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26 Abstract

27 Monoclonal antibodies (MAbs) obtained from a mouse immunised with *Clostridium*
28 *botulinum* type D toxoid were developed into a sandwich ELISA (sELISA) format that
29 was able to detect type D toxin and types C and D toxin complexes. The sELISA was
30 examined for its potential to replace the mouse bioassay as an alternative *in vitro* assay
31 for the diagnosis of cattle botulism. Its application directly to intestinal samples collected
32 from suspect cattle botulism cases and prepared for testing for the standard mouse
33 bioassay showed poor correlation and sensitivity with the mouse bioassay results.
34 However, anaerobic pre-enrichment of the samples after heat treatment at 80°C for 10
35 minutes to activate any residual *C. botulinum* spores greatly improved the sELISA
36 detection rate of the toxin by increasing the sample toxin levels. All of the mouse
37 bioassay positive cattle cases tested were detected by the sELISA from the heated and
38 pre-enriched samples tested after 24 h incubation. Toxin was detected by sELISA and
39 subsequently confirmed by mouse bioassay in samples from an additional 3 cases that
40 had been originally mouse bioassay negative. The results indicate that the application of
41 this procedure for screening intestinal samples for *C. botulinum* strains that produce types
42 C and D toxins from suspect cattle botulism cases would improve the diagnostic rate as
43 well as significantly reduce the number of mice involved in diagnosis.

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45 Key Words: *C. botulinum*, cattle botulism, ELISA, monoclonal antibodies

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51 1. Introduction

52 Since 2001, the number of suspect clinical cases submitted to the Veterinary Sciences
53 Division for diagnostic investigation has risen significantly, and from the 594 cases
54 recorded between 2001-2007, 97 (16.3%) have been confirmed as botulism by the mouse
55 bioassay (Scarlatos et al, 2005). Although the recommended test sample for confirmation
56 of bovine botulism is serum (Dowell et al, 1977), experience at this laboratory has shown
57 that botulinum neurotoxin (BoNT) is detected much more commonly by the examination
58 of intestinal contents (unpublished data, AFBINI). The mouse bioassay remains the 'gold
59 standard' test because of its high analytical sensitivity [(0.8×10^8 LD₅₀/mg protein for
60 type C toxin and 1.6×10^8 LD₅₀/mg protein for type D toxin (Sugiyama, 1986)],
61 however the use of live animals in the test raises obvious welfare considerations and
62 makes the test costly to perform.

63 The present study was undertaken to develop a monoclonal antibody (MAb) based BoNT
64 sandwich ELISA (sELISA) as a potential replacement for the mouse bioassay. The
65 samples obtained were tested for toxin as received and also after heat shock and
66 anaerobic enrichment. It was postulated that *C. botulinum* spores as well as the toxin
67 were likely to be present in the intestinal contents in botulism cases, and that a sample
68 enrichment procedure involving heat shock and anaerobic culture could raise the toxin
69 level to a level detectable by sELISA. The detection of toxin from the culture of spores
70 from these samples could be regarded as significant since the organism is not usually
71 found in the absence of botulism (Dowell et al, 1977, Princewell and Agba, 1981). The
72 sELISA results were compared with those of the mouse bioassay.

73 2. Materials and methods

74 2.1 Monoclonal antibodies

75 Purified spleen lymphocytes obtained from a type D toxoid (Biodesign International,
76 Saco, ME, USA) immunised BALB/c mouse [carried out under a project licence issued
77 by the Department of Health, Social Services and Public Safety in Northern Ireland in
78 compliance with the 'Animals (Scientific Procedures) Act 1986'] were fused with mouse
79 myeloma line SP2/0-Ag14 at a ratio of 5:1 (Galfre and Milstein, 1981; Teh et al., 1984).

80 The subsequent hybridomas were screened by ELISA in microtitre wells (Dynatech,
81 Chantilly, VA, USA) coated with the immunising toxoid antigen. Supernatants from the
82 hybridomas showing a strong optical density (OD) reading were titrated and the five
83 demonstrating the highest titres were selected for cloning. MAbs from the cloned
84 hybridomas were tested by ELISA against microtitre strips coated with BoNT types A, B,
85 C, D, E, F and G (Metabionics Inc., Wisconsin, USA) to check for specificity.

86 2.2 Sandwich ELISAs

87 Three of the five cloned hybridomas were adapted to grow in RPMI 1640 (Gibco, 21875)
88 medium containing only 5% (v/v) gamma-globulin-free horse serum (Labtech
89 International, Donor Horse Serum, IGG free, Cat.No. 4-101-500/IGG) and grown in bulk
90 in 200 ml triple layer flasks (Nunclon, Nalge Nunc International, Denmark). The MAbs
91 from these were purified by caprylic acid precipitation (McKinney and Parkinson, 1987)
92 and some of the purified immunoglobulin was biotinylated (Hofmann *et al.*, 1982).

93 Combinations of these three MAbs were examined in the following sELISA format using
94 the immunising D toxoid as antigen, their optimal working dilutions being determined by
95 chequerboard titration. Well volumes of 100 µl were used in all but the final step of the

96 procedure. Purified MAb in coating buffer (0.05 M carbonate/bicarbonate buffer, pH
97 9.5) was used to coat Maxisorp microtitre plate wells (Nalge Nunc International,
98 Denmark) at 37°C for 2 h. The coating antibody solution was then discarded and a
99 blocking solution of coating buffer containing 1 % (w/v) bovine serum albumin (BSA;
100 Initial fraction by heat shock, Sigma) was added and incubated overnight at 4°C. The
101 subsequent steps consisting of D toxoid antigen at 0.2 µg/ml, biotinylated MAb and
102 streptavidin-peroxidase (Sigma) were diluted in PTN buffer [0.01 M PBS pH 7.2 (27ml
103 of 0.2 M anhydrous NaH₂PO₄, 73ml of 0.2 M Na₂HPO₄, 17g NaCl made up to 2 L with
104 dH₂O), with 0.04% (v/v) Tween 80 and 2% additional NaCl (w/v)] containing 1% BSA
105 (w/v), incubated with shaking for 1 h at 37°C and washed between steps with ELISA
106 wash [0.01 M PBS, pH 7.2, containing 0.05% (v/v) Tween 20]. The final peroxidase
107 substrate (TMB, Millipore) stage was incubated for 10 min at 37°C without shaking and
108 stopped with 50 µl per well 2.5 M sulphuric acid. PTN buffer in four wells replaced the D
109 toxoid antigen as the negative control. The absorbance at 450 nm was determined using
110 an ELISA plate reader (Titertek Multiskan, Helsinki, Finland) and greater than three
111 times the average negative control (PTN) OD value for each plate was taken as a positive
112 reaction.

113 Combinations of MAbs were examined in the sELISA format for sensitivity for D toxoid
114 and for specificity against types A, B, C, D and E toxoid complexes and types C and D
115 toxin complexes (MetabioLogics Inc., Wisconsin, USA), and a selected combination was
116 subsequently used to examine clinical samples.

117 2.3 Immunoblotting

118 SDS-PAGE was carried out using NuPAGE 4-12% BisTris Gel 1.00 x 2D well
119 (Invitrogen Ltd, Paisley, UK) on *C. botulinum* type C toxin complex and *C. botulinum*
120 type D toxin complex (Metabio Inc., Wisconsin, USA) at 150 µg/ml, under
121 reducing conditions. Western blot transfer of the proteins onto nitrocellulose (Invitrogen)
122 was overnight at 30 mA with subsequent blocking with PTNE (100 ml 0.01 M PBS, 2 g
123 NaCl, 1 ml 0.1 M EDTA, 2.5 ml 20% Tween 80) containing 2% (w/v) BSA (BSA; Initial
124 fraction by heat shock, Sigma) for 1 h at 37°C. Strips of the nitrocellulose preparations of
125 both type C and type D complexes were used to test biotinylated MAbs 5B5 at 1/25,000
126 and 7A10 at 1/1000 dilutions in PTNE with 2% BSA for 1 h at 37°C and washed in
127 PTNE four times. Bound antibodies were detected by incubation of the strips with
128 streptavidin peroxidase at 1/2000 in PTNE and, after washing as above, developed using
129 DAB (3,3-diaminobenzidine tetrahydrochloride; Sigma-Aldrich Company Ltd, Dorset,
130 England) substrate in 0.02 M Tris-HCL buffer containing H₂O₂.

131 2.4 Test samples

132 Sixty three bovine samples collected from 21 clinically suspect cattle from 16 farms in
133 Northern Ireland during 2007 and 2008 were tested by the sELISA using MAb 7A10 for
134 capture and biotinylated MAb 5B5 for the detection stage. These samples consisted of
135 10% (w/v) homogenates in 0.01 M PBS of abomasum contents (n=10), faeces (n=21),
136 rumen contents (n=16) and small intestine contents (n=16) and had been stored at +4°C
137 for up to 3 months.

138 In addition to direct testing, the 63 bovine field samples were also heat treated and
139 cultured anaerobically for the possible presence of *C. botulinum* before testing by
140 sELISA. Briefly, 200 µl of each original sample [10% (w/v) homogenates in 0.01M PBS]

141 was heat treated at 80°C for 10 min and 100 µl of each was added to 20 ml of pre-reduced
142 1% (w/v) cooked meat medium (CMM, Difco) with Brain Heart Infusion (BHI, Oxoid)
143 and incubated anaerobically at 40°C. Two ml of broth was removed after overnight (Day
144 1) and 5 day (Day 5) enrichment and tested by sELISA.

145 2.5 Mouse bioassay

146 All samples submitted for diagnostic investigation from suspect cattle botulism cases had
147 been tested by the mouse bioassay, carried out under a project licence issued by the
148 Department of Health, Social Services and Public Safety in Northern Ireland in
149 compliance with the 'Animals (Scientific Procedures) Act 1986'.

150 Culture supernatants from heat shocked and cultured Day 1 or Day 5 samples that were
151 sELISA positive but which had been previously negative by the mouse bioassay when
152 tested directly were also tested by the mouse bioassay. Dilutions of 1/5 to 1/7, dependent
153 on available volume, were prepared by filter sterilisation before testing.

154 2.6 Statistics

155 The results were compared with those of the mouse bioassay by analysis in terms of
156 correlation (i.e. the percentage of sELISA results that were the same as the mouse
157 bioassay results), sensitivity (i.e. the percentage of the mouse bioassay positive samples
158 that were found positive by sELISA) and specificity (i.e. the percentage of the mouse
159 bioassay negative samples found negative by sELISA), with the mouse bioassay results
160 being taken as the gold standard (Altman and Bland, 1994).

161 3. Results

162 3.1 Antibodies

163 Forty seven of the 544 hybridomas that developed from the D toxin fusion were reactive
164 to the type D toxoid and the five with the highest antibody titre to the type D toxoid were
165 cloned. Three of these hybridomas, 7A10, 7D10 and 5B5, continued to produce high
166 levels of antibody after cloning and were grown in bulk. These antibodies were specific
167 for type D toxin when tested by direct ELISA against *C. botulinum* toxin types A, B, C,
168 D, E, F and G.

169 3.2 Sandwich ELISA

170 Monoclonal antibody reagents prepared from the three bulk cultured MAb 7A10, 7D10
171 and 5B5 were tested in various combinations in the sELISA format and 7A10 and 5B5
172 were selected for further work on the basis of their specificity and sensitivity. Two
173 sELISA combinations (MAb 7A10 capturing with MAb 7A10 biotinylated and MAb
174 7A10 capturing with MAb 5B5 biotinylated) were specific for type D when tested against
175 types A, B, C, D and E toxoid complexes but detected both types C and D toxin
176 complexes. The analytical sensitivity of a sELISA using MAb 7A10 for both capture
177 and detection was 12.5 ng per ml of the immunising D toxoid. The analytical sensitivity
178 of the sELISA using MAb 7A10 for capture and MAb 5B5 for detection was 3 ng per ml
179 of the immunising D toxoid and 1.5 ng per ml of D toxoid complex or D toxin complex
180 (Metabionics Inc), and also had a sensitivity of 1.5 ng per ml for the C toxin complex.

181 3.3 Immunoblots

182 The most prominent staining observed with MAb 5B5 was a 50 kDa protein
183 corresponding to the neurotoxin light chain, and 70 kDa and 33 kDa proteins

184 corresponding to non toxigenic haemagglutinating proteins, on each of the type C and
185 type D toxin complexes (Suzuki et al., 2005). MAb 7A10 detected proteins of the same
186 size as MAb 5B5 but also detected a prominent band at 15 kDa and faint bands of
187 approximately 100 kDa corresponding to the BoNT heavy chain on both the type C and D
188 complexes (Fig 1).

189 3.4 Test samples

190 Preliminary results using MAb 7A10 on both sides of the sELISA for the direct testing of
191 325 non-enriched samples collected from 115 clinically suspect cattle in Northern Ireland
192 during 2002 and 2003 showed a poor correlation of 72.3% with a sensitivity of 13.8%
193 and specificity of 93.7% relative to the mouse bioassay (results not shown).

194 Subsequently, the sELISA combination of MAbs 7A10 and 5B5 with improved
195 sensitivity was used to examine additional samples.

196 The results obtained with the mouse bioassay and this sELISA from the direct testing of
197 63 non-enriched samples collected from 16 farms during 2006 and 2007 are summarised
198 in Table 1. The comparative results between the mouse bioassay and these sELISA
199 results showed an overall correlation at sample level of 76.2% with a sensitivity and
200 specificity of 42.9% and 85.7% respectively.

201 3.5 Heat treatment and pre-enrichment culture

202 A total of 28 of the 63 individual samples collected during 2006-2007 were sELISA
203 positive on Day 1 post enrichment as summarised in Table 2. The sELISA detected 13 of
204 the 14 samples that had initially tested positive for toxin by the direct mouse bioassay. In
205 addition, the sELISA indicated the presence of type C or D toxin complexes in a further
206 15 samples at Day 1 and an additional 3 samples at Day 5 from samples that had been

207 negative for the mouse bioassay when originally tested directly as samples from clinically
208 suspect botulism cases. Eleven (8 from day 1 and the 3 from day 5) of these 18 samples
209 were from 7 individual animal cases where toxin had been detected by the mouse
210 bioassay before enrichment and 7 were from 3 mouse bioassay negative individual
211 animal cases. All 18 were confirmed as toxin positive by the mouse bioassay
212 neutralisation test.

213 4. Discussion

214 The MAb-based sELISA with the sensitivity of 3 ng per ml of D toxoid and 1.5 ng per
215 ml D and C toxin complexes that was developed in this study applied to 24 h culture
216 enriched samples appears to have the potential of providing a rapid and humane assay for
217 the detection of type C or D toxin producing *Clostridium botulinum* in intestinal contents
218 samples from suspect cases of cattle botulism. Although the sELISA lacked sufficient
219 sensitivity for the direct detection of toxin from most of the mouse bioassay positive
220 samples, the application of a procedure for the enrichment culture of any spores that may
221 be present in the test samples greatly improved the detection rate of the toxin by
222 increasing the sample toxin levels.

223 Although MAbs 7A10 and 5B5 were shown to be specific for type D toxin when tested
224 by ELISA against purified toxin, they demonstrated few individual immunoblot staining
225 differences against types C and D toxin complexes (Fig. 1), and did demonstrate a
226 reaction to type C as well as type D toxin complexes by sELISA. The detection of
227 several proteins by each of these MAbs indicates that the epitopes detected in the
228 immunoblots were common to several constituents of the toxin complex. However, the
229 reduction of these toxin complexes during SDS-PAGE may have exposed epitopes which

230 are not available to the MAbs in the intact toxin or toxin complex in the sELISA. It is
231 recognised that *C. botulinum* type C and D toxin complexes are antigenically similar
232 (Oguma et al., 1980), but since the BoNT in the mouse bioassay positive samples were
233 typed as D or a chimeric combination of C and D (data not shown), it is uncertain
234 whether this sELISA will detect BoNT C in suspect samples. As far as the diagnosis of
235 botulism in cattle is concerned, since both type C and D toxin are recognised causes
236 (Senturk and Cichan, 2007), the distinction between type C and D toxins may be regarded
237 as unnecessary at least for initial diagnosis of the disease.

238 The analytical sensitivity of the sELISA for the direct testing of suspect samples was
239 poor, with the majority of mouse bioassay positive samples not being detected by the
240 sELISA from the test samples. In addition, the sELISA detected 7 positive samples from
241 the directly tested samples of 2006-2007 (Table 1) that were not detected initially by the
242 mouse bioassay. Only two of these 7 positives were found to be positive by both sELISA
243 and mouse bioassay after heat treatment and enrichment. The sELISA positivity of the
244 non-enriched samples may be attributed to the sELISA antibodies identifying inactive
245 toxin fragments or to non-specific binding of the biotinylated MAb to anti-species
246 antibody or bacterial antigens such as *Staphylococcus aureus* protein A that may be
247 present in such a complex matrix as faeces.

248 Although toxin detection is typically used to diagnose botulism, the data presented here
249 demonstrate that the organism is also recoverable, as evidenced by toxin detection post
250 culture in 13/14 samples from 2007 to 2008 in which toxin was originally found by
251 mouse bioassay. Following enrichment, all 18 sELISA positive samples that had initially
252 been mouse bioassay negative were confirmed to be positive for *C. botulinum* toxin by

253 the mouse bioassay. In so far as no false positive sELISA results were obtained from the
254 post enrichment assay, the sample treatment involving heating and diluting appears to
255 reduce the potential for non-specific signals in the sELISA. In addition, three new
256 botulism cases were tentatively confirmed on the basis of the cultural presence of toxin
257 producing organism.

258 Botulism in cattle is considered to be caused mainly by intoxication due to ingestion of
259 pre-formed toxin, as opposed to toxicoinfection with neurotoxins derived from growth of
260 *C. botulinum* in the intestine (Smith, 1977). The ingested material that subsequently
261 causes botulism is likely to contain toxin and spores, and the results of the present study
262 indicate that the spores may be more persistent and readily detected in the intestinal
263 samples examined than the toxin. However, it is recognised that *C. botulinum* spores are
264 widely distributed at low levels in the environment, and the culture and testing of a range
265 of environmental and intestinal samples from cattle free from clinical suspicion of
266 botulism is required to fully evaluate the significance of this finding to determine
267 diagnostic specificity and sensitivity. This work is currently being undertaken at this
268 institute and initial results have demonstrated the low occurrence of *C. botulinum* spores /
269 toxin in intestinal samples collected from cattle free of clinical suspicion of botulism
270 examined by the methods used in the current work (unpublished results).

271 5. Conclusion

272 The mouse bioassay remains as the 'gold standard' assay for the detection of botulinum
273 toxin because of its greater sensitivity for the low levels of the toxins relative to *in vitro*
274 assays. Its continued use is certainly an animal welfare issue and replacement with
275 reproducible *in vitro* tests is long overdue. The present results demonstrate that although

276 the heat shock and culture process prior to sELISA failed to detect one botulism sample
277 that had been detected by the mouse bioassay, it did not miss any cases identified by the
278 initial mouse bioassay and was able to detect three new cases missed by the latter after
279 only 24 h enrichment. There remains the possibility that the sELISA positive results that
280 were detected after enrichment may have arisen from the culture of contaminating spores
281 of environmental origin, and further work is required to fully evaluate this approach. This
282 study does indicate that the use of culture enrichment for 24 h and subsequent sELISA for
283 screening samples for toxin produced from group III *C. botulinum* organisms, with the
284 mouse bioassay being used for confirmation, may improve the diagnostic rate as well as
285 significantly reduce the number of mice involved in the diagnosis of bovine botulism.

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318

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Table 1. Correlation between the direct mouse bioassay and the sELISA results on untreated samples collected from suspect cases of cattle botulism from 16 farms during 2006-2007.

Sample	N.	Mouse bioassay	sELISA		Correlation	Sensitivity	Specificity
			Positive	Negative			
Individual test sample	63	Positive	6	8	76.2	42.9	85.7
		Negative	7	42			
Individual animal	21	Positive	6	2	71.4	75.0	69.2
		Negative	4	9			

Table 2. Correlation between the direct mouse bioassay and the sELISA results following heat treatment and enrichment for 1 day on samples collected from suspect cases of cattle botulism from 16 farms during 2006-2007.

Sample	N.	Mouse bioassay	sELISA		Correlation	Sensitivity	Specificity
			Positive	Negative			
Individual test sample	63	Positive	13	1	74.6	92.8	69.3
		Negative	15	34			
Individual animal	21	Positive	8	0	85.7	100	76.9
		Negative	3	10			

Fig. 1 Immunoblot of *C. botulinum* type C and D complexes with MAbs 5B5 and 7A10.

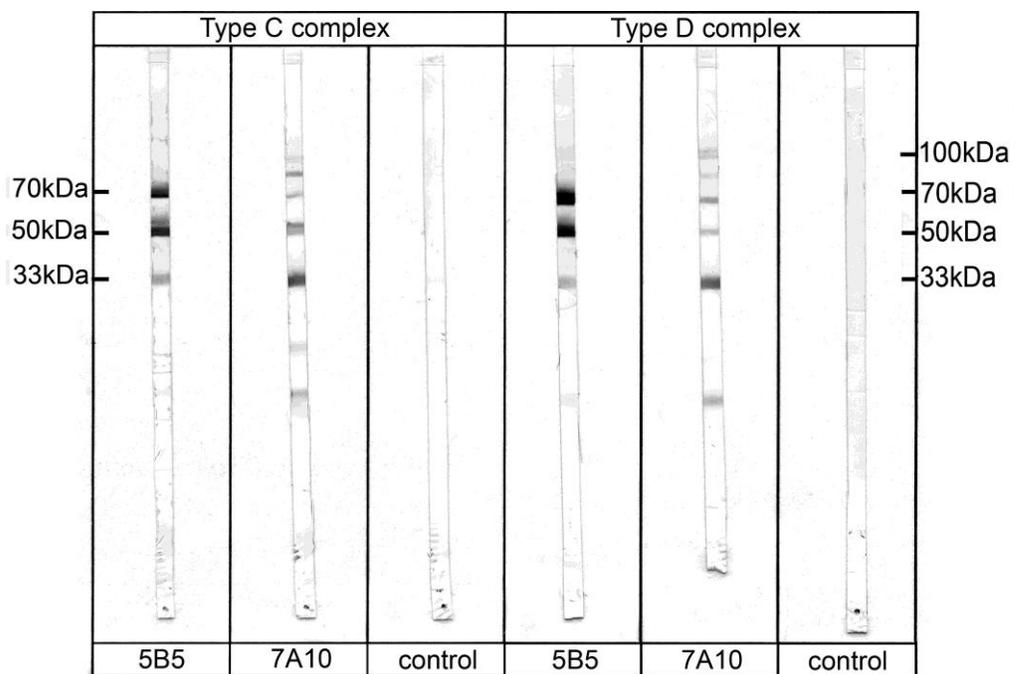


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