APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2001, p. 5694–5699 Vol. 67, No. 12
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.12.5694–5699.2001

Multiplex PCR Assay for Detection and Identification of *Clostridium botulinum* Types A, B, E, and F in Food and Fecal Material

MIIA LINDSTRÖM,* RIIKKA KETO, ANNUKKA MARKKULA, MARI NEVAS, SEBASTIAN HIELM, AND HANNU KORKEALA

Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, FIN-00014 Helsinki University, Finland

Received 21 May 2001/Accepted 22 September 2001

Botulism is diagnosed by detecting botulinum neurotoxin and *Clostridium botulinum* cells in the patient and in suspected food samples. In this study, a multiplex PCR assay for the detection of *Clostridium botulinum* types A, B, E, and F in food and fecal material was developed. The method employs four new primer pairs with equal melting temperatures, each being specific to botulinum neurotoxin gene type A, B, E, or F, and enables a simultaneous detection of the four serotypes. A total of 43 *C. botulinum* strains and 18 strains of other bacterial species were tested. DNA amplification fragments of 782 bp for *C. botulinum* type A alone, 205 bp for type B alone, 389 bp for type E alone, and 543 bp for type F alone were obtained. Other bacterial species, including *C. sporogenes* and the nontoxigenic nonproteolytic *C. botulinum*-like organisms, did not yield a PCR product. Sensitivity of the PCR for types A, E, and F was 10^2 cells and for type B was 10 cells per reaction mixture. With a two-step enrichment, the detection limit in food and fecal samples varied from 10^{-2} spore/g for types A, B, and F to 10^{-1} spore/g of sample material for type E. Of 72 natural food samples investigated, two were shown to contain *C. botulinum* type A, two contained type B, and one contained type E. The assay is sensitive and specific and provides a marked improvement in the PCR diagnostics of *C. botulinum*.

Clostridium botulinum is a spore-forming bacterium that produces lethal neurotoxin, the causative agent of a paralytic disease known as botulism (28). Based on the toxin type produced, *C. botulinum* strains are divided in groups I to IV, with groups I and II being the main human pathogens. Group I consists of proteolytic types A, B, and F, and group II consists of nonproteolytic types B, E, and F (5, 30). The two groups are completely different in their phenotypical characteristics, such as temperature requirements, biochemical profile, and production of metabolites (13, 14). The main taxonomic denominator is thus the production of the botulinum neurotoxin (13, 14).

Diagnosis of botulism is obtained by detecting the neurotoxin and *C. botulinum* cells in a patient or suspected food sample (24, 25). The standard method for detecting the toxin is the mouse bioassay (24), which is time-consuming and expensive and raises ethical concern due to the use of experimental animals. Conventional isolation and identification of *C. botulinum* is difficult unless the toxicity of the isolates is confirmed by the mouse assay. Commercial biochemical tests have been shown to fail in identifying both group I and II organisms as *C. botulinum* (20). The isolation of *C. botulinum* in environmental and food samples is frequently complicated by the presence of proteolytic and nonproteolytic nontoxigenic strains that both phenotypically and genetically resemble *C. botulinum* and exhibit a high relatedness with their toxigenic counterparts (4, 14, 19, 23).

* Corresponding author. Mailing address: Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, P.O. Box 57, FIN-00014 Helsinki University, Finland. Phone: 358-9-191 49702. Fax: 358-9-191 49718. E-mail: mklindst@mappi.helsinki.fi.

PCR provides high sensitivity and specificity in detection of a number of pathogenic microorganisms. For *C. botulinum*, several PCR-based detection methods have been reported during the last decade (1, 3, 6, 10, 11, 12, 17, 29, 31, 32). Following the current taxonomy of *C. botulinum*, these methods are based on the detection of the botulinum neurotoxin gene (*BoNT*). Compared to conventional methods, these protocols provide rapid and sensitive detection of *C. botulinum*. Most of these protocols employ toxin type-specific primers as a single pair in the PCR (12, 17, 29, 31, 32), and not more than one serotype may be detected at a time. Consequently, in an investigation of unknown samples, each *C. botulinum* type needs to be detected separately, which extends the detection time and increases the reagent costs. Some of the older primer pairs are also poorly designed with regard to their optimal annealing temperatures, resulting in the formation of unspecific amplification products. A different approach is to use a general primer pair common for more than one type of *C. botulinum* and to differentiate between the toxin types by a type-specific DNA probe (1, 3, 6, 10, 11). In this method, a limited number of essential oligonucleotides may be used, but the probing step required for the complete identification of the serotype extends the detection time.

The multiplex PCR method would provide a more sophisticated approach, enabling a simultaneous and specific detection of more than one serotype of *C. botulinum*. In general, this method employs more than one pair of specific primers added to the same PCR. Useful applications of multiplex PCR in the detection of other pathogenic bacteria have been previously reported (15, 21, 22), none of these in connection with *C. botulinum*. The *BoNT*-specific primers described in earlier papers (12, 17, 29, 31, 32) are highly variable in their melting

temperatures and may thus not be added to multiplex reaction mixtures.

In this study, four new pairs of *BoNT*-specific primers with equal melting temperatures were conducted. Furthermore, a multiplex PCR assay for the simultaneous detection of *C. botulinum* types A, B, E, and F in foods and fecal material was designed. The assay includes a two-step enrichment, being very sensitive and specific and providing a marked improvement in the PCR diagnostics of *C. botulinum*.

MATERIALS AND METHODS

Bacterial strains and culturing. A total of 11 *C. botulinum* type A, 9 type B, 16 type E, and 7 type F strains and 18 strains of other bacterial species were included in the study (Table 1). *Clostridium sporogenes* and nonproteolytic nontoxigenic *C. botulinum*-like strains (further referred to as *C. botulinum*-like strains) were used as negative controls. These strains have formerly been confirmed to be nontoxigenic by the mouse bioassay (20).

All clostridial strains were cultured in 10 ml of tryptose-peptone-glucose-yeast extract (TPGY) medium (Difco Laboratories, Detroit, Mich.) and incubated under anaerobic conditions (MK3 Anaerobic Work Station; Don Whitley Scientific Ltd., West Yorkshire, United Kingdom) at 37°C (*C. botulinum* group I, *C. sporogenes*, *Clostridium histolyticum*, *Clostridium perfringens*, and *Clostridium septicum*) or 30°C (*C. botulinum* group II and *C. botulinum*-like strains) for 24 to 48 h, followed by overnight culturing of 20 h at respective temperatures. *Listeria* spp. and *Yersinia enterocolitica* strains were cultured on blood agar plates at 30°C for 24 h before template preparation.

Template preparation. Cells from 1 ml of each clostridial overnight culture were washed with 1 ml of TE buffer (0.01 M Tris-HCl, 0.001 M EDTA) for 1 h at 37°C and suspended in 1 ml of distilled water. One to five typical colonies of *Listeria* spp. and *Y. enterocolitica* strains were picked from agar plates, washed with 100 μl of distilled water, and suspended in 100 μl of distilled water. In addition to the individual cell suspensions of each bacterial strain, three mixed suspensions containing proteolytic *C. botulinum* types A (ATCC 25763), B (126B), and F (ATCC 25764), the nonproteolytic types B (Eklund 2B), E (Dolman Beluga E), and F (Craig 610B8-6B), or all four serotypes were prepared by mixing the individual cell suspensions. All suspensions were heated at 99°C for 10 min to break up the cells and release the bacterial DNA and were centrifuged for 5 min at 10,000 × *g*. A volume of 1 μl of each supernatant was used as template in the PCR mixture.

Primers. Based on published DNA sequences of the *BoNT* gene (2, 8, 9, 27, 33, 34, 35), four new primer pairs with each being specific for either *C. botulinum* type A, B, E, or F were designed (Table 2). The primers were selected from the nonhomologous regions of the *BoNT* types A, B, E, and F gene by using the Primer 3 software (S. Rozen and H. J. Skaletsky, Primer 3, Whitehead Institute for Biomedical Research, Cambridge, Mass. [http://www-genome.wi.mit.edu/genome_software/other/primer3.html], 1998).

PCR. PCR was performed with 50 μl of reaction mixture containing 1 μl of template, 0.3 μM concentrations of each primer (Sigma-Genosys Ltd., Cambridgeshire, United Kingdom), 220 nM concentrations of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP; dNTP Mix; Finnzymes, Espoo, Finland), 32 mM Tris-HCl (pH 8.4), 80 mM KCl, 4.8 mM MgCl, and 2 U of DNA polymerase (DynaZyme; Finnzymes). The reaction mixture was overlaid with mineral oil before adding the template and amplification (PTC-200 Peltier Thermocycler; MJ Research Inc., Watertown, Mass.). Each PCR cycle consisted of denaturation at 95°C for 30 s, annealing at 60°C for 25 s, and extension at 72°C for 1 min 25 s and was repeated 27 times. Final extension at 72°C for 3 min followed. The amplified PCR products were visualized in 2% agarose gels (I.D.NA agarose; BioWhittaker Molecular Applications, Rockland, Maine) stained with ethidium bromide. Standard DNA fragments (DNA molecular weight marker VI; Boehringer Mannheim, Mannheim, Germany) were used as molecular weight markers to indicate the sizes of the amplification products.

Inhibition of PCR by sample material. Equal volumes of the overnight cultures of *C. botulinum* types A (Riemann 62A), B (126B), E (Beluga E), and F (ATCC 25764) were mixed, and 21 Eppendorf tubes were filled with 1 ml of the mixture. Raw minced beef, hot-smoked whitefish, and pig feces were each added to seven tubes at levels of 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, and 0.005 g/ml of the overnight culture, followed by the cell wash and PCR as described. The final concentrations of the sample materials were estimated to be correspondingly 500, 250, 100, 50, 25, 10, and 5 μg of sample material per 50-μl PCR mixture.

Sensitivity of the PCR. The overnight cultures of *C. botulinum* types A (Riemann 62A), B (Eklund 2B), E (Dolman Beluga E), and F (Craig 610B8-6F) were quantified by the five-tube most probable number (MPN) method (26). The cultures were serially diluted in peptone water, followed by the cell wash and resuspension in 1 ml of distilled water. Each dilution was heated and used as a template in the PCR.

Detection limit of multiplex PCR assay in inoculated food and feces samples. In order to test the applicability of the multiplex PCR protocol in investigation of food and fecal specimens, 10-g samples of raw minced beef, hot-smoked whitefish, and pig feces were inoculated with 10-fold dilutions of individual spore suspensions of either *C. botulinum* type A (ATCC 25763), type B (proteolytic strain 126B, or nonproteolytic strain Eklund 2B), type E (Dolman Beluga E), or type F (proteolytic strain ATCC 25764 or nonproteolytic strain Craig 610B8-6B) to yield final spore counts of 10^{-2} to 10^{3} spores/g of sample. In addition, one 10-g sample of minced beef was inoculated with a mixture of the above-mentioned strains, containing equal numbers of each of the four types at the level of 10^{3} spores/g of minced beef.

All samples were homogenized (Stomacher 400 Laboratory Blender; Seward Medical Ltd., London, United Kingdom) in sterile pouches with 90 ml (wt/vol [1:9]) of peptone water for 120 s. A total of two 5-g samples of each homogenate were then transferred to tubes containing 45 ml of TPGY broth and incubated at 30 and 37°C under anaerobic conditions for 5 days. The samples were incubated for 1 day (samples inoculated with individual strains), 3 days (all samples), or 5 days (samples inoculated with individual strains), and anaerobic overnight culturing in 10 ml of TPGY followed at respective temperatures. Templates were prepared and PCR was performed as described earlier. The final concentration of each sample material in the PCR was estimated to be approximately 1 μg/reaction mixture. The experiment was repeated once.

Investigation of natural food samples. A number of 36 vegetable sausages, 11 cans of Finnish wild deer meat, and 25 whitefish heads collected from a local fishery were investigated for the presence of *C. botulinum*. A total of 10 g of each sausage and deer sample was transferred to bottles containing 100 ml of TPGY medium, and the whitefish heads were each placed in tubes containing 45 ml of TPGY broth. The bottles and tubes were incubated anaerobically at 30°C for 3 days, followed by overnight culturing at 30°C. Cell washing and PCR were performed as described. The final concentration of sample material in the PCR was estimated to be 10 μg.

RESULTS

Multiplex PCR of bacterial cell suspensions. *C. botulinum* types A, B, E, and F alone yielded the expected amplification products (Table 1): type A, 782 bp; type B, 205 bp; type E, 389 bp; and type F, 543 bp (Fig. 1). The mixed-cell suspensions yielded the corresponding DNA fragments (Fig. 1). None of the *C. sporogenes* or *C. botulinum*-like strains or other bacterial species yielded a PCR product by this assay (Table 1). The PCR products were clearly visualized in agarose gels; a 150-to-200-bp difference in the size of each amplification product enabled an easy distinction between the fragments without the use of high-resolution agarose (Fig. 1).

Inhibition of PCR by sample material. Of the three sample materials tested, only minced beef inhibited the PCR at higher concentrations: the levels of 500 and 250 μg/reaction mixture yielded no PCR products, whereas 100 μg/reaction mixture allowed for the detection of type B only, and 50 and 25 μg/reaction mixture yielded types B and E (Table 3). At lower concentrations of minced beef, all four types were detected. Feces at the level of 250 μg/reaction mixture inhibited only the amplification of the type A-specific DNA fragment.

Sensitivity of multiplex PCR. The sensitivity of the multiplex PCR for *C. botulinum* types A, E, and F was approximately 10^{2} cells/reaction mixture, and for type B it was 10 cells/reaction mixture.

Detection limit of assay in inoculated food and feces. The detectable *C. botulinum* spore concentration in the individually inoculated food and fecal samples varied from 10^{-2} to 10^{3}

TABLE 1. Bacterial strains tested by the multiplex PCR assay

Species	Type	Strain(s)	Origin	Source[a]	Multiplex PCR result[b]
C. botulinum (group I)	A	ATCC 25763	Type strain	ATCC	A
		ATCC 19397	NK[c]	ATCC	A
		ATCC 3502	NK	ATCC	A
		62A, 69A	Cow liver infarct and stool; USA	Riemann/Lindroth[d]	A
		SL-2A, SL-3A, SL-4A, SL-6A	NK	Lindroth	A
		RS-3A, RS-4A	Pacific red snapper; USA	Lindroth	A
	B	ATCC 7949	Canned shallots; USA	ATCC	B
		ATCC 17841	NK	ATCC	B
		SL-1B	NK	Lindroth	B
		133–4803	NK	McClung/Lindroth	B
		126B	NK	IP	B
	F	ATCC 35415	Liver paste; Denmark	ATCC	F
		ATCC 25764	Crab; UK	ATCC	F
		F4VIF	Blue crab viscera; UK	Lindroth	F
		Crab F	Pasteurized crabmeat; USA	Lindroth	F
C. botulinum (group II)	B	ATCC 25765	Marine sediment; USA	ATCC	B
		ATCC 17844	NK	ATCC	B
		2B	Marine sediment; USA	Eklund/Lindroth	B
		706B	Salted salmon; USA	Hatheway/Lindroth	B
	E	ATCC 9564	Smoked salmon; Canada	ATCC	E
		Beluga E	Fermented white whale flippers; USA	Dolman/Lindroth	E
		92E	Marine environment; USA	Eklund/Lindroth	E
		211 E	Pickled herring; Canada	Dolman/Lindroth	E
		250E	Canned salmon; USA	Crowther/Lindroth	E
		4062E	Fermented whale blubber; USA	Hatheway/Lindroth	E
		KA-2E	Scola Creek strain; USA	Ricmann/Lindroth	E
		C-51E	Seal meat; Denmark	SSI	E
		C-60E	Dried mutton; Denmark	SSI	E
		K-6E, K-44E, K-45E	Rainbow trout; Finland	DFH	E
		K-115E	Hot-smoked whitefish; Finland and Canada	DFH	E
		S-3E	Mud; Finland	DFH	E
		S-4E, S-6E	Fishpond sediment; Finland	DFH	E
	F	ATCC 23387	Marine sediment; USA	ATCC	F
		610B8-6F	Salmon; USA	Craig/Lindroth	F
		FT10F	Marine sediment; UK	Hobbs/Lindroth	F
C. sporogenes		ATCC 19404	Gas gangrene	ATCC	—
C. botulinum-like	E-like	H11	Marine sediment; Finland	DFH	—
		P559	Marine sediment; Finland	DFH	—
C. histolyticum		105	Finland	EELA	—
C. perfringens		ATCC 3624	NK	ATCC	—
		ATCC 3626	Intestinal contents of lamb	ATCC	—
		CCUG 2036	Sheep	CCUG	—
		CCUG 2037	Sheep	CCUG	—
		CCUG 2038	NK	CCUG	—
		NCTC 8239	Boiled salt beef	NCTC	—
C. septicum		ATCC 12464	NK	ATCC	—
Listeria innocua		L434	Processing plant; Finland	DFH	—
		L435	Cheese product; Finland	DFH	—
Listeria monocytogenes		ATCC 7644	Human	ATCC	—
		NCTC 10527	Spinal fluid; Germany	NCTC	—
		NCTC 5105	Human	NCTC	—
Y. enterocolitica		YTSK 22K2, P102	Pig tonsil; Finland	DFH	—

[a] ATCC, American Type Culture Collection; IP, Institute Pasteur, Paris, France; SSI, Statens Serum Institut, Copenhagen, Denmark; DFH, Department of Food and Environmental Hygiene, University of Helsinki; CCUG, Culture Collection, University of Gothenburg, Gothenburg, Sweden; NCTC, National Collection of Type Cultures, London, United Kingdom; EELA, National Veterinary and Food Research Institute, Helsinki, Finland.

[b] A, B, E, or F, *BoNT/A*, *BoNT/B*, *BoNT/E*, or *BoNT/F* gene-specific amplification product obtained in multiplex PCR assay, respectively. —, no amplification products were obtained by the assay.

[c] NK, not known.

[d] Collected from various sources by Seppo Lindroth, University of California, Davis, Calif. The first name in each pair is the original source.

spores/g of sample material, depending on the inoculated *C. botulinum* strain, sample material, and enrichment time and temperature (Table 4). The optimal enrichment times ranged from 1 to 5 days followed by overnight culturing (Table 4), but with all strains being detectable within 3 days. In the minced beef sample inoculated with a mixture of *C. botulinum* types A, B, E, and F and enriched for 3 days, the type B, E, and F strains were detected at 30°C and type B and F strains were detected at 37°C. The type A strain was not detected in this sample after the 3-day incubation period at either temperature.

Presence of *C. botulinum* in natural food samples. One vegetable sausage and one can of deer meat were shown to contain

TABLE 2. Primers for multiplex PCR detection of *C. botulinum* types A, B, E, and F

Type[a]	Primer	Sequence (5′-3′)	Product size (bp)	Location on gene (coding region)	Temp (°C)	GC content (%)
A_f	CBMLA1	AGC TAC GGA GGC AGC TAT GTT	782	1788–1808	63.9	52
A_r	CBMLA2	CGT ATT TGG AAA GCT GAA AAG G		2569–2548	63.4	41
B_f	CBMLB1	CAG GAG AAG TGG AGC GAA AA	205	434–453	64.3	50
B_r	CBMLB2	CTT GCG CCT TTG TTT TCT TG		638–619	64.5	45
E_f	CBMLE1	CCA AGA TTT TCA TCC GCC TA	389	156–175	63.7	45
E_r	CBMLE2	GCT ATT GAT CCA AAA CGG TGA		544–525	63.6	43
F_f	CBMLF1	CGG CTT CAT TAG AGA ACG GA	543	185–194	64.1	50
F_r	CBMLF2	TAA CTC CCC TAG CCC CGT AT		727–708	63.3	55

[a] Subscript f, forward primer; subscript r, reverse primer.

C. botulinum type B. Type E was found in one vegetable sausage and, unexpectedly, type A was found in two fish heads.

DISCUSSION

A multiplex PCR assay for the simultaneous detection and identification of *C. botulinum* types A, B, E, and F in foods and fecal material was developed. The method is based on a primer cocktail consisting of four new pairs of oligonucleotide primers, each being specific for the botulinum neurotoxin type A, B, E, or F gene. This method provides a marked improvement in the PCR diagnostics of *C. botulinum*, since the previously described methods require more than one step for the complete detection and identification of several *C. botulinum* types (1, 3, 6, 10, 11, 12, 17, 29, 31, 32). The total time required by the multiplex PCR assay, including a two-step enrichment, was 2 to 6 days, depending on the sample material. The detection limit was 10^{-2} to 10^3 spores/g of sample material. All *C. botulinum* cultures yielded the expected amplification products that, due to differences of 150 to 200 bp in the product size, were easily differentiated in low-resolution agarose gels.

The oligonucleotide primers were designed for the nonhomologous regions of the botulinum neurotoxin types A, B, E, and F genes, and a limited variation in the selection criteria between the eight primers was allowed. This was not the case in the earlier studies on PCR detection of *C. botulinum* (12, 29, 31, 32), as the melting temperatures of those primers varied by up to 20°C, resulting in unspecific amplification. The melting temperatures of the present primers were almost equal, enabling the optimal annealing of all the eight primers at 60°C (Table 2). Amplification of unspecific products was thus predominantly avoided in the samples tested.

The sensitivity of the PCR varied from 10 to 10^2 cells/reaction mixture, corresponding to 10^4 to 10^5 vegetative cells/ml of bacterial culture. The PCR seemed to be the most sensitive for type B, which could be due to the smallest size (205 bp) of the four amplification fragments. The above-mentioned concentrations were easily obtained by the two-step enrichment employed in the present study, as was observed with the inoculated samples; 10^{-2} *C. botulinum* spores/g of sample material could be detected. Previous reports on PCR protocols designed for a single type of *C. botulinum* provide a variety of sensitivities, from 2.5 pg of purified DNA (32) to 10^4 cells/g of sample material (1), depending on the method of DNA purification and enrichment conditions of the target cells. In the latter study (1), they found that with an enrichment step of 18 h, they could detect as few as 1 cell/10 g of food sample when the DNA was recovered by a purification procedure. The

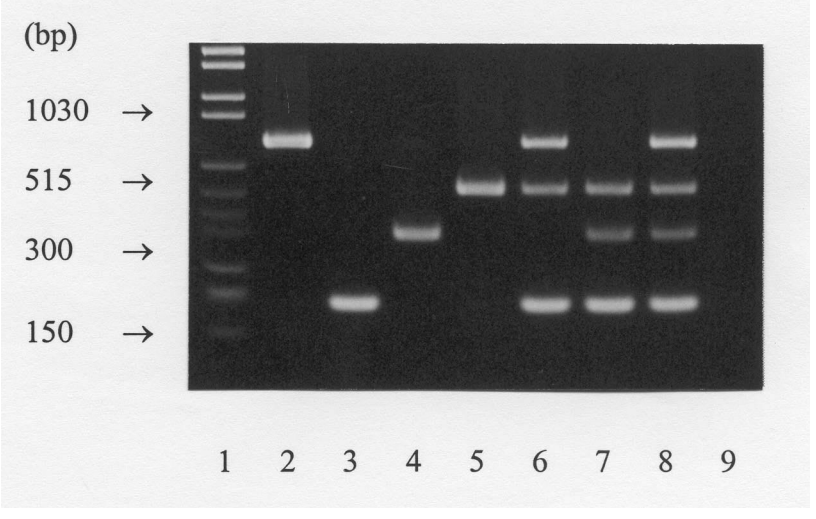

FIG. 1. Multiplex PCR detection of *C. botulinum*. Lanes: 1, molecular weight marker; 2, *C. botulinum* type A; 3, *C. botulinum* type B; 4, *C. botulinum* type E; 5, *C. botulinum* type F; 6, proteolytic *C. botulinum* types A, B, and F; 7, nonproteolytic *C. botulinum* types B, E, and F; 8, *C. botulinum* types A, B, E, and F; and 9, negative control.

TABLE 3. Inhibition of multiplex PCR by different concentrations of food and fecal material

Concn of sample material[a] (μg/PCR mixture)	*C. botulinum* type(s) detected		
	Minced beef	Fish	Feces
500		A, B, E, F	A, B, E, F
250		A, B, E, F	B, E, F
100	B	A, B, E, F	A, B, E, F
50	B, E, F	A, B, E, F	A, B, E, F
25	B, E	A, B, E, F	A, B, E, F
10	A, B, E, F	A, B, E, F	A, B, E, F
5	A, B, E, F	A, B, E, F	A, B, E, F

[a] The actual level of sample material in the PCR was estimated to be 1 μg with a dilution (1:9) step in peptone water and was 10 μg without the dilution.

detection limit in the present method was determined from washed and heated cell lysates, and it is likely that the sensitivity of this method could be further improved by DNA purification.

The multiplex PCR assay ensured a sensitive and specific tool for the detection of *C. botulinum* in the inoculated food and fecal samples. The optimal enrichment time varied from 1 to 5 days, depending on the inoculated *C. botulinum* strain and sample material (Table 4). In general, by extending the enrichment time by up to 5 days, the assay had an increased sensitivity for the types A and F strains, whereas the type B strains in all samples and type E strain in beef and fish were easily detected in 1 to 3 days. The optimal enrichment temperatures seemed to vary by the sample material rather than by the inoculated group I and II strains; optimal enrichment of the beef was obtained at 37°C, while that of the fish and feces was at 30°C. The lowest detection limits were observed in minced beef and hot-smoked fish, where 10^{-2} to 10^{-1} spore/g of sample material was detected. These spore counts correspond to the natural contamination level of *C. botulinum* in foods (7). For feces, the detection limit was higher; 10^{-1} to 10^{3} spores/g of feces were found, with the highest detection level being that for the type E strain. A number of sample materials, including feces and fatty foods, are generally known to cause failure in the PCR detection of microorganisms due to the inhibition of the DNA polymerase enzyme. However, it seems that this is not the case in this study, as the concentrations of all sample materials in the PCR were shown to be below the inhibitory level (Table 3).

The likelihood of PCR inhibition seemed to correlate with the size of the amplification product (Table 3). Relatively low concentrations of beef (25 to 100 μg) readily inhibited the amplification of types A and F, whereas higher concentrations (250 to 500 μg) were required to inhibit the amplification of types B and E. This may suggest that the DNA polymerase enzyme had a limited activity in the presence of lower concentrations of minced beef, being able to amplify the smallest fragments but not the larger ones.

TABLE 4. Detection limit and optimal enrichment time of multiplex PCR assay for *C. botulinum* types A, B, E, and F in food and fecal samples

C. botulinum type	Detection limit in three sample materials ([spores/g of sample material], optimal enrichment time [days])		
	Minced beef	Hot-smoked whitefish	Feces
A	10^{-2}, 5	10^{-1}, 3	10, 3
B	10^{-2}, 1	10^{-2}, 3	10^{-1}, 3
E	10^{-1}, 3	10^{-1}, 1	10^{3}, 5
F	10^{-1}, 3	10^{-2}, 5	10, 3

C. botulinum type E is naturally highly prevalent in aquatic environments and fish (16, 17, 18), but not as frequently found in meats or fecal material. In this study, the type E strain was more rapidly detected in fish samples than in beef and feces, which may indicate that beef and feces are not natural niches for type E. Furthermore, it is even possible that the germination and growth rate of the *C. botulinum* type E strain were reduced by the presence of these sample materials.

In the minced beef inoculated with the mixture of types A, B, E, and F spores, types B and F were detected at both incubation temperatures and type E was detected at 30°C. According to a common understanding of the optimal growth temperatures of group I and II organisms, the types B and F strains detected at 30°C were assumed to be the nonproteolytic ones, whereas those detected at 37°C were most probably the proteolytic strains. The type A strain did not seem to reach the detectable level in minced beef within 3 days at either temperature, which may be explained by a relatively slow growth rate observed in beef inoculated by the individual type A strain (Table 4). It can thus be expected that type A is better detected in meat by extending the incubation time to 5 days.

As for the natural food samples, *C. botulinum* type A was detected in fish within 3 days, which is in agreement with the individually inoculated food samples (Table 4). The presence of type A in fish was not expected, and it remains unknown whether the contamination occurred from the surroundings of the processing plant. More expected were the findings with type B being detected in a can of deer meat and types B and E being detected in a vegetable sausage.

As the primary sources of *C. botulinum* types A, B, E, and F are different, this assay may also be modified for the detection of either a single type of *C. botulinum* or the proteolytic or the nonproteolytic types alone (Fig. 1). Combining the types A-, B-, and F-specific primers with the incubation temperatures of 35 to 40°C would probably ensure the detection of the proteolytic types more likely than that of the nonproteolytics. Similarly, B-, E-, and F-specific primers in combination with a slightly lower incubation temperature would result in optimal detection of the nonproteolytic types (Fig. 1). This shows a high flexibility and usefulness of the assay in the detection of different types of *C. botulinum* in various types of sample materials.

In conclusion, the multiplex PCR assay in combination with the two-step enrichment is a sensitive and specific method to simultaneously detect several *C. botulinum* types present in food or clinical material. The sensitivity of the assay enables the detection of low numbers of spores present in natural samples. The study also demonstrated that a careful consideration of the appropriate enrichment conditions for each type of sample material is required to obtain optimal results. The multiplex PCR assay provides high flexibility in detection of several types of *C. botulinum* present in various types of environments. The assay thus markedly improves the PCR diagnostics of *C. botulinum*.

ACKNOWLEDGMENTS

We thank Kirsi Ristkari for excellent laboratory assistance.

This study was supported by the Finnish Research Programme on Environmental Health 1998-2001 and the National Technology Agency.

REFERENCES

1. **Aranda, E., M. M. Rodríguez, M. A. Asensio, and J. J. Córdoba.** 1997. Detection of *Clostridium botulinum* types A, B, E and F in foods by PCR and DNA probe. Lett. Appl. Microbiol. **25:**186–190.
2. **Binz, T., H. Kurazono, M. Wille, J. Frevert, K. Wernars, and H. Niemann.** 1990. The complete sequence of botulinum neurotoxin type A and comparison with other clostridial neurotoxins. J. Biol. Chem. **265:**9153–9158.
3. **Braconnier, A., V. Broussolle, C. Nguyen-The, and F. Carlin.** 2001. Screening for *Clostridium botulinum* type A, B, and E in cooked chilled foods containing vegetables and raw material using polymerase chain reaction and molecular probes. J. Food Prot. **64:**201–207.
4. **Broda, D. M., J. A. Boerema, and R. G. Bell.** 1998. A PCR survey of psychrotrophic *Clostridium botulinum*-like isolates for the presence of BoNT genes. Lett. Appl. Microbiol. **27:**219–223.
5. **Buchanan, R. E., and N. E. Gibbons.** 1974. Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Company, Baltimore, Md.
6. **Campbell, K. D., M. D. Collins, and A. K. East.** 1993. Gene probes for identification of the botulinal neurotoxin gene and specific identification of neurotoxin types B, E, and F. J. Clin. Microbiol. **31:**2255–2262.
7. **Dodds, K. L.** 1993. *Clostridium botulinum* in foods, p. 53–68. *In* A. H. W. Hauschild and K. L. Dodds (ed.), Clostridium botulinum. Ecology and control in foods. Marcel Dekker, Inc., New York, N.Y.
8. **East, A. K., P. T. Richardson, D. Allaway, M. D. Collins, T. A. Roberts, and D. E. Thompson.** 1992. Sequence of the gene encoding type F neurotoxin of *Clostridium botulinum*. FEMS Microbiol. Lett. **96:**225–230.
9. **Elmore, M. J., R. A. Hutson, M. D. Collins, N. J. Bodsworth, S. M. Whelan, and N. J. Minton.** 1995. Nucleotide sequence of the gene coding for proteolytic (Group I) *Clostridium botulinum* type F neurotoxin: genealogical comparison with other clostridial neurotoxins. Syst. Appl. Microbiol. **18:**23–31.
10. **Fach, P., D. Hauser, J. P. Guillou, and M. R. Popoff.** 1993. Polymerase chain reaction for the rapid identification of *Clostridium botulinum* type A strains and detection in food samples. J. Appl. Bacteriol. **75:**234–239.
11. **Fach, P., M. Gibert, R. Griffais, J. P. Guillou, and M. R. Popoff.** 1995. PCR and gene probe identification of botulinum neurotoxin A-, B-, E-, F-, and G-producing *Clostridium* spp. and evaluation in food samples. Appl. Environ. Microbiol. **61:**389–392.
12. **Franciosa, G., J. L. Ferreira, and C. L. Hatheway.** 1994. Detection of type A, B, and E botulism neurotoxin genes in *Clostridium botulinum* and other *Clostridium* species by PCR: evidence of unexpressed type B toxin genes in type A toxigenic organisms. J. Clin. Microbiol. **32:**1911–1917.
13. **Hatheway, C. L.** 1990. Toxigenic clostridia. Clin. Microbiol. Rev. **3:**66–98.
14. **Hatheway, C. L.** 1992. *Clostridium botulinum* and other clostridia that produce botulinum neurotoxin, p. 3–20. *In* A. H. D. Hauschild and K. L. Dodds (ed.), *Clostridium botulinum*—ecology and control in foods. Marcel Dekker, New York, N.Y.
15. **Herman, L. M. F., H. R. M. deRidder, and F. M. M. Vlaenmynck.** 1995. A multiplex PCR method for the identification of *Listeria* sp. and *Listeria monocytogenes* in dairy samples. J. Food Prot. **58:**867–872.
16. **Hielm, S., E. Hyytiä, A.-B. Andersin, and H. Korkeala.** 1998. A high prevalence of *Clostridium botulinum* type E in Finnish freshwater and Baltic Sea sediment samples. J. Appl. Microbiol. **84:**133–137.
17. **Hielm, S., E. Hyytiä, J. Ridell, and H. Korkeala.** 1996. Detection of *C. botulinum* in fish and environmental samples using polymerase chain reaction. Int. J. Food Microbiol. **31:**357–365.
18. **Hyytiä, E., S. Hielm, and H. Korkeala.** 1998. Prevalence of *Clostridium botulinum* type E in Finnish fish and fishery products. Epidemiol. Infect. **120:**245–250.
19. **Lee, W. H., and H. Riemann.** 1979. Correlation of toxic and non-toxic strains of *Clostridium botulinum* by DNA composition and homology. J. Gen. Microbiol. **60:**117–123.
20. **Lindström, M., H. Jankola, S. Hielm, E. Hyytiä, and H. Korkeala.** 1999. Identification of *Clostridium botulinum* with API 20 A, Rapid ID 32 A and RapID ANA II. FEMS Immunol. Med. Microbiol. **24:**267–274.
21. **Meer, R. R., and J. G. Songer.** 1997. Multiplex polymerase chain reaction assay for genotyping *Clostridium perfringens*. Am. J. Vet. Res. **58:**702–705.
22. **Nakajima, H., M. Inoue, T. Mori, K.-I. Itoh, E. Arakawa, and H. Watanabe.** 1992. Detection and identification of *Yersinia pseudotuberculosis* and pathogenic *Yersinia enterocolitica* by an improved polymerase chain reaction method. J. Clin. Microbiol. **30:**2484–2486.
23. **Nakamura, S., I. Okado, S. Nakashio, and S. Nishida.** 1977. *Clostridium sporogenes* isolates and their relationship to *C. botulinum* based on deoxyribonucleic acid reassociation. J. Gen. Microbiol. **100:**395–401.
24. **Nordic Committee on Food Analysis.** 1991. Botulinum toxin. Detection in foods, blood and other test materials. Method no. 79, 2nd ed. Nordic Committee on Food Analysis, Espoo, Finland.
25. **Nordic Committee on Food Analysis.** 1991. *Clostridium botulinum*. Detection in foods and other test materials. Method no. 80, 2nd ed. Nordic Committee on Food Analysis, Espoo, Finland.
26. **Oblinger, J. L., and J. A Koburger.** 1984. The most probable number technique. *In* M. L. Speck (ed.), Compendium of methods for the microbiological examination of foods, 2nd ed. American Public Health Association, Inc., Washington, D.C.
27. **Poulet, S., D. Hauser, M. Quantz, H. Niemann, and M. R. Popoff.** 1992. Sequences of the botulinal neurotoxin E derived from *Clostridium botulinum* type E (strain Beluga E) and *Clostridium butyricum* (strains ATCC 43181 and ATCC 43755). Biochem. Biophys. Res. Commun. **183:**107–113.
28. **Prévot, A. R.** 1953. Rapport d'introduction du président du sous-comité *Clostridium* pour l'unification de la nomenclature des types toxigéniques de *C. botulinum*. Int. Bull. Bacterial Nomencl. **3:**120–123.
29. **Sciacchitano, C. J., and I. N. Hirshfield.** 1996. Molecular detection of *Clostridium botulinum* type E neurotoxin gene in smoked fish by polymerase chain reaction and capillary electrophoresis. J. AOAC Int. **79:**861–865.
30. **Smith, L. D. S., and H. Sugiyama.** 1988. Botulism: the organism, its toxins, the disease. Charles C. Thomas, Springfield, Ill.
31. **Szabo, E. A., J. M. Pemberton, A. M. Gibson, R. J. Thomas, R. R. Pascoe, and P. M. Desmarchelier.** 1994. Application of PCR to a clinical and environmental investigation of a case of equine botulism. J. Clin. Microbiol. **32:**1986–1991.
32. **Takeshi, K., Y. Fujinaga, K. Inoue, H. Nakajima, K. Oguma, T. Ueno, H. Sunagawa, and T. Ohyama.** 1996. Simple method for detection of *Clostridium botulinum* type A to F neurotoxin genes by polymerase chain reaction. Microbiol. Immunol. **40:**5–11.
33. **Thompson, E. D., J. K. Brehm, J. D. Oultram, T.-J. Swinfield, C. C. Shone, T. Atkinson, J. Melling, and N. P. Minton.** 1990. The complete amino acid sequence of the *Clostridium botulinum* type A neurotoxin, deduced by nucleotide analysis of the encoding gene. Eur. J. Biochem. **189:**73–81.
34. **Whelan, S. M., M. J. Elmore, N. J. Bodsworth, J. K. Brehm, T. Atkinson, and N. P. Minton.** 1992. Molecular cloning of the *Clostridium botulinum* structural gene encoding the type B neurotoxin and determination of its entire nucleotide sequence. Appl. Environ. Microbiol. **58:**2345–2354.
35. **Whelan, S. M., M. J. Elmore, N. J. Bodsworth, T. Atkinson, and N. P. Minton.** 1992. The complete amino acid sequence of the *Clostridium botulinum* type-E neurotoxin, derived by nucleotide-sequence analysis of the encoding gene. Eur. J. Biochem. **203:**657–667.