APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 2001, p. 4781–4788 Vol. 67, No. 10
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.10.4781–4788.2001

Development of a Combined Selection and Enrichment PCR Procedure for *Clostridium botulinum* Types B, E, and F and Its Use To Determine Prevalence in Fecal Samples from Slaughtered Pigs

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Received 6 April 2001/Accepted 1 August 2001

A specific and sensitive combined selection and enrichment PCR procedure was developed for the detection of *Clostridium botulinum* types B, E, and F in fecal samples from slaughtered pigs. Two enrichment PCR assays, using the DNA polymerase *rTth,* were constructed. One assay was specific for the type B neurotoxin gene, and the other assay was specific for the type E and F neurotoxin genes. Based on examination of 29 strains of *C. botulinum*, 16 strains of other *Clostridium* spp., and 48 non-*Clostridium* strains, it was concluded that the two PCR assays detect *C. botulinum* types B, E, and F specifically. Sample preparation prior to the PCR was based on heat treatment of feces homogenate at 70°C for 10 min, enrichment in tryptone-peptone-glucose-yeast extract broth at 30°C for 18 h, and DNA extraction. The detection limits after sample preparation were established as being 10 spores per g of fecal sample for nonproteolytic type B, and 3.0×10^3 spores per g of fecal sample for type E and nonproteolytic type F with a detection probability of 95%. Seventy-eight pig fecal samples collected from slaughter houses were analyzed according to the combined selection and enrichment PCR procedure, and 62% were found to be PCR positive with respect to the type B neurotoxin gene. No samples were positive regarding the type E and F neurotoxin genes, indicating a prevalence of less than 1.3%. Thirty-four (71%) of the positive fecal samples had a spore load of less than 4 spores per g. Statistical analysis showed that both rearing conditions (outdoors and indoors) and seasonal variation (summer and winter) had significant effects on the prevalence of *C. botulinum* type B, whereas the effects of geographical location (southern and central Sweden) were less significant.

Clostridium botulinum is an obligate anaerobic, endospore-forming bacterium that is ubiquitous in the environment. The pathogenicity of the organism is associated with the production of serologically distinct neurotoxins, types A to G (15). Types A, B, E, and F, which are responsible for food-borne botulism in humans, contain both proteolytic and nonproteolytic strains. Only a limited number of surveys have been performed in Europe on the distribution of *C. botulinum* in the environment and in raw materials for foodstuffs (16, 18–20). In Sweden, the most recent survey was carried out in 1963 by Johannsen (22) in soil and coast sediment. Both *C. botulinum* types B and E were found.

Today, there is an increasing demand from the consumer for convenient foods of high quality. This has resulted in the development of refrigerated processed foods of extended durability that require minimal preparation time and contain low concentrations of preservatives. Nonproteolytic strains of *C. botulinum* are considered to be a hazard in these foods due to lower heat treatment than, for example, canned foods and the anaerobic atmosphere used during processing of the foods (29). These conditions favor germination of spores and toxin formation from nonproteolytic *C. botulinum*, which can multiply and produce neurotoxins of types B, E, or F at temperatures as low as 3.3°C (8, 9, 32). The potential presence of *C. botulinum* spores in these foods represents the most severe hazard of food-borne poisoning (15, 29). Therefore, information about the incidence and levels of spores in environmental samples and in foods is necessary for an assessment of the botulinum hazard.

Many previous investigations into the prevalence of *C. botulinum* in the environment and in food samples are based on enrichment for 5 to 10 days and subsequent detection by in vivo mouse bioassay of the toxin produced (24, 33). A drawback of the in vivo mouse bioassay (23) is the use of experimental animals. Conventional isolation and identification methods, based on phenotypic characteristics, have also been developed, but their performance is often time consuming and they have been found to be insufficient in identifying strains of *C. botulinum* correctly (7, 27). In recent years, a number of PCR assays have been developed for the detection of botulinal neurotoxin genes (5, 36). However, the amplification capacity of many PCR assays is limited due to the presence of PCR-inhibitory components, low concentration of target cells or DNA, and high concentration of microorganisms within the complex biological sample (25). Thus, the probability of detecting the target organism with a PCR assay at different cell concentrations needs to be established both in a pure system and in a system containing the biological sample to be analyzed.

The first objective of this study was to construct a highly

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specific and sensitive PCR detection method for *C. botulinum* types B, E, and F in pig fecal samples. As a sample preparation step prior to PCR, we developed a combined selection and enrichment procedure including activation, germination, and transformation of bacterial endospores into active vegetative bacteria by heat treatment and multiplication of the bacterium in enrichment broth to a PCR-detectable concentration. Two nested PCR assays were constructed using *rTth*, a DNA polymerase that has been found to exhibit high levels of resistance to various known PCR-inhibitory components (1–3). One PCR assay is specific for the type B neurotoxin gene, and the other assay is specific for both the type E and F neurotoxin genes. The second objective of this study was to determine the prevalence of *C. botulinum* types B, E, and F in slaughtered pigs in Sweden.

MATERIALS AND METHODS

Statistical analyses. In the development of the combined selection and enrichment PCR procedure, the data obtained from the different experiments were analyzed by replicate analyses. The number of replicates in the different experiments varied between 8 and 24. Each replicate resulted in an individual PCR detection pattern from which a probability of detecting the bacteria at different concentrations was calculated (P = number of positive PCR results/number of replicates tested). The detection probability curves were adjusted using a model described by Baranyi and Roberts (4). The statistical analysis of the results from the prevalence study was performed using the software SYSTAT 227 (version 7.0.1; SPSS INC., Evanston, Ill.). Both analysis of variance (ANOVA) and the Pearson chi-square test were used for statistical analysis. The following model, in which Y represents prevalence, was used when ANOVA was performed:

Y = season + rearing condition + geography + season × rearing condition + season × geography + rearing condition × geography + season × rearing condition × geography

Bacteria and culture conditions. Twenty-nine strains of *C. botulinum*, 16 strains of other *Clostridium* spp., and 48 non-*Clostridium* strains were used (Table 1). All the *C. botulinum* strains were grown anaerobically in tryptone-peptone-glucose-yeast extract (TPGY) broth at 30°C. The TPGY broth contained tryptone (50 g/liter; Oxoid Ltd., Basingstoke, United Kingdom), Proteose Peptone (5 g/liter; Oxoid Ltd.), yeast extract (20 g/liter; Oxoid Ltd.), D-glucose (4 g/liter; Merck, Darmstadt, Germany), sodium thioglycolate (1 g/liter; Merck), and soluble starch (1 g/liter; Merck). Before sterilization (121°C for 15 min), anaerobic conditions were created by boiling the medium for 10 min and, during cooling, flushing the medium with nitrogen gas. All the other strains of *Clostridium* spp. were grown anaerobically in TPGY broth at 37°C. The non-*Clostridium* strains were cultivated at 37°C in brain heart infusion broth (Oxoid Ltd.) except as follows. *Lactobacillus* strains were grown in MRS broth (Oxoid Ltd.); *Brochothrix*, *Pediococcus*, and *Streptococcus* strains were grown in APT broth (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.); and *Escherichia* strains were grown in tryptone soy broth (Oxoid Ltd.).

Preparation of spores. Spores from two proteolytic strains of *C. botulinum* type B (ATCC 17841 and ATCC 7949) were produced in a sporulation medium as described by Gaze and Brown (14). Spores from three nonproteolytic strains of *C. botulinum* type B (Eklund 2B, Eklund 17B, and Johannesson 105–66), *C. botulinum* type E (CB-S3-E), and *C. botulinum* type F (Craig 610B8–6F) were produced in a two-phase medium as described by Peck et al. (30). During the production of spores, contamination was checked on blood agar plates {blood agar base (37 g/liter; Lab M, Bury, United Kingdom) and citrate-treated horse blood (4% [vol/vol]; SVA, Uppsala, Sweden)} after aerobic and anaerobic incubation (Gas Pak Plus Anaerobic system; BBL, Becton Dickinson Microbiology Systems) at 30°C for 48 h. The spores were finally resuspended in water and stored at 1 to 2°C. The enumeration of vegetative cells and spores of *C. botulinum* was performed using a Bürker chamber in a phase-contrast microscope. The bacterium or spore suspension was diluted so that each square contained approximately 10 to 15 cells or spores. Counting was performed three times per suspension, and the mean value was calculated. The concentration was expressed as cells or spores per milliliter.

Primer design. Alignment (Clustal X 1.64b Multiple Sequence Alignment Program) (35) of sixteen published nucleotide sequences of the botulinal neurotoxin gene of all seven, A to G, serotypes was performed. The nucleotide sequences were collected from the GenBank Sequence Database (http://www.ncbi.nlm.nih.gov) for *C. botulinum* type A (accession no. X52066, X73423, and M30196), *C. botulinum* type B (accession no. X71343 and M81186), *C. botulinum* types C and D (accession no. D38442, X54254, and D49440), *C. botulinum* type E (accession no. X62683 and X62089), *C. botulinum* type F (accession no. L35496, X81714, and M92906), *C. botulinum* type G (accession no. X74162), *Clostridium butyricum* type E (accession no. X62088), and *Clostridium baratii* type F (accession no. X68262). Two sets of primers were designed, one specific for the type B neurotoxin gene and one specific for the type E and F neurotoxin genes (Table 2).

Conditions in the PCR assays. A nested PCR strategy was used for the two PCR assays, one specific for the type B neurotoxin gene and one specific for both the type E and F neurotoxin genes. In the first step of the assays, amplification was performed with the following set of primers: primers fB and rB for type B and primers fEF and rEF for types E and F (Table 2). The total volume of the PCR master mixture was 25 μl. The PCR mixture consisted of 1× chelating buffer (Perkin Elmer Applied Biosystems, Foster City, Calif.); 1.5 mM $MgCl_2$ (Perkin Elmer Applied Biosystems); 0.5 μM concentrations of each primer (Scandinavian Gene Synthesis AB, Köping, Sweden); 0.2 mM (each) dATP, dTTP, dCTP, and dGTP (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.); 1.25 U of *rTth* (Perkin Elmer Applied Biosystems); and 5 μl of template solution (DNA or cells). The water used in the assay was autoclaved Millipore water (Millipore S. A., Molsheim, France). Each amplification commenced with a denaturation step at 94°C for 5 min, followed by 30 cycles for the type B-specific primer and 35 cycles for the type E-and-F-specific primer, consisting of heat denaturation at 94°C for 40 s, primer annealing at 58°C for 40 s, and extension at 72°C for 40 s. Finally, extension was performed at 72°C for 7 min to complete the synthesis of all strands. The second step consisted of amplification with an internal set of primers, primers fBn and rBn for the first assay, and primers fEFn and rEFn for the second assay, using the same conditions as those described above. The template added to the second step (1 to 25 μl), was diluted 1:500 in autoclaved Millipore water. All amplifications were carried out in a Gene Amp 9700 thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). The PCR products were visualized by agarose gel electrophoresis. The 1.5% (wt/vol) agarose gel was obtained by dissolving ultrapure DNA grade agarose (Bio-Rad Laboratories, Hercules, Calif.) in 1× TBE buffer (108 g of Tris [ICN Biochemicals, Inc., Aurora, Ohio], 55 g of boric acid [Merck], 40 ml of 0.5 M EDTA [Sigma Chemical Co., St. Louis, Mo.], 10 liters of Millipore water) and stained with ethidium bromide (Sigma Chemical Co.) (31). Five microliters of 6× loading buffer (40% [wt/vol] D-sucrose [Sigma Chemical Co.] and 0.025% [wt/vol] bromophenol blue [Merck] dissolved together in 1× TBE buffer) was added to each PCR tube. The gel was analyzed using a gel documentation system (Bio-Rad Laboratories), and Molecular Analyst software (Bio-Rad Laboratories) was used to analyze the gel images. To determine the size of the amplicons, a DNA molecular size marker (100-bp ladder; Amersham Pharmacia Biotech, Inc.) was included in the gel.

In order to optimize the reaction conditions for the two assays, pure DNA was used as a template. DNA was extracted from *C. botulinum* Eklund 2B, *C. botulinum* CB-S3-E, and *C. botulinum* Craig 610B8–6F in accordance with standard protocol (31) and modified by the addition of 30 U of mutanolysine (Sigma Chemical Co.) per ml of lysis solution. The DNA was further purified by using phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma Chemical Co.) extraction. The DNA was resuspended in 500 μl of TE buffer containing 1 μl of 100-mg/ml RNase A (Sigma Chemical Co.) The concentration and purity of the DNA were measured spectrophotometrically at 260 and 280 nm. When *Taq* DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany) was added to the PCR mixture, the same reaction conditions were used as for *rTth*, except for the use of 1× PCR buffer (Roche Diagnostics GmbH) as the buffer system and a concentration of 0.75 U of *Taq* DNA polymerase in a total volume of 25 μl.

Preparation of standardized pig fecal samples. The PCR assay was evaluated using inoculated pig feces. Approximately 2.5 kg of pig feces was sampled at the slaughter line in a slaughterhouse. The feces were transported to the laboratory within 2 h at a temperature below 4°C. The feces were homogenized in a sterilized meat grinder, mixed, distributed into stomacher bags in portions of approximately 0.1 kg, and stored at −20°C. Before use, the samples were thawed at 4°C for 16 h.

Fecal samples were prepared by mixing 10 g of feces with 90 ml of TPGY broth in a stomacher for 2 min. Homogenates (9 ml) were transferred to six sterile test tubes. Large debris was avoided when withdrawing the samples. Each tube was spiked with cells of *C. botulinum* type B (Eklund 2B) to a final concentration of 1.6×10^2 to 1.6×10^7 cells per ml of homogenate. Ten 1-ml samples from each test tube were subjected to DNA extraction using a PrepMan kit for gram-

TABLE 1. Bacterial strains used in specificity tests of two PCR assays

Species	Strain(s)
C. botulinum type A (proteolytic)	ATCC 3502, ATCC 19397, ATCC 25763, Atlanta 2204-4, Johannesson 263
C. botulinum type B (proteolytic)	ATCC 7949, ATCC 17841, Atlanta 3025
C. botulinum type B (non proteolytic)	Eklund 17B, Eklund 2B, Johannesson 105-66
C. botulinum type E	CB-S3 E, CB-S4 E, CB-S21 E, CB-S25 E, CB-K27 E, CB-K44 E, CB-K58 E, CB-K83 E, CB-K114 E, CB-124 E, ATCC 9564
C. botulinum type F	ATCC 35415, ATCC 23387, ATCC 25765, Craig 610B8-6F, Atlanta 2821, Johannesson
Clostridium perfringens	SMRICC 81, SMRICC 146, SMRICC 147, SMRICC 148, ATCC 14810, ATCC 3629, ATCC 12925, ATCC 3624, ATCC 3626
Clostridium sporogenes	ATCC 19404, ATCC 11437
Clostridium butyricum	ATCC 19398
Clostridium tyrobutyricum	SMR 213, SMR 224, SMR 226
Clostridium limosum	SMR 191
Bacillus cereus	NCIB 9373, F 4635A/90, F 4632/90, SMR 747
Bacillus coagulans	NCIB 9365
Bacillus megaterium	NCIB 9376
Bacillus polymyxa	NCIB 8158
Bacillus pumilus	NCTC 10337
Bacillus subtilis	ATCC 6633, ATCC 9372
Brochothrix thermosphacta	SMRICC 347
Citrobacter freundii	SMRICC 373
Edwardsiella tarda	CCM 2238
Enterobacter amnigenus	CCM 3430
Enterobacter cloacae	NCIB 8272
Enterobacter sakazakii	1826-2, 1802
Escherichia coli	3359-1, 3359-2, SMRICC 378, SMRICC 380
Escherichia hermannii	SMRICC 381
Escherichia vulneris	SMRICC 384
Hafnia alvei	CCUG 15720
Klebsiella pneumoniae	102, 1790, 1826-1
Kluyvera ascorbata	CCUG 15716
Listeria monocytogenes	ATCC 7644
Listeria innocua	ATCC 33090
Morganella morganii	CCM 680
Neisseria meningitidis	MC 1:B312/82
Pediococcus pentosaceus	SMRICC 178
Pseudomonas fluorescens	SMRICC 445
Salmonella enteritidis	SMRICC 140
Serratia liquefaciens	CCM 2717
Serratia marcescens	ATCC 13880
Serratia rubidaea	CCM 3412
Streptococcus faecalis	NCIB 775
Yersinia enterocolitica	Y79/O:3
Lactobacillus brevis	LMG 12020
Lactobacillus plantarum	LMG 9210, LMG 1284
Lactobacillus buchneri	LMG 6892
Lactobacillus fermentum	LMG 6902
Lactobacillus pentosus	ATCC 8041
Lactobacillus delbrueckii	ATCC 9649

positive bacteria (Perkin Elmer Applied Biosystems). Finally, after DNA precipitation, the pellet was resuspended in 100 μl of autoclaved Millipore water.

Preparation of microorganisms from pig feces. Ten grams of feces was mixed with 90 ml of 0.9% (wt/vol) NaCl solution in a stomacher for 2 min. Eight samples of the homogenate (0.83 ml) were transferred to sterile Eppendorf tubes, and the microorganisms in each homogenate were extracted using a buoyant density centrifugation technique (Percoll; Amersham Pharmacia Biotech, Inc.) (26) with the following modifications. The bacterial cells were washed in 0.9% (wt/vol) NaCl at least five times. The remaining supernatant (83 μl) was prepared so that the final volume of 100 μl corresponded to 1 ml of feces homogenate (1:10 dilution).

Development of the combined selection and enrichment procedure. All enrichment was performed in TPGY medium at 30°C for up to 48 h. In order to select for *C. botulinum* in pig feces, two different methods were evaluated: (i) heat treatment of sample homogenate at 70°C for 10 min prior to enrichment and (ii) addition of D-cycloserine to the enrichment broth. When heat treatment was used, 90 ml of TPGY was preheated to 70°C and inoculated anaerobically with 10 g of feces to make a 1:10 dilution and the mixture was vortexed for 1 min and reheated. After the temperature reached 70°C, the homogenate was inoculated with spores of nonproteolytic *C. botulinum* type B (Eklund 2B and Eklund 17B) at a low concentration (10 spores per sample of homogenate) and heating was continued for 10 min. A logger in a reference bottle, connected to a computer, monitored the temperature. When D-cycloserine (25 mg/ml) was used, it was mixed in the TPGY medium before the feces and the spores were added. The control samples were enriched in TPGY medium at 30°C without additional selection. Two 1-ml samples were withdrawn after 0, 18, 24, 30, 36, and 48 h of enrichment and subjected to analysis with the PrepMan DNA extraction kit for gram-positive bacteria (Perkin Elmer Applied Biosystems). Each sample was amplified three times with the type B PCR assay.

An additional experiment was performed at 70°C for 10 min combined with enrichment for 18 h at 30°C. The homogenate samples were inoculated with spores from *C. botulinum* strain Eklund 2B and strain Eklund 17B (both nonproteolytic type B), *C. botulinum* strain ATCC 17841 (proteolytic type B), *C. botulinum* strain CB-S3-E (type E), and *C. botulinum* strain Craig 610B8–6F (nonproteolytic type F) at the following concentrations: 10^1, 10^2, 10^3, and 10^4 spores per homogenate sample. The growth experiment was performed twice with each strain. Three 1-ml samples were withdrawn after 18 h of incubation and

TABLE 2. Primers used for detection of BoNT gene types B, E, and F[a]

Primer	Nucleotide sequence	Location within the BoNT gene[b]	PCR product size (kb)
BoNT type B			
fB	5′-TGGATATTTTTCAGATCCAGCCTTG-3′	650–674	0.48[c]
rB	5′-TGGTAAGGAATCACTAAAATAAGAAGC-3′	1107–1133	
fBn	5′-AAAGTAGATGATTTACCAATTGTA-3′	723–746	0.22[d]
rBn	5′-GTTAGGATCTGATATGCAAACTA-3′	916–938	
BoNT type E and F			
fEF	5′-CAA(A/G)ATATGATTCTAATGG(A/T)ACAAGTGA-3′	1492–1519	0.63[c]
rEF	5′-TG(C/T)AAAGC(C/T)TGATACATTTG(C/T)TCTTTTC-3′	2095–2122	
fEFn	5′-CAGCA(C/T)TATTT(A/G)TA(A/G)(A/G)(C/T)TGGATA-3′	1702–1724	0.20[d]
rEFn	5′-TCTAA(C/T)AAAATACC(C/T)(A/G)C(C/T)CCTA-3′	1879–1900	

[a] BoNT, *C. botulinum* neurotoxin.
[b] *C. botulinum* neurotoxin genes are described in GenBank under accession no. M81186 for type B and under accession no. X62683 for type E. Positions correspond to the nucleotide numbers downstream from the ATG start codon of the respective *C. botulinum* neurotoxin gene.
[c] Size of PCR product using the external set of primers.
[d] Size of PCR product using the internal set of primers.

treated in the same way as described above. Each sample was amplified twice with the PCR assay.

Collection and analysis of naturally contaminated pig fecal samples. Seventy-eight pig fecal samples were collected at the slaughter line of two slaughterhouses, one in southern Sweden (37 samples) and one in central Sweden (41 samples). The two slaughterhouses are situated 600 km away from each other. Approximately 10 g of feces was collected from the rectum, transferred to sterile tubes, and subsequently stored at −20°C until analysis. Forty-five of the samples were collected during the summer (June to September), and 33 of the samples were collected during the winter (December to March). Thirty-six of the 78 fecal samples collected were obtained from pigs reared outdoors, and the other 42 samples were collected from pigs reared indoors.

Before analysis, the fecal samples were thawed at 4°C for 7 h and mixed thoroughly in each tube. Three grams of feces was subsequently inoculated into 27 ml of preheated TPGY broth (1:10 dilution) and analyzed according to the combined selection and enrichment PCR procedure: (i) selection at 70°C for 10 min, (ii) enrichment at 30°C for 18 h, (iii) DNA extraction with a PrepMan DNA extraction kit for gram-positive bacteria (Perkin Elmer Applied Biosystems), and (iv) amplification with the two nested PCR assays with the DNA polymerase *rTth* (Perkin Elmer Applied Biosystems). A spore suspension of 10^3 spores (Eklund 2B) was used to inoculate 30 ml of feces homogenate (1:10 dilution in TPGY broth) to be included as a positive control, and 30 ml of TPGY broth was included as a negative control in each experiment. Two 1-ml samples were withdrawn after enrichment, and each sample was amplified twice with the type B and type E and F PCR assays.

RESULTS

Specificity and detection limits of PCR assays. Two sets of primers were designed, one specific for part of the type B neurotoxin gene and the other specific for part of both the type E and F neurotoxin genes (Table 2). The specificity of the two nested PCR assays was evaluated using 29 strains of *C. botulinum* neurotoxin serotypes A, B, E, and F; 16 strains of other *Clostridium* spp.; and 48 non-*Clostridium* strains (Table 1). The assay for the type B neurotoxin gene resulted in a PCR product at the predicted size, 0.22 kb, for all six *C. botulinum* type B strains. The Atlanta 2204–4 strain, a type A strain, also resulted in a PCR product of the same size with the type B assay. The assay for type E and F neurotoxin genes resulted in a 0.20-kb PCR product, as predicted, when tested on all 12 type E strains and all 6 type F strains. None of the other *Clostridium* spp. strains or the non-*Clostridium* strains gave amplified PCR products with the two tested PCR assays.

The detection probability of *C. botulinum* types B, E, and F in Millipore water was established at different cell concentrations (Fig. 1). The probability curves display a difference of 2 log units in the detection limit between the two PCR assays at a detection probability of 95%. The detection limit for *C. botulinum* type B in water, with a detection probability of 95%, was 1.6×10^3 cells per ml of water, which is approximately 8 cells per reaction tube. The corresponding values for *C. botulinum* types E and F were 1.6×10^5 cells per ml of water and approximately 8.0×10^2 cells per reaction tube. By adding equal amounts of purified cells of the microorganisms from feces to an increasing concentration of *C. botulinum* Eklund 2B cells in water, a 3 log unit decrease in sensitivity, at the detection probability of 95%, to 1.6×10^6 cells per ml, was found, that is, 8.0×10^3 cells per reaction tube (Fig. 1). Cells of *C. botulinum* Eklund 2B were inoculated in fecal homogenates and subsequently treated with the PrepMan DNA extraction kit prior to PCR to determine the concentration of *C. botulinum* required to obtain detection with a probability of 95%. The concentration was established to be 1.6×10^6 cells per ml of homogenate.

Enrichment PCR procedure and detection limits in pig fecal samples. From the combinations of different selection procedures and different enrichment times, the probability of detecting 10 spores of *C. botulinum* type B per 10 g of pig feces was determined (Table 3). This low concentration of spores was used based on the predicted low numbers of spores in naturally contaminated fecal samples. Overall, heat treatment at 70°C for 10 min resulted in a higher probability of positive PCR results during 48 h of enrichment than did not performing selection or the addition of 25-mg/ml D-cycloserine to the enrichment medium. Regarding heat treatment, the highest probabilities (P) were calculated after the following incubations: 18 h ($P = 0.42$), 36 h ($P = 0.50$), and 48 h ($P = 0.42$). A similar probability ($P = 0.42$) of positive PCR results was observed with the combination of 24 h of enrichment and no selection.

The fecal samples were inoculated with spores of proteolytic and nonproteolytic *C. botulinum* types B, E, and F to determine the detection limit in pig fecal samples after analysis with the combined selection and enrichment PCR procedure. The inhibitory effect of the microorganisms in pig feces on the amplification capacity of two different DNA polymerases, *rTth*

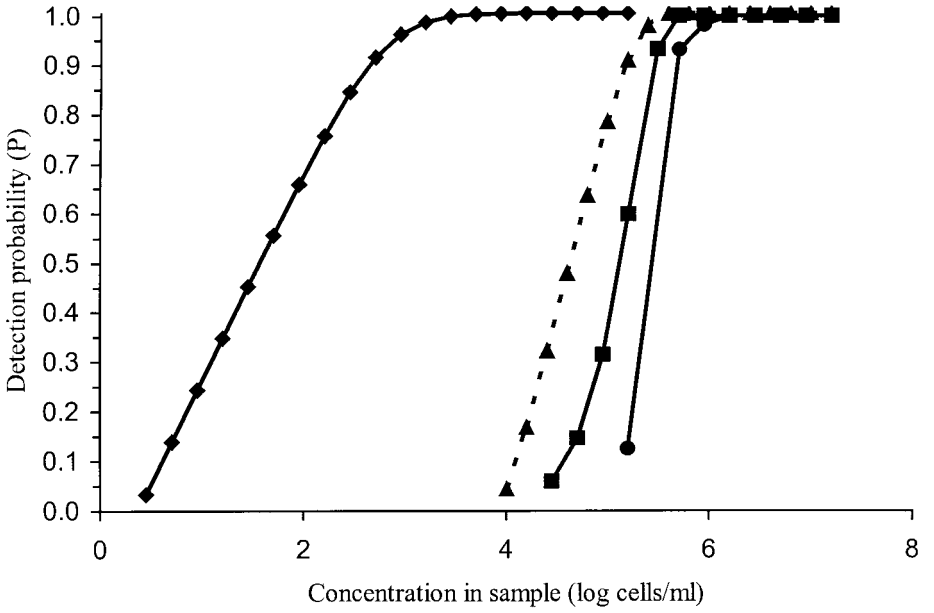

FIG. 1. Detection probability curves of *C. botulinum* types B, E, and F at different cell concentrations obtained using the two PCR assays and testing the effects of fecal microflora on the detection limit of the type B PCR assay. *P*, no. of positive PCR results/no. of replicates tested (n = 8 to 15); ◆, serial dilution of *C. botulinum* type B cells (Eklund 2B and Eklund 17B) in water (n = 15); ●, serial dilution of *C. botulinum* type B cells (Eklund 2B) in water with the addition of purified fecal microbial flora (n = 8); ■, fecal homogenate (1:10 in TPGY broth) spiked with a serial dilution of *C. botulinum* type B cells (Eklund 2 B) and subsequently treated with PrepMan DNA extraction kit (n = 10); ▲, serial dilution of *C. botulinum* type E and F cells (CB-S3-E and Craig 610B8-6F) in water (n = 15).

and *Taq*, in the PCR assay was studied (n = 9). The results showed a decrease in sensitivity by 1 log unit when using *Taq* DNA polymerase instead of *rTth*. All nine replicates gave the same result (data not shown). The detection limit when using *rTth* in the PCR assay for nonproteolytic *C. botulinum* type B with a detection probability of 95% was 10 spores per g of fecal sample (Fig. 2). The corresponding value for *C. botulinum* type E and nonproteolytic type F was 3.0×10^3 spores per g of fecal sample. For the proteolytic *C. botulinum* type B strain, no positive PCR detection was observed at any of the inoculated spore concentrations after 18 h of incubation. When increasing the enrichment temperature from 30 to 37°C and incubating the mixture for 18 h, a detection pattern similar to that of the nonproteolytic type B strains was observed (data not shown).

TABLE 3. Probability of detecting 10 spores of *C. botulinum*[a] type B per sample[b] using different selection methods and enrichment times prior to PCR

Enrichment time (h)	Detection probability[c] (%) with selection:		
	Not performed	By heat treatment (70°C, 10 min)	By D-cycloserine (25 mg/ml)
0	0/12 (0)	0/12 (0)	0/12 (0)
18	2/12 (0.17)	5/12 (0.42)	2/12 (0.17)
24	5/12 (0.42)	3/12 (0.25)	2/12 (0.17)
30	0/12 (0)	0/12 (0)	3/12 (0.25)
36	2/12 (0.17)	6/12 (0.50)	2/12 (0.17)
48	2/12 (0.17)	5/12 (0.42)	3/12 (0.25)

[a] Strains Eklund 2B and Eklund 17B.
[b] The sample was a homogenate of 10 g of pig feces in 90 ml of TPGY broth (1:10 dilution).
[c] No. of positive PCR results/no. of replicates tested (n = 12).

Prevalence of *C. botulinum* in pig fecal samples. Of the 78 pig fecal samples collected, 48 (62%) gave a positive PCR result with respect to the type B neurotoxin gene. No samples were positive regarding the type E and F neurotoxin genes, indicating that, if present, the prevalence of types E and F is less than 1.3%. ANOVA showed that both variation in season (summer and winter) and rearing conditions (outdoors and indoors) had significant effects on the prevalence of *C. botulinum* type B in pigs (Table 4). The distribution of positive samples in the groups from the southern and central parts of Sweden were similar. In the interplay between rearing conditions and seasonal variation a larger difference in prevalence was observed between the summer and winter periods in the group of outdoor pigs than between the corresponding periods in the group of indoor pigs (Table 5).

Four replicates of each of the 78 fecal samples were analyzed using PCR to semiquantify the number of spores for each sample category (Table 6). The probability of detection was determined for each positive fecal sample as described above. This ratio, combined with the detection probability curve of *C. botulinum* type B (Fig. 2), provided an indication of the spore load in each fecal sample. Thirty-four of the 48 positive fecal samples (71%) had a spore load of less than 40 spores per 10 g. Within each category (rearing condition, season) the distribution of positive samples, regarding the calculated probability, displayed a shift in the group of outdoor pigs from lower levels of spores during the summer ($\leq$15 spores/10 g) to higher levels during the winter ($\geq$40 spores/10 g). For the group of indoor pigs, the distribution of spore load remained approximately constant during the two sampling periods.

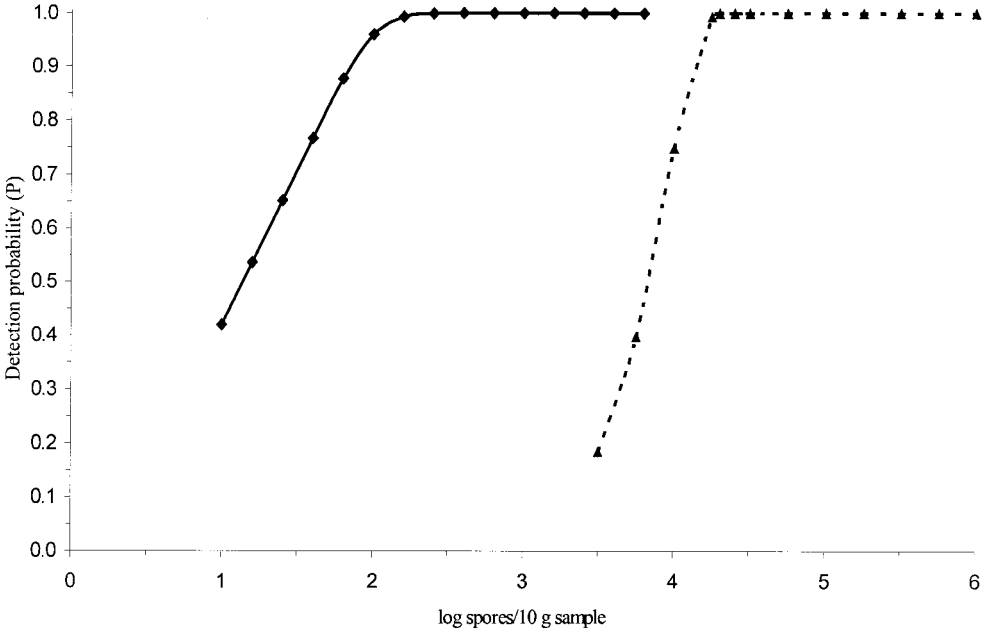

FIG. 2. Detection probability curves of *C. botulinum* types B, E, and F at different spore concentrations in pig fecal samples after analysis with the combined selection and enrichment PCR procedure. *P*, no. of positive PCR results/no. of replicates tested; ◆, fecal homogenate (1:10 in TPGY broth) spiked with a serial dilution of *C. botulinum* type B spores (Eklund 2B and Eklund 17B) ($n = 24$); ▲, fecal homogenate (1:10 in TPGY broth) spiked with a serial dilution of *C. botulinum* type E and F spores (CB-S3-E and Craig 610B8-6F) ($n = 24$).

DISCUSSION

Various sets of primers for the detection of *C. botulinum*, either degenerated primers (5, 10) or specific primers (34, 36), have been described previously. In recent years, a number of nucleotide sequences of the different types of the neurotoxin genes (A to G) have been published, increasing the possibility of designing more specific primers. The results of our study indicate that the two sets of primers, constructed for type B and types E and F, are specific. The Atlanta 2204–4 strain, a type A strain, resulted in a PCR product of the same size as the type B-specific primers (0.22 kb). In the literature, the presence of a silent or unexpressed type B gene is known to exist in several type A strains (6, 12), in which the presence of a stop codon and deletions has been identified (13, 21).

The detection limits for *C. botulinum* cells in water varied by 2 log units between the two PCR assays, at a detection probability of 95%. The slopes of the probability curves differed from each other (Fig. 1). At a detection probability of 50%, the range for detecting type B cells with high probability is broader than the corresponding range for types E and F. The variation in the probability curves and detection limits between the two assays is probably due to the different prerequisites for primer-target annealing efficiency. The primers for the type B assay were designed from highly conserved regions of the light chain of the type B neurotoxin gene, whereas the primers constructed for the type E and F assay were designed from less conserved regions of the heavy chain of the type E and F neurotoxin genes.

A number of components have been reported to be PCR inhibitors in feces, namely bile salts, complex polysaccharides, proteinases, and a high concentration of non-target DNA (25). In pig feces the number of microorganisms is estimated to be

TABLE 4. Prevalence of *C. botulinum* type B in pig feces regarding variations in geography, season, and rearing condition

Variable	No. of positive samples/ no. of samples examined	% Positive samples	*P* value
Geography			
Southern Sweden	23/37	62	
Central Sweden	25/41	61	0.105
Season			
Summer	21/45	47	
Winter	27/33	82	0.001
Rearing condition			
Outdoor	12/36	33	
Indoor	36/42	86	<0.0005

TABLE 5. Prevalence of *C. botulinum* type B in feces from outdoor and indoor pigs regarding seasonal variation

Season	No. of positive samples/no. of samples examined (%) for pigs reared:	
	Outdoors	Indoors
Summer		
Southern Sweden	0/12 (0)	6/6 (100)
Central Sweden	2/9 (22)	13/18 (72)
Total	2/21 (10)	19/24 (79)
Winter		
Southern Sweden	8/10 (80)	9/9 (100)
Central Sweden	2/5 (40)	8/9 (89)
Total	10/15 (67)	17/18 (94)

TABLE 6. Distribution of positive pig fecal samples (total 48 samples) within each category (rearing condition, season) with regard to detection probability[a]

Rearing condition	No. of positive fecal samples with a detection probability of:			
	1/4	2/4	3/4	4/4
Outdoor pigs				
Summer	0	2	0	0
Winter	0	1	6	3
Indoor pigs				
Summer	4	6	4	5
Winter	4	4	3	6
Total	8	13	13	14

[a] Detection probability = no. of positive PCR results/no. of replicates tested ($n = 4$).

around 10^{10} bacteria per g (28). To overcome these inhibitory effects when applying the PCR assays to inoculated fecal samples, an enrichment step, followed by a DNA extraction step, was included prior to PCR. By adding purified microorganisms from feces to the PCR, it was assessed that the decrease in sensitivity was mainly a consequence of the inhibitory effect constituted by the high concentration of microorganisms on the PCR amplification (Fig. 1).

Different combinations of selection procedures and different enrichment times were evaluated with inoculation of the feces homogenate using low concentrations of spores. This was because of the prediction of detecting spores, not bacteria, in the pig feces. From the results of the growth experiments, heat treatment at 70°C for 10 min and enrichment at 30°C for 18 h was identified as the best combination (Table 3). This was based on the highest number of positive PCR results out of the total number of 12 replicates tested ($P = 0.42$) together with the aspect of practicable working hours in the laboratory. Heat activation of spores is well described in the literature and is dependent on several factors, e.g., the heating temperature (15). In our study, the use of heat treatment at 80°C for 10 min resulted in no PCR product at all (data not shown), which is why the temperature was lowered to 70°C. A combined selection and enrichment procedure has several advantages. The heat treatment activates the spores present in the fecal samples and induces them to germinate and transform into vegetative bacteria. To some extent, the heat treatment also provides for a reduction in the numbers of competitive microorganisms. The enrichment in liquid broth allows the bacteria to grow and multiply to PCR-detectable levels. The only hitherto reported *C. botulinum* enrichment PCR protocol evaluated on spores involves incubating the biological samples for 5 days with no selection, allowing the spores to germinate and transform into vegetative cells in the enrichment broth (17). Two *C. botulinum* enrichment PCR procedures, based on detection after an 18-h enrichment step, have previously been described (10, 11), but these protocols were evaluated on food samples inoculated with bacteria, not spores. Although both *Taq* DNA polymerase and *rTth* worked well in the combined selection and enrichment PCR procedure, the detection limits were slightly different. A decrease in sensitivity by 1 log unit was observed when using *Taq* DNA polymerase instead of *rTth*, which could be due to the inhibitory effect of the microorganisms in pig feces on the amplification capacity. This finding is in accordance with previously performed investigations, in which *rTth* was found to exhibit a high level of resistance to PCR-inhibitory components in biological samples (1, 2, 3). The detection limit for the combined selection and enrichment PCR procedure, when using *rTth* in the PCR assay, was approximately 10 spores per g of fecal sample at a detection probability of 95% for nonproteolytic *C. botulinum* type B. The corresponding value for *C. botulinum* type E and nonproteolytic type F was 3.0×10^3 spores per g of fecal sample.

No surveys of *C. botulinum* types A, B, E, or F in pigs have, to our knowledge, been published. In Scandinavia, the incidence of the organism in terrestrial samples has been studied, and both types B and E were found (19, 22), indicating a potential risk of contamination of animals. In this study, a high prevalence of *C. botulinum* type B was established (62%). The reverse was true for types E and F, where no fecal sample was found to be positive using the combined selection and enrichment PCR procedure. This may indicate a prevalence of less than 1.3% of types E and F in the fecal samples. Another explanation may be the 2 log unit difference in the detection limits between the two PCR assays for type B and types E and F. Furthermore, *C. botulinum* type F is rarely found in the environment, which may also explain why no fecal samples were positive regarding this type. As for type E, this organism is most often found in aquatic environments and in fish samples (19).

The geographical area studied covers part of the lowlands of Sweden where almost all pigs are reared. Both rearing conditions and seasonal variation had a significant effect on the prevalence of *C. botulinum* type B in pigs. The prevalence of *C. botulinum* in the group of indoor pigs was markedly higher than in the group of outdoor pigs (Table 4). As for seasonal variation, a higher prevalence was observed in the fecal samples collected during the winter than in the samples collected during the summer. In this article, the term outdoor pigs refers to the ecological production of pigs; the major difference between this and conventional rearing is that the animals are always outdoors during the summer and may stay outdoors or in barns with conditions similar to the outdoor climate during the winter. In addition, we observed a correlation between rearing conditions and season. The prevalence of *C. botulinum* type B was higher in samples collected in the winter than in samples collected in the summer in both groups. However, the observed increase in prevalence was greater in the group of outdoor pigs, increasing from 10% during the summer to 67% during winter, than the corresponding increase, from 79 to 94%, in the group of indoor pigs. This may be due to several factors, such as the microbial environment of the barn and/or feed. One theory may be that spores ingested by the outdoor pigs were spread out again in the environment during the summer. However, as the pigs are more often found indoors in barns during the cold period of the year, the spores may be confined to the environment of the barn, increasing the risk of recontamination of the animal. This theory can also explain the higher prevalence in the group of indoor pigs. The shift in the distribution of positive fecal samples in the group of outdoor pigs, indicating a lower spore load during the summer and a higher spore load during the winter, is evidence of the recon-

tamination of the animal (Table 6). This difference could not be seen in the group of indoor pigs, where the level of spores was approximately constant between the summer and winter. Furthermore, a geographical difference was noticed among the samples (Table 6). In the southern part of Sweden, the prevalence of *C. botulinum* in the group of outdoor pigs increased from 0% during the summer to 80% during the winter, whereas the corresponding values in central Sweden were 22 and 40%, respectively. For the group of indoor pigs, the prevalence of *C. botulinum* remained constant at 100% in the southern part of Sweden during the summer and winter, whereas the values for central Sweden increased from 72% in the summer to 89% in the winter.

ACKNOWLEDGMENT

This work was financially supported by the Swedish Foundation for Strategic Research through a national, industry-oriented program for research and Ph.D. education, LiFT — Future Technologies for Food Production.

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