ORIGINAL ARTICLE

Optimization of Polymerase Chain Reaction for Detection of *Clostridium botulinum* Type C and D in Bovine Samples

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Summary

Botulism is a rare but serious paralytic illness caused by a nerve toxin that is produced by the bacterium *Clostridium botulinum*. The economic, medical and alimentary consequences can be catastrophic in case of an epizooty. A polymerase chain reaction (PCR)-based assay was developed for the detection of *C. botulinum* toxigenic strains type C and D in bovine samples. This assay has proved to be less expensive, faster and simpler to use than the mouse bioassay, the current reference method for diagnosis of *C. botulinum* toxigenic strains. Three pairs of primers were designed, one for global detection of *C. botulinum* types C and D (primer pair Y), and two strain-specific pairs specifically designed for types C (primer pair VC) and D (primer pair VD). The PCR amplification conditions were optimized and evaluated on 13 bovine and two duck samples that had been previously tested by the mouse bioassay. In order to assess the impact of sample treatment, both DNA extracted from crude samples and three different enrichment broths (TYG, CMM, CMM followed by TYG) were tested. A 100% sensitivity was observed when samples were enriched for 5 days in CMM followed by 1 day in TYG broth. False-negative results were encountered when *C. botulinum* was screened for in crude samples. These findings indicate that the current PCR is a reliable method for the detection of *C. botulinum* toxigenic strains type C and D in bovine samples but only after proper enrichment in CMM and TYG broth.

Importance of the paper's findings for a non-specialist:

1. Developing a fast diagnosis method for botulism.
2. To be able to prevent the disease.
3. Reduce massive economic losses and public health risk whenever contaminated meat or dairy products enter the food chain.

Introduction

Botulism is a deadly disease of humans and animals caused by the neurotoxins produced by the anaerobic bacteria *Clostridium botulinum*. These neurotoxins (BoNT) are considered to be the most potent of all bacterial toxins (Lamanna, 1959). In their active form, they consist of two subunits: a heavy chain (100 kDa) and a light chain (50 kDa) which are held together by a disulphide bridge (Niemann, 1991). Their mode of action relies on blocking the release of the acetylcholine neurotransmitter at the cholinergic synapses resulting in neuroparalytic disease that can lead to death (DasGupta, 1990). BoNTs are categorized into seven different types (A to G) according to their antigenic properties (Niemann, 1991).

Bovines are principally susceptible to type D toxin; however, they may also be affected by types C, B and rarely A. They are usually toxin-infected when grazing poultry litter-contaminated pastures. However, in developing countries, these animals may suffer from hypophosphorous conditions and get infected by grazing

spore-contaminated ground or carcasses (Carlier et al., 2001; Afssa, 2002).

The most important clinical signs of botulism in cattle include loss of tongue tone, decreased upper eyelid and tail tone, dilated pupils, dysphagia, and decreased ruminal motility (Whitlock, 1999). Whenever intoxication occurs in intensively farmed animals, high mortality rates can be observed (Abbitt et al., 1984; Trueman et al., 1992; Martin, 2003). Abbitt et al. (1984) found *C. botulinum* type D intoxication to be responsible for the death of 42 of 67 lactating cows in a south-east Texas dairy herd over an 11-day period. The economical, medical and alimentary consequences can be catastrophic in case of an epizooty. One gram of pure toxin dispersed in the food could kill 400 000 adult cows (Galey et al., 2000).

Symptoms alone are often insufficient to make a diagnosis of botulism and a laboratory confirmation is required. Because of its high sensitivity, the mouse bioassay including the identification of the responsible BoNT by (sero) neutralization, still remains the standard method in botulism diagnostics (Shone et al., 1985; Doellgast et al., 1993). However, animal testing remains highly disapproved and non-specific mouse mortality can blur the diagnosis (Szabo et al., 1994a).

Treatment of bovine botulism is symptomatic and supportive but not curative. Treating the animal with antitoxins only prevents progression of the disease and is considered only for animals of great commercial value (Kirk and Adaska, 1998; OVF- Office vétérinaire fédéral, 2002).

In order to prevent botulism epizootic spread both preventative measures and rapid detection of the causative agent are of immense importance. In this respect, polymerase chain reaction (PCR) seems to be a diagnostic method with major advantages including rapid, easy and cheap methodology.

The application of PCR for the detection of neurotoxin genes has been successful for several different BoNT types (A, B, C, D, E and F) (Fach et al., 1993, 1995, 2002; Franciosa et al., 1994, 1996; Szabo et al., 1994a,b; Takeshi et al., 1996; Aranda et al., 1997; Braconnier et al., 2001; Dahlenborg et al., 2001; Lindström et al., 2001; Chaffer et al., 2006). Sensitivity is high; it can detect as little as 35 clostridial bacteria in a sample. However, detection limits appear to be dependent on the sample type (Wictome and Shone, 1998). This can be attributed to the presence of PCR inhibitors and other bacterial species in the samples (Fach et al., 1993). Therefore, PCR is considered highly sensitive when used for detection of BoNT genes in pure cultures in contrast to the detection directly in crude samples where sensitivity cannot be guaranteed without prior culture enrichment (Wictome and Shone, 1998).

With the recent achievements in the field of PCR detection of BoNT genes, we aimed at creating a highly specific, sensitive, quick and cheap method for the diagnosis of *C. botulinum* types C and D in bovine samples. In this light, we designed three sets of primers, optimized the PCR conditions, tested the optimized conditions in bovine samples and compared these results with the standard method. Testing of crude samples as well as different enrichment broths was also incorporated.

Materials and Methods

Bacterial strains

A total of 26 reference strains (15 *C. botulinum* and 11 other *Clostridium* species) were included in this study (Table 1). All reference strains were tested for BoNT production in the mouse bioassay as described below.

Sixteen clinical and field samples were collected from suspected cases (liver, stomach contents, kidney, brain and feed) (Table 1). Thirteen of these 16 samples tested positive for toxin production in the mouse bioassay. The remaining three samples tested negative in the same assay and were used as negative controls.

Mouse bioassay

All procedures with animals were carried out in accordance to appropriate humane methods. Swiss OF1 conventional mice were used. Breeding was performed in the breeding facilities of the Pasteur Institute, agreement number LA2230389. Mice were feed *ad libitum* with commercial pellets. Temperature was kept at 21°C, ventilation at 15 refreshments per hour and light regimen was of 12 h light/12 h dark. Cages were of type II long mouse cages: height = 16 cm, surface = 21 × 37 cm, with 10 mice per cage. Mice were used when they reached a weight of about 20 g.

The mouse lethality test was performed as described previously (Kautter and Solomon, 1977). Briefly, after 5 days of anaerobic incubation in cooked meat medium (CMM) (Difco BD, Erembodegem-Aalst, Belgium) (Quagliaro, 1977) at 37°C, 2 ml of enrichment culture were filtered over a 0.22 μm Millipore filter (MillexGP, Millipore, Bruxelles, Belgium). A volume of 0.4 ml of filtered solution was subsequently injected intraperitoneally into mice (two mice per sample). For 4 days, the mice were inspected for clinical symptoms of botulism (pinching of the waist, laboured breathing, paralysis and death). The presence and type of botulinum toxin was confirmed by an *in vivo* seroneutralization test in mice. Therefore, 225 μl of specific botulinum antitoxins (Centre of Disease Control, Atlanta, GA, USA) were added to 900 μl of filtered culture medium and incubated for 30 min at 37°C.

Table 1. PCR and mouse bioassay results for the reference and routine samples

	Name	Origin	Mouse bioassay	Set of primers		
				Y	VC	VD
Routine samples	03B019	Bovine liver	D[a]	√[b]	×[c]	√
	03B020[d]	*Bovine feed*	D	√	×	√
	03B021	Bovine stomach contents	D	√	×	√
	03B027	Bovine stomach contents	D	√	×	√
	03B030	Bovine stomach contents	D	√	×	√
	03B033	Bovine stomach contents	D	√	×	√
	03B038	Bovine liver + kidney + brain	D	√	×	√
	03B039	Bovine stomach contents	D	√	×	√
	03B041	Bovine liver + stomach contents	D	√	×	√
	03B042	Bovine liver	D	√	×	√
	03B111	Bovine liver	D	√	×	√
	03B113	Bovine liver	D	√	×	√
	05B134	Bovine liver	D	√	×	√
	05B159	Bovine liver	–[e]	×	×	×
	05B166	*Duck Liver*	–	×	×	×
	05B167	*Duck Liver*	–	×	×	×
Reference strains	04I	*C. botulinum A* (CIP[f] 104.310)	A	×	×	×
	04V	*C. botulinum A* (ATCC 3502)[g]	A	×	×	×
	04VI	*C. botulinum A* (ATCC 19397)	A	×	×	×
	97I	*C. botulinum B* (IPB)[h]	B	×	×	×
	04IV	*C. botulinum B* (NCTC 7273)[i]	B	×	×	×
	05e	*C. botulinum B* (IPB)	B	×	×	×
	04II	*C. botulinum C* (CIP 104.983)	C	√	√	×
	98I	*C. botulinum C* (IPB)	C	√	√	×
	02II	*C. botulinum C* (IPB)	C	√	√	×
	02V	*C. botulinum D* (IPB)	D	√	×	√
	02VI	*C. botulinum D* (IPB)	D	√	×	√
	04III	*C. botulinum D* (CIP 105.256)	D	√	×	√
	05p	*C. botulinum E* (NCTC 08266)	E	×	×	×
	05o	*C. botulinum F* (NCTC 10281)	F	×	×	×
	05l	*C. novii A* (NCTC 13029)	A	×	×	×
	05a	*C. sordelii* (IPB)	–	×	×	×
	05b	*C. difficile* (IPB)	–	×	×	×
	05c	*C. bifermentans* (IPB)	–	×	×	×
	05c	*C. difficile* (IPB)	–	×	×	×
	05f	*C. fallax* (IPB)	–	×	×	×
	05g	*C. perfringens* (IPB)	–	×	×	×
	05k	*C. sporogenes* (NCTC 13020)	–	×	×	×
	05m	*C. butyricum* (NCTC 07423)	–	×	×	×
	05n	*C. hastiforme* (NCTC 11832)	–	×	×	×
	80I	*C. tetani* (ATCC 10779)	–	×	×	×
	02X	*C. sporogenes* (IPB)	–	×	×	×

[a]*C. botulinum* type detected.
[b]Fragment amplified with the expected size.
[c]No amplification.
[d]In italic: samples that are not bovine.
[e]Negative result.
[f]CIP, Collection de l'Institut Pasteur, Paris, France.
[g]ATCC, American Type Culture Collections, Rockville, MD, USA.
[h]IPB, Strain isolated at the Institut Pasteur de Bruxelles, Brussels, Belgium.
[i]NCTC, National Collection of Type Cultures, London, UK.

Table 2. Tested culture methods

Name	Description	% of positive detections		
		Y	VC	VD
TYG	Culture in TYG[a]	36	0	82
CMM	Culture in CMM[b]	0	0	0
CMM.10 min	Culture in CMM heated 10′ at 80°C	0	0	0
CMM.E	Culture in CMM treated with ethanol[c]	0	0	0
CMM.E.10 min	Culture in CMM heated 10′ at 80°C and treated with ethanol	0	0	0
TYG ← CMM	Culture in TYG inoculated with a culture of CMM	100	100	100
TYG ← CMM.10 min	Culture in TYG inoculated with a culture of CMM heated 10′ at 80°C	100	100	100
TYG ← CMM.E	Culture in TYG inoculated with a culture of CMM treated with ethanol	100	100	100

The last column gives the percentage of positive samples (tested in the mouse bioassay) that were detected positive by PCR for each type of culture and each primer.

[a]All cultures in TYG were performed overnight at 37°C.

[b]All cultures in CMM were performed for 5 days at 37°C.

[c]Treatment with ethanol at 50% (v/v) for 30 min at 37°C.

A volume of 0.5 ml was then injected intraperitoneally into mice. The animals were checked for symptoms as described above.

Culture methods

For PCR optimization stored reference strains were grown under anaerobic conditions in CMM broth for 5 days at 37°C followed by an overnight enrichment under similar conditions in trypticase yeast extract glucose (TYG) medium (Sebald and Petit, 1994).

Clinical and field samples were grown in various media (Table 2). Prior to inoculation, some cultures were either treated with ethanol, heat shocked for 10 min at 80°C or both to enhance *C. botulinum* recovery.

DNA extraction

DNA extractions were performed with the DNA extraction Kit GenElute^{TM} Bacterial Genomic DNA Kit (Sigma, Bornem, Belgium). Briefly, a volume of 1.8 ml of TYG culture was centrifuged at 16 060 ***g***. Cell pellets were lysed with 200 μl of a lysozyme solution (45 mg/ml) and subsequently treated with 20 μl of RNase A for 3 min at room temperature. When extractions were made directly from the sample, 0.25 g was grinded with lysozyme solution and treated as described above.

The solution was incubated at 37°C for 30 min. After addition of 20 μl of proteinase K and 200 μl of lysis solution the mix was incubated for 10 min at 55°C. The Sigma binding columns were prepared by adding 500 μl of column preparation solution and centrifuged at 13 000 rpm. The elute was discarded. Thereafter, a volume of 200 μl of ethanol (100%) was added to the lysate and mixed. The entire content was then transferred into the binding column and centrifuged at 6090 ***g*** for 1 min. Subsequently, the DNA recovered on the column was washed twice with 500 μl of wash solution 1 and 500 μl of wash solution respectively at 8000 rpm for 1 min and at 13 000 rpm for 3 min. DNA was eluted with 200 μl of elution solution and centrifuged at 8000 rpm for 1 min.

PCR assay

Primer design

The sequences of the different *C. botulinum* neurotoxin genes (BoNT/A, BoNT/B, BoNT/C, BoNT/D, BoNT/E, BoNT/F BoNT/G and the tetanus neurotoxin) were retrieved from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The primers were designed after alignment using the program Wemboss from the European Molecular Biology Network (http://www.embnet.org). The sequences and the locations of these primers as well as the expected amplicon sizes are listed in Table 3. One set was chosen in the most homologous regions of the sequences for BoNT/C and BoNT/D (primer set Y). Two sets of primers (VC and VD) were chosen for the specific BoNT/C and BoNT/D detection, respectively. The primers were tested with the program Amplify (version 1.2) for primer–dimer formation and specificity.

PCR optimization

Amplification was carried out in the GENEAmp® PCR system 2700 (Applied Biosystems, Lennik, Belgium). The cycling conditions were optimized in order to use all primers sets in a single run. For this, a variation of the annealing temperature from 40 to 65°C was tested as well as various concentrations of $MgCl_2$ (1.5, 2.5, 3.5 and 4.5 mM).

Table 3. Description of the primers

Primer	Primer set	Sequences 5′–3′	*BoNT* type	Position in the BoNT gene sequence	Accession number	Corresponding toxin chain	Size of amplicons
cds2	Y	tttatacgagaatgttcyg	C and D	2343–2362	AB037166 AB012112	Hc[a]	327 bp
cdas3		cattatatcctgatgtatcc	C and D	2650–2670			
cs1.2	VC	tcctcgagttacaagcc	C	182–199	AB037166	Lc[b]	169 bp
cas2		caggaaagggtatatctg	C	333–351			
ds2	VD	ttagactatacagcatccc	D	495–514	AB012112	Lc[b]	264 bp
das2		taacttgtggacgaatcc	D	741–759			

[a]Heavy chain.
[b]Light chain.

The sensitivity of the method was estimated with a large range of dilutions from 100 ng to 10 ag of the DNA from the reference strains (Table 1) in order to determine the lowest concentration allowing detection. The specificity of the primers was tested in the presence of DNA from other types of *BoNT* and with other *Clostridia* listed in Table 1.

The PCR cycling is a modification of the protocol described previously by Fach in 1993 (Fach et al., 1993). The cycling began with an initial step at 95°C for 15 min to activate the HotStart *Taq* polymerase (Eurogentec, Seraing, Belgium). Each following cycle consisted of a denaturation step at 94°C for 30 s, an annealing step at 55°C for 30 s, and an extension step at 72°C for 30 s. This was repeated 40 times. A final step at 72°C for 10 min followed to complete extension.

Positive controls with DNA from reference strains and a negative control containing all reagents except DNA template were included during amplification. The amplified PCR products were visualized in 1.8% agarose gels (Agarose ultra Pure; GibcoBRL, Merelbeke, Belgium) and stained with ethidium bromide (molecular weight marker: Smart Ladder SF^{TM}; Eurogentec). After optimization, clinical samples were tested by PCR.

Results

PCR optimization

The optimal PCR conditions were set as follows: a final reaction volume of 50 μl containing 25 μl of Mix PCR (Red'y'Star Mix from Eurogentec, PK-0073-02R), 3.5 mM of $MgCl_2$, 0.5 μM concentrations of each primer (Operon, Cologne, Germany) and 10 μl of template (100 ng of sample DNA). The optimal annealing temperature was set at 55°C. The lowest concentration of DNA detectable for primers Y, VC and VD was set at 100 fg, 1 pg and 10 fg respectively.

The specificity of the primers was confirmed. None of the other *C. botulinum* types and *Clostridia* tested were amplified with either of the three sets of primers (Fig. 1 shows some of the results). All *C. botulinum* type C strains tested positive for the amplification of a 327 bp and 169 bp DNA fragments when primers Y and VC were used, respectively. All *C. botulinum* type D strains were found positive for the amplification of a 327-bp fragment (primers Y) and a 264-bp fragment (primers VD) (Fig. 1). In order to confirm the results, most of the segments amplified in the PCR optimization were sequenced.

Application of the optimized PCR in routine samples

Sixteen routine samples enriched in CMM medium (5 days) followed by an overnight culture (16 h) in TYG broth were analysed by the mouse bioassay and subjected to molecular diagnosis by PCR amplification (Table 1 and Fig. 2).

Figure 2 shows the results of amplification of routine samples 03B019, 03B039 and 03B134 with primer set Y, VC and VD. These three samples were tested positive for

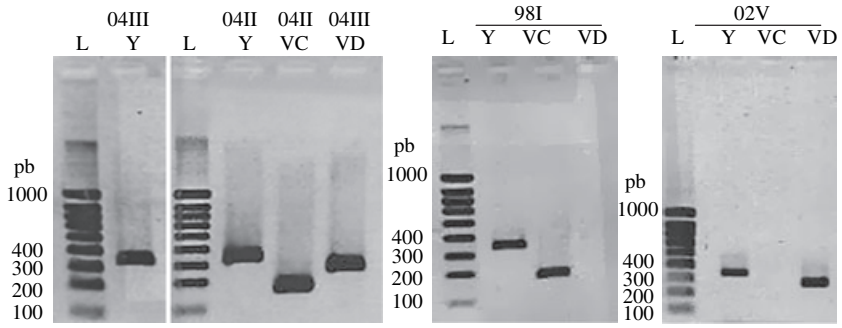

Fig. 1. Results of the PCR optimization: amplification of reference samples with primer set Y, VC and VD. L, ladder; 04II, type C; 04III, type D; 98I, type C; 02V, type D.

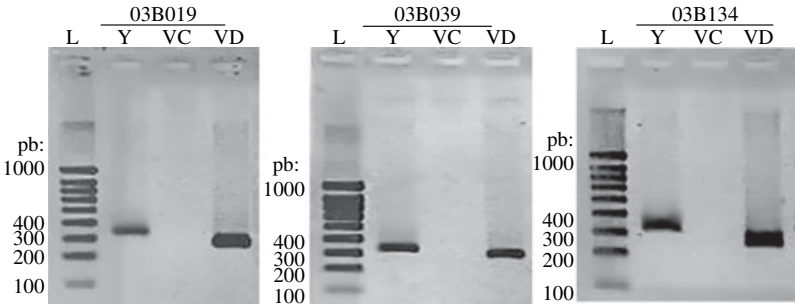

Fig. 2. Results of amplification of routine samples 03B019, 03B039 and 03B134 with primer set Y, VC and VD. L, ladder.

toxin production type D in the mouse bioassay, and pictures show that primers Y and VD amplify them. No pictures could be shown for sample amplification with primer set VC since we didn't dispose any natural positive bovine samples for *C. botulinum* type C.

Polymerase chain reaction testing confirmed the positive bioassay results of all 13 samples. Cross-reactivity between type C and D was not encountered. The three remaining samples that tested negative in the mouse bioassay were also found to be negative in PCR assay. Of the different culture methods used (Table 2), 5 days of enrichment in CMM at 37°C followed by overnight incubation in TYG at the same temperature proved to be the most appropriate. PCR performed on crude samples and other enrichment cultures were not reproducible. Only three and four positive results were confirmed in the PCR assay when crude samples or 16 h TGY enrichment were used, respectively. PCR results were also confirmed by sequencing.

Discussion

The prevention and rapid detection of bovine botulism is a worldwide problem. An epizooty can result in massive economic losses and public health could be at risk whenever contaminated meat or dairy products enter the food chain. In the search for rapid botulism detection methods, PCR has already proven its usefulness (Fach et al., 1993, 1995; Franciosa et al., 1994, 1996; Szabo et al., 1994a,b; Takeshi et al., 1996). However, as far as bovine samples are concerned, very little has been studied. In the studies performed by Fach et al. (1996) and Chaffer et al. (2006), PCR was used to optimize a diagnostic technique for bovine samples. Fach et al. (1996) employed a double PCR. They first amplified a fragment common to BoNT C1 and D, followed by a separate amplification based on two primers specifically for the latter toxin types. The primers were located in the N-terminal domain of the heavy chain, which is responsible for the translocation of the light chain into the cytosol. Enrichment of naturally contaminated animal and food samples was made in TYG for 48h. Fach et al. found a 89.4% correlation rate with the standard mouse bioassay and 4.3% false-negative results. In the study of Chaffer et al. (2006) the samples were enriched in fortified egg meat medium (FEM) for 5 days and their DNA was amplified with primers based on those of Takeshi et al. (1996). They were situated within the light chain of the neurotoxins (catalytic domain). For their bovine samples, they found 100% correlation between the bio assay and their PCR. However, only six bovine samples were incorporated in this study.

In contrast to the PCR methods described above, we set up a new approach to confirm the presence of the entire BONT/C or D genes. For this, three new pairs of primers were designed: a general set situated in the heavy chain (Y) and two specific sets of primers localized in the light chain of toxin type C (VC) and D (VD), respectively. The simultaneous detection of primer Y and primer VC or VD amplicons only endorse the presence of either two *C. botulinum* toxin genes, as both toxic activity (light chain) and binding/translocation (heavy chain) domain will be detected.

Bovine *C. botulinum* type C cases are rather rare in Belgium. They are more frequently encountered in countries that entail mixed bovine and avian husbandry like Australia and South Africa. PCR sensitivity and specificity for type BoNT/C was confirmed in our study using the reference strains; hence it is fair to assume that, under condition that recommended enrichments are made, bovine botulinum type C will be detectable. However the lack of natural positive bovine samples for *C. botulinum* type C did not allow us to confirm that bovine botulinum type C will be indeed detectable.

The primers designed and tested in this study proved to be highly specific and sensitive for the detection of BoNT/C and D genes. Results drew out of our PCR technique were concordant with those from the mouse bioassay and a 100% sensitivity level was reached. However, for this, samples required enrichment in CMM for 5 days followed by enrichment in TYG broth overnight. The application of the technique directly on crude samples and enrichment culture of just one night in TYG broth resulted in false-negative results. For these samples, our PCR hitherto remains inadequate. This could be due to a low concentration of target bacteria, cation concentration

or even the presence of large numbers of concomitant bacteria, proteases, chelators or other *Taq* polymerase inhibitory factors remaining in the crude sample (Thomas et al., 1991; Wilson et al., 1991). These factors are variable according to sample origin and conservation conditions. Therefore, sample treatment as well as culture method, depend on sample nature and conservation status (Fach et al., 1993).

Internal amplification controls should be routinely included in diagnostic PCR to control inhibition by the sample material. So far reports on their use in the molecular diagnostics of *C. botulinum* are limited: Fach et al. (2002) developed a synthetic internal control (IC) that was able to coamplify the *bont*/A, *bont*/B, *bont*/E, and *bont*/F genes in the same reaction. IC is a recombinant pMOS *Blue* plasmid DNA with CB1 and P261 primer-binding regions, flanking a DNA sequence of the chloramphenicol resistance gene (Cm^r) from Tn*9* (Fach et al., 2002).

In order to accelerate the rapidity of our PCR technique, it would be of interest to realize a reduction in or even an omission of sample enrichment time. In this light it would be necessary to find a technique that will eliminate all PCR inhibitors and impeding bacteria present in the sample. Different types of sample treatment (heat and/or ethanol) have shown to be useful for some samples but could not be extrapolated for others (F. Tweepenninckx, E. Van Nerom and V. Prévot, unpublished observations). We were unable to standardize sample treatment to be used systematically.

Unfortunately, until now no efficient selective media for isolation of *C. botulinum* are available (Fach et al., 2002). An alternative method to 'purify' samples has yet to be discovered. In this light, miscellaneous methods can be proposed: antibiotic treatment to eliminate contaminant bacteria (a mixture of cycloserine 1%, sulfomethoxazole 1.9% and trimethoprim 0.1% is already used in some cases; Dezfulian et al., 1981); development of a matrix capable of eliminating the *Taq* polymerase inhibitors directly from the samples or refining methods permitting the recovery of merely *C. botulinum* DNA after extraction.

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