



Clinical microbiology

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



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ABSTRACT

Two real-time PCR arrays based on the GeneDisc[®] cyclor platform (Pall-GeneDisc Technologies) were evaluated in a multicenter collaborative trial for their capacity to specifically detect and discriminate *Clostridium botulinum* types C, D and their mosaic variants C-D and D-C that are associated with avian and mammalian botulism. The GeneDisc[®] arrays developed as part of the DG Home funded European project 'AniBioThreat' were highly sensitive and specific when tested on pure isolates and naturally contaminated samples (mostly clinical specimen from avian origin). Results of the multicenter collaborative trial involving eight laboratories in five European Countries (two laboratories in France, Italy and The Netherlands, one laboratory in Denmark and Sweden), using DNA extracts issued from 33 pure isolates and 48 naturally contaminated samples associated with animal botulism cases, demonstrated the robustness of these tests. Results showed a concordance among the eight laboratories of 99.4%–100% for both arrays. The reproducibility of the tests was high with a relative standard deviation ranging from 1.1% to 7.1%. Considering the high level of agreement achieved between the laboratories these PCR arrays constitute robust and suitable tools for rapid detection of *C. botulinum* types C, D and mosaic types C-D and D-C. These are the first tests for *C. botulinum* C and D that have been evaluated in a European multicenter collaborative trial.

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1. Introduction

Botulism is a severe flaccid paralytic disease caused by seven different neuroparalytic toxins subtypes (BoNT A-G) which could affect animals and humans. Botulinum neurotoxins (BoNTs) are produced by the anaerobic gram-positive bacteria *Clostridium botulinum* group I to IV [1]. Groups I and II BoNT producing Clostridia are mainly

responsible for human botulism whereas toxin produced by group III (*C. botulinum* types C and D) are involved in animal botulism worldwide [2]. BoNT-producing clostridia can affect wild and domesticated animals such as poultry, birds, cattle, horses, sheep and minks. Outbreaks with high mortality have become an increasing environmental and economical problem [3] but the deliberate release of BoNT producing clostridia or isolated toxin for bioterrorism purposes is also of great concern [4,5]. Economical, medical and alimentary consequences could be catastrophic, underlying the necessity of rapid detection tests. The European project 'AniBioThreat' ("Bio-preparedness measures concerning prevention, detection and response to

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animal bio-terrorism threats”, www.anibiothreat.com) funded in 2010 for three years by the European commission (DG Home) has been set up for improving prevention, detection and response to animal bio-terrorism threats. This project focuses on animal bio-terrorism threats and raised the question of rapid detection of clostridia responsible for animal botulism [6].

The standard method to perform sample analysis of suspected animal botulism outbreaks is currently the mouse lethality bio-assay followed by sero-neutralization [7,8]. However this method presents several drawbacks. It is time consuming, expensive, and causes ethical considerations. Real-time PCR based assays using the GeneDisc[®] technology (Pall, GeneDisc Technologies, Bruz, France) have recently been developed within the framework of the European project ‘AniBioThreat’ for neurotoxin gene profiling of *C. botulinum* types C and D and their mosaic types C-D and D-C [9]. While not solving the shortcoming of the detection of the neurotoxin gene instead of the toxin, these real-time PCR-based assays have the advantage of being animal free, rapid and easy to use. Two GeneDisc[®] arrays were designed as follows: The *C. botulinum* types C and D GeneDisc[®] array (GD1 C&D) and the *C. botulinum* types C, D and mosaic GeneDisc[®] array (GD2 C, D & mosaic). The first PCR array (GD1) has been developed to detect *C. botulinum* types C and type D whereas the second array (GD2) allows for the differentiation between mosaic C-D and D-C strains from parental C and D strains. Both arrays include an appropriate external amplification control (EAC) to monitor PCR inhibitors. The importance of characterizing the BoNT genes is underlined by the higher toxic activity of the mosaic types C-D and D-C neurotoxins compared to normal types C and D [10,11]. There are a limited number of real-time PCR assays for *C. botulinum* types C and D that have been developed and reported in the scientific literature [12–14]. None of these are able to clearly discriminate *C. botulinum* subtypes C, C-D, D and D-C except the GeneDisc[®] arrays as described by Woudstra et al., 2012. In addition, none have been validated for use by a full-scale inter-laboratory collaborative trial. Proper validation based on consensus criteria is an absolute prerequisite for successful adoption of a PCR-based diagnostic methodology [15]. Due to lack of international validation and standardization protocols for *C. botulinum* detection, and control of quality reagents and equipment, the transfer of the assays from expert to end-user laboratories has met with great difficulties. As a step toward the recognition of a standard PCR-based method to detect and subtype *C. botulinum* C, C-D, D and D-C in clinical samples, the performance characteristics of the GeneDisc[®] arrays should be assessed with a special focus on their reproducibility. The aim of the present study was to evaluate the two GeneDisc[®] arrays developed as part of the European Research Project ‘AniBioThreat’ in a multicenter collaborative trial performed at eight different European laboratories.

2. Materials and methods

2.1. Participating laboratories

The laboratories participating in the evaluation trial were the French agency for food, environmental and occupational health & safety (Anses) which is the organizing laboratory; The Analysis and Development Laboratory 22 (LDA22), Ploufragan, France; The National Reference Centre for Botulism (NRCB) at the Istituto Superiore di Sanità, Rome, Italy; The Istituto Zooprofilattico Sperimentale delle Venezie (IZSve), Laboratorio di Treviso, Italy; The National Food Institute, Technical University of Denmark (DTU), Søborg, Denmark; The Central Veterinary Institute (CVI) of Wageningen University and Research center, Lelystad, The Netherlands; The Centre for Zoonoses and Environmental Microbiology, Bilthoven,

RIVM, The Netherlands; and the National Veterinary Institute (SVA), Uppsala, Sweden. Due to international restrictions on exchange and sending of BoNT-producing clostridia strains, only sterile DNAs were shipped to the participants.

2.2. GeneDisc[®] arrays

Two GeneDisc[®] arrays were used in the ring trial. The GeneDisc[®] array “GD1 C & D” was used for detection of *C. botulinum* types C and D. The GeneDisc[®] array “GD2 C, D & mosaic” was used for detection of *C. botulinum* types C, C-D, D and D-C. Both are identical to those previously described [9]. Each GeneDisc[®] array contains a calibrated EAC provided by Pall GeneDisc[®] Technologies which should give a positive signal with a Cq quantification cycle [16] value around 30. GeneDisc[®] spotting and manufacturing were performed by Pall GeneDisc[®] Technologies (Bruz, France) in one batch during summer 2011.

2.3. GeneDisc[®] cyclers and software

The V2 GeneDisc[®] cycler from Pall GeneDisc[®] technologies was used by each laboratory for the trial. Technical staffs from each participating laboratory were trained by Pall GeneDisc[®] technologies to use the GeneDisc[®] cycler and software. Results were analyzed using the GeneDisc[®] cycler software (V2.10.12) which is part of the V2 GeneDisc[®] cycler. Positive results and Cq values were recorded according to the GeneDisc[®] cycler software calculation.

2.4. Bacterial strains

Strains of *C. botulinum* types C, C-D, D and D-C used in this study are listed in Tables 1 and 2. The strains consisted of seven *C. botulinum* types C, eighteen *C. botulinum* mosaic types C-D, three *C. botulinum* types D and five *C. botulinum* mosaic types D-C. *C. botulinum* strains were grown overnight in Trypticase-Peptide-Glucose-Yeast extract (TPGY) extract broth [17], brain heart infusion medium (Difco, Paris, France), or in broth fortified cooked meat medium at and incubated, without homogenization, at 30 °C under anaerobic conditions [18].

2.5. Naturally contaminated samples

A total of 48 naturally contaminated samples (intestinal content, faeces and organs from birds and cattle), were collected from animal botulism outbreaks across Europe in the last few years. Each sample (1 g) was added to 9 ml of pre-reduced Trypticase-Peptide-Glucose-Yeast extract (TPGY) and incubated, without homogenization, in anaerobic conditions. After 48 h incubation in TPGY at 30 °C, 1 mL aliquots of the enrichment broths were collected and centrifuged at 9000 × g for 5 min. The supernatant was discarded and the cell pellet submitted to DNA extraction using either Phenol/Chloroform method [19], DNeasy blood and tissue kit (Qiagen, Hilden, Germany), Chelex 100 (Bio-Rad Life Science Research, Hercules, CA), and Automatic system Microlab Starlet (Hamilton, Nevada, USA) employing the MegMax Total Nucleic Acid Isolation Kit (Ambion, Austin, USA), according to manufacturer’s instructions for Gram positive bacteria. DNA was stored at –20 °C until shipment for further analysis.

2.6. Trial organization

Concordance and reproducibility of GD1 and GD2 GeneDisc[®] arrays were evaluated within a ring trial involving eight European laboratories. Each laboratory used the real-time PCR platform “GeneDisc cycler” V2 from Pall GeneDisc technologies for the trial.

Table 1
Strains of *C. botulinum* tested with the GeneDisc® “GD1, C & D”.

| Strain | Origin | GeneDisc® “GD1, C & D” | | | | | | | | |
|--------|-----------|------------------------|------------|-------------------|-------|------|--------------|-------|------|------|
| | | PCR | Lab 1 to 8 | Cq value | | | EAC Cq value | | | |
| | | | | Mean Cq | SD | RSD% | Mean Cq | SD | RSD% | |
| 1 | Intestine | Duck ^a | C | + 8/8 | 24.41 | 0.72 | 2.96 | 28.60 | 0.52 | 1.82 |
| 2 | Intestine | Duck ^a | C | +8/8 | 25.67 | 0.41 | 1.61 | 28.78 | 0.33 | 1.15 |
| 3 | Intestine | Duck ^a | C | +8/8 | 22.74 | 0.44 | 1.92 | 28.56 | 0.62 | 2.16 |
| 4 | Feces | Milch-cow | D | +8/8 | 22.67 | 0.87 | 3.85 | 28.72 | 0.35 | 1.23 |
| 5 | Intestine | Cow | D | +8/8 | 24.94 | 0.49 | 1.95 | 27.47 | 1.63 | 5.93 |
| 6 | Unknown | | C | +8/8 | 25.65 | 0.41 | 1.61 | 27.49 | 0.43 | 1.56 |
| 7 | Unknown | | C | +8/8 | 25.97 | 0.58 | 2.24 | 28.47 | 0.68 | 2.37 |
| 8 | Unknown | | C | +8/8 | 24.73 | 0.30 | 1.22 | 28.52 | 0.40 | 1.39 |
| 9 | Unknown | | C | +8/8 | 23.09 | 0.57 | 2.46 | 28.58 | 0.39 | 1.35 |
| 10 | Unknown | | C | +8/8 | 20.49 | 0.84 | 4.11 | 28.46 | 0.32 | 1.12 |
| 11 | Unknown | | C | +8/8 | 23.04 | 0.48 | 2.10 | 28.26 | 0.48 | 1.69 |
| 12 | Unknown | | C | +8/8 | 24.77 | 1.76 | 7.10 | 27.58 | 0.44 | 1.60 |
| 13 | Unknown | | C | +8/8 | 23.83 | 0.26 | 1.11 | 28.48 | 0.47 | 1.63 |
| 14 | Unknown | | C | +8/8 | 24.18 | 0.63 | 2.62 | 28.92 | 1.62 | 5.61 |
| 15 | Feed | | C | +8/8 | 26.35 | 0.88 | 3.35 | 28.49 | 0.43 | 1.52 |
| 16 | Unknown | | C | +8/8 | 24.08 | 0.90 | 3.72 | 28.36 | 0.37 | 1.30 |
| 17 | Unknown | | C | +8/8 | 21.55 | 0.86 | 3.99 | 28.30 | 0.43 | 1.50 |
| 18 | Unknown | Fox | C | +8/8 | 20.13 | 0.27 | 1.33 | 27.43 | 0.46 | 1.67 |
| 19 | Unknown | | C | +8/8 | 21.35 | 0.87 | 4.06 | 28.66 | 0.39 | 1.36 |
| 20 | Unknown | | C | +8/8 | 26.34 | 0.76 | 2.89 | 28.60 | 0.65 | 2.28 |
| 21 | Unknown | Chicken | C | +8/8 | 22.42 | 1.09 | 4.87 | 28.40 | 0.77 | 2.70 |
| 22 | Unknown | | D | +8/8 | 23.48 | 1.11 | 4.71 | 28.44 | 0.63 | 2.20 |
| 23 | Unknown | Cow | D | +8/8 | 26.30 | 0.59 | 2.24 | 28.25 | 0.79 | 2.79 |
| 24 | Unknown | Vaccine strain | D | +7/8 | 23.17 | 0.67 | 2.90 | 27.80 | 0.37 | 1.33 |
| 25 | Unknown | | D | +8/8 | 22.95 | 0.79 | 3.46 | 28.42 | 0.70 | 2.46 |
| 26 | Unknown | | D | +8/8 | 26.42 | 0.70 | 2.64 | 28.23 | 0.76 | 2.68 |
| 27 | Unknown | | D | +8/8 ^b | 25.30 | 0.56 | 2.21 | 28.56 | 0.53 | 1.85 |
| 28 | Intestine | Chicken | C | +8/8 | 26.28 | 0.93 | 3.53 | 28.19 | 1.11 | 3.93 |
| 29 | Unknown | | C | +8/8 | 22.74 | 0.98 | 4.31 | 28.31 | 0.52 | 1.85 |
| 30 | Unknown | | C | +8/8 | 21.03 | 0.54 | 2.56 | 27.78 | 0.27 | 0.96 |
| 31 | Unknown | | C | +8/8 | 27.59 | 1.40 | 5.07 | 28.76 | 1.17 | 4.05 |
| 32 | Unknown | | C | +7/8 | 27.42 | 0.97 | 3.55 | 28.52 | 0.84 | 2.93 |
| 33 | Unknown | | C | +8/8 | 33.49 | 1.58 | 4.72 | 28.40 | 1.17 | 4.11 |

^a *Oxyura leucocephala*.

^b One laboratory reported a weak positive result with very low fluorescence signal in regard to the magnitude of fluorescence recorded by other laboratories. Not taken into account for mean, SD and RSD calculation.

Each participating laboratory received 81 identical “blind” coded DNA samples: 33 originated from *C. botulinum* strains and 48 from naturally contaminated samples. All were equally diluted and distributed among the eight participating laboratories in cooled packages by the organizing laboratory and stored at -20°C until further analysis. The two GeneDisc® arrays tested were sent to each laboratory and stored at $+4^{\circ}\text{C}$ until analysis. Each participant received a standard operating procedure and a standardized test report form on which to record the results to return to the trial leader for analysis.

2.7. Terms and definitions

The following terms and definitions were used in the manuscript:

Relative accuracy (AC) is defined as the degree of correspondence between the response obtained with the PCR tests of Hill et al. [20] and Kouguchi et al. [21] used as reference for testing *C. botulinum* types C and D and the response obtained with the GeneDisc® arrays on an identical sample.

Relative sensitivity (SE) is the ability of the GeneDisc® arrays to detect a positive result when the reference real-time PCR methods as described by Hill et al. (2010) and Kouguchi et al. (2006) also produced a positive result on an identical sample.

Relative specificity (SP) is the ability of the GeneDisc® arrays to detect a negative result when the reference real-time PCR methods as described by Hill et al. (2010) and Kouguchi et al. (2006) produces a negative result on an identical sample.

Selectivity includes the evaluation of inclusivity and exclusivity. Inclusivity is the ability of the GeneDisc® arrays to detect the target bacteria from a wide range of bacterial strains. Exclusivity is the lack of interference from a relevant range of non-target strains of the GeneDisc® arrays. The relevant non-target strains used for this study have been reported in the recent study of Woudstra et al. [9].

Concordance is defined as the percentage chance of finding the same result, positive or negative, from the identical sample analyzed by the eight laboratories [22].

Reproducibility refers to the variation in Cq values among the eight laboratories on identical sample. Reproducibility is expressed (in %) as the relative standard deviation (RSD%).

3. Results

In a recent study we investigated the selectivity (inclusivity and exclusivity) of the GeneDisc® arrays using DNA extracts issued from 56 strains of *C. botulinum* types C, C-D, D, D-C and 63 non-BoNT-producing Clostridia. Results of selectivity showed 100% of inclusivity on the 56 target strains and 100% of exclusivity on the 63 non-target strains. Results of 292 real samples analyzed by the reference real-time PCR assays as described by Hill et al. (2010) and Kouguchi et al. (2006) and the GeneDisc® arrays gave a relative accuracy (AC) of 97.9%, relative sensitivity (SE) of 96.8% and relative specificity (SP) of 99.3% [9].

In the present study the results of a multicenter collaborative trial performed with eight European laboratories using the GeneDisc®

Table 2
Strains of *C. botulinum* tested with the GeneDisc® “GD2, C, D & mosaic”.

| Strain | Origin | GeneDisc® “GD2 C, D & mosaics C-D, D-C” | | | | | | | | |
|--------|-----------|---|------------|-------------------|-------|------|--------------|-------|------|------|
| | | PCR | Lab 1 to 8 | Cq value | | | EAC Cq value | | | |
| | | | | Mean Cq | SD | RSD% | Mean Cq | SD | RSD% | |
| 1 | Intestine | Duck ^a | C-D | + 8/8 | 24.13 | 0.63 | 2.61 | 29.49 | 1.70 | 5.76 |
| 2 | Intestine | Duck ^a | C-D | +8/8 | 25.28 | 0.61 | 2.43 | 29.33 | 1.55 | 5.30 |
| 3 | Intestine | Duck ^a | C-D | +8/8 | 22.35 | 0.81 | 3.64 | 29.05 | 1.39 | 4.79 |
| 4 | Feces | Milch-cow | D-C | +8/8 | 22.35 | 1.19 | 5.35 | 29.42 | 1.23 | 4.19 |
| 5 | Intestine | Cow | D-C | +8/8 | 25.25 | 0.92 | 3.64 | 29.14 | 1.24 | 4.25 |
| 6 | Unknown | | C-D | +8/8 | 25.36 | 0.55 | 2.18 | 27.95 | 1.85 | 6.64 |
| 7 | Unknown | | C-D | +8/8 | 26.52 | 1.20 | 4.54 | 29.17 | 1.25 | 4.29 |
| 8 | Unknown | | C-D | +8/8 | 24.92 | 1.20 | 4.81 | 28.48 | 2.15 | 7.54 |
| 9 | Unknown | | C-D | +8/8 | 23.13 | 0.72 | 3.11 | 29.27 | 1.42 | 4.86 |
| 10 | Unknown | | C-D | +8/8 | 21.00 | 0.33 | 1.58 | 29.21 | 1.33 | 4.56 |
| 11 | Unknown | | C-D | +8/8 | 22.59 | 0.81 | 3.57 | 29.04 | 1.31 | 4.52 |
| 12 | Unknown | | C-D | +8/8 | 24.38 | 0.91 | 3.73 | 28.64 | 1.41 | 4.92 |
| 13 | Unknown | | C-D | +8/8 | 23.75 | 0.51 | 2.15 | 29.52 | 1.29 | 4.36 |
| 14 | Unknown | | C-D | +8/8 | 23.94 | 0.93 | 3.88 | 29.22 | 1.27 | 4.34 |
| 15 | Feed | | C-D | +8/8 | 26.67 | 0.42 | 1.59 | 29.30 | 1.38 | 4.73 |
| 16 | Unknown | | C | +8/8 | 24.09 | 0.47 | 1.94 | 29.39 | 1.30 | 4.42 |
| 17 | Unknown | Fox | C-D | +8/8 | 21.50 | 0.49 | 2.26 | 28.73 | 1.74 | 6.05 |
| 18 | Unknown | | C-D | +8/8 | 19.75 | 0.67 | 3.38 | 28.35 | 1.33 | 4.70 |
| 19 | Unknown | | C | +8/8 | 21.33 | 0.49 | 2.31 | 29.62 | 1.25 | 4.22 |
| 20 | Unknown | | C | +8/8 | 25.50 | 0.64 | 2.49 | 29.32 | 1.51 | 5.15 |
| 21 | Unknown | Chicken | C-D | +8/8 | 23.09 | 0.87 | 3.75 | 29.14 | 1.67 | 5.74 |
| 22 | Unknown | | D | +8/8 | 23.98 | 1.49 | 6.21 | 28.61 | 1.96 | 6.87 |
| 23 | Unknown | Cow | D-C | +8/8 | 26.55 | 0.91 | 3.44 | 28.65 | 1.61 | 5.61 |
| 24 | Unknown | Vaccine strain | D-C | +8/8 | 23.17 | 0.83 | 3.60 | 27.60 | 1.41 | 5.10 |
| 25 | Unknown | | D-C | +8/8 | 23.39 | 0.93 | 3.96 | 29.19 | 1.31 | 4.49 |
| 26 | Unknown | | D | +8/8 | 26.99 | 0.75 | 2.80 | 28.75 | 1.19 | 4.14 |
| 27 | Unknown | | D | +8/8 ^b | 25.64 | 0.93 | 3.61 | 28.81 | 1.64 | 5.69 |
| 28 | Intestine | Chickens | C-D | +8/8 | 26.33 | 0.57 | 2.17 | 29.08 | 1.64 | 5.63 |
| 29 | Unknown | | C | +8/8 | 22.51 | 0.55 | 2.46 | 28.58 | 1.70 | 5.95 |
| 30 | Unknown | | C-D | +8/8 | 20.41 | 0.70 | 3.43 | 28.51 | 1.29 | 4.53 |
| 31 | Unknown | | C | +8/8 | 27.17 | 0.94 | 3.46 | 29.56 | 1.17 | 3.94 |
| 32 | Unknown | | C | +8/8 | 26.44 | 0.59 | 2.24 | 29.37 | 1.02 | 3.46 |
| 33 | Unknown | | C | +8/8 | 33.19 | 1.66 | 5.01 | 29.29 | 1.55 | 5.30 |

^a *Oxyura leucocephala*.

^b One laboratory reported a weak positive result with very low fluorescence signal in regard to the magnitude of fluorescence recorded by other laboratories. Not taken into account for mean, SD and RSD calculation.

arrays are presented. Tables 1 and 2 show the participants' results obtained with 33 *C. botulinum* isolates using the GeneDisc® arrays GD1 and GD2 respectively. The results showed a concordance of 99.4% and 100% for the GeneDisc® arrays GD1 and GD2 respectively among the eight participating laboratories. Data obtained with GD1 showed that two laboratories failed in the identification of one sample each: laboratory 7 failed to identify strain n°24 and laboratory 8 failed to identify strain n°32. Using GD2 all laboratories successfully identified the 33 *C. botulinum* strains. RSDs of the Cq values obtained for each *C. botulinum* strain were in the range of 1.1%–7.1%.

Tables 3 and 4 show the participants' results of the collaborative trial obtained with DNA from 48 naturally contaminated samples (clinical specimen mainly gathered from intestinal content, faeces, organ of birds and cattle) using the GeneDisc® arrays GD1 and GD2 respectively. The results showed 99.5% of concordance between each participant for GD1 and GD2 GeneDisc® arrays respectively. Using the GeneDisc® array GD1, two of the laboratories failed to identify one sample each, laboratory 7 failed to identify sample n°14 and laboratory 3 failed to identify sample n°33. Using the GeneDisc® array GD2, laboratory 7 failed to identify samples n°3 and n°4. The RSDs of Cq values obtained for each sample were in the range of 1.3%–4.4%. Regarding the Cq values related to the external amplification control (EAC), the RSD obtained with GD1 and GD2 on *C. botulinum* strains and naturally contaminated samples ranged from 1.0% to 7.9%. Cq values obtained with the EAC never

exceeded the expected value of 30 which indicated the absence of PCR inhibitors in strains and naturally clinical samples that were tested in this study.

4. Discussion

Botulism occurs worldwide, both as sporadic cases and massive outbreaks in wild and domestic animals [23,24]. However, as animal botulism is not a notifiable disease in all European Countries, it is probably under-diagnosed and under-reported in Europe. Botulism can cause high mortality, leading rapidly to significant economic loss in intensively farmed animals. In case of epizooty, the economical, medical and alimentary consequences could be catastrophic, underlying the necessity of rapid identification of the source of animal botulism. It is of major importance for veterinarians to quickly characterize the disease and identify the causative agent in order to take appropriate measures within a limited time frame. In the first stages of the European project 'AnibioThreat', PCR assays based on the GeneDisc® technology have been developed for the detection of *C. botulinum* types C, C-D, D and D-C. The assays were validated in-house against an extensive list of clostridia and other bacterial isolates and were shown to be highly specific and sensitive. The limit of detection of the tests was 38 fg of total DNA, corresponding to 15 genome copies. Artificially contaminated samples of cecum showed a limit of detection below 50 spores per gram [9]. With the aim to further evaluate the robustness and reproducibility of the assays as a step towards their routine utilization in veterinary laboratories

Table 3
Naturally contaminated samples tested with the GeneDisc® “GD1, C & D”.

| Sample | Origin | | GeneDisc® “GD1, C & D” | | | | | | | | |
|--------|------------------------|-----------------------------|------------------------|-------------------|----------|------|------|--------------|------|------|--|
| | | | PCR | Lab 1 to 8 | Cq value | | | EAC Cq value | | | |
| | | | | | Mean Cq | SD | RSD% | Mean Cq | SD | RSD% | |
| 1 | Liver | Pheasant | C | +8/8 ^a | 25.71 | 0.50 | 1.93 | 28.46 | 0.42 | 1.47 | |
| 2 | Spleen | Pheasant | C | +8/8 | 27.21 | 0.53 | 1.95 | 28.35 | 0.63 | 2.22 | |
| 3 | Intestinal content | Pheasant | C | +8/8 | 25.29 | 0.83 | 3.29 | 28.18 | 1.13 | 4.00 | |
| 4 | Intestinal content | Wild duck | C | +8/8 | 28.35 | 0.69 | 2.45 | 28.41 | 0.78 | 2.74 | |
| 5 | Liver | Mallard | C | +8/8 | 26.20 | 0.49 | 1.89 | 27.54 | 1.03 | 3.75 | |
| 6 | Intestinal content | Mallard | C | +8/8 | 27.08 | 0.35 | 1.29 | 27.38 | 0.85 | 3.09 | |
| 7 | Soil | NA | C | +8/8 | 27.89 | 0.80 | 2.88 | 27.83 | 1.43 | 5.15 | |
| 8 | Maggot | Egret | C | +8/8 | 28.22 | 0.98 | 3.47 | 28.36 | 1.03 | 3.62 | |
| 9 | Liver | Egret | C | +8/8 | 29.18 | 0.79 | 2.70 | 28.22 | 0.76 | 2.68 | |
| 10 | Intestinal content | Egret | C | +8/8 | 28.09 | 0.65 | 2.31 | 28.63 | 0.37 | 1.30 | |
| 11 | Intestinal content | Wild duck | C | +8/8 | 29.16 | 0.61 | 2.09 | 28.35 | 0.49 | 1.74 | |
| 12 | Liver | Peewit gull | C | +8/8 | 27.50 | 0.66 | 2.39 | 27.74 | 0.75 | 2.69 | |
| 13 | Intestinal content | Moorhen | C | +8/8 | 27.25 | 0.94 | 3.47 | 28.68 | 0.41 | 1.41 | |
| 14 | Intestinal content | Wild duck | C | +7/8 | 26.48 | 0.64 | 2.42 | 28.37 | 0.64 | 2.27 | |
| 15 | Intestinal content | Wild duck | C | +8/8 | 27.47 | 0.72 | 2.61 | 28.28 | 0.79 | 2.80 | |
| | | | D | +8/8 | 30.67 | 1.19 | 3.89 | | | | |
| 16 | Intestinal content | Wild duck | C | +8/8 ^b | 28.34 | 0.61 | 2.15 | 28.71 | 0.67 | 2.32 | |
| 17 | Intestinal content | Peewit gull | C | +8/8 | 27.59 | 0.37 | 1.34 | 28.19 | 0.60 | 2.14 | |
| 18 | Liver | Herring gull | C | +8/8 | 26.72 | 0.36 | 1.34 | 27.44 | 0.68 | 2.46 | |
| 19 | Maggot | NA | C | +8/8 | 26.64 | 0.39 | 1.45 | 28.61 | 0.49 | 1.70 | |
| 20 | Intestinal content | Peewit gull | C | +8/8 | 27.07 | 1.01 | 3.73 | 27.78 | 1.70 | 6.13 | |
| 21 | Intestinal content | Wild duck | C | +8/8 | 26.52 | 0.75 | 2.83 | 28.29 | 0.61 | 2.17 | |
| 22 | Liver | Wild duck | C | +8/8 ^c | 26.68 | 0.53 | 1.98 | 28.48 | 0.36 | 1.27 | |
| 23 | Stomach | Mallard | C | +8/8 | 27.63 | 0.82 | 2.95 | 28.34 | 0.59 | 2.10 | |
| 24 | Stomach | Mallard | C | +8/8 | 25.86 | 0.57 | 2.19 | 27.40 | 0.89 | 3.23 | |
| 25 | Liver | Rat | C | +8/8 | 25.80 | 1.01 | 3.92 | 28.33 | 0.67 | 2.35 | |
| 26 | Intestinal content | Rat | C | +8/8 | 26.48 | 0.49 | 1.83 | 28.50 | 0.44 | 1.55 | |
| 27 | Liver | Coypus | C | +8/8 | 27.63 | 0.84 | 3.04 | 28.18 | 0.77 | 2.75 | |
| 28 | Liver | Coot | C | +8/8 | 27.77 | 0.69 | 2.50 | 28.42 | 0.37 | 1.30 | |
| 29 | Liver | Wild duck | C | +8/8 | 29.44 | 0.84 | 2.85 | 28.10 | 0.40 | 1.43 | |
| 30 | Intestinal content | Wild duck | C | +8/8 | 28.54 | 0.39 | 1.37 | 27.90 | 0.27 | 0.97 | |
| 31 | Intestinal content | Coypus | C | +8/8 | 26.15 | 0.77 | 2.96 | 28.57 | 0.45 | 1.57 | |
| 32 | Feces | Dog | C | +8/8 | 29.82 | 0.80 | 2.69 | 28.45 | 0.54 | 1.91 | |
| | | | D | +8/8 | 30.24 | 0.81 | 2.68 | | | | |
| 33 | Feces | Dog | C | +8/8 | 29.56 | 0.85 | 2.87 | 28.08 | 0.93 | 3.32 | |
| | | | D | +7/8 | 30.17 | 1.19 | 3.95 | | | | |
| 34 | Liver | Bovine | D | +8/8 | 28.61 | 0.76 | 2.64 | 28.44 | 0.69 | 2.41 | |
| 35 | Feces | Bovine | D | +8/8 | 27.57 | 0.57 | 2.09 | 28.40 | 0.36 | 1.26 | |
| 36 | Ruminal content | Bovine | D | +8/8 | 27.97 | 0.77 | 2.77 | 27.96 | 0.71 | 2.53 | |
| 37 | Liver | Pochard | C | +8/8 | 29.51 | 0.66 | 2.25 | 28.24 | 0.67 | 2.39 | |
| 38 | Intestinal content | Coot | C | +8/8 | 28.93 | 0.71 | 2.46 | 28.22 | 0.68 | 2.41 | |
| 39 | Feed | Mink | D | +8/8 | 28.11 | 0.99 | 3.53 | 27.98 | 0.79 | 2.83 | |
| 40 | Intestinal content | Cow | D | +8/8 | 28.97 | 0.77 | 2.65 | 28.32 | 0.88 | 3.11 | |
| 41 | Control vaccine strain | <i>C. botulinum</i> CKIII-4 | C | +8/8 | 24.07 | 0.66 | 2.73 | 28.59 | 0.50 | 1.74 | |
| 42 | NA | Gallus | C | +8/8 | 31.76 | 0.74 | 2.32 | 28.02 | 0.33 | 1.17 | |
| 43 | NA | Pheasant | C | +8/8 | 29.65 | 0.70 | 2.36 | 28.54 | 0.59 | 2.06 | |
| 44 | Fecal culture | Bovine | D | +8/8 | 30.32 | 0.76 | 2.52 | 28.51 | 0.66 | 2.31 | |
| 45 | NA | Gallus | C | +8/8 | 26.96 | 0.84 | 3.11 | 27.94 | 1.13 | 4.04 | |
| 46 | Fecal culture | Bovine | D | +7/7 ^d | 27.18 | 0.67 | 2.47 | 28.43 | 0.47 | 1.65 | |
| 47 | Fecal culture | Bovine | D | +7/7 ^d | 28.38 | 0.64 | 2.24 | 27.97 | 0.80 | 2.84 | |
| 48 | Fecal culture | Bovine 2806 | D | +7/7 ^d | 28.58 | 0.85 | 2.99 | 27.80 | 0.47 | 1.71 | |

NA: not available.

^a One laboratory reported a weak positive result with very low fluorescence signal in regard to the magnitude of fluorescence recorded by other laboratories. Not taken into account for mean, SD and RSD calculation.

^b One laboratory recorded a positive result with a Cq value significantly different compared with other laboratories.

^c One laboratory showed a positive result but the software failed to calculate a Cq value for the corresponding sample.

^d One laboratory has been unable to test the corresponding samples.

across Europe, a multicenter collaborative trial was conducted with eight European laboratories and presented in this study.

Data obtained with DNA extracts from pure isolates and naturally contaminated samples using the GeneDisc® arrays “GD1 C & D” and “GD2 C, D & mosaic” were highly concordant, with agreement percentages ranging from 99.4% to 100%. The low (1.1%–7.9%) RSD of the Cq values recorded by the eight laboratories with both GD1 and GD2 is another indication of the accuracy and robustness of the kit prototypes evaluated in this study. RSD values obtained with the GeneDisc® arrays “GD1 C & D” and “GD2 C, D & mosaic” were in the same order of magnitude and often lower than those

reported in previously published evaluation trial using the GeneDisc® arrays for *C. botulinum* types A, B, E and F [25]. However, some drawbacks have been noted during the trial. The software which is part of the GeneDisc cyler failed to calculate an appropriate Cq value for two PCR determinations despite a strong positive amplification clearly visible when checking the amplification curves. It seems that the software was unable to calculate the Cq value due to the very unusual high fluorescence levels obtained for these samples. A new version of the software is under development to overcome this inconvenience. Conversely, three PCR determinations gave a positive signal of fluorescence two times lower

Table 4
Naturally contaminated samples tested with the GeneDisc® “GD2, C, D & mosaic”.

| Sample N° | Origin | | GeneDisc® “GD2 C, D & mosaics C-D, D-C” | | | | | | | | |
|-----------|------------------------|-----------------------------|---|-------------------|----------|------|------|--------------|------|------|--|
| | | | PCR | Lab 1 to 8 | Cq value | | | EAC Cq value | | | |
| | | | | | Mean Cq | SD | RSD% | Mean Cq | SD | RSD% | |
| 1 | Liver | Pheasant | C-D | +8/8 | 25.42 | 0.73 | 2.87 | 28.91 | 1.72 | 5.96 | |
| 2 | Spleen | Pheasant | C-D | +8/8 | 26.43 | 0.71 | 2.69 | 28.97 | 1.78 | 6.16 | |
| 3 | Intestinal content | Pheasant | C-D | +7/8 | 24.81 | 0.85 | 3.44 | 28.58 | 2.13 | 7.46 | |
| 4 | Intestinal content | Wild duck | C-D | +7/8 | 28.12 | 0.64 | 2.29 | 29.03 | 1.72 | 5.91 | |
| 5 | Liver | Mallard | C-D | +8/8 | 25.54 | 0.94 | 3.69 | 28.86 | 1.16 | 4.01 | |
| 6 | Intestinal content | Mallard | C-D | +8/8 | 26.39 | 1.02 | 3.86 | 28.41 | 1.43 | 5.04 | |
| 7 | Soil | NA | C-D | +8/8 | 27.64 | 0.83 | 3.02 | 28.95 | 1.84 | 6.36 | |
| 8 | Maggot | Egret | C-D | +8/8 | 27.80 | 0.73 | 2.63 | 28.88 | 1.56 | 5.41 | |
| 9 | Liver | Egret | C-D | +8/8 | 28.31 | 1.09 | 3.85 | 29.06 | 1.68 | 5.77 | |
| 10 | Intestinal content | Egret | C-D | +8/8 | 27.87 | 0.60 | 2.17 | 28.99 | 1.83 | 6.30 | |
| 11 | Intestinal content | Wild duck | C-D | +8/8 | 28.49 | 0.76 | 2.68 | 28.68 | 1.94 | 6.78 | |
| 12 | Liver | Peewit gull | C-D | +8/8 | 27.08 | 0.62 | 2.29 | 28.10 | 1.70 | 6.03 | |
| 13 | Intestinal content | Moorhen | C-D | +8/8 | 27.16 | 0.66 | 2.42 | 29.44 | 1.31 | 4.44 | |
| 14 | Intestinal content | Wild duck | C-D | +8/8 | 26.07 | 0.74 | 2.84 | 29.02 | 1.29 | 4.45 | |
| 15 | Intestinal content | Wild duck | C-D | +8/8 | 27.10 | 0.86 | 3.17 | 28.60 | 1.65 | 5.78 | |
| | | | D-C | +8/8 | 30.51 | 1.06 | 3.48 | | | | |
| 16 | Intestinal content | Wild duck | C-D | +8/8 | 27.54 | 1.58 | 5.73 | 28.94 | 1.87 | 6.45 | |
| 17 | Intestinal content | Peewit gull | C-D | +8/8 | 27.07 | 0.90 | 3.34 | 28.31 | 1.66 | 5.88 | |
| 18 | Liver | Herring gull | C-D | +8/8 | 26.35 | 0.50 | 1.90 | 28.83 | 1.04 | 3.59 | |
| 19 | Maggot | NA | C-D | +8/8 | 26.57 | 0.49 | 1.86 | 29.13 | 1.55 | 5.33 | |
| 20 | Intestinal content | Peewit gull | C-D | +8/8 | 26.38 | 0.69 | 2.62 | 28.46 | 1.37 | 4.83 | |
| 21 | Intestinal content | Wild duck | C-D | +8/8 | 26.17 | 0.91 | 3.47 | 28.82 | 2.22 | 7.71 | |
| 22 | Liver | Wild duck | C-D | +8/8 | 26.67 | 0.56 | 2.11 | 29.49 | 1.46 | 4.95 | |
| 23 | stomach | Mallard | C-D | +8/8 | 27.55 | 0.77 | 2.78 | 29.08 | 1.75 | 6.01 | |
| 24 | stomach | Mallard | C-D | +8/8 | 25.89 | 0.51 | 1.95 | 28.93 | 1.40 | 4.86 | |
| 25 | Liver | Rat | C-D | +8/8 | 26.18 | 0.57 | 2.20 | 29.47 | 1.14 | 3.87 | |
| 26 | Intestinal content | Rat | C-D | +8/8 | 25.98 | 0.86 | 3.32 | 29.10 | 1.44 | 4.93 | |
| 27 | Liver | Coypus | C-D | +8/8 | 27.63 | 0.72 | 2.61 | 28.87 | 1.61 | 5.58 | |
| 28 | Liver | Coot | C-D | +8/8 | 27.74 | 0.60 | 2.16 | 29.32 | 1.28 | 4.37 | |
| 29 | Liver | Wild duck | C-D | +8/8 | 29.10 | 1.00 | 3.43 | 27.89 | 1.78 | 6.37 | |
| 30 | Intestinal content | Wild duck | C-D | +8/8 | 28.10 | 0.71 | 2.51 | 27.65 | 1.44 | 5.22 | |
| 31 | Intestinal content | Coypus | C-D | +8/8 | 26.29 | 0.46 | 1.74 | 28.87 | 1.37 | 4.76 | |
| 32 | Feces | Dog | C-D | +8/8 | 29.41 | 1.01 | 3.45 | 28.23 | 0.53 | 1.88 | |
| | | | D-C | +8/8 | 30.15 | 1.15 | 3.81 | | | | |
| 33 | Feces | Dog | C-D | +8/8 | 29.27 | 0.94 | 3.20 | 28.91 | 1.80 | 6.23 | |
| | | | D-C | +8/8 | 29.90 | 1.17 | 3.92 | | | | |
| 34 | Liver | Bovine | D-C | +8/8 | 28.73 | 1.25 | 4.36 | 29.13 | 1.25 | 4.28 | |
| 35 | Feces | Bovine | D-C | +8/8 | 27.92 | 0.91 | 3.25 | 28.79 | 1.23 | 4.29 | |
| 36 | Ruminal content | Bovine | D-C | +8/8 | 28.23 | 1.03 | 3.67 | 27.86 | 0.88 | 3.14 | |
| 37 | Liver | Pocharde | C-D | +8/8 | 28.79 | 0.66 | 2.28 | 29.14 | 1.32 | 4.55 | |
| 38 | Intestinal content | Coot | C-D | +8/8 ^a | 28.41 | 0.96 | 3.37 | 28.72 | 1.59 | 5.53 | |
| 39 | Feed | Mink | D-C | +8/8 | 28.30 | 1.07 | 3.79 | 28.83 | 1.85 | 6.43 | |
| 40 | Intestinal content | Cow | D-C | +8/8 | 29.28 | 0.94 | 3.22 | 29.46 | 1.23 | 4.16 | |
| 41 | Control vaccine strain | <i>C. botulinum</i> CKIII-4 | C | +8/8 | 24.03 | 0.47 | 1.94 | 28.87 | 1.38 | 4.79 | |
| 42 | NA | Gallus | C-D | +8/8 | 31.09 | 1.23 | 3.96 | 28.65 | 1.31 | 4.56 | |
| 43 | NA | Pheasant | C-D | +8/8 | 29.78 | 0.68 | 2.30 | 28.88 | 1.95 | 6.77 | |
| 44 | Fecal culture | Bovine | D-C | +8/8 | 30.43 | 0.91 | 3.00 | 28.68 | 2.26 | 7.87 | |
| 45 | NA | Gallus | C-D | +8/8 | 26.88 | 0.96 | 3.58 | 29.17 | 1.57 | 5.39 | |
| 46 | Fecal culture | Bovine | D-C | +8/8 | 27.07 | 0.74 | 2.72 | 29.35 | 1.18 | 4.01 | |
| 47 | Fecal culture | Bovine | D-C | +8/8 | 28.70 | 0.59 | 2.06 | 29.22 | 1.54 | 5.28 | |
| 48 | Fecal culture | Bovine 2806 | D-C | +8/8 | 28.30 | 1.11 | 3.93 | 28.85 | 1.07 | 3.72 | |

NA: Not available.

^a One laboratory observed a positive result but the software failed to calculate a Cq value for the corresponding sample.

in regards to the other corresponding analyses performed in the trial. Also, six determinations were found negative despite being recorded positive by the other seven laboratories. Given that these false negative results were reported by different participating laboratories, a random degradation of DNA during shipment and storage can be hypothesized. The degradation pattern of DNA isolated from clinical samples has previously been shown to be dependent on sample type, target organism and storage temperature [26]. The false negative results observed were not found associated with a particular DNA extraction procedure. Primers and probes degradation could be another potential explanation of the observed false negative results. Among the 648 PCR determinations performed on positive samples during this multicenter collaborative trial, only six were recorded as false negative (0.9%). This

clearly indicates there is a very low risk of obtaining false-negative responses using these tests.

In conclusion the results of this multicenter collaborative trial showed that the method is effective in detecting *C. botulinum* types C, D and their associated mosaic variants in the sample types tested. The tests are robust, fast, easy to handle and can be readily applied in microbiological laboratories for the detection of *C. botulinum* in putative cases of animal botulism.

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