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SYBR Green Real-Time PCR Method To Detect *Clostridium botulinum* Type A[▽]

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Botulinum toxins (BoNTs) are classically produced by *Clostridium botulinum* but rarely also from neurotoxigenic strains of *Clostridium baratii* and *Clostridium butyricum*. BoNT type A (BoNT/A), BoNT/B, BoNT/E, and very rarely BoNT/F are mainly responsible for human botulism. Standard microbiological methods take into consideration only the detection of *C. botulinum*. The presumptive identification of the toxigenic strains together with the typing of BoNT has to be performed by mouse bioassay. The development of PCR-based methods for the detection and typing of BoNT-producing clostridia would be an ideal alternative to the mouse bioassay. The objective of this study was to develop a rapid and robust real-time PCR method for detecting *C. botulinum* type A. Four different techniques for the extraction and purification of DNA from cultured samples were initially compared. Of the techniques used, Chelex 100, DNeasy tissue kit, InstaGene matrix DNA, and boiling, the boiling technique was significantly less efficient than the other three. These did not give statistically different results, and Chelex 100 was chosen because it was less expensive than the others. In order to eliminate any false-negative results, an internal amplification control was synthesized and included in the amplification mixture according to ISO 22174. The specificity of the method was tested against 75 strains of *C. botulinum* type A, 4 strains of *C. botulinum* type Ab, and 101 nontarget strains. The detection limit of the reaction was less than 6×10^1 copies of *C. botulinum* type A DNA. The robustness of the method was confirmed using naturally contaminated stool specimens to evaluate the tolerance of inhibitor substances. SYBR green real-time PCR showed very high specificity for the detection of *C. botulinum* types A and Ab (inclusivity and exclusivity, 100%).

Botulism is a severe neuroparalytic disease characterized by flaccid paralysis. Seven antigenically distinct toxin types (A, B, C1, D, E, F, and G) of the botulinum neurotoxins (BoNTs) have been identified (12). These act preferentially on cholinergic nerve endings in both humans and lower animals to prevent the release of acetylcholine on neuromuscular junctions. BoNT type A (BoNT/A), BoNT/B, BoNT/E, and very rarely BoNT/F are mainly responsible for human botulism.

As reported previously, illness resulting from BoNT/A toxin is more severe than those from BoNT/B or BoNT/E (33) and the recovery time is longer (28).

In Italy the National Reference Center for Botulism (NRCB) performs an active surveillance of diseases, mainly by laboratory confirmation of suspected cases of food-borne and infective forms of botulism. About 90% of botulism cases in Italy are correlated with BoNT/B, with mild symptomatology, and the rare severe or fatal cases are correlated with BoNT/A (7).

Although BoNTs are classically produced by *Clostridium botulinum*, since 1979 other BoNT-producing species have been isolated. In particular, *Clostridium baratii*, which produces BoNT/F, has been isolated in the United States (31) and in Hungary (32). *Clostridium butyricum*, which produces BoNT/E, has been isolated in Italy (4, 8, 15; G. Franciosa, F. Anniballi, L. Fenicia, and P. Aureli, New recovery of neurotoxigenic *Clostridium butyricum* type E from case of infant botulism, presented at the European Clostridia Conference, Teistungen, Germany, 1998), China (27), India (11), and Japan (M. Chie, Y. Yanagawa, M. Shibata, H. Obata, S. Yamada, and T. Itoh, Botulism-Tokyo, presented at the 43rd Interagency Botulism Research Coordinating Committee Meeting, Silver Spring, MD, 14 to 17 November 2006).

Standard microbiological methods take into consideration only the detection of *C. botulinum*. The presumptive identification of the toxigenic strains and typing of BoNT have to be performed by mouse bioassay (10). This technique is highly sensitive and specific but laborious, time-consuming, and costly and raises ethical concerns with regard to the use of experimental animals. Efforts have been made to develop alternatives to animal testing, as recommended by international legislation (Directive 86/609/EEC). Molecular biological methods based on the detection of BoNT genes in any neurotoxigenic microorganisms would be an ideal substitute. Different PCR methods for detection of BoNT-producing clostridia in food and clinical samples have been described. Results obtained using PCR assays to detect neurotoxin gene fragments have shown a high level of agreement with those from mouse bioassay (3).

The International Organization for Standardization (ISO) has underlined the need to harmonize and standardize PCR-based methods to detect BoNT-producing *Clostridia* (Resolution 259, taken at the 24th Meeting of ISO TC34 SC9, Warsaw, Poland, June 2005). In recent years, real-time PCR has been used to detect pathogenic microorganisms in food, environmental, and clinical samples (22). Several fluorescent-probe-based real-time PCR studies to detect BoNTs using TaqMan

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probes have been carried out (3, 34). These fluorescent-probe-based assays require the availability of primers and probes that must be selected according to very rigid criteria. Use of the simple and less expensive double-stranded DNA-binding dye SYBR green I for detection of PCR amplicons overcomes this limitation. Therefore, real-time PCR could be applied without the need for fluorescent probes (1). In the absence of probes, the specificity of the reaction is determined on the basis of the melting temperature (T_m). Recently the application of SYBR green real-time PCR to the detection of pathogen microorganisms in different samples was increased (13, 16, 20). The objective of the present study was to establish simple and specific methods to detect BoNT/A gene coding using SYBR green real-time PCR and conventional (electrophoresis-based signal detection) PCR assays, each containing a competitive chimerical internal amplification control (IAC). The robustness of both methods was evaluated by determining the tolerance of inhibitor substances (26) using stool samples collected during suspected botulism cases.

TABLE 1. Results obtained using classic PCR and SYBR green real-time PCR

Strains	No. of strains tested	No. of strains with positive result by:	
		Conventional PCR	SYBR green real-time PCR
C. botulinum type A	75	75	75
C. botulinum type Ab	4	4	4
C. botulinum type B	69	0	0
C. botulinum type E	1	0	0
C. botulinum type F	4	0	0
C. butyricum type E	11	0	0
Others[a]	18	0	0

[a] *Clostridium perfringens* ATCC 13124, *Clostridium carnis* ATCC 10456, *Clostridium histolyticum* NCTC 503, *Clostridium butyricum* ATCC 19398, *Clostridium sporogenes ATCC 11437*, *Bacillus subtilis* ATCC 6633, *Campylobacter jejuni*, *Citrobacter freundii* ATCC 8090, *Enterobacter faecalis* ATCC 29212, *Escherichia coli* ATCC 25992, *Listeria innocua* ATCC 33090, *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enterica* serovar Enteritidis ATCC 13076, *Salmonella enterica* serovar Typhimurium, and *Staphylococcus aureus* ATCC 25923.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study were isolated at the NRCB and are listed in Table 1. *Clostridium* strains were cultured in Trypticase-peptone-glucose-yeast extract (TPGY) broth and incubated in anaerobic conditions for 24 h at 30°C. *Campylobacter jejuni* was cultured in Bolton broth (Oxoid, Basingstoke, United Kingdom) and incubated in microaerophilic conditions for 48 h at 41.5°C. All remaining strains were cultured in brain heart infusion broth (Oxoid) and incubated in aerobic conditions for 24 h at 37°C.

One milliliter of cultured sample was subjected to Chelex 100 (the selected DNA extraction and purification method, as described below), conventional PCR and SYBR green real-time PCR, and melting curve analysis.

Stool specimens, culture conditions, and preparation of DNA. Thirty stool specimens collected at NRCB from food-borne or infant botulism cases were used in the experiments. These specimens were previously tested for the presence of BoNT-producing clostridia according to the CDC method (10) and stored at −20°C. Ten fecal samples containing *C. botulinum* type A (7 samples from infant botulism cases and 3 from food-borne cases), 2 containing *C. botulinum* type Ab (food-borne cases), 4 containing *C. botulinum* type B (food-borne cases), 2 containing *C. butyricum* type E (infant botulism) (15; G. Franciosa et al., presented at the European Clostridia Conference), and 2 containing *C. botulinum* type F (food-borne cases) and 10 specimens in which BoNT-producing clostridia were not detected were tested.

To perform the PCR method, 1 gram of each specimen was cultured in 9 ml of TPGY broth and incubated in anaerobic conditions for 96 h at 30°C. One milliliter of cultured samples was subjected to Chelex 100 (Bio-Rad Life Science Research, Hercules, CA) as described below, conventional PCR and SYBR green real-time PCR, and melting curve analysis.

Comparison among different techniques for extraction and purification of DNA. *C. botulinum* type A ATCC 19397 was incubated in TPGY broth for 18 to 24 h at 30°C. Enrichment broth was subjected to four different DNA extraction procedures, briefly described below.

(i) Boiling. One milliliter of the enrichment broth was transferred to a microcentrifuge tube with a capacity of 1.5 ml. The cell suspension was centrifuged for 10 min at 14,000 × *g* at 4°C. The supernatant was discarded carefully. The pellet was resuspended in 200 μl of DNase-RNase-free distilled water by vortexing. The microcentrifuge tube was incubated for 15 min at 100°C and immediately chilled on ice and centrifuged for 5 min at 14,000 × *g* at 4°C. Five microliters of supernatant was used as the template DNA in the PCR (13).

(ii) Chelex 100. One milliliter of the enrichment broth was transferred to a microcentrifuge tube with a capacity of 1.5 ml. The cell suspension was centrifuged for 10 min at 14,000 × *g* at 4°C. The supernatant was discarded carefully. The pellet was resuspended in 200 μl of 6% Chelex 100 (Bio-Rad Life Science Research) by vortexing. The microcentrifuge tube was incubated for 20 min at 56°C and then for 8 min at 100°C. The suspension was immediately chilled on ice and centrifuged for 5 min at 14,000 × *g* at 4°C. Five microliters of supernatant was used as the template DNA in the PCR (25).

(iii) DNeasy tissue kit (QIAGEN, Hilden, Germany). DNA extraction was carried out according to the manufacturer's instructions. Five microliters of supernatant was used as the template DNA in the PCR.

(iv) InstaGene matrix (Bio-Rad). DNA extraction was carried out according to the manufacturer's instructions. Five microliters of supernatant was used as the template DNA in the PCR.

BoNT/A primer design. Primers were designed by alignment of the published DNA sequences of the *C. botulinum* neurotoxin A gene (GenBank accession numbers M73423, M52066.1, M30196.1, AY953275, AY327854, AF461540.1, and AF488749.1), by using the BioEdit sequence alignment editor (17). The primers were designed to target consensus sequences using Primer Express 1.5 software, generating a 101-bp product (Applied BioSystems, Foster City, CA).

IAC construction. In order to identify false-negative results, an IAC was constructed. The IAC was coamplified with the target DNA (BoNT/A).

The IAC was constructed according to the procedure of Abdulmawjood et al. (2), using commercial plasmid pUC19 (GenBank accession number L09137) as a template (Sigma). The primers used to amplify the IAC, designed using Primer Express 1.5 software, generate a 197-bp product. One microliter of pUC19 was transferred to a vial containing 50 μl of a mixture of 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 200 μM of each deoxynucleoside triphosphate, 1 μM each of primer IOA1-pUC19 F (5′-GGG CCT AGA GGT AGC GTA GTG TGA CGA GCA TCA CAA AAA TCG-3′) and IOA2-pUC19 R (5′-TCT TCA TTT CCA GAA GCA TAT TTT GAA GGG AGA AAG GCG GAC AG-3′) (M-Medical Genenco, Florence, Italy), and 2.5 U of *Taq* polymerase (Applied BioSystems, Roche Molecular Systems). A 30-cycle PCR was carried out using the following conditions: denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and elongation for 2 min at 72°C. After the 30th cycle, the extension reaction was continued for another 10 min at 72°C. To determine the optimal IAC concentration, 10-fold dilutions of IAC (ranging from 1.5×10^0 to 15×10^7 copies) were tested in the presence of 6×10^6 copies of the *C. botulinum* type A genome and analyzed using conventional PCR or SYBR green real-time PCR. The optimal IAC concentration was determined for both conventional and real-time PCR and was established when the amplicon-specific band in conventional PCR or melting peak in real-time PCR was always present in the BoNT/A-negative broth cultures and absent in the BoNT/A-positive broth cultures.

Conventional (electrophoresis-based signal detection) PCR. PCR was carried out in a programmable thermal cycler (model 9700; Applied BioSystems) and was performed in 50 μl containing 10× PCR buffer (Applied BioSystems), 4.0 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (100 mM), 0.3 μM primers IOA1a F (5′-GGGCCTAGAGGTAGCGTARTG-3′) and IOA2a R (5′-TCTTYATTTCCAGAAGCATATTTT-3′) (M-Medical Genenco), 1.25 U of *Taq* polymerase (Applied BioSystems, Roche Molecular Systems), 1,500 copies of IAC, and 5 μl of purified DNA. The reaction mixture was incubated at 95°C for 15 min to activate the *Taq* polymerase and then subjected to 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 55°C), and extension (30 s at 72°C). After the 30th cycle, the extension reaction was continued for another 7 min at 72°C. Products were analyzed by 2% agarose gel electrophoresis at 90 V for 70 min, and the sizes of the fragments were determined by relating their

TABLE 2. C_T values obtained from the four methods of DNA extraction and purification

Extraction method	Mean[a] C_T ± SD
Boiling	27.60 ± 0.80
Chelex 100	21.28 ± 0.20
InstaGene matrix	21.14 ± 0.06
DNeasy tissue kit	21.19 ± 0.13

[a] Values are means of three determinations.

positions on the gel to those of standard DNA fragments (EZ Load 100-bp molecular ruler, catalog no. 170-8352; Bio-Rad). To avoid contamination, sample preparation, DNA amplification, and electrophoresis were carried out in three different rooms.

SYBR green real-time PCR. Real-time PCR and data analysis were performed in a total volume of 50 μl using 96-well microwell plates and an ABI PRISM 7700 sequence detector (Applied BioSystems, Foster City, CA).

Five microliters of purified DNA, 25 μl SYBR green I PCR Master Mix (Applied BioSystems), 200 nM of primers IOA1a and IOA2a, and 150 copies of IAC were added to each microwell. To reach a total volume of 50 μl per well, DNase-RNase-free distilled water (Sigma) was added. The reaction was run online at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 55°C for 30 s and an extension phase of 1 cycle at 95°C for 60 s, 60°C for 60 s, and 95°C for 60 s (ramp time, 19.59 min).

All PCRs were performed in triplicate.

To evaluate the efficiency of the amplification, a standard curve was constructed using the threshold cycle (C_T) versus 10-fold dilution (6×10^1 to 6×10^7 copies of DNA) of *C. botulinum* type A. The specificity of the reaction is given by the detection of the T_ms of the amplification products immediately after the last reaction cycle. Results were analyzed with the melting curve analysis software (Dissociation Curve 1.0; Applied BioSystems) provided with the ABI PRISM 7700 sequence detector.

Statistical analysis. The inclusivity and exclusivity were calculated according to the Microval protocol (6). Inclusivity is the ability of the PCR method to detect the target analyte from a wide range of strains. Exclusivity is the lack of interference of a relevant range of nontarget strains in the PCR methods (24).

The amplification efficiency (E) was calculated using the following equation: $E = (10^{-1/\text{slope}}) - 1$ (21).

The means and standard deviations of the C_T and of the T_m were calculated for each target strain and nontarget strain, respectively.

To compare the results obtained by the four different methods of extraction, one-way variance with Bonferroni post hoc comparisons was used.

The statistical significance of the difference in the means of T_m values obtained by analyzing bacterial strains or stool samples was determined by Student's t test. A P value <0.05 was chosen as significant.

RESULTS

Table 2 shows the C_T values obtained by the four DNA extraction and purification techniques used to extract and purify DNA of *C. botulinum* type A. One-way analysis of variance of the mean differences in C_T values among the four DNA extraction and purification techniques showed a statistically significant difference ($F = 178.27$; $P < 0.0001$). The post hoc Bonferroni comparison showed that, compared to all other methods considered, the boiling techniques gave higher and significantly different C_T values. Chelex 100, Instagen matrix, and DNeasy tissue techniques were not significantly different from each other.

A standard curve constructed by plotting the mean C_T ($n = 3$) versus logarithmic concentrations of *C. botulinum* (ranging from 6×10^1 to 6×10^7 copies of DNA) displayed a good linearity of response ($R^2 = 0.9962$). The detection limit of the reaction was less than 6×10^1 genome copies of *C. botulinum* type A. Standard regression analyses of the linear part of the slope gave a coefficient of −3.3378, and the PCR efficiency calculated from the slope of the dilution curve was 0.9925.

SYBR green real-time PCR revealed that, when using a concentration of 6×10^6 genome copies of *C. botulinum*, a concentration of 150 copies of IAC is optimal. In fact the addition of 150,000 copies of IAC produced complete competition of the target BoNT/A gene with the amplification of only the IAC amplicon, while the addition of 15,000 and 1,500 copies of IAC produced a melting curve in which the presence of both amplicons is clear. When 150 and 15 copies were added, only the peak resulting from the BoNT/A amplicon was present (Fig. 1). When conventional (electrophoresis-based signal detection) PCR was used, 1,500 copies of IAC were found to be optimal (Fig. 2) when combined with a concentration of 6×10^6 copies of the *C. botulinum* genome.

The inclusivity and exclusivity were tested against a panel of 75 strains of *C. botulinum* type A, 4 strains of *C. botulinum* type Ab, 74 strains of *C. botulinum* non-type A (including type B, D, E, and F), 11 strains of *C. butyricum* type E, and 16 strains of other nontarget strains (Table 1).

SYBR green real-time PCR shows specific PCR products

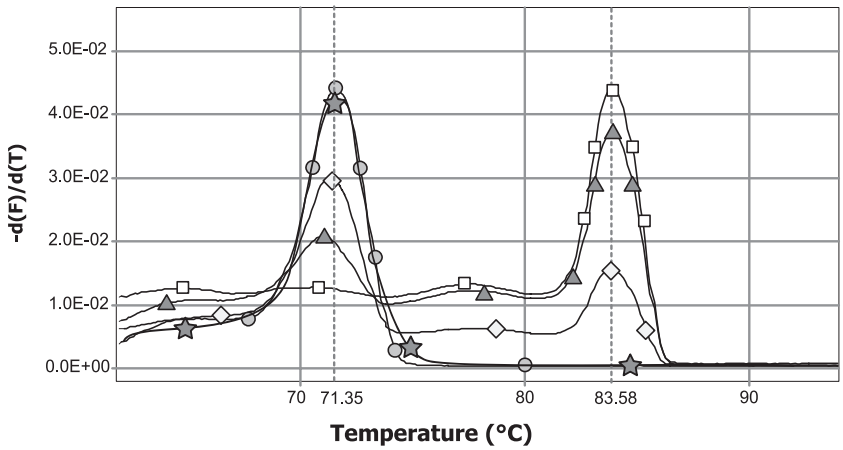

FIG. 1. Melting curves obtained after coamplification of 6×10^6 copies of *C. botulinum* ATCC 19397 and (★) 15 copies, (●) 150 copies, (◇) 1,500 copies, (▲) 15,000 copies, and (□) 150,000 copies of IAC.

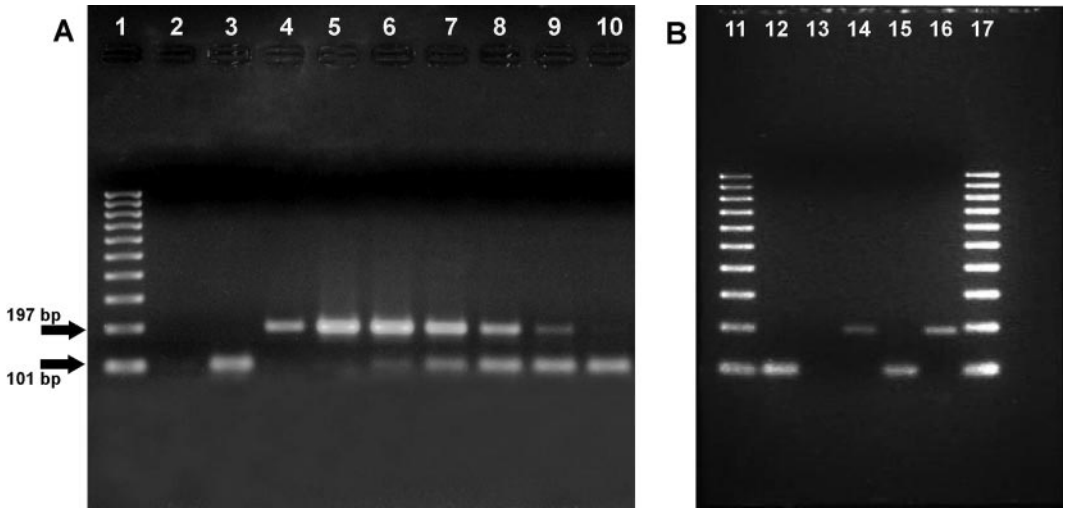

FIG. 2. Conventional (electrophoresis-based) PCR. (A) Coamplification of a 10-fold dilution of IAC (197 bp) in the presence of *C. botulinum* DNA (101 bp). Lane 1, marker (EZ Load 100-bp molecular ruler; Bio-Rad); lane 2, negative control; lane 3, positive *C. botulinum* DNA control; lane 4, positive IAC control; lane 5, 1.5×10^6 copies of IAC and 6×10^6 copies of *C. botulinum* DNA; lane 6, 1.5×10^5 copies of IAC and 6×10^6 copies of *C. botulinum* DNA; lane 7, 1.5×10^4 copies of IAC and 6×10^6 copies of *C. botulinum* DNA; lane 8, 1.5×10^3 copies of IAC and 6×10^6 copies of *C. botulinum* DNA; lane 9, 1.5×10^2 copies of IAC and 6×10^6 copies of *C. botulinum* DNA; lane 10, 1.5×10^1 copies of IAC and 6×10^6 copies of *C. botulinum* DNA. (B) Lanes 11 and 17, marker (EZ Load 100-bp molecular ruler; Bio-Rad); lane 12, positive control; lane 13, negative control; lane 14, positive IAC control; lane 15, positive stool sample; lane 16, negative stool sample.

identified by melting curve analysis, and a reproducible T_m of 71.35 ± 0.22°C was observed for all *C. botulinum* type A strains. Negative controls and non-*C. botulinum* type A strains showed an IAC-specific peak at 83.58 ± 0.16°C, giving evidence of the specificity of the method (Fig. 3A). Assay of all 79 *C. botulinum* type A and Ab strains gave a specific band of 101 bp, while assay of all 101 other strains gave only the IAC-specific 197-bp band (Fig. 1) using conventional (electrophoresis-based signal detection) PCR.

Of the 30 stool samples analyzed, 12 gave positive results with conventional (electrophoresis-based signal detection) PCR (Fig. 2) and SYBR green real-time PCR, confirming the presence of *C. botulinum* type A or Ab (Fig. 3B), while the other 18 showed negative results.

The mean peak obtained with the 36 curves specific for *C. botulinum* type A was 71.49 ± 0.35°C, and the mean peak obtained with the 54 curves specific for the IAC was 83.68 ± 0.17°C (Fig. 3B).

Student's *t* test showed no significant difference either between the results obtained from the mean peaks obtained analyzing strains and stool specimens or between the mean peaks obtained from nontarget strains and from negative stool specimens.

DISCUSSION

The use of the PCR in microbial diagnostics is a valuable alternative to time-consuming traditional methods, especially when PCR could be applied alternatively to animal-based assays. In fact, PCR testing offers the possibility to improve detection and characterization of pathogenic bacteria, since one can target species-specific DNA regions and specific traits of pathogenicity (23) represented in BoNT-producing clostridia by the genes coding for toxins.

Different aspects have to be evaluated in developing new PCR methods, such as the DNA extraction and purification step, the inclusion of new epidemiological strains in the design of the primers, and the inclusion of the appropriate positive and negative controls.

In the evaluation of the DNA extraction and purification step, the boiling extraction method, in contrast to its application to a gram-negative target like *Salmonella enterica* (13), did not show good recovery when applied to gram-positive clostridia and was therefore excluded. This result confirms that more-destructive methods are needed to break the cell walls of gram-positive bacteria and release bacterial DNA (9, 29). Therefore, Chelex was chosen over InstaGene matrix and DNeasy tissue kit techniques for its simplicity, rapidity of execution, and low cost.

The primers selected showed very high specificity for the detection of *C botulinum* type A and Ab (inclusivity and exclusivity, 100%), using a large number of strains (79 strains of *C. botulinum* type A and Ab and 101 nontarget strains); this specificity was the consequence of the use of a significant number of published sequences of the BoNT/A gene code in the design of the primers. The effectiveness of the proposed method was, furthermore, confirmed by the analysis of 30 stool specimens, for which neither false-positive nor false-negative results were obtained.

Furthermore, notwithstanding the use of two primers with one degenerate base, nonspecific amplicons were observed; this confirmed the high specificity of the amplification.

A major drawback of most published PCR-based methods for detection of the BoNT gene of *C. botulinum* is the absence of an IAC (22). The inclusion of an IAC in these methods prevents false-negative results due to the inhibition of the polymerase enzyme or the malfunction of the thermal cycler (18). The lack of IAC detection indicates that the analysis has

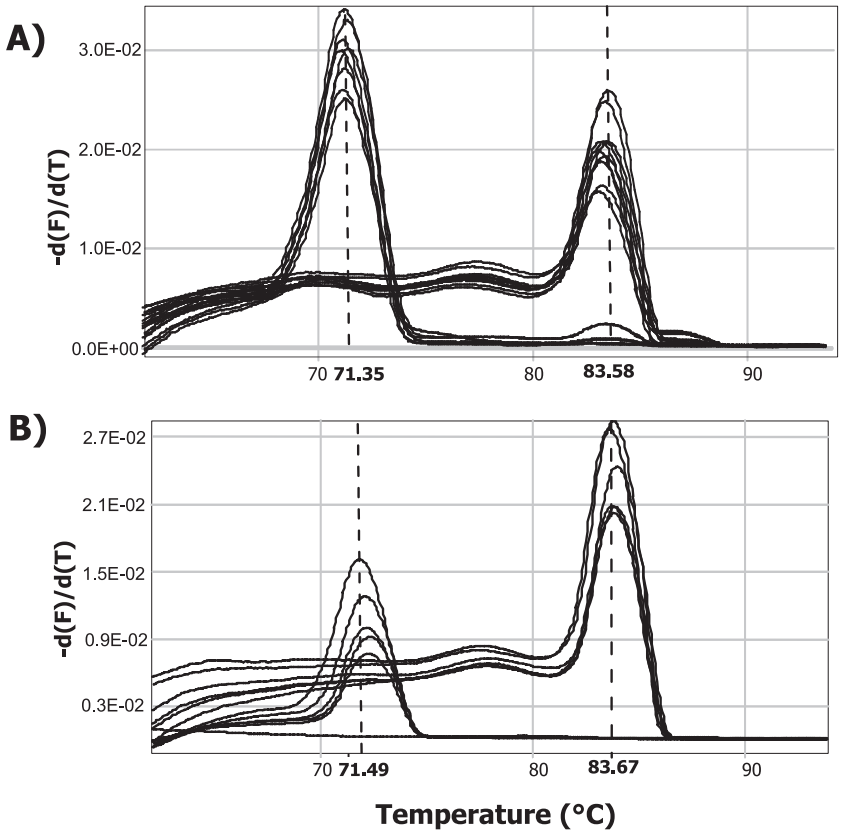

FIG. 3. Melting curve after 35 cycles. (A) Pure culture of strains of *C. botulinum* type A (T_m = 71.35°C) and non-*C. botulinum* type A (T_m = 83.58°C) related to the IAC amplicon. (B) Cultures of enrichment broths of five naturally contaminated stools containing *C. botulinum* type A (T_m = 71.49°C) and six stools that did not contain BoNT clostridia (T_m = 83.67°C) related to IAC amplicons.

to be repeated, for example, using new reagents or an alternative method of DNA extraction and purification (19).

Recently the publication of ISO 22174 has made mandatory the inclusion of an IAC in any international standard using PCR based-methods to detect pathogenic microorganisms in food (5). The use of a competitive IAC was proposed for an enzyme-linked immunosorbent assay-PCR (14), while a noncompetitive exogenous internal control was recently proposed for a real-time PCR assay to detect BoNT/A, BoNT/B, and BoNT/E fragment genes (3).

The main disadvantage of the use of a noncompetitive IAC is that it will not accurately reflect amplification of the primary target due to the differences in the primer sequences. However, the use of a competitive IAC is strongly recommended to avoid the risk of undesired interaction of multiple primers; both PCRs (the target specific and the IAC specific) work with the same primer set and under identical reaction conditions. Determination of the optimal concentration of the competitive IAC in the PCR-based methods is a crucial step, because the optimal concentration should be kept as low as possible to avoid inhibition of the target-specific reaction (30). The use and the optimization of a competitive internal control for application in a SYBR green real-time PCR method have been proposed in this paper.

The presence of the IAC-specific peak in all the negative results demonstrated the absence of reaction inhibition. A reaction efficiency of 0.9925 is proof that all the conditions chosen for the reaction (primer design, buffer composition, and amplification condition) allowed a twofold increase in amplification product after every cycle (21).

We have confirmed that methods developed for SYBR green real-time PCR could be easily applicable to conventional PCR. In fact, unlike other published methods developed specifically for real-time PCR or conventional PCR, this protocol can be applied to either technique with only slight modification This could facilitate the introduction of real-time PCR equipment to laboratories that are already using standard protocols for conventional PCR and thus avoid the use of laborious and time-consuming electrophoresis in the detection phase.

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