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Authors: Rocke, Tonie E., Smith, Susan R., and Nashold, Sean W.

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PRELIMINARY EVALUATION OF A SIMPLE *IN VITRO* TEST FOR THE DIAGNOSIS OF TYPE C BOTULISM IN WILD BIRDS

Tonie E. Rocke,[1,2] Susan R. Smith,[1] and Sean W. Nashold[1]

[1] U.S. National Biological Survey, National Wildlife Health Center, 6006 Schroeder Road, Madison, Wisconsin 53711, USA

[2] Corresponding author (e-mail: Tonie_Rocke@usgs.gov)

ABSTRACT: An enzyme-linked immunosorbent assay (ELISA) was developed for the detection of type C botulinum toxin (*Clostridium botulinum*) in wild birds. This simple, antigen-capture ELISA utilizes polystyrene immunosticks as the solid substrate, chicken antitoxin (IgY) as the coating antibody, rabbit antitoxin as the primary antibody, and peroxidase-labeled goat-anti-rabbit as the secondary antibody. To evaluate the immunostick ELISA as a diagnostic test for avian botulism, known concentrations of toxin were added to heparinized blood collected from healthy birds and tested by both the ELISA and mouse bioassay. Also, blood samples from 236 bird carcasses submitted to the National Wildlife Health Center (NWHC) for cause of death determinations were tested by both procedures. Using ≤ 0.5 ml as the test volume for both procedures, the ELISA was less sensitive, detecting 0.25 ng/ml of toxin compared to 0.12 ng/ml for the mouse bioassay. Using the same volume of test sample for diagnostic submissions (≤ 0.5 ml), the ELISA was positive for 60% of the 149 clinically-diagnosed cases of botulism, whereas the mouse bioassay was positive for 79%. However, we demonstrated that with larger sample volumes (≥ 1.0 ml), the sensitivity of the ELISA may be equivalent or better than the mouse test due to the concentrating effect of the ELISA procedure. These preliminary results suggest that when adequate sample volumes are available, the immunostick ELISA can replace the mouse test for the diagnosis of botulism in wild birds.

Key words: Avian botulism, *Clostridium botulinum*, enzyme-linked immunosorbent assay, immunostick, type C botulinum toxin.

INTRODUCTION

Type C botulism, a neuroparalytic disease caused by ingestion of toxin produced by *Clostridium botulinum*, is an important disease of wild waterfowl, annually killing thousands to millions of birds throughout the world. Sporadic outbreaks of type C botulism also occur in domestic animals, particularly poultry, cattle, and horses (Rocke, 1993).

The traditional test for the detection of type C botulinum toxin in the blood of affected animals is the mouse neutralization test (Quortrup and Sudheimer, 1943). However, in recent years, the use of mice has become very costly and inconvenient in some facilities, and public pressure has been increasing to find alternatives to live animal bioassays. In addition, the mouse bioassay typically requires several days to complete and can be inconclusive if other pathogens are present in the specimens being tested. An *in vitro* alternative to the mouse bioassay for diagnosing botulism in animals is highly desired.

Enzyme-linked immunosorbent assays (ELISA) have been previously described and used with varying success for the detection of botulinum neurotoxins, including type C toxin (Thomas, 1991). However, these tests are performed using microplates, which require a plate reader and other specialized equipment, must be performed in a laboratory setting by trained personnel, and are generally less sensitive than the mouse bioassay. In addition, blood samples from dead animals and other environmental samples are not always suitable for testing on microplates, because they often result in high background staining and non-specific reactions [National Wildlife Health Center (NWHC), unpublished data]. In this paper, we describe a simple ELISA for the diagnosis of type C botulism that requires no special equipment or facilities and can be completed in the field if necessary. A preliminary evaluation of the ELISA as a diagnostic tool for type C botulism was conducted using known concentrations of tox-

in added to blood samples from healthy birds and diagnostic specimens submitted to NWHC for cause of death determinations.

MATERIALS AND METHODS

Toxin

Semi-purified type C botulinum toxin (Wako Chemicals USA, Inc., Richmond, Virginia, USA) was purchased as a 1 mg/ml solution in 0.05 M phosphate buffer (pH 6.0), with a molecular weight of 500,000 and a toxicity of 4×10^6 mouse LD_{50}/mg. The preparation consisted of C1 toxin, the neurotoxin that causes botulism, and C2 toxin, an enzyme with ADP-ribosylating activity but no neurotoxic effects (Simpson, 1989), as well as nontoxic components.

Toxoid

Formaldehyde was added to the semi-purified toxin to reach a final concentration of 0.6%. This solution was incubated at 37 C for 10 days, after which the toxoid was dialyzed for 24 hr against succinate buffer. To test for toxicity, 0.5 ml (100 μg) of the toxoid was injected intraperitoneally (IP) into each of two mice (ICR, Harlan Sprague Dawley, Madison, Wisconsin, USA), and the mice were observed for signs of botulism intoxication for 5 days.

Antitoxin

Two white Leghorn chickens (laying hens) were each immunized by intramuscular injection of 1.2 ml of type C toxoid (150 μg) mixed with an equal portion of Freund's incomplete adjuvant (Gibco, Grand Island, New York, USA). Booster injections of 150 to 250 μg of toxoid were given on weeks 1, 8 and 16 after the initial immunization. Eggs were collected daily from the immunized hens, and immunoglobulin was harvested from the yolk (IgY) by polyethylene-glycol (PEG) precipitation according to a modification of Polson et al. (1980). After separating the yolks from the whites, the yolks were blended with two volumes of 0.01 M phosphate buffer (pH 7.5) containing 0.1 M NaCl and 0.01% merthiolate. Then 3.5% PEG was added to the yolks, and the solution was centrifuged for 10 min at 14,000 × *g*. The supernatant was poured through cheesecloth, 8.5% PEG was added, and the solution centrifuged again for 10 min. After discarding the supernatant, the resulting pellet was resuspended in phosphate buffer at a volume equivalent to the original yolk volume. Then 12.5% PEG was added, and the solution was centrifuged again for 10 min. The resulting pellet was resuspended in Tris-buffered saline (TBS; 50 mM Tris-HCl, 200 mM NaCl, 3 mM KCl) containing 0.01% merthiolate. The IgY from several eggs from the same hen were pooled, aliquoted, and stored frozen. The presence of antibody to type C botulism in the pooled extractions was confirmed and quantified by the mouse neutralization test as described below.

Pooled PEG-precipitated IgY (with a titer > 1:1,024) was affinity-purified on a 5-ml Actigel ALD (aldehyde-activated agarose; Sterogene Biochemicals, Arcadia, California, USA) column prepared by coupling 0.5 mg type C toxin (Wako Chemicals) per ml of resin. The antibody was loaded onto the column at a flow rate of 1 ml/min, after which the column was washed with 10 bed volumes of borate buffer (0.1 M boric acid, 0.025 M sodium borate, 1.0 M NaCl, 0.1% Tween 20, pH 8.3), followed by five bed volumes of TBS at a flow rate of 60 ml/hr. We then applied one bed volume of Actisep elution medium (Sterogene Biochemicals) to the column, stopped the flow for 1 hr, resumed the flow with TBS at 60 ml/hr, and collected 1 to 2 ml fractions. IgY-containing fractions were detected and quantified by UV absorption at 280 nm, pooled, and immediately dialyzed against TBS overnight at 4 C and then stored in aliquots at −20 C. The presence of antibody was confirmed by membrane immunoassay with enzyme-labeled goat-anti-chicken IgG (Kirkegaard and Perry, Gaithersburg, Maryland, USA). The column was washed and regenerated with TBS containing 0.1% Tween 20 (TBST), followed by TBS containing 0.01% sodium azide.

Two New Zealand white rabbits were each immunized with 100 μg of type C botulinum toxoid mixed with the Ribi 730 adjuvant system (Ribi Immunochem Research, Inc., Hamilton, Montana, USA), according to the manufacturers recommended protocol. Booster injections were given on weeks 2, 4, 8, 12, 16, and 20 after the initial immunization. Blood was collected from the median ear artery of each rabbit on week 5 and monthly thereafter. Serum was harvested by centrifugation, and the presence of antibody was confirmed and quantified by the mouse neutralization test as described below. Immunoglobulin (IgG) was purified by applying serum (with a titer > 1:1,024) to a column of protein A-sepharose 4B (Sigma Chemical Co., St. Louis, Missouri, USA) and washing with 10 column volumes of 0.1 M Tris (pH 8.0) and 10 column volumes of 0.01 M Tris (pH 8.0) at a flow rate of 60 ml/hr. Upon elution with 0.1 M glycine (pH 3.0), IgG-containing fractions were detected and quantified by

UV absorption at 280 nm and were confirmed by membrane immunoassay with enzyme-labeled goat-anti-rabbit IgG (Kirkegaard and Perry). The fractions were pooled, aliquoted and stored frozen.

Mouse neutralization tests were performed to confirm the presence of and quantify antitoxin in chicken and rabbit immunoglobulin preparations. Four-fold serial dilutions of the immunoglobulins were made in gelatin phosphate buffer; an equal volume of toxin (1 μg/ml) was added to each dilution and incubated at room temperature for 1 hr. Four, 6-wk-old, Swiss/ICR mice (Harlan Sprague Dawley, Madison, Wisconsin, USA) were injected intraperitoneally (IP) with 0.2 ml amounts of each immunoglobulin dilution. The mice were observed for 4 days, and the total deaths were recorded. The antitoxin titer was considered to be the dilution at which 50% of the mice survived the inoculation.

ELISA Procedure

An indirect, antigen-capture ELISA was developed using Nunc MaxiSorp Immuno-sticks and CryoTube Vials (Nunc Inc., Naperville, Illinois, USA). Optimal test conditions and incubation times were determined using known concentrations of toxin diluted in either milk diluent or in heparinized blood from healthy ducks. The immunosticks were placed in 1.8 ml vials (10 mm × 40 mm) and each coated with 0.5 ml affinity-purified chicken antitoxin (5 μg/ml) diluted in carbonate buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6). The vials with the immunosticks were then refrigerated overnight with gentle agitation. After pouring off the antibody, the coated immunosticks were blocked by adding milk diluent/blocking solution (Kirkegaard and Perry) to the vials (1.5 ml/vial) and shaking them gently for 2 hr at room temperature. Next, the immunosticks were washed. For this and all subsequent wash steps, 1.5 ml wash solution (Kirkegaard and Perry) was placed in the vials containing immunosticks, and the vials were shaken gently for 5 min; this was repeated for a total of three times. Antibody-coated, blocked immunosticks were stored in the refrigerator for ≤ 6 mo prior to use. A sample from each lot of antibody-coated immunosticks was evaluated prior to general use by titration with the semi-purified toxin, and the whole lot was rejected and discarded if the ELISA failed to detect at least 0.25 ng/ml toxin in 1 ml samples.

Test samples (heart blood from carcasses, culture supernatants, and toxin standards) in volumes of 0.5 to 5.0 ml (optimal 1.0 ml or more) were placed in fresh vials, and antibody-coated immunosticks were inserted into the sample and refrigerated overnight with gentle agitation. The next day, the immunosticks were rinsed thoroughly using a wash bottle to remove clinging particles (blood, etc.), placed in fresh vials, and washed as before. Affinity-purified rabbit antitoxin diluted to 10 μg/ml in milk diluent was added to each vial (1 ml/vial), and the immunosticks were incubated for 90 min at 37 C with gentle agitation. After washing, peroxidase-labeled goat-anti-rabbit (1.0 mg/ml; Kirkegaard and Perry) diluted 1:1,000 in milk diluent was added to each vial (1 ml), and the immunosticks were incubated for 90 min at 37 C with gentle agitation. After washing, TMB peroxidase substrate (Kirkegaard and Perry) was applied to the immunosticks (1 ml/vial). Vials were shaken gently at room temperature for 30 min. The reaction was stopped by immersing the immunosticks in tubes containing distilled water. Positive reactions were evidenced by purple coloration only on the antibody-coated bottom half of the immunostick, ranging from slight (1) to strong (4). If color was observed on the entire immunostick, the reaction was considered non-specific.

Specificity of the immunostick ELISA

Stock cultures of several strains of *C. botulinum* types C and D (Table 3), as well as one strain each of *C. botulinum* types A, B, and E (NWHC stocks) and one strain each of *C. novyi* and *C. sporogenes* (American Type Culture Collection, Rockville, Maryland, USA) were grown in chopped-meat-carbohydrate medium (Carr-Scarborough Microbiologicals, Stone Mountain, Georgia, USA). Culture fluids were collected and filtered through 0.20 μm syringe filters. Each *C. botulinum* culture filtrate was confirmed to contain botulinum toxin using the standard mouse bioassay. All culture filtrates (1.0 ml) were then tested by the ELISA.

Mouse bioassays for toxin

Mouse bioassays were performed on blood samples and culture filtrates with 6-wk-old, Swiss/ICR mice (Harlan Sprague-Dawley) according to the general methods described by Quortrup and Sudheimer (1943). Briefly, blood samples were mixed 4:1 with an antibiotic solution (15,000 units penicillin G, 1,000 units mycostatin, 5% glycerin, 15 mg streptomycin sulfate, 200 μg gentamicin, 1 L Hanks balanced salt solution; ph 7.6) to inhibit bacterial pathogens. Two mice were required for each test sample. One was inoculated IP with 0.2 ml type C botulinum antitoxin produced in horses (NWHC). After 30 min, both the antibody-protected and a non-protected mouse were inoc-

ulated IP with 0.5 ml of the test sample. Mice were observed for signs of botulism, such as hind-limb paralysis, wasp waist, labored breathing, and death for 5 days post inoculation. A positive test was recorded if the non-protected mouse died or showed signs of botulism and the antibody-protected mouse remained healthy. Samples that produced clinical signs of botulism in non-protected mice that survived were considered weakly positive. A negative test was recorded if both mice survived, and, if both mice died, the test was considered inconclusive. A similar procedure was used for testing culture supernatants, although for botulinum toxin types other than C, the corresponding antitoxin used in the bioassay was obtained from the Centers for Disease Control (Atlanta, Georgia, USA).

Comparison of the immunostick ELISA and mouse bioassay detection limits

Semi-purified toxin was diluted to specific concentrations (0.06 to 2.0 ng/ml) in heparinized blood from healthy Pekin ducks (Abendroth Waterfowl Hatchery, Waterloo, Wisconsin, USA) to evaluate the detection limits of both the ELISA and mouse bioassay and to determine the effect of sample volume on the performance of the ELISA. For each toxin concentration tested, two antibody coated immunosticks were placed in vials containing volumes of the test samples ranging from 0.5 ml to 4.0 ml. The vials were then refrigerated overnight with gentle shaking, and the ELISA was performed as described previously. The reactions were scored for their color reaction on a scale from 1 to 4, with 4 being the strongest reaction observed. The remaining test sample at each toxin concentration was then used for mouse bioassays, which were performed on the same day as the ELISA. For each toxin concentration, 0.5 ml of the test sample was inoculated IP into each of four mice, and the number of animals that died or developed clinical signs of botulism were recorded.

Comparison of the immunostick ELISA and mouse bioassay using diagnostic specimens

In order to compare the immunostick ELISA with the standard mouse bioassay for the diagnosis of type C botulism, heart blood was collected from 236 birds (from 50 birds species, representing nine orders) that were found sick or dead and sent to NWHC for necropsy and cause of death determinations (NWHC, unpublished data). Because the mouse bioassay was the diagnostic test of choice in these cases, for this comparison, only a small volume of the heart blood sample ($\leq$ 0.5 ml) could be set aside for the immunostick ELISA. The remainder was centrifuged and the supernatant used for mouse bioassays as described above. The two procedures were performed blind, i.e. without knowledge of the results of the other test or of other diagnostic findings, and the results were compared by the kappa test (Thrusfield, 1995).

Of the 236 birds necropsied and tested, NWHC diagnosticians determined that 149 died or were suspected to have died from type C botulism in 51 separate events; this determination was based on mouse bioassay results, clinical history, lack of other significant diagnostic findings, and/or the occurrence of botulism in other birds submitted from the same location at the same time (NWHC, unpublished data). The other 87 samples were from birds in which the cause of death was undetermined (24) or was something other than type C botulism [including emaciation (17), organophosphate poisoning (13), trauma (13), lead poisoning (6), carbamate poisoning (5), avian cholera (2), external oiling (2) steatitis (2), air sacculitis (1), parasitic enteritis (1), and zinc poisoning (1); NWHC, unpubl. data].

RESULTS

Optimal reagent concentrations and incubation times for the immunostick ELISA were determined using known toxin standards diluted in heparinized blood from mallard ducks or milk diluent. The optimal concentrations of chicken antitoxin, rabbit antitoxin, and enzyme-conjugated goat-anti-rabbit antibody was 5 μg/ml, 10 μg/ml, and 1 μg/ml, respectively. Optimal sample incubation was overnight at 4 C (Table 1), although 4 hr at 25 C was almost as good and could provide a same-day result. The poorest results (less intense color reaction with positive samples) were obtained with incubation at 37 C.

All toxigenic type C cultures that we tested were positive by the immunostick ELISA, including strains that produce only C2 toxin (Table 2). No cross-reactivity was noted with culture filtrates of *C. botulinum* types A, B, E, F, and type D-VHD, nor with *C. novyi*, or *C. sporogenes*. However, cross reactivity was evident with the Boroff strain of *C. botulinum* type D.

Using a sample volume of 0.5 ml, the lowest concentration of semi-purified toxin

TABLE 1. Results of the immunostick enzyme-linked immunosorbent assay (ELISA) for type C botulinum toxin run in duplicate using the same concentration of botulinum toxin (2.5 ng/ml) in 1.0 ml of heparinized duck blood incubated with gentle shaking at different times and temperatures. No differences were noted in the color reaction on duplicate tests.

Time (hr)	Temperature (C)	ELISA reaction[a]
24	4	3
24	25	2
4	4	1
4	25	2
4	37	1
1.5	25	1
1.5	37	1

[a] Reaction was judged on a scale of 1–4, with 4 being the strongest reaction.

detected by the immunostick ELISA was 0.25 ng/ml compared to 0.12 ng/ml by the mouse bioassay (Table 3). However, by increasing the sample volume to ≥ 1 ml, the ELISA detected toxin concentrations as low (0.12 ng/ml) and even lower (0.06 ng/ml) than the mouse bioassay, with stronger reactions evident with greater sample volumes. No difference was noted in the color reaction of duplicate tests.

TABLE 2. Results of immunostick enzyme-linked immunosorbent assay (ELISA) for type C botulinum toxin on 1 ml culture filtrates of various *Clostridium* spp. and strains.

Species	Toxin type	Strain[a]	ELISA reaction[b]
C. botulinum	A	A-62	0
	B	169B-2	0
	C	468	4
	C	6812	4
	C	SAC-96	4
	C2 only	AO28	4
	D	Boroff	2
	D	VHD	0
	E	026-080	0
C. novyi		19402	0
C. sporogenes		3584	0

[a] Most strains were from the stock collection located at the National Wildlife Health Center with the exception of *C. novyi* and *C. sporogenes*, which were obtained from the American Type Culture Collection.

[b] Reaction was judged on a scale of 1–4, with 4 being the strongest reaction.

TABLE 3. Results of the immunostick enzyme-linked immonosorbent assay (ELISA) for type C botulinum toxin performed in duplicate with known concentrations of botulinum toxin in various sample volumes of heparinized blood in comparison with the mouse bioassay using a 0.5 ml volume of inoculum. No differences were detected in the color reaction on duplicate tests.

Toxin concentration (ng/ml)	ELISA reaction[a] Sample volume (ml)				Mouse bioassay Number dead/number inoculated
	0.5	1.0	2.0	4.0	
2.00	4	4	4	4	4/4
1.00	2	4	4	4	4/4
0.50	1	2	4	4	3/4[b]
0.25	1	1	2	3	3/4[b]
0.12	0	1	2	2	1/4[b]
0.06	0	0	1	1	0/4[c]

[a] Reaction was judged on a scale of 1–4, with 4 being the strongest.

[b] Surviving mice showed clinical signs of botulism.

[c] Mice showed no clinical signs of botulism.

For the 236 diagnostic samples that were tested by both the ELISA and mouse bioassay, the overall concordance was 74% (Table 4), and the calculated kappa statistic of 0.48 indicated a moderate level of agreement between the two tests (Thrusfield, 1995). For the 149 birds that were determined to or suspected to have died from botulism, 118 (79%) were positive by the mouse bioassay and 90 (60%) were positive by ELISA. Of the 40 samples that were mouse bioassay positive but ELISA negative, 27 were considered only weakly positive by mouse bioassay, i.e., unprotect-

TABLE 4. Results of mouse bioassays and immunostick enzyme-linked immunosorbent assays (ELISA) for blood samples from birds submitted to the National Wildlife Health Center for diagnostic evaluation. A calculated kappa statistic of 0.48 indicated a moderate level of agreement between the two tests.

	Immunostick ELISA		
Mouse bioassay	Positive	Negative	Total
Positive	78	42	120
Negative	19[a]	97	116
Total	97	139	236

[a] For six of these samples, the ELISA was positive, but the mouse bioassay was inconclusive, because both the protected and unprotected mice died.

ed mice showed clinical signs of botulism but survived. Also, for 12 samples considered positive by ELISA, 6 were negative by mouse bioassay, but the other 6 were considered inconclusive because both the protected and unprotected mice died in the test. Interestingly, of the 51 separate events for which botulism was diagnosed or suspected in this study (single and multiple case submissions), positive results were obtained with the immunostick ELISA 75% of the time. In 21 of those events, three or more birds were submitted for diagnosis, and positive results were obtained with the ELISA 100% of the time. In the other 30 events, only one or two birds were submitted, and positive ELISA results were obtained only 57% of the time.

For the 87 birds in which the cause of death was considered something other than botulism, 78 (90%) were negative by both the mouse bioassay and the immunostick ELISA. In two cases for which the mouse bioassay was positive, but the ELISA was negative, carbamate poisoning was considered the primary cause of death. In seven cases for which the ELISA was positive, but the mouse bioassay was negative, the causes of death were considered organophosphate poisoning (5), emaciation (1), and steatitis (1).

DISCUSSION

The efficiency of a new diagnostic test and the degree of concordance between a new test and an existing test is generally measured using animals known to have the disease and a corresponding control group. Because detection of botulinum toxin in the blood of affected animals depends directly on the amount of toxin present, an effective comparison between the immunostick ELISA and mouse bioassay for type C botulinum toxin would require inoculation of a number of birds with type C botulism toxin at different dosage levels. To minimize the use of live animals and to provide an analysis of the detection limits for each procedure, we chose first to compare the ELISA and mouse bioassay using known concentrations of toxin added to heparinized blood collected from healthy birds, and second, to compare the mouse bioassay and immunostick ELISA using typical diagnostic specimens. Admittedly, this approach precludes the calculation of standard parameters of sensitivity and specificity, because the actual status of the birds submitted for diagnostic evaluation was unknown, and the mouse bioassay, although currently considered the gold standard, is not infallible. Nonetheless, this preliminary evaluation of the immunostick ELISA suggests that this test may be a useful alternative to the mouse bioassay in some situations.

The kappa test indicated a moderate agreement between the two tests, however the overall efficiency of the immunostick ELISA (true negatives plus true positives/total) based on NWHC diagnoses was somewhat lower (72%) than the mouse bioassay (86%). The immunostick ELISA was most useful in diagnosing type C botulism as the cause of an epizootic when three or more birds were submitted for testing, but it was less useful for single case submissions. In botulism epizootics, when many sick or dead birds are available for testing, our results suggest the ELISA could be expected to detect the toxin in at least 60% of the birds tested with blood sample volumes $\leq$ 0.5 ml compared to 79% for the mouse bioassay. However, we must emphasize that this comparison between the mouse test and the ELISA was conducted with less than optimal blood volumes for the ELISA ($\leq$ 0.5 ml), which significantly affects the sensitivity of the test. With blood volumes of 0.5 ml, which is typically used for the mouse bioassay, the lowest limit of toxin detection of the ELISA was 0.25 ng/ml. However, because the antibody-coated immunosticks essentially act to capture and concentrate toxin, as demonstrated by the results presented in Table 3, sample volumes of $\geq$ 1.0 ml could result in detection of lower concentrations of toxin (as low as 0.06 ng/ml). Perhaps a comparison of the two tests us-

ing greater sample volumes for the ELISA would reveal little difference in their diagnostic efficiencies. Unfortunately, from most bird carcasses, it is difficult to collect a sufficient quantity of blood to run both tests.

Of 87 blood samples from birds in which something other than type C botulism was considered the primary cause of death based on other diagnostic findings, seven reacted positively by immunostick ELISA and two others were positive by mouse bioassay. Assuming the clinical diagnoses were accurate, these nine positive reactions might be due to the presence of toxin formed post-mortem in the carcasses; all of the birds in question were found dead in various stages of decomposition and clinical signs prior to death were not observed. Type C botulism spores are frequently carried by healthy birds and can readily germinate and produce toxin in bird carcasses upon death, regardless of the cause of death (Reed and Rocke, 1992). Alternatively, these nine reactions could be false positives. After conducting this study, we have since determined that certain chemical intoxications, such as pentobarbital poisoning and organophosphate poisoning, apparently can cause the formation of endogenous peroxidase in the blood that results in a non-specific color reaction on the immunostick when substrate is applied. To control for this non-specific reaction, blood sample volumes > 0.5 ml should be used so that a clear demarcation can be observed between the bottom half of the immunostick, which is coated with antibody (0.5 ml), and the upper half, which is un-coated. If the entire immunostick reacts, then the reaction should be regarded as non-specific and the test as invalid. The five cases that were positive by the immunostick ELISA, but diagnosed as organophosphate poisoning in our study, may have been non-specific reactions that could have been differentiated if larger sample volumes had been available.

By testing culture filtrates, the immunostick ELISA was shown to detect toxin from 4 different strains of *C. botulinum* type C, but no reactions were observed with a number of other clostridial cultures and neurotoxin types, with the exception of type D-Boroff. Previous investigators have demonstrated that some type C1 and D botulinum toxins share common antigenic determinants (Oguma et al., 1984) that may serologically cross-react even in mouse bioassays. Also, it should be recognized that the ELISA will detect inactive as well as active toxin, whereas the mouse bioassay will only detect toxin that is biologically active. In some situations, this might be an advantage. Other investigators have demonstrated that type C botulinum toxin antigen detectable by ELISA can persist in the blood stream of chickens for several days after the serum has lost its toxicity in the mouse bioassay (Sakaguchi et al. 1987).

Several factors influence the sensitivity and performance of the immunostick ELISA, including length and temperature of incubation of the test sample, pH of the initial coating buffer, and adequate agitation of the samples. Overnight incubation at 4 C with gentle shaking provided the best results on the immunostick ELISA, with room temperature incubation for at least 4 hr the next best. Incubation at 37 C for 90 min to 4 hr resulted in the poorest performance, perhaps due to thermal degradation of toxin in the sample at the higher temperature (Hubalek and Halouzka, 1988). We also determined that the pH of the carbonate buffer used for coating the chicken antibody to the immunosticks is extremely critical. A coating buffer pH of 9.8 to 10.2 is optimal, and above and below that, poor results were obtained. Samples that contain particulate matter or that are heavily contaminated with bacteria should be centrifuged prior to testing with the immunostick ELISA. Also, after each lot of immunosticks are coated and prior to use, it is imperative to titrate their sensitivity with known toxin standards to ensure adequate coating.

To summarize, we have developed an immunostick ELISA that can replace the in vivo mouse bioassay for the diagnosis of type C botulism in some situations. At less than half the cost of the mouse test, the immunostick ELISA is fairly inexpensive. It also is simple to perform, requiring virtually no special equipment, and can easily be conducted in the field if necessary. In addition, because live animals are not involved, the ELISA is not subject to interference by other, non-specific pathogens as is the mouse test. In this study, six cases that were considered equivocal by mouse bioassay, because both mice died, were found to be positive by ELISA. Volume to volume, the ELISA is not as sensitive as the mouse test, but with larger sample volumes ($\geq$ 1.0 ml), the ELISA may be even more sensitive. However, in cases where only minimal sample volumes are available ($\leq$ 0.5 ml), the mouse test might be preferred. We are continuing work to improve the sensitivity of the immunostick ELISA for type C botulinum toxin, remove the reactivity to C2 toxin, and add the capability of simultaneous detection of type E botulinum toxin, another type of botulism that occasionally afflicts wild waterbirds.

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