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Detection of *Clostridium botulinum* types A, B, E and F in foods by PCR and DNA probe

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E. ARANDA, M.M. RODRÍGUEZ, M.A. ASENSIO AND J.J. CÓRDOBA. 1997. A PCR procedure was developed for the detection of *Clostridium botulinum* in foods. PCR products were detected in agarose gels and by Southern hybridization. The sensitivity of PCR was tested in broth cultures and in canned asparagus, dry cured ham and honey. The sensitivity of the method in broth was high (2·1–8·1 cfu ml^{-1}) for types A and B, but rather low (10^4 cfu ml^{-1}) for types E and F. However, after enrichment at 37°C for 18 h, it was possible to detect *Cl. botulinum* types A, B, E and F in food samples at initial levels of about 1 cfu 10 g^{-1} of food. This PCR detection protocol provides a sensitive and relatively rapid technique for the routine detection of *Cl. botulinum* in foods.

INTRODUCTION

Clostridium botulinum produces seven different neurotoxins (BoNT) that are designated A to G (Dasgupta 1990). BoNT A, B, E and F cause human botulism, while BoNT C and D are the causative agents of animal botulism. In addition to the 'classical' food-borne botulism caused by the toxin preformed in the foods, 'infant' botulism due to the toxin produced in the intestine after ingestion of spores has been described (Hatheway 1992).

Low concentrations of *Cl. botulinum* pose a high potential risk for both classical and infant botulism (Dodds 1992). Thus, extremely sensitive methods are needed to detect *Cl. botulinum* in foods.

The standard method for detection of *Cl. botulinum*, using bacteriological characteristics and confirmation of BoNT production by mouse bioassay (AOAC 1990), is sensitive but time-consuming, very expensive and requires handling of laboratory animals.

Since BoNT genes have been characterized (Binz *et al*. 1990 ; Thompson *et al*. 1990 ; East *et al*. 1992 ; Poulet *et al*. 1992), DNA-based methods, such as PCR and gene probes, can be used for detection of *Cl. botulinum*. A single set of degenerate primers and a DNA probe have been used for the specific detection of the toxin genes of *Cl. botulinum*, *Cl. barati* and *Cl. butyricum*, (Campbell *et al*. 1993 ; Córdoba *et al*. 1995). The aim of this work was to develop a sensitive PCR procedure using these primers and a DNA probe to detect *Cl. botulinum* in foods.

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MATERIALS AND METHODS

Cultures

The organisms used in the present study included four strains of *Cl. botulinum* and also *Cl. sporogenes* (four strains), *Cl. perfringens* (five strains), *Cl. butyricum*, *Cl. histolyticum*, *Cl. limosum*, *Cl. tyrobutyricum*, *Cl. chauvoei* and *Cl. spiroforme* as controls (Table 1). Cultures were grown under anaerobic conditions at 37°C for 24 h in trypticase, yeast extract, peptone, cysteine, glucose (TYPCG) broth containing trypticase (30 g l^{-1}), yeast extract (12 g l^{-1}), peptone (10 g l^{-1}), cysteine (0·5 g l^{-1}), glucose (5 g l^{-1}), sodium chloride (5 g l^{-1}), starch (1 g l^{-1}) and sodium acetate (3 g l^{-1}). Overnight cultures were serially diluted to obtain eight inocula containing from about 1 to 10^7 cfu ml^{-1}. The bacterial loads were determined on TYPCG agar under anaerobic conditions at 37°C for 48 h.

Food inoculation

Samples (10 g) of canned asparagus, dry cured ham and honey were homogenized separately with 90 ml of TYPCG broth for 2 min in a Stomacher Lab blender 400 (Seward Medical UAC House, London, UK) and inoculated with 1 ml each of dilutions 1 : 10^5, 1 : 10^6, 1 : 10^7 of overnight cultures of *Cl. botulinum* types A, B, E and F. Food samples inoculated

Table 1 Species tested by PCR with primers BoNT1·1 and BoNT2 and their PCR products with probe Con.BoNT

Strains	PCR amplification with primers BoNT1.1 and BoNT2	Signal with probe Con.BoNT
Clostridium botulinum type A NCTC 2916	+	+
Cl. botulinum type B NCTC 7273	+	+
Cl. botulinum type E NCTC 8266	+	+
Cl. botulinum type F ATCC 10281	+	+
Cl. sporogenes NCTC 532	−	−
Cl. sporogenes NCTC 8594	−	−
Cl. sporogenes ATCC 11437	−	−
Cl. sporogenes ATCC 3584	−	−
Cl. perfringens type A NCTC 8237	−	−
Cl. perfringens type B NCTC 8533	−	−
Cl. perfringens type C NCTC 3180	−	−
Cl. perfringens type D NCTC 8346	−	−
Cl. perfringens CECT 4110	−	−
Cl. butyricum NCTC 7423	−	−
Cl. histolyticum CECT 4109	−	−
Cl. limosum CECT 4329	−	−
Cl. tyrobutyricum CECT 4011	−	−
Cl. chauvoei CECT 4108	−	−
Cl. spiroforme CECT 4322	−	−

NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection; CECT, Spanish Type Culture Collection.

with 1 ml of dilution $1:10^1$ (*ca* 10^6 cfu ml^{-1}) of overnight culture of *Cl. sporogenes* NCTC 532 were tested as controls. The inoculated food suspensions were incubated under anaerobic conditions at 37°C for 18 h for enrichment, prior to further analysis.

In addition, samples (10 g) of honey were inoculated with 1 ml of a $1:10^6$ dilution (*ca* 10 cfu ml^{-1}) of both *Cl. sporogenes* and each type of *Cl. botulinum*. Honey samples inoculated with the same load of only *Cl. sporogenes* NCTC 532 were tested as controls. The inoculated honey samples were incubated at 37°C for 18 h in anaerobic conditions, before DNA recovery.

DNA recovery

Cells were collected from 1·5 ml of TYPCG broth or inoculated food suspensions by centrifugation at 9840 *g* for 10 min. The pelleted cells were suspended in 500 μl of TES (Tris, EDTA, NaCl) buffer containing 2 μg μl^{-1} lysozime (Sigma Chemical Co., St Louis, MO, USA). To remove proteins and RNA, 25 μl proteinase K (10 μg μl^{-1}) (Sigma) and 25 μl RNAase (10 μg μl^{-1}) (Sigma) were added, and the mixture was heated at 65°C for 30 min. Then, 50 μl 20% sodium dodecyl sulphate (SDS) were added. DNA was further purified by using phenol–chloroform–isoamyl alcohol (25 : 24 : 1) (Lawson *et al*. 1989) and dissolved in 50 μl pure water. This solution (10 μl) was used for PCR amplification.

PCR amplification

For amplification of BoNT genes, primers BoNT1·1 [5′-CC(C/A)TAT(A/G)TAGG(A/T)C(T/C)TGCTTTAAA TATAGG(A/T)A(A/T)T-3′] and BoNT2 [5′-TTAGT (T/A)ATAGTTACAAAAATCCA(T/C)(T/C)T(A/G)T TTATATATA-3′] were used (Campbell *et al*. 1993; Córdoba *et al*. 1995). PCR amplification was performed in a 50 μl volume containing 5 μl of a deoxyribonucleotide mixture (1·25 mmol l^{-1} each), 1 μl of each primer (20 mmol l^{-1}), and 10 μl of the extracted DNA solution. The mixture was denatured at 94°C for 3 min before the addition of 1 U of Taq DNA polymerase (Pharmacia Biotech, Uppsala, Sweden). PCR amplification was performed by using a thermal cycler from Biometra (Maidstone, UK) following the method described by Córdoba *et al*. (1995) with some modifications: 29 cycles of 92°C for 1 min, 47°C for 1 min, and 58°C for 5 min. A final extension was performed at 58°C for 5 min. As a result of PCR amplification with the above primers, a DNA fragment of 1·1 kbp is expected.

Detection of PCR products

Agarose gels. PCR products were analysed by submarine gel electrophoresis in a 1% agarose gel. Gels were run for 60 min at 90 V in Tris-acetate buffer and stained with ethidium bromide (0·5 μg ml^{-1}). The electrophoresed products were visualized by u.v. transillumination and photographed. DNA molecular size marker VI from Boehringer Mannheim (Mannheim, Germany) was used to determine the size of PCR products.

Probe hybridization. The PCR products generated with BoNT1·1 and BoNT2 primers were tested by DNA hybridization with the specific DNA probe Con.BoNT (Campbell *et al*. 1993). For this, amplified DNA fragments from BoNT genes were denatured by boiling for 5 min in 1 × SSC (0·15 mol l^{-1} NaCl and 0·015 mol l^{-1} trisodium citrate). The denatured DNA fragments were transferred to a Nylon membrane Hybond H^+ (Amershan International, Amersham, UK) with a slot blot apparatus (Millipore, Bedford, USA) and fixed in 0·4 mol l^{-1} NaOH. The blot was hybridized with probe Con.BoNT labelled with fluorescein-dUTP for 2 h and washed in 1 × SSC–0·1% SDS (w/v) at 42°C for 30 min. The hybridization was detected using an enhanced chemi-

luminescence kit (Amersham International), exposing the blot to X-ray film for 5 min.

RESULTS AND DISCUSSION

PCR amplification products of 1·1 kbp were obtained from all four strains of *Cl. botulinum*, but no amplification was detected for the remaining species used as negative controls (Table 1).

Sensitivity of PCR amplification

The sensitivity of the method was tested in TYPCG broth using eight dilutions of overnight cultures of *Cl. botulinum* types A, B, E and F, starting from $2{\cdot}1 \times 10^7$, $8{\cdot}1 \times 10^6$, $9{\cdot}3 \times 10^6$ and $1{\cdot}1 \times 10^7$ cfu ml^{-1}, respectively. Agarose gel electrophoresis revealed PCR products of 1·1 kbp from TYPCG dilutions down to 2·1 cfu ml^{-1} for type A and 8·1 cfu ml^{-1} for type B. However, amplified products of 1·1 kbp from *Cl. botulinum* types E and F were detected only when counts were higher than 10^4 cfu ml^{-1} (Fig. 1).

All PCR products revealed by agarose gel electrophoresis were also detected by hybridization with probe Con.BoNT. In addition, hybridization with this probe allowed a 10-fold increase in the sensitivity of detection of *Cl. botulinum* type B, but not for types E and F (Fig. 2). Thus, the sensitivity of the method is high for *Cl. botulinum* types A and B, but rather low for types E and F. Primers BoNT1·1 and BoNT2 showed less differences in the nucleotide sequence with BoNT E and BoNT F genes (Poulet *et al.* 1992; East *et al.* 1992) than with BoNT A and BoNT B genes (Binz *et al.* 1990; Whelan *et al.* 1992). Therefore, the lower sensitivity of the method for types E and F cannot be explained by a higher mismatching rate of the primers with BoNT E and BoNT F genes.

Efficiency of PCR amplification in foods

Given that the sensitivity of PCR amplification for *Cl. botulinum* types E and F was rather low, an enrichment step of 18 h at 37°C was used, prior to the detection of *Cl. botulinum* in foods. This enrichment had proved to be efficient for the detection of *Cl. botulinum* by PCR (Fach *et al.* 1993; Szabo *et al.* 1994), since inhibitory substances present in foods are diluted and false positive results due to non-culturable bacteria are eliminated.

Counts for the first of the three dilutions used for con-

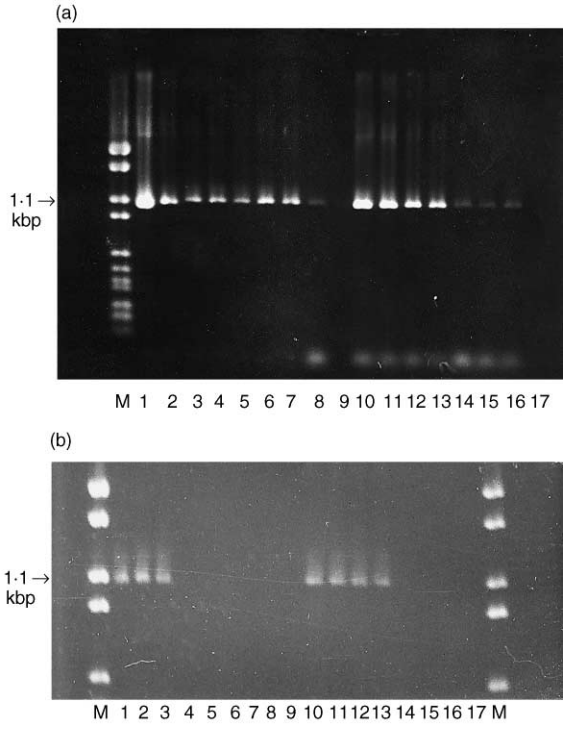

Fig. 1 Agarose gel electrophoresis of amplification products obtained from decimal dilutions in TYPCG broth of (a) *Clostridium botulinum* type A starting at $2{\cdot}1 \times 10^7$ cfu ml^{-1} (lanes 1–8) and type B starting at $8{\cdot}1 \times 10^6$ cfu ml^{-1} (lanes 10–17), and (b) type E starting at $9{\cdot}3 \times 10^6$ cfu ml^{-1} (lanes 1–8) and type F starting at $1{\cdot}1 \times 10^7$ cfu ml^{-1} (lanes 10–17). M, DNA molecular weight marker VI (Boehringer Mannheim)

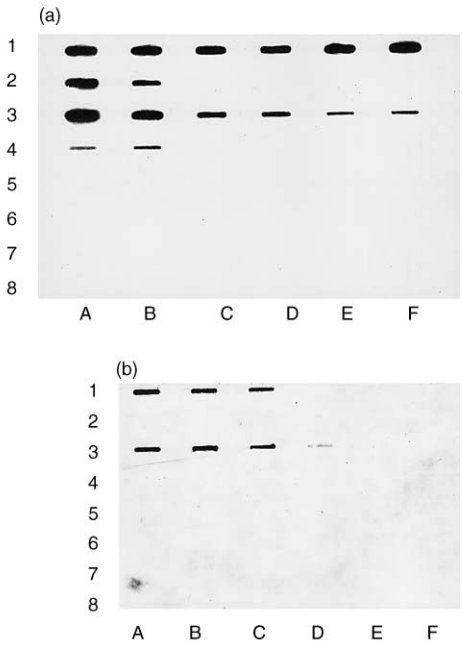

Fig. 2 Slot blot hybridization with probe Con.BoNT of PCR products of (a) type A from dilutions containing $2{\cdot}1 \times 10^7$ to 2·1 (A1–B2) and type B from dilutions containing $8{\cdot}1 \times 10^6$ to 0·81 cfu ml^{-1} (A3–B4), and (b) type E from dilutions containing $9{\cdot}3 \times 10^6$ to 0·93 cfu ml^{-1} (A1–B2) and type F from dilutions containing $1{\cdot}1 \times 10^7$ to 1·1 cfu ml^{-1} (A3–B4)

taminating 10 g food samples were $2{\cdot}1 \times 10^2$ cfu ml^{-1} for type A, $8{\cdot}1 \times 10^1$ cfu ml^{-1} for type B, $9{\cdot}3 \times 10^1$ cfu ml^{-1} for type E and $1{\cdot}1 \times 10^2$ cfu ml^{-1} for type F. From the three foods assayed, PCR products of 1·1 kbp were detected on agarose gels, irrespective of the dilution used and the *Cl. botulinum* type (results for type E are shown in Fig. 3). In all cases PCR products were also detected by hybridization with Con.BoNT probe. Thus, with the enrichment step, the PCR method allowed detection of an initial contamination of about 1 cfu of *Cl. botulinum* types A, B, E and F per 10 g of food. As a consequence, the method is sensitive enough to detect the lowest count (10 cfu g^{-1}) of *Cl. botulinum*, referred to as being able to cause infant botulism (Dodds 1992).

Several PCR methods to detect *Cl. botulinum* in foods have been described. Most are able to detect only one type of *Cl. botulinum* (Ferreira *et al*. 1993 ; Fach *et al*. 1993 ; Sciacchitano and Hirshfield 1996). Szabo *et al*. (1994) used a specific pair of primers for each of the types A, B and F. However, with the PCR procedure described in this work, it is possible to detect at least types A, B, E and F using a single set of primers. Fach *et al*. (1995) also described a single set of primers to amplify BoNT A, B, E, F and G genes detecting initial contamination of 1–10 cfu of *Cl. botulinum* per gram of food. However, in the latter work specific DNA probes for each type of *Cl. botulinum* were used, while just the Con.BoNT probe is enough to detect PCR products of *Cl. botulinum* types A, B, E and F.

The ability of the enrichment step to support growth to detectable levels from very low numbers of *Cl. botulinum* when closely related organisms are present was also assessed. For this, honey samples were inoculated simultaneously with *Cl. botulinum* and *Cl. sporogenes*. Each type of *Cl. botulinum* was detected both in the agarose gel and by hybridization with Con.BoNT probe when *Cl. sporogenes* was present (Fig. 4).

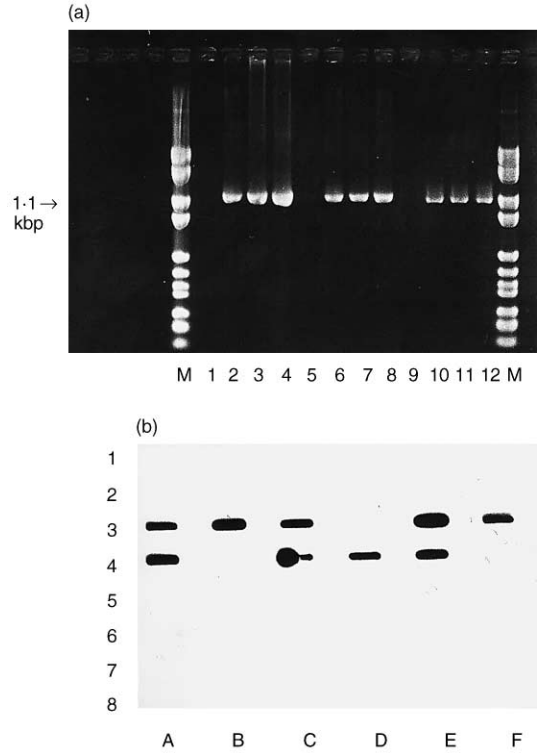

Fig. 3 (a) Agarose gel electrophoresis of PCR products obtained from canned asparagus (lanes 2–4), dry-cured ham (lanes 6–8) and honey (lanes 10–12) inoculated with *Clostridium botulinum* type E at 0·93, 9·3 and 93 cfu 10 g^{-1} of foods. Lanes 1, 5 and 9, PCR products of the above foods inoculated only with *Cl. sporogenes*. M, DNA molecular weight marker VI (Boehringer Mannheim). (b) Slot blot hybridization with probe Con.BoNT of the above PCR products (canned asparagus, A3–C3 ; dry-cured ham, E3–A4, and honey, C4–E4. D3, B4 and F4 contain PCR products of foods samples inoculated only with *Cl. sporogenes*

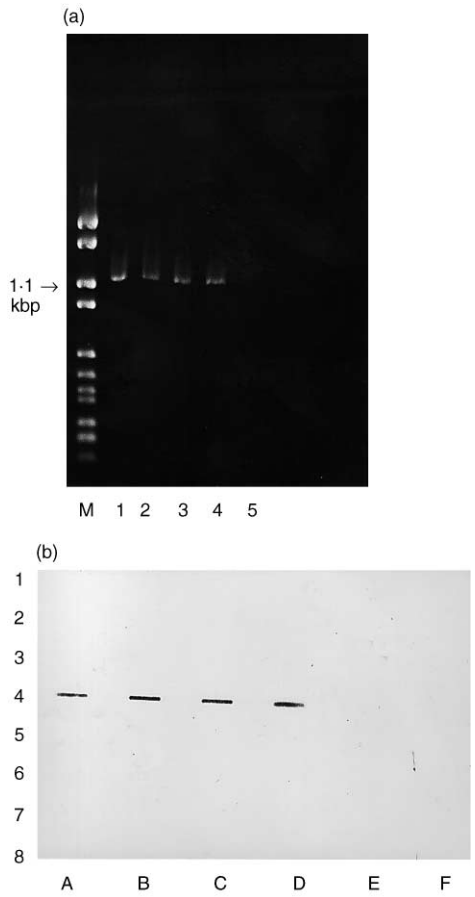

Fig. 4 (a) Agarose gel electrophoresis of PCR products obtained from honey inoculated both with *Clostridium sporogenes* and *Cl. botulinum* types A (lane 1), B (lane 2), E (lane 3), F (lane 4) or inoculated only with *Cl. sporogenes* (lane 5). M, DNA molecular weight marker VI (Boehringer Mannheim). (b) Slot blot hybridization with probe Con.BoNT of the above PCR products (*Cl. sporogenes* and *Cl. botulinum* types A(A4), B(B4), E(C4), F(D4), and *Cl. sporogenes* only (E4))

There was no amplification when honey samples were inoculated only with *Cl. sporogenes* (Fig. 4).

The PCR method described in this work provides a reliable and simple means to detect *Cl. botulinum* in foods, even at levels of about 1 cfu 10 g^{-1}. This procedure is moderately time consuming, given that at least four types of *Cl. botulinum* can be investigated in around 26 h when the detection is done by agarose gel, or 30 h when probe hybridization in included. Although it may need to be optimized for other foods, this PCR detection protocol provides a sensitive and reasonably fast technique for routine analysis of *Cl. botulinum*.

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