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Confirmation of botulism diagnosis in Australian bird samples by ELISA and RT rtPCR

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Abstract. We developed a sandwich ELISA that detects *Clostridium botulinum* C and D toxins and reverse-transcription real-time PCRs (RT-rtPCRs) that detect botulinum C and D toxin genes, respectively, to replace the mouse bioassay. The toxin genes were closely associated with the toxin molecules and used as surrogates for the presence of toxin. Samples (638) from 103 clinical cases of birds (302) with suspected botulinum toxicity came from wild birds and poultry (9 cases). Samples tested included blood serum, other body fluids, various tissues, gut contents, maggots, water, and sediment. Botulism was diagnosed in 34 cases (all of which had positive samples in the ELISA, the C toxin gene RT-rtPCR, or both assays). Botulism was suspected in 16 cases (each of which had 1 positive sample either in the ELISA or the C toxin gene RT-rtPCR). In the remaining 53 cases, no samples were positive, but botulism could not be excluded in 32 of these cases, whereas there was no indication of botulism or another diagnosis in 21 cases. The D toxin gene was not detected in any of the clinical samples. No C or D toxin genes were detected in 71 pooled cloacal swabs from 213 healthy migratory birds. The use of an ELISA that detects botulinum C and D toxins in combination with a RT-rtPCR for the botulinum C toxin gene can help confirm the diagnosis of botulism in birds.

Keywords: birds; botulism; ELISA; PCR.

Avian botulism occurs in wild birds, particularly in waterbirds, and in domestic poultry.[3,36,49] Botulism in birds can be the result of ingestion of food containing preformed toxin, for example the carcass-maggot cycle, or in other cases may occur as a toxicoinfection.[3,18,38,49,52] Some authors also consider botulism in birds, or at least in chickens, to occur as toxicoinfections associated with coprophagy.[13,28,51,55] Botulism in animals is usually associated with toxin types C or D or mosaic toxins C/D or D/C and occasionally type E toxin; botulism in humans is usually associated with types A, B, or E, and occasionally F.[20,43,60]

The gold standard for detecting botulinum toxin in biological samples and foods is the mouse protection bioassay, but as a result of animal welfare considerations and the cost and time involved, alternative assays are required. The mouse protection bioassay requires the use of a minimum of 4 mice per sample (2 protected and 2 unprotected mice, and, if dilution of the sample is required, 4 for each additional dilution). In our laboratory, we have received >80 samples per year for botulinum toxin assays over the last 7 y. Replacement of the mouse protection bioassay with an alternative in vitro assay is desirable from an animal ethics point of view. The mouse bioassay involves considerable staff commitment given that it is a 3–4 d assay with observations of mice required every 4 h. In addition, a secure, dedicated facility must be maintained for this assay. Botulinum toxins are the most toxic biological substances known; toxicity is associated with very small amounts of these toxins.[12] The mouse bioassay can detect <1 minimum mouse lethal dose (MLD)/mL (i.e., 30 pg of C toxin/mL or 10 pg of D toxin/mL for a 25-g mouse).[21]

Various in vitro assays for detection of botulism have been described since ~1990. These include tests based on the specific protease activity of the toxins, detected either by ELISA or other immunologic recognition methods, by MALDI-TOF, and by PCR.[1,8,27,29,30,32,35,36,48,50,52,53,60] The majority of these reports describe the detection of botulinum type A toxin and the other toxin types associated with botulism in humans.[22,37]

The presence of botulinum toxin in various food sources spiked with toxin has been revealed by detecting the DNA or

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Table 1. Summary of avian botulism cases and Australian regions of origin.

Bird category/State of origin	Median of mortalities*	Cases (*n*)	Median of birds submitted/case*	Total no. of samples†
Poultry				
WA	8 (5–50)	7	3 (2–26)	61 (6, 3–31)
NSW	13 (1–24)	2	1 (1–1)	2 (1, 1–1)
Wild birds				
WA	8 (1–3,000)	61	2 (1–19)	408 (4, 1–69)
NSW	5 (1–10)	17	2 (1–10)	93 (3, 1–23)
VIC	5 (1–250)	11	2 (1–6)	51 (3, 2–10)
QLD	8 (5–10)	5	2 (1–10)	22 (3, 2–10)

NSW = New South Wales; QLD = Queensland; VIC = Victoria; WA = Western Australia.
* Ranges in parentheses.
† Parentheses contain median number of samples per case, followed by range of sample numbers per case.

RNA of the botulinum toxin gene associated with the botulinum toxin complex. Because the DNA/RNA is tightly bound to the toxin during toxin formation, it can be used as a reliable surrogate for the presence of botulinum neurotoxin.[27] However, this molecular test for the detection of botulinum toxin and many of the other assays described above have, to our knowledge, not been reported in a veterinary laboratory setting.

We report here on the detection of botulinum toxin complex–associated toxin gene RNA/DNA as a surrogate test for the presence of toxin in clinical samples. We also developed an in-house antigen ELISA for the detection of C and/or D toxin and applied it to the same clinical material. We used a simple and robust toxin capture immunoassay, modified to avoid false-positive readings caused by matrix interference by using a pre-immunization normal goat serum as capture antibody negative control. To estimate the assay inhibition for each sample and to flag possible false-negative results, we also included a matrix inhibition control by spiking each sample type with a known amount of botulinum toxoid.

Materials and methods

This work was performed in a laboratory appropriately accredited for dealing with high-risk pathogens.

Samples

Bird samples for testing were received at the Department of Primary Industries and Regional Development (DPIRD), Western Australia, Diagnostics and Laboratory Services. Samples included blood, serum, crop or gizzard contents, gut contents, feces, and various fresh tissues, and various environmental samples such as sediment, maggots, and water.

We analyzed 638 samples (from 103 clinical cases) with the ELISA and both PCRs described below. Just over half of the samples were from ducks of various species (pacific black duck, wood duck, pink-eared duck, freckled duck, hardhead duck, wandering whistling duck, and other species), but there were also samples from chickens, seagulls, silver gulls, crested tern, ibis, pelicans, magpies, black swans, a white swan, pheasants, geese, corellas, lorikeets, coots, a grebe, a raven, a heron, a noisy miner, a sandpiper, a cormorant, and an emu. The cases with the largest number of birds submitted were 26 commercial chickens and 19 wild ducks with typical signs of botulism.

Most cases were submitted from Western Australia, but cases were also received from other states of Australia (Table 1). Reported mortalities associated with these cases ranged from a few animals to several hundred dead birds. One case in the north of Western Australia involved the death of >3,000 ducks as the result of a cyanobacterial bloom. In addition, 71 pooled cloacal swab samples (each pooled sample being a group of 3 swabs) collected from 213 migratory birds during National Avian Influenza Wild Bird surveillance were analyzed to determine the prevalence of detectable botulinum C or D toxin genes in feces of healthy migratory birds with no clinical signs of botulism. Unfortunately, given a lack of sufficient sample material, these samples could not be tested for the presence of botulinum toxin by ELISA.

Sample preparation

Serum and body fluids were diluted 2-fold or 4-fold in 10 mM Tris-HCl, 0.5 M NaCl, 0.05% v/v Tween 20, and 0.01% thimerosal containing 1.4% fat-free soy protein (soy blocking buffer). Gut contents were diluted 3-fold in soy blocking buffer, vortex-mixed thoroughly, and centrifuged at 16,000 × *g* for 15 min at <10°C. The clear supernatant was recovered for assay. The pH was adjusted to 6.5–7.5 with 1 M Tris pH 8.5 or 1 M citrate pH 5, as required. Tissues and maggots (200 mg plus 1 mL of soy blocker) were sliced and homogenized (Fast Prep with lysing matrix D; MP Biomedicals) for ≥40 s at 6 m/s, then centrifuged at 16,000 × *g* for 15 min at <10°C, and the clear supernatant was assayed. Water and sediment samples were first centrifuged to remove solid particles (1,500 × *g* for 30 min), then concentrated 50–100 times by using a centrifugal filter unit with a molecular weight cutoff of 10^5 Da (Amicon Ultra-15; Merck).

Antibodies

All animal experimentation was approved by the DPIRD institutional Animal Ethics Committee under the guidelines of the National Health and Medical Research Council's Australian code of practice for the care and use of animals for scientific purposes (approvals 4-06-30; 3-07-32; 3-10-20; 3-13-13).

Goat antiserum to C toxoid for the ELISA was raised by immunizing a goat with partially purified C toxoid. Prior to immunization, pre-immune serum was collected from the same goat for the matrix background control protocol of the ELISA. Initial immunization occurred with botulinum C toxoid eluted from a commercial alum-based vaccine against C and D toxins for cattle (Commonwealth Serum Laboratories). The vaccine was mixed with 1.2 M potassium phosphate to elute C and D toxoids from the alum.[4] Dynabeads protein G (Dynal) were cross-linked with rabbit antibody to C toxin (Statens Serum Institute) according to the manufacturer's instructions. The eluted C and D toxoids were incubated with the rabbit anti-C~Dynabeads, then C toxoid was eluted from the beads and concentrated to 1 mL before immunizing the goat; ~50 µg of toxoid was injected intramuscularly with adjuvant as a water in oil emulsion (Montanide ISA 50 V; SEPPIC). The subsequent 4 booster injections (at least 6 wk apart) of similar amounts consisted either of the same toxoid preparation or of purified toxoid prepared from *C. botulinum* type C culture supernatant (kindly provided by Fort Dodge, New South Wales, Australia) as follows: crude culture supernatant was precipitated with ammonium sulfate, and the precipitate was redissolved, dialyzed, and partially purified by isoelectric focusing (Micro-Rotofor; Bio-Rad) as described.[41] The purified toxin was converted to a toxoid by dialysis against 0.4% formalin for 2 wk and mixed at a ratio of 1:1 with aluminum hydroxide gel (Alhydrogel; Millipore Sigma) as an adjuvant. Serum was collected 6 wk after the last booster immunization. The produced antiserum cross-reacted in the ELISA with purified botulinum C and D toxin.

Monoclonal antibody (mAb) to C and D toxin was raised as described.[19,25] Botulinum C and D toxins were purified as described, converted to toxoid, and used to immunize mice.[23] Hybridomas were selected, cloned, and re-cloned by limiting dilution based on ELISA reactivity to purified botulinum type C or D toxin. The selected mAb (clone 4D11, isotype IgM) reacted to a common epitope present in purified type C and D preparations and was stored frozen at a concentration of 5 mg/mL in phosphate-buffered saline (PBS) containing 0.25 M trehalose.

ELISA

We developed and optimized in-house an indirect antigen (toxin) sandwich ELISA for botulinum C and D toxins. In previous studies in our laboratory, various antibodies to C and D toxins had been raised. During the ELISA development, 30 possible combinations of capture (coating) and primary detection antibodies, and 4 secondary labeled antibody conjugates, were compared by testing 10-fold dilutions of C and D toxins in 50% soy blocker to find the combination that detected the smallest amount of the toxins. The trialed antibodies included 2 commercial antibodies, 2 neutralizing antisera from cattle, in-house polyclonal and monoclonal antibodies raised in sheep and mice, respectively, during other projects,[17] and the in-house–produced goat polyclonal antibody described above.[23] The 2 commercial capture or detection antibodies were rabbit α-C toxin (Statens Serum Institute; Abcam). The most sensitive combination (data on combinations not shown) as described below was used for testing of all cases described in our study.

In addition, the ELISA deviated from standard ELISA formats by the following modifications (described in detail below):

- Determination of background value for each sample by replacing the capture antibody (in-house–produced goat anti–botulinum toxin antibody) with the pre-immune serum from the same goat. This was done on the same ELISA plate and served as a matrix background control.
- Inclusion of wells containing each sample spiked with a known botulinum toxoid concentration to assess assay inhibitions caused by matrix effects.

Sample or reagent additions to ELISA plates were all 100 µL per well. Wells of ELISA plates (Microlon high binding microplates; Greiner) were coated with goat hyperimmune serum to C and D toxoids (one half of the ELISA plate), or pre-immune serum from the same goat (the other half of the ELISA plate), each diluted 1:500 in 0.1 M glycine buffer (pH 9.6) and incubated overnight (or up to 7 d) at 4°C. An equal number of wells was coated with pre-immune and hyperimmune goat serum depending on the number of samples to be tested. Wells were washed 4 times with PBS (pH 7.2) containing 0.05% Tween 20 (PBST). Unoccupied spaces on the surface of the wells were blocked by incubating with soy blocking buffer at 37°C for 45–60 min with shaking at 1,000 rpm. After 6 washes with PBST, samples prepared as above in soy blocking buffer were added in duplicate, both to the wells coated with pre-immune serum and the wells coated with immune serum. Positive reference and blank samples were also added to each side of the plate. The positive reference consisted of crude D toxoid (1:500) in duplicate, and the blank samples consisted of at least 10 wells containing only 50% soy blocking buffer (final concentration of 0.7% soy protein in 10 mM Tris-HCl, 0.5 M NaCl, 0.05% v/v Tween 20, 0.01% thimerosal). In addition, "inhibition checks" were included for each sample, on each side of the plate. The inhibition checks consisted of each sample spiked with crude D toxoid at 1:500 dilution. The plate was incubated for 60 min at

37°C with shaking at 1,000 rpm. Four washes with PBST were followed by the addition of mAb (4D11) to C and D toxoids (0.5 µg in 100 µL of 50% soy blocking buffer per well). Incubation with mAb was for 3 h with shaking at 37°C followed by overnight incubation at room temperature (without shaking) to maximize the sensitivity of the assay. After 4 washes with PBST, monoclonal rat anti-mouse IgM conjugated to horseradish peroxidase (Southern Biotech) diluted 1:500 in 50% soy blocking buffer was added and incubated at 37°C with shaking for 60 min. The plate was washed 6 times with PBST, tapped dry, and wells were loaded with tetramethylbenzidine ('K Blue' ready-to-use substrate; ELISA Systems). After incubation at 20–25°C with shaking for 15–25 min, 0.5 M H_2SO_4 was added to stop color development. Absorbance readings were taken at 450 nm on a spectrophotometer (Multiskan MS; Labsystems). The average absorbance values for the blank wells on each side of the plate were calculated separately and subtracted from all corresponding absorbance readings on the same side of the plate (i.e., pre-immune or immune serum–coated wells) to create blank-corrected readings for pre-immune and immune serum–coated wells. The blank-corrected readings for the samples in the wells coated with the pre-immune goat serum were subtracted from the corresponding blank-corrected readings for the matching samples in the immune wells to create final readings for the samples. The average final blank reading plus 5 SDs was the designated cutoff between negative and positive results. Percentage inhibition for each sample was calculated as follows: $[1 - (Abs_{450}$ of inhibition check/ Abs_{450} of positive reference)] × 100%.

Optimal sample pH for the ELISA

Samples of contents from the gut can vary in pH from <4 to >8. Gut contents (that had tested negative previously) from an ibis were spiked with toxin, and aliquots were mixed with 1 M citrate or Tris buffer to adjust pH to 4.7, 5.1, 5.5, 6.4, 7.8, and 8.1, then tested in the ELISA to determine the effect of pH.

Sensitivity, specificity, and optimal incubation times of the ELISA

Samples of C and D toxins of known toxicity as determined by the mouse bioassay were tested in serial 3-fold dilutions. We compared incubations with sample (1, 2, and 3 h at 37°C), mAb (1, 2, and 3 h at 37°C, 1 or 2 h at 37°C plus overnight at room temperature), and rat anti-mouse IgM~HRP conjugate (1, 2, and 3 h at 37°C). Incubation times were optimized to detect the smallest amount of toxin.

RT-rtPCR

Prior to nucleic acid extraction, samples were prepared as for the ELISA, but with PBS as the diluent. RNA/DNA extractions were performed on the clear supernatants (MagMAX viral RNA isolation kit, Applied Biosystems; KingFisher instrument, Thermo Fisher) for automated nucleic acid extraction.

Primers and probes used for targeting the botulinum C and D toxin genes were as described.[27] These primers also detect C/D and D/C mosaic toxins, respectively, by comparison with sequences in GenBank. RT-rtPCR was performed (AgPath-ID one-step RT-rtPCR kit, Thermo Fisher; Rotor-Gene Q, Qiagen). Reverse transcription was at 45°C for 10 min, followed by inactivation of the reverse transcriptase at 95°C for 10 min. Thermocycling conditions for the PCRs were 50 cycles of denaturation at 95°C for 15 s and annealing/extension at either 59°C for 30 s (for the C gene) or 52°C for 45 sec (for the D gene).

It was of interest to determine whether toxin samples contain RNA or DNA. Five nucleic acid samples (from avian cases) that were positive by C toxin RT-rtPCR were pretreated with DNase for 25 min at 37°C (DNA-free DNA removal kit; Thermo Fisher), according to the manufacturer's instructions, then assayed in the C toxin gene RT-rtPCR.

Sensitivity and specificity of C and D toxin gene RT-rtPCRs

Dilutions of C and D toxin samples (crude culture supernatants) from 200 down to 1 MLD/mL were made in PBS. Nucleic acid was extracted from the dilutions as described above. The extracted nucleic acid was assayed in triplicate in both the C and the D toxin gene RT-rtPCRs.

Diagnostic case definitions

We defined a case returning a positive botulinum test result in at least one sample as a positive case. All cases originally diagnosed by various duty pathologists were critically reviewed by one of the authors (D.G. Palmer) using consistent criteria as outlined below. The case diagnosis of botulism was confirmed if:

- the toxin (ELISA) or associated type C or D toxin gene RNA/DNA (RT-rtPCR) was demonstrated in serum collected from live birds, or
- the toxin (ELISA) was demonstrated in any environmental sample (including maggots collected from other carcasses), or the toxin (ELISA) **and** associated type C or D toxin gene RNA/DNA (RT-rtPCR) was demonstrated in at least one fresh sample (not necessarily in the same sample, or from the same bird included in the case submission), or the toxin (ELISA) **or** the associated type C or D toxin gene RNA/DNA (RT-rtPCR) was demonstrated in at least 2 unrelated samples (e.g., gastrointestinal tract content and liver from the same bird, or any samples from different birds of the same case submission).

Table 2. C toxin gene RT-rtPCR results before and after DNase treatment.

Sample	No treatment (Ct)	DNase treatment (Ct)	Interpretation: sample contained
A	29.3	31.9	RNA + DNA
B	31.4	No amplification	DNA only
C	24.5	34.1	RNA + DNA
D	23.5	31.1	RNA + DNA
E	29.4	No amplification	DNA only

Ct = cycle threshold.

The diagnosis of botulism was suspected if the toxin (ELISA) or the associated type C or D toxin gene RNA/DNA (RT-rtPCR) was demonstrated in only one of the samples submitted. Botulism could not be excluded in cases involving several dead birds with typical signs of botulism in which all tests were negative but only a limited number of samples or birds were submitted.

No indication for type C or D botulism was reported in cases with or without clinical history of paralysis in which an extensive number of appropriate samples returned a negative test result for botulinum toxin (ELISA) and associated type C or D toxin gene RNA/DNA (RT-rtPCR). Given the lack of appropriate assays and positive controls, botulism caused by type E toxin could not be excluded in any of the cases tested.

Statistics

The chi-square test was used to analyze the frequency of positive results with the ELISA compared with the C toxin gene RT-rtPCR.

Results

The sample pH range over which the ELISA detected toxin in a spiked sample of gut content was quite broad: at least pH 4.7–8.1, with the optimal range of pH 5.0–7.0. Sample incubation times of 2 and 3 h improved the sensitivity of the assay (e.g., at a dilution of D toxin containing ~15 MLD/mL, the corrected ODs were 0.401, 0.354, and 0.280 at 3, 2, and 1 h, respectively). This effect was less pronounced at higher dilutions. The optimized ELISA detected both C and D toxins down to <4 MLD/mL of sample (provided the sample was <30% inhibitory) when the incubation time with the mAb was extended to 1–3 h at 37°C with shaking followed by overnight incubation at room temperature without shaking. Extending the incubation time with the monoclonal rat anti-mouse IgM~HRP conjugate, when used at a dilution of 1:500, beyond 1 h at 37°C did not provide any increase in sensitivity.

We decided to maintain the cutoff at "mean of the corrected blank readings plus 5 SDs" because, although reducing it to "mean corrected blank plus 3 SDs" increased the sensitivity (20 more positive samples in positive cases [i.e., botulism confirmed or suspect]), it slightly decreased the specificity, resulting in 2 "positive" samples, from 2 cases in the category "Other diagnosis" and 3 positive samples from only 1 case in the category "No indication of botulism." Thus, in the interest of avoiding false-positive cases, the mean plus 5 SDs was applied as a cutoff to all samples.

Percentage inhibition of samples in the ELISA was 0–100%. Gut content samples were the most variable and included the most inhibitory samples (mean: 41%, range: 0–100%, n = 99); the least inhibitory samples were blood sera (<35% inhibitory).

Nucleic acid samples that were positive previously for the C toxin gene, when treated with DNase, were shown to contain either DNA (no amplification after DNase treatment) or both RNA and DNA resulting in a later cycle threshold (Ct) after DNase treatment (Table 2).

The C toxin gene RT-rtPCR did not detect the D toxin gene and vice versa. The C toxin gene could be detected reliably in diluted toxin >20 MLD/mL. At 1–20 MLD/mL, the C toxin gene could only be detected in ~50% of the PCR tubes. Ct values for the latter samples were 36–39. The lowest concentration of toxin diluted in PBS that could be detected reliably in the D toxin gene RT-rtPCR was 2 MLD/mL (Ct = 40.4–42.6). A quantity of 1 MLD/mL of D toxin was detected in 2 of 3 PCR tubes (Ct = 44–45). The botulinum D toxin gene was not detected in any of the samples in our study.

The pooled cloacal swab samples from healthy migratory birds were all negative for both the C and the D toxin genes by RT-rtPCR.

The diagnosis of botulism was confirmed in 34 cases; botulism was suspected in 16 cases. The sensitivities of the ELISA or RT-rtPCR alone to detect suspect and confirmed cases of botulism were 74% and 76%, respectively. If both a positive ELISA and positive RT-rtPCR were deemed to be necessary for a case diagnosis of confirmed or suspected botulism, then only 50% of cases were detected (Table 3).

In a case in which 19 ducks with typical signs of botulism were submitted, 8 of 19 gizzard contents, 12 of 19 duodenal contents, 11 of 19 livers, and 5 of 19 sera were positive in the C toxin gene RT-rtPCR, and only 2 of 19, 0 of 19, 3 of 19, and 0 of 19 were ELISA positive, respectively. The results in this duck case suggested that the C gene RT-rtPCR is more useful diagnostically, and when the overall results were analyzed, there was very little difference in the frequency of positive results between the 2 assays for body fluids

Table 3. Case diagnosis and in vitro assay results of all cases (toxin ELISA and type C toxin gene RT-rtPCR).

	Cases (*n*)		
Case diagnosis	With ELISA positive samples only	With RT-rtPCR positive samples only	With both ELISA & RT-rtPCR positive samples
Botulism confirmed (*n* = 34)	6	3	25
Botulism suspected (*n* = 16)	6	10	0
Total (*n* = 50)	12 (24%)	13 (26%)	25 (50%)
Botulism not excluded (*n* = 32)	0	0	0
No indication of botulism (*n* = 13)	0	0	0
Other diagnosis (*n* = 8)	0	0	0

Table 4. Sample results by sample type and assay (toxin ELISA and type C toxin gene RT-rtPCR).

	Sample results (number of samples and/or %)*			
No. of samples from all cases or positive cases† by sample type	ELISA positive only	RT-rtPCR positive only	ELISA & RT-rtPCR positive	Total of positive samples
Body fluids, *n* = 158‡	11 (7%)	13 (8%)	3 (2%)	27 (17%)
From positive cases, *n* = 113§	10%	11%	3%	24%
GI contents, *n* = 250	14 (6%)	39 (16%)	18 (7%)	71 (28%)
From positive cases, *n* = 175	8%	22%	10%	41%
Tissues/organs excluding livers, *n* = 49	2 (4%)	2 (4%)	1 (2%)	5 (10%)
From positive cases, *n* = 37	5%	5%	3%	14%
Livers, *n* = 163	5 (3%)	17 (10%)	9 (6%)	31 (19%)
From positive cases, *n* = 90	6%	19%	10%	34%
Environmental samples (water, sediment, maggots), *n* = 18	1 (6%)	0 (0%)	6 (33%)	7 (39%)
From positive cases, *n* = 15	7%	0%	40%	47%
Total no. of samples, *n* = 638	33 (5%)	71 (11%)	37 (6%)	141 (22%)
From positive cases, *n* = 430	8%	17%	9%	33%
Positive samples only, *n* = 141¦	33 (23%)	71 (50%)	37 (26%)	141 (100%)

GI = gastrointestinal content.
* Entries are number of positive samples of each sample type, followed by percentage of all samples of that type, followed by (in next row) percentage of samples of that type in positive cases†.
† Cases with at least one positive ELISA or RT-rtPCR sample (i.e., cases with confirmed or suspect botulism diagnoses).
‡ Sera, bloods or plasma; 4 vitreous humor; 1 body cavity fluid; 1 pericardial fluid.
§ Sera, bloods or plasma; 2 vitreous humor; 2 pericardial fluid.
¦ Samples positive in at least one of the assays. Number of positive samples in each category is followed by percentage of the positive samples in parentheses.

(χ^2 [1, *n* = 113] = 0.15, *p* = 0.7) but the RT-rtPCR was more frequently positive overall (χ^2 [1, *n* = 638] = 9.4, *p* = 0.002) and in gastrointestinal content (χ^2 [1, *n* = 175] = 9.4, *p* = 0.002) and liver (χ^2 [1, *n* = 90] = 9.4, *p* = 0.03; Table 4) samples. The liver was the most useful tissue for detection of the toxin (Table 4) but was also the tissue submitted most frequently. Other tissues that tested positive were spleen (1 of 7 positive by C gene RT-rtPCR), lung (1 of 9 positive by ELISA, and 1 of 5 positive by C gene RT PCR), skeletal muscle (1 of 7 positive by C gene RT-rtPCR), gall bladder (1 of 2 positive by ELISA), and sciatic nerve (2 of 5 positive by ELISA). Kidney (4 samples), brain (7), and stomach wall (2) were all negative in both tests.

In positive cases, samples of gastrointestinal tract contents from the upper end of the tract appeared to be more frequently positive with the C toxin gene RT-rtPCR (contents of gizzards + proventriculus + stomach: 41% positive, *n* = 42) than samples from the lower end (contents of "gut" + duodenum + small and large intestines + feces: 32%, *n* = 134) but this was not statistically significant. There was little difference in the frequency of positive results along the gastrointestinal tract by ELISA (upper end 21%, *n* = 42, vs. lower end 20%, *n* = 134).

Toxin was rarely detected in every sample from an individual bird suffering from botulism. Only 2 of the 103 cases received had positive results in both the ELISA and the C toxin gene RT-rtPCR for every sample received: a duck (serum, stomach contents, feces, liver, lung) and a black duck (stomach and intestinal contents, liver, maggots). Similarly, in cases with a diagnosis of botulism or suspect botulism in which samples from multiple birds were submitted (36 cases with an average number of 5 birds/case), the same

diagnosis could only be confirmed in less than half of the birds submitted. The percentage of birds with identical individual diagnoses in cases in which larger numbers of birds were submitted (5 birds or more) was even lower. The confirmed botulism diagnosis of the biggest case with 26 commercial chickens (26 serum samples and 5 gizzard contents submitted) relied on the results from only 3 birds (1 serum and 2 gizzard contents positive by ELISA). The diagnosis of confirmed botulism in a case involving wild waterfowl with 19 ducks collected (serum, gastrointestinal content, liver) could only be made in 12 ducks (only 3 of which were positive by both ELISA and RT-rtPCR). Environmental samples (maggots, water) were only submitted in 13 cases with a diagnosis of botulism or suspect botulism, and were only positive in 6 cases (5 by RT-rtPCR and ELISA, and 1 by ELISA only). Only 3 environmental samples were received for the remaining cases, and all tested negative. Overall, in positive cases in which multiple birds were submitted, the same diagnosis as the case diagnosis was made in 62 of 171 (36%) birds submitted. All of the samples from 93 of the remaining 109 birds tested negative by both tests.

In our series of 103 cases in which 638 samples were examined, 11% of samples were positive by ELISA, 17% by RT-rtPCR, and 6% positive with both tests. The 50 cases of confirmed or suspect botulism comprised of 430 samples only had 141 (33%) samples with a positive test result by either the ELISA or RT-rtPCR. Of these, 16% were positive by ELISA, 25% by RT-rtPCR, and 9% by both assays. The sensitivity of the detection of positive samples by RT-rtPCR or ELISA was 50% for the ELISA only, 77% for the RT-rtPCR only, and 26% by ELISA and RT-rtPCR (Table 4).

Discussion

The monoclonal detection antibody used for the ELISA detected an antigen common to toxin types C and D, and was able to detect toxin complexes as well as purified neurotoxins and their corresponding toxoids. The improvement in sensitivity with longer sample incubation times in the ELISA had to be balanced against the possibility of enzymic degradation of toxin or coating antibody when incubating samples of gut contents. In practice, the sample incubation time used was ~60–90 min. The sensitivity of the assay was improved by lengthening the incubation time with the monoclonal detection antibody. The longest practicable incubation time was used at this step. The ELISA cutoff between positive and negative results (average corrected blank reading + 5 SDs) was chosen to reduce the risk of false-positive results. There is, therefore, a slight risk of false-negative results by the ELISA, particularly with inhibitory samples. If an ELISA result is just below the cutoff, and the sample has >50% inhibition, it is likely to be a false negative. This can be flagged in reporting.

Toxin-associated nucleic acid was used as a surrogate for the presence of toxin rather than for the presence of bacterial spores or vegetative cells.[27] The sample extraction method of preparing a clear supernatant from a homogenate prior to ELISA or nucleic acid extraction was to ensure that toxin, not bacterial cells, was detected. This approach differs from that of other authors, who either extracted nucleic acids directly from homogenates or cultured the sample homogenates prior to nucleic acid extraction, or compared both of these methods.[3,27,35,58] We used RT-rtPCR because it has been reported that both RNA and DNA are bound to botulinum toxins[27,31,33,42]; we confirmed this experimentally.

The sensitivity of the C toxin gene RT-rtPCR appeared to be less than that of the ELISA when diluted toxin was extracted and assayed, but in practice using clinical samples, it was similar or better, depending on the sample type. The analytical sensitivity of the ELISA was similar to the mouse bioassay for the detection of both C and D toxin to achieve a practical sensitivity of $<10\,MLD_{50}$/mL, which is comparable with the mouse bioassay.[39] Although the mouse protection bioassay is very sensitive at detecting low concentrations of botulinum toxin, it requires a total amount of at least 5–10 MLD of toxin per sample to run a full mouse bioassay (i.e., 2 mice each protected for C and D toxin, 2 unprotected mice, and 2 mice challenged with heat-inactivated sample). Avian samples are often very small and insufficient for the full mouse bioassay. Provided that the same amount of toxin is contained in 1 mL of sample, it can also be detected with an ELISA. However, the mouse bioassay is less likely to be affected by matrix interference. The RT-rtPCR detection of the toxin-associated RNA/DNA, as a surrogate for the presence of C toxin, showed slightly less analytical sensitivity than the ELISA. The sensitivity of both assays, however, should be sufficient to confirm botulism in birds. Birds, in general, are not very sensitive to type C toxin, and the lethal dose, particularly for waterbirds, can be up to 1,000 times higher than the lethal dose for mice.[49,51] Birds often recover from intoxications with appropriate supportive therapy, and C toxin can be demonstrated in serum of asymptomatic chickens by the mouse bioassay.[2,14,51] The RT-rtPCRs that we used detect C or D toxins, including the associated mosaic forms of C/D or D/C, respectively (by comparison of the primer sequences used, with C/D and D/C gene sequences in GenBank, EMBL, and DNA Database of Japan). There was no evidence that any of our cases were associated with type D or the mosaic D/C toxin. This is not surprising, given that birds are generally not considered to be susceptible to type D toxin, with the possible exception of turkeys.[24,34,35,57] The presence of E toxin or associated RNA/DNA was not excluded given the lack of specific tests; the in-house ELISA was unlikely to cross-react with E toxin. Botulism outbreaks caused by botulinum type E toxin have never been reported in Australia, and the presence of the organism in Australia is debatable.[9]

Our study indicated that samples of gastrointestinal content and liver are good sources of toxin. Similar observations were made by others with and without the use of pre-enrichment

culture.[3,7,14,34,35,55] It has been suggested that toxin accumulates in liver and spleen, but there are no data available to suggest that toxin is cleared through the liver.[54,56] Liver seems to be better than other tissues for the demonstration of bacterial spores, vegetative cells, and toxin in botulism outbreaks.[6,7,34,35,55] Surprisingly, *C. botulinum* spores were also observed in liver and cecum of a large proportion of sentinel mallards in an experimental study.[47] *Clostridium* spp. have the capability to hide as spores in animal tissues, especially liver, possibly entering through the gastrointestinal route.[61] It is unclear whether this mode of entry also occurs commonly with *C. botulinum* or if it is possibly only a perimortem phenomenon. Toxic maggots can be found on carcasses within 3 d of death, irrespective of whether the carcasses originated from birds that died of botulism or were from euthanized healthy birds. Carcasses play an important epidemiologic role in botulism outbreaks in wild birds. Carcass removal is therefore an important control measure.[47] The etiologic and epidemiologic role of *C. botulinum* spores in botulism outbreaks in wild bird populations requires further in-depth studies to assist with sample selection and interpretation of laboratory test results.

Environmental samples including the likely food source, especially maggots, are recommended for testing in botulism outbreaks because maggots usually contain concentrations of toxin well above the detection limit of laboratory tests; a single maggot can contain enough botulinum toxin to be lethal for a duck.[49] However, maggots are rarely submitted in botulism outbreaks. Only 17 environmental samples (41% of which tested positive for botulinum toxin), including 5 samples of maggots, were submitted in our case series. Maggots were rarely observed in the gastrointestinal tract of the submitted birds, contrary to other reports.[14] Toxic maggots are most likely found after the initial phase of an outbreak when large numbers of carcasses are present.[47]

Demonstrating botulinum toxin in pre-enrichment culture supernatant by ELISA or PCR has been claimed as the method of choice for the diagnosis of botulism in birds and mammals.[3,6,10,11,34,35,58] We made no attempts to use the pre-enrichment culture technique of *C. botulinum* for biosafety reasons. The success of pre-enrichment culture demonstration of *C. botulinum* spores indicates a close association of the presence of spores and toxin in relevant samples during a botulism outbreak. Others, on the other hand, consider the detection of spores in animal samples of botulism either as rare or only successful in the gastrointestinal content of approximately one-third of the birds during an outbreak investigation.[3,45] The diagnosis of botulism *sensu stricto* requires the demonstration of the relevant toxin rather than the bacterium. The high potency of the toxin makes mass intoxications without the presence of bacterial vegetative forms or spores possible. The demonstration of spores can also be the cause of false-positive results. Shedding of *C. botulinum* in feces or presence in gastrointestinal content of normal birds has been demonstrated during non-outbreak periods in up to 50% of birds.[2,47,59] We found no evidence of the presence of the C or D toxin gene in the gastrointestinal tract of normal healthy migratory birds, although the presence of spores only would not be detected by the sample preparation used.

After ingestion, toxic food passes along the gastrointestinal tract; toxin traverses the intestinal epithelium to the lymph, portal vein, and liver, enters the general circulation, and then the muscles and motor end-plates. If the toxic food or substance was consumed on one occasion only, the meal may pass from one end of the gut to the other before signs are noted and samples are collected. Food retention time in waterbirds is only about 24 h, but, depending on the species and length of the ceca, *C. botulinum* can be excreted for up to 7 d after treatment and hospitalization.[3] It is therefore important to test several different samples from each bird and, when possible, more than one bird, to maximize the chance of detecting this toxin. Likewise, to increase the chance of detection, we found that it is worth performing both the ELISA and the C/D toxin RT-rtPCR in birds. It is unclear, but diagnostically important, where and when the toxin complex–associated RNA/DNA dissociates from the actual neurotoxin. The RNA/DNA is tightly bound to the neurotoxin, difficult to separate, and detectable even in highly purified neurotoxin preparations; special steps during purification are required to remove it.[31,33,38,40,42] Dissociation of the neurotoxin from the nontoxic proteins of the toxin complex occurs as soon as the toxin complex has passed the intestinal and vascular endothelium. According to some studies, only neurotoxin (and no non-toxin proteins) can be found in blood or lymph, but other studies found undissociated toxin complex in the lymph.[15,26,31,44,51] The fate of the associated non-toxin proteins is not known but re-association to form the whole active toxin complex is a possibility.[15,44] It is unclear if the same applies to toxin-associated nucleic acid, but we have found toxin-associated RNA/DNA in tissue and blood samples either with or without detectable neurotoxin.

Despite the use of highly sensitive assays, the dispersion of the toxin and its nontoxic components through the animal body and tissues might account for the generally poor detection rate in individual birds of larger botulism outbreaks. In our cases with multiple bird submissions (average of 5 birds), less than half the birds in each case had an identical diagnosis. Confirmation or exclusion of botulism, therefore, requires the submission of samples collected from at least 4 to 5 live symptomatic birds. Similarly, using cultural pre-enrichment for the diagnosis of botulism in birds, previous studies recommended the submission and testing of samples from at least 4 birds in an outbreak.[34] Others found that *C. botulinum* spores were carried in the gastrointestinal content of only 37% of birds during an outbreak, explaining the relatively poor sensitivity of pre-enrichment culture.[3] The demonstration of toxin in the serum of live symptomatic birds is considered to be diagnostic but seems to be very unreliable even in clinically obvious cases. In our case with the largest

number of birds submitted, the toxin could only be demonstrated in serum of 1 of 26 chickens. Similarly, in our case of botulism in ducks, only half of the 12 sera tested were positive for botulinum toxin by RT-rtPCR. Toxin could also only be demonstrated in serum of a few birds during a massive botulism outbreak involving several hundred birds, but others reported up to 77% positive detection rates in bird serum using the mouse lethality assay.[3,34] The observation that positive results in sera are not common may indicate that the levels in serum are often below the limit of detection, possibly because of dispersion. The dynamic distribution and fate of the botulinum toxin and complex are not well known. The retention of toxin in serum over time is reported to be stable, and the half-life of the toxin in mouse serum is up to 300 min.[46,54] In only ~20% of human botulism patients can toxin be demonstrated in serum, but in some cases, it is detectable for up to 30 d after the onset of symptoms.[5,16,54]

The diagnosis of avian botulism, therefore, remains a diagnostic challenge despite the availability of new immunologic and molecular tests that are as sensitive as the mouse inoculation bioassay. Fresh samples from birds showing clinical signs are not always available for testing and are often compromised by matrix interference. The variable kinetic distribution of the toxin in the body over time also makes optimal sample selection for the detection of toxin difficult. However, in contrast to the mouse protection bioassay, immunologic and molecular assays for botulinum toxin allow the testing of a large number of samples at a reasonable cost. Furthermore, as we demonstrated, the detection of toxin-associated RNA/DNA, as a surrogate for the presence of botulinum toxin, in particular, is a very useful addition to the molecular testing arsenal of veterinary diagnostic laboratories. All reagents are easily obtainable, and the development of specific antibodies produced in-house is not required.

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References

1. Anniballi F, et al. Multiplex real-time PCR SYBR Green for detection and typing of group III *Clostridium botulinum*. Vet Microbiol 2012;154:332–338.
2. Anza I, et al. Eutrophication and bacterial pathogens as risk factors for avian botulism outbreaks in wetlands receiving effluents from urban wastewater treatment plants. Appl Environ Microbiol 2014;80:4251–4259.
3. Anza I, et al. Differences in the vulnerability of waterbird species to botulism outbreaks in Mediterranean wetlands: an assessment of ecological and physiological factors. Appl Environ Microbiol 2016;82:3092–3099.
4. Barteling SJ, et al. Innocuity testing of foot-and-mouth disease vaccines. I. Formaldehyde-inactivated alhydrogel vaccines. J Biol Stand 1983;11:297–304.
5. Böhnel H, Gessler F. Botulinum toxins—cause of botulism and systemic diseases. Vet Res Commun 2005;29:313–345.
6. Böhnel H, et al. Tonsils—place of botulinum toxin production: results of routine laboratory diagnosis in farm animals. Vet Microbiol 2008;130:403–409.
7. Böhnel H, et al. Presumptive diagnostic of an intrauterine transmission of *Clostridium botulinum* in a bovine fetus. Int J Vet Med Res Reports 2013:747291.
8. Boyer AE, et al. From the mouse to the mass spectrometer: detection and differentiation of the endoproteinase activities of botulinum neurotoxins A–G by mass spectrometry. Anal Biochem 2005;77:3916–3924.
9. Brandis KJ, et al. Avian-botulism risk in waterbird breeding colonies and implications for environmental water management. Mar Freshw Res 2019;71:179–190.
10. Brooks CE, et al. Culture enrichment assists the diagnosis of cattle botulism by a monoclonal antibody based sandwich ELISA. Vet Microbiol 2010;144:226–230.
11. Brooks CE, et al. Diagnosis of botulism types C and D in cattle by a monoclonal antibody-based sandwich ELISA. Vet Rec 2011;168:455.
12. Coffield JA, Whelchel DD. Botulinum neurotoxin. In: Gupta RC, ed. Veterinary Toxicology: Basic and Clinical Principles. 1st ed. Elsevier, 2007:755–770.
13. Cope RB. Botulinum neurotoxins. In: Gupta RC, ed. Veterinary Toxicology: Basic and Clinical Principals. 3rd ed. Elsevier, 2018:743–757.
14. Defillippo F, et al. Outbreak of type C botulism in birds and mammals in the Emilia Roma Region, Northern Italy. J Wildl Dis 2013;49:1042–1046.
15. Dorner MB, et al. Complexity of botulinum neurotoxins: challenges for detection technology. In: Rummel A, Binz T, eds. Botulinum Neurotoxins. (Current Topics in Microbiology and Immunology, Book 364). Springer, 2013;219–255.
16. Dowell VR Jr, et al. Copro-examination for botulinal toxin and *Clostridium botulinum*. A new procedure for laboratory diagnosis of botulism. JAMA 1977;238:1829–1832.
17. Ellis TM. Botulism in pastoral cattle, an investigation of improved methods of detection: final report for the Cattle

Industry Compensation Act–Research Advisory Committee. Western Australia Department of Agriculture, 1989.
18. Espelund M, Klaveness D. Botulism outbreaks in natural environments—an update. Front Microbiol 2014;5:287.
19. Galfrè G, Milstein C. Preparation of monoclonal antibodies: strategies and procedures. Method Enzymol 1981;73:3–46.
20. Gessler F, Böhnel H. Production and purification of *Clostridium botulinum* type C and D neurotoxin. FEMS Immunol Med Microbiol 1999;24:361–367.
21. Gill DM. Bacterial toxins: a table of lethal amounts. Microbiol Rev 1982;46:86–94.
22. Grate JW, et al. Advances in assays and analytical approaches for botulinum toxin detection. Trends Anal Chem 2010;29:1137–1156.
23. Gregory AR, et al. Use of enzyme-linked immunoassays for antibody to types C and D botulinum toxins for investigations of botulism in cattle. Aust Vet J 1996;73:55–61.
24. Gross WB, Smith LDS. Experimental botulism in gallinaceous birds. Avian Dis 1971;15:716–722.
25. Harlow E, Lane D. Antibodies. A laboratory manual. 1st ed. Cold Spring Harbor Laboratory Press, 1988.
26. Heckly RJ, et al. On the size of the toxic particle passing the intestinal barrier in botulism. J Exp Med 1960;111:745–759.
27. Hill BJ, et al. Universal and specific quantitative detection of botulinum neurotoxin genes. BMC Microbiol 2010;10:267–283.
28. Hyun S-H, Sakaguchi G. Implication of coprophagy in pathogenesis of chicken botulism. Nihon Juigaku Zasshi 1989;51:582–586.
29. Jones RGA, et al. Development of improved SNAP25 endopeptidase immuno-assays for botulinum type A and E toxins. J Immunol Methods 2008;329:92–101.
30. Kalb SR, et al. The use of endopep-MS for the detection of botulinum toxins A, B, E and F in serum and stool samples. Anal Biochem 2006;351:84–92.
31. Kitamura M, et al. Purification and some properties of *Clostridium botulinum* type-E toxin. Biochim Biophys Acta 1968;168:207–217.
32. Kouguchi H, et al. Quantitative detection of gene expression and toxin complex produced by *Clostridium botulinum* serotype D strain 4947. J Microbiol Methods 2006;67:416–423.
33. Kozaki S, et al. Purification and some properties of progenitor toxins of *Clostridium botulinum* Type B. Infect Immun 1974;10:750–756.
34. Le Maréchal C. Livers provide a reliable matrix for real-time PCR confirmation of avian botulism. Anaerobe 2016;38:7–13.
35. Le Maréchal, et al. Development and validation of a new reliable method for the diagnosis of avian botulism. PLoS One 2017;12:1–17.
36. Lindberg A, et al. Real-time PCR for *Clostridium botulinum* type C neurotoxin (BoNTC) gene, also covering a chimeric C/D sequence—application on outbreaks of botulism in poultry. Vet Microbiol 2010;146:118–123.
37. Lindström M, Korkeala H. Laboratory diagnostics of botulism. Clin Microbiol Rev 2006;19:298–314.
38. Miyazaki S, Sakaguchi G. Experimental botulism in chickens: the cecum as the site of production and absorption of botulinum toxin. Jpn J Med Sci Biol 1978;31:1–15.
39. Nakamura K, et al. Improved detection methods by genetic and immunological techniques for botulinum C/D and D/C mosaic neurotoxin. Vet Microbiol 2013;162:881–890.
40. Norwood DA, et al. Molecular and immunological detection of botulinum toxins, a comparative study. Presented at the Annual Interagency Botulinum Research Coordinating Committee Meeting; Silver Spring, MD; 14–17 Nov 2006.
41. Oguma K, et al. Homogeneity and heterogeneity of toxins produced by *Clostridium botulinum* type C and D strains. Infect Immun 1981;34:382–388.
42. Ohishi I, Sakaguchi G. Purification of *Clostridium botulinum* type F progenitor toxin. Appl Microbiol 1974;28: 923–928.
43. Piazza TM, et al. In vitro detection and quantification of botulinum neurotoxin type E activity in avian blood. Appl Environ Microbiol 2011;77:7815–7822.
44. Popoff MR, Connan C. Absorption and transport of botulinum neurotoxin. In: Foster KA, ed. Molecular Aspects of Botulinum Neurotoxin. (Current Topics in Neurotoxicity 4). Springer, 2014:35–68.
45. Prévot AR, Brygoo ER. Nouvelle recherches sur le botulisme et ses cinq types toxiniques [New investigations on botulism and its five toxic types]. Ann Inst Pasteur Paris 1953;85:544–575. French.
46. Ravichandran E, et al. An initial assessment of the systemic pharmacokinetics of botulinum toxin. J Pharmacol Exp Ther 2006;318:1343–1351.
47. Reed TM, Rocke TE. The role of avian carcasses in botulism epizootics. Wildlife Soc Bull 1992;20:175–182.
48. Reiss J, Reiss K. Recent advances in detection and identification of botulinum neurotoxins (BoNTs). J Med CBR Def. 2007;5.
49. Rocke TE. The global importance of avian botulism. In: Boere GC, et al., eds. Waterbirds Around the World. Scottish National Heritage, 2006:422–426. https://data.jncc.gov.uk/data/08cfb4da-4c5a-4bef-b45d-8f2f87dc8070/waterbirds-around-the-world.pdf
50. Ruge DR, et al. Detection of six serotypes of botulinum neurotoxin using fluorogenic reporters. Anal Biochem 2011;411: 200–209.
51. Sakaguchi G. *Clostridium botulinum* toxins. Pharmacol Ther 1982;19:165–194.
52. Sánchez-Hernández L, et al. Detection of *Clostridium botulinum* neurotoxin coding genes: analysis of PCR products by real time versus capillary gel electrophoresis methods. Eur Food Res Technol 2008;227:495–502.
53. Simon S, et al. Recommended immunological strategies to screen for botulinum neurotoxin-containing samples. Toxins 2015;7:5011–5034.
54. Simpson L. The life history of a botulinum toxin molecule. Toxicon 2013;68:40–59.
55. Skarin H. Investigations into an outbreak of botulism caused by *Clostridium botulinum* type C/D in laying hens. Avian Dis 2015;59:335–340.
56. Takahashi T, et al. Localisation of the sites and characterisation of the mechanisms by which anti-light chain antibodies neutralise the actions of the botulinum holotoxin. Vaccine 2009;27:2616–2624.
57. Takeda M, et al. Characterisation of the neurotoxin produced by isolates associated with avian botulism. Avian Dis 2005;49:376–381.
58. Vidal D, et al. Real-time polymerase chain reaction for the detection of toxigenic *Clostridium botulinum* type C1 in waterbird

and sediment samples: comparison with other PCR techniques. J Vet Diagn Invest 2011;23:942–946.
59. Vidal D, et al. Environmental factors influencing the prevalence of *Clostridium botulinum* type C/D mosaic strain in non-permanent Mediterranean wetlands. Appl Environ Microbiol 2013;79:4264–4271.
60. Woudstra C, et al. Neurotoxin gene profiling of *Clostridium botulinum* types C and D native to different countries within Europe. Appl Environ Microbiol 2012;78:3120–3127.
61. Zachary JF. Mechanisms of microbial infections. In: Zachary JF, ed. Pathologic Basis of Veterinary Disease. 6th ed. Elsevier, 2017:132–241.