

**QUALITATIVE CORRELATION OF THE MOUSE  
NEUROTOXIN AND ENZYME-LINKED IMMUNOASSAY  
FOR DETERMINING *CLOSTRIDIUM BOTULINUM*  
TYPES A AND B TOXINS<sup>1</sup>**

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**ABSTRACT**

*The efficacy of an enzyme-linked immunosorbent assay for botulinum toxin using polyclonal antibodies was evaluated in relation to the standard mouse assay. Qualitative tests for toxin in a meat system inoculated with Clostridium botulinum and pure cultures of various aerobic and anaerobic bacteria showed that both the mouse neurotoxin and enzyme-linked immunosorbent assays detected toxin in the samples. However the ratios of mouse:ELISA activity of culture supernatant toxins of C. botulinum showed wide disparity among strains of types A and B. Trypsin treatment resulted in a slight loss of ELISA activity but the mouse response increased.*

**INTRODUCTION**

Enzyme-linked immunosorbent assays (ELISA) for botulinum toxin types A, B, and E were first reported by Notermans *et al.* (1978), Kozaki *et al.* (1979) and Notermans *et al.* (1979), respectively. Dezfulian *et al.* (1984) used the test for detecting types A and B toxin in stools of infants with botulism; the ELISA detected toxin in every case where positive results had been found by the mouse neurotoxin test. Dezfulian and Bartlett (1985) used antibodies prepared from immunotolerant rabbits (neonate rabbits were injected for 14 days with culture filtrates of *C. sporogenes*, 7 weeks later the rabbits received injections of *C. botulinum*) for the assay of botulinum toxin from cases of infant botulism that showed nonspecific

deaths of mice injected with stool samples. Antibodies from immunologically tolerant rabbits also were used by Dezfulian and Bartlett (1984) for an ELISA of culture filtrates of *C. botulinum* strains. They found that antibody prepared against type A cross-reacted with type B toxin but not with any other type or with other clostridial supernatants. Betley and Sugiyama (1979) reported on a comparison of a radioimmunoassay and the mouse neurotoxin test and found good agreement when fresh culture preparations were tested. However, after standing at room temperature neurotoxicity decreased faster than antigenicity.

The purpose of the studies reported here was to assess, in comparison to the mouse neurotoxin test, the effectiveness of a polyclonal antibody based ELISA for detection of toxin production by strains of type A and B *C. botulinum*.

## MATERIALS AND METHODS

### Meat Inoculation

Mechanically deboned meat was obtained from a local poultry processor; some was radiation sterilized with  $^{137}\text{Cs}$  (42 kGy) to eliminate the indigenous flora. Ten gram portions were weighed into sterile petri dishes in a biohazard hood. Cultures of aerobic and facultative bacteria isolated previously from chicken meat were grown at 30°C for 2 days in NT broth (equal volumes of nutrient and tryptic soy broths). These were used to inoculate the meat at levels of 0.1 mL/10 g (approximately  $10^7$ /g) together with a mixture of equal numbers of spores of proteolytic *C. botulinum* (20 strains, 11 type A and 9 type B) for a final spore concentration of 100/g. The plates were incubated under anaerobic conditions at 25°C for 10 days; the meat was then removed, placed into centrifuge tubes with gelatin phosphate buffer (0.2% gelatin, 0.36%  $\text{KH}_2\text{PO}_4$  and 0.15%  $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ ), mixed and centrifuged at 3000 x g for 5 min. The supernatants were used for the toxin and serological tests.

### ELISA

Multiwelled microtitre plates were used for the ELISA. Each step of the assay was given 60 min incubation at 45°C. The plates were washed 4 times after each incubation step with PBS : Tween buffer (0.2 g KCl, 8.0 g NaCl, 0.2 g  $\text{NaN}_3$ , 0.2 g  $\text{KH}_2\text{PO}_4$ , 2.90 g  $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ , 1.0 g Tween 20 and 1000 ml  $\text{H}_2\text{O}$ ) with 1% fetal bovine serum added as a blanking protein. The specific procedure follows:

Plates were coated (100  $\mu\text{L}$ /well) with anti-A botulinum toxin goat antiserum (Environmental Diagnostics, Burlington, NC) diluted 1/1000 in carbonate buffer (1.59 g  $\text{Na}_2\text{CO}_3$ , 2.93 g  $\text{NaHCO}_3$ , 0.2 g  $\text{NaN}_3$  and 1000 mL distilled water). Toxins from culture supernates were diluted with gelatin phosphate buffer and 100  $\mu\text{L}$  added per well. A secondary antibody, anti-rabbit A/B, was prepared by the

method of Dezfulian and Barlett (1984) (obtained from W.H. Lee, Food Safety and Inspection Service, USDA, Beltsville, MD). This was diluted 1:4000 in PBS : Tween buffer and was added at a level of 100  $\mu\text{L}$ /well. Anti-rabbit alkaline phosphate conjugate (Sigma # A8025), diluted 1/500 in PBS : Tween buffer containing 1% normal horse serum, was added at a level of 100  $\mu\text{L}$ /well. Finally, 100  $\mu\text{L}$ /well substrate (p-phenyl phosphate, disodium salt, Sigma # 104) dissolved in substrate buffer (1.88 g glycine, 0.05 g  $\text{MgCl}_2$ , 0.014 g  $\text{ZnCl}_2$  with 250 ml  $\text{H}_2\text{O}$ ; adjusted to pH 10.4 with 1.0 N NaOH) was added. Optical densities (410 nm) were read with a Dynatech Minireader II spectrophotometer. Sample dilutions were considered positive by the ELISA if the optical densities of duplicate wells were 0.10 or higher, corrected for blanks.

### Mouse Test

Samples were diluted serially 1:1 in gelatin phosphate buffer and pairs of mice, 15-20 g each, were inoculated intraperitoneally with 0.5 mL diluted sample. Positive dilutions were those that caused deaths (with typical symptoms of botulism) of at least 1 of 2 mice with the next higher dilution causing no symptoms or deaths. The mouse test and ELISA were run within one day of each other using the same series of dilutions. The ratio of mouse to ELISA activity was based on the highest dilutions giving positive responses.

## RESULTS AND DISCUSSION

The qualitative responses in the mouse test and ELISA for the samples of chicken meat inoculated with *C. botulinum* spores and one of the aerobic or facultative cultures showed that, in each case where the 1:20 dilution was positive in the mouse test, the ELISA was also positive (Table 1). In a related experiment with nonsterilized chicken meat inoculated with the *C. botulinum* spore mix and incubated for 18 h at 28°C, 29/30 samples giving ELISA ODs of 0.08 to 0.24 were positive in the mouse test. One sample gave an OD of 0.04 in the ELISA but it also was positive in the neurotoxin assay. That the two tests are not of equal sensitivity was also demonstrated in another test (in cooperation with Dr. M. Dezfulian, data not shown) where the minimum lethal dose in the mouse test was first determined and the next more concentrated ten-fold dilution was used for the ELISA. Only 10 out of 46 samples positive by the mouse test were positive by the ELISA. However those tests were performed with a less sensitive polyvalent antibody (burro, CDC) and the ELISA was done a week after the mouse test, the samples having been stored at refrigerator temperature. These factors may account for the decreased sensitivity between our results and those of Dezfulian and Bartlett (1984) who reported that the ELISA could detect less than 10 mouse  $\text{LD}_{50}$ s.

The quantitative discrepancy between the two tests for botulinum toxin type A

**TABLE 1.**  
**COMPARISON OF ELISA AND MOUSE TESTS FOR BOTULINAL TOXIN: CHICKEN**  
**MEAT INOCULATED WITH *C. BOTULINUM* AND AEROBIC AND FACULTATIVE BACTERIA**

Culture <sup>a</sup>	ELISA OD <sup>b</sup>	Mouse Toxin <sup>c</sup>	Culture	ELISA OD	Mouse Toxin
43-16	0.37	+	14-1	1.25	+
112-3	1.17	+	13-4	1.07	+
112-1	1.04	+	117-1	1.19	+
112-2	0.73	+	117-14	0.77	+
112-4	1.01	+	117-13	1.49	+
112-6	1.04	+	42-4	1.44	+
112-7	0.90	+	117-7	1.28	+
112-8	0.75	+	42-6	0.96	+
6-K	1.01	+	43-13	1.17	+
14-4	1.25	+	13-5	0.90	+
6F	0.87	+	117-11	1.48	+
13-9	1.01	+	117-5	0.92	+
117-6	0.50	+	117-2	1.10	+
none	1.30	+			

<sup>a</sup> Cultures were aerobic and facultative isolates from chicken meat.

<sup>b</sup> Samples were 1:20 dilutions in gel-phosphate, read at 410 nm.

<sup>c</sup> Intraperitoneal injection of 2 mice with 1:20 dilution.

is shown by the results in Table 2. Supernatants of the cultures of the seven strains of type A *C. botulinum*, grown in tryptic soy broth at 35°C in an anaerobic chamber, were diluted in 1:1 increments until no activity was detected in the two tests. The ratios of the highest dilutions giving positive responses in the mouse test and ELISA showed that the mouse test was 1 to 32 times as sensitive as the ELISA. Subcultures of strains 33, 62 and 69 were single colony isolates from stock cultures streaked onto Brewer anaerobic agar and incubated in an anaerobic chamber for two days. These showed a tendency to revert to a nontoxic state. Clones selected from strain 69, which showed a strong tendency to dissociate into toxic and nontoxic substrains (unpublished observations), showed wide differ-

TABLE 2.  
COMPARISON OF ELISA AND MOUSE TESTS FOR BOTULINAL TOXIN:  
TYPE A CULTURES

	Highest active dilution		
	Mouse	ELISA	Mouse/ELISA <sup>a</sup>
33-1 <sup>b</sup>	1/64000	1/4000	16
33-2	1/64000	1/8000	8
69-1	1/8000	1/4000	2
69-2	1/32000	1/2000	16
69-3	1/8000	1/4000	2
69-4	1/2000	1/2000	1
62-1	1/32000	1/4000	8
62-2	1/32000	1/4000	8
78	1/32000	1/4000	8
429	1/128000	1/4000	32
426	1/128000	1/8000	16
ATCC 25763	1/64000	1/4000	16

<sup>a</sup> Highest dilution giving mouse response divided by highest dilution giving positive ELISA.

<sup>b</sup> Cultures 33, 69 and 62 were from single colony isolates.

ences in the ratio of mouse:ELISA activity. These differences may be related to its propensity for dissociation into the nontoxic state.

A similar comparison was made for type B strains, Table 3. Several strains showed greater activity in the ELISA than in the mouse test. For example, strains 17409 and 53, were at least 8 times more active in the ELISA than in the mouse assay. The ratios of mouse:ELISA activities for strains 642, 999, 8688 and ATCC 7949 were close to unity but one strain, 770, was 16 times more active in the mouse. Four isolates from type B parent strains that were toxin negative by a preliminary ELISA screening of 1:2 dilutions were also toxin negative by the mouse test.

The effect of trypsin treatment on the toxins of 3 strains of type A and 8 strains of type B is shown in Table 4. Trypsin treatment decreased slightly the ELISA activity of 7 strains and there was no effect on the other 4 strains. In the mouse test, trypsinization resulted in increased activity of most of the types A and B

**TABLE 3.**  
**COMPARISON OF ELISA AND MOUSE TESTS FOR BOTULINAL TOXIN:**  
**TYPE B CULTURES**

Culture <sup>a</sup>	Highest active dilution		
	Mouse	ELISA	Mouse/ELISA
17409-1	1/2000	1/32000	0.06
17409-2	1/2000	1/16000	0.12
642-1	1/16000	1/16000	1
642-2	1/16000	1/16000	1
770-1	1/512000	1/32000	16
770-2	1/512000	1/32000	16
999-1	1/16000	1/16000	1
999-2	1/32000	1/8000	4
8688-1	1/16000	1/16000	1
8688-2	1/2000	1/1000	2
53	1/2000	1/16000	0.12
ATCC 7949	1/4000	1/4000	1
17409 <sup>b</sup>	< 1/2	< 1/2	
53 <sup>b</sup>	< 1/2	< 1/2	
770 <sup>b</sup>	< 1/2	< 1/2	
8688 <sup>b</sup>	< 1/2	< 1/2	

<sup>a</sup> All cultures were single colony isolates.

<sup>b</sup> These were non-toxic (ELISA) variants selected from isolated colonies grown in anaerobic chamber.

<sup>c</sup> No activity in 1:1 dilution.

strains; one strain, 383, after trypsin treatment was 32 times as active as the untreated. Based on the mouse/ELISA activity ratios, trypsin treatment resulted in a greater difference between the mouse test and ELISA in most strains.

Our results indicate that the ELISA using polyclonal antibodies readily detects botulinum toxin although some strains of *C. botulinum* may produce antigens that have no neurotoxicity. For example the ELISA may react to the hemagglutinins associated with botulinum toxin. This does not necessarily invalidate the use of the

TABLE 4.  
EFFECT OF TRYPSIN ON THE RATIO OF MOUSE/ELISA BOTULINAL  
TOXIN ACTIVITY

Culture	Type	Ratio of activity <sup>a</sup>			
		Trypsin/no trypsin <sup>b</sup>		Mouse/ELISA	
		ELISA	Mouse	Trypsin	No trypsin
429	A	1	2	32	16
17409	B	0.5	8	4	0.25
7949	B	0.5	2	8	1
69	A	0.5	0.5	2	2
999	B	0.5	4	64	8
642	B	0.5	4	16	2
383	B	1	32	8	0.5
4	B	0.5	8	4	0.25
53	B	1	1	0.5	0.5
8688	B	0.5	2	8	2
33	A	1	2	4	4

<sup>a</sup> Ratio of highest dilutions showing activity. Culture supernatants diluted in gelatin phosphate buffer 1:100, 1:1000, then serially 1:1 until no activity was found.

<sup>b</sup> Trypsin (Sigma # T-8128, type II crude, porcine) added at level of 1 mg/ml to samples of culture supernatants diluted 1:100 with gelatin phosphate buffer.

test for toxin determination since hemagglutinins associated with the toxins serve to stabilize them. The effect of trypsin on the culture supernates indicates that it increases the activity of the neurotoxin and has only a minimal effect on the ELISA.

Monoclonal antibodies may also be used in the ELISA assay for botulinal toxin; these however are not generally available and require special expertise in their preparation. Monoclonal antibodies have been prepared against type A toxin by Ferreira *et al.* (1987) and Shone *et al.* (1985); these did not cross react with other *C. botulinum* types. Gibson *et al.* (1987) evaluated a type A monoclonal antibody in a meat system and found that 14/15 strains positive by the mouse neurotoxin test were also positive by the ELISA test. The single exception was

NCTC 2012 which was negative by the ELISA. Gibson *et al.* (1987) also evaluated a monoclonal antibody against type B toxin in a meat system; 73/101 samples were positive by both the mouse and ELISA tests, the others had toxin at or below the detection level for the ELISA. Ferreira *et al.* (1990) prepared monoclonal antibodies against type F; these cross-reacted slightly to type D toxin but not to any other. Monoclonal antibodies, with their extreme specificity, may produce false negative results if the toxin moiety has been altered or damaged. Further evaluation of monoclonal antibody use is necessary.

The widely disparate results from different strains of *C. botulinum* by the ELISA using polyclonal antibodies, indicates that the production of toxin and antigen is not uniform among strains of the same serotype. In each case however, the ELISA with polyclonal antibody from goat gave positive responses in samples containing the neurotoxin. Although ELISA results should be verified by the mouse test, the serological test is a very useful tool for laboratory investigations where known strains are used and where fast results are desired. The utility of the ELISA is in its promise of giving rapid quantitative results for a large number of samples, thus eliminating the need for the many mice needed for calculation of minimum lethal doses or LD<sub>50</sub>s.

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