JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1994, p. 1986–1991
0095-1137/94/$04.00+0

Vol. 32, No. 8

Application of PCR to a Clinical and Environmental Investigation of a Case of Equine Botulism

E. A. SZABO,[1] J. M. PEMBERTON,[1] A. M. GIBSON,[2] R. J. THOMAS,[3] R. R. PASCOE,[4] AND P. M. DESMARCHELIER[5]*

Department of Microbiology[1] and Tropical Health Program,[5] University of Queensland, St. Lucia, Queensland 4072, Division of Food Science and Technology, Food Research Laboratory, Commonwealth Scientific and Industrial Research Organisation, North Ryde, New South Wales 2113,[2] Animal Research Institute, Yeerongpilly, Queensland 4105,[3] and Oakey Veterinary Hospital, Oakey, Queensland 4401,[4] Australia

Received 2 March 1994/Returned for modification 6 April 1994/Accepted 4 May 1994

PCR for the detection of botulinum neurotoxin gene types A to E was used in the investigation of a case of equine botulism. Samples from a foal diagnosed with toxicoinfectious botulism in 1985 were reanalyzed by PCR and the mouse bioassay in conjunction with an environmental survey. Neurotoxin B was detected by mouse bioassay in culture enrichments of serum, spleen, feces, and intestinal contents. PCR results compared well with mouse bioassay results, detecting type B neurotoxin genes in these samples and also in a liver sample. Other neurotoxin types were not detected by either test. *Clostridium botulinum* type B was shown to be prevalent in soils collected from the area in which the foal was raised. Four methods were used to test for the presence of botulinum neurotoxin-producing organisms in 66 soil samples taken within a 5-km radius: PCR and agarose gel electrophoresis (types A to E), PCR and an enzyme-linked assay (type B), hybridization of crude alkaline cell lysates with a type B-specific probe, and the mouse bioassay (all types). Fewer soil samples were positive for *C. botulinum* type B by the mouse bioassay (15%) than by any of the DNA-based detection systems. Hybridization of a type B-specific probe to DNA dot blots (26% of the samples were positive) and PCR-enzyme-linked assay (77% of the samples were positive) were used for the rapid analysis of large numbers of samples, with sensitivity limits of 3×10^6 and 3,000 cells, respectively. Conventional detection of PCR products by gel electrophoresis was the most sensitive method (300-cell limit), and in the present environmental survey, neurotoxin B genes only were detected in 94% of the samples.

Toxicoinfectious botulism in horses presents clinically as progressive neuromuscular paralysis. Horses of all ages are susceptible, but the disease most commonly occurs in fast-growing foals at 2 to 4 weeks of age (9). The condition, previously referred to as "shaker foal syndrome," was first described in 1967 (7). The etiologic mechanism of the disease remained obscure until it was established experimentally that *Clostridium botulinum* infection of necrotic lesions such as gastric ulcers and foci of necrosis in the liver and subsequent intoxication caused the disease syndrome (10).

Laboratory confirmation of toxicoinfectious botulism is often difficult, and the diagnosis is usually based on clinical presentation alone. Direct testing of serum samples from affected animals for toxin often yields negative results in the mouse bioassay, presumably because circulating toxin is bound quickly at the neuromuscular junction and the serum contains only a minute amount of toxin that usually cannot be detected by conventional methods (9). *C. botulinum* has been isolated from feces, gastric ulcers, and areas of necrosis in the livers, spleens, lungs, skin, and muscles of diseased animals (9). The presence of other bacteria in feces and necrotic lesions often prevents the isolation of *C. botulinum* and detection of the neurotoxin in the mouse bioassay.

Contaminated soil and fecal material are the likely sources of the organism. The type of *C. botulinum* involved in outbreaks of equine botulism seems to correlate with the geographic prevalence of a specific type in the region. MacKay and Berkhoff (4) reported on the incidence of type C equine botulism in Florida and the isolation of *C. botulinum* type C in soils and sediments in that region. *C. botulinum* type B is prevalent in the Kentucky area, where numerous cases of type B toxicoinfectious botulism have been reported (9). This disease has been reported in horse studs on the Darling Downs, approximately 130 km west of Brisbane, Queensland, Australia (6, 13). In 1985, a 17-day-old foal with signs of botulism was received at the Animal Research Institute at Yeerongpilly. *C. botulinum* type B was isolated from the foal's intestinal contents and feces. A head lesion yielded a similar isolate not confirmed to be *C. botulinum* type B because of low toxicity in the mouse bioassay.

The purpose of the study described here was to reanalyze this case of equine botulism by using DNA-based methods for the detection of *C. botulinum* neurotoxin genes and to compare these methods with the conventional mouse bioassay. Soil samples taken from the Darling Downs region were tested to determine the prevalence of *C. botulinum*. PCR and ethidium bromide-stained agarose gel electrophoresis for the visualization of amplified PCR products were compared with PCR and then an enzyme-linked assay (PCR-ELA) (2). The latter is a simplified protocol for the detection of PCR products suited to the screening of a large number of samples.

MATERIALS AND METHODS

Bacteria. The strains which were used in the study are listed in Table 1. All clostridial isolates were inoculated into cooked meat medium (CMM; Oxoid) rehydrated with either trypticase

* Corresponding author. Mailing address: Department of Microbiology, University of Queensland, Queensland 4072, St. Lucia, Australia. Phone: 61 7 365 4641. Fax: 61 7 365 4620. Electronic mail address: Trish@biosci.uq.edu.au.

TABLE 1. Isolates used in the study and their toxin production

Species	Toxin[a]	Reference	Source[b]
Clostridium botulinum	A	ATCC 441[c]	ATCC
		A726	ACH
	B	NCTC 7273	NCTC
		A625, A688, A777, A805	ACH
		ARI J6621-11, ARI P8568-3A, CSL 1787	ARI
		17B	CSIRO
	C	NCTC 8264, NCTC 3732	NCTC
	D	M91, Oderstepoort	ARI
		NCTC 8265	NCTC
	E	ATCC 17855	ATCC
	E	Abashiri, Alaska, Beluga, EK, Iwannai, Minneapolis, Rakefisk, VH, 16/63	IFR-UK
Clostridium butyricum	E	ATCC 43755	ATCC
Clostridium botulinum	F	NCTC 10281	NCTC
Clostridium baratii	F	ATCC 43756	ATCC
Clostridium argentinense	G	ACM 3690	ACM
Clostridium tetani	Tetanus	ACM 58	ACM
Clostridium sporogenes		ACM 54, ACM 60	ACM
Clostridium novyi type A		ACM 349	ACM
Clostridium perfringens		ACM 55	ACM
Clostridium butyricum		ACM 2646	ACM
Bacillus cereus		ATCC 14579	ATCC
Listeria innocua		ACM 3178	ACM
Listeria monocytogenes		ACM 527	ACM
Escherichia coli		ACM 1803	ACM

[a] Only clostridial neurotoxins are recorded.
[b] ATCC, American Type Culture Collection; ACH, Adelaide Children's Hospital, Adelaide, Australia; NCTC, National Collection of Type Cultures, London, United Kingdom; ARI, Animal Research Institute, Yeerongpilly, Australia; IFR-UK, Institute of Food Research, Reading Laboratory, Reading, United Kingdom; ACM, Australian Collection of Microorganisms, Brisbane, Australia.
[c] ATCC Deaccessioned from the American Type Culture Collection in 1964; available as strain NCTC 88738.

peptone yeast extract glucose cysteine (TPYGC) broth (3) or peptone yeast extract glucose (PYG) (12). Proteolytic isolates were incubated at 37°C and nonproteolytic strains were incubated at 30°C for 24 to 48 h. When cooked meat granules were omitted from the broth, anaerobic conditions were established by using H_2–CO_2-generating envelopes placed in anaerobic jars (BBL GasPak systems). Nonclostridial isolates were grown in PYG broth or on nutrient agar plates (Oxoid) aerobically at 37°C for 24 h. DNA was extracted as described previously (11).

Animal and soil samples. Autopsy samples from a 17-day-old foal with signs of toxicoinfectious botulism had been stored at −20°C since 1985 at the Animal Research Institute, Yeerongpilly. The animal presented with signs including muscular tremors, unsteady gait, inability to rise unaided, paralysis of the tongue with inability to swallow, dilated pupils, and respiratory distress. The samples examined in the present investigation were the intestinal contents, feces, liver, spleen, serum, and a head lesion swab. Two grams of each specimen was added to 20 ml of CMM rehydrated with TPYGC for 48 h at 32°C.

Soil samples were collected from three horse studs (properties 1 to 3) in the Darling Downs area with a history of toxicoinfectious botulism (5) and one property (property 4) on which horses were not bred and which had no recorded cases of botulism. Six samples were taken from one paddock on property 1, 5 samples were taken from one paddock on property 2, 30 samples were taken from five fields on property 3, and 25 samples were taken from five paddocks on property 4. Approximately 100-g samples, taken with a trowel from 10 cm below the turf, were placed in sterile plastic bags, sealed, and transported to the laboratory. Ten grams of each soil sample was added to 90 ml of TPGYC broth, and the mixture was incubated for 48 h at 32°C.

The liquid broth of culture enrichments from all specimens

TABLE 2. Oligonucleotide primers selected for neurotoxin types A to E

Type	Primer	Sequence	Region[a]	T_m (°C)[b]
A	A1	5′-TATGGAATAGCAATTAATCC-3′	697–1154 (457)	47
	A2	5′-GTGTAATTTACCTTAGGTAC-3′		49
B	B3	5′-AGATAGACGTGTTCCACTCG-3′	414–1141 (727)	55
	B4	5′-CTGCTATATTAGTTTCTG-3′		43
C	C1	5′-ATATACTCCGGTTACGGCG-3′	1353–2351 (998)	53
	C2	5′-CCTGGATAACCACGTTCCC-3′		55
D	D1	5′-TAAGTAAACCGCCCAGACC-3′	220–623 (403)	53
	D2	5′-TAGTATAGATAATGTTCCA-3′		43
E	E1	5′-TATATATTAAACCAGGCGG-3′	283–728 (445)	47
	E2	5′-TAGAGAAATATTGGAACTG-3′		45

[a] Region refers to the position of the first and last nucleic acid in the targeted fragment, and the size (in parentheses) refers to the number of nucleotides that comprise the fragment.

[b] The melting temperature (T_m) was calculated by the following formula: T_m = [4°C(G+C content of oligonucleotide) + 2°C(A+T content of oligonucleotide)] − 5°C (1).

was centrifuged at 3,500 × *g* for 15 min to sediment the particulate matter, including bacterial cells, and the particulate matter was held for PCR analysis and DNA dot blot hybridizations. The supernatants were collected and examined immediately for the presence of any botulinum toxins by using the mouse bioassay as described by the Standards Association of Australia (8).

DNA amplification and colorimetric detection of PCR products. DNA was extracted from the cell pellets of sample enrichments by a simple alkaline lysis procedure, and both DNA extraction and PCR for neurotoxin genes A and C to E proceeded as described previously (11). All oligonucleotide primers (Table 2) were synthesized either by the Queensland Institute of Medical Research or at the University of Queensland Centre for Molecular Biology and Biotechnology. Primers specific for neurotoxin type B were synthesized by Bresatec Ltd., Adelaide, Australia, for use with the PCR-ELA detection protocol. Primer B3.1 had the same sequence as primer B3 except for a DNA-binding protein recognition sequence attached to the 5′ end (GGATGACTCA + primer B3), while primer B4.1 was identical to primer B4 with a biotin moiety at the 5′ end (biotin + B4). Optimization of the PCR format and the specificity and sensitivity of the B3.1-B4.1 primer pair were determined by previously described procedures (11), and PCR was evaluated by both agarose gel electrophoresis and PCR-ELA.

The colorimetric analysis of PCR products (PCR-ELA) was performed according to the manufacturer's instructions (Captagene-GCN4; AMRAD Corporation Ltd., Melbourne, Victoria, Australia). The DNA-binding protein recognition sequence incorporated in primer B3.1 allowed amplified DNA to be bound to wells of a microtiter dish coated with the protein GCN4. The bound DNA could then be detected colorimetrically by using avidin-peroxidase, which binds to the biotin label incorporated into the PCR product via primer B4.1. Results were interpreted by eye or with a microtiter plate reader (Titertek Multiskan MCC/340) according to the manufacturer's instructions.

Hybridization with a specific probe. DNA dot blots were prepared by using a microfiltration apparatus (Bio-Rad) according to the manufacturer's instructions. Two hundred microliters of alkaline cell lysate of soil enrichment samples, held at room temperature for 5 min, were neutralized with an equal volume of 2 M ammonium acetate (pH 7.0). DNA was transferred to a nylon membrane pretreated with 1 M ammonium acetate. The filter was air dried and the DNA was fixed to the membrane by exposure to UV light for 3 min. A digoxigenin-labelled probe specific for neurotoxin type B (11) was used for hybridization. Hybridization was carried out at 65°C for 4 h. The hybridization solution contained 100 ng of probe per ml. Posthybridization washes and visualization of the probe-target hybrid were done in accordance with the manufacturer's instructions. Sensitivity was determined by hybridization of the type B probe to DNA dot blots of various amounts of *C. botulinum* NCTC 7273 type B genomic DNA (100 ng to 1 fg).

RESULTS

Specificity and sensitivity of primers B3.1-B4.1 and neurotoxin B probe. The reaction conditions for B3.1-B4.1 primers that gave the optimal sensitivity and specificity required 20 pmol of each primer, 3.0 mM $MgCl_2$, and annealing, extension, and denaturation temperatures and times of 55°C and 20 s, 60°C and 2 min, and 90°C and 20 s, respectively, for 25 cycles. The specificity of the B3.1-B4.1 primer pair was assessed by using purified DNA extracted from neurotoxic clostridial strains, nontoxic clostridial species, and unrelated strains (Table 1). Only the DNAs from strains of *C. botulinum* type B produced positive PCR results that were detected by agarose gel electrophoresis (data not shown) and PCR-ELA (Fig. 1). Nonspecific amplification products were not detected following gel electrophoresis following PCR at DNAs from genotypically related microorganisms or different genera (data not shown).

Serial 10-fold dilutions of genomic DNA from *C. botulinum* type B were used to determine the sensitivities of PCR and gel electrophoresis, PCR-ELA, and DNA dot blot hybridizations for the detection of neurotoxin B genes. The level of sensitivity observed following DNA amplification and gel electrophoresis was 1 pg. The lowest amount of template that produced an observable color reaction in the ELA after the optimal number of PCR cycles was 10 pg. Only 10 ng of DNA was detected by using the type B-specific probe. No false-positive reactions that could be attributed to molecular contamination with DNA or the constituents of the enrichment medium were observed. Given that the molecular mass of *C. botulinum* is 1.8×10^9 Da

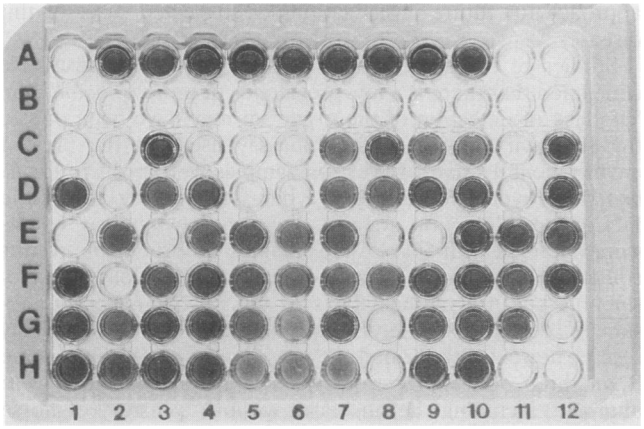

FIG. 1. Detection of botulinum neurotoxin type B PCR products by an ELA (Captagene-GCN4; Amrad). Row A, reactions with DNAs from *C. botulinum* type A ATCC 441 (position 1); *C. botulinum* type B strains NCTC 7273 (position 2), 17B (position 3), A625 (position 4), A688 (position 5), A777 (position 6), A805 (position 7), ARI J6621-11 (position 8), ARI P8568-3A (position 9), and CSL 1797 (position 10); *C. botulinum* type C NCTC 8264 (position 11); and *C. botulinum* type D M91 (position 12). Row B, reactions with DNAs from *C. botulinum* type E ATCC 17855 (position 1), *C. butyricum* type E ATCC 43755 (position 2), *C. botulinum* type F NCTC 8265 (position 3), *C. baratii* type F ATCC 43756 (position 4), *C. argentinense* ACM 3690 (position 5), *C. tetani* ACM 58 (position 6), *C. novyi* type A ACM 349 (position 7), *C. sporogenes* ACM 54 (position 8), *C. perfringens* ACM 55 (position 9), *C. butyricum* ACM 2646 (position 10), *B. cereus* ATCC 14579 (position 11), and *L. innocua* ACM 3178 (position 12). Row C, reactions with DNAs from *L. monocytogenes* ACM 527 (position 1), *E. coli* ACM 1803 (position 2), positive plate control (position 3), negative plate control (position 4), negative PCR control (position 5), soil samples from Darling Downs property 4, field 1 (positions 6 to 10), field 2 (positions 11 and 12). Row D, reactions with DNAs from Darling Downs property 4, field 2 (positions 1 to 3), field 3 (positions 4 to 8), and field 4 (positions 9 to 12). Row E reactions with DNAs from Darling Downs property 4, field 4 (position 1) and field 5 (positions 2 to 6) and property 1 (positions 7 to 12). Row F, reactions with DNAs from property 2 (positions 1 to 5) and property 3, field 1 (positions 6 to 10) and field 2 (positions 11 and 12). Row G, reactions with DNAs from property 3, field 2 (positions 1 to 8) and field 3 (positions 9 to 12). Row H, reactions with DNAs from property 3, field 3 (position 1), field 4 (positions 2 to 6), and field 5 (positions 7 to 11); position 12 is a blank well. The detection system was specific for pure cultures of *C. botulinum* type B only. No cross-reactions with other organisms were observed.

(1), theoretically, PCR and gel electrophoresis could detect 300 type B cells and PCR-ELA could detect 3,000 type B cells.

Original laboratory results. In 1985, samples were examined, without enrichment, for *C. botulinum* toxin by the mouse bioassay, with negative results (Table 3). Bacteria with morphological and biochemical features of *C. botulinum* were simultaneously isolated from intestinal contents, feces, and head lesion enrichments. Culture supernatants of the isolates from the intestinal contents and feces killed mice, which had characteristic signs of botulism, and the toxicity was neutralized by antitoxin to type B but not antitoxin to type A, C, D, or E. Mice injected with culture supernatant from the head lesion isolate showed signs of botulism but did not die, and antitoxin tests were not done.

Retesting of stored samples. Anaerobic bacterial growth was observed in all sample enrichments with the exception of the head lesion material. Positive mouse bioassay results were observed when culture supernatants of serum, feces, spleen,

TABLE 3. Detection of *C. botulinum* type B in autopsy samples from a foal with toxicoinfectious botulism by PCR and the mouse bioassay

Sample	1985 results			1992 results	
	Direct toxin testing[a]	Isolation[b]	Toxin type B confirmed[c]	Mouse bioassay[d]	PCR[e]
Feces	−	+	+	+	+
Head lesion	NT[f]	+	−	−	−
Intestinal contents	−	+	+	+	+
Liver	NT	−	NA[g]	−	+
Serum	−	−	NA	+	+
Spleen	NT	−	NA	+	+
C. botulinum type B (control)				+	+

[a] Direct testing of a sample for botulinum neurotoxin by the mouse bioassay.
[b] Isolation of an organism resembling *C. botulinum*.
[c] Confirmation of neurotoxin type B production from the culture supernatant of a suspect isolate.
[d] Detection of neurotoxin following sample enrichment by the mouse bioassay.
[e] Detection of type B neurotoxin genes by PCR.
[f] NT, not tested.
[g] NA, not applicable.

and intestinal content enrichments were used (Table 3). The PCR protocol was able to detect the presence of type B neurotoxin genes in these samples and additionally from the liver enrichment (Table 3; Fig. 2). Other neurotoxin types were not detected in the samples by either PCR (types A to E only) or the mouse bioassay (all types). Negative and positive controls reacted accordingly.

Soil sampling. *C. botulinum* type B was detected by PCR and agarose gel electrophoresis, PCR-ELA, hybridization with a type B-specific probe, and the mouse bioassay on properties 1, 3, and 4 (Table 4). Only the two PCR-based methods detected the organism on property 2 (Table 4). PCR and gel electrophoresis were the most sensitive methods, detecting *C. botulinum* type B in 94% of the samples; this was followed by PCR-ELA (77%), probing of DNA dot blots of crude alkaline cell lysates with the type B-specific probe (26%), and the mouse bioassay (15%) (Table 4). Definitive positive or negative results were not obtained by the mouse bioassay for more than two-thirds of the soil sample enrichments either because reproducible symptoms were not observed or because neutral-

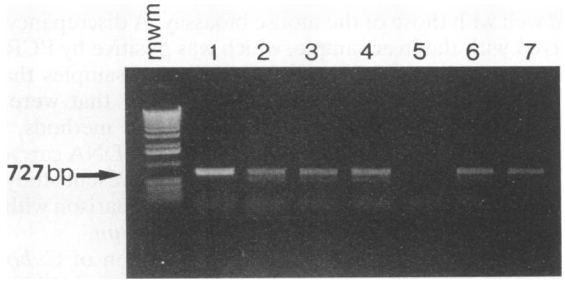

FIG. 2. PCR detection of type B botulinum neurotoxin genes in autopsy samples from a foal with clinical symptoms of toxicoinfectious botulism. The molecular mass marker (MWM) was a commercially available 1-kb DNA ladder (Bethesda Research Laboratories). The amount of material loaded into each well of the gel represented 1/10th of the PCR mixture volume. The samples were a positive PCR control (lane 1), feces (lane 2), intestinal contents (lane 3), liver (lane 4), head lesion (lane 5), serum (lane 6), and spleen (lane 7). Only the head lesion sample produced negative PCR results.

TABLE 4. Occurrence of *C. botulinum* type B in soil samples from four properties in the Darling Downs region

Property no.[a]	% Positive by the following detection method			
	PCR	PCR-ELA	Hybridization with type B probe	Mouse bioassay
1 (n = 6)	100	67	34	17
2 (n = 5)	100	80	0	0
3 (n = 30)	100	87	37	20
4 (n = 25)	84	68	16	12
Total (n = 66)	94	77	26	15

[a] n denotes the number of samples.

izations could not be confirmed from successive tests. No other neurotoxin types were detected by PCR or the mouse bioassay.

DISCUSSION

Toxicoinfectious botulism in foals has been clinically diagnosed in horses from horse farms in a well-defined area of the Darling Downs region for the past 27 years. Between 1967 and 1977 on one particular thoroughbred horse farm, 91 cases occurred among foals, yearlings, and adult horses, which showed typical clinical signs of botulism, and only two animals survived (6). Several foals autopsied at the end of the period were found to be infected with *C. botulinum* type B (13). On the basis of these observations, a vaccine for *C. botulinum* type B was developed and administered to mares prior to foaling (13). Examination of soil samples from the region in the present study confirmed a prevalence of *C. botulinum* type B. Other neurotoxin types were not detected by PCR or mouse bioassay, indicating that the use of a monovalent type B vaccine was appropriate in this area. We used PCR and the mouse bioassay to reexamine a foal from the region with a clinical diagnosis of botulism. When this foal was first investigated in 1985, microbial analysis of autopsy samples from the foal supported the clinical diagnosis of botulism by isolation and subsequent confirmation in the mouse bioassay of *C. botulinum* type B from the intestinal contents and feces. Upon reexamination of the specimens in the present study, the mouse bioassay again detected the organism in enrichments from the intestinal contents and feces and, in addition, in enrichments from the spleen and serum. PCR results compared well with those of the mouse bioassay. A discrepancy was observed with the liver sample, which was positive by PCR but negative by the mouse bioassay. Head lesion samples that in 1985 produced signs of botulism in mice but that were not lethal were negative upon retesting by both methods. The integrity of the PCR was confirmed because no DNA carryover effects were detected. From the results of the present study, we conclude the PCR is a sensitive method in comparison with the mouse bioassay for the detection of *C. botulinum*.

An unexpected observation was the detection of *C. botulinum* in serum samples by both PCR and the mouse bioassay. Botulinum neurotoxin is rarely detected in the serum of horses with toxicoinfectious botulism (9). However, serum is not usually enriched prior to the mouse bioassay, and this may explain the discrepant result on retesting. Serum would contain fewer contaminating bacteria than necrotic lesions, which are usually used in the microbial investigation of horse botulism cases. The analysis of serum both directly and following enrichment should be considered an alternative diagnostic approach for future investigations of suspected cases of this disease.

Studies of the environment in Australia in the past have concluded that there is a low prevalence of or no *C. botulinum*. The lack of detection of the organism may have been due to the sensitivity of the mouse bioassay used. In the present environmental survey, fewer soil samples were positive for *C. botulinum* type B by the mouse bioassay than by any of the DNA-based detection systems. Inconclusive bioassay results were obtained with 66% of the samples. Reproducible results could not be obtained from successive mouse bioassays, presumably because of the effects of unknown interfering substances present in the natural soil sample. The mouse bioassay results, however, confirmed the presence of *C. botulinum* type B on three of the properties from which soil samples were obtained, including the one farm without a history of horse botulism.

The number of soil samples positive by each DNA-based method reflected the order of sensitivity of each test. Conventional detection of PCR products by gel electrophoresis was the most sensitive method, and in the soil survey, 94% of the samples contained the genes for neurotoxin B; this was followed by PCR-ELA, which detected type B neurotoxin genes in 77% of the samples, and direct probing of crude cell lysates, the least sensitive technique, which detected type B neurotoxin genes in only 26% of the samples, which was slightly better than the mouse bioassay results. However, unlike the mouse bioassay, the results from PCR-based methods were reproducible in three successive tests and after 6 months of storage of the purified DNA extract (data not shown). Of the three DNA-based detection methods used, hybridization of the type B-specific probe to DNA dot blots and PCR-ELA were particularly suited for the analysis of large sample numbers. By PCR-ELA, up to 96 DNA amplifications were analyzed on a single microtiter plate within 2 h, which was more rapid than the DNA blot hybridization techniques and analysis of that number of PCR products by gel electrophoresis. Results were easily distinguished by eye and were confirmed spectrophotometrically. Although a convenient method for mass screening, PCR-ELA was less sensitive than the visualization of PCR products by gel electrophoresis, which remains the preferred procedure, especially when detection of small spore numbers is expected. Attempts were made to improve the sensitivity of ELA detection of PCR products by increasing the DNA binding time from 30 to 60 min and then 90 min, without success (data not shown).

The environmental survey described here provided the opportunity to compare PCR with the mouse bioassay. Unfortunately, components of the soil samples interfered with the bioassay, and inconclusive results were obtained from two-thirds of the samples. The mouse bioassay is considered the standard with which to compare new methods; however, that bioassay is not suitable for use on environmental samples. Nevertheless, our results demonstrate that PCR is a convenient alternative for the detection of *C. botulinum* from environmental samples. Use of both PCR and the mouse bioassay demonstrated the prevalence of *C. botulinum* type B in the soils of properties where equine botulism cases had occurred and in those of properties with no history of botulism. A more extensive quantitative survey for *C. botulinum* in soils in areas used for horse breeding and those used exclusively for agricultural purposes and a survey of animal husbandry practices on the properties used for horse breeding are now required. This would help to determine how widespread the bacterium is in the Darling Downs region and the potential risk factors associated with the disease.

ACKNOWLEDGMENTS

The project was supported by CSIRO/University of Queensland grant 111 and an Australian Research grant, grant 1028.

REFERENCES

1. **Durban, E., E. M. Durban, and N. Grecz.** 1974. Production of spore spheroplasts of *Clostridium botulinum* and DNA extraction for density gradient centrifugation. Can. J. Microbiol. **20:**353–358.
2. **Kemp, D. J., M. J. Churchill, D. B. Smith, B. A. Biggs, S. J. Foote, M. G. Peterson, N. Samaras, N. J. Deacon, and R. Doherty.** 1990. Simplified colorimetric analysis of polymerase chain reactions: detection of HIV sequences in AIDS patients. Gene **94:**223–228.
3. **Lilly, T., Jr., S. M. Harmon, D. A. Kautter, H. M. Solomon, and R. K. Lynt, Jr.** 1971. An improved medium for detection of *Clostridium botulinum* type E. J. Milk Food Sci. **34:**492.
4. **MacKay, R. J., and G. A. Berkhoff.** 1982. Type C toxicoinfectious botulism in a foal. J. Am. Vet. Assoc. **180:**163–164.
5. **Pascoe, R.** Unpublished data.
6. **Pascoe, R. R.** Personal communication.
7. **Rooney, J. P., and M. E. Prickett.** 1967. Shaker foal syndrome. Modern Vet. Pract. **48:**44–45.
8. **Standards Association of Australia.** 1991. Food microbiology. Method 2.7. Examination for specific organisms—*Clostridium botulinum* and *Clostridium botulinum* toxin. Standards Association of Australia, Sydney.
9. **Swerczek, T. W.** 1980. Toxicoinfectious botulism in foals and adult horses. J. Am. Vet. Assoc. **176:**217–220.
10. **Swerczek, T. W.** 1980. Experimentally induced toxicoinfectious botulism in horses and foals. Am. J. Vet. Res. **41:**348–350.
11. **Szabo, E. A., J. M. Pemberton, and P. M. Desmarchelier.** 1993. Detection of the genes encoding botulinum neurotoxin types A to E by the polymerase chain reaction. Appl. Environ. Microbiol. **59:**3011–3020.
12. **Thomas, R. J.** 1991. Detection of *Clostridium botulinum* types C and D toxin by ELISA. Aust. Vet. J. **68:**111–113.
13. **Thomas, R. J., D. V. Rosenthal, and R. J. Rogers.** 1988. A *Clostridium botulinum* type B vaccine for prevention of shaker foal syndrome. Aust. Vet. J. **65:**78–80.