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## Validation of the Endopep-MS method for qualitative detection of active botulinum neurotoxins in human and chicken serum

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#### Abstract

Botulinum neurotoxins (BoNTs) are highly toxic proteases produced by anaerobic bacteria. Traditionally, a mouse bioassay (MBA) has been used for detection of BoNTs, but for a long time, laboratories have worked with alternative methods for their detection. One of the most promising *in vitro* methods is a combination of an enzymatic and mass spectrometric assay called Endopep-MS. However, no comprehensive validation of the method has been presented.

The main purpose of this work was to perform an in-house validation for the qualitative analysis of BoNT-A, B, C, C/D, D, D/C, E, and F in serum. The limit of detection (LOD), selectivity, precision, stability in matrix and solution, and correlation with the MBA were evaluated. The LOD was equal to or even better than that of the MBA for BoNT-A, B, D/C, E, and F. Furthermore, Endopep-MS was for the first time successfully used to differentiate between BoNT-C, D and their mosaics C/D and D/C by different combinations of antibodies and target peptides. In addition, sequential antibody capture was presented as a new way to multiplex the method when only a small sample volume is available. In the comparison with the MBA, all the samples analyzed were positive for BoNT-C/D with both methods. These results indicate that the Endopep-MS method is a good alternative to the MBA as the gold standard for BoNT detection based on its sensitivity, selectivity, speed, and that it does not require experimental animals.

#### INTRODUCTION

Botulism is a serious disease that affects both humans and animals. It is rare in humans but thousands of animals are afflicted each year [1]. The symptoms of botulism are characterized by a descending flaccid paralysis. This will, if left untreated, eventually affect the respiratory system and cause death [2]. The paralysis is an effect of inhibition of acetyl

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choline release into synaptic clefts, effectively blocking muscle contractions [3]. The inhibition is caused by botulinum neurotoxins (BoNTs) produced by the bacteria *Clostridium botulinum* group I–IV, *C. baratii* and *C. butyricum* [4]. BoNTs are some of the most toxic substances known. The toxins have an approximate molecular weight of 150 kDa. They are comprised of a light (50 kDa) and a heavy (100 kDa) chain, where the heavy chain is responsible for cell internalization and the light chain carries the proteolytic activity [5,6]. BoNTs exhibit toxicity by, very specifically, cleaving at least one of the 3 proteins (i.e. SNAP-25, VAMP-2, and syntaxin) involved in the SNARE complex in motor neurons [7–12].

So far 7 confirmed serotypes of BoNTs, designated A–G, have been described [13]. Different species are not equally susceptible to all the serotypes, e.g. BoNT-A, B, E, and F mainly affect humans [2], BoNT-C and C/D frequently intoxicate birds [14,15], whereas BoNT-D and D/C are most common in cattle [14].

The classical form of human botulism emanates from the intake of preformed toxin from improperly preserved food [16]. However, as the food preservation techniques have improved, the most frequent type of this illness in humans today is infant botulism where bacteria colonize the gut and produce toxin which is absorbed and distributed systemically [17]. In animals, however, the predominant route of infection is through feed, containing preformed toxin [18] An exception is represented by broiler chickens where toxin producing bacteria have been identified in the intestines [15]. Apart from natural contamination, intoxication can occur through a hostile deliberate act. BoNTs are recognized as potential biological weapons and terrorist attacks using BoNTs have occurred [19]. To use such an agent on food producing animals, in so called agro terrorism, may result in high costs and spread fear in the population [20].

Several of the BoNT serotypes also have subtypes, e.g. BoNT-A1-5 [21], B1–7 [22], E1–9 [23], F1–7 [24]. For BoNT-C and D, the mosaic forms BoNT-C/D and D/C have been described [25]. In BoNT-C/D the light chain share 97.6% amino acid sequence identity with the light chain of BoNT-C and the amino acid sequence of the heavy chain is 95.3 % identical to that of BoNT-D. Comparison of the BoNT-D/C light chain with BoNT-D light chain and BoNT-D/C heavy chain with the BoNT-C heavy chain reveals amino acid sequence similarities of 98.3% and 92.0%, respectively [26]. This means that BoNT-C/D and C share the same target for their proteolytic action, as do D/C and D [25]. In most cases all subtypes within a serotype exhibit the same proteolytic cleavage, apart from the only known exception which is BoNT-F5 [27].

In order to confirm a botulism diagnosis, the presence of BoNT in bodily fluids must be established [16,28]. Owing to the extreme potency of the toxin, a very sensitive detection method is required. The historic standard for this purpose is the mouse bioassay (MBA), where a sample is injected into mice which are observed for symptoms of botulism [29]. The method is both ethically challenged and time consuming, and many laboratories seek to replace it.

Polymerase chain reaction (PCR)-based methods [30,15,31,32] can identify the presence of the BoNT encoding genes. They do, however, suffer from the drawback that they do not actually prove that the toxin has been expressed [29]. An alternative PCR using a DNA fragment coupled to sialyllactose with binding affinity to BoNT-B was recently presented by Kwon et al. [33]. This method could demonstrate the presence of BoNT-B, but not its enzymatic acitivity. The use of enzyme-linked immunosorbent assay (ELISA) for analysis of BoNTs has several advantages, e.g. great sensitivity, speed and ease of use [29,34]. However, cross reactivity may lead to false positives and/or false negatives and it may also detect inactive BoNT [29,34].

An approach termed endopeptidase assay, described by Hallis et al. [35], utilized the specific proteolytic characteristics of the BoNTs. In this method, the samples were incubated together with synthetic peptide substrates mimicking the natural BoNT target proteins. The resulting cleavage products were then detected by ELISA. Moreover, a gold nano particle-based assay employing colorimetric detection of the light chain of BoNT-A was recently described by Liu et al. [36]. This approach also utilized synthetic peptides as substrates for the toxin but they were not a part of the detection step.

Mass spectrometric analysis of BoNTs has recently been reviewed [37]. This technique in conjunction with the endopeptidase approach, termed Endopep-MS, has proven to be a powerful tool for the analysis of active BoNTs, both in terms of selectivity and sensitivity [37]. The selectivity of the Endopep-MS is achieved on two levels, 1) by using serotype specific antibodies directed towards the BoNTs, and 2) by using a specific substrate for each serotype. The sensitivity has been proven to be as good as, or better, than that of the MBA [38,39]. Furthermore, Endopep-MS detects active toxin down to serotype level. Also, it has been demonstrated that the subtype can be determined by a subsequent proteomics approach applied on the antibody captured toxin after the Endopep-MS for the detection of BoNTs in different matrices [42,38]. However, a comprehensive description of a validation of the method is lacking. The main aim of this study was to in-house validate the Endopep-MS method for the qualitative analysis of BoNTs A, B, C, C/D, D, D/C, E, and F in serum.

The main focus was on BoNT-C, D and their mosaics since the method then was applied on avian serum samples. To the best of the authors' knowledge, this is the first comprehensive validation of the Endopep-MS assay including serotypes A–F, encompassing limit of detection (LOD), selectivity, precision, stability, and correlation with the MBA. Thus, this paper provides the necessary basis for the replacement of the mouse bioassay. We also present the first application of Endopep-MS together with antibody capture of BoNTs to differentiate BoNT-C from the mosaic BoNT-C/D and BoNT-D from the mosaic BoNT-D/C. In addition, we examined a novel way to multiplex the Endopep-MS method in cases where only a small sample volume is available.

#### MATERIAL AND METHODS

#### Chemicals and reagents

Botulinum neurotoxins A, B, C, D/C, E, and F were purchased as complexes from Metabiologics (Madison, WI, USA). The  $MLD_{50}$ /mg of the BoNTs were  $3.5 \times 10^7$ ,  $9.0 \times 10^6$ ,  $6.0 \times 10^6$ ,  $3.0 \times 10^7$ ,  $3.0 \times 10^7$ ,  $5.0 \times 10^6$  for BoNT-A, B, C, D/C, E, and F respectively, as determined by the MBA. Recombinant BoNT-D and C/D (purified, not as complexes) produced in *E. coli* were purchased from Toxogen GmbH (Hannover, Germany). These toxins were not tested with the MBA.

Monoclonal antibodies targeting the different BoNTs (Table 1) were purchased from Dr. James Marks at the University of California San Francisco (San Francisco, CA, USA) as 1 mg/mL solutions in different buffers (e.g. mixtures of glycine, tris, and phosphate buffered saline). Magnetic beads of the type Dynabeads M-280 Streptavidin were acquired from Life Technologies (Stockholm, Sweden) and EZ-link sulfo-NHS-biotin, No-Weigh<sup>TM</sup> format was purchased from Thermo Scientific (Waltham, MA, USA). Hepes buffered saline solution with EDTA and surfactant P20 pH 7.4 (HBS-EP) was purchased from GE Healthcare Europe (Uppsala, Sweden). Human and chicken serum, phosphate buffered saline (PBS), dithiotreitol (DTT), bovine serum albumin (BSA), zinc chloride (ZnCl<sub>2</sub>),  $\alpha$ -cyano-4-hydroxy cinnamic acid (CHCA), ammonium citrate, tri fluoro acetic acid (TFA), and Tween<sup>®</sup>20 were purchased from Sigma-Aldrich (St. Louis, MO, USA). All peptides in Table 2 were custom synthesized by Xaia Custom Peptides (Gothenburg, Sweden). The water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA) and all other chemicals used were of analytical grade or better and used without further purification.

Due to the high toxicity of BoNTs, the toxins were handled within a class 2 biosafety cabinet equipped with HEPA filters.

#### Endopep-MS

**Coupling of antibodies to magnetic beads**—A buffer exchange was performed on the antibody solutions by the use of Sephadex columns (illustra<sup>TM</sup> NAP<sup>TM</sup>-5 Sephadex<sup>TM</sup> G-25 DNA Grade, GE Healthcare Life Science, Uppsala, Sweden) in accordance with the instructions from the manufacturer, resulting in 0.5 mg/mL solutions in HBS-EP buffer pH 7.4. The antibodies were then biotinylated by adding 1.0  $\mu$ L of 1 mM sulfo-NHS-biotin (aq) solution for every 5.0  $\mu$ g of antibody. The reaction was carried out over night at room temperature. The magnetic beads (250  $\mu$ L per 5  $\mu$ g of antibody) were first washed twice with HBS-EP buffer and then 6.0  $\mu$ L of the antibody-biotin solution was added together with 1 mL of HBS-EP. The mixture was incubated for 1 h at room temperature, fixed to a rotator (speed 30 rpm). Afterwards the beads were washed twice with 1 mL of HBS-EP buffer and then suspended in 250  $\mu$ L of HBS-EP buffer.

**Extraction of toxin from serum samples**—The extraction of BoNTs with antibodies on magnetic beads was performed on a KingFisher Flex (Thermo Scientific, Waltham, MA, USA). Twenty  $\mu$ L of vortexed antibody-coupled bead suspension were used to extract toxin

from a 500  $\mu$ L serum sample, and 100  $\mu$ L of PBST (0.1 M PBS in water with 0.2% Tween<sup>®</sup>20) was added to prevent aggregation of the magnetic beads during the procedure. The volume of sample used corresponds to that normally used in the MBA. The binding of the toxin was carried out by mixing the beads and the serum sample for 1 h. Thereafter, beads were removed from the serum sample and washed twice with 1 mL HBS-EP buffer, once with 150  $\mu$ L of water and finally released into 150  $\mu$ L of water.

**The endopep reaction**—The 150 µL of bead suspension in water was transferred to 0.2 mL 24-well PCR plates (Thermo Fisher Scientific, Gothenburg, Sweden) and placed on a DynaMag<sup>TM</sup>-96 Side magnetic stand (Life Technologies AS, Oslo, Norway). The water was removed and 20.0 µL of reaction buffer (10 mM DTT, 200 µM ZnCl<sub>2</sub>, 1.0 mg/mL BSA, and 50 µM substrate peptide in 20 mM HEPES buffer pH 7.3) was added. Since the first Endopep-MS article published by Boyer et al. [43], the substrate peptides used for the different BoNT serotypes have been continuously improved [44–48,39,38]. The different substrate peptides that were used in this study are listed in Table 2. The reaction buffer and bead mixture was incubated in an Arktik PCR thermo cycler (Thermo Fisher Scientific, Gothenburg, Sweden) at 42°C for BoNT-C, C/D, D/C, and D and at 37°C for BoNT-A, B, E, and F. The higher incubation temperature for BoNT-C, D and their mosaics was optimized by Moura et al. [26] and might reflect the fact that birds have a higher body temperature than humans [49]. A 2.0 µL aliquot was taken out at 4 and 24 h, to be used for mass spectrometric analysis.

**MALDI-Q-TOF MS**—The 2.0 µL reaction buffer aliquot was mixed with 18 µL of MALDI matrix (5 mg CHCA/mL in MilliQ/Acetonitrile/10% TFA (aq)/1 M ammonium citrate (aq) 98/98/2/2 v/v/v/v) containing 0.1 nmol IS (see Table 2). Each sample was spotted onto a MALDI target plate 3 times and 1.0 µL was used for each spot. The mass spectrometric analysis was performed on a Synapt G2 instrument (Waters Corporation, MA, USA) with a MALDI interface operated in positive potential and MS resolution mode. The mass range was set to m/z 100–8000, the laser firing rate was 1000 Hz, the acquisition time 30 s, the scan time 1 s and the laser was moving in a spiral pattern to get a good representation of the spot. Every spectrum was a result of 1000 laser shots. Thirty spectra were combined to get one spectrum representing the whole spot. The combined spectrum was processed by the automatic peak detection and background subtraction option in the software (MassLynx V4.1). The instrument was calibrated with red phosphorus in the range m/z 100–8000 about once a month.

#### Validation parameters and procedures

**Limit of Detection (LOD)**—The intensities of the peaks representing the C terminal (CTP) and N terminal (NTP) cleavage products were divided by the intensity of the internal standard (IS) in the same spectrum (see Table 2 for information on the peptide sequences and m/z). The mean value of the CTP/IS and NTP/IS ratios for the three spots of each sample were calculated and divided by the same mean ratio of the blank samples from the same sequence of analysis, called "x blank" throughout this paper. The LOD was defined as the BoNT concentration in MLD<sub>50</sub>/500 µL resulting in a x blank value of 3, for at least one of the cleavage products.

**Selectivity**—BoNT-A, B, C, D/C, E, and F were incubated in reaction buffer containing either C or D substrate to evaluate the selectivity of the different BoNT serotypes in terms of proteolytic cleavage. The samples were incubated in triplicates for 4 and 24 h. The amount of toxin per 500  $\mu$ L sample were 10 MLD<sub>50</sub> for A, B, D/C, and F, 25 MLD<sub>50</sub> for E, and 250 MLD<sub>50</sub> for C.

The selectivity of the antibodies (RAZ1, B12.2, 8DC1.2, 4E17.1, and 6F5.1) was evaluated by analysis of blank chicken or human serum spiked with BoNT-A, B, C, D/C, E, and F in triplicates (10 MLD<sub>50</sub>/500  $\mu$ L of A, B, D/C and F, 25 MLD<sub>50</sub>/500  $\mu$ L for E, and 250 MLD<sub>50</sub>/500  $\mu$ L for C). The samples were incubated with all the different antibodies, individually, for 4 h.

The affinity for binding C, D, C/D, and D/C, respectively, was evaluated for five different antibodies; 8DC1.2, 4C2, 4C2.2, 4C10.2, and 1C1.1 to determine if it would be possible to differentiate C from C/D and D from D/C. The concentrations were 250 and 10 MLD<sub>50</sub>/500  $\mu$ L for C and D/C, respectively, and 33 and 0.9 ng/500  $\mu$ L of C/D and D, respectively. Three chicken serum samples were analyzed for each toxin and antibody.

**Precision**—The intra-day precision was investigated for BoNT-A, B, E and F in human serum and for BoNT-C, D, D/C, and C/D in chicken serum. Blank serum was spiked at three different toxin levels (n=6 for each level) and six replicates of blank serum were analyzed in the same run. The concentrations were 0.5, 1 and 10 MLD<sub>50</sub>/500  $\mu$ L for BoNT-A, B, and F; 5, 10 and 20 MLD<sub>50</sub>/500  $\mu$ L for BoNT-E; 50, 250 and 500 MLD<sub>50</sub> (i.e. 8, 42 and 83 ng) for BoNT-C; 1, 10, and 20 MLD<sub>50</sub> (i.e. 0.03, 0.3 and 0.7 ng) for BoNT-D/C; 23, 33 and 49 ng for BoNT-C/D; and 0.1, 0.9 and 1.8 ng for BoNT-D. For BoNT-C and D/C the experiment was repeated on three different days to evaluate the inter-day precision. For calculation of the intra- and inter-day precisions the relative standard deviations (RSD%) of the ratios between the relative intensities of the cleavage products and the IS were used. The antibody used for toxin capture in these experiments were RAZ1, B12.2, 8DC1.2, 4E17.1, and 6F5.1.

**Stability**—BoNT standards were stored as 10  $\mu$ L aliquots in PBST in protein LoBind eppendorf tubes (VWR International, Stockholm, Sweden) at -80°C. The freeze-thaw stability in buffer solution was evaluated for BoNT-C and D/C by comparing freshly prepared samples where the toxin had been spiked directly into the reaction buffer with samples that had been thawed 3 and 5 times (two hours at room temperature and a refreezing time between the thawings of at least 24 h) during a total period of 2 weeks. From each thaw cycle, six replicates were analyzed and the results were compared with those of the freshly prepared samples. The toxin amount/sample used for all the replicates were 250 and 10 MLD<sub>50</sub> for BoNT-C and D/C, respectively. All replicates from the freeze-thaw cycles were, together with blank samples, incubated at the same time and the mass spectrometric analyses of the samples were performed in the same batch.

The freeze-thaw stability of BoNT C and D/C in chicken serum was evaluated at the intended storage temperature  $-20^{\circ}$ C, which is the standard storage temperature used for the MBA. Samples (3.5 mL) were spiked (250 and 10 MLD<sub>50</sub>/500 µL for BoNT-C and D/C, respectively) and underwent 1, 3 or 5 freeze thaw cycles of 2 h in room temperature and a

refreezing time between the thawings of at least 24 h during a total period of two weeks before analysis. From each thaw cycle, six replicates were analyzed. All replicates from the thaw cycles were, together with blank chicken serum, subjected to the full workflow of the Endopep-MS method at the same occasion. The results of the samples that had gone through 3 and 5 freeze-thaw cycles were compared to those from the samples that had only been thawed once.

#### Correlation with mouse bioassay

To evaluate the concordance between the Endopep-MS method and the MBA, clinical samples (11 serum/blood samples; 1 from a wild mallard and 10 from domestic fowl) were analyzed. The BoNT serotype had been previously determined by MBA neutralization according to the established method by the Nordic Committee on Food Analysis [50], and the samples had been stored in  $-20^{\circ}$ C until Endopep-MS analysis. The oldest sample was collected in 2005 and the newest one in 2013.

Botulism outbreaks among wild birds and poultry in Sweden are mainly caused by BoNT-C/D [30,51]. When the samples were analyzed by Endopep-MS, the 8DC1.2 and 4C2 antibodies were used for the toxin capture and the C substrate was used in the endopep reaction in order to determine whether the samples contained BoNT-C or the C/D mosaics. In each analytical run, chicken serum was analyzed, both blank and spiked with BoNT-C (50, 250, and 500 MLD<sub>50</sub>/sample) or C/D (23, 33 and 66 ng/sample). In order to further confirm the findings in the clinical samples, culture supernatants of *Clostridium botulinum* strains BKT015925 (type C/D), and C-Stockholm (type C) as well as a non-toxin producing *C. botulinum* were also analyzed as additional reference materials. Culture of bacteria and collection of supernatant, as well as confirmation of the strain identities by PCR, were performed as described by Skarin et al. [51].

#### Multiplexing

Since the sample volume is sometimes limited, the possibility of performing the antibody extraction procedure multiple times on the same serum sample was evaluated. First, the RAZ1 antibody was used (specific for BoNT-A) and when the antibody extraction procedure on the KingFisher Flex was completed, the serum sample was moved to a new position in the 96 well plate and the procedure was repeated with the B12.2 antibody (for BoNT-B), followed by the 6F5.1 antibody (for BoNT-E and F), the 4C2 antibody (for BoNT-C and D/C), and finally the 8DC1.2 antibody (for BoNT-C/D and D,). Human serum spiked with the different BoNTs were used in the experiments. Each toxin was spiked in 3.5 mL of serum and  $3 \times 500 \,\mu\text{L}$  of it was analyzed by the multiplex approach, while  $3 \times 500 \,\mu\text{L}$ of the same spiked serum was kept as a reference sample at room temperature until the time came for "the right" antibody when it was extracted in parallel with the sample analyzed with the multiplexed method. The spiking concentration per 500  $\mu$ L sample was 1 MLD<sub>50</sub> for BoNT-A, B, and F, 10 MLD<sub>50</sub> for BoNT-D/C, 20 MLD<sub>50</sub> BoNT-E, and 250 MLD<sub>50</sub> for BoNT-C. The reaction buffer used for magnetic beads with antibody 6F5 contained 25 µM each of the peptide substrate for BoNT-E and F. The reaction buffer used with antibodies 4C2 and 8DC1.2 also contained double peptide substrates, 25  $\mu$ M each of the substrates for BoNT-C and D.

#### **RESULTS AND DISCUSSION**

The Endopep-MS method with MALDI-Q-TOF-MS detection was implemented for BoNT serotypes A–F in human and chicken serum. Representative mass spectra for the different serotypes at the lowest spiking concentration (see Table 3) after 24 h of incubation are shown in Figure 1. For BoNT-A, B, and F at least one of the expected peptide cleavage products gave a response higher than LOD (see Materials and Methods for a definition of LOD) after only 4 h of incubation at 0.5 MLD<sub>50</sub> and for BoNT-D/C this was achieved at 1 MLD<sub>50</sub> per sample, indicating that the Endopep-MS method has an equal or better sensitivity compared to that of the MBA for these serotypes. For BoNT-E the LOD was at 5 MLD<sub>50</sub> which is just slightly above that of the MBA whereas for BoNT-C, 50 MLD<sub>50</sub> was needed to reach the LOD. However, it has been demonstrated that high amounts of BoNT-C is needed to affect birds [52,53]. Thus, the higher LOD for this serotype can be considered defensible.

#### **Method validation**

Endopep-MS has a great potential to replace the MBA as the gold standard for the diagnosis of botulism. However, a comprehensive validation is necessary in order to demonstrate the method's fitness for purpose. The parameters selected for the validation of the qualitative mode of this technique were: precision, stability, selectivity, correlation with the MBA, and multiplexing.

**Selectivity**—In order for the Endopep-MS method to challenge the MBA, it is important that it can distinguish between the different BoNT serotypes. The method contains two levels of selectivity, i.e., the antibody capture and the substrate cleavage. This is especially important for the mosaics C/D and D/C, since the cleavage reaction alone cannot differentiate these toxin types from C and D, respectively. Since the method was applied on avian samples in this project, the main focus was on BoNT-C, D, and their mosaics which are most frequent in animal botulism.

First, the selectivity of the C and D substrate cleavage was evaluated. BoNT-A, B, E, and F were incubated with the peptide substrates for BoNT-C and D directly in reaction buffer. None of the cleavage products expected from BoNT-C or D could be observed above the blank level after 4 or 24 h, confirming the selectivity of the substrates (results not shown).

Then, antibody selectivity was assessed for all the BoNTs studied. Separate serum samples were spiked with BoNTs A, B, C, D/C, E, and F in triplicates (10 MLD<sub>50</sub>/500 µL of A, B, D/C, and F, 25 MLD<sub>50</sub>/500 µL for E, and 250 MLD<sub>50</sub>/500 µL for C) and analyzed by the Endopep-MS procedure with antibodies RAZ1, B12.2, 8DC1.2, 4E17.1, and 6F5.1 The samples were spotted onto a MALDI plate and analyzed after 4. It was concluded from the collected data that the 8DC1.2 antibody did not bind any of the BoNT-A, B, E or F nor did the antibodies RAZ1, B12.2, 4E17.1, and 6F5.1 have any affinity for BoNT-C or D/C (results not shown). Hence, the antibody 8DC1.2 proved to be selective for the capture of BoNT-C and D/C and there was no cross reactivity for these BoNT serotypes with the other antibodies. BoNT-A was captured by antibodies RAZ1, 4E17.1 and 6F5. BoNT-E was bound by antibody B12.2 and to a small extent by antibody 6F5. BoNT-E was captured

equally well by the 6F5.1 and 4E17.1 antibodies while BoNT-F was captured by antibody 6F5.1, and to a small extent also by 4E17.

Furthermore, five different antibodies were evaluated regarding their abilities to capture BoNT-C, C/D, D/C and D from chicken serum, as presented in Figure 2. Antibodies 8DC1.2, 4C4.2, and 4C10.2 captured all four toxin types studied, cf. Table 1. However, the 8DC1.2 antibody was chosen as the primary antibody for a general Endopep-MS method of BoNT-C and D and their mosaics. BoNT-C and C/D cleave the peptide substrate at the same position and hence cannot be differentiated by their proteolytic selectivity. The same is true for BoNT-D and the mosaic D/C. Therefore we investigated the possibility to differentiate them using antibodies. The 1C1.1 antibody, which binds to the N terminal of the heavy chain of BoNT-C, proved to be BoNT-C specific while the 4C2 antibody that binds to the C terminal of the heavy chain captured both BoNT-C and D/C. Thus, we have for the first time demonstrated that by combining the results from the antibodies 8DC1.2 and the 4C2 in the Endopep-MS approach using the C and D peptide substrates we can differentiate the mosaics, BoNT-C/D and D/C, from pure BoNT-C and D, see Table 4. With this approach a much lower amount of toxin is needed for differentiating between BoNT-C, D, C/D, and D/C compared to the proteomics approach, less than 25 ng compared to 10 µg [26].

**Precision**—Even though the Endopep-MS method is only used for qualitative purposes in the version presented in this paper, the precision is an important parameter to validate. The ability to repeat a certain response, especially close to the LOD, may determine if a sample will be reported as positive or negative. The intra-day precision was determined for three concentrations each of the BoNT-A, B, E, and F in human serum and C/D, and D toxins in chicken serum (see Table 5). According to the European Medicines Agency's Guideline on bioanalytical method validation the estimated precision for quantitative ligand binding assays should not exceed 20% (25% at the lower and upper limit of quantification) [54]. However, since the Endopep-MS method is more complex than a conventional ligand binding assay, these acceptance criteria were expected to be too strict for the presented study. In this validation study, the relative standard deviation between the samples (six of each concentration, three spots of each) was found to be below 25% for a majority of the concentrations tested. There were some exceptions where the RSD was higher, e.g. for BoNT-D. The large spread between spots for this serotype could be explained by the extremely high intensities of the C and N terminal cleavage products of the peptides in the spectra giving rise to high and unrobust ratios with the IS. However, the high RSD in this case would not cause a risk to miss a positive sample, owing to the high sensitivity of the Endopep-MS method for BoNT-D. Another example with high spread was the N terminal peptide for BoNT-F. In this case the peptide was poorly ionized resulting in low intensities. However, the C terminal peptide for BoNT-F ionized well and gave an intense signal with lower RSD. At the lowest concentration where imprecision might result in false negative samples, the RSDrs were lower than 25% after 4 h in all cases but for BoNT-D. After 24 h there was an increase in the imprecision but the overall signal compared to blank also increased (except for the NTP of BoNT-F), diminishing the risk for false negatives at this concentration. A comparison of the precision with the MBA is not possible since the MBA is not quantitative or even semi-quantitative.

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Parks et al. have earlier described the quantification of BoNT-A and B in serum with an electrospray MS/MS set-up of Endopep-MS [45]. To evaluate the linear correlation between the response and the BoNT concentration for all the serotypes studied here, three concentrations were evaluated for precision, where one of them was at the proposed LOD. The linearity was satisfactory (coefficient of regression between 0.85 and 0.97, data not shown). These results imply that the method has potential to be validated also for quantification, or at least semi-quantification, in the future.

Since the method was to be applied on avian samples, BoNT-C and D/C were selected as examples for an evaluation of the inter-day precision. The experiment was repeated on three different days for the commercially available BoNT-C and D/C (see Tables 6 and 7). All the samples were spotted and analyzed by MALDI-Q-TOF MS after both 4 and 24 h. The results for BoNT-C are presented in Table 6. Up to 4 h of incubation, the spread in the cleavage product/IS ratio was fairly consistent, both within (RSD<sup>r</sup> 7–27%) and between (RSD<sup>R</sup> 13–19%) analytical runs. However, after 24 h of incubation a substantial increase in imprecision was observed. The ratio between both the N terminal (NTP) and the C terminal (CTP) peptide cleavage products and the IS doubled between 4 and 24 h of incubation. However when compared to the blank samples the CTP/IS or NTP/IS ratios for BoNT-C were not significantly improved by incubation overnight, probably due to unspecific cleavage. The samples were prepared and spiked separately on the day of the analysis, which is likely an additional contributing factor to the inter-day precision.

Table 7 presents the intra- and inter-day precision for BoNT-D/C after 4 and 24 h of incubation. For BoNT-D/C the effect of prolonging the incubation had a higher impact than for BoNT-C on the cleavage product/IS ratio, compared to that of the blank samples. For the lowest concentration, 1 MLD<sub>50</sub>, an overnight incubation was needed to reach the LOD requirement. Even though the spread also increased with incubation time the gain in LOD was highly desirable. As for BoNT-C, the fact that samples analyzed on different days were spiked separately is likely a contributing factor to the inter-day precision for BoNT-D/C. All in all, the method precision was considered satisfactory for all the toxins as the scope was merely qualitative.

**Stability**—Since the Endopep-MS method contains an enzymatic reaction, toxin activity is a prerequisite for the assay to be functional. Hence, proper handling and storage of the toxin standards and samples are necessary to avoid denaturation. In this study, BoNT-C and D/C standards were stored in PBST at  $-80^{\circ}$ C and spiked chicken serum samples were stored at  $-20^{\circ}$ C. The freeze-thaw stability was evaluated for up to 5 freeze-thaw cycles both for toxin standards at  $-80^{\circ}$ C and for the spiked serum samples at  $-20^{\circ}$ C. As before, cleavage product/IS ratio of samples containing toxin were compared to the corresponding ratio for the blank samples. For toxin standards freeze-thawed multiple times, all ratios were deviating less than 1 SD of the samples only freeze-thawed once (results not shown). Hence, we conclude that 5 freeze-thaw cycles can be performed with no significant loss in toxin activity. For the serum samples an increase in the cleavage product/IS ratios, compared to blank, was observed for those samples freeze-thawed multiple times compared to samples freeze-thawed once. Thus there is no risk of false negatives.

#### Correlation with the mouse bioassay

To evaluate the concordance with the mouse bioassay, Endopep-MS was applied to 11 clinical avian blood and serum samples that previously had been found positive for BoNT with the MBA. Broth samples, from cultures of *C. botulinum* confirmed by PCR to contain BoNT-C or BoNT-C/D genes, were also included as well as positive and negative control samples in both serum and broth. The results are presented in Table 8.

All the serum samples tested were neutralized by both the C and D antitoxin in the MBA. With the Endopep-MS method they tested positive for BoNT-C/D using the 8DC1.2 and the 4C2 antibodies for differentiation of BoNT-C, C/D, D, D/C as described in Table 4. These results demonstrate the strength of this new mode of combination of antibodies and substrate peptides as illustrated by sample 9 in Table 6 that was previously analyzed with the Endopep-MS method with a less selective antibody as described in Hedeland et al. [44]. In that study only BoNT C cleavage could be determined whereas it could be shown in the presented work that the sample actually contained the mosaic BoNT-C/D. Furthermore, in the broth samples the mosaic C/D was easily distinguished from BoNT-C by the use of the two antibodies 8DC1.2 and 4C2.

In summary, the results of the Endopep-MS analysis of the clinical samples as well as the bacteria culture samples conclude that the method gave results that were in agreement with those obtained from the MBA or PCR. Thus, the Endopep-MS is an attractive alternative to the mouse bioassay.

#### Multiplexing

The Endopep-MS method can provide information about the serotype of the BoNT within a workday [46] and by automation of the magnetic bead antibody capture procedure the hands-on work is reduced. However, this approach demands many milliliters of sample, since 500  $\mu$ L is needed for each type of antibody. Kalb et al. have worked on multiplex alternatives of the method, e.g. a monoclonal antibody (4E17.1) to capture BoNT-A, B, E, and F has been tested [45]. In this paper we present another multiplex mode of the Endopep-MS assay that can be used when the sample volume is limited. In this setup, a sequential separate capture with one antibody after the other is performed until all the BoNT serotypes have been captured and separately analyzed. The order of the antibodies was determined after the selectivity had been evaluated. In this study, we successfully detected all the BoNTs by antibody capture in the following order: RAZ1 (captures BoNT-A), B12.2 (captures BoNT-B), 6F5.1 (captures BoNT-E and F), 4C2 (captures BoNT-C and D/C), and finally 8DC1.1 (captures BoNT-C/D and D). The results, as a comparison of cleavage product/IS ratios divided by the same ratio of the blank samples, for samples that were extracted with the correct antibody directly compared to the same sample that had been subject to the multiplex process, are illustrated in Figure 3. The responses of the multiprocessed samples were comparable to those that were not multiprocessed for all the toxins. This means that unspecific binding of the toxin to the magnetic beads and the "wrong" type of antibody using the above-mentioned sequence was negligible. This multiplex set up takes longer time to perform but it can still be accomplished during one day and we believe this approach to be superior, to e.g. dilution, if the sample volume is limited.

Here, only 500  $\mu$ L of sample was needed to do the serotyping, compared to 10–15 mL of sample which is recommended for MBA neutralization [16]. Additionally, to differentiate BoNT/C from CD and BoNT/D from D/C has, to the authors knowledge, not been proven possible with MBA neutralization.

#### CONCLUSIONS

The Endopep-MS method has for the first time been in-house validated for qualitative analysis of active BoNT-A, B, C, C/D, D, D/C, E, and F in serum. The parameters LOD, selectivity, precision, stability in matrix and solution, and correlation with the MBA were evaluated with successful results. The Endopep-MS can challenge the mouse bioassay as the gold standard for the diagnosis of botulism, as the sensitivity is in the same order of magnitude or better than that of the MBA for most of the toxin serotypes, it measures toxin activity, it is faster and it does not require any experimental animals. Furthermore, it has for the first time been demonstrated that Endopep-MS can differentiate between BoNT-C, D and their mosaics C/D and D/C by a combination of different antibodies and different target peptides. Additionally, a new way of multiplexing has been presented, based on sequential capture with antibodies directed against the different serotypes.

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The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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#### Figure 1.

Spectra of BoNT A–F spiked serum samples (500  $\mu$ L) after 24 h of incubation. The amount of toxin in the different samples was: 0.5 MLD<sub>50</sub> BoNT-A (A); 0.5 MLD<sub>50</sub> BoNT-B (B); 50 MLD<sub>50</sub> BoNT-C (C); 1 MLD<sub>50</sub> BoNT-D/C (D); 5 MLD<sub>50</sub> BoNT-E (E); 0.5 MLD<sub>50</sub> BoNT-F (F). For more information about the peptides, see Table 2; sub=peptide substrate, CTP= C terminal cleavage product, NTP= N terminal cleavage product, IS=internal standard peptide.



#### Figure 2.

CTP/IS or NTP/IS ratio in relation to the blank samples (y axis) after 4 h of incubation for BoNT-C, C/D, D, and D/C using 5 different antibodies (x axis). For a summary of antibody selectivities, see Table 1. Antibodies 8DC1.2, 4C4.2 and 4C10.2 capture all toxins. Antibody 4C2 captures BoNT-C and D/C. Antibody 1C1.1 captures BoNT-C only. Error bars represent  $\pm$  1 SD.



#### Figure 3.

CTP/IS or NTP/IS ratio in relation to the blank samples (y axis) for BoNT-B, F, E, C, D/C, C/D, and D (x-axis) after 4 h of incubation. The samples were analyzed with regular Endopep-MS and with the multiplexed set up described in Materials and methods. Error bars represent  $\pm 1$  SD.

Specificity of the monoclonal antibodies used in the assay.

Antibody	Serotypes	Specificity
RAZ1	А	НС
B12.2	В	HC
8DC1.2	C, C/D, D, D/C	HN
4C4.2	C, C/D, D, D/C	HN
4C10.2	C, C/D, D, D/C	LC
4C2	C and D/C	HC
1C1.1	С	HN
4E17.1	E (A, F)	HN
6F5.1	F (A, B, E)	HN

HC = C terminal domain of the heavy chain; HN = N terminal domain of the heavy chain; LC = light chain

Substrate peptides and the different cleavage products

BoNT serotype	Substrate peptide and cleavage products	m/z
	Ac-RGSNKPKIDAGN <b>QR</b> ATRXLGGR-NH2	2 406.4
А	Ac-RGSNKPKIDAGNQ	1 426.7
	RATRXLGGR-NH <sub>2</sub>	998.6
	LSELDDRADALQAGAS <b>QF</b> ESSAAKLKRKYWWKNLK	4 024.1
В	LSELDDRADALQAGASQ	1 759.9
	FESSAAKLKRKYWWKNLK	2 283.3
	BoNT-C substrate*	2 868.6
C and C/D	BoNT-C N terminal cleavage product *	1 424.7
	BoNT-C C terminal cleavage product*	1 462.9
	AQVDEVVDIMRVNVDKVLERDQ <b>KL</b> SELDDRADALQAGAS	4 311. 2
D and D/C	AQVDEVVDIMRVNVDKVLERDQK	2 698.4
	LSELDDRADALQGAS	1 631.8
	BoNT-E substrate*	3 611.9
Е	BoNT-E N terminal cleavage product *	2 498.3
	BoNT-E C terminal cleavage product <sup>*</sup>	1 132.6
	TSNRRLQQTQAQVDEVVDIMRVNVDKVLERD <b>QK</b> LSELDDRADALQAGAS	5 523.8
F	TSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQ	3 783.0
	KLSELDDRADALQAGAS	1 759.9
IS (internal standard)	LRTAQADITNSK-Biotin	1543.8

Bold characters indicate cleavage site for the toxin. Ac=acetylated terminus, NH2=amidated terminus, X=norleucine, hR=homo arginine,

\* =manuscript with amino acid sequence in preparation.

The lowest spiking levels in serum for the different BoNT serotypes.

BoNT serotype	Concentration (MLD50/500 µL)
А	0.5
В	0.5
С	50
D/C	1
Е	5
F	0.5

Differentiation between BoNT-C, C/D, D, and D/C using different combinations of antibody and peptide substrate.

	А	ntibody-substr	ate combinatio	n
	8D0	C1.2	40	C2
Serotype	C substrate	D substrate	C substrate	D substrate
BoNT C	POS	-	POS	-
BoNT C/D	POS	-	-	-
BoNT D	-	POS	-	-
BoNT D/C	-	POS	-	POS

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The intra-day precision for BoNTs C/D, D, A, B, E, and F after 4 and 24 h of incubation. BoNT C/D and D was spiked in chicken serum and the others in human serum.

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		NTP/IS			<b>CTP/IS</b>	
	Mean	$RSD^{r}$ (%)	x blank	Mean	RSD <sup>r</sup> (%)	x blank
Blank 4h (24h)	0.22 (0.24)	9 (21)		0.20 (0.32)	17 (23)	
23ng BoNT-C/D 4h (24h)	0.59 (1.02)	18 (18)	2.6 (4.2)	1.08 (2.13)	9 (12)	5.5 (6.7)
33ng BoNT-C/D 4h (24h)	0.77 (1.37)	14 (14)	3.4 (5.6)	1.41 (2.97)	8 (17)	7.2 (9.4)
49ng BoNT-C/D 4h (24h)	0.96 (1.91)	15 (7)	4.3 (7.8)	2.16 (4.30)	13 (11)	11 (14)
Blank 4h (24h)	0.02 (0.01)	37 (23)		0.03 (0.03)	10 (17)	'
0.1ng BoNT-D 4h (24h)	0.12 (0.67)	26 (47)	7.4 (50)	0.24~(0.90)	36 (50)	8.8 (35)
0.9ng BoNT-D 4h (24h)	1.04 (5.79)	25 (48)	66 (434)	2.35 (4.77)	18 (40)	87 (185)
1.8ng BoNT-D 4h (24h)	2.34 (7.35)	35 (55)	149 (551)	3.60 (6.03)	18 (36)	133 (234)
Blank 4h (24h)	0.17 (0.17)	12 (18)		0.35 (0.31)	15 (19)	1
BoNT-A 0.5MLD50 4h (24h)	1.19 (1.96)	5 (5)	6.8 (11.2)	2.07 (2.55)	6 (6)	6.0 (8.3)
BoNT-A 1MLD50 4h (24h)	2.16 (3.28)	15 (15)	16 (24)	3.40 (3.83)	16 (20)	14 (17)
BoNT-A 5MLD50 4h (24h)	9.71 (15.34)	13 (10)	73 (114)	15.36 (19.88)	14 (12)	65 (86)
Blank 4h (24h)	0.01 (0.01)	12 (14)		0.11 (0.07)	21 (15)	1
BoNT-B 0.5MLD50 4h (24h)	0.05 (0.22)	6 (7)	5.0 (21)	$0.10\ (0.10)$	9 (23)	1.0 (1.4)
BoNT-B 1MLD50 4h (24h)	0.07 (0.33)	6 (5)	7.1 (32)	0.11 (0.10)	19 (19)	1.1 (1.4)
BoNT-B 5MLD50 4h (24h)	0.24 (1.08)	12 (8)	25 (103)	0.13 (0.17)	21 (24)	1.3 (2.3)
Blank 4h (24h)	0.013 (0.011)	28 (20)	'	0.10 (0.13)	24 (12)	, '
BoNT-E 5MLD50 4h (24h)	0.018 (0.026)	15 (34)	1.4 (2.3)	$0.16\ (0.36)$	17 (16)	1.7 (2.7)
BoNT-E 10MLD50 4h (24h)	0.024 (0.034)	12 (42)	1.8 (3.1)	0.23 (0.55)	15 (39)	2.4 (4.1)
BoNT-E 20MLD50 4h (24h)	0.031 (0.068)	20 (27)	2.3 (6.1)	0.34 (1.04)	8 (13)	3.4 (7.8)
Blank 4h (24h)	0.002 (0.003)	29 (124)	:	0.04 (0.04)	11 (11)	1
BoNT-F 0.5MLD50 4h (24h)	0.008 (0.006)	24 (25)	5.1 (2.2)	0.24 (0.26)	10 (12)	6.2 (6.9)
BoNT-F 1MLD50 4h (24h)	0.017 (0.012)	45 (25)	11 (4.4)	0.37~(0.46)	15 (15)	9.8 (12)
BoNT-F 5MLD50 4h (24h)	$0.055\ (0.048)$	39 (87)	35 (17)	1.33 (1.55)	12 (10)	35 (41)

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NTP/IS = The ratio between the intensities of the N terminal cleavage product and the internal standard peptide in the same spectrum.

CTP/IS = The ratio between the intensities of the C terminal cleavage product and the internal standard peptide in the same spectrum.

RSD<sup>T</sup>= the relative standard deviation in percent calculated from 6 replicates, each spotted and analyzed 3 times.

x blank = the mean NTP/IS (or CTP/IS) in a sample (6 replicates, 3 spots each) divided by the mean of the same ratio of the blank samples (6 replicates, 3 spots each).

The intra- and inter-day precision for BoNT-C spiked in chicken serum after 4 and 24 h of incubation.

			ATN	SI/			CTP	SI/	
	Day	Mean	$RSD^{r}$ (%)	RSD <sup>R</sup> (%)	x blank	Mean	RSD <sup>r</sup> (%)	RSD <sup>R</sup> (%)	x blank
Blank 4 h (24 h)	-	0.23 (0.35)	13 (17)		'	0.26 (0.58)	14 (37)		'
	7	0.24 (0.32)	9 (15)	16 (21)	'	0.29 (0.48)	8 (16)	19 (28)	
	б	0.18 (0.32)	14 (27)		'	0.22 (0.47)	23 (15)		'
50 MLD <sub>50</sub> BoNT-C 4 h (24 h)	-	0.43 (1.1)	12 (12)		1.9 (3.1)	0.78 (2.3)	9 (11)		3.0 (4.0)
	7	0.42 (0.71)	27 (29)	18 (26)	1.8 (2.0)	0.74 (1.1)	27 (34)	17 (34)	2.6 (2.0)
	33	0.43 (0.83)	9 (15)		2.4 (2.4)	0.78 (1.5)	10 (15)		3.6 (2.7)
250 MLD <sub>50</sub> BoNT-C 4 h (24 h)	-	1.5 (4.5)	7 (26)		6.5 (13)	3.9 (12)	10 (28		15 (21)
	2	1.5 (3.1)	11 (13)	14 (40)	6.1 (8.7)	3.8 (6.7)	4 (14)	16 (42)	13 (12)
	б	1.3 (2.0)	20 (23)		7.4 (5.8)	3.1 5.4)	22 (14)		15 (9.5)
500 MLD <sub>50</sub> BoNT-C 4 h (24 h)	-	2.4 (5.5)	15 (15)		10 (16)	6.3 (13)	16 (24)		24 (24)
	7	2.2 (4.6)	10 (15)	15 (19)	9.2 (13)	6.4 (12)	7 (13)	13 (19)	22 (21)
	с	2.4 (4.0)	17 (12)		13 (11)	6.2 (11)	14(7)		29 (19)
NTP/IS = The ratio between the in	tensitie	s of the N term	inal cleavage	product and the	e internal st	andard peptide	in the same s	pectrum.	
CTP/IS = The ratio between the in	tensitie	s of the C termi	inal cleavage p	product and the	e internal st	indard peptide	in the same s	pectrum.	
RSD <sup>T</sup> = the relative standard deviat	tion in J	percent calculat	ed from 6 repl	licates, each sp	otted and a	nalyzed 3 time	·s		

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x blank = the mean NTP/IS (or CTP/IS) in a sample (6 replicates, 3 spots each) divided by the mean of the same ratio of the blank samples (6 replicates, 3 spots each).

RSDR= the relative standard deviation in percent calculated from 6 replicates, each spotted and analyzed 3 times on 3 different days.

The intra- and inter-day precision for BoNT-D/C spiked in chicken serum after 4 and 24 h of incubation.

			ATP	SI			CTP	SI	
	Day	Mean	RSD <sup>r</sup> (%)	RSD <sup>R</sup> (%)	x blank	Mean	RSD <sup>r</sup> (%)	RSD <sup>R</sup> (%)	x blank
Blank 4 h (24 h)	-	0.01 (0.02)	15 (14)		I	0.02 (0.03)	10 (12)		'
	2	0.01 (0.02)	15 (12)	29 (31)	ı	0.03 (0.04)	6 (7)	12 (24)	'
	3	0.02 (0.03)	35 (14)		'	0.02 (0.03)	14 (35)		
1 MLD <sub>50</sub> BoNT-D/C 4 h (24 h)	-	0.02 (0.06)	12 (22)		1.6 (3,8)	0.04 (0.14)	16 (23)		1.6 (4.2)
	2	0.02 (0.09)	17 (23)	31 (38)	1.9 (5,9)	0.05 (0.22)	7 (19)	21 (48)	1.9 (5.7)
	б	0.03 (0.11)	28 (33)		2.0 (4,1)	0.05 (0.10)	26 (77)		1.9 (3.7)
10 MLD <sub>50</sub> BoNT-D/C 4 h (24 h)	-	0.09 (0.41)	35 (35)		7.3 (26)	0.17 (0.99)	31 (40)		7.0 (30)
	7	0.11 (0.60)	11 (24)	32 (47)	8.9 (39)	0.28 (1.8)	4 (19)	35 (56)	10 (46)
	б	0.14 (0.64)	29 (58)		8.8 (24)	0.19 (0.53)	42 (51)		7.6 (21)
20 MLD <sub>50</sub> BoNT-D/C 4 h (24 h)	-	0.20 (1.5)	16 (33)		16 (92)	0.42 (2.9)	23 (27)		17 (86)
	7	0.20 (1.2)	13 (30)	45 (33)	16 (78)	0.51 (2.8)	7 (20)	38 (55)	19 (73)
	З	0.42 (1.5)	24 (31)		26 (54)	0.27 (0.70)	64 (43)		11 (27)
NTP/IS = The ratio between the inte	ensities	of the N termin	al cleavage pi	roduct and the	internal star	idard peptide i	n the same spe	ctrum.	
CTP/IS = The ratio between the inte	ensities	of the C termin	al cleavage pr	oduct and the j	internal star	idard peptide i	n the same spe	ctrum.	
$RSD^{T}$ = the relative standard deviation	on in pe	rcent calculated	l from 6 replic	cates, each spo	tted and and	ılyzed 3 times.			
RSDR= the relative standard deviati	ion in p	ercent calculate	d from 6 repl	icates, each spo	otted and an	alyzed 3 times	s on 3 different	t days.	

x blank = the mean NTP/IS (or CTP/IS) in a sample (6 replicates, 3 spots each) divided by the mean of the same ratio of the blank samples (6 replicates, 3 spots each).

The results after analysis of samples with the MBA and the Endopep-MS method.

Sample number	Sample ID	Sample type	Result of MBA	<b>Result of Endopep-MS</b>
1	07-BKT024891	Serum, poultry	C and D	C/D
2	09-BKT103381	Blood, poultry	C and D	C/D
3	10-BKT001917	Blood/serum, poultry	C and D	C/D
4	10-BKT062061	Blood, poultry	C and D	C/D
5	11-BKT028437	Blood, poultry	C and D	C/D*
6	11-BKT031326	Blood, poultry	C and D	C/D
7	11-BKT051704	Serum, poultry	C and D	C/D
8	13-BKT067648	Blood/serum, poultry	C and D	C/D
9	AN2339	Blood/serum, mallard	C and D	C/D
10	08-BKT015386	Blood/serum, poultry	C and D	C/D
11	08-BKT037465	Blood, poultry	C and D	C/D
12	Broth HD/C5 negative	Broth, bacteria without BoNT gene	-	Negative
13	Broth C Stockholm	Broth, C Stockholm strain (BoNT-C)	-	С
14	Broth 15925	Broth, BKT015925 strain (BoNT-C/D)	-	C/D

MBA = Mouse bioassay, the result is presented as the antitoxin(s) used that neutralized the BoNT.

\*Positive after 24 h (but not after 4 h).