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Accuracy of a Mouse Bioassay for the Diagnosis of Botulism in Horses

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Background: The laboratory diagnosis of botulism in horses traditionally has relied upon the mouse bioassay (MBA). The accuracy of this test for the diagnosis of botulism in horses is unknown.

Hypothesis/Objectives: Our goal was to determine the sensitivity, specificity, positive predictive value, and negative predictive value of the MBA on laboratory-processed fecal and gastrointestinal samples for foals and adult horses.

Animals: Cases included all horses with a final clinical diagnosis of botulism that were admitted between 1986 and 2011 and had MBA testing performed. Controls included horses without botulism that were admitted during the same time period and had MBA testing performed.

Methods: Retrospective study. Horses suspected of having botulism had fecal or (less commonly) gastrointestinal content samples tested using MBA. For every hospitalized botulism suspect, control samples were obtained from ≥1 additional hospitalized horses not suspected to have botulism.

Results: One hundred and twenty-nine adult horses and 253 adult controls were identified. Overall sensitivity of the MBA was only 32% but specificity was 97%. Forty-three foal cases and 21 foal controls were evaluated; sensitivity of the MBA was 53% and specificity was 100%. Positive predictive value was substantially higher (100% for foals and 89% for adults) than negative predictive value (51% for foals and 67% for adults).

Conclusions and Clinical Importance: Mouse bioassay has low sensitivity but high specificity for the diagnosis of botulism in horses. Positive results are highly suggestive of botulism but negative results do not exclude the diagnosis. Unaffected horses and foals rarely shed *C. botulinum* in their feces.

Key words: Botulinum; Clostridium; Fecal; Foal; Manure.

Botulism, caused by the *Clostridium botulinum* neurotoxin (BoNT), is a disease characterized by progressive muscle weakness and cranial nerve deficits, particularly dysphagia. Horses are most commonly affected by type B botulism, which in adult horses is acquired primarily through ingestion of preformed toxin in feed, and in foals generally is a toxicoinfection.[1–3] Disease course is related to total toxin exposure, and most commonly results in death unless the horse is treated promptly with specific antitoxin.[1–3]

The necessity of prompt treatment and threat of outbreaks linked to a common feed source make fast and accurate diagnosis crucial. Unfortunately, laboratory diagnosis has proven difficult in horses, and botulism generally is considered to be a clinical diagnosis.[1–3] Available laboratory tests include the mouse bioassay (MBA) and polymerase chain reaction (PCR) assays for BoNT genes.[4,5] These tests have been compared to each other, but there has been no evaluation of the sensitivity and specificity of either assay in a clinical population of horses, and the largest retrospective study to date indicates that the sensitivity of the MBA for botulism in horses is likely to be low.[3] This finding is in agreement with data from foodborne botulism in humans, for which the sensitivity of laboratory tests for clinical specimens can be as low as 33–44%.[6,7] In addition to concerns about low sensitivity, the specificity of the MBA has been questioned. Because this test is typically performed on culture-enriched manure samples, false-positive results may occur because of "pass-through" spore contamination of samples, whereby patients ingest and excrete *C. botulinum* spores without germination, toxin production, or any adverse effect.[8] The likelihood of a clinically normal horse having a positive MBA

Abbreviations:

AUC	area under the curve
BoNT	*Clostridium botulinum* neurotoxin
CI	confidence interval
CMGS	chopped meat-glucose-starch
ELISA	enzyme-linked immunosorbent assay
GI	gastrointestinal
GPB	gelatin phosphate buffer
IQR	interquartile range
MBA	mouse bioassay
NBC	New Bolton Center
NPV	negative predictive value
OR	odds ratio
PCR	polymerase chain reaction
PPV	positive predictive value
ROC	receiver operating characteristic

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result on a culture-enriched manure sample has not been investigated, but previous work indicated a false-positive MBA result for one per 20 clinically normal horses.[5]

The primary goal of this retrospective study was to determine the accuracy of the MBA for the diagnosis of botulism in a hospitalized population of horses in an endemic region. Secondary aims were to determine whether having a positive test result was predictive of outcome for affected horses, and whether fecal samples from unaffected horses contain *C. botulinum* spores.

Materials and Methods

Study Population and Sample collection

This investigation was a retrospective study of MBA results from foals (<6 months of age) and adult horses (≥6 months of age) that were clinic or pathology cases at New Bolton Center (NBC), University of Pennsylvania School of Veterinary Medicine, and had a final diagnosis of botulism with laboratory testing performed from 1986 to 2011. Cases were identified by searching the laboratory records system of the Botulism Reference Laboratory at NBC for all test results from horses with NBC clinic or pathology medical record numbers. Horses were considered cases if the final clinical diagnosis was botulism, regardless of MBA results. A clinical diagnosis of botulism was made if the horse had characteristic signs of diffuse neuromuscular weakness affecting the head and remainder of the body. These signs included decreased tongue tone, decreased eyelid tone, decreased tail tone, dysphagia, muscle tremors, short-strided gait, decreased anal tone, mydriasis with slow pupillary light reflexes, increased time in recumbency, and inability to rise. Specific clinical tests performed included the tongue stress test and the grain test; abnormal results were considered supportive for botulism. The former involves gently withdrawing the horse's tongue from the mouth while holding the jaws closed, and assessing the horse's ability to retract the tongue (normal horses retract after 1–2 attempts). The latter involves offering the horse 8 ounces of sweet feed in a flat feeding tub and timing how quickly the horse consumes the feed (normal is < 2 minutes). Alternative diagnoses were excluded using appropriate diagnostic tests when indicated. For example, esophageal obstruction and guttural pouch disease were excluded using endoscopy for horses showing predominately dysphagia. When postmortem results were available, horses with botulism were expected to have no gross or histologic lesions that could explain the clinical signs. Horses were considered controls, if an alternative diagnosis was reached either clinically or after postmortem examination. Also, during this study period, a protocol was in place to collect additional control samples from horses hospitalized at the same time as botulism suspects; these control horses all had final diagnoses other than botulism.

The standard protocol for sample collection from horses that were hospitalized as botulism suspects was to collect fecal samples on 3 consecutive days, starting on the day of admission. Horses that died or were euthanized had gastrointestinal (GI) content samples collected during postmortem examination. Occasionally, horses had serum, feed, wound exudate, or other samples submitted for testing. Control horses were identified for most hospitalized botulism suspects; standard protocol was to use 2 hospitalized patients as controls for each botulism suspect and to collect fecal samples from them on the same 3 consecutive days, as samples were collected from the botulism suspect.

Mouse Bioassay

The MBA protocol was approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC number 804158) and performed pre- and postculture enrichment to allow detection of preformed BoNT, *C. botulinum* spores, or both in the original sample.[2,9] In brief, samples (2–5 g) were diluted 1:2 (W:V) with gelatin phosphate buffer (GPB; 0.2% gelatin, 0.4% Na_2PO_4; pH 6.4), homogenized, and refrigerated at 4°C overnight. On the second day, 6 mL of this suspension were transferred to a sterile tube and centrifuged. Supernatant was injected intraperitoneally (IP) into four 5–6 week old Swiss Webster mice weighing 20–30 g each (0.4 mL each) and 2 of the mice also received 0.1 mL *C. botulinum* type B antitoxin. Mice were observed for clinical signs of botulism for 4 days. Positive results, along with successful neutralization of the toxin, indicated the presence of BoNT (pre-formed toxin) in the original sample and confirmed the serotype as type B.

In addition, the original samples were culture-enriched for 5–7 days before MBA to allow detection of *C. botulinum* spores. A 1 mL aliquot of the original suspension was inoculated into a degassed tube containing chopped meat-glucose-starch (CMGS) media. The samples in CMGS media were transferred to an anaerobic chamber (88–90% N_2, 5–7% H_2, 5% CO_2) at 37°C for 5–7 days. Culture samples then were vortexed to remove toxin from chopped meat particles and 4 mL of the liquid portion was transferred into tubes containing 4 mL GPB. The resulting mixture was centrifuged and the supernatant (containing any toxin elaborated by *C. botulinum* during the culture period) was used for MBA as described above. Positive results indicated the presence of *C. botulinum* type B spores in the original sample.

Data Collection

Data were retrieved from laboratory, hospital, and pathology records for cases and controls. Information collected included age, admission and discharge dates, final diagnosis, survival status, types and timing of samples submitted for MBA, and all MBA results. Horses were classified into 5 categories: foal case, foal control, adult case, adult control, and control of unknown age. The MBA results were classified into 4 categories: negative, positive for preformed BoNT only, positive for *C. botulinum* spores only, and positive for both preformed BoNT and *C. botulinum* spores.

Statistical Analysis

Descriptive statistics and additional analyses were performed using a commercially available statistical software package.[a] Foal data were analyzed separately from adult data, such that foal cases were compared to foal controls and adult cases were compared to adult controls. However, because of a limited number of foal controls, foal cases also were compared to a mixed control group of both foals and adults. The adults in this mixed control group were different horses than those in the adult control group compared to adult cases. Controls of unknown age were excluded from analysis. Several control horses were used repeatedly; only the first 2 uses were included in analysis, and subsequent uses were excluded. Data for continuous variables were evaluated for normality using a Shapiro–Wilk test. Results for cases and controls were compared using the nonparametric Wilcoxon rank–sum (Mann–Whitney) test for non-normally distributed continuous variables. Fisher's exact test or chi-squared test was used for categorical data. All of these tests identified variables that were significantly different between cases and controls. For these significantly different variables,

logistic regression with robust variance estimation (to control for within-horse repeated measures) was used to establish odds ratios and quantify the magnitude of the difference.

Descriptive statistics, including sensitivity, specificity, overall accuracy, positive predictive value (PPV), negative predictive value (NPV), and 95% exact binomial confidence intervals (CI), were calculated for the MBA separately for foal and adult populations. The area under the receiver operating characteristic (ROC) curve was determined as a summary statistic of discriminatory ability in each population. Somers' *D* statistic also was determined as an indicator of the predictive ability of the test. The Somers' *D* statistic is the ratio of probabilities of concordance and discordance between methods. Values range from −1 (all pairs disagree) to 1 (all pairs agree); larger values indicate better predictive value. The closer the Somers' *D* statistic approaches 1, the better the alternate test's predictive ability, with 1 being it predicts the outcome perfectly. The null hypothesis is that compared to the definitive test, which in this study was the final diagnosis, the alternate test is not predictive. For all methods, statistical significance was inferred when $P < .05$.

Results

Population Demographics and MBA Results

Forty-three foal botulism cases were identified. Eighteen of these cases were included in a retrospective analysis of foals treated for botulism.[10] Fifty-three percent of these foals (23/43) had at least 1 positive MBA result when all sample types (feces, GI content, serum, and wound exudate) were considered; all positive results were *C. botulinum* type B. Twenty-one foal controls were linked to the foal cases, representing 20 animals (1 foal was used twice). None of the foal controls had a positive MBA result. Because foal controls were not always available for the foal botulism cases, adult controls were also linked to the foal cases. A total of 73 adult controls were used for foal cases, representing 70 animals (3 horses were used twice). Three of these adult controls had positive MBA results. For 3 unknown controls enough identifying information was not available to ascertain whether the animal was a foal or an adult; all had negative MBA results but were excluded from analysis.

One-hundred and twenty-nine adult botulism cases were identified. Eighty-three of these cases were included in a recent retrospective analysis of adult horses treated for botulism.[3] Thirty-two percent of these horses (41/129) had at least 1 positive MBA result when all sample types (feces, GI content, serum, wound exudate) were considered; all positive results were *C. botulinum* type B. One-hundred and eighty adult controls were linked to the adult botulism cases. Because 8 horses were used as controls on 2 occasions, this control set represented 172 animals. Five of these adult control horses had positive MBA results.

Table 1 shows a summary of MBA results by patient category. Although the recommended protocol was to collect fecal samples on the first 3 days of hospitalization for all potential botulism cases (along with corresponding control samples), not every case had a complete set of samples. Gastrointestinal samples generally were only submitted when potentially affected horses died or were euthanized, and these samples were tested instead of fecal samples. Serum samples (not shown in Table 1) were collected sporadically and only tested in 12 animals. Wound exudate (not shown in Table 1) was tested only if wound botulism was suspected (12 animals).

Of the 43 foal botulism cases, 38 had fecal samples submitted, and 20/38 (53%) had at least 1 positive fecal sample. Seventeen of the 20 were positive on the day 1 sample, 1 foal was negative on day 1 but positive on day 2, and 2 foals were negative on days 1 and 2 but positive on day 3. Two foal cases had fecal samples submitted on day 4, and 1 was positive; this foal was also positive on the first 3 days. Therefore, 41/97 (42%) of all fecal samples from foal cases were positive on MBA. Thirty-four of the positive fecal samples were only positive after culture, indicating that they contained *C. botulinum* spores but BoNT concentrations were below the limit of detection before culture

Table 1. Summary of MBA results by patient category.

Patient Classification	Sample Sets, n	Any positive MBA Result[a], n (%)	Positive Day 1 Fecal, n (%)	Positive Day 2 Fecal, n (%)	Positive Day 3 Fecal, n (%)	Positive GI Content, n (%)
Foal case	43	23/43 (53%)	17/38 (45%) *14 spores* *3 toxin + spores*	12/33 (36%) *10 spores* *2 toxin + spores*	11/24 (46%) *10 spores* *1 toxin + spores*	2/4 *2 spores*
Adult case	129	41/129 (32%)	20/85 (24%) *2 toxin* *14 spores* *4 toxin + spores*	7/62 (11%) *1 toxin* *5 spores* *1 toxin + spores*	4/51 (8%) *4 spores*	8/40 (20%) *2 toxin* *6 spores*
Overall case	172	64/172 (37%)	37/123 (30%)	19/95 (20%)	15/75 (20%)	10/44 (23%)
Foal control	21	0/21 (0%)	0/16	0/11	0/7	na
Adult control	253	8/253 (3%)	3/201 (1%) *1 toxin* *2 spores*	3/159 (2%) *3 spores*	1/112 (1%) *1 spores*	0/7
Overall control	274	8/274 (3%)	3/217 (1%)	3/170 (2%)	1/119 (1%)	0/7

[a]"Any" positive MBA result indicates that at least 1 sample from the patient was positive on MBA; these samples could include feces, GI content, or wound swabs.

enrichment. Seven of the positive fecal samples were positive for both BoNT and spores. Four foals had GI content samples submitted and 2 were positive for spores only. Two foal cases had wound swabs submitted and 1 was positive for spores. Five foal cases had serum samples submitted and all were negative for BoNT.

Of the 129 adult botulism cases, 89 had fecal samples submitted, and 26/89 (29%) had at least 1 positive fecal sample. Twenty of the 26 were positive on the day 1 sample, 4 horses were negative on day 1 but positive on day 2, and 2 horses were negative on days 1 and 2 but positive on day 3. Eight adult cases had fecal samples submitted on day 4, but none were positive. Six adult cases had fecal samples submitted on day 5, and 1 was positive; this horse also had a positive day 1 fecal result. Therefore, 32/212 (15%) of all fecal samples from adult cases were positive on MBA. Twenty-four of the positive fecal samples were only positive after culture, indicating that they contained *C. botulinum* spores but BoNT concentrations were below the limit of detection before culture enrichment. Five of the positive fecal samples were positive for both BoNT and spores, and 3 were positive for BoNT alone.

Forty adult horses had GI content samples submitted and 8 (20%) were positive, 2 for toxin and 6 for spores. The number of GI content samples tested per horse was variable, as was whether the sample(s) were pooled (from multiple areas within the GI tract) or unpooled (from a single area). Of the 8 adult cases with at least 1 positive GI content sample, 1 had a single unpooled sample tested, 2 had a single pooled sample tested, 3 had a single unspecified sample tested, and 2 had multiple pooled samples tested, with 3/8 and 2/13 pooled samples yielding positive results for the latter 2 horses. For the 32 adult horses with negative results for all GI content samples, 5 had a single unpooled sample tested, 8 had a single pooled sample tested, and 19 had multiple samples tested, with a range of 2–15 samples per horse. Seven adult horses had serum samples submitted and none were positive. Ten adult horses had suspicious feed samples submitted and 2 were positive for spores. Ten adult cases had wound swabs submitted and 4 were positive, 3 for spores and 1 for toxin and spores. Fourteen adults (11 cases and 3 controls) had additional samples submitted; these samples varied and included milk, feces from later time points, syringes, and environmental samples (eg, bedding, water, grass roots, soil, mud). Four adults (3 cases and 1 control) had positive results for at least 1 additional sample. These samples all were positive for spores and consisted of feces from later time points or environmental samples (eg, grass roots, shavings, soil, mud).

Statistical Analysis

Foals. There was no difference in duration of hospitalization between foal cases and foal controls ($P = .098$). However, foal cases were hospitalized for a significantly longer duration than the mixed control group ($P = .045$), with a median of 12 days (interquartile range [IQR], 7–19 days) compared to 4 days (IQR, 2–7 days). There was no significant difference in discharge status (dead or alive) between foal cases and controls. Foal cases were significantly more likely to have positive MBA results regardless of whether a foal control group ($P < .001$) or mixed control group (OR, 36.03; 95% CI, 9.81–132.35; $P < .001$) was used. The Somers' *D* statistic could not be determined for foal cases and foal controls, but when the foal cases and mixed group controls were examined, compared to the final clinical diagnosis, the MBA had good predictive value (Somers' $D = 0.504$, $P < .001$). Complete analysis of MBA test performance is shown in Table 2. When applied to foals, the MBA showed very high specificity (100%) and PPV (100%), but only fair sensitivity (53%) and NPV (51%). When only foal botulism cases were considered, MBA results did not accurately predict survival (ROC area under the curve [AUC] = 0.478; 95% CI, 0.264–0.693). The Somers' *D* statistic also indicated that the MBA had no ability to predict survival in foal botulism cases (Somers' $D = 0.044$; 95% CI, −0.372 to 0.460; $P = .837$).

Adults. There was no difference in duration of hospitalization between adult cases and adult controls ($P = .988$), but cases were less likely to survive than controls ($P < .001$). Adult cases were significantly more likely than adult controls to have positive MBA results (OR, 16.31; 95% CI, 6.21–42.79; $P < .001$). Analysis of MBA test performance in shown in Table 2. When

Table 2. Overall MBA test performance.

	Foal Cases, Foal Controls	Foal Cases, Mixed Age Controls	Adult Cases, Adult Controls
Sensitivity % (95% CI)	53 (38–69)	53 (38–69)	32 (24–41)
Specificity % (95% CI)	100 (84–100)	97 (91–99)	97 (94–99)
Positive predictive value % (95% CI)	100 (85–100)	88 (70–98)	89 (76–96)
Negative predictive value % (95% CI)	51 (35–67)	82 (74–89)	67 (60–72)
Overall accuracy % (95% CI)	69 (56–80)	84 (76–89)	70 (64–75)
ROC AUC (95% CI)	0.767 (0.692–0.843)	0.752 (0.675–0.829)	0.645 (0.603–0.687)
Somers' *D* statistic (95% CI)	Could not be determined	0.504 (0.349–0.659)	0.290 (0.206–0.374)

applied to adults, the MBA showed high specificity (97%) and good PPV (89%), but poor sensitivity (32%) and moderate NPV (67%). The Somers' *D* statistic indicated that, compared to the final clinical diagnosis, the MBA had only modest predictive ability (Somers' *D*, 0.290), which was nonetheless significant ($P < .001$). When only adult botulism cases were considered, MBA results did not accurately predict survival (ROC AUC, 0.517; 95% CI, 0.436–0.598). In addition, as with foals, the Somers' *D* statistic supported the inability of the MBA to predict the survival of adult botulism cases (Somers' *D*, 0.033; 95% CI, −0.129 to 0.196; $P = .688$).

Discussion

Although the MBA is considered the gold standard laboratory test for botulism, its performance varies widely for different patient populations and types of botulism. Lack of a true gold standard has hindered botulism research for decades, affecting both clinical investigation and assessment of new laboratory tests. Recent studies[8,11] in humans with botulism have used clinical diagnosis as the gold standard against which to measure the limited sensitivity of the MBA.[12] Proponents of this approach, which also was used in our study, argue that the resultant estimation of the sensitivity of the MBA reflects test performance in a clinical setting rather than under ideal laboratory conditions.[12] Results of our study demonstrate a low overall sensitivity of 32% for adult horses and a fair overall sensitivity of 53% for foals. These results are higher than those obtained for a population of cattle clinically suspected to have botulism, in which MBA sensitivity was estimated at 12.9%,[13] which is lower than that reported for people, where approximately 66% of botulism suspects will test positive.[6,11] Correspondingly, approximately one-third of people clinically diagnosed with botulism do not have a positive laboratory test result. Factors contributing to these false-negative results in human patients include timing and type of sample collection, ingested dose of toxin, amount of wound contamination, kinetics of toxin absorption into the bloodstream, and uptake of toxin by the extravascular compartment.[12]

Similar factors likely influence MBA testing in horses, and might even be magnified by the size of the horse and susceptibility to disease. Unlike in people, BoNT concentrations in pre-enriched equine samples are usually below the detection limit of the MBA. Several reasons for this phenomenon have been postulated, including the high sensitivity of horses to BoNT and relatively smaller toxin doses that produce clinical signs,[14] rapid binding of circulating toxin to receptors on motor endplates with subsequent internalization,[14] and degradation of toxin in the GI tract by microbial organisms and their enzymes.[15] Therefore, commonly the MBA is applied to equine samples after a period of culture enrichment, which allows *C. botulinum* spores present in the initial sample to germinate and elaborate toxin. Even after culture enrichment, the majority of samples from horses clinically diagnosed with botulism yield negative MBA results. Because this study showed no relationship between survival and MBA test result, amount of toxin ingested is unlikely to be the primary explanation for false-negative results. Instead, type and timing of sample collection are probably more important.

Although the ideal type and timing of samples was not specifically investigated in this study, some recommendations can be made. Serum is not recommended; none of the serum samples from 12 horses and foals diagnosed clinically with botulism were positive. Likewise, only 1% of infants in the US with botulism have positive MBA results on serum samples, despite the usual detection of toxin in feces samples.[16] In contrast to people with foodborne or toxicoinfectious botulism, testing equine fecal samples for BoNT before culture enrichment is unlikely to yield positive results. Without culture enrichment, only 3/38 (8%) foals and 8/89 (9%) adults were positive on MBA. Therefore, culture enrichment is imperative because it increases test sensitivity from 8% to 53% in foals and from 9% to 29% in adults. Testing multiple fecal samples on sequential days leads to more modest increases in detection rates. For foals with fecal testing, 17/38 (45%) were positive on day 1, but by day 3, 20/38 (53%) had a positive test result. For adults with fecal testing, 20/89 (22%) were positive on day 1, but by day 3, 26/89 (29%) had a positive test result. Whenever possible, several fecal samples should be submitted to maximize sensitivity.

Although *C. botulinum* is not considered a normal inhabitant of the equine GI tract,[1] this supposition has not been specifically investigated for horses in the mid-Atlantic region, where type B *C. botulinum* spores are commonly found in the soil.[17] Performing MBA after culture enrichment of samples, so that positive results indicate the presence of *C. botulinum* spores in the initial sample, could lead to false-positive results if nonaffected horses excrete spores in their manure. This would most likely occur because of "pass-through," whereby spores from the environment contaminate forage consumed by the horse and resist degradation in the GI tract, with subsequent passive excretion in the manure. Results of this study indicate that clinically normal horses from the mid-Atlantic region rarely have *C. botulinum* in their feces, with a false-positive rate on MBA of approximately 3%. This observation parallels findings in other species. For example, researchers have observed a low occurrence of *C. botulinum* spores in intestinal samples from clinically normal cattle.[13,18,19] In addition, isolation of *C. botulinum* from fecal or GI content samples from people is considered confirmatory evidence for botulism, because the organism is rarely encountered in specimens from humans in the absence of disease.[7,9] Therefore, in the presence of clinical signs compatible with botulism in horses, positive MBA results on culture-enriched samples should be considered confirmatory.

One potential reason for false-positive MBA results in clinically normal horses would be antimicrobial-induced changes in GI flora that led to colonization and shedding of *C. botulinum*. Previous research has shown that metronidazole treatment can predispose mice to developing botulism,[20,21] which has led to the recommendation that metronidazole not be given to

animals with botulism.[1] Although predisposing factors for false-positive MBA results were not specifically investigated in this study, records from the 8 clinically normal adult horses with positive test results were reviewed. There were 2 adult control horses receiving antibiotic treatment that tested positive; 1 received trimethoprim-sulfamethoxazole for a high-risk pregnancy and the other received penicillin and gentamicin followed by trimethoprim-sulfamethoxazole for an infected tendon sheath. Two additional horses were healthy mares accompanying foals being treated for botulism; neither mare received antibiotics. One of the foals also had a positive MBA result; the other did not. These mare and foal pairs could have been housed in areas with particularly high spore loads resulting in a "pass-through" effect in the mares and development of botulism in the foals. Alternatively, perhaps the immunosuppression of pregnancy contributed to fecal passage of spores in these mares. However, dams frequently were used as control animals for foals with botulism, and most were not positive. Full medical records for the other 4 control animals with positive results were unavailable, and their antibiotic treatment history is unknown. Therefore, we are unable to provide information about whether antimicrobial use, specifically metronidazole, contributed to false-positive MBA results in these horses.

Our study highlights the limitations of using MBA, the current gold standard test, for the diagnosis of botulism in horses. Because clinical diagnosis is widely used, one could question whether laboratory testing should ever be pursued. However, laboratory testing remains an important tool for several reasons. Clinical examination findings cannot predict toxin type, which has important implications for treatment and prevention strategies, as well as provision of epidemiologic information, particularly in outbreak situations.[2] Furthermore, if unusual new botulism syndromes, toxin types, or modes of transmission were to occur, their full description would require clinicopathologic data.[12]

Several new tests, including PCR assays[4,5] and ELISAs,[13,18] have been developed in the hope of overcoming the limitations of the MBA by improving sensitivity, decreasing time for results, decreasing costs, and eliminating live animal use. During test development, these methods are almost invariably compared to the MBA, often using archived laboratory samples. However, very limited information is available regarding the performance of the MBA for clinical cases. Our study assessed the performance of the MBA for botulism in horses in a clinical setting and provides useful information for comparison with future assessments of new diagnostic tests. Limitations include the study's retrospective nature, the need to rely on clinical diagnosis as the gold standard test for botulism, and less than ideal control groups, particularly with respect to the limited number of foal controls available.

In summary, this study specifically examined the accuracy of the MBA in the diagnosis of botulism in horses. Results indicate that the test has low sensitivity but very high specificity, such that positive results are highly likely to indicate botulism but negative results do not exclude the diagnosis. Sensitivity is improved by testing culture-enriched samples and by submitting multiple fecal samples from a clinically suspect animal. Manure samples from horses that live in endemic regions but are not showing clinical signs of botulism are highly unlikely to contain *C. botulinum* spores.

Footnote

[a] STATA IC version 13.1, StataCorp LP, College Station, TX.

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