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

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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 cycler platform (Pall-GeneDisc 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 botulism 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-

Received 16 November 2011 **Accepted** 7 February 2012
Published ahead of print 17 February 2012
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Supplemental material for this article may be found at http://aem.asm.org/.

doi:10.1128/AEM.07568-11

TABLE 1 Primers and probes

Primer or probe[a]	Sequence (5′–3′)[b]	Target *bont* 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]AACCCAGTTGTTACCTTGTCTAGTTT[BHQ2]		441–416	
C-II_F	GGGTCAAAATTTATCTCG	C, C-D	1233–1250	135
C-II_R	AGCTCTCTACAATCTAATG		1367–1349	
C-II_P	[ROX]ATCCAGCATTAAGAAAAGTCAATCCT[BHQ2]		1253–1278	
C-III_F	TCAGCTTAATCCAATATTTCC	C, D-C	2697–2717	77
C-III_R	GGGTTACTATAACTTTACCTC		2773–2753	
C-III_P	[ROX]CCCTGAACTACCTAATTTAAAGTCAAA[BHQ2]		2745–2719	
D-C_F	GACTGATTTAGTTCCACTAG	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	TCCTCAAATTGTACGTTG		806–789	
D-I_P	[ROX]AAATCCCTCGCTAACTTGTGGAC[BHQ2]		771–749	
D-II_F	GAAGCATTAGGTTATAGCAATAAG	D, D-C	1696–1719	116
D-II_R	TCCTCAACTACTTCATTCG		1811–1793	
D-II_P	[ROX]AGAATAAACCTGCTTGAACACCTTT[BHQ2]		1783–1759	
D-III_F	ATGGCAATATAGAATGGA	D, C-D	2949–2966	138
D-III_R	ACCCCATTATATTATTAGTTATAG		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 areAB012112, 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-

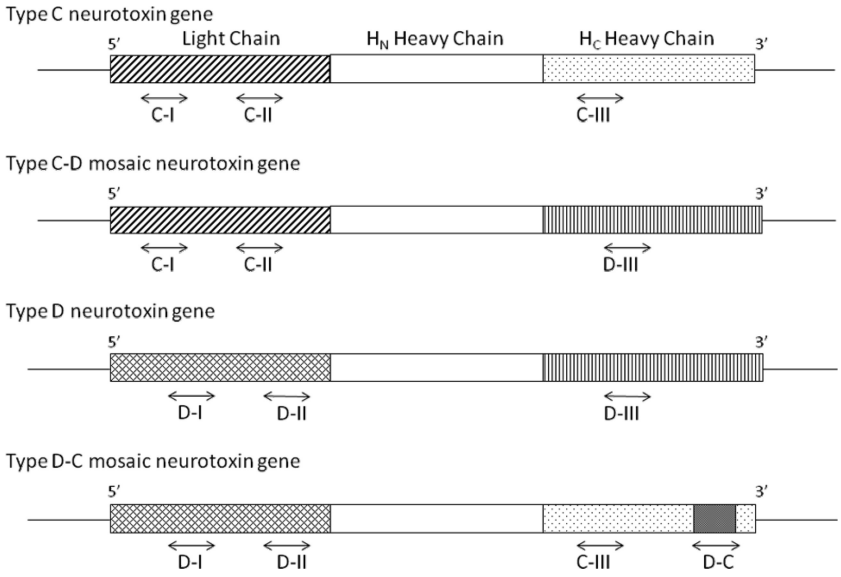

FIG 1 Position of oligonucleotides. Highly homologous domains are represented with the same pattern. GD1 C&D contains the C-I, C-II, D-I, and D-II oligonucleotides, together with the IAC and the negative control. This allows the detection of N-term part of the neurotoxin genes and also allows the detection 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 μg 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]		
		GD1 C&D	GD2 C,D&mosaic	Reference PCR[b]
Clostridium botulinum				
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 *Clostridium butyricum* type E	2	–	–	–
Clostridium botulinum type F	1	–	–	–
Toxic *Clostridium baratii* type F	1	–	–	–
Clostridium botulinum type G	1	–	–	–
Non-BoNT-producing clostridia[c]	20	–	–	–
Non-*Clostridium* 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. bifermentans*, *C. chauvoei*, *C. difficile*, *C. mangenotii*, *C. edematiens*, *C. perfringens* type A, *C. perfringens* type E, *C. perfringens* type C, *C. perfringens* type D, *C. septicum*, *C. sordellii*, *C. spirogenes*, *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 prereduced 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^{2c}	Slope[d]	Efficiency (%)[e]
C. botulinum 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
C. botulinum 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
C. botulinum 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
C. botulinum 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 prereduced 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 toxinotype) 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 results, 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.

ACKNOWLEDGMENTS

This research was supported by the framework of the EU AniBioThreat (grant agreement Home/2009/ISEC/AG/191) project with financial support from the Prevention of and Fight against Crime Programme of the European Union, European Commission–Directorate General Home Affairs. We also acknowledge the DIM malinf Ile de France for financial support of the project.

This publication reflects the views only of the authors, and the European Commission cannot be held responsible for any use which may be made of the information contained therein.

We thank Sophie Roussel for helpful comments during the writing of the paper. We also thank Shunji Kozaki and Keiji Nakamura, Osaka Prefecture University, Osaka, Japan, who kindly provided the DNAs of *C. botulinum* types C (strain CB-19), C-D (strain 6813,003-9), D (strain 1873), and D-C (strain OFD-05).

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