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Multiplex real-time PCR SYBR Green for detection and typing of group III *Clostridium botulinum*

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ABSTRACT

Clostridium botulinum type C and type D belonging to the group III organisms, are mainly responsible for animal botulism outbreaks. Clinical signs alone are often insufficient to make a diagnosis of botulism and a laboratory confirmation is required. Laboratory confirmation can be performed by demonstrating the presence of botulinum neurotoxins in serum, gastrointestinal contents, liver, wound of sick or dead animals, or by demonstrating the presence of *C. botulinum* in gastrointestinal contents, liver, and wound. Demonstration of spores in gastrointestinal contents or tissue of animals with clinical signs indicative of botulism reinforces the clinical diagnosis. With the aim of detecting and typing *C. botulinum* group III organisms, a multiplex real-time PCR SYBR Green was developed and *in-house* validated. Selectivity, limit of detection, relative accuracy, relative specificity, relative sensitivity, and repeatability of the method were investigated. The multiplex real-time PCR SYBR green used showed a 100% selectivity, 100% relative accuracy, 100% relative specificity, 100% relative sensitivity and a limit of detection of 277 and 580 DNA copies for *C. botulinum* type C and *C. botulinum* type D, respectively. The method reported here represents a suitable tool for laboratory diagnosis of type C and D botulism and for testing a large number of samples collected during the animal botulism surveillance and prevention activities.

1. Introduction

Clostridium botulinum strains are classified into 4 groups on the basis of their phenotypical and biochemical characteristics. Group III organisms, mainly responsible for animal botulism, can produce botulinum neurotoxin type C (BoNT/C), BoNT/D, and C/D and D/C mosaic neurotoxins (Takeda et al., 2005; Takeda et al., 2006; Peck, 2009; Nakamura et al., 2010). BoNT/C is generally responsible for botulism in birds, horses, cattle, fur animals, and carnivores, whereas BoNT/D is mainly related to botulism in cattle and sheep. In addition, animals are also susceptible to BoNT/A, BoNT/B and BoNT/E (Deprez, 2006).

Clinical signs alone are often insufficient to make a diagnosis of botulism, and a laboratory confirmation is required. Laboratory confirmation can be performed demonstrating the BoNTs in serum, feed material or intestinal content, by *in vivo* standard mouse bioassay (SMB) (Deprez, 2006). However, a negative mouse bioassay does not eliminate the botulism diagnosis as the toxin may still be present, but not detectable due to it being below the limit of detection or biodegraded by microbes in the intestinal tract of animals (Allison et al., 1976). Demonstration of botulinum spores in gastrointestinal contents or tissue of animals with clinical signs indicative of botulism supports the clinical diagnosis,

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Table 1
Selectivity study: origins of strains and extracted DNAs, and results.

Organism or DNA	No. of isolates	Origin	Real-time PCR		
			bont type		IAC
			C	D	
C. botulinum type C	11	10 NRCB[a], NCTC 8264[b]	+	–	+
C. botulinum type D	2	1 NRCB, NCTC 8265	–	+	+
C. botulinum type A	22	20 NRCB, NCTC 7272, NCTC 2012	–	–	+
C. botulinum type A(B)	1	NCTC 2916	–	–	+
C. botulinum type Ab	4	NRCB	–	–	+
C. botulinum type B	21	20 NRCB, NCTC 3815	–	–	+
C. botulinum type F	4	3 NRCB, NCTC 10281	–	–	+
C butyricum type E	7	ATCC 43755[c], 6 NRCB	–	–	+
Extracted DNA C/D mosaic	5	2 ANSES[d], 3 SVA[e]	+	–	+
Extracted DNA D/C mosaic	1	ANSES	–	+	+
Non-BoNT producing clostridia[f]	25		–	–	+

[a] NRCB, National Reference Centre for Botulism, Istituto Superiore di Sanità, Italy.
[b] NCTC, National Collection of Type Cultures, UK.
[c] ATCC, American Type Culture Collection, USA.
[d] ANSES, French Agency for Food, Environmental and Occupational Health, Food Safety Laboratory, Maisons-Alfort, France.
[e] SVA, National Veterinary Institute, Department of Bacteriology, Uppsala, Sweden.
[f] *Clostridium butyricum* ATCC19398 *Clostridium butyricum* NRCB CL19, *Clostridium carnis* ATCC10456, *Clostridium difficile* ISS-WPI10463, *Clostridium histolyticum* NCTC503, *Clostridium novyi* ATCC7659, *Clostridium paraputrificum* ISS8, *Clostridium perfringens* ISSD1632, *Clostridium sordelli* ISS1, *Bacillus cereus* ATCC11778, *Bacillus coagulans* ATCC7050, *Bacillus subtilis* ATCC6633, *Campylobacter jejuni* ISS, *Citrobacter freundii* ATCC8090, *Cronobacter zakazakii* ATCC12868, *Enterococcus faecalis* ATCC29212, *Escherichia coli* ATCC25922, *Listeria innocua* ATCC33090, *Listeria monocytogenes* ATCC7644, *Micrococcus luteus* ATCC9341, *Pseudomonas aeruginosa* ATCC9027, *Rhodococcus equi* ATCC6939, *Salmonella choleraesuis* ISS/129/27/05, *Staphylococcus aureus* ATCC13565, *Streptococcus thermophilus* ATCC19258.

together with the demonstration of the presence of neutralizing antibodies that may permit a retrospective diagnosis in surviving animals (Deprez, 2006).

C. botulinum is currently detected by microbiological methods combined with SMB, and more recently, by PCR (Szabo et al., 1994; Fach et al., 1996; Franciosa et al., 1996; Williamson et al., 1999; Chaffer et al., 2006; Prévot et al., 2007; Lindberg et al., 2010). Among the PCR techniques, the real-time approach combined with the multiplexing, has several advantages (Edwards and Gibbs, 1994; Heid et al., 1996). To the best of our knowledge, multiplex real-time PCR for detection and typing of *C. botulinum* group III has not been published yet. Consequently, we have developed, and also *in-house* validated, a multiplex real-time PCR SYBR Green method capable of detecting *bont*/C, and *bont*/D.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacteria strains used in this study were from the Italian National Reference Centre for Botulism (NRCB) collection and are listed in Table 1. Clostridia strains were cultured in Trypticase-Peptone-Glucose-Yeast extract (TPGY) broth and incubated under anaerobic conditions for 24 h ± 2 h at 30 °C ± 1 °C. *Campylobacter jejuni* was cultured in Bolton broth (Oxoid, UK) and incubated in microaerophilic condition for 48 h ± 2 h at 41.5 °C ± 1 °C. All remaining strains were cultured in Brain Heart Infusion (BHI) broth (Oxoid, UK) and incubated under aerobic conditions for 24 h ± 2 h at 37 °C ± 1 °C. *C. botulinum* type C strain NCTC 8264 and *C. botulinum* type D strain NCTC 8265 were used as reference strains.

DNA extraction was performed using 1 mL of each cultured strain, as reported elsewhere (Fenicia et al., 2007).

2.2. Samples and culture conditions

One hundred eight samples, collected during animal botulism surveillance performed by NRCB in the period 2006 to 2009, were tested in this study (Table 2). All samples were tested, on arrival in the NRCB laboratory for botulinum neurotoxin-producing clostridia and their toxins, using the culture-based standard method (CDC, 1998), and stored at −20 °C. In the study, 1–2 g of each defrosted sample was cultured in 9 mL of pre-reduced TPGY broth and incubated under anaerobic conditions at 30 °C ± 1 °C. After 24 h ± 2 h of incubation, 1 mL aliquot of each enrichment broth was subjected to DNA extraction and used as template for the real-time PCR (first PCR step). The broths were re-incubated under anaerobic condition at 30 °C ± 1 °C for an additional 72 h. At the end of the incubation period (96 h), all cultures resulted negative from the first PCR step were again submitted to a real-time PCR. In addition, all enrichment broths were centrifuged at 12,000 × *g* for 20 min at 4 °C and the upper phase was subjected to SMB for the confirmation of BoNT production.

2.3. Standard mouse bioassay

The detection and typing of BoNTs in surnatants of samples and cultures were performed by SMB according to CDC manual with some modifications (CDC, 1998). The heptavalent (type A to G) antitoxin and monovalent type C and type D antitoxins were used in the neutralization step of the protocol. The SMB was performed in accordance with European law on the protection of animals used for experimental and other scientific purposes, and approved

Table 2
Origin of tested samples and results obtained by SMB and multiplex real-time PCR SYBR Green.

	Number of tested samples (number of positive samples – type of *bont* gene and BoNT)*							
	Faeces	Intestinal content	Liver	Stomach/ ruminal content	Spleen	Maggots	Mood/soil	Vegetables/ forage
Bovine	7 (2 type D)	1 (0)	2 (1 type D)	1 (1 type D)	2 (1 type D)			5 (0)
Coot		5 (1 type C)	4 (1 type C)					
Dog	2 (2 type C)							
Wild Duck	1 (1 type C)	13 (10 type C)	6 (5 type C)				7 (2 type C)	
Sea Gull		8 (4 type C)	9 (5 type C)			1 (1 type C)		
Heron		2 (1 type C)						
Moorhen		2 (0)	1 (0)					
Nutria		1 (1 type C)	1 (1 type C)					
Pheasant		1 (1 type C)	1 (1 type C)		1 (1 type C)			
Rabbit		2 (0)						
Rat		1 (1 type C)	1 (1 type C)					
Mallard		4 (4 type C)	2 (2 type C)	3 (3 type C)		1 (1 type C)		
Gloose			1 (0)					
Egret		1 (1 type C)	1 (1 type C)			1 (1 type C)		
Pochard		3 (1 type C)	3 (2 type C)					

* Detection and typing of bont genes and BoNTs provide 100% of concordance for all tested samples.

by Istituto Superiore di Sanità (Italy) Committee on the use of Animals in research and teaching.

2.4. Multiplex real-time PCR SYBR Green

2.4.1. Primers design

Primers for detection of *bont*/C were designed on a consensus sequence generated by the alignment of the light chain domain of neurotoxin gene. This was based on published *C. botulinum* type C DNA sequences, which included: strain 6813 (GenBank accession number D49440), strain CB-19 (AB200358), strain 573 (AB200359), strain 003-9 (AB200360), strain OFD-01 (AB200361), strain 005-1 (AB200362), strain OFD-02 (AB200363), strain OTZ-01 (AB200364), and strain 468C (X73751). This region is conserved in both type C and C/D mosaic strains. Primers for detection of *bont*/D were designed on a consensus sequence generated by the alignment of light chain and heavy chain aminoterminal domains of neurotoxin gene. This was based on published *C. botulinum* type D DNA sequences, which included: strain South Africa (D38442), strain CD16 (S49407), strain 1873 (AB012112), and strain D-4947 (AB037920). These regions are conserved in both type D and D/C mosaic strains. Internal Amplification Control (IAC) was designed in competition with the primers amplifying *bont*/D according to the procedure suggested by Abdulmawjood and colleagues (Abdulmawjood et al., 2002) using commercial

Table 3
Primers.

Target gene	Primer	Primer sequence (5′ → 3′)	Product size (bp)	Position	GeneBank acc. no.
bont/C	FABO_c_F	gag cct gaa aaa gcc ttt cgc a	108	239–260	D49440
	FABO_c_R	tag ggc ttg taa ctc gag gag gtt		324–347	
bont/D	FABO_d_F	tta tgg gag att caa gta cgc ct	422	509–531	D38442
	FABO_d_R	atc cct cgc taa ctt gtg gac gaa		908–931	
IAC	FABO_iac_F	tta tgg gag att caa gta cgc ctT GAT GAG CAT CAC AAA AAT CG[a]	153+46[b]	888–1008	L09137
	FABO_iac_R	atc cct cgc taa ctt gtg gac gaa GAA GGG AGA AAG GCG GAC AG[a]		1021–1040	

[a] Lower case letters refer to bont/D sequence, upper case letters refer to pUC 19 sequence.
[b] The size of PCR product of the first PCR (the PCR in which IAC is amplified by pUC 19 as template) is 153 bp; the size of the PCR product of the IAC during sample analysis is 199 bp as reported by Abdulmawjood et al. (2002).

plasmid pUC19 (GenBank accession number L09137) as template. All primers (Table 3) were designed using free Fast PCR software (Primer Digital Ltd., Helsinki, Finland).

2.5. Multiplex real-time PCR SYBR Green run

Multiplex real-time PCR SYBR Green was performed using Stratagene Mx3005p spectro-fluorometric thermal cycler (Agilent, USA) using MxPro PCR software. Each assay was performed in a total volume of 25 μL containing 3 μL of template DNA, 12.5 μL of 2× QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany), 600 nM of each primer (Table 3), and 3 μL of IAC (150 copies/well). The real-time PCR conditions consisted of initial denaturation and Taq polymerase activation at 95 °C for 15 min, followed 40 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s and followed by a melting curve analysis. All PCRs were performed at least twice. Each PCR run included positive control of *bont*/C, *bont*/D, and IAC, and no template control used as negative control to check for no reagent contamination.

2.6. Determination of the performance parameters of the method

Selectivity study (inclusivity and exclusivity) was performed twice testing DNA extracted from enrichment broths of bacterial strains. Inclusivity was calculated as the ratio between positive results by multiplex real-time PCR and positive results obtained by SMB. Exclusivity was calculated as the ratio between negative results obtained by multiplex real-time PCR and by SMB.

Linearity and limit of detection (LOD) studies were performed testing in triplicate, serial dilutions of DNA from reference strains. A standard curve was obtained plotting the Log of the number of DNA copies (*x*-axis) against the Ct value for these copies (*y*-axis). Amplification efficiency for the different targets was calculated according to the following equation: $E = 10^{(-1/\text{slope})} - 1$. DNA copies were calculated using the following equation: genomes = [(g/μL of DNA spectrophotometrically measured)/(660 × genome size)] × 6.023×10^{23}. Correct quantification of DNA copies, can be done using this equation, assuming that each reference strain cell contains only one bacteriophage harboring *bont* gene, and that the genome sizes are 2.96 Mb and 2.54 Mb for *C. botulinum* type C and *C. botulinum* type D, respectively.

According to the ISO 16140:2003, relative accuracy (AC), relative sensitivity (SE) and relative specificity (SP) were determined comparing the results obtained by the analysis of the samples with SMB (used as a reference method) and multiplex real-time PCR.

AC was calculated for positive and negative samples by the following equation: $AC = [(PA + NA)/N] \times 100\%$, where PA represents the positive results and NA represents the negative results obtained by both SMB and multiplex real-time PCR methods, and *N* represents the total number of tested samples.

SE was calculated for negative samples with the following equation: $SE = (NA/N_-) \times 100\%$, where NA represent the negative results obtained by both SMB and multiplex real-time PCR methods, and N_- represents negative results obtained by SMB.

SP was calculated for positive samples with the equation: $SP = (PA/N_+) \times 100\%$, where PA represents the positive results obtained by both SMB and multiplex real-time PCR methods, and N_+ represents positive results obtained by SMB.

Repeatability of the multiplex real-time PCR was evaluated by determination of intra-assay variation of Ct values obtained analyzing the samples as reported in the additional file. The coefficient of variance (CV = SD/arithmetic mean of Ct) was calculated for results of two replicated in the same run.

2.7. Statistical analysis

Arithmetic means and standard deviations of melting temperature (Tm) values were calculated for multiplex assay to definite the expected Tm for *bont*/C, *bont*/D and IAC. A positive result was assigned in a sample that generated a positive Ct value at the expected Tm for *bont*/C or *bont*/D. A negative result was assigned in a sample that generated a positive Ct value at expected Tm for IAC. The statistical significance of the difference in the means of Tm values obtained by analyzing samples was determined by Student's *t*-test. A P value < 0.05 was chosen as significant.

3. Results

3.1. Design and optimization of multiplex real-time PCR SYBR Green

Primers pairs were designed to obtain distinguishable Tm for the *bont*/C and *bont*/D genes and IAC. These primers resulted highly specific for *bont*/C and *bont*/D once submitted to *in silico* analysis by BLAST (data not shown). Simplex real-time PCR SYBR Green amplification of DNA from *C. botulinum* type C strain NCTC 8264, *C. botulinum* type D strain NCTC 8265, and pCU19 generated PCR products of 108 bp, 422 bp and 199 bp, respectively (data not show), and produced dissociation curves at Tm of 74.34 °C, 76.68 °C and 84.00 °C. The combination of the designed primers pairs in one multiplex real-time PCR SYBR Green produced, for the same templates, dissociation curves at Tm of 74.40 °C, 76.40 °C and 84.40 °C, using 2× QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany), 600 nM of each primers, and thermal profile recommended by Taq polymerase manufacturer. One hundred and fifty copies of IAC/well resulted suitable to evaluate PCR inhibitors in this multiplex real-time PCR SYBR Green assay, also confirmed the results reported elsewhere (Fenicia et al., 2007).

3.2. Performance parameters of multiplex real-time PCR SYBR Green

Selectivity study conducted on 13 target strains (11 *C. botulinum* type C and 2 *C. botulinum* type D), 6 mosaic DNAs (5 type C/D and 1 type D/C), 84 non-target strains (59 non-type C and non-type D BoNT-producing clostridia, 9 other clostridia, 16 non-clostridia strains) showed 100% inclusivity and 100% exclusivity (Table 1). All tested stains

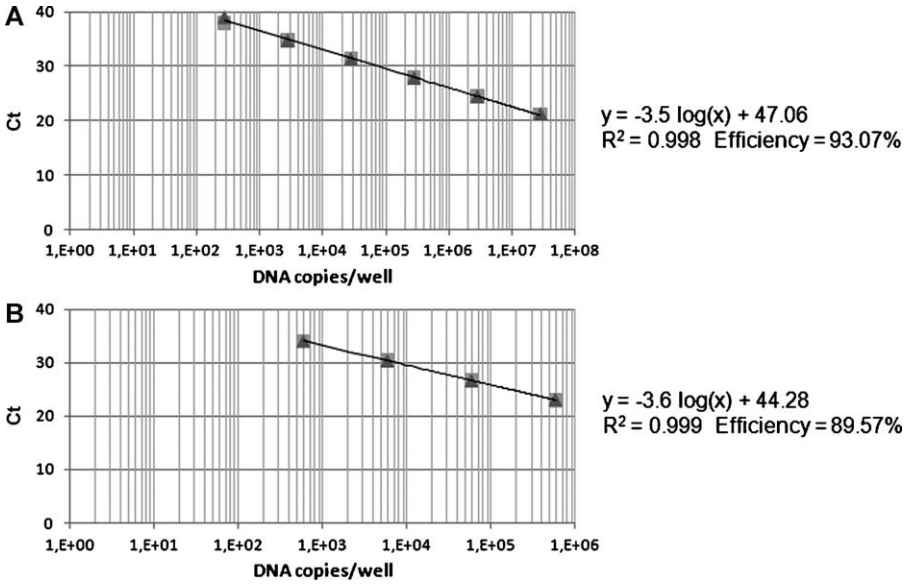

Fig. 1. (A) Standard curve (plot of the Ct values against the DNA copies of *C. botulinum* type C) indicating the linearity of multiplex real-time PCR SYBR Green. The curve is linear overall 6 order of magnitude with R^2 value of 0.998, slope of−3.5 and amplification efficiency of 93.07%. (B) Standard curve (plot of the Ct values against the DNA copies of *C. botulinum* type D) indicating the linearity of multiplex real-time PCR SYBR Green. The curve is linear overall 4 order of magnitude with R^2 value of 0.999, slope of −3.6 and amplification efficiency of 89.57%.

produced Ct values at expected Tm for IAC. Only strains used for inclusivity gave Ct values at expected Tm for target templates. Unspecific amplifications and presence of primers dimers were not observed during selectivity study.

Serial dilutions of DNA from reference strains were used to test the response linearity and LOD of the method. A standard curve was plotted over a range of 2.77×10^7 to 2.77×10^2 copies/well for *C. botulinum* type C, and a range of 5.8×10^5 to 5.8×10^2 copies/well of *C. botulinum* type D. The linear portion of the standard curve was deduced from 2.77×10^7 to 2.77×10^2 copies/well for *C. botulinum* type C (Fig. 1A) and from 5.8×10^5 to 5.8×10^2 copies for *C. botulinum* type D (Fig. 1B), therefore a LOD of 277 and 580 copies per well were established.

Ct cut-off values of 38.31 for *bont*/C, and 34.03 for *bont*/D were established through the LOD determination. These Ct values allow a correct determination of gene targets at 95% confidence intervals (Rodrìguez-Lázaro et al., 2003). The samples with a Ct value greater than the cut-off value were considered negative. As shown in Fig. 1, the linearity of the response was demonstrated in a range of six orders of magnitude for *bont*/C and four orders of magnitude for *bont*/D, with excellent correlation coefficients (R^2) of 0.998 (for *bont*/C) and 0.999 (for *bont*/D). The slopes of the

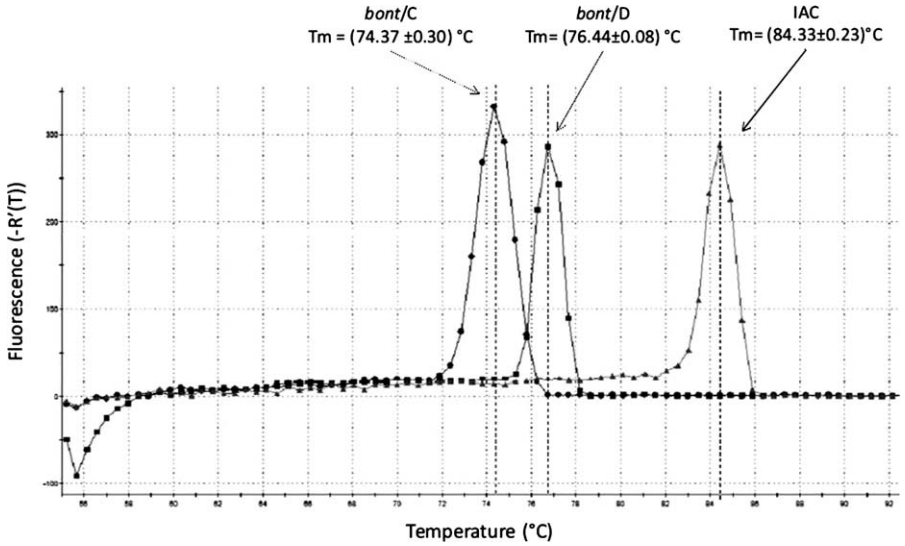

Fig. 2. Dissociation curves of *bont*/C, *bont*/D and IAC indicating, respectively, the melting temperatures of 74.37 ± 0.30 °C, 76.44 ± 0.08 °C, 84.33 ± 0.23 °C.

standard curves (−3.5 and −3.6) were equivalent to PCR efficiencies of 93.07% for *bont*/C and 89.57% for *bont*/D.

One hundred eight samples were tested simultaneously with multiplex real-time PCR SYBR Green and SMB to evaluate AC, SE, and SP. The results obtained testing identical samples using the two methods showed a 100% AC, 100% SE and 100% SP (Table 2). All *bont*/C positive samples generated an average Tm of 74.37 ± 0.30 °C and Ct values in the range of 22.21 ± 0.06 to 33.37 ± 0.29. All *bont*/D positive samples generated an average Tm of 76.44 ± 0.08 °C and Ct values in the range of 26.91 ± 0.16 to 31.74 ± 0.06. Positive and negative samples generated an average Tm of 84.33 ± 0.23 °C for IAC (Fig. 2). A lack of amplification was only observed in a single sample of vegetables that resulted negative by SMB. Student's *t*-test showed no significant difference ($P > 0.05$) either between the Tm values obtained from reference strains or positive samples.

Repeatability of the method was evaluated testing all samples at least twice, reporting a coefficient of variation (CV) of Ct values ranging of 0.00–7.29% with a midpoint of 0.47% (see additional file).

4. Discussion

Animal botulism represents a severe environmental and economical concern due to the high mortality during outbreaks. An epizooty could also result as a public health problem whenever meat or animal products enter in the food chain. To this end, an early clinical diagnosis and a rapid laboratory confirmation are crucial for veterinary public health measures, to contain problems related to the zoonosis. In order to perform laboratory diagnosis of animal botulism cases, all type A, B, C, D, and E toxins have to be considered. Currently, multiplex real-time PCR is unable to detect all *bont* genes involved in animal botulism in a single well and at least two different experiments have been performed. The NRCB currently performs laboratory diagnosis of animal botulism cases using two multiplex real-time PCR protocols, one for type A, B, E, and F and the other for type C and type D genes.

In the study, a multiplex real-time PCR SYBR Green was developed and *in-house* validated for detection and typing of BoNT-producing clostridia harboring type C and type D *bont* genes, in clinical and environmental samples. This method is capable to detect also C/D and D/C mosaic genes; however, they are not differentiated from typical *bont*/C and *bont*/D genes. To the best of our knowledge, it is the first time that a multiplex real-time PCR SYBR Green method for detection and typing *C. botulinum* group III organisms has been used. Considering that the complex matrices tested for laboratory confirmation of animal botulism could contain PCR inhibitors, in avoiding false negative results due to the lack of amplification, an IAC was constructed and included in the protocol, as suggested by Abdulmawjood et al. (2002). However, the wide range of matrices tested, only one sample of vegetables showed a lack of IAC amplification demonstrating that the DNA extraction and culturing used proved to be suitable tools in eliminating or restricting PCR inhibitors.

Results obtained from testing 108 samples with multiplex real-time PCR SYBR Green demonstrated 100% of correlation with the SMB results.

Main advantages of the PCR respect to SMB are the more rapid time of response and the replace of the use of laboratory animals. In fact, SMB needs of 4 days for samples enrichment and at least further a day for animal observation. PCR analysis could be performed on 24 h cultures and final results can be obtained after few hours.

In summary, a rapid, specific and reproducible multiplex real-time PCR SYBR Green method was developed and in-house validated, for the detection and typing of BoNT-producing clostridia group III in animal botulism. Considering the performance parameters and the low cost of this SYBR Green approach, the reported method may be considered as suitable tool, for laboratory diagnosis of type C and type D botulism. In particular, it represents an inexpensive method for testing a large numbers of samples, collected especially during avian botulism outbreaks.

Conflict of interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2011.07.018.

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