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Detection, differentiation, and identification of botulinum neurotoxin serotypes C, CD, D, and DC by highly specific immunoassays and mass spectrometry

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1 **Full paper**

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3 Detection, differentiation, and identification of 4 botulinum neurotoxin serotypes C, CD, D, and DC 5 by highly specific immunoassays and mass spectrometry

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Abstract

Botulinum neurotoxins (BoNT) serotypes C and D and their mosaic variants CD and DC cause severe cases of botulism in animal husbandry and wildlife. Epidemiological data on the exact serotype or toxin variant causing outbreaks are rarely available, mainly because of their high sequence identity and the lack of fast and specific screening tools to detect and differentiate the four similar toxins. To fill this gap, we developed four highly specific sandwich enzyme-linked immunosorbent assays (ELISAs) able to detect and differentiate botulinum neurotoxins type BoNT/C, D, CD, and DC based on four distinct combinations of specific monoclonal antibodies targeting both conserved and divergent subdomains of the four toxins. Here, highly sensitive detection with detection limits between 2 and 24 pg/mL was achieved. The ELISAs were extensively validated and results were compared with data obtained by quantitative real-time PCR using a panel of *Clostridium botulinum* strains, real sample materials from veterinary botulism outbreaks, and non-BoNT-producing *Clostridia*. Additionally, in order to verify the results obtained by ELISA screening, the new monoclonal antibodies were used for BoNT enrichment and subsequent detection (i) on a functional level by endopeptidase mass spectrometry (Endopep-MS) assays and (ii) on a protein sequence level by LC-MS/MS spectrometry. Based on all technical information gathered in the validation study, the four differentiating ELISAs turned out to be highly reliable screening tools for the rapid analysis of veterinary botulism cases and should aid future field investigations of botulism outbreaks and the acquisition of epidemiological data.

Introduction

Botulism, a potentially life-threatening paralytic disease in men and animals, is caused by the complex family of high molecular weight botulinum neurotoxins (BoNTs) which cleave and thereby functionally inactivate essential molecules within the neurotransmitter release machinery. All BoNTs are synthesised as single molecules of 150 kDa. Upon activation by a protease they form di-chain toxins composed of a 50 kDa light chain (LC) with zinc-dependent protease activity, linked via a disulphide bond to the 100 kDa heavy chain (HC), comprising an *N*-terminal translocation domain (H_N) and a *C*-terminal receptor binding domain (H_C).[1] The BoNTs can be distinguished into seven confirmed serotypes, A through G. While BoNT/A, B, E, and F predominantly cause botulism in humans, serotypes BoNT/C and D have been attributed to veterinary botulism.[2, 3] Rare evidence points towards naturally occurring botulism in man to be associated with serotypes C or D, but the relevance of the historic data is questionable.[4] Although natural intoxication with BoNT/C and D does not seem to occur in man, it has been shown that experimental intoxication with BoNT/C can cause botulism symptoms in humans.[5, 6] On the other hand, serotypes C and D can cause mass outbreaks in farm animals and frequently in waterfowls.[7-9] Animal botulism can even threaten already endangered species.[10-12] Genetic analysis of the neurotoxin genes of BoNT/C and D revealed that, apart from BoNT/C and BoNT/D, two highly related mosaic variants called BoNT/CD and BoNT/DC exist[13-15] in which the first letter designates the catalytic activity and the second letter the receptor-binding activity[13]. For BoNT/C and CD, two thirds of the molecules are highly identical (LC and H_N domains, identity >92%), while the H_C domains are less conserved (>40%, Figure 1). Similarly, BoNT/D and DC show a high identity in their LC and H_N domains (>94%) and reduced conservation in the H_C domains (>37%). Conversely, the H_C domains of BoNT/D and CD are highly homologous (>91%, Figure 1), while the H_C domains of BoNT/C and DC are less conserved (overall 74%), containing a region of lower homology (58%) at nucleotide positions 3485–3800 encoding the *C*-terminal portion of BoNT/C H_C (H_C/C).[14] These mosaic structures explain many of the puzzling observations of cross-reactivity of antisera and antibodies in the past. With respect to epidemiologic investigation of veterinary botulism outbreaks, only very few studies have been published, demonstrating that BoNT/CD and DC seem to be predominant in Europe and Japan in avian and bovine botulism.[16-24]

The BoNTs are produced by the Gram-positive, anaerobic, spore-forming organism *Clostridium (C.) botulinum* and strains of *C. baratii* and *C. butyricum*. For more than 100 years morphological, physiological, and biochemical differences between *C. botulinum* strains have been recognised, and modern genomic analyses have revealed that *C. botulinum* can be separated into four Groups I to IV[25]: The proteolytic strains of Group I

produce BoNT/A, B, and F, while the non-proteolytic Group II strains produce BoNT/B, E, and F. Group III strains are linked to serotypes C and D and their mosaics, while BoNT/G is produced by Group IV.[26] On the basis of 16S rRNA homology, genomic and phenotypical relatedness all four groups have non-BoNT-producing counterparts lacking the neurotoxin gene cluster but are assigned to different species names.[26] Depending on the particular strain, the neurotoxin gene cluster can be encoded in mobile genetic elements such as plasmids or phage, but can also be incorporated into the bacterial chromosome.[27] Loss of the neurotoxin gene cluster which has been reported for Groups I, II, and III transforms *C. botulinum* to a different species. In particular for Group III where the BoNT gene cluster is located on a pseudo-lysogenic phage, loss of the BoNT gene cluster and thus loss of neurotoxicity has been described frequently.[14, 28, 29] Additionally, interconversion between *C. botulinum* serotypes C and D and conversion of *C. novyi* (non-toxigenic) into *C. botulinum* (toxigenic) by infection with the corresponding phage are possible.[30-32] Due to the similarities on genomic and phenotypic level, the organisms compiling *C. botulinum* Group III, *C. novyi*, and *C. haemolyticum* have been designated *C. novyi sensu lato*.[29]

After reaching the neuromuscular junction, BoNT first accumulates via binding to complex polysialo-gangliosides on the surface of motoneurons and subsequently endocytose via interaction with protein receptors into small synaptic vesicles.[33] For all serotypes except for BoNT/C, a proteinaceous cell surface receptor on the neuronal cell surface has been identified that is bound with high affinity. Noteworthy, while the H_C/DC has been shown to bind synaptotagmin I and II, no such interaction was found for the H_C/C despite their high homology.[34] In contrast, the H_C/C fragment recognises two gangliosides employing two distinct binding pockets.[35-37] Also H_C of BoNT/D and CD bind two gangliosides but with much lower affinity than H_C/C.[38, 39] One glycolipid interaction might be substituted by binding synaptic vesicle protein 2 (SV2).[40] After internalisation, the H_N domain of HC conveys the translocation of the LC into the cytosol. The LC harbours the catalytic endopeptidase activity towards the so-called soluble NSF attachment protein receptor (SNARE) molecules, thus preventing fusion of the neurotransmitter-loaded vesicles with the synaptic membrane. Unlike other BoNTs which cleave either the synaptic proteins synaptosomal-associated protein of 25 kDa (SNAP-25) or synaptobrevin-2/vesicle-associated membrane protein-2 (VAMP-2) at distinct, individual positions, BoNT/C and BoNT/CD are the only known neurotoxins that target two different synaptic molecules: SNAP-25 and syntaxin.[41] BoNT/C and BoNT/CD hydrolyse SNAP-25 between amino acid positions Arg198 and Ala199 and syntaxin between positions Lys253 and Ala254.[41] In the case of BoNT/D and DC, the enzymatic cleavage site has been localised between positions Lys59 and Leu60 of VAMP-2.[41]

123 *C. botulinum* strains are found ubiquitously in soil and marine sediments and are able to
124 induce human and veterinary botulism cases worldwide. Whereas numerous assays have
125 been established[42] for the detection and discrimination of the BoNT serotypes pathogenic to
126 humans (serotypes A, B, E, and F), targeting either the coding DNA or the BoNT proteins,
127 the situation is different for the detection of serotypes BoNT/C and D. In particular, assays
128 distinguishing between BoNT/C, D, and the mosaic variants CD and DC are sparse. Only
129 very few conventional and quantitative PCR methods have been reported to be able to
130 discriminate between these four toxin variants.[19, 20, 43] Convenient detection and
131 differentiation of BoNT/C, D, and their mosaic variants on protein level is an unresolved issue
132 so far, but this would be highly important for a number of reasons: (i) DNA-based assays
133 cannot distinguish between a low-level contamination with the bacterium and the growth and
134 active toxin production; (ii) the level of toxin produced cannot be determined based on
135 genetic data; (iii) certain *C. botulinum* cultures or individual suspected samples may have lost
136 their phage carrying the BoNT gene locus, thus turning PCR negative while the culture
137 supernatant might still be toxic; (iv) in some strains, silent genes have been described that
138 encode a non-functional toxin.[44] Therefore, genetic information is indicative of toxin-
139 producing *Clostridia*, but results can be ambiguous. The gold standard for toxin detection is
140 still the mouse bioassay, which is time consuming, expensive, unable to deal with high
141 sample numbers, and questionable under ethical considerations. Additionally, the clear
142 differentiation of BoNT/C or D as well as discrimination of mosaic forms has been impaired
143 due to cross-reactivity of antibodies used for neutralisation. To date, only a few
144 immunoassays have been described which can detect some, but not all of the four BoNTs
145 relevant in the context of veterinary botulism. Immunochromatographic assays and sandwich
146 enzyme-linked immunosorbent assays (ELISAs) have been described, performed either
147 plate-bound or on magnetic beads, detecting both BoNT/C and/or D.[45-49] Recently, Nakamura
148 *et al.* reported the development of rapid immunochromatographic tests for the detection and
149 differentiation of BoNT/CD and DC[18] and Zhang *et al.* a multiplex ELISA for six BoNT
150 serotypes including BoNT/C and DC.[50] A specific combination of appropriate sandwich
151 ELISAs for detection and differentiation of all four BoNTs relevant in the context of veterinary
152 botulism is lacking so far.

153 As an alternative approach, assays addressing the functional activity of BoNT have been
154 developed which detect the endopeptidase activity of the LC coupled to different read-outs.
155 Very promising is the combination of immunoaffinity enrichment of BoNT by antibody-
156 coupled magnetic beads plus detection of the *in vitro* cleavage of artificial SNARE substrates
157 by mass spectrometry (MS), the Endopep-MS assay.[51] The assay is highly sensitive, and –
158 by using cross-reactive antibodies for enrichment – can determine the type of LC activity
159 (BoNT/C- or D-type activity). A different access for toxin detection lies in its identification by

160 tryptic peptide fingerprint and its amino acid sequence using an MS/MS proteomics
161 approach.[51] However, the proteomics approach is often hampered by its lower analytic
162 sensitivity compared to the Endopep-MS assay. In this context, Björnstad and co-workers
163 very recently described the use of two parallel immunocapture reactions based on two
164 monoclonal antibodies (mAbs) either recognising all four BoNT variants (BoNT/C, D, CD, and
165 DC) or recognising BoNT/C and BoNT/DC only. In combination with the Endopep-MS assay,
166 they were able to enrich and distinguish between the four veterinary BoNTs with good
167 sensitivities.[22] The sophisticated approach is a clear technological advancement; still it might
168 be restricted to expert laboratories with access to expensive MS-based instrumentation and
169 dedicated expertise.

170 The aim of our study was to develop fast, easy, and precise ELISA-based screening tools for
171 the detection and differentiation of BoNT/C, D, and their respective mosaic variants that can
172 be applied in routine veterinary laboratories. To this end, highly specific monoclonal
173 antibodies were developed which differentially bind to LC or H_C of the four BoNT proteins.
174 Proper combinations of the antibodies led to four individual ELISAs, one of them specifically
175 detecting BoNT/C, the second detecting BoNT/CD, the third detecting BoNT/D, and the
176 fourth detecting BoNT/DC only. The four ELISAs were thoroughly validated on a panel of
177 *C. botulinum* strains and real veterinary samples with respect to sensitivity and specificity,
178 and no cross-reactivity was observed. In a second approach, the set of monoclonal
179 antibodies was used to corroborate the ELISA-based screening results by Endopep-MS
180 assays which were able to functionally discriminate between BoNT/C, D, CD, and DC. In a
181 validation study, the ELISA data were correlated with PCR data and Endopep-MS data on
182 the same specimens, and a 100% correlation of results was obtained.

Experimental

Toxins, bacterial supernatants, and primary culture enrichments

BoNTs are highly toxic and therefore require appropriate safety measures. All toxins were handled by trained personnel under a class II vertical laminar flow cabinet (Heraeus Herasafe, Thermo Scientific, Dreieich, Germany) in a dedicated toxin laboratory. Toxin-containing solutions were inactivated in 5% sodium hydroxide overnight, liquids containing *Clostridia* and/or spores as well as solid waste containing traces of toxin or *Clostridia*/spores were inactivated by autoclaving (134 °C, 1 h).

Purified BoNT/A, B, C, DC, E, F, and G as well as the corresponding neurotoxin protein complexes were obtained from Metabiologics (Madison, WI, USA). The commercially available BoNT/DC is sold as “BoNT/D” but has been identified as BoNT/DC by mass spectrometry[51].

All recombinant BoNT/C proteins are based on the coding DNA sequence in the phage from *C. botulinum* strain 468, and BoNT/D proteins are based on the coding DNA sequence in the phage from strain BVD/-3 identical to that of strain 1873. Non-toxic H_CCS (aa 867–1291 fused to a *C*-terminal Strep-tag[52]), ‘H_C/C’ in this manuscript, BoNTCS-Thro wildtype (active single chain [sc] BoNT/C fused to a Strep-tag and thrombin cleavage site[53]), ‘scBoNT/C’ in this manuscript, non-toxic H_C/DS (aa 863–1276 fused to a *C*-terminal Strep-tag[54]), ‘H_C/D’ in this manuscript and sc BoNT/D wild type with native loop sequence (scBoNTDSL[38]) were produced as described previously.[38] In order to eliminate catalytic activity, three mutations each were introduced into scBoNTCS-Thro (= E230A/R372A/Y375F) and scBoNTDSL (= E230A/R372A/Y375F), yielding inactive scBoNT/C_i and scBoNT/D_i. Absence of biological activity was examined employing the mouse phrenic nerve hemidiaphragm assay. Recombinant BoNT/CD (scH6tBoNTCDtS; strain 003-9), LC/C6xHN (aa 1–436), and LC/D6xHN (aa 1–436) were purchased from toxogen GmbH (Hannover, Germany).

Bacterial cell culture supernatants were prepared from *C. botulinum* (Groups I, II, III, and IV), *C. baratii*, *C. berijerinckii*, *C. botulinum* Group II atoxic, *C. butyricum*, *C. bifermentans*, *C. difficile*, *C. glycolicum*, *C. hiranonis*, *C. innocuum*, *C. novyi*, *C. paraputrificum*, *C. perfringens*, *C. sartagoforme*, *C. scindens*, *C. septicum*, *C. sordellii*, *C. sporogenes*, *C. subterminale*, *C. tertium*, and *C. tetani* raised in tryptone-peptone-glucose-yeast extract (TPGY) medium for 1 to 7 days at 30 °C under anaerobic conditions in an anaerobic workstation (MACS 500, Don Whitley Scientific, Shipley, West Yorkshire, United Kingdom). Supernatants were collected by removing vegetative bacteria, using centrifugation (12,000 × *g*, 5 min, 4 °C) and two filtration steps (0.45 µm and 0.2 µm). A total of 23 primary culture enrichments from swabs, spleen, intestine, liver, and faeces from birds and cattle were collected from animal botulism outbreaks mainly from France and Germany over the last few years and were prepared as described earlier.[43]

220

221 **Isolation and sequence analysis of strain 08084L-60 Eppendorf**

222 From a confirmed outbreak of botulism among cattle in Eppendorf, Germany, in 2008, tissue
223 biopsy material was obtained from the Institut für Lebensmittel, Arzneimittel und
224 Tierseuchen, Berlin. Cooked meat medium (Oxoid, Basingstoke, United Kingdom) was
225 inoculated with organ sample material, and subsequently the *C. botulinum* group III strain
226 08084L-60 Eppendorf was isolated. Organ material and the isolated strain were initially
227 tested positive for BoNT/D or DC by TaqMan-PCR[55], a test which is not distinctive between
228 both forms. Genomic DNA was isolated as described before[56] and subjected to whole
229 genome sequencing on an Ion Torrent platform (Life Technologies, Darmstadt, Germany).
230 Sequencing reads were imported into Geneious 7.1.4 (Biomatters, Auckland, New Zealand)
231 and assembled on the neurotoxin gene cluster of strain CB-19 (Genbank AB745665).[18]
232 Reads were re-assembled on the resulting neurotoxin gene cluster sequence. Comparison of
233 the final sequence of the BoNT gene with Genbank entries revealed that it belongs to the DC
234 mosaic type. The sequence of the BoNT/DC gene of strain 08084L-60 Eppendorf is available
235 from Genbank under accession number KT355686.

236

237 **Sequence analysis**

238 For sequence comparison, the following Genbank entries were used for BoNT/C: AP008983
239 (Stockholm), X71126 (A028), X53751 (468C); for BoNT/CD: AB745659 (OTZ07), FN436022
240 (S19), AY251553 (TW/2003), CP002411 (BKT015925), D49440 (6813), AB745666 (003–9),
241 AB745667 (348); for BoNT/DC: AB037920 (D-4947), AB745668 (OFD05), D38442 (South
242 African), AB745669 (OFD16), LC008286 (OFD40), KT355686 (Eppendorf); and finally for
243 BoNT/D: AB012112 (1873), S49407 (CB16). Sequences were uploaded into the Geneious
244 7.1.4 software package, protein sequences were aligned and distances calculated using
245 built-in Geneious Multiple Sequence Alignment algorithm (Global alignment with free end
246 gaps; BLOSUM 62[57]). For amino acid sequence comparison of BoNT domains, whole
247 alignment was tailored to contain the LC (aa 1–446), HC (aa 446–1293), H_N (aa 446–867), or
248 H_C (aa 868–1293) based on sequence D49440.

249

250 **Quantitative real-time PCR**

251 DNA was purified from culture supernatants with the DNeasy Blood & Tissue Kit (Qiagen,
252 Hilden, Germany) according to the manufacturer's recommendation for Gram-positive
253 bacteria. Alternatively, 200 µL of culture were centrifuged (12.000 × *g*, 5 min at 4 °C), the
254 sediment washed with 200 µL of PBS and boiled for 15 min in 200 µL of H_2O to release DNA.
255 Quantitative real-time TaqMan PCR for *bont/*A–G was performed as described earlier.[55] For

256 the detection of *bont*/G, the following primers and probe were selected based on Genbank
257 X74162[58]: forward primer: 5'-TGGCCATTCCCCAATATCA-3',
258 reverse primer: 5'-GGATCGCTATGTTGCATGAAAAA-3',
259 and probe: FAM-TAGACGCGCGTATTTTGCAGATCCAGC-BHQ1. In order to discriminate
260 *bont/*C, CD, DC, and D at the genomic level, quantitative PCR was performed on a GeneDisc
261 cycler (Qiagen) as described before. [20, 43]
262

263 **Animal experiments**

264 Animal experiments were performed in compliance with the German Animal Welfare Act and
265 European legislation for the protection of animals used for scientific purposes (Directive
266 2010/63/EU). Experiments were evaluated and approved by the State Office for Health and
267 Social Affairs in Berlin (LaGeSo Berlin, Germany) under registration numbers H109/03
268 (production of antibodies) and A0073/08 (mouse bioassay).
269

270 **Generation of monoclonal antibodies**

271 Monoclonal antibodies (mAb) were generated according to standard procedures.[59] Briefly,
272 BALB/c or NMRI mice bred under pathogen-free conditions at Charles River (Sulzfeld,
273 Germany) were used at the age of 8 weeks. Four female mice were immunised
274 intraperitoneally (i.p.) with 25 µg or 50 µg of recombinant H_C/C and/or 10 µg of scBoNT/C_i
275 and 10 µg of scBoNT/D_i in adjuvant as indicated in Table 1. Mice were boosted several times
276 with similar doses of the same antigen i.p. in adjuvant at four-week intervals. On day –3, –2,
277 and –1 before fusion, 25 µg or 50 µg of H_C/C or 10 µg of scBoNT/C_i, and 10 µg of scBoNT/D_i
278 in phosphate-buffered saline (PBS) were applied i.p. daily. Hybridomas were produced by
279 fusing spleen cells from immunised mice with myeloma cells (P3-X63-Ag8.653, American
280 Type Culture Collection) at a ratio of 2:1 in polyethylene glycol 1500 (PEG, Roche
281 Diagnostics, Mannheim, Germany) according to standard procedures.[59] Antigen-specific mAb
282 were screened by an indirect ELISA against the corresponding toxins at days 10 to 14 post-
283 fusion, and positive hybridoma clones were subcloned at least twice (Table 1).
284 Immunoglobulins (IgG) were purified from hybridoma supernatants grown in RPMI media
285 supplemented with IgG-free foetal bovine serum by affinity chromatography over a HiTrap
286 MabSelect SuRe column using an Äkta Protein Purification System (GE Healthcare Bio-
287 Sciences AB, Uppsala, Sweden). L. Bellanger, CEA, kindly provided mAbs which were
288 generated by immunising BALB/c mice (Charles River, Lyon, France) with recombinantly
289 expressed H_C/C and H_C/D fragments purified from *E. coli*. The isotype of all purified
290 monoclonal antibodies (mAbs) was determined using an IsoStrip mouse monoclonal
291 antibody isotyping kit (Roche Applied Science, Mannheim, Germany). Antibodies were
292 coupled to biotin according to the manufacturer's instructions (EZ-Link Sulfo-NHS-LC-biotin;

293 Pierce, Rockford, IL, USA). Biotinylated antibodies were stored in PBS with 0.2% (w/v)
294 bovine serum albumin (BSA; Serva, Heidelberg, Germany) and 0.05% (w/v) NaN_3 (Carl Roth,
295 Karlsruhe, Germany).
296

297 **Surface plasmon resonance measurements (Biacore)**

298 Kinetics and affinity of antibody–antigen interaction were analysed by surface plasmon
299 resonance (SPR) technology using a Biacore X100 unit (GE Healthcare, Freiburg, Germany)
300 at 25 °C. For all experiments HBS-EP+ buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA,
301 0.05% Tween-20, pH 7.4) was used as running buffer. mAbs were immobilised as ligands
302 onto flow cell 2 (Fc2) of a biosensor chip using a mouse antibody capture kit (GE
303 Healthcare), resulting in 100 to 300 resonance units (RU). Fc1 channel (anti-mouse capture
304 antibody only) was set as blank control. Three-fold serial dilutions of analytes (scBoNT/C or
305 scBoNT/D_i) ranging from 200 to 0.82 nM were injected over both flow cells for 60 s at
306 30 µL/min; time course of binding dissociation was monitored for 600 or 1200 s after running
307 buffer injection. Microchip sensor surfaces were regenerated by injection of 10 mM glycine-
308 HCl buffer pH 1.7 (GE Healthcare) for 180 s with 10 µL/min over both flow cells prior to every
309 new injection cycle. Reproducibility and retained activity of the sensor surface was controlled
310 by duplicate measurement at the highest analyte concentration. Before data analysis, RUs of
311 Fc2 were referenced to Fc1; in addition, three blank injections without antigen were used as
312 a second reference. Kinetic binding parameters were determined by fitting the double
313 referenced binding curves to the 1:1 Langmuir binding model (A + B = AB) by using the
314 Biacore X100 evaluation software 1.1 (GE Healthcare, Uppsala, Sweden). To account for
315 small differences in maximum binding capacity due to the usage of the mouse capture kit,
316 R_{max} was fitted locally.
317

318 **Indirect enzyme-linked immunosorbent assay (ELISA)**

319 MaxiSorp microtitre plates (F96; Nunc, Wiesbaden, Germany) were coated with antigen (500
320 ng/mL, 50 µL per well) in PBS containing 1 µg/mL of BSA at 4 °C overnight and blocked with
321 blocking buffer (PBS containing 0.1% Tween 20, 2% skimmed milk [Merck, Darmstadt,
322 Germany]) for 1 h[59]. After washing with PBS containing 0.1% Tween 20 (PBST), the antibody
323 was added (10 µg/mL in blocking buffer) for 1 h and was detected by horseradish peroxidase
324 (HRP)-labelled goat anti-mouse IgG (Fc-γ specific; Dianova, Hamburg, Germany) and
325 3,3',5,5'-tetramethylbenzidine (TMB, SeramunBlau slow, Seramun, Heidesee, Germany).
326 The average absorbance was measured at 450 nm and referenced to absorbance of
327 impurities at 620 nm ($A_{450\text{-}620\ nm}$).
328
329

330 **Sandwich ELISA**

331 MaxiSorp microtitre plates were coated with primary mAb C394 (anti-LC/C), H_CC10S or
332 H_CC2378 (both anti-H_C/C), or D63 (anti-LC/D) at 5 µg/mL in 50 µL of PBS (pH 7.2) at 4 °C
333 overnight. After blocking with casein buffer (Diavita, Heidelberg, Germany) at room
334 temperature for 1 h and a following washing step with PBST, 50 µL of toxin was added in
335 duplicate in serial dilutions from 1 mg/L to 1 ng/L or, for bacterial supernatants, diluted 1:10
336 or 1:100 in assay buffer (PBS, 0.1% BSA). After 2 h at room temperature, cavities were
337 washed and incubated at room temperature for 1 h with biotinylated secondary mAb C9 (anti-
338 H_C/C), D63 (anti-LC/D), or D967 (anti-H_C/D) diluted in casein buffer at 1 µg/mL, followed by
339 washing and amplified detection with streptavidin-coupled horseradish peroxidase (SA-
340 PolyHRP40 [Senova, Weimar, Germany] or SA-POD [Dianova, Hamburg, Germany]) at
341 0.2 µg/mL for 30 min. TMB was used as substrate and measurement was performed as
342 above.

343

344 **Validation of sandwich ELISA**

345 For statistical analysis, we measured standard curves for each sandwich ELISA in ten
346 independent runs over 10 days, with three replicates per concentration. Differences in
347 absorbance at 450 and 620 nm were plotted against the log concentration of the BoNT
348 standard and fitted against a sigmoidal dose–response curve (4-parametric non-linear
349 regression analysis) in Prism 5.04 (GraphPad, La Jolla, CA, USA). The limit of detection
350 (LOD) was calculated from the regression curve by adding 3.29-fold of the standard
351 deviations to the mean of the absorbance ($A_{450\text{-}620\ nm}$) of ten determinations of blanks
352 performed in duplicate on one microtitre plate. The lower and upper limits of quantification
353 (LLOQ and ULOQ) flank the linear range of the sigmoidal curve between the inflection points
354 of the 1st derivative of the sigmoidal regression curve and were computed as the maxima and
355 minima of the 2nd derivative. The intra-assay coefficient of variation (CV% intra at EC_{50}) was
356 determined as the standard deviation divided by the mean of concentrations of ten double
357 determinations of the half maximal effective concentrations (EC_{50}) within plates, multiplied by
358 100. The inter-assay coefficient of variation (CV% inter at EC_{50}) was calculated
359 correspondingly with concentrations measured between ten separate and independent runs.
360 Statistically outlying concentration values were identified by Grubbs tests[60] on a significance
361 level of 0.05 using the R package 'outliers'[61] and excluded from the calculation of CVs.

362

363 **Immunoblot**

364 LC and H_C domains of BoNT/C and BoNT/D were separated by 10% SDS-PAGE under non-
365 reducing conditions and transferred onto an Immuno-Blot 0.45 µm PVDF membrane
366 (Invitrogen, Karlsruhe, Germany). After blocking the membrane in blocking buffer (2%

skimmed milk in PBST) at 4 °C overnight, appropriately diluted primary antibody in blocking buffer was added to the membrane for 1 h. After three washing steps, the membrane was incubated with biotin-labelled goat anti-mouse IgG (Dianova, Hamburg, Germany) in blocking buffer at room temperature for 30 min and was developed with avidin-alkaline phosphatase and CDP-Star (Perkin Elmer, Waltham, MA, USA).

Mouse bioassay

Culture supernatants from primary enrichment cultures or isolated strains were diluted in PBS containing 0.1% BSA and injected i.p. into female BALB/c mice. Animals were closely monitored for typical botulism symptoms (wasp-like narrowed waist, laboured breathing, and beginning paralysis) for up to 4 days. When typical symptoms of botulism were observed animals were sacrificed according to human end-points.

Toxin extraction with antibody-coated magnetic beads

Different mAbs were immobilised to M-280 tosyl-activated paramagnetic Dynabeads (Invitrogen, Karlsruhe, Germany) following the manufacturer's protocol as described earlier[62]: mAb C394 directed against the LC of BoNT/C and CD; mAb D63 (anti-LC of BoNT/D and DC); and mAb H_CC2378 (anti-H_C of BoNT/C and DC) or mAb D967 (anti-H_C of BoNT/D and CD). To determine the catalytic activity (C-type or D-type) by Endopep-MS assay without differentiation of mosaics, 200 µL of bacterial culture supernatant or primary enrichment culture was added to 300 µL of PBST containing a mixture of both anti-LC antibody-coated beads (10 µL of mAb C394 plus 10 µL of mAb D63 beads). For differentiation of BoNT/C, CD, DC, and D, two independent antibody-based enrichment reactions were performed in parallel using the anti-H_C antibody-coated beads (either mAb H_CC2378 or mAb D967, 20 µL each). As negative control TPGY medium without toxin was used. After end-over-end incubation at room temperature for 1 h, the beads were washed twice in 1 mL of PBST, twice in PBS, and finally in 100 µL of distilled water. For naturally contaminated samples, beads were additionally washed twice in 1 mL of PBST with 2 M NaCl after PBST washing to remove unspecific bindings.[63] The beads were reconstituted in 20 µL of Endopep reaction buffer.

Endopep-MS analysis

Endopep-MS analysis was performed according to Moura *et al.* and Hedeland *et al.*[24, 51] with slight modifications. In brief, beads were reconstituted in 20 µL of reaction buffer containing 20 mM HEPES (pH 7.4), 0.2 M $ZnCl_2$, 10 mM dithiothreitol, 1 mg/mL BSA, and 50 pmol/µL of each BoNT/C and BoNT/D peptide substrate. Peptides (HPLC-purified, purity > 95%) were synthesised by peptides & elephants (Potsdam, Germany) or Dr Petra Henklein (Institute for

404 Biochemistry, Charité Universitätsmedizin, Berlin, Germany). The reaction solution was
405 started at 47 °C for 10 min, followed by incubation at 42 °C for 17 h using a thermocycler.
406 The cleavage substrate for light chain activity of BoNT/C or BoNT/CD was modified from the
407 human SNAP-25 protein sequence (Biotin-KGSNRTRIDEANQRATRMLGGK-biotin; *m/z*
408 2911)[51] and yielded the *N*-terminal (NT) cleavage product at *m/z* 1871 (Biotin-
409 KGSNRTRIDEANQR) and *C*-terminal (CT) one at *m/z* 1059 (ATRMLGGK-biotin). For light
410 chain activity of BoNT/D and BoNT/DC, peptide substrate based on the sequence of the
411 VAMP-2 protein (LQQTQAQVDEVVDIMRVNVDKVLERDQKLSELDDRADAL; *m/z* 4496)[64]
412 was used; NT cleavage product is *m/z* 3297 (LQQTQAQVDEVVDIMRVNVDKVLERDQK);
413 and CT cleavage product is *m/z* 1217 (LSELDDRADAL).
414
415 **Mass spectrometry detection**
416 MS-based Endopep analysis was done in MS-positive ion reflector mode utilizing an autoflex
417 speed matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass
418 spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a smartbeam laser.
419 Sample (2 µL) was mixed with 18 µL of MALDI-Matrix (5 mg/mL of α-Cyano-4-
420 hydroxycinnamic acid, Bruker Daltonics) in 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich,
421 Seelze, Germany) and 50% acetonitrile (Carl Roth, Karlsruhe, Germany) in HPLC water,
422 1 mM ammoniumphosphate (Merck, Darmstadt, Germany). Of this mixture 1 µL was spotted
423 onto a MTP 384 polished steel target plate (Bruker Daltonics). For matrix suppression,
424 deflection was set to *m/z* 500, mass spectra were acquired over the mass range *m/z* 600 to
425 4800. External mass calibration was performed with peptide calibration standard II (Bruker
426 Daltonics). Each spectrum represents an average of 4,000 laser shots. Spectra were
427 processed by flexAnalysis 3.4 software (Bruker Daltonics).
428
429 **LC-MS/MS analysis of tryptic digest**
430 For liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, 500 µL of
431 bacterial culture supernatant was mixed with 50 µL of 10× PBST, 20 µL of C394 beads or
432 D63 beads and incubated under rotation at room temperature for 1 h. Beads were washed
433 twice in 1 mL of HBS-EP buffer (GE Healthcare), once in 1 mL of distilled water and were
434 reconstituted in 15 µL of 50 mM ammonium bicarbonate (pH 7.5). Tryptic digestion was
435 performed at 52 °C for 5 min with 5 µL of 0.2 µg/µL trypsin (Sigma-Aldrich, Munich,
436 Germany). After digestion, beads were removed and 2 µL of 10% TFA was added to the
437 peptide solution. Prior to LC-MS/MS analysis 10 µL of peptide solution was loaded onto a
438 trap column (Proxeon Thermo Scientific EASY-nLC C18, 5 µm nano LC column, 100 µm ID,
439 and 2 cm long) and separated on an analytic column (Acclaim PepMap100 C18, 3 µm nLC
440 column, 75 µm ID, and 15 cm long [Thermo Fisher Scientific, Dreieich, Germany]). Elution

441 was achieved by formation of a binary gradient of buffer A (water with 0.1% formic acid) and
442 buffer B (99.9% acetonitrile with 0.1% formic acid) with a flow rate of 300 nL/min: B = 2% at 0
443 min; B = 40% at 60 min; B = 80% at 62 min; hold for 10 min; B = 2% at 74 min; hold for 5
444 min. The nano LC was coupled online to an LTQ-Orbitrap Discovery mass spectrometer
445 equipped with a nanoelectrospray ion source (both from Thermo Fisher Scientific, Bremen,
446 Germany). For all measurements, the analyser was operated in data-dependent acquisition
447 mode. After a survey scan in the Orbitrap (resolution of 30,000 from *m/z* 300 to 1,700)
448 MS/MS fragmentation data was recorded for the seven most intensive precursor ions in the
449 linear ion trap at collision-induced energy of 35%. Database search was performed using
450 MASCOT Server 2.4 software (Matrix Science Ltd., London, United Kingdom). A database
451 was compiled by extracting entries from the UniProt database using “clostridium botulinum”
452 as search term and from the NCBI non-redundant database specific for BoNT/C, CD, D, and
453 DC. Database search was performed with up to two missed tryptic cleavages, and
454 methionine oxidation was set as variable modification. Peptides with up to +4 charge ions
455 were used, precursor tolerance was set to 10 ppm and tolerance for fragment ions was set to
456 0.6 Da.

457

458 **Results and Discussion**

459 **Generation and characterisation of monoclonal antibodies specific for fragments of**
460 **BoNT/C and D**

461 In order to develop ELISA-based assays for detection and differentiation of all four BoNTs
462 pathogenic for animals, we developed monoclonal antibodies (mAb) specifically binding to
463 the LC or the H_C-fragment of BoNT/C and BoNT/D, respectively. Technically challenging is
464 the fact that, depending on the fragment analysed, BoNT/C, D, and their mosaic variants
465 show a high degree of identity on the protein level[65], as summarised in Figure 1 on the basis
466 of different GenBank entries (three sequences for BoNT/C, seven sequences for BoNT/CD,
467 two sequences for BoNT/D, and six sequences for BoNT/DC). BoNT/C and BoNT/CD are
468 96.9–97.7% identical in their LC and 92.6–93.6% in their H_N-domain, while the H_C-domain of
469 BoNT/C is only 40.0–40.4% identical to the H_C-domain of BoNT/CD, which is 91.6–95.6%
470 identical to H_C/D. Similarly, BoNT/D and BoNT/DC are 97.8–98.2% identical in their LC and
471 94.8–95.5% in their H_N-domain, while the H_C-domain of BoNT/D is 37.9–38.9% identical to
472 the H_C-domain of BoNT/DC, the latter being more closely related to the H_C-domain of
473 BoNT/C (73.8–74.0% identity).

474 Classically, immunisation strategies to produce antibodies started by using formaldehyde-
475 inactivated purified toxins.[45, 66, 67] While this allowed the application of sufficient amounts of
476 antigen to trigger an immune response, the altered 3-dimensional structure often resulted in
477 antibodies preferentially recognising the denatured toxoid over the native toxin. To overcome
478 this drawback, non-toxic subdomains and here particularly the H_C-fragments of BoNT/C or D
479 have been used to generate monoclonal or polyclonal antibodies[68] or to identify vaccine
480 candidates.[69-72] Only BoNT/C and BoNT/DC were commercially available as purified proteins
481 from *C. botulinum* culture supernatants (see Materials and Methods), but BoNT/D and
482 BoNT/CD were important as well for differential immunisation, screening, and for validation
483 studies. To address this issue, single-chain derivatives of BoNT/C and D with drastically
484 reduced biological activity (scBoNT/C_i, ~500,000-fold less active; scBoNT/D_i, ~10,000-fold
485 less active in an hemidiaphragm assay) as well as isolated H_C-fragments of BoNT/C and
486 BoNT/D (H_C/C and H_C/D) were recombinantly expressed in *E. coli* and purified to
487 homogeneity. BALB/c and NMRI mice were repeatedly immunised with recombinant
488 scBoNT/C_i, scBoNT/D_i, H_C/C, H_C/D, or mixtures thereof. Serum antibody titres were regularly
489 tested by indirect ELISA against the purified, natural toxins or single-chain recombinant
490 proteins. In total, four fusions were performed and more than 12,000 hybridoma supernatants
491 were screened for the production of specific mAbs (Table 1). Out of 55 positive clones, 11
492 most promising hybridoma clones providing superior results in indirect ELISA were selected,
493 purified by affinity-chromatography, and further characterised. In addition to the newly

494 generated mAbs, a panel of mAbs available at L. Bellanger's laboratory (CEA, France),
495 which were produced by immunisation against recombinant H_C/C or H_C/D and screening
496 against natural BoNT/C and BoNT/DC, were included in the study for further experiments.
497 The isotype of all antibodies was determined as either IgG1 or IgG2b by using an antibody
498 isotyping kit (Table 2).

499 In order to narrow down the location of the epitopes recognised by the panel of antibodies,
500 recombinant LC- and H_C-fragments as well as the full-length BoNTs – either natural toxins or
501 single-chain recombinant proteins – were tested for binding of the mAbs in Western blot and
502 indirect ELISA experiments. Here, both technologies delivered concurrent results (Figure 2,
503 Table 2 and data not shown). As exemplarily shown in Figure 2A and D for Western blotting,
504 it turned out that the LC/C and LC/D were recognised by mAbs C394 and D63, respectively.
505 Antibody clone D967 was able to interact with H_C/D (Figure 2E) and the corresponding part
506 of BoNT/CD. Antibody clone C9 specifically bound to H_C/C (Figure 2B), but was
507 unexpectedly not able to bind the related H_C-fragment of BoNT/DC. Indeed, among 42 clones
508 initially identified to be specific for H_C/C, only two mAbs, clones H_CC10S and H_CC2378, were
509 able to bind to the H_C-fragment of both BoNT/C (Figure 2C and F) and the corresponding part
510 of BoNT/DC. A possible explanation could be that BoNT/C and DC show a reduced
511 sequence identity of approximately 74% in their H_C-fragments, this being a considerably
512 lower homology than the sequence identities observed among the other related LC- and H_C-
513 fragments (> 91.6%) for which cross-reacting antibodies could be obtained. This lower
514 sequence identity between H_C/C and H_C/DC is paralleled by a divergent binding pattern to
515 cell surface receptors: While H_C/C has been shown to interact with two ganglioside residues,
516 H_C/DC binds one ganglioside plus the protein receptor synaptotagmin.[34-36, 40]

517 For all antibodies, binding affinity was analysed by surface plasmon resonance (SPR)
518 against serial dilutions of scBoNT/C or scBoNT/D_i, respectively. Association (k_a) and
519 dissociation (k_d) rate constants were calculated from a 1:1-model-based fit.[73] All mAbs
520 showed high binding affinities with equilibrium dissociation constants (K_D) between 10^{-9} to
521 10^{-12} M towards their corresponding recombinant scBoNT (Table 2). Interestingly, two mAbs
522 (C1352 and C2574) were identified that recognised both the H_C/C- and the H_C/D-fragment in
523 indirect ELISA (Table 2). For both mAbs, SPR analysis showed a preference for BoNT/C
524 over BoNT/D, and the results were in line with a higher reactivity towards H_C/C than H_C/D in
525 the indirect ELISA experiments (not shown). As specificity control, all antibodies were also
526 tested against BoNT serotypes A, B, E, F, and G and their corresponding high molecular
527 weight complexes, always with negative results.

528

529 **Establishing sandwich ELISAs for specific detection and differentiation of BoNT/C, D,**
530 **and their mosaic variants**

531 Based on the results of the antibody characterisation, combinations of mAbs were tested for
532 the specific and differential recognition of BoNT/C, D, CD, and DC. Here, in each case the
533 mosaic structure of the four toxins was taken into account by including one antibody directed
534 against the LC of the toxin and the other antibody directed against the H_C-domain. The
535 principle is schematically displayed in Figure 3A where the different mAbs binding to related
536 sub-fragments of the four BoNTs are indicated in the same colour. We determined the ideal
537 antibody pair for each individual toxin in buffered solution. An antibody pair was considered
538 optimal when it specifically detected its corresponding antigen with high sensitivity, without
539 any cross-reactivity to any of the other four toxins. As antigens, the commercially available
540 purified BoNT/C and BoNT/DC (the latter sold as BoNT/D[51]) or recombinantly expressed
541 scBoNT/C, scBoNT/CD, and scBoNT/D were used.

542 For specific detection of BoNT/C, it turned out that a combination of mAb C394 which binds
543 at the LC of BoNT/C with biotinylated mAb C9 which recognises H_C/C was optimal (Figure
544 3B). The combination of the same capture mAb C394 with biotinylated mAb D967, an
545 antibody recognising the H_C/D domain, delivered a specific sandwich ELISA for BoNT/CD
546 (Figure 3C). BoNT/D was optimally detected by a combination of mAb D63 binding to LC/D
547 as capture antibody and biotinylated mAb D967 recognising H_C/D as detection antibody
548 (Figure 3D). Finally, for specific detection of BoNT/DC, mAb H_CC10S was used for capturing,
549 which binds to the H_C-domain of BoNT/C or BoNT/DC, in combination with biotinylated mAb
550 D63 binding to LC/D (Figure 3E). The different antibody combinations resulted in four highly
551 specific sandwich ELISAs, each recognising one of the four related BoNTs – either BoNT/C,
552 D, CD, or DC – with virtually no cross-reactivity among each other (Figure 3B–E, left panel).
553 Figure 3 (3 B–E, right panel) displays standard curves of three experiments for each of the
554 four toxins, including a four-parametric curve fit. For all sandwich ELISAs investigated, the
555 dynamic range of the assay spanned about two to three orders of magnitude above the limit
556 of detection (LOD). As indicated in Table 3, excellent LODs were reached by the four
557 individual ELISAs: For BoNT/C, CD, D, or DC, the LOD was determined to be 11.5 pg/mL,
558 23.8 pg/mL, 2.1 pg/mL, and 16.1 pg/mL, respectively. Thus, all four ELISAs exceed the
559 sensitivity of the current gold standard, the mouse bioassay, which was reported to detect
560 167 or 33 pg/mL for BoNT/C or BoNT/DC, respectively.[22]

561 In terms of validation, the lower and upper limits of quantification (LLOQ and ULOQ) were
562 characterised as concentrations where the curvature in the four-parametric curve fit was
563 maximal, and for each of the four ELISAs the numbers are given in Table 3. The variability of
564 the four ELISAs was determined at the half-maximal effective concentration (EC_{50}), the point

565 of highest precision in the linear range of the ELISA. The intra- and inter-assay precision for
566 the determination of the toxins were determined as coefficient of variation (Table 3, CV%-
567 intra assay and CV%-inter assay, see Experimental). For the four ELISAs, the mean intra-
568 assay precision was determined as between 3.3 and 6.4% (Table 3), similar to or better than
569 within-run precisions reported for other BoNT-specific ELISAs.[59, 74-78] The mean inter-assay
570 precision for the four ELISAs ranged from 8.2 to 24.4% (Table 3), again similar to other
571 BoNT-specific ELISAs.[59, 74, 76-78]

572 The high specificity and sensitivity of the four ELISAs developed in this work represent a
573 significant technical improvement over other immunoassays described for BoNT/C, CD, D, or
574 DC that are non-discriminatory on the mosaic variants. Some do not even properly
575 differentiate between BoNT/C and D – and mostly provide LODs in the range of 250–1500
576 pg/mL.[46-48, 79, 80] An exception was the most recent ELISA described as multiplex approach to
577 detect six BoNT serotypes including BoNT/C and DC which reached sensitivities similar to
578 those of the ELISAs established in this work (LODs: 2.2 and 0.9 pg/mL for BoNT/C and DC,
579 respectively).[50] Some of the older assays were relying solely on polyclonal antibodies that
580 may not necessarily detect the BoNT molecule itself but accessory proteins or unrelated
581 proteins found in *Clostridia* supernatants.[49, 81]

582

583 **Different mass spectrometric approaches for differentiation and identification of**
584 **BoNT/C, D, and their mosaic variants**

585 To confirm the ELISA-based data on a functional level, our newly developed mAbs specific
586 for LC- or H_C-fragments of BoNTs were evaluated as tools for Endopep-MS assays. In
587 different approaches, specific immunoaffinity enrichment steps were followed by incubation
588 with artificial SNAP-25 (for BoNT/C and CD[51]) or VAMP-2 (for BoNT/D and DC[64]) substrates
589 and MS-based detection of cleavage products. To this end, it has to be taken into account
590 that BoNT/C and CD cleave the SNAP-25 substrate at the very same position, so that they
591 cannot be differentiated by their endopeptidase activity alone. Similarly, BoNT/D and DC
592 cleave the VAMP-2 peptide at the very same position.
593 On this background, two different Endopep-MS approaches were tested: in the first setting,
594 the BoNTs were captured with a mixture of two mAbs specifically binding to the LC-domain
595 of either BoNT/C and CD (mAb C394) or BoNT/D and DC (mAb D63), and LC activity was
596 detected as described[51, 64]. In this approach, the light chain activity can be monitored by
597 determining the BoNT/C-type or BoNT/D-type endopeptidase activity, but without
598 differentiation of the mosaic variants. This is highlighted in Table 4 by the exemplary analysis
599 of bacterial supernatants from BoNT/C-, CD-, DC-, and D-producing strains. Here, we used a
600 representative group of four *C. botulinum* Group III strains, one producing BoNT/C complex

601 (strain NCTC8548), one producing BoNT/CD complex (strain C6814), one producing BoNT/D
602 complex (strain D4947), and one producing BoNT/DC complex (strain Eppendorf). Using the
603 Endopep-MS approach described, the four strains could be divided into two strains with
604 either BoNT/C-type cleavage activity or BoNT/D-type cleavage activity, but without
605 discrimination of the mosaics. The LOD of this type of assay was determined to be 5 pg/mL
606 for BoNT/D, 50 pg/mL for BoNT/DC, and 1 ng/mL for BoNT/C and BoNT/CD on toxin-spiked
607 buffer samples (data not shown). This endopeptidase assay format reproduced data
608 obtained by Moura *et al.* on BoNT/C and DC using a set of polyclonal capture antibodies.[51]
609 With respect to sensitivity, our assay reached similar results using monoclonal antibodies.[51]
610 Only very recently the same group was able to improve the limited sensitivity of the BoNT/C-
611 type activity assay by a factor of 200 by introducing a novel artificial peptide substrate.[82]

612 In the second setting, the veterinary BoNTs were captured in two parallel reactions using
613 either a mAb specifically binding to the H_C-fragment of BoNT/C and DC (mAb H_CC2378, anti-
614 H_C/C) or BoNT/D and CD (mAb D967, anti-H_C/D), and the endopeptidase activity was
615 detected after incubation with a mixture of BoNT/C and D substrates by mass spectrometry.
616 This Endopep-MS format delivered an LOD similar to that of the previously mentioned
617 Endopep-MS format when purified toxins or recombinant single chain toxins were measured
618 (data not shown). Based on the mosaic structure of the four BoNTs, theoretically this
619 approach of capturing the toxins via the H_C-domain plus detection of endopeptidase activity
620 of LC should directly allow for the determination of the different BoNT mosaic variants.
621 Indeed, as shown in Table 4 and Figure 4B, the approach delivered correct results on the
622 four representative bacterial supernatants from BoNT/C-, CD-, DC-, and D-producing strains.
623 A combination of capturing via anti-H_C/C plus detection of C-type cleavage activity identifies
624 strain NCTC8548 as BoNT/C-producing strain; capturing via anti-H_C/D plus detection of C-
625 type cleavage activity identifies strain C6814 as BoNT/CD. Similarly, the capturing via anti-
626 H_C/D plus detection of D-type cleavage activity identifies strain D4947 as BoNT/D producer;
627 finally, capturing via anti-H_C/C plus detection of D-type cleavage activity identifies strain
628 Eppendorf as BoNT/DC producer. The results perfectly confirm the ELISA data presented
629 above (Figure 4A).

630 This differentiating Endopep-MS approach is only the second assay published that is able to
631 discriminate the four closely related veterinary BoNTs, including discrimination of the mosaic
632 variants *in vitro* on a functional level. A related approach was very recently published by
633 Björnstad *et al.* who used one mAb to capture all four toxin types by a common epitope on
634 the H_N-domain, followed by determination of BoNT/C- or BoNT/D-type cleavage activity.[22] In
635 a second reaction, a different mAb was used for enrichment that specifically bound to the
636 H_C-fragment of two of the four toxin types, allowing the discrimination of the mosaic variants.

In our approach, the capturing step via two mAbs directly targets the different H_C-domains and is combined with the detection of the LC activity, thus delivering straightaway the serotype including discrimination of mosaic variants.

To finally verify the results obtained by ELISA and Endopep-MS assay, we aimed at identifying BoNT/C, D, and their mosaic variants by protein sequencing using liquid chromatography (LC)-MS/MS analysis of the four representative culture supernatants from BoNT/C-, CD-, D-, and DC-producing strains. To this end, the BoNTs were immunocaptured by their LC with mAb C394 to enrich for BoNT/C and CD or with mAb D63 to enrich for BoNT/D and DC. After tryptic digest, they were subjected to LC-MS/MS analysis. As shown in Table 4 and in Supplementary Information Fig. S1, the four neurotoxins were unambiguously identified on the protein level with a sequence coverage of 81% for BoNT/C (strain NCTC8548), 61% for BoNT/CD (strain C6814), 65% for BoNT/D (strain D4947), and 71% for BoNT/DC (strain Eppendorf), respectively. This high sequence coverage has been obtained on the basis of the concentration of toxins present in the four representative culture supernatants, as determined by our newly developed sandwich ELISAs (Table 4, concentration range between 0.4 mg/mL and 11.1 mg/mL).

To our knowledge, not all of the four veterinary BoNTs have been analysed by protein sequencing using LC-MS/MS analysis before; this is probably due to the lack of purified BoNT/CD and BoNT/D or the respective toxin-producing strains. Moura *et al.* obtained amino acid sequence coverage of 44% for BoNT/C and 18% for BoNT/DC,[51] which could be increased in a later work to 90% and higher when a sequential triple endoprotease in-gel digestion protocol was introduced.[83]

Validation study: Analysis of bacterial supernatants and comparison of results obtained with different methods

In the course of a botulism outbreak, laboratory diagnostics depends on a proper combination of technical approaches to detect both the pathogenic organism and the toxin. A successful strategy combines fast and easy screening methods with confirmation assays providing information on the activity and/or identity of the toxin. The detection, isolation, and genetic characterisation of the toxin-producing strain all deliver important additional information in an epidemiological investigation.[42] With respect to BoNT/C, CD, D, and DC, the main problem addressed in this work was the lack of easy and still highly specific and sensitive screening tools that allowed for direct discrimination of mosaic variants. It was important to put the ELISA results obtained in a larger context and to ask for the applicability and reliability of the ELISAs in the context of alternative genetic (real-time PCR) or activity-

672 based assays (mouse bioassay [MBA], Endopep-MS assays). To this end, the representative
673 panel of four *C. botulinum* Group III strains producing either BoNT/C, CD, D, or DC in their
674 naturally occurring complexed form was tested with a set of different methods including
675 different real-time quantitative (q)PCR protocols and MBA. Data obtained before by the four
676 ELISAs, by Endopep-MS, and LC-MS/MS approaches were corroborated on the genetic
677 level by qPCR[20, 43] and by MBA, and 100 % concordance of results was obtained (Table 4).

678 While these results were encouraging, they had to be challenged with a larger panel of
679 *C. botulinum* supernatants. To this end, blinded bacterial culture supernatants were analysed
680 from 39 *C. botulinum* strains isolated in the course of veterinary botulism outbreaks in
681 Europe. Based on the four ELISAs developed in this work, the 39 strains could clearly be
682 assigned to 6 BoNT/C-, 27 BoNT/CD-, two BoNT/D-, and four BoNT/DC-producing strains
683 (Table 5). An identical assignment was obtained by the technically independent qPCR, and
684 the presence of functionally active toxin was shown by either MBA and/or Endopep-MS
685 assay.

686 In a second step, we tested the four ELISAs for cross-reactivity against a number of other
687 *Clostridia* strains. These included *C. botulinum* Group I, II, and IV strains expressing
688 serotypes A, B, E, F, and G, their non-toxic counterparts, and strains of *C. novyi sensu lato*
689 (Table 6). All had been checked by qPCR[55] for the presence of BoNT genes, and neurotoxin
690 production was also confirmed by mouse bioassay, if applicable. All ELISA results were
691 found to be in perfect agreement with the results obtained by qPCR, with no cross-reactivity
692 observed against other bacterial supernatants.

693 While the first validation involved culture supernatants from isolated, pure strains, we went a
694 step further and tested anaerobic enrichment cultures from veterinary botulism case material
695 which represent relatively crude materials. Inoculating suspicious sample material into an
696 anaerobic enrichment culture often allows spore germination and toxin production even if
697 *C. botulinum* was present in the inoculum in low spore numbers. Such an enrichment step
698 has been aiding botulism diagnosis for a long time[84, 85] and has been proposed for the
699 detection of Group III cases either via PCR[43, 86] or by immunoassays[45]. Apart from
700 *C. botulinum*, other bacteria might also grow and release their products of metabolism into
701 the medium. Therefore such culture supernatants are more challenging because the toxin is
702 often present in lower concentrations. Moreover, the cultures contain additional compounds
703 which could possibly interfere with the assay. We analysed 23 primary enrichment cultures
704 from natural veterinary botulism cases occurring in France and Germany, all tested
705 previously by qPCR.[20, 43] All 21 avian botulism cases were found to be positive for BoNT/CD,
706 while two cases in cattle were positive for BoNT/DC in concordance with the qPCR and
707 Endopep-MS results, while no false negative results were observed (Table 7).

708 Taken together, the validation study showed a 100% concordance of results obtained by the
709 four newly developed sandwich ELISAs specific for BoNT/C, D, and their mosaic variants
710 with data obtained on genetic (qPCR) and functional level (Endopep-MS).

711 **Conclusions**

712 The majority of veterinary botulism cases are caused by *C. botulinum* Group III strains which
713 can produce the closely related toxins BoNT/C, BoNT/D, and their mosaic variants BoNT/CD
714 and BoNT/DC. Based on novel mAbs, in the current work we have established a set of
715 technically independent approaches that allow for the correct and precise determination and
716 quantification of the toxin type, including differentiation of mosaic variants at different levels.
717 Basically, the different methods are intended to be placed in an optimised workflow as step-
718 by-step approach and can be applied in routine and/or expert laboratories. (i) In a first
719 screening step, the four highly specific ELISAs deliver information on which specific
720 veterinary toxin is present in a suspicious sample and allow for its quantification down to a
721 few pg/mL. This is a clear advantage over genetic methods identifying potentially toxin-
722 containing samples, which, however, do not detect the toxin itself. The four ELISAs can be
723 readily performed in routine clinical or microbiological laboratories and do not need
724 sophisticated technical equipment. The current work has shown that the ELISAs developed
725 are highly specific, highly sensitive, and robust tools that give correct results on real
726 veterinary sample materials. (ii) In a second step, the results obtained by ELISA screening
727 can be corroborated on the functional level, showing that active toxin is present in a
728 suspicious sample. Here, one Endopep-MS format presented gives evidence for either C-
729 type or D-type activity in the sample, and this method can be used as replacement method
730 for the classical MBA in botulism diagnostics. This format is easier to perform technically and
731 requires less hands-on time (one enrichment and cleavage reaction per sample with both
732 artificial substrates to demonstrate either C-type or D-type activity) than the second
733 Endopep-MS format presented that allows for the discrimination of all four veterinary BoNTs.
734 In this second format, the veterinary toxins are captured in two parallel enrichment reactions
735 either with an anti-H_C/C or an anti-H_C/D mAb, followed by a cleavage reaction with both
736 artificial substrates (corresponding to two parallel tests per sample). As to the applicability of
737 the two Endopep-MS approaches, the first approach might be more appropriate if the overall
738 toxicity of a sample should be demonstrated, while the second approach delivers more
739 detailed information e.g. in the course of an epidemiological investigation. Due to the
740 technical equipment and expertise necessary, both methods might be restricted to expert
741 laboratories. In future, both formats could be further developed into quantitative Endopep-MS
742 methods by implementation of stable isotope-labelled peptides for quantification.[51, 87] (iii)
743 Finally, if unambiguous identification of toxin is necessary, e.g. in the case of a forensic
744 investigation, a proteomic approach can be applied: an appropriate combination of mAbs for
745 enrichment plus tryptic digest and LC-MS/MS analysis resulted in a high sequence coverage
746 of all four veterinary toxins (61–81%) from bacterial culture supernatants. This method could
747 be very helpful in case little, degraded, or no DNA is present in a suspicious sample material

748 and genomic sequencing is prevented. Alternatively, if DNA is available, this step can be
749 substituted or complemented by a sequencing approach. Again, these approaches are
750 presumably applicable in highly specialised laboratories. While the different methods (i) to
751 (iii) vary with respect to sensitivity, specificity, and unambiguousness, they are appropriate
752 for complementing each other on a technically independent level to deliver preliminary,
753 confirmed, and – all of them combined – unambiguous results. Our comprehensive validation
754 study including 39 culture supernatants from toxin-producing *C. botulinum* Group III strains,
755 13 toxin-producing *C. botulinum* Group I, II, or IV strains, 21 non-neurotoxin-producing
756 *Clostridia*, and 23 primary enrichment cultures resulted in conclusive results on all technical
757 levels. Specifically, the four differentiating ELISAs already yielded correct results on BoNT/C,
758 D, and their mosaic variants on all sample materials tested, with no false-positive or false-
759 negative results. Therefore, the differentiating ELISAs represent highly reliable, sensitive,
760 and easy screening tools for the fast analysis of veterinary botulism cases, which should aid
761 future field investigations of botulism outbreaks, the acquisition of epidemiological data, and
762 support routine veterinary quality control measures, e.g. feed lot analysis.

763

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771

772 **Author contribution**

773 E-MH, MS, TE, DS, FF, JeW, MBD and BGD designed the experiments involved in antibody
774 generation and characterization; E-MH, MS, TE, DS, FF, JeW and MBD performed the
775 experiments; MS and E-MH designed and performed the mass-spectrometry based
776 experiments; JaW and AR contributed essential recombinant proteins; WL performed the
777 statistical analysis; UM, LB, CW and PF provided essential antibodies or strains. E-MH, MS,
778 AR, MBD and BGD wrote the manuscript.

779

780

781 **References**

782

783 1. T. Binz and A. Rummel, *J. Neurochem.*, 2009, **109**, 1584-1595.
784 2. O. Rossetto, M. Pirazzini and C. Montecucco, *Nat. Rev. Microbiol.*, 2014, **12**, 535-
785 549.
786 3. E. A. Johnson and C. Montecucco, *Handb. Clin. Neurol.*, 2008, **91**, 333-368.
787 4. T. A. Roberts and A. M. Gibson, *J. Food Technol.*, 1979, **14**, 211-226.
788 5. R. Eleopra, V. Tugnoli, O. Rossetto, C. Montecucco and D. De Grandis, *Neurosci.*
789 *Lett.*, 1997, **224**, 91-94.
790 6. R. Eleopra, V. Tugnoli, R. Quatrale, O. Rossetto, C. Montecucco and D. Dressler,
791 *Neurotox. Res.*, 2006, **9**, 127-131.
792 7. D. W. Trampel, S. R. Smith and T. E. Rocke, *Avian Dis.*, 2005, **49**, 301-303.
793 8. M. Lindström, M. Nevas, J. Kurki, R. Sauna-aho, A. Latvala-Kiesila, I. Polonen and H.
794 Korkeala, *J. Clin. Microbiol.*, 2004, **42**, 4718-4725.
795 9. M. N. Egyed, A. Shlosberg, U. Klopfer, T. A. Nobel and E. Mayer, *Refuah Vet.*, 1978,
796 **35**, 93-99.
797 10. T. Rocke, K. Converse, C. Meteyer and B. McLean, *Waterbirds*, 2005, **28**, 87-94.
798 11. W.-C. Chuang, Y.-C. Hsieh, S.-H. Chen, Y.-F. Lee, K.-Y. Tsai and S.-S. Tsai, *Taiwan*
799 *Vet. J.*, 2005, **31**, 267-273.
800 12. T. M. Work, J. L. Klavitter, M. H. Reynolds and D. Blehert, *J. Wildl. Dis.*, 2010, **46**,
801 499-506.
802 13. K. Moriishi, M. Koura, N. Abe, N. Fujii, Y. Fujinaga, K. Inoue and K. Ogumad,
803 *Biochim. Biophys. Acta*, 1996, **1307**, 123-126.
804 14. H. Skarin and B. Segerman, *PLoS One*, 2014, **9**, e107777.
805 15. K. Moriishi, B. Syuto, S. Kubo and K. Oguma, *Infect. Immun.*, 1989, **57**, 2886-2891.
806 16. R. Souillard, C. Woudstra, C. Le Maréchal, M. Dia, M. H. Bayon-Auboyer, M.
807 Chemaly, P. Fach and S. Le Bouquin, *Avian Pathol.*, 2014, **43**, 458-464.
808 17. S. P. Hardy and M. Kaldhusdal, *Vet. Microbiol.*, 2013, **165**, 466-468.
809 18. K. Nakamura, T. Kohda, Y. Seto, M. Mukamoto and S. Kozaki, *Vet. Microbiol.*, 2013,
810 **162**, 881-890.
811 19. K. Nakamura, T. Kohda, K. Umeda, H. Yamamoto, M. Mukamoto and S. Kozaki, *Vet.*
812 *Microbiol.*, 2010, **140**, 147-154.
813 20. C. Woudstra, H. Skarin, F. Anniballi, L. Fenicia, L. Bano, I. Drigo, M. Koene, M. H.
814 Bayon-Auboyer, J. P. Buffereau, D. De Medici and P. Fach, *Appl. Environ. Microbiol.*,
815 2012, **78**, 3120-3127.
816 21. F. Anniballi, B. Auricchio, C. Woudstra, P. Fach, A. Fiore, H. Skarin, L. Bano, B.
817 Segerman, R. Knutsson and D. De Medici, *Biosecur. Bioterror*, 2013, **11 Suppl 1**,
818 S207-214.
819 22. K. Björnstad, A. Tevell Åberg, S. R. Kalb, D. Wang, J. R. Barr, U. Bondesson and M.
820 Hedeland, *Anal. Bioanal. Chem.*, 2014, **406**, 7149-7161.
821 23. I. Anza, H. Skarin, D. Vidal, A. Lindberg, V. Baverud and R. Mateo, *Anaerobe*, 2014,
822 **26**, 20-23.
823 24. M. Hedeland, H. Moura, V. Baverud, A. R. Woolfitt, U. Bondesson and J. R. Barr, *J.*
824 *Med. Microbiol.*, 2011, **60**, 1299-1305.
825 25. T. J. Smith, K. K. Hill, G. Xie, B. T. Foley, C. H. Williamson, J. T. Foster, S. L.
826 Johnson, O. Chertkov, H. Teshima, H. S. Gibbons, L. A. Johnsky, M. A. Karavis and
827 L. A. Smith, *Infect. Genet. Evol.*, 2015, **30**, 102-113.
828 26. M. W. Peck, *Adv. Microb. Physiol.*, 2009, **55**, 183-265, 320.
829 27. H. Skarin and B. Segerman, *Mob. Genet. Elements*, 2011, **1**, 213-215.
830 28. Y. Sakaguchi, T. Hayashi, K. Kurokawa, K. Nakayama, K. Oshima, Y. Fujinaga, M.
831 Ohnishi, E. Ohtsubo, M. Hattori and K. Oguma, *Proc. Natl. Acad. Sci. USA*, 2005,
832 **102**, 17472-17477.
833 29. H. Skarin, T. Hafstrom, J. Westerberg and B. Segerman, *BMC Genomics*, 2011, **12**,
834 185.
835 30. M. W. Eklund and F. T. Poysky, *Appl. Microbiol.*, 1974, **27**, 251-258.

836 31. M. W. Eklund, F. T. Poysky, J. A. Meyers and G. A. Pelroy, *Science*, 1974, **186**, 456-
837 458.
838 32. K. Inoue and H. Iida, *Jpn. J. Med. Sci. Biol.*, 1971, **24**, 53-56.
839 33. A. Rummel, *Curr. Top. Microbiol. Immunol.*, 2013, **364**, 61-90.
840 34. L. Peng, R. P. Berntsson, W. H. Tepp, R. M. Pitkin, E. A. Johnson, P. Stenmark and
841 M. Dong, *J. Cell Sci.*, 2012, **125**, 3233-3242.
842 35. J. Strotmeier, S. Gu, S. Jutzi, S. Mahrhold, J. Zhou, A. Pich, T. Eichner, H. Bigalke, A.
843 Rummel, R. Jin and T. Binz, *Mol. Microbiol.*, 2011, **81**, 143-156.
844 36. A. P. Karalewitz, Z. Fu, M. R. Baldwin, J. J. Kim and J. T. Barbieri, *J. Biol. Chem.*,
845 2012, **287**, 40806-40816.
846 37. N. Nuemket, Y. Tanaka, K. Tsukamoto, T. Tsuji, K. Nakamura, S. Kozaki, M. Yao and
847 I. Tanaka, *Biochem. Biophys. Res. Commun.*, 2011, **411**, 433-439.
848 38. J. Strotmeier, K. Lee, A. K. Volker, S. Mahrhold, Y. Zong, J. Zeiser, J. Zhou, A. Pich,
849 H. Bigalke, T. Binz, A. Rummel and R. Jin, *Biochem. J.*, 2010, **431**, 207-216.
850 39. Y. Zhang, G. W. Buchko, L. Qin, H. Robinson and S. M. Varnum, *Biochem. Biophys.*
851 *Res. Commun.*, 2011, **404**, 407-412.
852 40. L. Peng, W. H. Tepp, E. A. Johnson and M. Dong, *PLoS Pathog.*, 2011, **7**, e1002008.
853 41. T. Binz, *Curr. Top. Microbiol. Immunol.*, 2013, **364**, 139-157.
854 42. M. B. Dorner, K. M. Schulz, S. Kull and B. G. Dorner, *Curr. Top. Microbiol. Immunol.*,
855 2013, **364**, 219-255.
856 43. C. Woudstra, H. Skarin, F. Anniballi, B. Auricchio, D. De Medici, L. Bano, I. Drigo, T.
857 Hansen, C. Löfström, R. Hamidjaja, B. J. van Rotterdam, M. Koene, M. H. Bäyon-
858 Auboyer, J. P. Buffereau and P. Fach, *Anaerobe*, 2013, **22**, 31-37.
859 44. R. A. Hutson, Y. Zhou, M. D. Collins, E. A. Johnson, C. L. Hatheway and H.
860 Sugiyama, *J. Biol. Chem.*, 1996, **271**, 10786-10792.
861 45. C. E. Brooks, H. J. Clarke, D. A. Finlay, W. McConnell, D. A. Graham and H. J. Ball,
862 *Vet. Microbiol.*, 2010, **144**, 226-230.
863 46. F. Gessler, K. Hampe and H. Böhnel, *Appl. Environ. Microbiol.*, 2005, **71**, 7897-7903.
864 47. F. Gessler, K. Hampe, M. Schmidt and H. Böhnel, *Diagn. Microbiol. Infect. Dis.*, 2006,
865 **56**, 225-232.
866 48. R. J. Thomas, *Aust. Vet. J.*, 1991, **68**, 111-113.
867 49. S. Notermans, J. Dufrenne and S. Kozaki, *Jpn. J. Med. Sci. Biol.*, 1982, **35**, 203-211.
868 50. Y. Zhang, J. Lou, K. L. Jenko, J. D. Marks and S. M. Varnum, *Anal. Biochem.*, 2012,
869 **430**, 185-192.
870 51. H. Moura, R. R. Terilli, A. R. Woolfitt, M. Gallegos-Candela, L. G. McWilliams, M. I.
871 Solano, J. L. Pirkle and J. R. Barr, *FEMS Immunol. Med. Microbiol.*, 2011, **61**, 288-
872 300.
873 52. A. Rummel, T. Karnath, T. Henke, H. Bigalke and T. Binz, *J. Biol. Chem.*, 2004, **279**,
874 30865-30870.
875 53. A. Rummel, K. Hafner, S. Mahrhold, N. Darashchonak, M. Holt, R. Jahn, S.
876 Beermann, T. Karnath, H. Bigalke and T. Binz, *J. Neurochem.*, 2009, **110**, 1942-
877 1954.
878 54. S. Bade, A. Rummel, C. Reisinger, T. Karnath, G. Ahnert-Hilger, H. Bigalke and T.
879 Binz, *J. Neurochem.*, 2004, **91**, 1461-1472.
880 55. S. Kirchner, K. M. Krämer, M. Schulze, D. Pauly, D. Jacob, F. Gessler, A. Nitsche, B.
881 G. Dorner and M. B. Dorner, *Appl. Environ. Microbiol.*, 2010, **76**, 4387-4395.
882 56. S. Kull, K. M. Schulz, J. W. Strotmeier, S. Kirchner, T. Schreiber, A. Bollenbach, P.
883 W. Dabrowski, A. Nitsche, S. R. Kalb, M. B. Dorner, J. R. Barr, A. Rummel and B. G.
884 Dorner, *PLoS One*, 2015, **10**, e0116381.
885 57. S. Henikoff and J. G. Henikoff, *Nucleic Acids Res.*, 1991, **19**, 6565-6572.
886 58. K. Campbell, M. D. Collins and A. K. East, *Biochim. Biophys. Acta*, 1993, **1216**, 487-
887 491.
888 59. D. Pauly, S. Kirchner, B. Stoermann, T. Schreiber, S. Kaulfuss, R. Schade, R.
889 Zbinden, M. A. Avondet, M. B. Dorner and B. G. Dorner, *Analyst*, 2009, **134**, 2028-
890 2039.
891 60. F. E. Grubbs, *Ann Math Statist*, 1950, **21**, 27-58.

892 61. Ł. Komsta, *Journal*, 2011.
893 62. S. Kull, D. Pauly, B. Störmann, S. Kirchner, M. Stämmler, M. B. Dorner, P. Lasch, D.
894 Naumann and B. G. Dorner, *Anal. Chem.*, 2010, **82**, 2916-2924.
895 63. D. Wang, J. Baudys, S. R. Kalb and J. R. Barr, *Anal. Biochem.*, 2011, **412**, 67-73.
896 64. S. R. Kalb, H. Moura, A. E. Boyer, L. G. McWilliams, J. L. Pirkle and J. R. Barr, *Anal.*
897 *Biochem.*, 2006, **351**, 84-92.
898 65. Y. Sakaguchi, T. Suzuki, Y. Yamamoto, A. Nishikawa and K. Oguma, *Res. Microbiol.*,
899 2015, **166**, 318-325.
900 66. H. W. Bennetts and H. T. B. Hall, *Aust. Vet. J.*, 1938, **14**, 105-118.
901 67. J. D. Marks, *Anesthesiol. Clin. North America*, 2004, **22**, 509-532, vii.
902 68. R. M. Curran, E. Fringuelli, D. Graham and C. T. Elliott, *Vet. Immunol.*
903 *Immunopathol.*, 2009, **130**, 1-10.
904 69. L. A. Gil, C. E. da Cunha, G. M. Moreira, F. M. Salvarani, R. A. Assis, F. C. Lobato,
905 M. Mendonca, O. A. Dellagostin and F. R. Conceicao, *PLoS One*, 2013, **8**, e69692.
906 70. J. C. Lee, H. J. Hwang, Y. Sakaguchi, Y. Yamamoto, H. Arimitsu, T. Tsuji, T.
907 Watanabe, T. Ohyama, T. Tsuchiya and K. Oguma, *Microbiol. Immunol.*, 2007, **51**,
908 445-455.
909 71. C. Stahl, L. Unger, C. Mazuet, M. Popoff, R. Straub and J. Frey, *Vaccine*, 2009, **27**,
910 5661-5666.
911 72. R. P. Webb, T. J. Smith, P. M. Wright, V. A. Montgomery, M. M. Meagher and L. A.
912 Smith, *Vaccine*, 2007, **25**, 4273-4282.
913 73. D. G. Myszka, *J. Mol. Recognit.*, 1999, **12**, 279-284.
914 74. H. Volland, P. Lamourette, M.-C. Nevers, C. Mazuet, E. Ezan, L.-M. Neuburger, M.
915 Popoff and C. Créminon, *J. Immunol. Methods*, 2008, **330**, 120-129.
916 75. M. Szílagyi, V. R. Rivera, D. Neal, G. A. Merrill and M. A. Poli, *Toxicon*, 2000, **38**,
917 381-389.
918 76. S. E. Maslanka, C. Lúquez, B. H. Raphael, J. K. Dykes and L. A. Joseph, *Botulinum*
919 *J.*, 2011, **2**, 72-92.
920 77. K. L. Jenko, Y. Zhang, Y. Kostenko, Y. Fan, C. Garcia-Rodriguez, J. Lou, J. D. Marks
921 and S. M. Varnum, *Analyst*, 2014, **139**, 5093-5102.
922 78. E. A. Garber, K. V. Venkateswaran and T. W. O'Brien, *J. Agric. Food Chem.*, 2010,
923 **58**, 6600-6607.
924 79. T. E. Rocke, S. R. Smith and S. W. Nashold, *J. Wildl. Dis.*, 1998, **34**, 744-751.
925 80. C. E. Brooks, H. J. Clarke, D. A. Graham and H. J. Ball, *Vet. Rec.*, 2011, **168**, 455.
926 81. G. Sakaguchi, S. Sakaguchi, S. Kozaki, S. Sugii and I. Oishi, *Jpn. J. Med. Sci. Biol.*,
927 1974, **27**, 161-172.
928 82. D. Wang, J. Krilich, J. Baudys, J. R. Barr and S. R. Kalb, *Bioorg. Med. Chem.*, 2015,
929 **23**, 3667-3673.
930 83. D. Wang, J. Baudys, J. Rees, K. M. Marshall, S. R. Kalb, B. A. Parks, L. Nowaczyk,
931 2nd, J. L. Pirkle and J. R. Barr, *Anal. Chem.*, 2012, **84**, 4652-4658.
932 84. V. R. Dowell, Jr., L. M. McCroskey, C. L. Hatheway, G. L. Lombard, J. M. Hughes and
933 M. H. Merson, *J. Am. Med. Assoc.*, 1977, **238**, 1829-1832.
934 85. C. L. Hatheway and L. M. McCroskey, in *Biomedical aspects of botulism*, ed. G. E.
935 Lewis, Academic Press, Inc., New York, USA, 1981, pp. 165-180.
936 86. P. Fach, M. Gibert, R. Griffais and M. R. Popoff, *FEMS Immunol. Med. Microbiol.*,
937 1996, **13**, 279-285.
938 87. B. A. Parks, J. D. Shearer, J. Baudys, S. R. Kalb, D. C. Sanford, J. L. Pirkle and J. R.
939 Barr, *Anal. Chem.*, 2011, **83**, 9047-9053.

940

941

942 **Figure Legends**

943 **Figure 1:**

944 Schematic domain organisation of BoNT/C, BoNT/D, and their related mosaic variants. All
945 BoNT serotypes are composed of a light chain (LC) and a heavy chain (HC), connected via a
946 disulphide bond. The HC can be further subdivided into N-terminal translocation domain (H_N)
947 and C-terminal receptor binding domain (H_C). BoNT/C, BoNT/D, and their mosaic variants
948 BoNT/CD and BoNT/DC show a high degree of identity on the protein level. Genbank entries
949 of 18 different amino acid sequences were aligned and compared using Geneious 7.1.4. as
950 described within the Experimental section. For each of the four toxins, two to seven
951 sequences available in the database were compared with each other, and percent identity is
952 indicated. Highly homologous domains are indicated in similar colours.

953

954 **Figure 2:**

955 Immunoblots demonstrating mAb specificity. Recombinantly expressed light chains (LC) and
956 H_C fragments of BoNT/C and D were separated on 10% SDS-PAGE and transferred onto
957 PVDF membranes. The membranes were incubated with the following antibodies: C394 (A),
958 C9 (B), H_CC2378 (C), D63 (D), D967 (E), and H_CC10S (F), followed by detection with
959 biotinylated anti-mouse antibody and a streptavidin-alkaline phosphatase-conjugate and
960 CDP-Star as substrate.

961

962 **Figure 3:**

963 Highly specific sandwich ELISAs detecting and differentiating BoNT/C, CD, D, or DC. The
964 principle of the four differentiating ELISAs for the veterinary BoNTs is schematically
965 displayed in (A). The following combination of antibodies was used in an amplified sandwich
966 ELISA setting: (B) BoNT/C ELISA: mAb C394 plus biotinylated mAb C9; (C) BoNT/CD
967 ELISA: mAb C394 plus biotinylated mAb D967; (D) BoNT/D ELISA: mAb D63 plus
968 biotinylated mAb D967; and (E) BoNT/DC ELISA: mAb H_CC10S plus biotinylated mAb D63.
969 For each of the four ELISAs, the first-mentioned mAb was immobilised onto microtitre plates.
970 After blocking of non-specific binding, 200 ng/mL of each toxin or BSA (left panel) or serial
971 dilutions of the corresponding toxin (from 1 µg/mL to 1 pg/mL, right panel) were added.
972 Detection was performed using a biotinylated detection antibody as indicated above, followed
973 by streptavidin-peroxidase conjugate and TMB. For the right panel, three representative

974 measurements performed in triplicate are overlaid. The left panel summarises three
975 independent experiments.

976

977 **Figure 4:**

978 Detection and differentiation of four representative BoNT/C-, CD-, D-, and DC-producing
979 bacterial culture supernatants by sandwich ELISA and Endopep-MS reaction.
980 (A) Differentiating sandwich ELISA. Bacterial supernatants containing BoNT/C (NCTC8548),
981 BoNT/CD (C6814), BoNT/D (D4947), and BoNT/DC (Eppendorf) were tested in 1:10 dilutions
982 by the four differentiating sandwich ELISA described in Fig. 3. (B) MALDI mass spectra of
983 BoNT/C, D, and their related mosaic variants by differentiating Endopep-MS. The four
984 representative bacterial supernatants described above were enriched by mAb H_CC2378
985 directed against the H_C of BoNT/C and BoNT/DC (α-H_C/C) and in a second independent
986 reaction by mAb D967 directed against H_C of BoNT/D and BoNT/CD (α-H_C/D).
987 Immunocaptured toxins were incubated with synthetic SNAP-25 (C-Sub$^+$, *m/z* 2911) and
988 VAMP2-based (D-Sub$^+$, *m/z* 4496) peptide substrates to detect the LC proteolytic activity of
989 BoNT/C, CD, or BoNT/D, DC, respectively. Samples containing active BoNT/C and CD lead
990 to specific *C*-terminal (C-CT) and *N*-terminal (C-NT) cleavage peptide products at *m/z* 1059
991 and *m/z* 1871, whereas BoNT/D and DC cleavage products are detected at *m/z* 1217 (D-CT)
992 and *m/z* 3297 (D-NT).

993

994 **Graphical entry figure:**

995 Novel monoclonal antibodies enable highly specific immunoassays for the detection,
996 differentiation, and quantification of related botulinum neurotoxin serotypes C, CD, D, and
997 DC followed by mass-spectrometry-based functional characterisation and identification.

998 **Table 1. Generation of monoclonal antibodies specific for fragments of BoNT/C and D.**

Fusion No.	Mouse strain	Immunisation	Hybridoma supernatants tested	Hybridoma subcloned	Hybridoma clones isolated*
1	BALB/c	scBoNT/C_i, scBoNT/D_i	5366	21	**C394**, **D63**, C1352, C2574, C1916
2	BALB/c	scBoNT/C_i, scBoNT/D_i, H_C/C, H_C/D	1450	18	**D967**
3	NMRI	H_C/C	19	1	**C9**
4	BALB/c	H_C/C	4700	15	**H_CC2378**, H_CC141, H_CC515, H_CC1304

999 * The indicated hybridoma were characterised in detail in Table 2; hybridoma clones indicated in bold
1000 were used for setting up different ELISAs to detect BoNT/C, D, and their mosaic variants in Figure 3 or
1001 for Endopep-MS analysis.

1002

1003

1004 **Table 2. Characterisation of mAb generated against fragments of BoNT/C and D.**

Antibody	Species	Isotype	Affinity K_D [M]	Source	Specificity*			
					BoNT/C		**BoNT/D**	
					LC	**H_C**	**LC**	**H_C**
C394	mouse	IgG2b, λ	$< 10^{-12}$ (a)	RKI	+			
C9	mouse	IgG2b, κ	4×10^{-10} (a)	RKI		+		
H_CC10S	mouse	IgG1, κ	1×10^{-10} (a)	CEA		+		
H_CC2378	mouse	IgG1,κ	2×10^{-10} (a)	RKI		+		
D63	mouse	IgG2b, κ	4×10^{-10} (b)	RKI			+	
D967	mouse	IgG1, λ	1×10^{-10} (b)	RKI				+
C1916	mouse	IgG1, κ	6×10^{-12} (a)	RKI	+			
C1352	mouse	IgG2b, κ	5×10^{-10} (a)/ 1×10^{-7} (b)	RKI		+		+
C2574	mouse	IgG1, κ	2×10^{-9} (a)/ 7×10^{-8} (b)	RKI		+		+
H_CC141	mouse	IgG1, κ	4×10^{-11} (a)	RKI		+		
H_CC515	mouse	IgG2b, κ	5×10^{-10} (a)	RKI		+		
H_CC1304	mouse	IgG1, κ	1×10^{-9} (a)	RKI		+		

1005 * Specificity is shown as derived from indirect ELISA and Western blot experiments on recombinant
1006 fragments of BoNT/C or BoNT/D (see Figure 2). Affinity was measured by surface plasmon resonance
1007 on (a) scBoNT/C or (b) scBoNT/D_i, respectively. Hybridoma clones indicated in bold were used for
1008 setting up different ELISAs to detect BoNT/C, D, and their mosaic variants in Figure 3 or for Endopep-
1009 MS analysis. RKI: Robert Koch Institute; CEA: Commissariat à l'énergie atomique et aux énergies
1010 alternatives
1011
1012
1013

1014 **Table 3. Validation of sandwich ELISA specific for BoNT/C, CD, D, or DC.**

ELISA*	antigen	LOD [pg/mL]	LLOQ [ng/mL]	ULOQ [ng/mL]	EC_{50} [ng/mL]	CV% intra at EC_{50}	CV% inter at EC_{50}
C394 vs. C9-bio	BoNT/C	11.5	0.4	4.4	1.3	5.8	24.4
C394 vs. D967-bio	scBoNT/CD	23.8	0.2	2.4	0.7	3.3	17.1
D63 vs. D967-bio	scBoNT/D	2.1	0.2	2.5	0.7	3.6	8.2
H_CC10 vs. D63-bio	BoNT/DC	16.1	0.3	4.3	1.1	6.4	24.0

1015 * For each specific ELISA, the LOD determined on purified or recombinant toxins, the lower and upper
1016 limit of quantification, the EC_{50}, as well as coefficients of variation are shown. Indicated in the first
1017 place is the mAb used as capture antibody, and in the second place the mAb used as biotinylated
1018 (bio) detection antibody. LOD: Limit of detection; LLOQ: Lower limit of quantification; ULOQ: Upper
1019 limit of quantification; EC_{50}: half maximal effective concentration; CV: coefficient of variation
1020 determined as intra-assay precision and inter-assay precision at the EC_{50}, respectively.

1021

Table 4. Exemplary analysis of bacterial culture supernatants analysed in detail by sandwich ELISA, real-time quantitative PCR, MBA, different Endopep-MS formats, and LC-MS/MS analysis.

		Results of serotyping as obtained by different methods					
BoNT serotype	**Strain designation**	**ELISA**[a]	**qPCR**[b]	**MBA**[c]	**Endopep-MS (LC activity)**[d]	**Endopep-MS (differentiation)**[e]	**LC-MS/MS**[f]
C	NCTC8548	**C**	C	+	C-type	C	BoNT/C (81%)
CD	C6814	**CD**	CD	+	C-type	CD	BoNT/CD (61%)
D	D4947	**D**	D	+	D-type	D	BoNT/D (65%)
DC	Eppendorf	**DC**	DC	+	D-type	DC	BoNT/DC (71%)

[a] Serotype identified by application of the four ELISAs developed in this work. Quantification of the representative supernatants yielded: 1.8 mg/mL (NCTC8548), 0.4 mg/mL (CD6814), 6.7 mg/mL (D4947), and 11.1 mg/mL (Eppendorf) (mean of three independent measurements).

[b] Real-time PCR according to Woudstra *et al.*[43]

[c] Supernatant tested positive by mouse bioassay (MBA), no serotyping performed.

[d] Endopep-MS assay based on immunoaffinity enrichment using LC-specific mAb C394 specific for the LC of BoNT/C and CD and mAb D63 specific for BoNT/D and DC, respectively. This type of assay delivers information on the C-type cleavage activity (without discrimination of C and CD) or D-type cleavage activity (without discrimination of D and DC).

[e] Endopep-MS assay based on immunoaffinity enrichment using H_C-specific mAbs H_CC2378 or D967, respectively. This type of assay allows discrimination of BoNT/C, D, and their respective mosaic variants on a functional level.

[f] Protein sequence coverage obtained by enrichment with mAbs C394 or D63 followed by tryptic digest and LC-MS/MS analysis using an Orbitrap instrument (for more information on sequence coverage, see Supplementary Information Fig. S1).

1045 **Table 5. Bacterial culture supernatants from a panel of *C. botulinum* strains tested by**
1046 **sandwich ELISA, real-time quantitative PCR, MBA, and Endopep-MS.**

Lab. no.	Source	ELISA[a]	qPCR[b]	MBA[c]	Endopep-MS[d] (LC activity)
14-044	strain	**C**	C	+	C-type
14-045	strain	**C**	C	+	C-type
14-046	strain	**C**	C	+	C-type
14-047	strain	**C**	C	+	C-type
NCTC8548	strain	**C**	C	+	C-type
12-149-01	strain	**C**	C	n.a.	C-type
14-048	poultry	**CD**	CD	+	C-type
14-049	duck, swab	**CD**	CD	+	C-type
14-050	turkey, swab	**CD**	CD	+	C-type
14-051	turkey, spleen	**CD**	CD	+	C-type
14-052	poultry	**CD**	CD	+	C-type
14-053	unknown	**CD**	CD	+	C-type
14-054	chicken	**CD**	CD	+	C-type
14-055	chicken	**CD**	CD	+	C-type
14-056	duck, faeces	**CD**	CD	+	C-type
14-057	chicken	**CD**	CD	+	C-type
14-058	unknown	**CD**	CD	n.a.	C-type
14-059	poultry	**CD**	CD	n.a.	C-type
14-060	poultry	**CD**	CD	n.a.	C-type
14-061	poultry	**CD**	CD	n.a.	C-type
14-062	poultry	**CD**	CD	+	C-type
14-063	poultry	**CD**	CD	+	C-type
14-064	poultry	**CD**	CD	+	C-type
14-065	turkey, faeces	**CD**	CD	+	C-type
14-066	duck, faeces	**CD**	CD	+	C-type
14-067	poultry	**CD**	CD	+	C-type
14-068	poultry	**CD**	CD	+	C-type
14-069	duck, faeces	**CD**	CD	+	C-type
14-070	poultry	**CD**	CD	+	C-type
C6814	strain	**CD**	CD	+	C-type
14-006-01	cattle	**CD**	CD	n.a.	C-type
14-007-01	cattle	**CD**	CD	n.a.	C-type
14-008-01	cattle	**CD**	CD	n.a.	C-type
14-071	unknown	**D**	D	+	D-type
D4947	strain	**D**	D	+	D-type
14-072	bovine, faeces	**DC**	DC	+	D-type
14-073	bovine, faeces	**DC**	DC	+	D-type
14-074	bovine, faeces	**DC**	DC	+	D-type
Eppendorf	strain	**DC**	DC	+	D-type

1047 [a] Serotype identified by application of the four ELISAs developed in this work. [b] Real-time PCR according to Woudstra *et al.*[43].
1048 [c] Mouse bioassay (MBA, no serotyping performed). n.a.: not analysed. [d] Endopep-MS assay to determine LC activity (see Table
1049 4 for details).

1050 **Table 6. Specificity of ELISA against supernatants of related *Clostridia* species tested**
1051 **by sandwich ELISA, real-time quantitative PCR, and MBA.**

Strain name	Species	Results of serotyping obtained by different methods		
		ELISA[a]	qPCR[b]	MBA[c]
NCTC 7272	*C. botulinum* (Group I)	–	A	+
NCTC 2012	*C. botulinum* (Group I)	–	A	+
Chemnitz	*C. botulinum* (Group I)	–	A	+
Friedrichshain	*C. botulinum* (Group I)	–	A	+
NCTC7273	*C. botulinum* (Group I)	–	B	+
2293	*C. botulinum* (Group I)	–	B	+
KL34/08	*C. botulinum* (Group I)	–	B	+
Wittenmoor	*C. botulinum* (Group II)	–	E	+
Beluga	*C. botulinum* (Group II)	–	E	n.a.
REB1718B	*C. botulinum* (Group II)	–	E	+
H092.2.01	*C. botulinum* (Group II)	–	E	+
NCTC10281	*C. botulinum* (Group I)	–	F	+
CECT4615	*C. botulinum* (Group IV)	–	G	n.a.
13-119-01	*C. baratii*	–	–	n.a.
H173852A-01	*C. berijerinckii*	–	–	n.a.
11-053-20	*C. botulinum, Group II atoxic*	–	–	n.a.
13-028-01	*C. butyricum*	–	–	n.a.
13-040-01	*C. butyricum*	–	–	n.a.
13-118-01	*C. bifermentans*	–	–	n.a.
14-005-05	*C. difficile*	–	–	n.a.
12-108-01	*C. glycolicum*	–	–	n.a.
13-159-02	*C. hiranonis*	–	–	n.a.
12-109-01	*C. innocuum*	–	–	n.a.
12-019-01	*C. novyi*	–	–	n.a.
12-135-05	*C. paraputrificum*	–	–	n.a.
14-003-01	*C. perfringens*	–	–	n.a.
13-123-04	*C. sartagoforme*	–	–	n.a.
12-007	*C. scindens*	–	–	n.a.
09-038	*C. septicum*	–	–	n.a.
13-039-01	*C. sordellii*	–	–	n.a.
DSM1734	*C. sporogenes*	–	–	n.a.
13-151-02	*C. tertium*	–	–	n.a.
14-081-01	*C. subterminale*	–	–	n.a.
3642	*C. tetani*	–	–	n.a.

1052 [a] tested by application of the four ELISAs developed in this work.
1053 [b] RT-PCR according to Kirchner *et al.*[55] (serotypes BoNT/A, B, E, and F).
1054 [c] Mouse bioassay (MBA).
1055 n.a.: not analysed.

1056 **Table 7. Primary enrichment cultures of veterinary botulism cases tested by sandwich**
1057 **ELISA, real-time quantitative PCR, and Endopep-MS.**

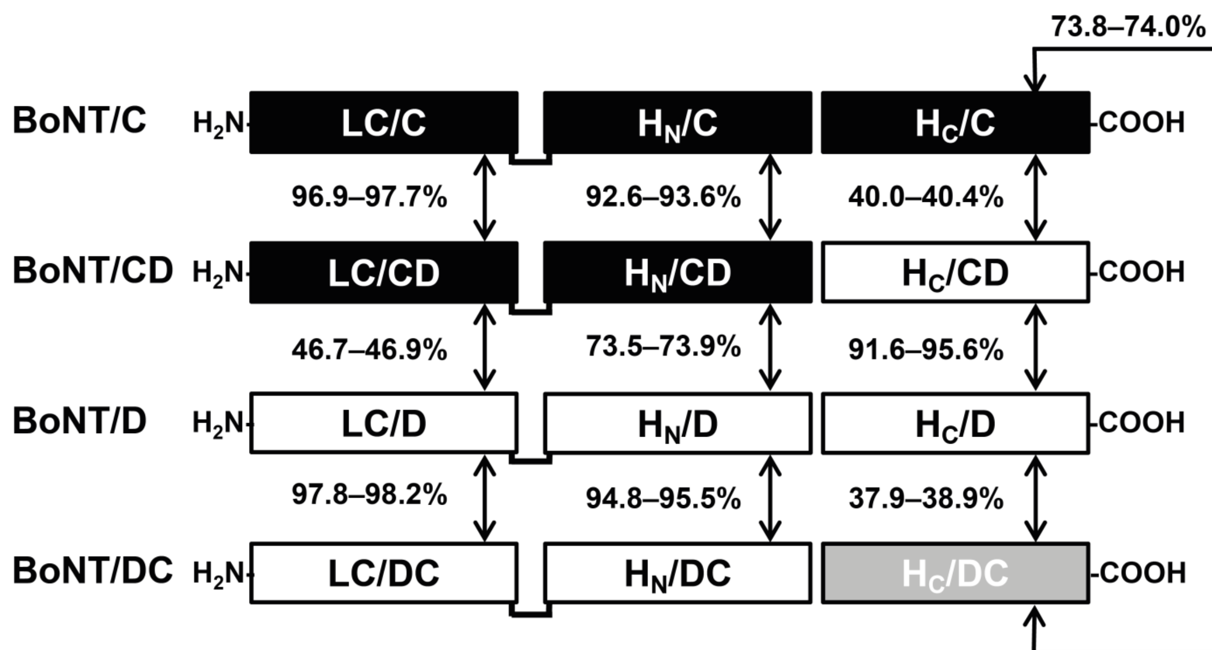
1058

		Results of serotyping obtained by different methods		
Lab. no.	**Source of enrichment culture**	**ELISA[a]**	**qPCR[b]**	**Endopep-MS (LC activity)[c]**
14-220	duck, swab	**CD**	CD	C-type
14-221	turkey, spleen	**CD**	CD	C-type
14-222	chicken, intestine	**CD**	CD	C-type
14-223	turkey, liver	**CD**	CD	C-type
14-225	chicken, faeces	**CD**	CD	C-type
14-226	chicken	**CD**	CD	C-type
14-228	chicken	**CD**	CD	C-type
14-229	duck, faeces	**CD**	CD	C-type
14-230	chicken	**CD**	CD	C-type
14-231	chicken, faeces	**CD**	CD	C-type
14-232	chicken, faeces	**CD**	CD	C-type
14-233	chicken	**CD**	CD	C-type
14-234	chicken	**CD**	CD	C-type
14-235	chicken	**CD**	CD	C-type
14-236	guinea fowl, faeces	**CD**	CD	C-type
14-237	duck, faeces	**CD**	CD	C-type
14-238	turkey	**CD**	CD	C-type
14-240	chicken	**CD**	CD	C-type
14-241	chicken	**CD**	CD	C-type
14-242	chicken	**CD**	CD	C-type
14-243	duck, faeces	**CD**	CD	C-type
14-224	bovine, faeces	**DC**	DC	D-type
14-239	bovine, faeces	**DC**	DC	D-type

1059 [a] Serotype identified by application of the four ELISAs developed in this work.
1060 [b] Real-time PCR according to Woudstra *et al.*[43].
1061 [c] Endopep-MS assay to determine LC activity (see Table 4 for details).
1062

1063 **Figure 1**

1064



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1067 Figure 2

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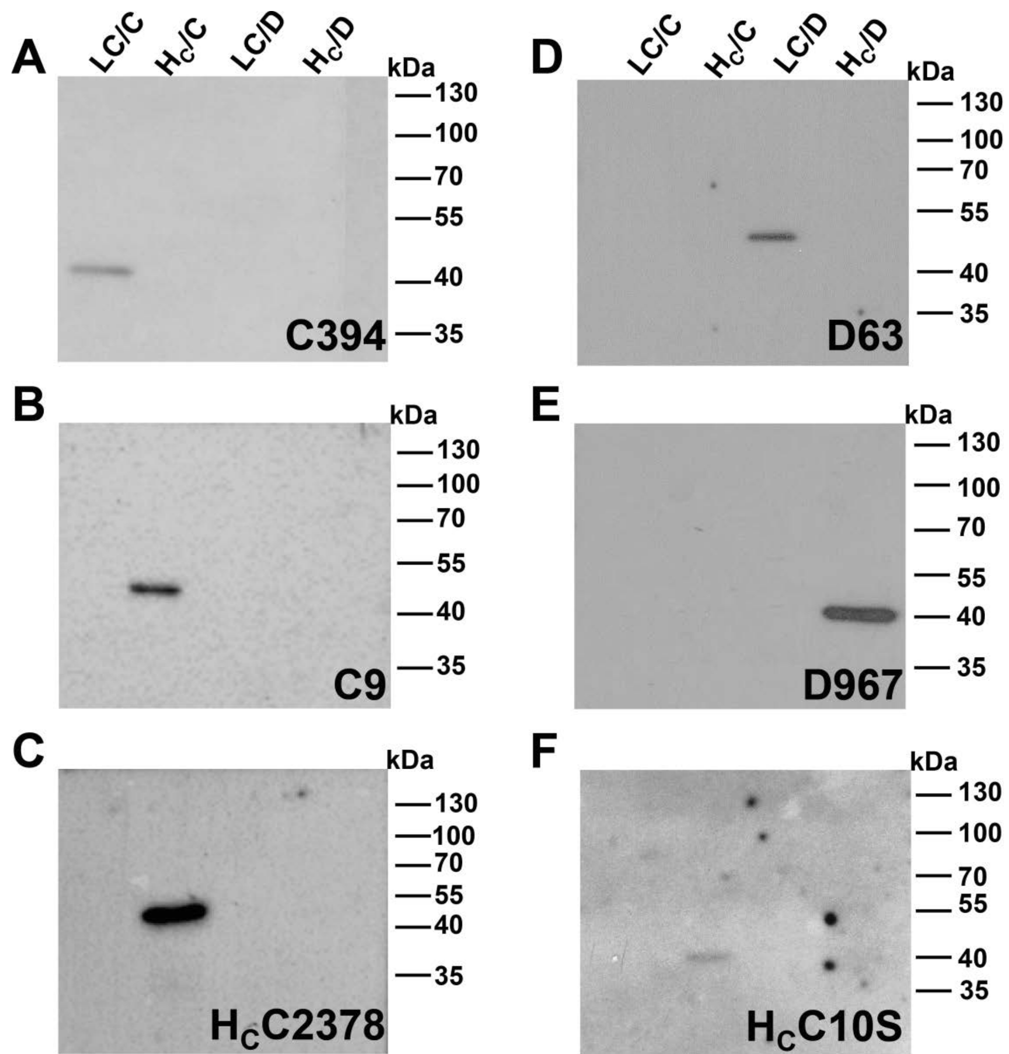

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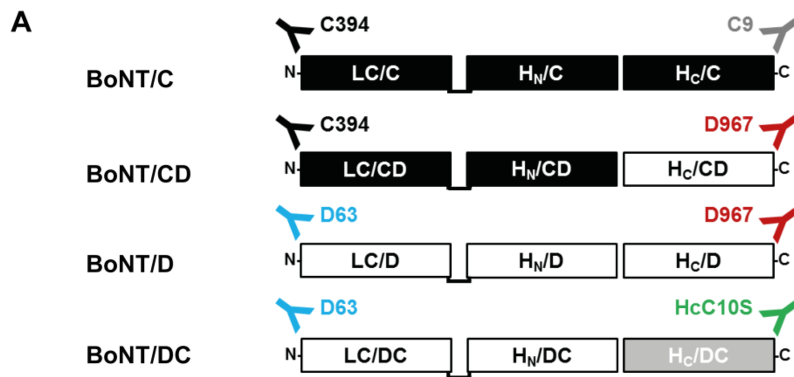
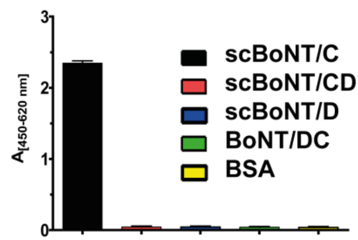
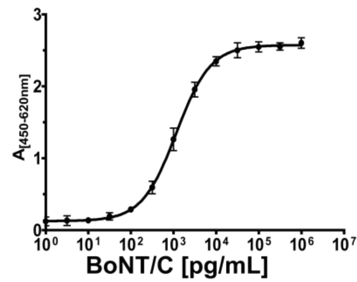
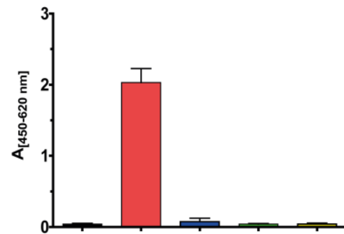
1071 **Figure 3**

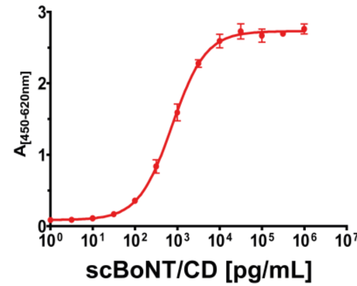
1072

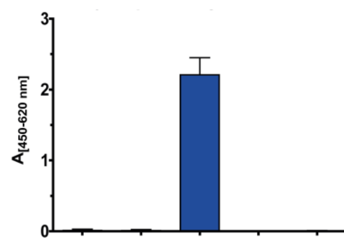


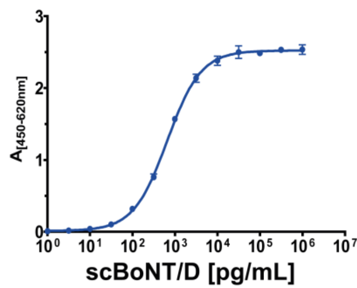


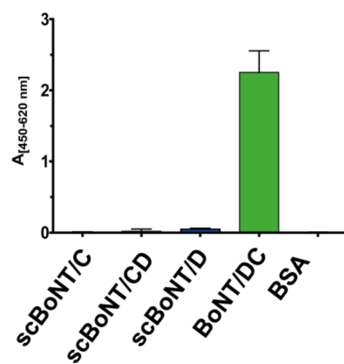


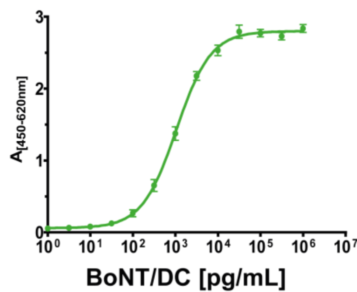












1073

1074 **Figure 4**

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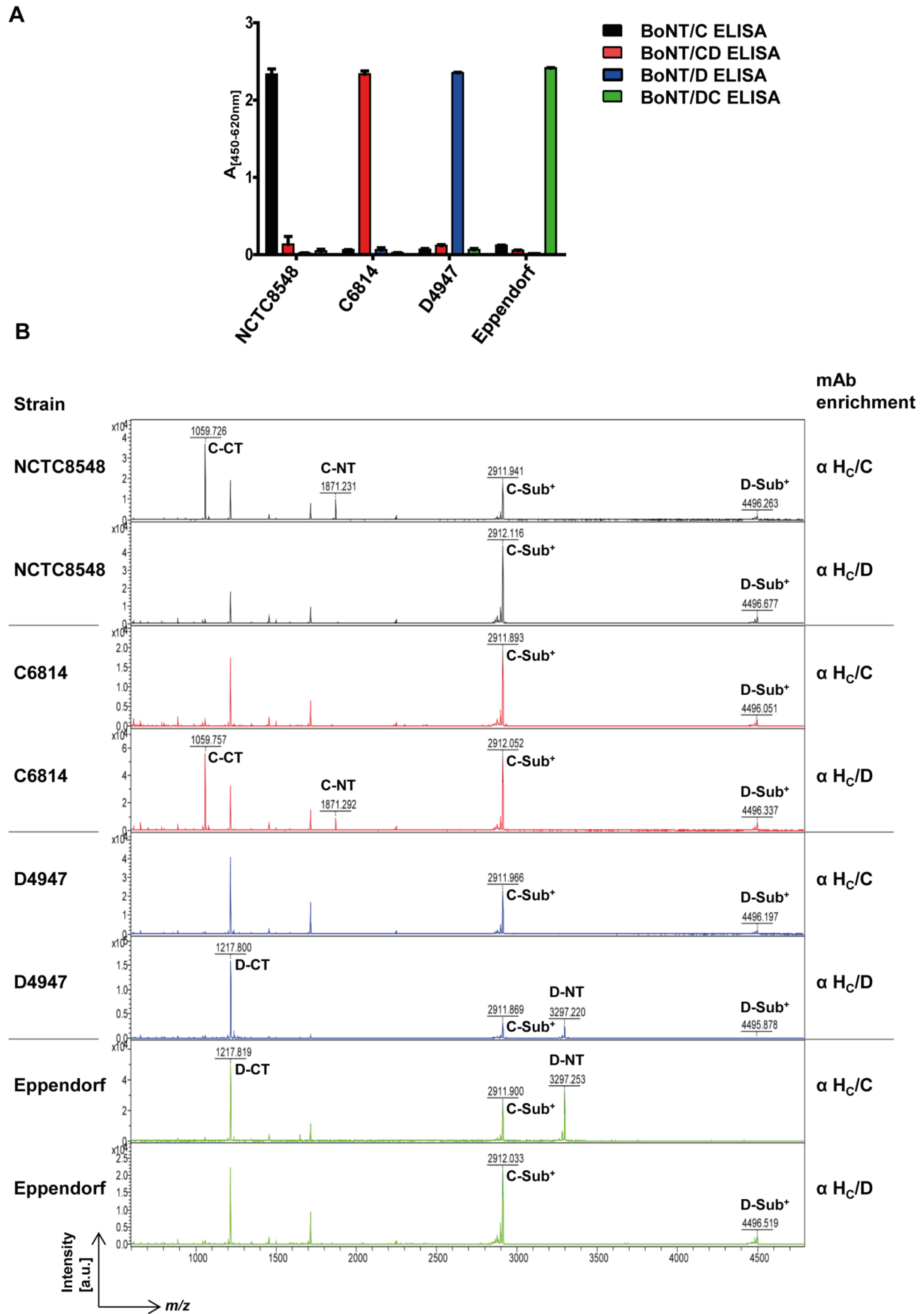

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Detection, differentiation, and identification of botulinum neurotoxin serotypes C, CD, D, and DC by highly specific immunoassays and mass spectrometry

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Supplementary Fig: S1

A) Amino acid sequence coverage of BoNT/C after tryptic digest and tandem mass spectrometry analysis

1 M**PITINNFNY SDPVDNKNIL YLDTHLNTLA NEPEKAFRIT GNIWVIPDR**F

51 SR**NSNPNLNK PPR**VTSPK**SG YYDPNYLSTD SDKDTFLKEI IK**LFK**RINSR**

101 **EIGEELIYRL STDIPFPGNN NTPINTFDFD VDFNSVDVKT RQGNNWVKTG**

151 **SINPSVIITG PRENIIDPET STFKLTNNTF AAQEGFGALS IISISPRFML**

201 **TYSNATNDVG EGR**FSKSEFC MDPILILMHE LNHAMHNLYG IAIPNDQTIS

251 SVTSNIFYSQ YNVK**LEYAEI YAFGGPTIDL IPK**SAR**KYFE EKALDYYR**SI

301 AK**RLNSITTA NPSSFNKYIG EYKQKLIR**KY R**FVVESSGEV TVNR**NKFVEL

351 YNELTQIFTE FNYAK**IYNVQ NRKIYLSNVY TPVTANILDD NVYDIQNGFN**

401 **IPKSNLNVLF MGQNLSRNPA LRKVNPENML YLFTK**FCHKA IDGRSLYNK**T**

451 **LDCRELLVKN TDLPFIGDIS DVKTDIFLRK DINEETEVIY YPDNVSVDQV**

501 **ILSKNTSEHG QLDLLYPSID SESEILPGEN QVFYDNRTQN VDYLNSYYYL**

551 **ESQKLSDNVE DFTFTRSIEE ALDNSAKVYT YFPTLANK**VN AGVQGGLFLM

601 WANDVVEDFT TNILR**KDTLD KISDVSAIIP YIGPALNISN SVRR**GNFTEA

651 FAVTGVTILL EAFPEFTIPA LGAFVIYSK**V QERNEIIKTI DNCLEQR**IKR

701 **WKDSYEWMMG TWLSRIITQF NNISYQMYDS LNYQAGAIKA KIDLEYKKYS**

751 **GSDKENIKSQ VENLKNSLDV KISEAMNNIN K**FIR**ECSVTY LFKNMLPKVI**

801 **DELNEFDRNT K**AK**LINLIDS HNIILVGEVD KLKAKVNNSF QNTIPFNIFS**

851 **YTNNSLLKDI INEYFNNIND SKILSLQNRK NTLVDTSGYN AEVSEEGDVQ**

901 **LNPIFPFDFK LGSSGEDRGK** VIVTQNENIV YNSMYESFSI SFWIR**INKWV**

951 **SNLPGYTIID SVK**NNSGWSI GIISNFLVFT LK**QNEDSEQS INFSYDISNN**

1001 **APGYNK**WFFV TVTNNMMGNM K**IYINGKLID TIKVKELTGI NFSKTITFEI**

1051 **NKIPDTGLIT SDSDNINMWI RDFYIFAKEL DGKDINILFN SLQYTNVVKD**

1101 **YWGNDLRYNK EYYMVNIDYL NRYMYANSRQ IVFNTRRNNN DFNEGYKIII**

1151 **KRIRGNTNDT RVRGGDILYF DMTINNKAYN LFMKNETMYA DNHSTEDIYA**

1201 **IGLREQTKDI NDNIIFQIQP MNNTYYYASQ IFKSNFNGEN ISGICSIGTY**

1251 **RFRLGGDWYR HNYLVPTVKQ GNYASLLEST STHWGFVPVS E**

B) Amino acid sequence coverage of BoNT/CD after tryptic digest and tandem mass spectrometry analysis

1 M**PITINNFNY SDPVDNKNIL YLDTHLNTLA NEPEKAFRII GNIWVIPDRF**

51 **SRDSNPNLNK PPR**VTSPK**SG YYDPNYLSTD SEKDTFLKEI IK**LFK**RINSR**

101 **EIGEELIYRL ATDIPFPGNN NTPINTFDFD VDFNSVDVK**T R**QGNNWVKTG**

151 **SINPSVIITG PRENIIDPET STFKLTNNTF AAQEGFGALS IISISPRFML**

201 **TYSNATNNVG EGR**FSKSEFC MDPILILMHE LNHAMHNLYG IAIPNDQR**IS**

251 **SVTSNIFYSQ YNVKLEYAEI YAFGGPTIDL IPK**SAR**KYFE EKALDYYR**SI

301 AK**RLNSITTA NPSSFNKYIG EYK**QKLIRKY R**FVVESSGEV AVDRNKFAEL**

351 **YKELTQIFTE FNYAKIYNVQ NRKIYLSNVY TPVTANILDD NVYDIQNGFN**

401 **IPKSNLNVLF MGQNLSRNPA LRKVNPENML YLFTK**FCHKA IDGRSLYNKT

451 LDCR**ELLVKN TDLPFIGDIS DIKTDIFLSK DINEETEVID YPDNVSVDQV**

501 **ILSKNTSEHG QLDLLYPIIE GESQVLPGEN QVFYDNRTQN VDYLNSYYYL**

551 **ESQKLSDNVE DFTFTTSIEE ALDNSGKVYT YFPK**LADKVN TGVQGGLFLM

601 WANDVVEDFT TNILR**KDTLD KISDVSAIIP YIGPALNISN SVR**RGNFTEA

651 FAVTGVTILL EAFQEFTIPA LGAFVIYSK**V QERNEIIK**TI DNCLEQRIKR

701 **WKDSYEWMIG TWLSRITTQF NNISYQMYDS LNYQADAIKD KIDLEYKKYS**

751 **GSDKENIKSQ VENLKNSLDI KISEAMNNIN K**FIRECSVTY LFK**NMLPKVI**

801 **DELNKFDLKT KTELINLIDS HNIILVGEVD RLK**AK**INESF ENTIPFNIFS**

851 **YTNNSLLK**DI INEYFNSIND SK**ILSLQNKK NALVDTSGYN AEVR**LEGDVQ

901 VNTIYTNDFK LSSSGDKIIV NLNNNILYSA IYENSSVSFW IKISKDLTNS

951 HNEYTIINSI KQNSGWKLCI R**NGNIEWILQ DINR**KYKSLI FDYSESLSHT

1001 GYTNKWFFVT ITNNIMGYMK LYINGELKQS ER**IEDLDEVK LDK**TIVFGID

1051 ENIDENQMLW IRDFNIFSKE LSNEDINIVY EGQILRNVIK DYWGNPLKFD

1101 TEYYMINYNY IDRYIAPK**NN ILVLVQYSDI SK**LYTKNPIT IK**SAANKNPY**

1151 **SRILNGDDIM FHMLYDSR**EY MIIRDTDTIY ATQGGQCSKN CVYALKLQSN

1201 LGNYGIGIFS IKNIVSQNKY CSQIFSSFMK NTMLLADIYK PWRFSFENAY

1251 TPVAVTNYET K**LLSTSSFWK** FISRDPGWVE

C) Amino acid sequence coverage of BoNT/D after tryptic digest and tandem mass spectrometry analysis

1 M**TWPVKDFNY SDPVNDNDIL YLR**IPQNK**LI TTPVKAFMIT QNIWVIPERF**
51 **SSDTNPSLSK PPRPTSKYQS YYDPSYLSTD EQKDTFLK**GI IKLFKRINER
101 DIGK**KLINYL VVGSPFMGDS STPEDTFDFT RHTTNIAVEK FENGSWK**VTN
151 IITPSVLIFG PLPNILDYTA SLTLQGQQSN PSFEGFGTLS ILK**VAPEFLL**
201 **TFSDVTSNQS SAVLGK**SIFC MDPVIALMHE LTHSLHQLYG INIPSDKRIR
251 PQVSEGFFSQ DGPNVQFEEL YTFGGLDVEI IPQIERSQLR **EKALGHYKDI**
301 **AKRLNNINKT IPSSWISNID K**YK**KIFSEKY NFDKDNTGNF VVNIDK**FNSL
351 YSDLTNVMSE VVYSSQYNVK **NRTHYFSRHY LPVFANILDD NIYTIRDGFN**
401 **LTNKGFNIEN SGQNIERNPA LQKLSSESVV DLFTK**VCLRL TK**NSRDDSTC**
451 **IK**VK**NNRLPY VADKDSISQE IFENK**IITDE TNVQNYSDKF SLDESILDGQ
501 VPINPEIVDP LLPNVNMEPL NLPGEEIVFY DDITK**YVDYL NSYYYLESQK**
551 **LSNNVENITL TTSVEEALGY SNKIYTFLPS LAEK**VNKGVQ AGLFLNWANE
601 VVEDFTTNIM K**KDTLDKISD VSVIIPYIGP ALNIGNSALR** GNFNQAFATA
651 GVAFLLEGFP EFTIPALGVF TFYSSIQER**E KIIKTIENCL EQR**VKR**WKDS**
701 **YQWMVSNWLS RITTQFNHIN YQMYDSLSYQ ADAIKAKIDL EYKKYSGSDK**
751 **ENIKSQVENL KNSLDVKISE AMNNINK**FIR **ECSVTYLFKN MLPKVIDELN**
801 **KFDLRTKTEL INLIDSHNII LVGEVDRLKA KVNESFENTM PFNIFSYTNN**
851 **SLLKDIINEY FNSINDSKIL SLQNKKNALV DTSGYNAEVR VGDNVQLNTI**
901 **YTNDFK**LSSS GDKIIVNLNN NILYSAIYEN SSVSFWIK**IS KDLTNSHNEY**
951 **TIINSIEQNS GWK**LCIR**NGN IEWILQDVNR K**YK**SLIFDYS ESLSHTGYTN**
1001 **KWFFVTITNN IMGYMKLYIN GELKQSQKIE DLDEVKLDK**T IVFGIDENID
1051 ENQMLWIR**DF NIFSKELSNE DINIVYEGQI LRNVIKDYWG NPLKFDTEYY**
1101 **IINDNYIDRY IAPESNVLVL VQYPDR**SK**LY TGNPITIKSV SDKNPYSRIL**
1151 **NGDNIILHML YNSRKYMIIR** DTDTIYATQG GECSQNCVYA LK**LQSNLGNY**
1201 **GIGIFSIK**NI VSKNKYCSQI FSSFRENTML LADIYKPWRF SFK**NAYTPVA**
1251 **VTNYETKLLS TSSFWK**FISR DPGWVE

D) Amino acid sequence coverage of BoNT/DC after tryptic digest and tandem mass spectrometry analysis

1 M**TWPVKDFNY SDPVNDNDIL YLRIPQNKLI TTPVKAFMIT QNIWVIPERF**

51 **SSDTNPSLSK PPRPTSKYQS YYDPSYLSTD EQKDTFLK**GI IKLFK**RINER**

101 **DIGKKLINYL VVGSPFMGDS STPEDTFDFT RHTTNIAVEK FENGSWK**VTN

151 IITPSVLIFG PLPNILDYTA SLTLQGQQSN PSFEGFGTLS ILK**VAPEFLL**

201 **TFSDVTSNQS SAVLGK**SIFC MDPVIALMHE LTHSLHQLYG INIPSDKR**IR**

251 **PQVSEGFFSQ DGPNVQFEEL YTFGGSDVEI IPQIER**LQLR **EKALGHYKDI**

301 **AKRLNNINKT IPSSWSSNID KYKKIFSEKY NFDKDNTGNF VVNIDKFNSL**

351 **YSDLTNVMSE VVYSSQYNVK NRTHYFSK**HY LPVFANILDD NIYTIINGFN

401 LTTK**GFNIEN SGQNIERNPA LQKLSSESVV DLFTK**VCLRL TR**NSRDDSTC**

451 **IQVK**NNTLPY VADKDSISQE IFESQIITDE TNVENYSDNF SLDESILDAK

501 **VPTNPEAVDP LLPNVNMEPL NVPGEEEVFY DDITKDVDYL NSYYYLEAQK**

551 **LSNNVENITL TTSVEEALGY SNKIYTFLPS LAEKVNK**GVQ AGLFLNWANE

601 VVEDFTTNIM K**KDTLDKISD VSAIIPYIGP ALNIGNSALR** GNFKQAFATA

651 GVAFLLEGFP EFTIPALGVF TFYSSIQER**E KIIKTIENCL EQR**VKR**WKDS**

701 **YQWMVSNWLS RITTQFNHIS YQMYDSLSYQ ADAIKAKIDL EYKKYSGSDK**

751 **ENIKSQVENL KNSLDVKISE AMNNINK**FIR ECSVTYLFK**N MLPKVIDELN**

801 **KFDLKTKTEL INLIDSHNII LVGEVDRLKA KVNESFENTI PFNIFSYTNN**

851 **SLLKDMINEY FNSINDSKIL SLQNKKNTLM DTSGYNAEVR VEGNVQLNPI**

901 **FPFDFKLGSS GDDRGK**VIVT QNENIVYNAM YESFSISFWI R**INKWVSNLP**

951 **GYTIIDSVK**N NSGWSIGIIS NFLVFTLK**QN ENSEQDINFS YDISK**NAAGY

1001 NKWFFVTITT NMMGNMMIYI NGK**LIDTIKV KELTGINFSK TITFQMNKIP**

1051 **NTGLITSDSD NINMWIRDFY IFAKELDDKD INILFNSLQY TNVVKDYWGN**

1101 **DLRYDKEYYM INVNYMNR**YM SK**KGNGIVFN TRKNNNDFNE GYKIIIKR**II

1151 GNTNDTR**VRG ENVLYFNTTI DNKQYSLGMY KPSRNLGTDL VPLGALDQPM**

1201 **DEIRK**YGSFI IQPCNTFDYY ASQLFLSSNA TTNR**IGILSI GSYSFKLGDD**

1251 **YWFNHEYLIP VIKIEHYASL LESTSTHWVF VPASE**

Amino acid sequence coverage of immunoextracted BoNT/C, CD, D, and DC after tryptic digest and liquid chromatography-tandem mass spectrometry analysis. Culture supernatants of *Clostridium botulinum* strains producing BoNT/C (NCTC8548) and BoNT/CD (C6814) were enriched by mAb C394 and BoNT/D (D4947) and BoNT/DC (Eppendorf) were enriched by mAb D63 (both mAb are directed against the light chain of the toxins, see Figure 2). Immunocaptured toxins were digested by trypsin and resulting peptides were analysed by LC-MS/MS. MS/MS evidence was obtained for amino acids marked in red comprising 81% sequence coverage for BoNT/C (Q93HT3_CLOBO) (A), 61% for BoNT/CD (Q5DW55_CLOBO) (B), 65% for BoNT/D (BXD_CLOBO) (C), and 71% for BoNT/DC (C6KZT4_CLOBO) (D).