Confirmation of botulism in birds and cattle by the mouse bioassay and Endopep-MS

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There have been several outbreaks of botulism among poultry and wild birds in Sweden in recent years. The National Veterinary Institute of Sweden (SVA) has identified botulinum neurotoxin (BoNT)/C1 or the mosaic BoNT/C1D using the mouse bioassay. This is believed to be the first report on the application of the Endopep mass spectrometry (Endopep-MS) method to selected clinical animal (serum and liver) samples and a feed sample that had previously given positive test results with the mouse bioassay. In the mouse bioassay eight of the eleven samples were found to be neutralized by both BoNT/C1 and /D antitoxins; the other three were neutralized only by BoNT/C1 antitoxin, but the mice showed a prolonged survival time when the samples had been treated with /D antitoxin. The Endopep-MS analysis, on the other hand, demonstrated only BoNT/C1 activity for all eleven samples. This suggests that at least eight of the samples were of the chimeric toxin type BoNT/C1D, where the enzymically active site is identical to that of BoNT/C1, while other parts of the protein contain sequences of BoNT/D. This is the first step of a cross-validation between the established mouse bioassay and the Endopep-MS of serotypes BoNT/C1 and /C1D. Endopep-MS is concluded to have potential as an attractive alternative to the mouse bioassay.

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INTRODUCTION

Botulinum neurotoxins (BoNTs) are the most potent toxins known (Schiavo *et al.*, 2000). They are produced under anaerobic conditions by some species of the genus *Clostridium*, particularly *C. botulinum*, *C. butyricum*, *C. baratii* and *C. argentinense*. BoNTs cause the disease botulism, which is manifested by flaccid paralysis (Johnson & Montecucco, 2008). This is a severe condition that may be fatal without proper treatment and aggressive supportive care (Schiavo *et al.*, 2000). There are at least seven serotypes of BoNT, which are denoted with the letters A to G. Human illness is usually caused by the types /A, /B, /E and /F, whereas /C and /D have been shown to affect mainly animals. Botulism can be acquired through the ingestion of food containing the toxin, or colonization

of the bacteria in either the gastrointestinal tract or a wound (Quinn *et al.*, 1994).

The BoNTs are proteins of about 150 kDa, consisting of one light chain of 50 kDa and one heavy chain of 100 kDa, linked together by a disulfide bridge (Wictome & Shone, 1998). The toxin is released from the bacteria as a non-covalent complex with other, non-toxic proteins. The non-toxic associated proteins are thought to enable systemic exposure of the toxin by promoting its absorption through the intestinal mucosal epithelium (Fujinaga *et al.*, 1997). However, animal experiments have demonstrated that pure BoNT/A can survive passage through the stomach and can be absorbed, although to a lower extent (Maksymowych *et al.*, 1999). The BoNTs are zinc-dependent proteases and their mechanism of action is based on cleavage of different proteins in the SNARE complex which are involved in acetylcholine release from motor neurons (Schiavo *et al.*, 2000). BoNT/A, /C1 and /E cleave at different sites of SNAP-25, and BoNT/B, /D, /G and /F are selective for VAMP-2. BoNT/C1 also has syntaxin A1 as an additional

Abbreviations: BoNT, botulinum neurotoxin; MALDI, matrix-assisted laser desorption ionization.

Supplementary figures showing mass spectra from all the samples analysed are available with the online version of this paper.

target. The light protein chain contains the enzymically active site, while the heavy chain is responsible for the binding and transport into the neuron. The unique target proteins and cleavage sites have been characterized for all seven toxin types (Johnson & Montecucco, 2008).

In the past 10 years, there have been many cases of botulism among both wild and farm animals. One large outbreak caused by BoNT/C1 occurred in Finland in 2002 among fur production animals such as foxes and mink (Lindström *et al.*, 2004). In Korea, at least five outbreaks have been described affecting wild birds in the period 2004–2008 (Shin *et al.*, 2010). Botulism has also afflicted the Swedish poultry population during this same time period. The first case was reported in 2003, and during 2008 outbreaks were confirmed in 13 different broiler farms (Blomqvist *et al.*, 2009; Skarin *et al.*, 2010). Furthermore, botulism was also reported in seabirds, primarily herring gulls, in the south-eastern part of Sweden in 2000–2004 (Neimanis *et al.*, 2007).

A real-time PCR assay was developed for the BoNT/C1 gene as an alternative to the mouse bioassay for detection and identification of *C. botulinum* serotype /C (Lindberg *et al.*, 2010). Interestingly, sequencing of the amplicons obtained from caecum samples from Swedish outbreaks gave data consistent with a chimeric /C1D sequence, earlier described in Japan (Takeda *et al.*, 2005).

It is required that the presence of toxin is demonstrated in serum of a suspected animal for confirmation of botulism (Quinn *et al.*, 1994). The reference method for the detection and identification of the active toxin is still the mouse bioassay, where mice are injected with the sample or an extract of the sample (Quinn *et al.*, 1994; Kautter & Solomon, 1977). The mouse bioassay is a time-consuming and expensive method requiring use of laboratory animals. The development of a comparable alternative analytical method for this purpose has not been straightforward, owing to the chemical nature of the BoNTs in combination with their extreme toxicity. Very high detection sensitivity and specificity, and the ability to exclusively measure the active toxin, are thus requirements for a new method to complement the mouse bioassay. A few alternative approaches have been described, such as activity-based methods using synthetic substrate peptides and ELISA detection of cleavage products (Evans *et al.*, 2004; Hallis *et al.*, 1996; Wictome *et al.*, 1999a, b). A peptidase activity method selective for BoNT/C1 with immunological detection has also been presented (Jones *et al.*, 2009). Furthermore, Evans *et al.* (2009) presented a new concept in which synaptosomes from rat brain were used for *in vitro* capture and activity measurement of BoNTs. Recently, a method for the detection of BoNT/B activity in foodstuffs and human serum using antibodies coupled to microchips was presented (Ferracci *et al.*, 2011).

In 2005, another concept was reported, based on BoNT cleavage of synthetic peptides followed by detection of the product peptides by matrix-assisted laser desorption

ionization (MALDI) and/or electrospray ionization (ESI) mass spectrometry (MS) (Barr *et al.*, 2005; Boyer *et al.*, 2005). The method, denoted Endopep-MS, was proven to be successful for detection of all seven BoNT serotypes in buffer solution, and the detection limits for BoNT/A, /B, /E and /F were lower than those obtained by the mouse bioassay. Further development of the Endopep-MS method, including an immunoaffinity purification step, enabled detection of BoNT/A, /B, /E and /F in human sera and stool samples (Kalb *et al.*, 2006). The Endopep-MS method has also been applied for the detection of BoNT/A in spiked milk samples (Kalb *et al.*, 2005). Recently, a paper on optimization of reaction conditions for BoNT/C1 and /DC1 in buffer solution was published (Moura *et al.*, 2011).

In the present study, selected clinical samples (serum and liver) and one feed sample, all of which tested positive for BoNT/C1 or /C1D using the mouse bioassay, were analysed by Endopep-MS. The purpose was for the first time to apply the Endopep-MS method to real samples of animal origin and thereby to take the initial step towards a cross-validation with the mouse bioassay.

METHODS

Materials. BoNT/A, /B, /C, /D, /E and /F complexes were obtained from Metabionics and were provided at 1 mg total protein ml⁻¹ in 50 mM sodium citrate buffer, pH 5.5. The toxin activities in mouse LD₅₀ (the dose which kills 50% of the mice) per mg protein obtained from the supplier were 3.6 × 10⁷ BoNT/A, 1.6 × 10⁷ BoNT/B, 4.6 × 10⁶ BoNT/C1, 2.4 × 10⁷ BoNT/D, 2.8 × 10⁷ BoNT/E and 5.5 × 10⁷ BoNT/F. Because BoNT is very toxic and requires appropriate safety measures all neurotoxins were handled within a class 2 biosafety cabinet equipped with HEPA filters. The antitype-specific BoNT polyclonal rabbit IgGs were supplied by Metabionics in 150 mM potassium phosphate (pH 7.4) at the supplier-indicated levels: anti-/A at 4.61 mg ml⁻¹, anti-/B at 5.46 mg ml⁻¹, anti-/E at 8.26 mg ml⁻¹ and anti-/F at 7.35 mg ml⁻¹. Custom-made peptide-based affinity-purified IgGs against specific linear sequences of BoNT/C1, /C1D, /D and /DC1 were obtained from GenScript. These IgGs have been used in a previous study (Moura *et al.*, 2008). Peptides were synthesized by Los Alamos National Laboratory, and the sequences of the substrates and the cleavage products were the same as the ones described by Boyer *et al.* (2005), with the addition of peptides 128 and 171 (see Table 1) for increased certainty in the detection of BoNT/C1. Dynabeads Protein G were purchased from Invitrogen at 1.3 g ml⁻¹ in PBS (pH 7.4) containing 0.1% Tween 20 (PBST) and 0.02% sodium azide. All other chemicals were from Sigma-Aldrich except where indicated otherwise.

For the mouse bioassay, antitoxins to BoNT/A, /B, /D and /E (National Institute for Biological Standards and Controls, UK) and to BoNT/C1 (developed from *C. botulinum* strain Stockholm by the Department of Bacteriology, National Veterinary Institute, Uppsala, Sweden) were used.

Samples. Blood was collected fresh in sterile tubes without anticoagulant from euthanized chickens having clinical signs of botulism. Samples from 10 animals per farm were centrifuged and pooled into one sample to obtain sufficient material to test by the mouse bioassay. The pelleted poultry feed sample was taken from the 'feeding machine' near a dead bird in a flock with a confirmed outbreak of botulism. Liver samples (20 g) were taken from two

Table 1. Peptide substrates and cleavage products for BoNT/C1 and /D

Peptide no.	Description	Sequence	m/z
4	BoNT/C1 substrate	Biotin-KGSNRTRIDEANQRATRLGGK-biotin	2911.5
	BoNT/C1 NT product	Biotin-KGSNRTRIDEANQR	1870.9
	BoNT/C1 CT product	ATRLGGK-biotin	1059.5
	BoNT/C1 internal standard	Biotin-KGSNRTRIDE (A + 7*) NQR	1877.9
	BoNT/C1 internal standard	(A + 7*) TRMLGGK-biotin	1066.5
128	BoNT/C1 substrate	YVERAVSDSKKAVKYQSKARRKK-perfluoro†	3099.5
	BoNT/C1 NT product	YVERAVSDSKK	1281.7
	BoNT/C1 CT product	AVKYQSKARRKK-perfluoro†	1836.9
171	BoNT/C1 substrate	Biotin-KGSNRTRIDEANQRATRLGGK-PEG-amine‡	3151.6
	BoNT/C1 NT product	Biotin-KGSNRTRIDEANQR	1870.9
	BoNT/C1 CT product	ATRLGGK-PEG-amine‡	1299.8
29	BoNT/D substrate	AQVDEVVDIMRVNVDKVLERDQKLSELDADRADALQAGAS	4312.16
	BoNT/D NT product	AQVDEVVDIMRVNVDKVLERDQK	2698.4
	BoNT/D CT product	LSELDADRADALQAGAS	1631.8

*Isotopically labelled on C and N in 7 positions.

†Attached to Lys ϵ -NH-CO-CH₂-CH₂-(CF₂)₅-CF₃.

‡Attached to Lys ϵ -NH-CO-CH₂-O-CH₂-CO-CH₂-NH-CH₂-CH₂-(O-CH₂-CH₂)₆-O-CH₂-CH₂-NH₂.

euthanized wild birds (one jackdaw and one mallard) and one cow at autopsy, all with clinical signs of botulism. The study included sera from six poultry outbreaks, liver or serum samples from three wild birds, liver from one cow and one feed sample. The samples are listed in Table 2.

The toxin was eluted from 20 g of liver and feed samples using 0.01 M phosphate buffer (sample:buffer 1:1, w/w). The sample was homogenized with a scalpel in the phosphate buffer. Thereafter the sample was centrifuged with cooling (+5 °C) at 4000 r.p.m. for 1 h. The supernatant (the extract of the sample) was used for injection. Sera were used without pretreatment. All samples were mixed with 0.1 M PBST (sample:PBST 9:1, v/v) prior to toxin extraction for Endopep-MS. Positive control samples were prepared by spiking reference toxin complexes (BoNT/A, /B, /C1, /D, /E and /F) into normal chicken serum and liver. Negative controls consisting of pure PBST, serum and chicken liver were also included in the analysis.

Mouse bioassay. Pooled sera, liver extract from affected animals and extract from the feed were tested for *C. botulinum* toxins /A to /E by the mouse bioassay performed according to the Nordic Committee on Food Analysis (NMKL, 1991). Mice weighing approximately 20 g were used. For each test, two mice were injected intraperitoneally (i.p.) with 0.5 ml of serum or liver or feed extract and observed for up to 4 days for clinical signs of botulism. Mice were euthanized if signs of botulism developed. Controls consisting of 0.5 ml boiled (heated to 100 °C for 10 min) serum or extract (from liver or feed) were each injected into two mice to determine whether the toxic agent was thermolabile.

The sera or extract were diluted 1:5, 1:10 and 1:100 (in 0.9% NaCl) to determine the lowest lethal dilution of the toxin, and 0.5 ml of each dilution was injected i.p. into two mice each.

Identification was done by neutralization with specific antibodies to BoNT/A, /B, /C1, /D and /E. The mixture with 0.1 ml antitoxin

Table 2. Sample information and detection of BoNT by mouse bioassay and Endopep-MS

Sample no. and sampling place	Lab ID	Date	Type	Specimen	Dilution factor	Neutralized with <i>C. botulinum</i> antitoxin by mouse bioassay	Result Endopep-MS (activity)
1. Farm A	AN2095/04	June 2004	Broiler	Sera	1/10	/C and /D	/C
2. Farm A	AN 2545/04	July 2004	Jackdaw	Liver	1/10	/C and /D	/C
3. Farm A	AN 3327/04	November 2004	Broiler	Sera	1/10	/C	/C
4. Farm A	AN 799/05	2005	Broiler	Sera	1/10	/C	/C
5. Farm B	AN 2897/04	2004	Broiler	Sera	Not diluted	/C	/C
6. Farm B	AN 3156/04	2004		Feed (pellets)	Not diluted	/C and /D	/C
7. Farm C	AN 2138/04	2004	Broiler	Sera	1/10	/C and /D	/C
8. Farm D	AN 1295/06	2006	Broiler	Sera	1/10	/C and /D	/C
9. Farm E	AN 317/06	2006	Swedish black and white cow	Liver	1/10	/C and /D	/C
10. Park pond	AN 1927/04	2004	Mallard	Liver	Not diluted	/C and /D	/C
11. Park pond	AN 2339/05	2005	Mallard	Sera	1/100	/C and /D	/C

solution and 1 ml toxic serum or extract of the lowest lethal dilution was allowed to stand at room temperature for 1 h before a 0.5 ml injection i.p. Two mice were used for each mixture. Simultaneously, sera or extract without antitoxin were injected into two other mice. Mice injected with a mixture of the homologous antitoxin and the toxic material survived and mice injected with mixtures of heterologous antitoxins and the toxic material developed clinical signs of botulism. The testing was approved by the Swedish Ethical Committee on Animal Experiments.

IgG binding to Protein G beads for the Endopep-MS method.

The specific IgGs were immobilized and cross-linked to the Dynabeads Protein G according to the supplier's protocol and as described before (Kalb *et al.*, 2006). Briefly, 50–200 µg of IgG (anti-BoNT/A, /B, /E and /F separately and anti-/C1, /C1D, /DC1 and /D in a mixture) was diluted into 500 µl PBS for every 100 µl of Dynabeads Protein G. Cross-linked IgG-coated Dynabeads were stored in PBST at 4 °C for up to 4 weeks.

BoNT extraction and Endopep-MS. Samples were first incubated for 1 h with 20 µl of anti-BoNT/C1, /CD, /D, /DC antibody-coated magnetic beads. The samples were then incubated separately with anti-BoNT/A, /B, /E and /F antibody-coated magnetic beads. After mixing for 1 h with constant agitation at room temperature, the respective beads were washed twice in 1 ml each of PBST and then washed once in 100 µl water. The Endopep-MS reaction was performed as previously described (Barr *et al.*, 2005; Boyer *et al.*, 2005; Kalb *et al.*, 2006) with the modifications described below. Beads were reconstituted in a 20 µl solution containing 0.02 M HEPES (pH 7.3), 10 mM dithiothreitol, 0.2 mM ZnCl₂, 1 mg BSA ml⁻¹ and 50 pmol of the substrate peptide µl⁻¹ [separate reactions for BoNT/A, /B, /E and /F substrates respectively, and a mixed reaction with three peptides (#4, #128 and #171) for /C1 and one peptide (#29) for /D]. The target peptide sequences and the cleavage products for BoNT/A, /B, /E and /F are the same as those described by Boyer *et al.* (2005). Table 1 lists the peptide sequences used to detect and differentiate BoNT/C1 and /D serotypes along with the specific cleavage products, including stable-isotope peptides used as internal standards and their masses. A mixture of three substrate peptides was used in the reaction for testing BoNT/C1 (#4 and #171 derived from the protein SNAP-25 and #128 derived from Syntaxin) and one peptide was used for BoNT/D (#29 derived from VAMP-2). All samples then were incubated at 37 °C for 4–16 h with no agitation.

MALDI-TOF MS detection. From each reaction supernatant, 2.0 µl was added to 18 µl MALDI matrix solution consisting of α -cyano-4-hydroxycinnamic acid (CHCA) at 5 mg ml⁻¹ in 50% acetonitrile, 0.1% trifluoroacetic acid and 1 mM ammonium phosphate. Aliquots of 0.8 µl of this mixture were pipetted onto each spot of a 384-spot MALDI plate (AB Sciex). Mass spectra of each spot were obtained by scanning from 1000 to 4800 *m/z* in MS-positive ion reflectron mode on an Applied Biosystems 4800 Proteomics Analyser (AB Sciex). The instrument uses an nd:YAG laser at 337 nm and 200 MHz repetition rate. Pulsed ion extraction was set to zero. Acceleration voltage was set to 20 kV, and each spectrum was an average of 2400 laser shots.

RESULTS

The results from the mouse bioassay of the 11 samples tested are given in Table 2. Eight samples were found to be neutralized by both BoNT/C1 and /D antitoxins, whereas three samples were neutralized only by BoNT/C1 antitoxin, although there was a delayed toxic reaction for samples treated with the /D antitoxin, i.e. onset of symptoms after about 2 days compared to a few hours for the other

subtypes. The dilution factor is an indirect measure of the concentration of the active toxin in the sample, as it denotes the volume factor by which the samples could be diluted and still give a positive result (for the dilution procedure, see Methods). Most of the samples had a maximal lethal dilution of 1/10. However, samples 5 and 6 from farm B as well as sample 10 from a park pond seemed to be less concentrated as they only showed toxicity in their undiluted form. Sample 11 appeared to be the most toxic, as a 1/100 dilution could still give rise to botulism in the mice.

The clinical samples together with positive and negative controls were also analysed using the Endopep-MS method. All the clinical samples (Table 2) gave positive results for BoNT/C1 activity, based on the detection of the peptide #4 cleavage products at *m/z* 1059.5 and *m/z* 1870.9, the peptide #128 cleavage products at *m/z* 1281.7 and *m/z* 1836.9, and the peptide #171 cleavage products at *m/z* 1299.8 and *m/z* 1870.9. The latter one is isobaric with one of the fragments of peptide #4. The mass spectrum of sample 8 is shown as an example in Fig. 1(a). There was no evidence for activity of any of the other toxin serotypes above the respective limits of detection (results not shown). All the positive controls consisting of avian serum and liver tissue spiked with BoNT/A to /F turned out to be positive in terms of the presence of the anticipated peptide cleavage products (data not shown). The result of a typical positive control sample containing BoNT/C1 at 5 LD₅₀/500 µl in serum is shown in Fig. 1(b). The matrix-based blanks containing blank serum and chicken liver did not show any specific cleavage of the substrate peptides; a typical mass spectrum from a blank serum sample is shown in Fig. 1(c). Mass spectra from all the samples described in Table 2 are available as supplementary figures with the online version of this paper.

The jackdaw (sample 2, Table 2) was found dead on farm A, which had ongoing botulism among its poultry. It is thus apparent that both wild and domestic birds can be affected during an outbreak. Even though farm A is geographically separated from farms B and C, it cannot be ruled out that the outbreaks emanated from a common source, as the cases occurred within a quite narrow time-frame. The cow (sample 9) had been fed with silage. However, it was never proven that this was the source of the toxin.

DISCUSSION

There have been many outbreaks of botulism among wild birds and poultry during recent years (Lindström *et al.*, 2004; Shin *et al.*, 2010). The method widely used for detection has been the mouse bioassay. Although this method is sensitive and detects active toxin, it suffers from major disadvantages such as a time scale of at least 4 days, the need for relatively large sample volumes, and a large consumption of laboratory animals (Cai *et al.*, 2007). Thus there has been a great need for a faster, equally sensitive method which is not based on animal use.

Fig. 1. BoNT/C1 proteolytic activity detected by MALDI-TOF MS: representative Endopep-MS spectra in avian sera after extraction with antibody-coated magnetic beads. Peptide-based antibodies were prepared against BoNT/C1, /C1D, /D and /DC1. Three substrate peptides for BoNT/C1 (m/z 2911.4, 3099.5 and 3151.6), and one substrate peptide for BoNT/D (m/z 4312.6), were included in the reactions. Typical spectra depict the expected peptide products and respective isotope-labelled standards (ISTD) in the positive reactions with a typical positive serum sample (a), and a positive control consisting of serum spiked with BoNT/C1 at 5 mouse $LD_{50}/500\ \mu l$ (b). BoNT/C1 hydrolyses the synthetic peptide substrate #4 (m/z 2911.4) to yield peptide products at m/z 1870.9 (NT) and 1059.5 (CT). ISTD peaks are at m/z 1877.9 and 1066.5 respectively. The substrate peptide #128 yielded products at m/z 1281.7 (NT) and 1836.9 (CT), whereas substrate peptide #171 yielded products at m/z 1870.9 (NT – isobaric with the NT product of substrate #4) and 1299.8 (CT). No cleavage product was detected for BoNT/D. In (c) only intact substrate peptides are detected in the absence of toxin.

In this study, the Endopep-MS method has for the first time been applied to detect BoNT activity in selected clinical samples of animal origin that previously tested positive with the mouse bioassay. This is the beginning of cross-validation studies between the two methods. The previously tested substrate peptide (#4, derived from

SNAP-25, Table 1; Boyer *et al.*, 2005; Moura *et al.*, 2011) for detection of BoNT/C1 in buffer solutions sometimes gave unspecific cleavage, thus creating false positives. In order to overcome this problem, two additional substrates that proved to be more stable were included in this study (#128 derived from syntaxin and #171 derived from

SNAP-25, Table 1). However, the sensitivity for BoNT/C1 was slightly lower using the latter two peptides separately, and a mixture of all three substrates was used to improve the consistency of the data.

It is interesting to note that all the samples reacted as being BoNT/C1 in the Endopep-MS method, whereas the majority of them had been neutralized by antibodies against both BoNT/C1 and /D in the mouse bioassay. A probable explanation for this phenomenon is the occurrence of the above-mentioned BoNT/C1D mosaic, where the sequence of the light protein chain of the toxin is similar to that of BoNT/C1 while the heavy chain has sequences similar to both /C1 and /D (Takeda *et al.*, 2005). As the enzymically active site resides within the light chain, the BoNT/C1D mosaic is expected to cleave the substrate peptide identically to BoNT/C1 and thus gives a pure /C1 response in the Endopep-MS assay. Using specific antibodies to BoNT/C1 and /D heavy chains in combination with the specific enzymic activity of these two toxins should permit the differentiation of BoNT/C1 from the /C1D mosaic. We are currently preparing antibodies that should aid the differentiation of BoNT/C1 from the /C1D mosaic along with BoNT/D from the /DC1 mosaic. In a previous study, intestinal contents from broilers from the same farm as the ones giving rise to the serum samples 1 and 8 were also analysed by PCR and concomitant sequencing of the amplicons (Lindberg *et al.*, 2010). The results were consistent with the presence of the BoNT/C1D mosaic gene.

There are discrepancies in the qualitative mouse test results between samples from the same farm (A and B) taken at different time points (Table 2). For farm A, samples 1 and 2 gave /C1 and /D in the mouse bioassay, whereas samples 3 and 4 gave only /C1 as the result. Furthermore, for farm B, sample 6 gave the result /C1 and /D and sample 5 gave only /C1. However, all the samples that qualitatively had pure /C1 based on mouse test results in this study also gave a delayed toxic reaction after neutralization with antitoxin /D, i.e. onset of clinical signs of botulism after 2 days compared to the usual few hours. This observation suggests that antitoxin /D had some degree of affinity to the toxin serotype present in these samples (3, 4 and 5). Thus, the seeming paradox of qualitatively different serotypes in samples from the same outbreak may be explained by a quantitative factor such as the toxin level in relation to the neutralizing efficiency of the anti-/D antibodies. The corresponding phenomenon of a partial effect of antitoxin /C on the BoNT/DC1 mosaic was described by others (Nakamura *et al.*, 2010).

In conclusion, this study is the initial step of a cross-validation between the Endopep-MS method and the mouse bioassay and has, to the best of our knowledge, for the first time demonstrated that an enzymic-mass spectrometric technique can detect BoNT/C1 activity in clinical samples of animal origin. The use of Endopep-MS has given evidence that the enzymically active sites and

thereby the light protein chains of all the samples tested were of /C1 serotype, whereas the mouse bioassay has given important information on the influence of BoNT/D structures, probably in the heavy chain as previously described for the BoNT/C1D mosaic (Takeda *et al.*, 2005). Thus, in this study the Endopep-MS alone would not have been able to differentiate between BoNT/C1 and /C1D, whereas the mouse bioassay has been unable to tell BoNT/C1D and /DC1 apart. However, further development of the antibody capture technique in the Endopep-MS method may increase the degree of selectivity for this technique against different toxin mosaics. Thus, Endopep-MS is anticipated to be an attractive alternative to the mouse bioassay.

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