+	+	IV		
Gun	Unknown	D	D/C	+	+	IV		
12LNR8	Swan intestine	+	C/D	+	+	I		
12LNR13	Chicken feces	+	C/D	+	+	I		
ISS Animal 2A	Unknown	C	C/D	+	+	I		
ISS Animal 2B	Unknown	C	C/D	+	+	I		
LDA22 29401	Chicken	+	C/D	+	+	I		
LDA22 30607	Chicken feces	+	C/D	+	+	I		
LDA22 276	Chicken	+	C/D	+	+	I		
LDA22 38028	Chicken	+	C/D	+	+	I		
LDA22 72870	Chicken	+	C/D	+	+	I		
LDA22 48212	Duck feces	+	C/D	+	+	I		
LDA22 29401	Chicken	+	C/D	+	+	I		
LDA22 49511	Chicken feces	+	C/D	+	+	I		
LDA22 48751	Chicken	+	C/D	+	+	I		
LDA22 58752	Chicken	+	C/D	+	+	I		
LDA22 69285	Chicken	+	C/D	+	+	I		
LDA22 71840	Chicken feces	+	C/D	+	+	I		
LDA22 52859	Chicken	+	C/D	+	+	I		
LDA22 69285	Chicken	+	C/D	+	+	I		
LDA22 71840	Chicken feces	+	C/D	+	+	I		
LDA22 52859	Chicken	+	C/D	+	+	I		
LDA22 69285	Chicken	+	C/D	+	+	I		
LDA22 43243	Guinea fowl feces	+	C/D	+	+	I		
LDA22 55741	Turkey	+	C/D	+	+	I		
LDA22 60979	Duck feces	+	C/D	+	+	I		
LDA22 50867	Chicken	+	C/D	+	+	I		
LDA22 52859	Chicken	+	C/D	+	+	I		
LDA22 65304	Duck feces	+	C/D	+	+	I		
NCTC 8265	Unknown	D	D/C	+	+	IV		
96564	Cow	D	D/C	+	+	IV		
LDA22 47295	Bovine feces	+	D/C	+	+	IV		

(Continued on following page)

TABLE 1 (Continued)

Strain	Origin	Assay result					GenBank accession no. ^f	
		SMB ^a	<i>bont</i> type ^{b,g}	<i>C. novyi sensu lato</i> ^{c,g}	Ntnh group III ^{d,g}	<i>fliC</i> type ^{e,g}	Flagellin	NTNH
LDA22 51714	Bovine feces	+	D/C	+	+	IV		
Eppendorf	Unknown	D/C	D/C	+	+	–	KP452499	

^a SMB, standard mouse bioassay; serotypes are shown; + indicates a positive mouse lethal test without serotyping results.

^b Differentiation of *bont*/C, *bont*/C/D, *bont*/D, and *bont*/D/C genes was determined by real-time PCR assay according to the method of Woudstra et al. (9).

^c The *C. novyi sensu lato* assay targets the conserved chromosomal gene of the *C. novyi sensu lato* group 50S ribosomal protein L10.

^d The *ntnh_grpIII* assay is specific for the *ntnh* gene of *C. botulinum* group III.

^e *fliC* flagellin characterization was obtained using the *fliC*-I to *fliC*-V assays developed in this study.

^f The GenBank accession numbers for strains determined in this study begin with KM.

^g –, negative result; +, positive result.

Nucleotide sequence accession numbers. The sequences of the *C. botulinum fliC* and *ntnh* genes were deposited in GenBank with accession numbers KM496343 to KM496410, KP452499, and KP452500 for the flagellin genes and KM496411 to KM496460 for the *ntnh* genes.

RESULTS

***C. botulinum* group III flagellin gene sequences.** *Clostridium* species are known to harbor flagellin gene sequences with high genetic diversity, and *C. botulinum* group III may be characterized by at least three different flagellin genes (807, 816 to 819, and 834 bp long, respectively) based on the sequences available in GenBank (20). The 816- to 819-bp sequence has been determined to be more closely related to *C. botulinum* group I and II flagellins FlaA1/A2. Therefore, we investigated the 816- to 819-bp flagellin gene sequences from *C. botulinum* type C/D (strains BKT015925, BKT028387, V-891, Sp77, and Eklund), type C (strain Stockholm), type D (strains 1873 and 16868), and type D/C (strains It1 and DC5) obtained from GenBank and PATRIC (21). Sequencing primers targeting the 5'- and 3'-end sequences of the 816- to 819-bp flagellin gene (here referred to as *fliC*) were used to successfully sequence the *fliC* genes of 12 *C. botulinum* type C, 39 type C/D, 4 type D, and 13 type D/C strains (for GenBank accession numbers, see "Nucleotide sequence accession numbers" above). The presence of potential multiple copies of *fliC* was investigated as previously described (13). We observed that of the 68 strains tested, half contained tandem copies of *fliC*. The genetic diversity of the flagellin gene sequences among *C. botulinum* group III strains investigated in the present study is shown in Table 3. The *fliC* gene sequences were clustered into four subgroups named *fliC*-I to *fliC*-IV. *fliC*-I was present in all *C. botulinum* type C/D strains investigated and three type D strains (strains 2639-2, 16868, and 16983), *fliC*-II is linked to *C. botulinum* type C and type D strain 1873, *fliC*-III is related to *C. botulinum* type C/D strain Eklund, and *fliC*-IV is specific to *C. botulinum* type D/C. The *fliC* gene sequences obtained for *C. botulinum* type D/C were longer than (1,239 bp) and significantly different from those of *C. botulinum* type C, C/D, or D.

Development of PCR assays for molecular characterization of *C. botulinum* group III reference strains. The 50S ribosomal protein L10 PCR assay previously developed (22) was modified to be used with TaqMan chemistry and used as the *C. novyi sensu lato* positive control, amplifying a conserved chromosomal target. Affiliation with *C. botulinum* group III was confirmed using both *ntnh_grpIII* and *bont* assays, providing a double detection system that targets the toxin-related phage (9, 18). To specifically detect the *C. botulinum* group III *ntnh* gene, a specific real-time PCR

assay was designed based on the sequenced *ntnh* genes of 48 strains (8 type C, 27 type C/D, 2 type D, and 11 type D/C). Alignment of *ntnh* sequences (for accession numbers, see "Nucleotide sequence accession numbers" above) was performed using SIAS sequence identity and similarity software (<http://imed.med.ucm.es/Tools/sias.html>). The results confirmed previously published findings (18) and revealed sequence similarities ranging from 96.96% to 99.97% (data not shown), showing that *ntnh* in *C. botulinum* group III is highly conserved.

Based on *fliC* sequencing results, four assays targeting the identified flagellin gene subgroups *fliC*-I to *fliC*-IV were developed. *fliC* typing was concordant with the sequencing results: all *C. botulinum* type C and *C. botulinum* type D-1873 strains were found positive for *fliC*-II. All *C. botulinum* type C/D and three *C. botulinum* type D strains were found positive for *fliC*-I. All *C. botulinum* type D/C strains were found positive for *fliC*-IV. Only two isolates of types D (strain D-4947) and D/C (strain Eppendorf) could not be typed. The *fliC* gene from strain D-4947 was sequenced and was assigned as a new flagellin type (called *fliC*-V), related to *fliC*-II with 72.67% sequence similarity (data not shown). The *fliC* sequence of strain Eppendorf (accession no. KP452499 and KP452500) was related to the *fliC*-II type with 93% sequence identity and thus was not assimilated to a new type. We were not able to assign it as *fliC*-II because of several mutations that impacted hybridization of the *fliC*-II probe.

Comparison of *fliC*-I to *fliC*-V translated protein sequences using CLC software (Fig. 1) showed that the N- and C-terminal parts are well conserved, unlike the internal region, as previously described (4). Additionally, five strains isolated from animal botulism outbreaks (RKI-12, RKI-13, RKI-18, RKI-21, and RKI-23) were negative in *ntnh_grpIII* and *bont* PCR assays but were successfully identified as type *fliC*-I. The performance criteria of each PCR assay were determined and are reported in Table 4. The efficiencies of the PCR assays ranged from 81% to 118%. The limit of detection of each assay was assessed using reference strains and showed sensitivities from 3 to 30 genome equivalents in the PCR (Table 4). All PCR assays were evaluated for their specificity on *C. botulinum* strains (consisting of types A, B, Ab, E, F, and G), non-*C. botulinum* clostridia, and non-*Clostridium* bacteria. The strains all tested negative, except for the *C. novyi sensu lato* PCR assay, which displayed the expected positive results on *C. novyi* (strains ATCC 7659 and CIP 60.44) and *C. haemolyticum* (strains NCTC 9693 and CIP 60.15). The results of selectivity, AC, SE, and SP studies showed 100% inclusivity, 100% exclusivity, 100% AC, 100% SE, and 100% SP.

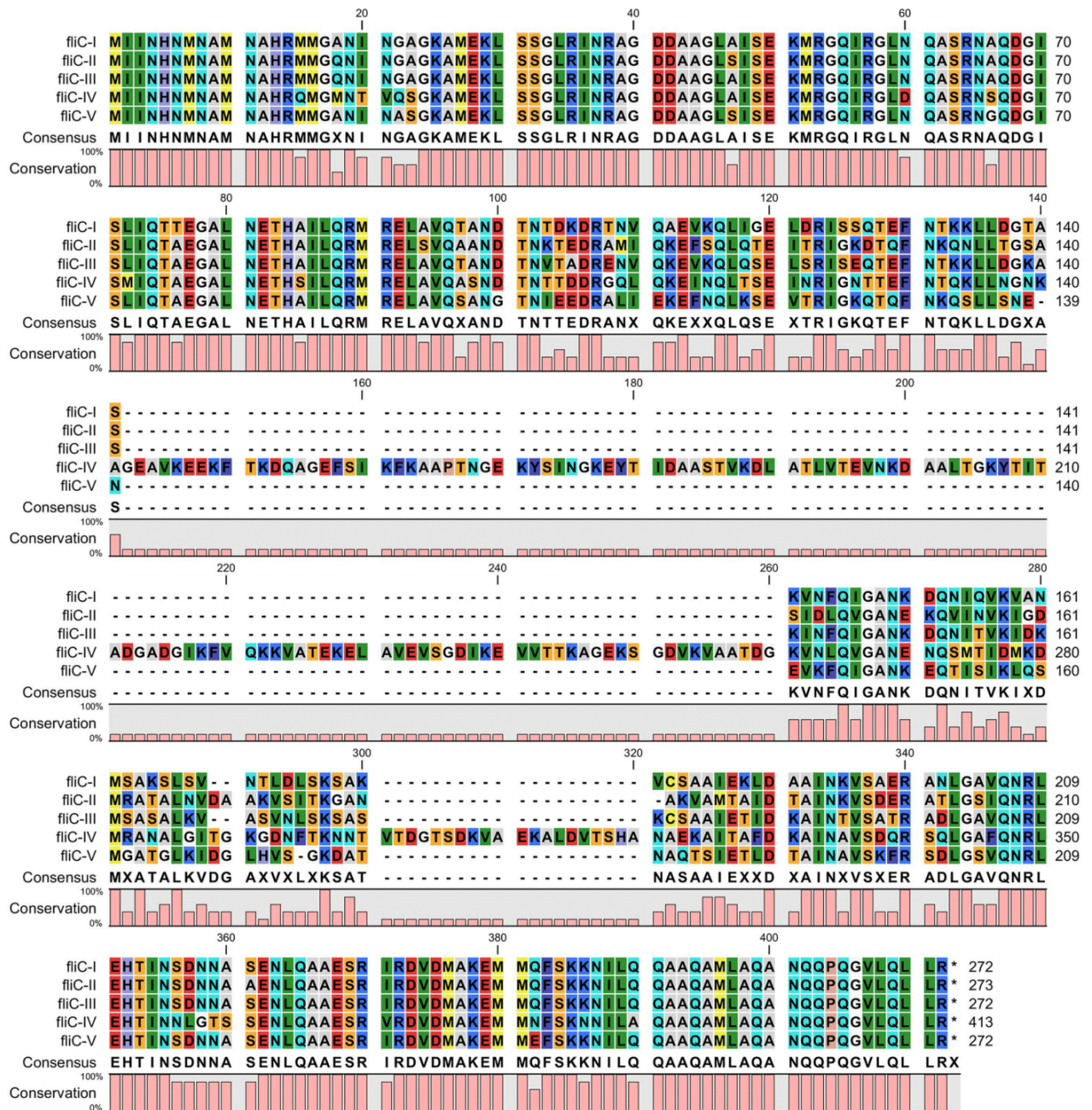


FIG 1 Alignment of *C. botulinum* group III flagellin amino acid sequences. The *fliC* protein sequence comparison was made using consensus translated sequences for each flagellin type using CLC Genomics Workbench software version 6.6.2.

PCR screening of genetic markers associated with *C. botulinum* group III in naturally contaminated samples originating from European countries. A collection of 560 samples from outbreaks of animal botulism from France ($n = 427$), Italy ($n = 64$), The Netherlands ($n = 31$), New Caledonia ($n = 30$), and Germany ($n = 8$) were characterized using high-throughput real-time PCR targeting the *C. novyi sensu lato* group, *ntnh_grpIII* and *bont* genes related to the toxin phage, and *fliC* flagellin types of *C. botulinum*

group III. As an internal amplification control (IAC) to check for PCR inhibition, 1 pg of DNA from strain *C. botulinum* type B (ISS-244) was inoculated into each sample, and a detection assay was performed as previously described (19). The results are summarized in Tables 5 and 6. All samples tested positive for the IAC, with an average cycle threshold value of 30.58 and a standard deviation of 0.73, confirming the absence of PCR inhibitors. The *C. novyi sensu lato* assay provided 361 positive hits. Among these,

TABLE 2 Primers and probes

Primer or probe ^a	Sequence 5'→3' ^b	Amplicon size (bp)
<i>fliC</i> -I_F	ACATGAGTGCTAAATCTTTATCAG	122
<i>fliC</i> -I_R	GTTAGCTCTTTCTGCTGATAC	
<i>fliC</i> -I_P	TTGCTGCTGAACAAACCTTAGCACTCT	
<i>fliC</i> -II_F	ATCAGCTTCAAGCATAGAC	111
<i>fliC</i> -II_R	CTAACTTTAGCTGCATCAAC	
<i>fliC</i> -II_P	AAGCAGTGGCTCTCATATCACCAATT	
<i>fliC</i> -III_F	AGCAAACAAAGATCAAAACATCAC	106
<i>fliC</i> -III_R	CTGCTGAACATTTACTCGCAC	
<i>fliC</i> -III_P	ACAGATGCTACCTTTAATGCTGAAGCACT	
<i>fliC</i> -IV_F	AAAGATGCTGCTTTAACTGG	117
<i>fliC</i> -IV_R	ATCACCCTAACTTCTACTGC	
<i>fliC</i> -IV_P	TAATTCCATCTGCACCATCAGCTGTTAT	
<i>fliC</i> -V_F	AATCAATGGGCGCAACAGGA	141
<i>fliC</i> -V_R	GAACTGATCCATAATCTGATCTG	
<i>fliC</i> -V_P	TGAGCATTGTTGCATCTTTACCAGAAACA	
Novyi-sensu-lato_F	GGAACCAACCTACCGAG	100
Novyi-sensu-lato_R	GTAGCCACCTCCTTAACA	
Novyi-sensu-lato_P	TCCAGACACGAAGAGGCTTAATATATCCA	
<i>ntnh</i> -grpIII_F	TACTGATTATTTAGACCTGATTG	122
<i>ntnh</i> -grpIII_R	CTTTAGGCACCTTGTATTG	
<i>ntnh</i> -grpIII_P	TCATCCAATTATAATCTCCATCTCTAAGTGA	

^a F, forward (primer); R, reverse (primer); P, probe.

^b All probes were labeled with 6-HEX or 6-FAM and BHQ1 (Black Hole Quencher).

158 were detected as positive in *ntnh*_grpIII and *bont* assays, and 172 were flagellin typed as *fliC*-I ($n = 140$), *fliC*-II ($n = 6$), or *fliC*-IV ($n = 26$). Eleven samples showed a dual-positive signal for either *fliC*-I and *fliC*-II (4 *C. botulinum* type C/D samples) or *fliC*-I and *fliC*-IV (5 *C. botulinum* type C/D and 2 type C/D and D/C samples). Nine samples detected as positive for the *C. novyi sensu lato* group and subtyped for their flagellin genes (7 *fliC*-I and 2 *fliC*-IV) were negative in the *ntnh*_grpIII or *bont* assay. Seven samples (*C. botulinum* type D/C) were detected as positive for the *C. novyi sensu lato* group and the *ntnh*_grpIII and *bont* assays; however, their flagellin gene types were not determined. No samples tested were determined to be type *fliC*-III or *fliC*-V. Overall, the data showed that samples positive for *bont*/C/D were highly associated with *fliC*-I (100%; confidence interval [CI], 97% to 100%) whereas those positive for *bont*/D/C are mainly associated with *fliC*-IV (55%; CI, 38% to 71%) and to a lesser extent with *fliC*-I (19%; CI, 9% to 36%) (Table 5). The frequencies of the genetic markers per sample origin showed that the *C. novyi sensu lato* genetic marker dominates in all the samples tested. The genetic markers *bont*/C/D and *fliC*-I are predominant in poultry, chicken, duck, turkey, goose, and guinea fowl. In bovines, the genetic markers *bont*/D/C dominate, together with *fliC*-I and *fliC*-

IV. Mink samples were shown to contain both *bont*/C/D and *bont*/D/C, together with *fliC*-I and *fliC*-IV genetic markers (Table 6).

DISCUSSION

Flagellin genes and their products have been used as genotypic and phenotypic typing methods for foodborne pathogens (23). The flagellar glycosylation island represents one of the major areas of divergence in the *Clostridium* genome (24), reflecting the genomic diversity. To date, the flagellar genetics of *Clostridium* species have not been investigated as extensively as those of Gram-negative bacteria. Nevertheless, some studies have referred to the flagellin genetic diversity of *Clostridium* species: Sasaki et al. (13) described a multiplex PCR assay for the rapid identification of the pathogenic clostridia *C. chauvoei*, *C. haemolyticum*, *C. novyi* types A and B, and *C. septicum*. Paul et al. (4) reported the first study showing the genetic diversity of the flagellin genes of *C. botulinum* strains belonging to groups I and II. In a previous study (14), multiple real-time PCR assays were developed to assess the genetic diversity of *C. botulinum* groups I and II based on the flagellin gene variable sequences. While *C. botulinum* groups I and II are well studied, little is known about group III. In the present study, we aimed to develop real-time PCR genotyping assays for discriminating *C. botulinum* group III strains based on their flagellin genetic diversity. Another objective was to analyze with these PCR assays a large number of samples from outbreaks of animal botulism, primarily from France and a few other European sites.

We report here the sequence of the *fliC* flagellin genes from 68 strains of *C. botulinum* group III types C, C/D, D, and D/C. The *fliC* genes clustered into four genetically different groups identified as *fliC*-I to *fliC*-IV. Based on the *fliC* gene sequences determined in this study, specific real-time PCR assays targeting each *C. botulinum* group III flagellin type were designed. After sequencing the *ntnh* genes from 48 *C. botulinum* group III strains, an

TABLE 3 *C. botulinum* group III *fliC* identities

<i>fliC</i> gene	Identity (%) with ^a :			
	<i>fliC</i> -I	<i>fliC</i> -II	<i>fliC</i> -III	<i>fliC</i> -IV
<i>fliC</i> -I	100			
<i>fliC</i> -II	87.27	100		
<i>fliC</i> -III	92.51	88.16	100	
<i>fliC</i> -IV	50.88	50.96	50.24	100

^a *fliC* sequence comparison were made using flagellin consensus sequences for each type and using SIAS sequence identity and similarity software (<http://imed.med.ucm.es/Tools/sias.html>).

TABLE 4 Performance criteria of PCR assays

PCR assay	Criterion	Value for strain (<i>bont</i> type)					
		Stockholm (C)	BKT015925 (C/D)	Eklund (C/D)	1873 (D)	D-4947 (D)	1585-19-11 (D/C)
<i>C. novyi sensu lato</i>	Efficiency (%) ^a	94	97	96	103	81	87
	R ^{2b}	0.9935	0.9973	0.9942	0.9937	0.9936	0.9972
	Slope ^c	-3.47	-3.40	-3.42	-3.25	-3.88	-3.68
	LOD (fg) ^d	10	100	100	20	100	10
	Genome eq. ^e	3	30	30	6	30	3
<i>ntnh_grpIII</i>	Efficiency (%)	116	106	104	118	99	106
	R ²	0.9962	0.9953	0.9962	0.9964	1.0000	0.9957
	Slope	-2.99	-3.19	-3.23	-2.95	-3.35	-3.19
	LOD (fg)	100	100	100	20	100	10
	Genome eq.	30	30	30	6	30	3
<i>fliC-I</i>	Efficiency (%)		115				
	R ²		0.9998				
	Slope		-3.01				
	LOD (fg)		100				
	Genome eq.		30				
<i>fliC-II</i>	Efficiency (%)	90			103		
	R ²	0.9780			0.9958		
	Slope	-3.59			-3.25		
	LOD (fg)	10			20		
	Genome eq.	3			6		
<i>fliC-III</i>	Efficiency (%)			92			
	R ²			0.9885			
	Slope			-3.53			
	LOD (fg)			100			
	Genome eq.			30			
<i>fliC-IV</i>	Efficiency (%)						107
	R ²						0.9938
	Slope						-3.16
	LOD (fg)						10
	Genome eq.						3
<i>fliC-V</i>	Efficiency (%)					98	
	R ²					0.9952	
	Slope					-3.37	
	LOD (fg)					20	
	Genome eq.					6	

^a Efficiency was calculated based on the following formula: $E = -1 + 10^{-1/\text{slope}}$.

^b R², regression correlation coefficient.

^c The slope of the linearity curve was calculated based on log dilution plotted against their corresponding threshold cycles (C_T).

^d LOD, limit of detection.

^e Genome equivalents (eq.) were calculated according to each genome size on the assumption that the average mass of a base pair is 650 Da, using the website <http://cels.uri.edu/gsc/cndna.html>.

ntnh_grpIII real-time PCR assay was developed and used in combination with the *bont*-typing assays for *C. botulinum* group III (9) as a *C. botulinum* group III toxin phage control. The assays were evaluated using a panel of 112 *C. botulinum* group III strains, 91 *C. botulinum* group I and II strains, 20 other *Clostridium* species, and 18 non-*Clostridium* species. The results showed the *C. novyi sensu lato* assay to be specific for *C. novyi*, *C. haemolyticum*, and *C. botulinum* group III, in accordance with previously published data (22). The *ntnh_grpIII* and *fliC-I* to *fliC-IV* assays were found to be specific for *C. botulinum* group III only. Of the strains investigated, two strains (D-4947 type D and Eppendorf type D/C, the latter originating from Germany) were not typeable based on the

flagellin type. The *fliC* gene for the type D strain D-4947 was sequenced and identified as a new flagellin type, named in this work *fliC-V*, and as genetically close to *fliC-II*. The *fliC* gene of strain Eppendorf was retrieved by whole-genome sequencing (unpublished data) and showed a DNA sequence affiliation with the *fliC-II* flagellin type. The flagellin genes of five strains isolated from animal botulism outbreaks (RKI-12, RKI-13, RKI-18, RKI-21, and RKI-23) and testing positive in the *C. novyi sensu lato* assay and negative in the *ntnh_grpIII* and *bont* PCR assays were sequenced. The flagellin sequences of these strains were identical to those of *fliC-I* *C. botulinum* type C/D strains. These strains have probably lost their toxin phages during cultivation, making them

TABLE 5 Distribution of *fliC*-I to *fliC*-V genes in *bont*-positive samples

<i>bont</i> type	No. of positive samples	% gene determinant presence (95% CI)				
		<i>fliC</i> -I	<i>fliC</i> -II	<i>fliC</i> -III	<i>fliC</i> -IV	<i>fliC</i> -V
C	1	50 (2–100)	0 (0–79)	0 (0–79)	0 (0–79)	0 (0–79)
C/D	123	100 (97–100)	3 (1–8)	0 (0–3)	4 (2–9)	0 (0–3)
D/C	31	19 (9–36)	6 (2–21)	0 (0–11)	55 (38–71)	0 (0–11)
C/D and D/C	3	100 (42–100)	0 (0–56)	0 (0–56)	67 (21–94)	0 (0–56)

undetectable by the *ntnh_grpIII* and *bont* assays, but they still test positive in the chromosome-based flagellin PCR assay. The flagellin gene from *C. botulinum* type D/C (*fliC*-IV) was shown to be significantly different from those of other group III strains, with only 50% sequence identity to the other *fliC* types. It is longer (1,239 bp), with a different internal variable region that does not match any available sequences using BLASTn software and the nr/nt nucleotide collection database. By comparing the translated protein sequence software to the nr protein collection database with BLASTx, a match with the *C. haemolyticum* NCTC 9693 genome up to 82% identity and 86.76% similarity was identified, reinforcing the affiliation of *C. botulinum* type D/C with the newly defined *Clostridium novyi sensu lato* group.

The PCR assays developed in this study were tested on 560 samples from botulism outbreaks across Europe (mainly France). Of the samples tested, 361 were positive for the *C. novyi sensu lato* assay, showing a high prevalence (64%) of *C. novyi*, *C. haemolyticum*, or *C. botulinum* group III in the samples investigated during animal botulism outbreaks. *C. botulinum* group III was confirmed using *ntnh_grpIII* and *bont* PCR assays in 158 samples (28%) that tested positive for *C. novyi sensu lato*. Among these, 151 samples were positively *fliC* typed: 140 samples were identified as *C. botulinum* type C/D and were all positive for *fliC*-I. Of the 140, 5 were also identified as *fliC*-II and seven as *fliC*-IV. Three samples were identified as both *C. botulinum* type C/D and type D/C, with a double *fliC* type (*fliC*-I and *fliC*-IV) in two of the samples. These results support the hypothesis that some samples could contain more than one *C. botulinum* group III strain at the same time. Regarding the 31 samples positively identified as *C. botulinum* type D/C, 17 were identified as *fliC*-IV, 6 as *fliC*-I, and 2 as *fliC*-II, and 7 provided no match with the flagellin detection assays developed (one sample was positive for both *fliC*-I and *fliC*-II). This finding suggests that *C. botulinum* type D/C shows greater flagellin genetic diversity than *C. botulinum* type C/D. All seven samples positive for *C. botulinum* type D/C but negative for their flagellin type originated from New Caledonia. The most probable explana-

tion is that the *C. botulinum* group III strains present in the samples carry a new flagellin type specific to the geographical area. Strain isolation and genetic analysis of the samples would be worthwhile. Of the samples that tested positive for *C. novyi sensu lato* but were found negative in the *ntnh_grpIII* and *bont* assays, nine were *fliC* positive. It is hypothesized that these samples contain strains that belong to *C. botulinum* group III but have lost their toxin phages during the enrichment process, or they may be transient carriers (11). Of the samples that tested positive for their flagellin genes, none were detected as *fliC*-III or *fliC*-V types, possibly because those flagellin types are less commonly encountered or, more probably, because the samples tested are not representative of the geographical area where they were isolated. Of the samples that tested positive for the *C. novyi sensu lato* assay but negative for the *ntnh_grpIII*, *bont*, and *fliC* assays, the samples are either *C. novyi* or *C. haemolyticum*. In addition, we acknowledge that these samples could be *C. botulinum* group III strains that have lost their toxin phages and carry a new flagellin type. The development of new markers to better characterize the *C. novyi sensu lato* group would be a prerequisite to better answer this question. We partially explored this possibility by sequencing the *C. botulinum* group III 807-bp and 834-bp flagellin genes, but they appeared not to be present in all strains and segregated identically to *fliC* (data not shown).

Regarding the *fliC* distribution, *fliC*-I dominates in *C. botulinum* type C/D, suggesting the clone has spread in different geographical areas. Type *fliC*-II was found to be represented in only six samples, indicating the flagellin gene group is not predominantly represented in Europe. Type *fliC*-III was not detected in any sample tested, which is not surprising, as *C. botulinum* type C/D strain Eklund, which represents this flagellin type, was isolated in the 1970s and originates from the United States. Type *fliC*-IV was encountered only in *C. botulinum* type D/C and has a particularly large variable internal sequence. Since *C. botulinum* type D/C is predominantly found in samples that originate from

TABLE 6 Frequencies of the genetic markers per sample origins

Origin	No. of samples	% gene determinant presence (95% CI)									
		<i>C. novyi sensu lato</i>	<i>ntnh_grpIII</i>	<i>bont</i> /C	<i>bont</i> /C/D	<i>bont</i> /D/C	<i>fliC</i> -I	<i>fliC</i> -II	<i>fliC</i> -III	<i>fliC</i> -IV	<i>fliC</i> -V
Poultry	135	67 (59–75)	7 (4–13)	0 (0–3)	7 (4–13)	0 (0–3)	9 (5–15)	1 (0–4)	0 (0–3)	0 (0–3)	0 (0–3)
Chicken	101	76 (67–83)	47 (37–56)	0 (0–4)	44 (34–53)	3 (1–8)	45 (35–54)	2 (5–7)	0 (0–4)	7 (3–14)	0 (0–4)
Duck	98	70 (61–78)	48 (38–58)	0 (0–4)	47 (37–57)	4 (2–10)	47 (37–57)	0 (0–4)	0 (0–4)	3 (1–9)	0 (0–4)
Turkey	95	56 (46–65)	19 (12–28)	0 (0–4)	19 (12–28)	0 (0–4)	19 (12–28)	0 (0–4)	0 (0–4)	0 (0–4)	0 (0–4)
Bovine	78	56 (45–67)	38 (28–49)	1 (1–7)	4 (1–11)	31 (22–42)	15 (9–25)	3 (1–9)	0 (0–5)	17 (10–26)	0 (0–5)
Mink	28	43 (26–61)	11 (4–27)	0 (0–12)	7 (2–23)	4 (1–18)	7 (2–23)	0 (0–12)	0 (0–12)	11 (4–27)	0 (0–12)
Goose	14	36 (16–61)	7 (1–31)	0 (0–21)	7 (1–31)	0 (0–21)	7 (1–31)	0 (0–21)	0 (0–21)	0 (0–21)	0 (0–21)
Guinea fowl	11	73 (43–90)	18 (5–48)	0 (0–26)	18 (5–48)	0 (0–26)	18 (5–48)	9 (2–38)	0 (0–26)	0 (0–26)	0 (0–26)

bovines, the *fliC-IV* flagellin type may be related to the bovine environment. In summary, the occurrence of different *fliC* types might indicate independent evolutionary traits within different regions of the planet and/or might be subject to certain host specificities. Among the samples investigated, flagellin type *fliC-V* was not detected. However, this flagellin type was identified in a unique *C. botulinum* type D (D-4947) strain presumably isolated by A. R. Prévot (Pasteur Institute). Prévot was involved in the analysis of human and animal botulism cases mainly in France and Africa. In light of the observed similarities between D-4947 and the D-South African strain (25), it is possible that D-4947 also originates from Africa, which could be a further indication of different regional evolutionary development. However, this is speculative and can be elucidated only with more group III strains available for analysis. If we compare the genetic diversity of FlaA1/A2 found in *C. botulinum* groups I and II to that of *fliC* in *C. botulinum* group III, it appears that group III is less genetically diverse. Similar results have been observed regarding the genetic diversity of the *bont* cluster, supporting slow genetic evolution within the group (11) or clonal spread. To confirm these assumptions, we would like to expand the publicly available whole-genome sequences of *C. botulinum* group III to explore its full genetic phylogeny.

Isolation of *C. botulinum* group III strains is particularly difficult due to the instability of the prophage carrying the BoNT genes under laboratory culture conditions. The *C. novyi sensu lato* and flagellin PCR assays are valuable tools to overcome this problem, making genotype profiling an interesting tool allowing the detection and characterization of *C. botulinum* group III strains that have lost their BoNT phages during the isolation process. The novelty of this study is also based on analysis of *fliC* sequence heterogeneity using a novel PCR typing scheme for *C. botulinum* group III. Very little was known regarding the genetic relatedness of strains of *C. botulinum* group III, even though it is an important animal pathogen that is widely distributed in the environment. Our findings suggest strain differences that are strongly geographically restricted, indicating this information may be useful if outbreaks are due to (accidentally or deliberately) imported sources but may be less valuable for epidemiologic source tracing within a given geographic area. Nonetheless, this is a useful start for genomic-diversity characterization of *C. botulinum* group III strains.

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Main results:

- Sequencing of *fliC* gene in 68 *C. botulinum* group III strains allowed the identification of 5 flagellin groups, with *C. botulinum* type C/D strains associated with *fliC*-I and type D/C associated with *fliC*-IV.
- Investigation of 560 polymicrobial samples from animal botulism cases (from France, Italy, Netherlands, Germany, New Caledonia) with specific *fliC* PCR assays revealed:
 - *fliC*-I, -II and -IV to be present while *fliC*-III and -V types are absent
 - Few *bont* negative samples are *fliC* positive maybe indicating these strains loss their phage.
 - Few *bont* D/C samples are also detected as *fliC*-I or -II types, maybe indicating a phage exchange.
 - 6 *bont* D/C samples are *fliC* negative which could be related to a new *fliC* type (all the sample originated from New Caledonia).

Main conclusions:

- *C. botulinum* group III *fliC* diversity is less important than flagellin diversity encountered in *C. botulinum* group I and II.
- *Bont* type of *C. botulinum* group III strains mainly associate with *fliC* type.
- Only 3 flagellin groups are detected in the European samples tested.
- The low flagellin diversity confirms the hypothesis of a clonal and suggests geographical and host specificity.

Perspectives:

It appears that flagellin genetic diversity from *C. botulinum* group III is less important than what we previously found for *C. botulinum* group I and II. Similar results have been observed regarding the genetic diversity of the *bont* cluster, supporting slow genetic evolution within the group or clonal spread. To confirm these assumptions, we would like to expand the publicly available whole-genome sequences of *C. botulinum* group III to explore its full genetic phylogeny.

V.3. Investigation of *C. botulinum* Group III French isolated strains by Next Generation Sequencing (NGS).

Since the easier access of the NGS technologies, whole-genome sequencing data provides the necessary information to perform advanced genetic investigations. The release of the first complete genome of *C. botulinum* Group III type C/D and several other *C. botulinum* Group III drafts genomes by Dr. H. Skarin, gave the scientific community invaluable new insights (Skarin et al., 2011; Skarin and Segerman, 2014). These developments revealed the genetic relatedness between *C. botulinum* Group III, *C. novyi*, and *C. haemolyticum* which showed to be so close that the new genospecies name *Clostridium novyi sensu lato* was proposed (Skarin et al., 2011). The genome sequences included *C. botulinum* type C, C/D, D and D/C, originating from several geographical areas, but none from France.

V.3.1. Draft genome sequences of 17 French *Clostridium botulinum* Group III strains.

My objective was to investigate the genomic diversity of *C. botulinum* Group III strains originating from France. With the help of the French National Reference Laboratory for avian botulism, I was able to gather and isolate strains from poultry and bovine farms samples involved in animal botulism events between 2008 and 2013. Fourteen strains type C/D originated from poultry and three types D/C originated from bovine (one was from New Caledonia) were sequenced using an Illumina MiSeq platform. The number of contigs by *de novo* assembly using CLC® genomic workbench software ranged from 75 to 146 (contigs under 1kbp were not considered), with a median read depth of 39X to 226X, and a N₅₀ values between 36 kbp and 68 kbp. The genome size was close to the reference genome BKT015925 (around 3.1 Mbp), with a low GC% content (close to 28%) as expected for *Clostridium botulinum* strains. The results of the sequenced genomes were published in 2015 in the scientific journal *Genome Announcement* (Article n°4).

Article n°4:

Woudstra C, Le Maréchal C, Souillard R, Bayon-Auboyer MH, Mermoud I, Desoutter D, Fach P. Draft Genome Sequences of 17 French *Clostridium botulinum* Group III Strains. **Genome Announc. 2015 Oct 1;3(5).**

Article n°4

Draft genome sequences of 17 French *Clostridium botulinum* Group III strains.

**Woudstra C, Le Maréchal C, Souillard R, Bayon-Auboyer MH,
Mermoud I, Desoutter D, Fach P.**

Genome Announc. 2015 Oct 1;3(5).

Draft Genome Sequences of 17 French *Clostridium botulinum* Group III Strains

Cédric Woudstra,^a Caroline Le Maréchal,^b Rozenn Souillard,^c Marie-Hélène Bayon-Auboyer,^d Isabelle Mermoud,^e Denise Desoutter,^e Patrick Fach^a

Université Paris-Est, Anses, Food Safety Laboratory, Platform IdentityPath, Maisons-Alfort, France^a; Anses, UEB, Ploufragan-Plouzané Laboratory, Hygiene and Quality of Poultry and Pig Products Unit, Ploufragan, France^b; Anses, UEB, Ploufragan-Plouzané Laboratory, Avian and Rabbit Epidemiology and Welfare Unit, Ploufragan, France^c; LABOCEA, Ploufragan, France; LNC, Veterinary Diagnostic Laboratory, Païta, New Caledonia^d

Animal botulism is mainly associated with *Clostridium botulinum* group III strains producing neurotoxin types C, C/D, D, and D/C. In this report, we present the draft genome sequences of fourteen strains of *Clostridium botulinum* producing type C/D and two strains producing type D/C isolated in France, and one strain producing type D/C that originated from New Caledonia.

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Address correspondence to Cédric Woudstra, cedric.woudstra@anses.fr.

Animal botulism is caused by group III *C. botulinum* strains that produce type C and D toxins, or a chimeric fusion of C and D termed C/D or D/C toxin (1). Animal botulism is considered an emerging disease in Europe, notably in poultry production (2), where it could lead to significant economic losses (3). The actual development of molecular tools allows rapid detection (4) and characterization (5) of the strains involved in an outbreak. We previously showed (4) that animal botulism in Europe is mainly due to mosaic type C/D for avian species, and type D/C for bovine.

Currently fourteen genomes of *Clostridium botulinum* group III are available in the public database: two type C, seven type C/D,

three type D, and two type D/C. They originate from different countries but none originate from France.

In order to investigate the epidemiological genetic relationship of strains originating from different geographical areas, we sequenced seventeen strains of *Clostridium botulinum* group III: fourteen type C/D and three type D/C. They all originate from France, except for one type D/C, which was isolated from New Caledonia. Isolates of type C/D originate from chicken, duck, guinea fowl, and turkey, whereas type D/C comes from bovine.

Genomic DNA was extracted from a 48-h culture, incubated at 37°C under anaerobic conditions, in TPGY medium, using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) accord-

TABLE 1 NCBI accession numbers and assembly metrics of *Clostridium botulinum* group III draft genomes

Isolate	Origin	Year	BoNT ^a type	No. of contigs	Genome size (Mbp)	G+C %	N ₅₀ (Kbp)	Median read depth (×)	No. of coding sequences (per PGAAP)	GenBank accession no.
12LNR1	Duck	2012	C/D	131	3.00	27.8	42	62	2,768	LGVR000000000
12LNR10	Turkey	2012	C/D	132	3.04	27.9	40	77	2,798	LGVQ000000000
12LNR13	Chicken	2012	C/D	140	3.07	27.9	42	63	2,831	LGVT000000000
29401	Chicken	2008	C/D	112	3.04	27.9	57	123	2,806	LGVP000000000
38028	Chicken	2008	C/D	104	3.12	27.9	48	70	2,861	LGVO000000000
43243	Guinea fowl	2009	C/D	111	3.00	27.9	57	57	2,764	LGVU000000000
48212	Duck	2008	C/D	75	3.00	27.9	68	101	2,763	LGVS000000000
49511	Chicken	2008	C/D	85	3.09	27.9	41	83	2,837	LHYP000000000
50867	Chicken	2008	C/D	134	3.08	27.8	36	90	2,849	LHYQ000000000
55741	Turkey	2008	C/D	103	3.04	27.9	57	50	2,801	LHYR000000000
58272	Chicken	2008	C/D	128	3.07	27.9	40	73	2,845	LHYS000000000
58752	Chicken	2008	C/D	114	2.82	28.0	42	73	2,581	LHYT000000000
69285	Chicken	2008	C/D	146	2.94	28.0	57	39	2,717	LHYU000000000
71840	Chicken	2008	C/D	145	3.00	27.9	40	128	2,764	LHYV000000000
LNC5	Bovine	2013	D/C	83	2.89	28.0	56	81	2,579	LHYW000000000
47295	Bovine	2009	D/C	108	3.18	27.9	51	223	2,899	LHYX000000000
51714	Bovine	2009	D/C	101	3.18	27.9	59	116	2,889	LHYZ000000000

^a BoNT, botulinum neurotoxin.

ing to the manufacturer's instructions for Gram-positive bacteria, with an additional RNase A (Roche) treatment. Libraries were prepared using the Nextera XT kit (Illumina). Whole-genome sequencing was performed using an Illumina MiSeq platform (Illumina) according to the manufacturer's instructions. Three MiSeq runs were carried out, two with paired-end 150-nucleotide (nt) reads on MiSeq V2 and V2 microchemistry, another with paired-end 300-nt reads on V3 chemistry. The raw reads were trimmed (minimum length 35 bp, quality score 0.03) and assembled in CLC Genomics Workbench version 7.5.1 by *de novo* assembly (minimum contig length 1,000 bp), producing 75 to 146 contigs (Table 1). The median read depth of the assemblies ranged from 39× for isolate 69285 to 223× for isolate 47295 with N_{50} values between 36 kbp and 68 kbp (Table 1). The sequences were annotated with the National Center for Biotechnology Information (NCBI) Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) at http://www.ncbi.nlm.nih.gov/genome/annotation_prok.

The average size of the genomes in this study is 3.09 Mb, 2.82 Mb being the smallest genome size, with an average G+C content of 28.9% (isolate 58752, Table 1), and 3.18 Mb being the largest genome size (isolate 47295 and 51714, Table 1). On average, 2,785 coding sequences were identified in the genomes (Table 1). A detailed report on further analyses of the draft genome sequences will be released in a future publication.

Nucleotide sequence accession numbers. The annotated draft whole-genome sequences of these *Clostridium botulinum* group III strains were deposited in DDBJ/ENA/GenBank under the accession numbers listed in Table 1. The versions described in this paper are the first versions.

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Main results:

- The genome sequences of seventeen *C. botulinum* group III strains were obtained (14 type C/D and 3 type C/D) using an Illumina MiSeq technology.
- The evaluation of the data generated regarding the contigs no., the N₅₀ (the average size of the contigs generated) and the median read depth are concordant with draft genome quality requirement for Illumina technology.
- The genome size, GC content and no. of coding sequences are concordant with *C. botulinum* group III reference strain BKT015925.

Main conclusions:

- The data generated are of good quality and could be used for further analyses.
- The properties of the genome sequences produced are concordant with *C. botulinum* group III type C/D reference genome.

Perspective:

This work was preliminary. The sequences produced need to be thoroughly analysed by looking for differences/similarities between French and European *C. botulinum* group III strains.

V.3.2. Analysis by Whole Genome Sequencing (WGS) of 17 French strains of *Clostridium botulinum* from Group III.

The work undertaken was based on the genome sequences published in article n°4. The objective was to investigate the genetic diversity of *C. botulinum* Group III using WGS. Previous investigation of *C. botulinum* Group III type C/D diversity (Anza et al., 2014a; Woudstra et al., 2015a) revealed a very high level of genetic similarity, supporting that one clade of closely related strains could be mainly responsible for causing avian botulism in Europe. This assumption is based on information gathered on strains isolated from outbreaks and typed using methods such as PFGE, or flagellin gene detection by real-time PCR. These methods have been universally used to check for strains' diversity. However, using these techniques the level of discrimination could be insufficient for the differentiation of highly genetically related strains. Given this situation the genome sequences of 17 *C. botulinum* Group III strains, originating from France, were analysed to gain insight in their genetic diversity and to complete the genetic picture of European *C. botulinum* Group III strains. Genome sequences from *C. botulinum* C/D and D/C were separately analysed. The reads produced by high throughput sequencing technology were treated and assembled either with mapping or *de novo* process, before being investigated for their genetic diversity using bioinformatic tools. The information gathered was used to assess *in silico* typing methods such as Clustered Regularly Interspaced Short Palindromic Repeats (Crispr) and Single Nucleotide Polymorphism (SNP) genotyping.

The results of this study were published in 2016 in the scientific journal *Frontiers in Microbiology*, in the section *Evolutionary and Genomic Microbiology* (Article n°5).

Article n°5:

Woudstra C, Le Maréchal C, Souillard R, Bayon-Auboyer MH, Mermoud I, Desoutter D, Fach P. New Insights into the Genetic Diversity of *Clostridium botulinum* Group III through Extensive Genome Exploration. **Front. Microbiol., 19 May 2016.**

Article n°5

New Insights into the genetic diversity of Clostridium botulinum Group III through extensive genome exploration.

**Woudstra C, Le Maréchal C, Souillard R, Bayon-Auboyer MH,
Mermoud I, Desoutter D, Fach P.**

Frontiers Microbiology, 19 May 2016.



New Insights into the Genetic Diversity of *Clostridium botulinum* Group III through Extensive Genome Exploration

Cédric Woudstra^{1*}, Caroline Le Maréchal^{2,3}, Rozenn Souillard^{3,4}, Marie-Hélène Bayon-Auboyer⁵, Isabelle Mermoud⁶, Denise Desoutter⁶ and Patrick Fach¹

¹ Laboratory for Food Safety, French Agency for Food, Environmental and Occupational Health & Safety – Université Paris-Est, Maisons-Alfort, France, ² Hygiene and Quality of Poultry and Pig Products Unit, Ploufragan-Plouzané Laboratory, UEB, French Agency for Food, Environmental and Occupational Health & Safety, Ploufragan, France, ³ I'UBL Université Bretagne Loire, Rennes, France, ⁴ Avian and Rabbit Epidemiology and Welfare Unit, Ploufragan-Plouzané Laboratory, UEB, French Agency for Food, Environmental and Occupational Health & Safety, Ploufragan, France, ⁵ Laboratoire Public Conseil, Expertise et Analyse en Bretagne, Ploufragan, France, ⁶ Veterinary Diagnostic Laboratory, Laboratoires Officiels Vétérinaires, Agroalimentaires et Phytosanitaires, La Direction des Affaires Vétérinaires, Païta, New Caledonia

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*Correspondence:

Cedric Woudstra
cedric.woudstra@anses.fr

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Animal botulism is caused by group III *Clostridium botulinum* strains producing type C and D toxins, or their chimeric forms C/D and D/C. Animal botulism is considered an emerging disease in Europe, notably in poultry production. Before our study, 14 genomes from different countries were available in the public database, but none were from France. In order to investigate the genetic relationship of French strains with different geographical areas and find new potential typing targets, 17 strains of *C. botulinum* group III were sequenced (16 from France and one from New Caledonia). Fourteen were type C/D strains isolated from chickens, ducks, guinea fowl and turkeys and three were type D/C strains isolated from cattle. The New Caledonian strain was a type D/C strain. Whole genome sequence analysis showed the French strains to be closely related to European strains from *C. botulinum* group III lineages Ia and Ib. The investigation of CRISPR sequences as genetic targets for differentiating strains in group III proved to be irrelevant for type C/D due to a deficient CRISPR/Cas mechanism, but not for type D/C. Conversely, the extrachromosomal elements of type C/D strains could be used to generate a genetic ID card. The highest level of discrimination was achieved with SNP core phylogeny, which allowed differentiation up to strain level and provide the most relevant information for genetic epidemiology studies and discrimination.

Keywords: animal botulism, *Clostridium botulinum* group III, CRISPR, phage, whole genome sequencing

INTRODUCTION

Clostridium botulinum is the aetiological agent of botulism, a deadly paralytic disease that can affect both humans and animals. The symptoms are caused by botulinum neurotoxins (BoNTs) typically produced by *C. botulinum*, a Gram-positive bacterium. The *C. botulinum* species can be divided into four groups (I to IV) based on physiological and genomic traits (Sobel, 2005). Animal botulism is mainly due to *C. botulinum* group III, which belongs to the *C. novyi sensu lato* species

(Skarin et al., 2011). They produce toxin types C and D in addition to their mosaic variants C/D and D/C (Takeda et al., 2005). Animal botulism is considered an emerging disease in Europe, notably in poultry production (Souillard et al., 2014), where it can lead to significant economic losses (Lindberg et al., 2010). We previously showed that animal botulism in Europe is mainly due to mosaic type C/D in avian species, and type D/C in cattle (Woudstra et al., 2012). The development of new methods for the rapid molecular detection (Woudstra et al., 2012) and characterization (Anza et al., 2014; Woudstra et al., 2015a) of strains involved in outbreaks have so far revealed marked genetic similarity. This supports the hypothesis that one clade of closely related strains responsible for animal botulism dominates in Europe. This assumption is based on information gathered on strains isolated from outbreaks and typed using methods such as PFGE or flagellin gene detection by real-time PCR (Anza et al., 2014; Woudstra et al., 2015a). These methods have been universally used to check for strain diversity. However, the level of discrimination provided by these techniques may be insufficient to differentiate closely related genetic strains.

Since the arrival of next-generation sequencing technologies, the investigation of whole genome sequences of *C. botulinum* has given the scientific community invaluable new insights and provided genetic information of relevance for epidemiological investigations (Skarin and Segerman, 2014; Giordani et al., 2015; Weedmark et al., 2015a). At the time of our study, 14 genomes of *C. botulinum* group III were available in the public database: two of type C, seven of type C/D, three of type D, and two of type D/C. These genomes mostly originated from Sweden, and none were from France. Given this situation, our objective was to investigate *C. botulinum* group III genome sequences from France to complete the genetic picture of European *C. botulinum* group III strains and to find new potential genetic targets for typing. The genome sequences of 17 *C. botulinum* group III strains were analyzed: 14 type C/D strains from chickens, ducks, guinea fowl, and turkeys, plus three type D/C strains from cattle, all from France except for one type D/C strain which was from New Caledonia (Woudstra et al., 2015b). The sequences produced were processed and assembled either with a mapping or *de novo* process before being investigated for their genetic diversity using bio-informatic tools.

MATERIALS AND METHODS

Isolates

All but one of the *C. botulinum* strains investigated in this study are from France. They were provided by LABOCEA in Brittany in the framework of Anses's work as the French National Reference Laboratory for avian botulism. One isolate originated from New Caledonia following collaboration with the National Laboratory of New Caledonia. Isolates producing type C/D toxin mainly originated from chicken samples, whereas all BoNTs producing type D/C isolates were from bovine sample material. The samples were collected in different geographical areas, mainly from the north-western part of France, during botulism outbreaks that occurred between 2008 and 2013 (Figure 1).

Culture Conditions, DNA Isolation, and Genome Sequencing

Genomic DNA was extracted from a 48-h culture incubated at 37°C under anaerobic conditions (A35 station, Don Whitley) in TPGY medium using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions for Gram-positive bacteria, with an additional RNase A (Roche) treatment. Libraries were prepared using the Nextera XT kit (Illumina). Whole genome sequencing was performed using an Illumina MiSeq platform (Illumina) according to the manufacturer's instructions. Three MiSeq runs were carried out, two with paired-end 150-nucleotide (nt) reads on MiSeq V2 and V2 microchemistry, another with paired-end 300-nt reads on V3 chemistry (Woudstra et al., 2015b).

Nucleotide Sequence Accession Numbers

The sequence reads of these *C. botulinum* group III strains were deposited in the Sequence Read Archive (SRA) under accession numbers SRR2120181, SRR2124854, SRR2124859, SRR2124863, SRR2124865, SRR2124866, SRR2124867, SRR2124869, SRR2124870, SRR2124883, SRR2124884, SRR2124885, SRR2124886, SRR2124887, SRR2124888, SRR2124889, and SRR2124890.

Genome Assembly

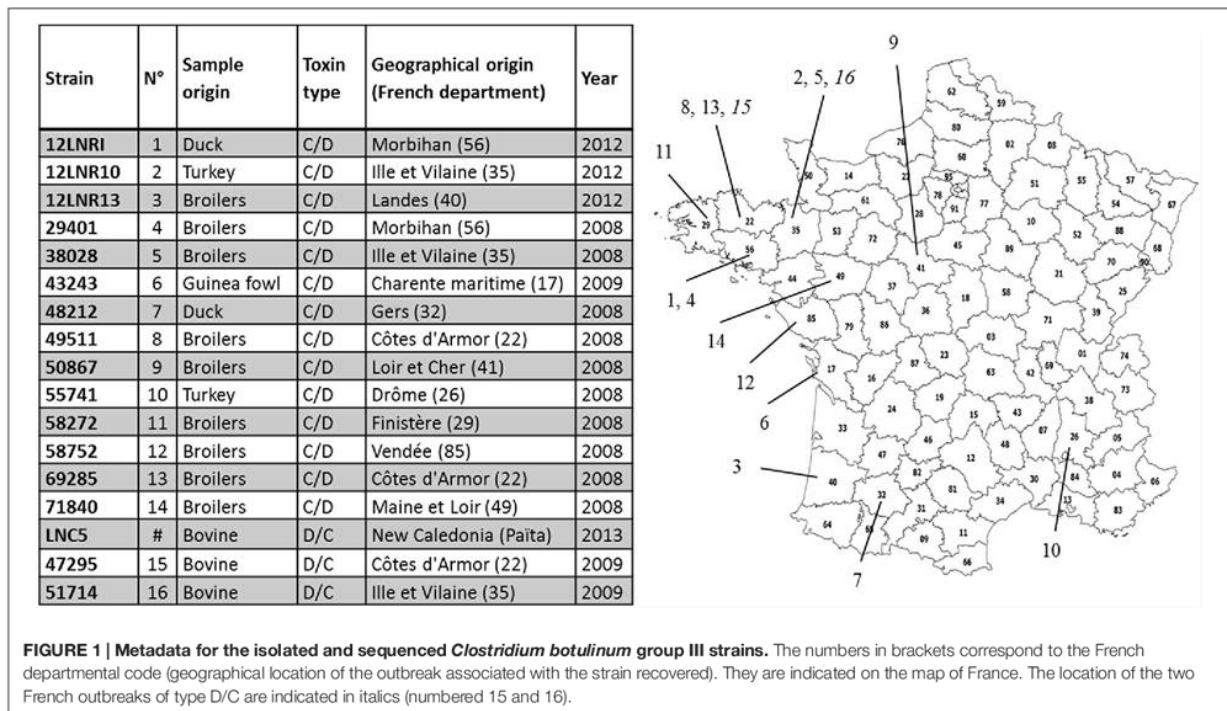
De novo assembly was performed as previously described (Woudstra et al., 2015b). Raw reads were trimmed (minimum length 35 bp, quality score 0.03) and assembled with the CLC Genomics Workbench version 7.5.1 (Qiagen, Aarhus, Denmark). The contigs created were ordered using *C. botulinum* C/D BKT015925 as a reference (GenBank accession number CP002411).

Mapping was performed using the NGS core "Map reads to reference" tools from CLC® Genomics Workbench software version 7.5.1. Sequences of *C. botulinum* group III type C/D strains were mapped using *C. botulinum* type C/D BKT015925 as a reference (GenBank accession number CP002411). Mapping sequences were considered successful only if the depth was more than 10X. Over 30X was considered a good sequencing depth (Ekblom and Wolf, 2014; Sims et al., 2014). As no reference sequence was available for type D/C, *C. botulinum* group III type D/C isolates were mapped using draft genome CCUG7971 type D as a reference for strain LNC5 and draft genome DC5 type D/C for strains 47295 and 51714.

Comparative Genome Analyses

Gegenees software (Agren et al., 2012; version 2.2.1¹), a comparative analysis tool for whole genome sequence data, was used to provide a phylogenetic overview of the microbial genome sequences investigated. The software uses a fragmented approach to make all-against-all BLASTN whole genome comparisons. A fragment length of 200 bp and step size of 100 bp were used. Dendrograms were produced in SplitsTree4 (Huson and

¹<http://www.gegenees.org>



Bryant, 2006; version 4.13.1²) using the neighbor-joining method from a Nexus file exported from GENECON. BLAST Ring Image Generator (BRIG) software (Alikhan et al., 2011; version 0.95³) was used to further compare the genome sequences.

Core SNP Phylogeny Profiling

De novo-assembled contigs of the French *C. botulinum* group III isolates and publicly available group III genome sequences produced by Skarin and Segerman (2014) were submitted to the CSI Phylogeny 1.1 web server⁴ for analyses. This server identifies SNPs from whole genome sequencing data, filters and validates the SNP positions, and then infers phylogeny based on concatenated SNP profiles (Kaas et al., 2014). *C. botulinum* BKT015925 type C/D was used as the reference strain. SNPs were excluded if they were in regions with a minimum fold coverage of <10, within 10 bp of another SNP or <15 bp from the end of a contig. A maximum likelihood tree was created from the concatenated SNP sequences and images were rendered in FigTree (v1.4.1⁵).

Phage Sequence Search

The phage search tool web server (Zhou et al., 2011; PHAST⁶), designed to rapidly and accurately identify, annotate and

graphically display prophage sequences within bacterial genomes or plasmids, was used to investigate the sequences produced.

CRISPR Identification

CRISPR-Cas genes were identified using PROKKA annotation pipeline (Seemann, 2014). The CRISPR recognition tool software (Bland et al., 2007) version 1.0 was used to investigate sequences for clustered regularly interspaced short palindromic repeats. The settings were chosen to search for a minimum number of three repeats with a minimum repeat length of 19 nucleotides and a maximum repeat length of 38 nucleotides, within a search window of eight. Spacer settings were for a minimum length of 19 nucleotides and a maximum of 48.

RESULTS

Assemblies and Genome Sequences Data

The genomes of the 14 strains of *C. botulinum* group III type C/D and the three *C. botulinum* type D/C strains were *de novo*-assembled. A table containing the information related to assembly statistics of the sequences produced was previously published (Woudstra et al., 2015b). The sequences were successfully checked to meet the requirements of genome quality for Illumina technology (Mavromatis et al., 2012). Genome size, GC content, and the number of coding sequences were concordant with reference strains *C. botulinum* type C/D BKT015925 (Skarin et al., 2011). Mapping assembly was also

²<http://www.splitstree.org/>

³<http://brig.sourceforge.net/>

⁴<https://cge.cbs.dtu.dk/services/CSIPhylogeny/>

⁵<http://tree.bio.ed.ac.uk/software/figtree>

⁶<http://phast.wishartlab.com/>

performed using reference genome BKT015925 for type C/D strains (Table 1). The analysis of the mapped sequences show that plasmid p1 containing the botulinum locus and plasmids p2 and p3 are always associated with-type C/D isolates. The sequence depth associated with plasmid p1 shows that isolates 12LNRI, 58752, and 69285 were not sequenced deeply enough (respectively, 22X, 10X, and 5X). This could be because there are fewer prophage copies in the strains than chromosome copies. This is an illustration of the pseudolysogeny phenomenon, the prophage being known to be unstable during cultivation (Oguma, 1976). Plasmids p4 and p5 were not systematically present. P4 was found in strains 38028, 48212, 50867, 58272, and 69285 but absent in the other strains. P5 was found in all the strains except 12LNRI3, 49511, and 69285. Regarding type D/C sequences, in the absence of a complete reference genome for *C. botulinum* type D/C, strains 47295, 51714, and LNC5 were mapped against the most genetically closely related draft genomes available, meaning CCUG7971 type D for LNC5 and DC5 type D/C for strains 47295 and 51714. Plasmids p1, p2, and p3 were identified, but no sequences related to plasmid p4 and p5 were retrieved. These results highlight the genetic difference between *C. botulinum* type C/D and type D/C.

The unused reads from the mapping assembly of type C/D and D/C isolates were collected and *de novo*-assembled to search for genetic elements that differ from the reference genomes (data not shown). The results show that for some isolates, *de novo* assembly of unused mapping sequences gathers into contigs long enough to be considered true genetic elements. Strains 12LNRI, 43243, 69285, 71840, 47295, and 51714 produced relatively short contig assemblies between 1300 and 8300 bp, which were related to 16S and 23S RNAs (data not shown). The other isolates gave contigs ranging up to 45 kbp, reflecting the presence of large genetic sequences that may be related to extrachromosomal elements. This emphasizes the benefits of investigating unmapped reads while doing resequencing analyses (Gouin et al., 2015).

Comparative Genome Analyses

As previously published by Skarin and Segerman (2014), *C. botulinum* group III is part of the larger group *C. novyi sensu lato* which also encompasses *C. novyi* and *C. haemolyticum*. It appears to be divided into four different lineages, with *C. botulinum* type C/D and type D/C mainly clustering into lineage Ia and Ib, respectively. Figure 2 represents the phylogenetic relationship between the *de novo*-assembled genome sequences of French *C. botulinum* group III and the publicly available *C. novyi sensu lato* genome sequences of lineage Ia and Ib, using Gegenees matrix comparison. The French type C/D isolates are clearly associated with group Ia, while those of type D/C are related to group Ib. None of the French isolates investigated were associated to *C. novyi* or *C. haemolyticum*.

The comparison matrix produced using BKT015925 as a reference value clearly distinguishes *C. botulinum* types C/D and D/C, with an average sequence identity of 70%. BKT015925 was shown to be genetically closely related to the French C/D isolates (94–100% identity). Twelve of these isolates showed 99–100% sequence identity with BKT015925, emphasizing their close genetic relationship. Strains 38028, 12LNRI3, and 49511

could be considered more distantly related to BKT015925 (95% genetic identity) and to the other French isolates (87–96%). Alignment of these sequences with BKT015925 using BRIG software show that isolates 38028, 12LNRI3, and 49511 lack a specific region of 16 kbp (data not shown). This missing region contains eight coding sequences (CbC4-2081 to CbC4-2088) that encode five hypothetical proteins and three proteins known to be involved in phosphorothioation DNA modification (DndD, DndE and a cysteine desulfurase). This mechanism modifies the DNA backbone, with sulfur replacing non-bridging phosphate oxygen (Wang et al., 2007). It was found to be responsible for the DNA degradation phenotype that renders modified DNA susceptible to cleavage during PFGE migration runs (Zhou et al., 2005; Ho et al., 2015). The intact genetic locus comprises five Dnd genes (DndA/B/C/D/E) and a regulator gene, *IscS* (that could be replaced by a cysteine desulfurase). A recent investigation has shown that DndA is not indispensable in *Streptomyces lividans*, but all the other Dnd genes are necessary for the locus to be functional (Xu et al., 2009). Thus, strains 38028, 12LNRI3, and 49511—which lack DndD, DndE and the cysteine desulfurase—should have a dysfunctional DNA phosphorothioation mechanism and thus be able to be typed by PFGE, while other strains containing the intact Dnd locus would probably not be typeable. Investigation of the presence of this Dnd locus in strains from Skarin et al. (2011) study showed BKT015925, Sp77 and V891 to be Dnd positive, while strains 16868, CCUG7978, DC5, BKT75002, and BKT2873 were negative. Such a phenotype could explain the PFGE typing difficulties encountered with certain *C. botulinum* group III strains (Anza et al., 2014).

Genome sequences of *C. botulinum* type D/C (strains 47295, 51714, and LNC5) were observed to belong to cluster Ib (Skarin and Segerman, 2014). Isolates 47295 and 51714 were highly genetically related to strain DC5 type D/C originating from Italy. On another hand, isolate LNC5 was more closely related to strain CCUG7971 type D isolated in South Africa. The whole genome size of strains LNC5 and CCUG7971 were smaller than other type D/C strains (2.89 and 2.81 Mbp, respectively, compared to ≥ 3.18 Mbp); it may thus be hypothesized that this could reflect differences in the content of mobile genetic elements. A closed reference genome of *C. botulinum* type D/C and further thorough analyses of more genome sequences would be necessary to fully investigate the genetic relatedness of type D/C strains.

SNP Profiling

To determine whether closely related isolates could be resolved by outbreak or region of origin, SNP profiling was performed on both French *C. botulinum* group III isolates and publicly available genome sequences (Skarin and Segerman, 2014). The botulinum locus sequences (botR to *bont*/C, 11575 bp) were investigated for SNPs. All the isolates investigated harbor the same ha+ locus sequence. BotR, HA17, HA70, HA33, ntnh, and *bont* sequences are identical to the BKT015925 botulinum locus, except for isolates 58752 and 69285 for which the locus was not totally covered (missing 574 and 908 bp, respectively). This could be the consequence of the poor depth coverage of plasmid p1 for these two isolates (respectively, 10X and 5X). Only four SNPs

TABLE 1 | Clostridium botulinum type C/D mapping assembly details.

Strain	Origin	Mapping	Chr	p1	p2	p3	p4	p5
12LNRI	Duck	Coverage	98%	98%	98%	95%	2%	100%
		Depth (X)	90	23	193	195	0	143
12LNR10	Turkey	Coverage	98%	98%	98%	95%	6%	100%
		Depth (X)	88	151	119	146	8	439
12LNR13	Chicken	Coverage	96%	88%	85%	90%	0%	17%
		Depth (X)	65	57	58	86	0	0
29401	Chicken	Coverage	98%	98%	99%	95%	5%	100%
		Depth (X)	101	120	83	118	6	426
38028	Chicken	Coverage	96%	82%	97%	91%	82%	72%
		Depth (X)	92	97	97	124	183	419
43243	Guinea fowl	Coverage	98%	98%	97%	95%	0%	100%
		Depth (X)	69	57	57	91	0	261
48212	Duck	Coverage	97%	98%	97%	94%	100%	100%
		Depth (X)	88	88	101	134	66	374
49511	Chicken	Coverage	95%	91%	90%	91%	6%	1%
		Depth (X)	46	48	42	60	1	0
50867	Chicken	Coverage	97%	98%	98%	96%	100%	100%
		Depth (X)	55	30	110	135	97	519
55741	Turkey	Coverage	98%	99%	98%	96%	5%	100%
		Depth (X)	95	91	65	102	4	68
58272	Chicken	Coverage	97%	98%	98%	94%	95%	100%
		Depth (X)	72	104	131	97	94	223
58752	Chicken	Coverage	97%	93%	98%	95%	7%	100%
		Depth (X)	99	10	171	212	15	775
69285	Chicken	Coverage	98%	94%	98%	96%	55%	9%
		Depth (X)	76	5	93	142	399	0
71840	Chicken	Coverage	97%	98%	97%	94%	0%	100%
		Depth (X)	44	43	48	61	0	241

All strains belong to *C. botulinum* group III type C/D. The chromosome sequence (Chr) is 2773157 bp long; p1 corresponding to the botulinum prophage, contains botulinum and C3 toxin, and is 203287 bp long; p2 contains alpha-toxin and is 98732 bp long; p3 contains C2 toxin and phospholipase C, and is 80365 bp long; p4 contains phage sequence phiCD6356 (Horgan et al., 2010) and is 39648 bp long; p5 contains *C. perfringens* putative epsilon toxin (Skarin and Segerman, 2014) and is 12403 bp long.

were retrieved on the entire botulinum locus, all identical for strains 49511, 12LNR13, and 38028 type C/D. Two are located in gene HA70 and the other two in gene HA17 (position 2395 G-A, 2457 T-C, 2529 C-T, and 2575 T-C). This is in accordance with the results of the whole genome comparison made with Gegenees software (see Figure 2) that showed strains 49511, 12LNR13, and 38028 to cluster slightly differently from other type C/D strains.

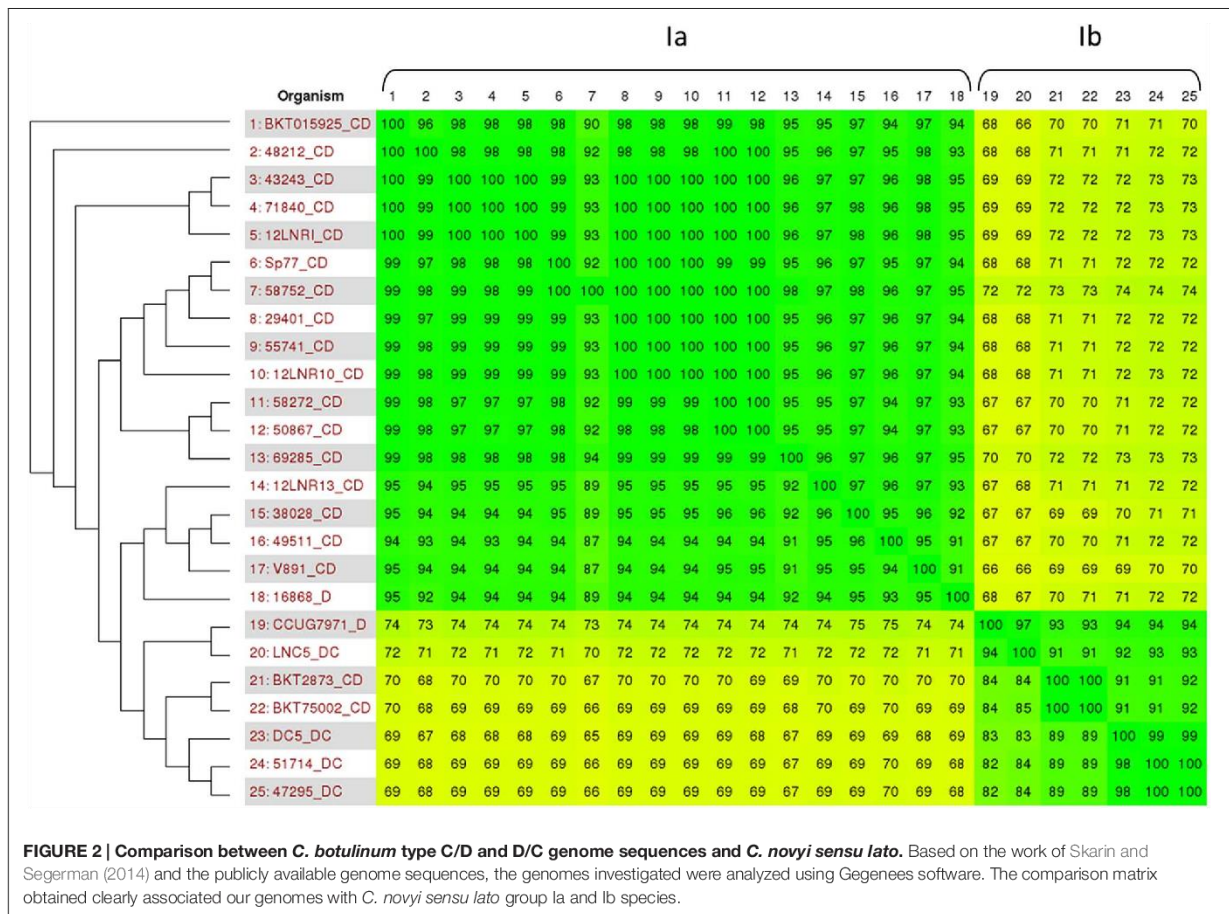
Core SNP profiling of both the French type C/D and D/C strains and the strains from the Skarin and Segerman (2014) study, were investigated using the CSI Phylogeny web server of the Centre for Genomic Epidemiology of DTU in Denmark (Kaas et al., 2014) (Figure 3). The reference sequence used was *C. botulinum* type C/D BKT015925. The SNP tree distinguishes three major groups (Figure 3A). The results showed a clear distinction between the French type C/D strains 12LNR13, 38028, and 49511 which clustered together with strain V891 type C/D (from Sweden) and strain 16868 type D (from The Netherlands), and remained distant from the other isolates. All the other French type C/D strains clustered together with the reference strain BKT015925 and Sp77 type C/D (originating from Sweden and Spain, respectively). As expected, *C. botulinum* type C/D isolates

clustered together at a distance from type D/C isolates, which is in accordance with previous results. The LNC5 type D/C and CCUG7971 type D strains clustered together, while strains 47295, 51714, and DC5 type D/C clustered separately, which is in accordance with the Gegenees matrix comparison results.

To further enhance discrimination, we focused on the genetically closely related French *C. botulinum* group III type C/D isolates (Figure 3B). French strains 48212/43243/58272/71840 and to a lesser extent 12LNRI, were related to strain Sp77 (originating from Spain). Strains 29401/55741/58752/12LNR10/69285 and to a lesser extent strain 50867 were related to reference strain BKT015925 (originating from Sweden). The results showed no correlation between toxin type, geographical and source origin. Despite their marked genetic degree of relatedness, SNP core phylogeny was able to clearly differentiate the French *C. botulinum* group III strains investigated even up to the outbreak origin.

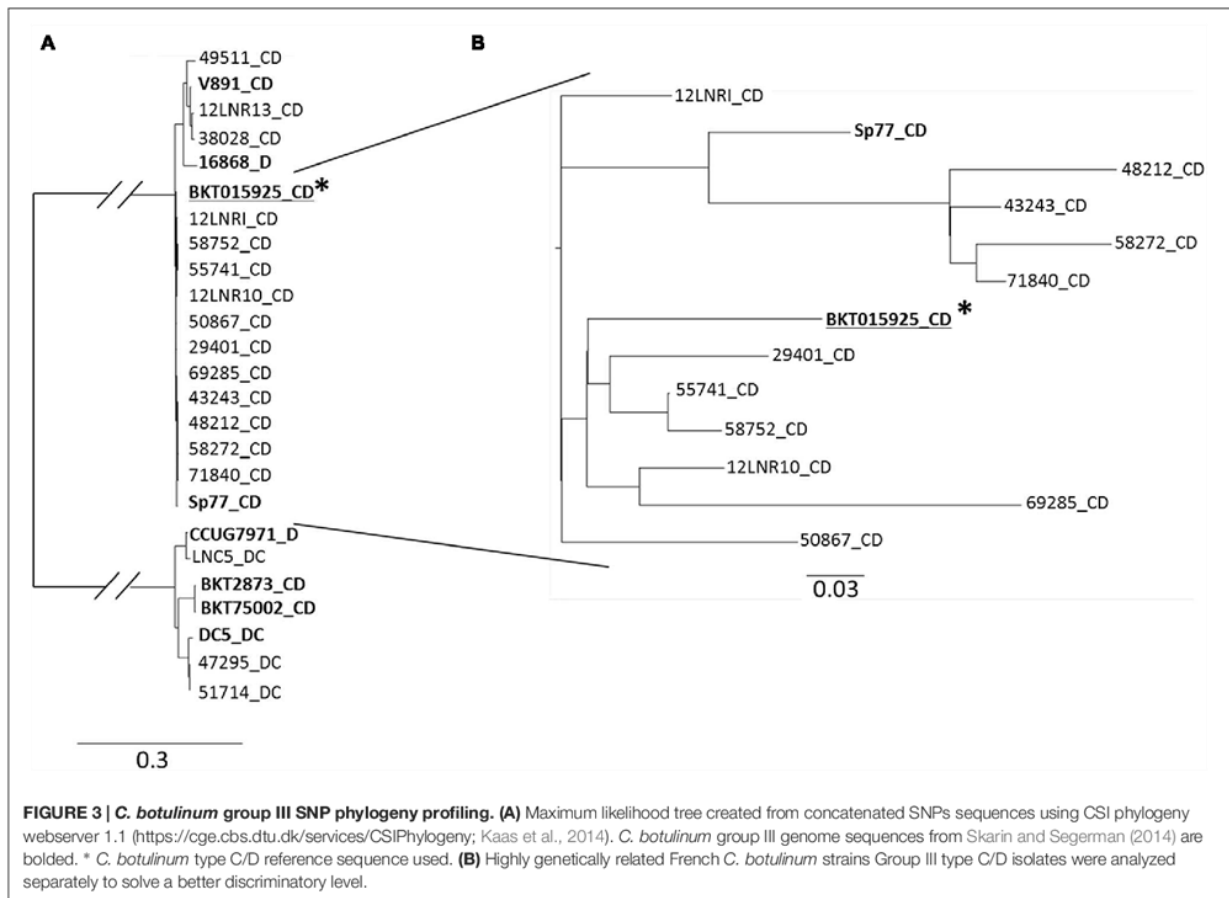
Comparative Phage Typing

The SNP core phylogeny proved to be highly discriminant but requires the analysis of whole genome sequences. Although,



nowadays benchtop sequencers are more affordable and easy to use, the analysis of extensive sequencing data produced still presents a challenge and prevents the routine application of WGS in veterinary laboratories. Another issue to develop this approach is the relative high difficulty to get *C. botulinum* group III isolated. Thus, there is a need to develop typing methods that could reach the same level of discrimination without the need of a whole genome sequence. The previous work of Skarin and Segerman (2011) showed *C. botulinum* group III to contain several phage sequences. We postulate it would be possible to use the mobile genetic element content of *C. botulinum* group III for typing purposes. We searched for the presence of extrachromosomal elements differing from reference strain BKT015925 using the PHAST web server (Zhou et al., 2011). PHAST identified three family phage sequences—phiSM101, phiS63, and phi3626—which had never previously been characterized in *C. botulinum* group III. Using this information, together with the presence of BKT015925 plasmids and phages, it was then possible to create a genetic ID card of the mobile genetic elements present in the investigated isolates (Table 2). Regarding type C/D, isolate 48212 contains the same mobile element as reference strain

BKT015925. Isolates 50867 and 58272 also contain the same five mobile elements as BKT015925, plus a sixth one which is attributed to phage sequence family phiSM101, a 38-kbp phage encountered in *C. perfringens* (Myers et al., 2006). Strain 38028 contains the five mobile elements of BKT015925, plus phiSM101 sequences and a seventh attributed phage sequence, phi3626, a bacteriophage of 33.5 kbp characterized in *C. perfringens* (Zimmer et al., 2002). Strains 12LNR10, 29401, 55741, and 58752 lack the plasmid p4 sequence [phiCD6356, encountered in *C. difficile* (Horgan et al., 2010)] from BKT015925, but harbor phiSM101. Isolate 49511 lacks elements phi6356 and plasmid p5, but contains phiSM101. Strains 12LNR1, 43243, and 71840 lack phiCD6356 element from BKT015925. Strain 12LNR13 lacks sequences phiCD6356 and p5 from BKT015925, but contains a sequence attributed to phiS63, a 33.6-kbp phage from *C. perfringens* not found in any other isolates. Finally, strain 69285 lacks phiCD6356 and p5 from BKT015925 and does not contain any other mobile genetic elements. Thus, using the presence/absence of mobile genetic element sequences in *C. botulinum* type C/D isolates, it is possible to differentiate eight groups, which constitutes a non-negligible improvement in the typing of these strains



compared to previously developed flagellin typing method (Woudstra et al., 2015a). The search for extrachromosomal elements in type D/C isolates showed them to contain sequences related to p1, p2, and p3. Isolates 47295 and 51714 contain also phiSM101, whereas LNC5 does not, and no other phage related sequences except for the botulinum prophage. It was quite surprising to see such difference in the mobilome content between type C/D and type D/C isolates. We thus asked ourselves the reason of such difference. We hypothesized that some mechanism regulating the presence or absence of extrachromosomal elements could be incriminated, such as the CRISPR–Cas (clustered regularly interspaced short palindromic repeats–CRISPR-associated proteins) system.

Comparative CRISPR Analyses

The CRISPR–Cas mechanism is an adaptive immunity system that is present in many archaea and bacteria and which act against invading genetic elements. It consists of distinct arrays of short repeats interspersed with unique spacer's content, with Cas proteins encoded by putative operons adjacent to CRISPR sequences. One hypothesis to explain the diversity of extrachromosomal elements encountered in type C/D strains and the absence of such elements in type D/C, would be the

CRISPR loci to be different between them. Analysis of the CRISPR–Cas sequences of the reference genome *C. botulinum* type C/D BKT015925, showed to contain CRISPR–Cas genes Cas3/Cas5 (located between the IS transposase proteins) and Cas3/Cas5/Cas6, present on the prophage (p1) and on plasmid p2, respectively (Skarin et al., 2011). Based on a review by Makarova et al. (2011), we could assume that these CRISPR loci belong to CRISPR type I-B and are incomplete. It therefore could be hypothesized to be non-functional for BKT015925 type C/D. Investigation of the genome sequences for the French type C/D strains revealed that CRISPR loci were of type I-B also and located on plasmid p2. It contains Cas3/Cas5/Cas6/Cas7 and a homolog gene to Cas8b1, lacking Cas1/Cas2 and Cas4 genes. Cas1 is involved in the integration of spacer DNA into CRISPR repeats, Cas2 facilitates spacer selection and/or integration, and Cas4 might be involved in spacer acquisition (Makarova et al., 2011). We can therefore suggest that this CRISPR locus is deficient in integrating new foreign DNA. The CRISPR-associated (Cas) proteins that provide acquired immunity against foreign DNA are inactive in type C/D strains, which could explain the presence of so many extrachromosomal elements. On the contrary, type D/C strains contain a fully functional type I-B CRISPR system and a partially deficient type

TABLE 2 | C. botulinum type C/D genetic mobile element ID card.

Strain	Origin	p1	p2	p3	p4	p5	Phage attributed sequence			Phage group
							phiSM101	phiS63	phi3626	
BKT015925	Chicken	+	+	+	+	+	-	-	-	I
48212	Duck	+	+	+	+	+	-	-	-	I
12LNR1	Duck	+	+	+	-	+	-	-	-	II
43243	Guinea fowl	+	+	+	-	+	-	-	-	II
71840	Chicken	+	+	+	-	+	-	-	-	II
50867	Chicken	+	+	+	+	+	+	-	-	III
58272	Chicken	+	+	+	+	+	+	-	-	III
38028	Chicken	+	+	+	+	+	+	-	+	IV
12LNR10	Turkey	+	+	+	-	+	+	-	-	V
29401	Chicken	+	+	+	-	+	+	-	-	V
55741	Turkey	+	+	+	-	+	+	-	-	V
58752	Chicken	+	+	+	-	+	+	-	-	V
12LNR13	Chicken	+	+	+	-	-	-	+	-	VI
69285	Chicken	+	+	+	-	-	-	-	-	VII
49511	Chicken	+	+	+	-	-	+	-	+	VIII

C. botulinum type C/D strains are grouped according to their mobile genetic content. P1 contains botulinum and C3 toxin, p2 contains alpha-toxin, p3 contains C2 toxin and phospholipase C, p4 contains phage sequences attributed to phiCD6356 and finally p5 contains C. perfringens putative epsilon toxin (Skarin and Segerman, 2014). PHAST web server-attributed phage sequences (phi SM101, phiS63, phi3626) were identified.

III-B system (lacking *cmr1* and *cmr3* genes, which could be involved in RNA-guided RNA cleavage), chromosomally located. It was furthermore possible to identify a fully functional CRISPR sequence related to system I-D, located on plasmid p2. Type D/C strains were shown to contain two active CRISPR systems and one potentially inactive system; this would explain why phage elements were so scarce in the genome of the type D/C strains investigated.

CRISPR regions are also known in other bacteria to be an appropriate target for high-resolution strain typing (Delannoy et al., 2012), the spacer sequences being representative of the historical encounter of the bacteria with extrachromosomal mobile genetic elements. As we were not able to use the mobile content in type D/C strains as typing targets, we investigated the CRISPR sequences diversity to achieve typing resolution. Isolates 47295 and 51714, which were highly genetically related with strain DC5 from Italy, showed their CRISPR content to be quite different, with only two common CRISPR loci. It was also possible to differentiate between strain 47295 and 51714 by one CRISPR loci. Based on their genome sequences comparison we could hypothesize that strains 47295, 51714, and DC5 are closely related. The analysis of their CRISPR content confirms this assumption but could also help differentiate them. Also, CRISPR sequences of strain LNC5 type D/C was observed to differ greatly from that of strains 47295 and 51714 with only one CRISPR locus that could be related to strain 51714, all other CRISPR sequences being totally unrelated, supporting the hypothesis that strain LNC5 is of a different origin. Although strain LNC5 was closely related to strain CCUG7971 from South Africa, they share only one CRISPR loci. On another hand, investigation of French type C/D isolates for their CRISPR genetic diversity revealed it to be not discriminatory enough to differentiate between them,

as expected due to the presence of a deficient CRISPR-Cas mechanism.

DISCUSSION

Seventeen isolates of C. botulinum group III types C/D and D/C were characterized *in silico* using whole genome sequence data. No genome data from French C. botulinum group III strains was available beforehand. The previous work of Skarin and Segerman (2014) was the first to investigate and to grant insights into the genomics of C. botulinum group III. It pointed out the high degree of genetic relatedness between isolates from different sources and geographical origins. The present work investigated French isolates in order to shed light on the genomic diversity of C. botulinum group III. The analyses confirmed marked similarity among the investigated strains and the publicly available genome sequences originating from Europe, clustering French strains into lineage Ia and Ib. This had already been reported when comparing strains from Spain and Sweden (Anza et al., 2014). No correlation was observed between geographical origin and source of the strains. It was possible to clearly discriminate type C/D from type D/C. Interestingly, there was a marked genetic correlation between strain LNC5 type D/C, isolated from cattle, and historical strain CCUG7971 type D, isolated in South Africa in 1929. This appears to show that LNC5 is more closely related to CCUG7971—despite being a type D/C and not D strain like CCUG7971—than to European type D/C strains. It might be interesting to compare group III C. botulinum from parts of the world outside Europe to investigate such potential geographical differences. Our investigation also revealed genomic diversity into the Dnd locus responsible for phosphorothioation DNA modification of the

DNA backbone, with sulfur replacing non-bridging phosphate oxygen, and being responsible for a degradation pattern observed during PFGE electrophoresis. It is hypothesized that endowment of DNA with sulfur gives it an oxidation-reducing chemical property that protects the hosting bacteria against peroxide (Xie et al., 2012). Therefore, bacteria lacking this functional mechanism would be typeable by PFGE, but probably more sensitive to oxidative stress.

A huge rise in the number of avian botulism outbreaks in poultry production was observed in France between 2007 and 2011. The origin of this upsurge is typically unknown. Gegenees and SNP analysis show that strains 49511, 38028, and 12LNR13 differ from the other type C/D strains studied here. These strains were isolated from broiler outbreaks in 2008 and 2012. This result seems to show that at least two groups of type C/D strains were involved in poultry botulism outbreaks between 2008 and 2012 in France. Interestingly, BKT015925—which is very similar to 12 C/D strains included in our study—was also isolated in 2008 from broilers in Sweden. Strain V891, which is very similar and related to strains 49511, 38028, and 12LNR13, was isolated in 2007 from a wild herring gull that had died from botulism in Sweden. This result implies that at least two groups of *C. botulinum* type C/D strains are involved in avian botulism outbreaks in Europe. It appears that there is no correlation between avian species and the type C/D group, but this tends to demonstrate that the same strains are involved in poultry and wild bird outbreaks. It is now necessary to analyze the genome of strains isolated from botulism outbreaks in wild birds to confirm this initial observation.

More detailed investigations of mapping assembly sequences, and particularly the unmapped reads, using the PHAST web server, revealed new genetic elements related to phage sequences in type C/D strains. These results helped discriminate closely related isolates into eight different groups. Interestingly, type D/C strains showed to harbor few mobile elements in comparison. The presence of new mobile genetic elements, which could be phage related, is in accordance with the large plasmidome content already characterized in *C. novyi sensu lato* (Skarin and Segerman, 2014). Therefore, by using specific genetic targets for each plasmid or phage sequence, it may be possible to generate an ID card, which would constitute an improvement in typing development. Yet this method only constitutes a pre-screening step that would be applicable only to type C/D strains. While investigating the extrachromosomal content we showed that the difference in the mobilome content between type C/D and type D/C strains could be attributed to a deficient CRISPR-Cas mechanism in strains type C/D. The investigation of CRISPR sequences diversity in type D/C strains for typing purposes helped differentiating between highly genetically related strains, while it did not improve matters in discriminating between type C/D strains. In conclusion, the mobilome content could be used for typing C/D strains, whereas CRISPR loci would be preferable to differentiate between type D/C strains.

In silico MLST could also be an option to investigate the genetic diversity of *C. botulinum* group III. The latest work of Weedmark et al. (2015b) investigated the possibility of

using whole genome sequence data to resolve phylogenetic profiling of *C. botulinum* group II by outbreak or location of origin. A preliminary *in silico* test of the French *C. botulinum* group III genome sequences produced showed it was only possible to discriminate between type C/D and type D/C strains using Weedmark's MLST-12 pattern (data not shown). This finding is not surprising as *C. botulinum* group III is genetically distantly related to groups I and II. It does not mean the method is irrelevant, but it will have to be adapted by defining specific locus targets for *C. botulinum* group III.

Regarding SNP genotyping, the phylogenetic trees obtained while investigating the French type C/D and D/C strains, compared with the closest publicly available genome sequences, allow three major groups to be differentiated. The French type D/C strain was shown to be very distant from type C/D, which may imply a different historical origin. The French type C/D strains were closely related, which corroborates the hypothesis of a clonal spread throughout Europe. Yet using SNP core phylogeny, it was possible to achieve the deepest characterization level, resolving strains by their outbreak origins. It is now necessary to sequence strains isolated from other countries and other outbreaks to identify discriminating SNPs that could be used for routine genotyping analysis. This opens up new avenues for epidemiological investigations and will be particularly useful for identifying sources of outbreaks.

CONCLUSION

The comprehensive analyses of phage, plasmid, CRISPR, and SNP content revealed significant differences between strains of *C. botulinum* group III, showing plasticity in both the chromosomal core and mobilome of these strains, providing discriminative information for future genetic studies. In the light of the results presented here, we may speculate these strains to have probably different capacity to adapt and evolve in new environments.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: CW and PF. Performed the experiments: CW. Analyzed the data: CW. Contributed reagents/materials/analysis tools: CW, CL, RS, M-HB-A, IM, DD, and PF. Wrote the paper: CW and PF. Critical revision of the paper for important intellectual content: CW, CL, RS, M-HB-A, IM, DD, and PF.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Main results:

- *De novo* and mapping genome assembly methods complement one another.
- Unmapped reads contained interesting genetic elements which differ from the reference genome used.
- French *C. botulinum* Group III strains are highly genetically related by toxin type and clustered with phylogenic group Ia and Ib together with European strains.
- Genome investigation revealed a high number of mobile genetic elements; their presence could be used to generate a strain-specific genetic ID card.
- Investigation of Crisprs sequences as potential typing targets was inconclusive for type C/D but remain to be thoroughly investigated for type D/C.
- Core SNP profiling is able to differentiate even the highest genetically related strains.

Main conclusions:

- The analyses confirmed the high degree of genetic relationship between French and European strains, supporting the hypothesis of a clonal spread for *C. botulinum* group III.
- The presence of a high number of mobile genetic elements is in accordance with the large plasmidome content already characterized in *C. novyi sensu lato*.
- *C. botulinum* type C/D Crispr loci are incomplete and therefore non-functional for incorporating new genetic element while type D/C strains showed to contain at least two complete Crispr loci.
- The presence of several extrachromosomal elements in type C/D strains could be explained by the presence of a deficient Crispr mechanism.

Perspectives:

C. botulinum Group III characterization could be improved using altogether *bont*, flagellin and phage genotyping. Also, it would be helpful to use the genome sequences generated to develop MLST scheme or specific SNPs detection method, to trace back the source of the contamination during an outbreak situation.

General Discussion and Conclusion

Chapter VI

Discussion

Botulism has been intensively studied since its discovery, and it is not presumptuous to say that the disease is today well understood. A lot of attention focuses on the toxins, which are the real danger. *C. botulinum* classification was indeed based on the ability of the organism to produce these deadly toxins responsible for the disease. The organisms responsible for botulism are different at the phenotypic level, which was confirmed by the recent advances in genome sequencing (Carter and Peck, 2015). Their physiological characteristics are so different (Group I are proteolytic, Group II are not, Group III is only found in animals, Group IV contains only strains producing BoNT serotype G), from a taxonomic point of view it is recognized that a nomenclature based on BoNT production is unsatisfactory and that in most circumstances, the different metabolic Groups of *C. botulinum* would be assigned to entirely different species (Collins and East, 1998). Furthermore, at the Group level *C. botulinum* shows close genetic relationship with other non-toxigenic bacteria species (e.g. *C. botulinum* Group I and *C. sporogenes*, *C. botulinum* Group II and *C. beijerinckii*, *C. botulinum* Group III with *C. novyi*, *C. botulinum* Group IV and *C. subterminale*). The only characteristic shared by the organisms responsible for botulism is the production of botulinum toxins, which were most presumably acquired by horizontal genetic transfer (Hill et al., 2007). One of the actual challenges for scientists consists in characterizing and differentiating in-between the same toxin producing Clostridia, to trace back to the outbreak level and find the origin of the contamination.

VI.1. Genetic signatures for characterizing strains of *C. botulinum* from Groups I to III

One of the challenges of my PhD work was to look for strain specific genetic signatures for outbreak source tracing of *C. botulinum* Group I, Group II and Group III. The findings I obtained, while investigating flagellin genes *flaA*, *flaB* and *fliC* in *C. botulinum* strains of Group I-II and III respectively, suggest the organism may vary with geographical origin and toxin type. Five major groups and fifteen subgroups shaped *C. botulinum* Group I and II organization, while only five groups were unravelled investigating *C. botulinum* Group III. Using flagellin as genetic target for characterizing *C. botulinum* strains seemed a

promising choice for genetic and epidemiological surveillance of the organism. Yet, it was not possible to differentiate up to the strain level. The differences of the flagellin encoding genes observed between the Groups could be explained by the bacterial life cycles. *C. botulinum* is a spore forming organism naturally present in soil and water. Because of the anaerobic nature of *C. botulinum*, the bacterium is unable to survive in aerobic environments as vegetative form. The dormant spore stage constitutes the main form of dissemination. Therefore, their genetic rate of evolution at the strain level could be less important than other non-spore-forming bacteria. This theory is supported by Weller et al. work, which showed evidence for an effect of spore formation on evolutionary rate (Weller and Wu, 2015). While comparing the genetic diversity of flagellins observed between Group I and II, it appeared that Group I showed the greatest flagellin diversity (Paul et al., 2007). Flagellin genes from strains of Group III revealed on the contrary a particularly low genetic diversity which is in accordance with previous work (Anza et al., 2014a; Skarin and Segerman, 2014). One hypothesis to explain such difference in *C. botulinum* Group III would be to consider a bacterial clonal spread. It is supported by the results I obtained during the course of my PhD regarding investigation of European biological material for *C. botulinum* Group III. The results brought out that mosaic type C/D and D/C are predominantly represented in the samples investigated. Nevertheless, it is still possible to encounter *C. botulinum* type C and D strains responsible for animal botulism outbreaks (Bano et al., 2015), but it is more and more uncommon in Europe (Woudstra et al., 2012). This phenomenon could be explained by the increased toxicity of mosaic types (Nakamura et al., 2010), which could have led these mosaic toxins to become predominant in *C. botulinum* Group III bacteria.

The next question which then comes into mind is when and how the formation of these mosaic toxins happened. Unfortunately, as no big scale study was done before 2012 about the prevalence of mosaic types in samples from animal botulism origin, it is only possible to speculate. When we look at *C. botulinum* Group III timeline, BoNT/C and D were discovered in the 1920s (Bigalke, 2012). The discovery of bacteriophages which contain the toxin gene and the interconversion mechanism are dating back to the 1970s (Eklund and Poysky, 1974; Inoue and Iida, 1971). Cross neutralization between type C and type D antiserum were demonstrated already in the 1980s (Oguma et al., 1981; Oguma et al., 1980). While investigating this particularity, Oguma et al. found out that *C. botulinum* Group III could be resolved in four different groups, based on their toxin antigenicity (Oguma et al.,

1986). Finally, toxin type C and D gene sequences were solved in 1990 (Binz et al., 1990; Hauser et al., 1990). The first C/D and D/C mosaic gene sequences to be published dated back to 1996 (Moriishi et al., 1996b). They were respectively related to strain 6813, isolated in 1971 from marine sediment (Segner et al., 1971), and strain D-Sa isolated from south Africa in the 1920s (Bigalke, 2012). It means that the mosaic strains were already circulating at that time. Thus, the genetic events which have led to the formation of *bont* type C/D and D/C should have taken place long ago. The thorough study of Skarin et al., about the genetic diversity of *C. novyi sensu lato*, enlightened the genetic organization of the mosaic genes at the sequence level (Skarin and Segerman, 2014). By comparison of type C, C/D, D and D/C it revealed the approximate position where the probable recombination event may have arisen. The chimeric *bont* genes are probably the result of a co-infection of two *bont*-encoding phages of different serotypes, where homologous recombination events have led to the chimeric *bont* genes. The authors hypothesize type C/D *bont* gene to be a recombination product between an existing type D BoNT-encoding phage and a *bont* gene from a transiently co-infected type C phage, and the other way around for serotype D/C. Type C is predominantly associated with avian botulism whereas type D is associated to cattle. Several studies showed that cattle could be contaminated by type C after an exposure to contaminated poultry litter (Bienvenu et al., 1990; Kennedy and Ball, 2011). In the presented work (Woudstra et al., 2012) several samples from avian or bovine origin (faeces, spleen, intestinal content) were showed to contain both type C/D and type D/C at the same time. It constitutes strong evidence that more than one strain of *C. botulinum* Group III, of different toxin type, could be present at the same time, in the same sample. Such conditions could leave the door open for genetic recombination events which could have led to the creation of the mosaic toxin types.

What was also revealed by the genetic study of *C. botulinum* Group III by Skarin et al. (Skarin and Segerman, 2014) is the presence of a large plasmidome consisting of plasmids and circular prophages. The investigation of *C. novyi sensu lato* strains revealed sixty one plasmids distributed in thirteen groups. The characterized reference strain BKT015925 showed to contain not less than five mobile genetic elements. This study enlightens the genetic complexity of these bacteria mainly driven by prophages. Considering this point, it was conceivable to characterize strains of *C. botulinum* Group III on the basis of their mobile genetic signature that might be genus- or species-specific. The French genome sequences investigated in my PhD work were thus search for phage sequences, and three new phage

DNA sequences were unravelled. The constituted genetic ID card based on phage sequences allowed dividing the fourteen types C/D French strains in eight groups. Such approach was already used for typing Brucellosis outbreaks in Sweden, showing phage analysis to be a useful tool for successful assignment of strains of the same origin within a single outbreak (Kaden et al., 2014). Therefore, it would be interesting to develop real-time PCR detection system, specific for each phage that was shown to be associated with *C. botulinum* Group III. Also, it is interesting to underline that one strain (type C/D 38028) even showed to contain up to seven mobile genetic elements. It constitutes a particularly large mobile genetic content which would worth to be investigated. Regarding strains from type D/C, the few genome sequences available do not allow a deep investigation of their prophage genetic content. Also, no reference genome of *C. botulinum* type D/C is yet available. Such material would be of great help while investigating cattle botulism. Unpublished experiments conducted in our laboratory showed that samples containing both *C. botulinum* prophage type C/D and D/C at the same level after 24h of culture in TPGY at 37°C, see their population change. Type D/C disappears while type C/D persists. As the detection was made using real-time PCR targets designed for *bont* genes, it means *C. botulinum* type D/C is more prompt to lose its phage than type C/D in the same cultural conditions. It could reflect a different behaviour linked to the prophage. Considering its size (200 kbp), it is not surprising that the bacteria would not keep it in an environment where it is unnecessary. The full sequence of the *bont*-phage was published in 2005 (Sakaguchi et al., 2005). It revealed that most of the coding DNA sequences (CDS) present on the *bont*-phage are coding for hypothetical protein of unknown functions. Still, little is known about the basic biology mechanisms of the phage. Knocking out phage genes using the Clostron (Heap et al., 2010) or the *pyrE* universal mariner transposon system (Zhang et al., 2015) would constitute a good start to investigate the mechanism underlying the biology of *C. botulinum* Group III phages.

VI.2. Whole genome sequencing and SNP genotyping of *Clostridium botulinum* Group III strains from France

Classical detection and typing methods, using real time PCR, developed to investigate the genetic diversity of *C. botulinum* Group I, II and III presented in this work, brought some insight in the bacterium diversity. But they were finally not accurate enough to resolve up to the strain level relatedness. On another hand, the investigation of the genetic diversity of

French *C. botulinum* Group III strains by whole genome sequencing gave much more interesting results. French isolated strains were originated from outbreaks that occurred across a wide area of France; therefore we were particularly interested in determining whether these strains have genetic relationship or regional differences. One interesting finding is the absence in some strains of a functional phosphorothioation DNA modification system (Dnd). It explains the encountered problem of DNAs degradation during PFGE runs, and why some strains could be typed using PFGE and some could not, using a standard protocol. Using these Dnd genes as genetic targets, it could be possible to develop a preliminary assay to test for this mechanism to be functional, therefore knowing which PFGE protocol to use for investigating strains diversity.

Full genome comparison using the Gegenees software, which is able to create a matrix comparison based on Blast analysis of genome sequences, show a high degree of genetic similarity in-between the *C. botulinum* Group III French strains investigated, but also with the publicly available genome sequences from other *C. botulinum* Group III. This kind of analyses is useful to get a first idea of the level of genetic identity shared by bacterial strains, but it is not accurate enough to differentiate up to the strain level. These results are concordant with the assumption that strains of *C. botulinum* Group III could be the result of a clonal spread (Anza et al., 2014).

Analyses of the extrachromosomal elements present in the genome sequences investigated confirm the presence of a large plasmidome in strains type C/D. Considering the high number of extrachromosomal elements present in several strains, it suggests genome evolution is mediated by a plastic plasmidome, which contain the pathogenicity factors and could therefore allow faster environmental adaptation. Furthermore, sequence comparisons of p1 to p3 elements, which are always associated with type C/D strains, show to contain conserved regions (e.g. BoNT, C2, C3, Phospholipase toxin encoding regions), but miss also several sequences. But as most of the encoding genes are of unknown function, it is not possible to conclude whether or not these missing sequences could result from an adaptive response to environment conditions, or an ongoing size reducing mechanism to remove non-essential DNA. What could be taken for certain is that these elements are going under active genomic evolution, which may be link to a better adapted genome for toxicoinfection.

Investigations of whole genome sequences of strains type C/D show that Crisprs loci do not show a high degree of genetic variability. The first practical application of CRISPR-Cas

systems was typing of bacterial diversity (Kamerbeek et al., 1997). Several typing methods were developed based on CRISPR locus size, spacer content of a given locus, or point mutations within specific spacer and/or repeat sequences CRISPR arrays, which constitute a record of past infections (Shariat and Dudley, 2014). One objective, while sequencing the whole genomes of French *C. botulinum* Group III strains, was to investigate Crisprs sequences as potential genetic targets for the development of typing scheme. It appeared that Crisprs loci present on extrachromosomal element were not discriminative. The deep investigation of Crispr-Cas loci of strains type C/D and D/C revealed an inactive mechanism for type C/D, while type D/C strains contain two active and one inactive systems. The inactive system in type C/D strains is concordant with the presence of numerous phages and extrachromosomal elements. Also on the other hand, presence of two active Crispr systems in type D/C explains the low number of extrachromosomal elements detected. As type D/C strains showed to have active Crisprs system, it could be hypothesized these strains would be typeable using a Crisprs based sequencing approach. This remains to be investigated.

While exploring SNPs core typing, a deeper level of discrimination was achieved compared to all other methods used. French type C/D strains clearly showed to be highly genetically related, which support the hypothesis of a clonal spread. Still, using SNPs phylogeny it was possible to distinguish between each strain, and even differentiate up to the outbreak level. French type D/C show to be clearly separated from type C/D, which is concordant with the previous observation made. Also it was possible to clearly differentiate between the two French strains and the one from New Caledonia, which is closely related to the historical strain CCUG7971 type D originating from South Africa. Yet, the low number of genome sequences of strains type D/C does not allow a thorough study of its genetic diversity, which remains to be investigated. Extraction of core genome SNP markers may be useful for future epidemiological studies of *C. botulinum* Group III that require high resolution for strain discrimination. With the level of discrimination achieved there is no doubt that in the next decade more and more phylogenetic study will be resolved using this method.

Assuming that next generation sequencing technology will be routinely used in the future, we can expect more genome sequences to be publicly available in the next years, which would improve the understanding of *C. botulinum* Group III behaviour. One challenge will be the development of new integrated bioinformatic tools that will allow deeper analyses of these massive amounts of generated data, that would still need to be correlated to

biological phenotype. Another important point is the availability of reference genome sequences of good quality. Here we could rely on *C. botulinum* type C/D BKT015925 as reference, but such reference is still missing for type D/C. Usually type D/C is correlated with bovine botulism. As botulism outbreaks are less frequent in bovine than in poultry, and therefore assumed to be less important, type D/C investigation was not made a priority. But with the further development of detection and typing methods, it was possible to detect and correlate type D/C also with poultry botulism, even detecting both types C/D and D/C co-circulating during an outbreak (Woudstra et al., 2012). In the light of this information, solving type D/C full genome sequence should now be considered a priority.

VI.3. Main achievements

The main outcomes of the PhD work I achieved, through the four main scientific articles and my last results are sum up here:

- Development of flagellin genetic typing method to characterize *C. botulinum* Group I, II and III appeared to be a promising tool for epidemiological purposes. Nevertheless it failed to discriminate between highly genetically related strains.
- Investigation of *C. botulinum* Group III isolates originating from various European countries revealed mosaic types C/D and D/C to be predominant in the samples investigated.
- Analyses of the genome sequences of *C. botulinum* Group III strains from French origin showed them to be genetically related to other Group III European strains.
- *C. botulinum* Group III mobile genetic elements present in French strains showed to be highly variable. Presence of such elements that constitute the 'mobilome' could be used as a genetic ID card for deeper strains characterization.
- Crispr typing was not relevant for differentiating *C. botulinum* Group III type C/D strains due to an inactive Crispr loci, while type D/C contain two active Crispr systems and remains to be thoroughly investigated.
- Core SNP phylogeny profiling constitutes the most powerful tool to investigate highly genetically related strains.

VI.4. Next perspectives

This PhD work brought out some new insight in the research field of *C. botulinum*, nevertheless several research points still need to be investigated:

Typing *C. botulinum* Group III is still difficult due to the absence of standardised methods discriminatory enough. One of the first points that would worth new research would be the development of detection method for characterizing the genetic mobile elements of *C. botulinum* Group III. Using specific genetic targets derived from these different mobile genetic elements, it would thus be possible to generate a strain-specific genetic ID card. *C. botulinum* Group III type C/D strains always contain at least three mobile elements: the botulinum prophage (plasmid p1) and plasmid p2 and p3. Plasmid p1 would be targeted using *bont* specific gene. For plasmid p2, Alphatoxin or Clostripain analogue genes would constitute good target candidates. C2 toxin component I and II, or phospholipase C present on plasmid p3 would worth the investigation as potential targets. The other mobile genetic elements, meaning plasmids p4 and p5, and the newly detected phage sequences phiSM101, phiS63 and phi3626, constitute the variable part of the mobile elements whose presence or absence will shape the genetic ID card. Sequencing more genomes of *C. botulinum* Group III in the next coming months will help to narrow down such genetic ID card identification system. In addition to these genetic targets that are related to the 'mobilome' some chromosomal signatures could be added and our work on the molecular typing of the *fliC* genes would be used to complete the genetic ID card.

Another point that needs to be addressed would be the development of new MLST typing profile specific for *C. botulinum* Group III. To date PFGE is still used as reference typing method but it is not discriminatory enough for strains of Group III. As demonstrated for *C. botulinum* Group I and II, the level of discrimination between related strains using MLST is far more interesting than PFGE. Furthermore it is possible to create an allele database with the obtained profile that could be easily exchanged between laboratories. This perspective is very interesting with regard to the development of a network of reference labs and the creation of a European reference laboratory for animal botulism. Selection of specific gene targets and their alleles for MLST typing of *C. botulinum* Group III would certainly represent a significant improvement in the future.

Using the whole genome sequencing expertise I acquired during my PhD work, one of my next objectives would be to sequence and analyse more genomes of *C. botulinum* Group III from different European origin, to compare them at the phylogenetic level. Also it would

be of great interest to complete and annotate the full length genome sequence of a *C. botulinum* Group III type D/C strain. Finally, even if the mosaic types are now predominantly responsible for botulism outbreaks in avian and cattle, some rare cases of type C and type D are still detected. Comparing their genome sequences with the historical strains type C Stockholm (originating from Sweden, 1949, from a mink outbreak) and type D 1873 (originating from Chad, 1958, from the only known human outbreak, related to ham consumption) would certainly help to understand how *C. botulinum* Group III has evolved in the last century. The genome sequences available after such work could also help to understand basic biology of the phage. The massive p1 mobile genetic element (200 Kbp) containing botulinum prophage needs to be better characterized. Indeed, still little is known about the natural factors that control phage infection and replication within the bacteria. A large majority of the genes present on this mobile element are of unknown function, and no doubt characterizing them would help. To achieve this objective the Clostron or the pyrE knock-out gene system could be used to inactivate specific targets. As I do not have enough knowledge on phage biology and gene inactivation, it would be a good opportunity for me to expand my skills on the subject by participating to conference, or to be trained by skilled teams. It would be interesting to meet other scientists and exchange about this subject, maybe extending our collaboration. One of the best options would be to participate in a European project. Precisely, our agency, Anses, applied for a 36-month collaborative project entitled "Animal botulism: innovative tools for diagnosis, prevention, control and epidemiological investigation, ANIBOTNET". The work is actually lead by Dr. Caroline Le Maréchal from Anses-Ploufragan. The project is granted by EU and is planned to start beginning of April 2016. This project, under the ANIWH (Animal Health and Welfare) ERA-Net, will involve eight research groups from different European countries, including our Anses group, with complementary expertise in *C. botulinum*, BoNT, mass spectrometry, veterinary diagnostics, genomic studies, epidemiology, and animal experiments. It will promote diagnosis, detection and epidemiology development to improve management of botulism in poultry and bovine production in Europe. No doubt this project would be a perfect opportunity to extend the understanding of *C. botulinum* Group III bacteria.

Chapter VII

General Conclusion

As I discovered during my thesis, the scientific community worldwide working on *C. botulinum* is mostly composed of small teams, scattered all around the world. The subject is not driving as much attention as others, like cancer or major disease studies do (e.g. diabetes), but scientists working on *C. botulinum* are as competent as any others. I had the chance to work under the supervision of Dr. Patrick Fach, who worked himself during his PhD on *C. botulinum*. It was of great help to begin my work, as I was able to retrieve strains and DNAs collection from his thesis. The next challenge was to update and grow the materials at my disposal. And for that there is no better solution than to collaborate, share and exchange with the other teams working on the same subject. My first successful contact was with the Japanese team of Dr. Shunji Kozaki, from the university of Osaka prefecture, who characterized the mosaic toxins types C/D and D/C involved in animal botulism. I remember my thought while I was doing my bibliographic research and reading their work published in 2005: "*C. botulinum Group III produce toxin types C, D, and mosaic toxins C/D and D/C. The catalytic part responsible for toxic activity is conserved between type C and mosaic C/D, and type D and mosaic D/C. The genetic detection tests available were developed to detect bont type C and type D. Those tests still work for the detection of the mosaic genes. But it is not possible to make the distinction between strains of C. botulinum type C and type C/D, or between type D and type D/C. The Japanese team of Kozaki already showed that mosaic toxins were more toxic than the original ones. They also proposed a PCR detection test to distinguish between the normal and mosaic bont genes, but they did not study at a larger scale the proportion of C. botulinum Group III harbouring normal or mosaic genes.*" And this is how begun my first collaboration. I contacted Dr Masato Takeda from Dr. Kozaki's team, asked him for DNA samples, which he nicely sent to me. I then started to work on the development of a real time PCR detection test to differentiate between normal and mosaic genes. The Japanese team already developed conventional PCR method for that, so my work was not bringing much novelty, but the real-time PCR I developed could facilitate large epidemiological investigations. What was important, except for the convenience of real time detection, was to test for the presence of these mosaics compared to the normal *bont/C* and *bont/D* elsewhere than Japan. I had the reference DNAs from

Japan, what I needed was some botulism DNA samples from the field, to put to the test the real time PCR method. Again, it was possible to get them with the help of Dr. Jean-Philippe Buffereau and his team, from LDA22 (today renamed LaboCEA) in Brittany (France). They were already testing their samples for animal botulism, but with the nested-PCR system developed by my superior Dr. Patrick Fach, during his PhD. They were really interested in switching from conventional nested-PCR to real time PCR, and my request was warmly welcomed. It was nice thinking that I could continue the work Dr. Patrick Fach started several years ago and improve it. With these samples it was possible to get an overview of mosaic *bont* genes in France. What I found was that only mosaic types were present in the samples investigated. It was scientifically intriguing. At that time, we were working on a collaborative proposal at the European level, regarding bioterrorism in animals. I had the chance to participate to this project, AnibioThreat (granted by the DG Justice), and even to lead a work package together with him. It was a great opportunity to collaborate with many other European teams working on *C. botulinum* Group III, and for that I would like to thank Dr. Rickard Knutsson, the leader of this project. Together with the other team we were able to show that animal botulism outbreaks we investigated in Europe were mainly associated with mosaic types. The results of this big collaborative work were published in *Applied and Environmental Microbiology* journal (paper N°2). Later on we also organized a ring trial proficiency test to assess the detection method, which led also to another publication in *Anaerobe* journal. Further studies were also connected to the work we undertook during the AnibioThreat collaborative project, and led to several publications in scientific journals. This project was the keystone that opened up many collaborative possibilities about animal botulism. It is how my work on botulism started to be acknowledged at the European level. It gave me also the opportunity to participate in the French biodefense project NRBC (Nuclear, Radiological, Biological and Chemical hazards) financed by the CEA (Commissariat à l'énergie atomique), which participated in helped me to move my research forward. The next collaboration was with a Canadian team from Ottawa, led by Dr. John Austin. A collaborator of Dr. Patrick Fach working on *E. coli* introduced us. During one of my personal travel through Canada, I met them and discuss about the possibility to collaborate together. We investigated *C. botulinum* Group I and II genetic diversity, using flagellin genes. They had DNA samples; I provided the technology and the typing system. This collaborative work led to another publication in *Applied and Environmental Microbiology* journal (paper N°1). As I was moving forward my work on *C. botulinum*, I contributed to the creation of the National

Reference Laboratory (NRL) for avian botulism. The work was led by Dr. Caroline Le Maréchal, my colleague from Anses Ploufragan. As the Identypath platform had the scientific background on *C. botulinum* Group III, we were naturally associated in the project. The samples gathered thanks to the NRL helped, together with the DNA samples of previous collaborations, to investigate genetic diversity of *C. botulinum* Group III, using flagellin genes. This collaborative work led to another publication in *Applied and Environmental Microbiology* journal (paper N°3). Later on I was also requested by colleagues from New Caledonia, to help them with bovine botulism cases. I transferred them the method for detection of Group III, and got some positive samples in exchange. I was able to isolate one strain out of them. I used it in the last part of my project, together with other isolated strains from the NRL. I investigated the whole genome sequences of *C. botulinum* Group III isolates involved in animal botulism in France (which led to paper N°4 in *Genome Announcement* journal). These *C. botulinum* Group III genomes sequences constitute the next step forward to my work. The amount of data out of a genome sequence could be tremendous. As would be the time necessary to analyse it. Today different software are available for sequences analysis. But it requires time to learn how to use them and how to get a proper interpretation out of the results. This was the last part of my work, which is not yet published but will be soon enough.

This PhD work would not have been possible without the human collaboration I had the chance to be part of. All the skilled scientists I met, all the meeting I participated, all the talk I had, shaped my work and helped me to move forward. Everything I learnt constitutes the base of my scientific philosophy, which I would like to expand and share with the entire community.

Related publications

Additional publications have been produced during this thesis, which are also devoted to the topic:

1) Development and validation of *C. botulinum* detection methods:

- Eva-Maria Hansbauer, Martin Skiba, Tanja Schreiber, Daniel Stern, Martin B. Dorner, Friedrich Finkenwirth, Jessica Wolf, Werner Luginbühl, Andreas Rummel, Ute Messelhäuser, Laurent Bellanger, Cédric Woudstra, Patrick Fach, Brigitte G. Dorner. Detection, differentiation, and identification of botulinum neurotoxin serotypes C, CD, D, and DC by highly specific immunoassays and mass spectrometry. *Analyst*, 2016. In revision.
- Le Maréchal C, Ballan V, Rouxel S, Bayon-Auboyer MH, Baudouard MA, Morvan H, Houard E, Poëzevara T, Souillard R, **Woudstra C**, Le Bouquin S, Fach P, Chemaly M. Livers provide a reliable matrix for real-time PCR confirmation of avian botulism. *Anaerobe*. 2015 Nov 3;38:7-13.
- Anniballi F, Auricchio B, **Woudstra C**, Fach P, Fiore A, Skarin H, Bano L, Segerman B, Knutsson R, De Medici D. Multiplex real-time PCR for detecting and typing *Clostridium botulinum* group III organisms and their mosaic variants. *Biosecur Bioterror*. 2013 Sep;11 Suppl 1:S207-14.
- **Woudstra C**, Skarin H, Anniballi F, Auricchio B, De Medici D, Bano L, Drigo I, Hansen T, Löfström C, Hamidjaja R, van Rotterdam BJ, Koene M, Bayon-Auboyer MH, Buffereau JP, Fach P. Validation of a real-time PCR based method for detection of *Clostridium botulinum* types C, D and their mosaic variants C-D and D-C in a multicenter collaborative trial. *Anaerobe*. 2013 Aug;22:31-7.
- Fenicia L, Fach P, van Rotterdam BJ, Anniballi F, Segerman B, Auricchio B, Delibato E, Hamidjaja RA, Wielinga PR, **Woudstra C**, Agren J, De Medici D, Knutsson R. Towards an international standard for detection and typing botulinum neurotoxin-producing *Clostridia* types A, B, E and F in food, feed and environmental samples: a European ring trial study to evaluate a real-time PCR assay. *Int J Food Microbiol*. 2011 Mar 1;145 Suppl 1:S152-7.
- Fach P, Fenicia L, Knutsson R, Wielinga PR, Anniballi F, Delibato E, Auricchio B, **Woudstra C**, Agren J, Segerman B, de Medici D, van Rotterdam BJ. An innovative molecular detection tool for tracking and tracing *Clostridium botulinum* types A, B, E, F and other botulinum neurotoxin producing *Clostridia* based on the GeneDisc cyler. *Int J Food Microbiol*. 2011 Mar 1;145 Suppl 1:S145-51.

2) Epidemiological studies:

- Bano L, Drigo I, Tonon E, Berto G, Tavella A, **Woudstra C**, Capello K, Agnoletti F. Evidence for a natural humoral response in dairy cattle affected by persistent botulism sustained by non-chimeric type C strains. *Anaerobe*. 2015 Dec;36:25-9.
- Souillard R, Maréchal CL, Hollebecque F, Rouxel S, Barbé A, Houard E, Léon D, Poëzévara T, Fach P, **Woudstra C**, Mahé F, Chemaly M, Bouquin SL. Occurrence of *C. botulinum* in healthy cattle and their environment following poultry botulism outbreaks in mixed farms. *Vet Microbiol*. 2015 Jul 29. pii: S0378-1135(15)00294-1.
- Souillard R, **Woudstra C**, Le Maréchal C, Dia M, Bayon-Auboyer MH, Chemaly M, Fach P, Le Bouquin S. Investigation of *Clostridium botulinum* in commercial poultry farms in France between 2011 and 2013. *Avian Pathol*. 2014;43(5):458-64.

3) Prevention and management of animal botulism outbreaks :

- Anniballi F, Fiore A, Löfström C, Skarin H, Auricchio B, **Woudstra C**, Bano L, Segerman B, Koene M, Båverud V, Hansen T, Fach P, Tevell Åberg A, Hedeland M, Olsson Engvall E, De Medici D. Management of animal botulism outbreaks: from clinical suspicion to practical countermeasures to prevent or minimize outbreaks. *Biosecur Bioterror*. 2013 Sep;11 Suppl 1:S191-9.
- Skarin H, Tevell Åberg A, **Woudstra C**, Hansen T, Löfström C, Koene M, Bano L, Hedeland M, Anniballi F, De Medici D, Olsson Engvall E. The workshop on animal botulism in Europe. *Biosecur Bioterror*. 2013 Sep;11 Suppl 1:S183-90.
- **Woudstra C**, Tevell Åberg A, Skarin H, Anniballi F, De Medici D, Bano L, Koene M, Löfström C, Hansen T, Hedeland M, Fach P. Animal botulism outcomes in the AniBioThreat project. *Biosecur Bioterror*. 2013 Sep;11 Suppl 1:S177-82.

4) Book :

- Uzal, F., Songer, G., Prescott, Popoff M., 2016 in press. Clostridial Diseases of Animals. Animal botulism chapter, Caroline Le Maréchal, **Cédric Woudstra**, Patrick Fach. Pp 303-330. Ames, Iowa: Wiley. DOI:10.1002/9781118728291.ch26.

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Abstract

Clostridium botulinum is the etiologic agent of botulism, a deadly paralytic disease that can affect both human and animals. Different bacteria, producing neurotoxins type A to G, are responsible for the disease. They are separated into different groups (I to VI) on the basis of their phenotypical and biological characteristics. My PhD work was structured by the different projects I was involved in, which were related to *C. botulinum* detection and typing, like BIOTRACER and AniBioThreat European projects, the French national CBRN program, or the French NRL mandate for avian botulism. The main transversal objective I followed led me to develop new methods to trace back the origin of *C. botulinum* contamination, in case of a deliberate, accidental or naturally occurring botulism outbreak. I investigated the flagellin genes as potential genetic targets for typing *C. botulinum* Group I-II and III, responsible for human and animal botulism respectively. Flagellin genes *flaA* and *flaB* showed *C. botulinum* Group I and II strains to cluster into 5 major groups and up to 15 subgroups, some being specific for certain geographical areas, and *flaB* being specific to *C. botulinum* type E. Flagellin *fliC* genes of *C. botulinum* Group III showed to cluster into five groups, with *fliC*-I and *fliC*-IV associated to type C/D and D/C respectively, being not discriminative enough to differentiate highly genetically related strains. I also studied the prevalence of mosaic toxin genes in *C. botulinum* Group III in animal botulism, mainly in poultry and bovine. The results brought out the mosaic toxin types C/D and D/C to be predominant in the samples investigated throughout Europe. Finally, I explored the full genome sequences of 14 types C/D and 3 types D/C *C. botulinum* Group III strains, mainly originating from French avian and bovine botulism outbreaks. Analyses of their genome sequences showed them to be closely related to other European strains from Group III. While studying their genetic content, I was able to point out that the extrachromosomal elements of strains type C/D could be used to generate a genetic ID card. Investigation of Crispr typing method showed to be irrelevant for type C/D, due to a deficient Crispr-Cas mechanism, but deserve more investigation for type D/C. The highest level of discrimination was achieved while using SNP core phylogeny, which allowed distinguishing up to the strain level.

Résumé

Le botulisme est une maladie nerveuse, commune à l'homme et aux animaux, due à l'action de la neurotoxine botulique produite par *Clostridium botulinum*. Il existe 7 types de toxines dénommées A à G. Les bactéries capables de produire cette toxine se différencient en six groupes sur la base de leurs caractéristiques phénotypiques et biologiques. Mon projet de doctorat s'est organisé autour de plusieurs projets de recherche visant à développer des méthodes de détection et de typage du germe et de sa toxine (projets Européens BIOTRACER et AniBioThreat ; projets NRBC-bio ; mandat du LNR botulisme aviaire en France). Lors de mes recherches j'ai concentré mon travail sur le développement de méthodes capables de suivre et remonter à la source d'une contamination, qu'elle soit délibérée, accidentelle ou naturelle. Afin d'y parvenir j'ai investigué les gènes codant pour les flagellines de *C. botulinum* groupe I à III, responsables du botulisme humain et animal. L'analyse des gènes *flaA* et *flaB* a mis en évidence 5 groupes majeurs et 15 sous-groupes, certains étant spécifiques de régions géographiques. *FlaB* s'est montré spécifique de *C. botulinum* type E. Les gènes flagellines *fliC*, spécifiques à *C. botulinum* du groupe III, se divisent en 5 groupes, avec *fliC*-I et *fliC*-IV associés aux types mosaïques C/D et D/C. J'ai étudié la prévalence des souches productrices de toxine de type mosaïques chez les volailles et les bovins. Les résultats montrent que les types C/D et D/C sont majoritaires en Europe. Enfin, j'ai séquencé le génome de 17 souches associées à des cas de botulisme animal en France (14 types C/D et 3 types D/C). Leur analyse montre que ces souches sont très proches génétiquement, entre elles et avec les souches Européennes. Grâce à ces données j'ai mis en évidence un large contenu extra chromosomique dans les souches C/D, qui peut être utilisé pour créer une carte d'identité génétique. D'autre part, l'étude des séquences Crisps à des fins de typage ne s'est pas avérée suffisamment résolutive, du fait de système Crispr-Cas déficient chez les souches C/D. Enfin, un très haut degré de discrimination a été atteint par typage SNP, qui a permis de distinguer jusqu'à l'origine de chaque souche.