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

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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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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. bifementans*; *C. chauvoei*; *C. difficile*; *C. mangenotii*; *C. novyi*; *C. haemolyticum*; *C. edematiensis*; *C. perfringens* types A, C, D, and E; *C. septicum*; *C. sordellii*; *C. sporogenes*; *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 prereduced 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, TTAATAATTGAAGTACACCTTGTTGG. 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

		Assay result					GenBank accession no. ^f	
Strain	Origin	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	ABDQ000000000.1	ABDQ000000000.1
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 ^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
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. Amplification 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	GAACTGATCCTAAATCTGATCTG	
<i>fliC</i> -V_P	TGAGCATTGTTGTCATCTTTACCAGAAACA	
Novyi-sensu-lato_F	GGAACCAACCTACCGAG	100
Novyi-sensu-lato_R	GTAGCCACCTCCTTAACA	
Novyi-sensu-lato_P	TCCAGACACGAAGAGGCTTAATATATCCA	
<i>ntnh</i> -grpIII_F	TACTGATTTATTTAGACCTGATTG	122
<i>ntnh</i> -grpIII_R	CTTTAGGCACCTTGTTATTG	
<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 R^2 , 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 explanation

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</i> _grpIII	<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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