Type C waterborne botulism outbreaks in buffaloes (*Bubalus bubalis*) in the Amazon region[1]

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Botulism is a poisoning caused by botulinum neurotoxins (BoNTs). BoNTs serotypes C and D are involved in botulism outbreaks in cattle in several countries. Despite the high number of buffaloes worldwide, the real impact of botulism in buffaloes is not known, because it is not a notifiable disease in Brazil and only few studies have evaluated the occurrence of the disease in buffaloes. Those studies did not conduct diagnostic tests to confirm the presence of BoNTs. The objective of the present study was to describe three outbreaks of botulism in buffaloes in the Brazilian Amazon region considering epidemiological and clinical data as well as laboratory diagnosis to confirm the presence of BoNTs. The results of the bioassay were negative in the tissues and in feed samples, but positive for BoNT C in water samples. Confirmation of the occurrence of botulism in buffaloes allows the implementation of preventive strategies in susceptible herds. Waterborne botulism in buffaloes is prevented by ensuring the constant circulation of water collections and restricting the presence of dead animals and bones in order to prevent the accumulation of organic matter and the development of anaerobic conditions, which might favor the replication of *Clostridium botulinum*. Another measure that can be adopted is the shading of the pasture, in order to maintain the thermal comfort for the buffaloes and to avoid the excess of permanence of them in the water pools.

INDEX TERMS: Botulism, buffaloes, *Bubalus bubalis,* Amazon, *Clostridium botulinum,* BoNT, mice bioassay.

RESUMO.- [**Surtos de botulismo hídrico tipo C em búfalos (*Bubalus bubalis*) na região amazônica.**] Botulismo é uma intoxicação causada por neurotoxinas botulínicas (BoNTs). Os sorotipos C e D de BoNTs estão envolvidos em surtos de botulismo em bovídeos em vários países. Apesar do elevado número de búfalos em todo o mundo, o real impacto do botulismo em búfalos não é conhecido; pois não é uma doença de notificação obrigatória no Brasil e poucos estudos avaliaram a incidência desta doença em búfalos. Além disso, estes estudos não realizaram testes diagnósticos para confirmar a presença de BoNTs. O objetivo do presente estudo foi descrever três surtos de botulismo em búfalos na região amazônica brasileira, considerando dados epidemiológicos e clínicos, bem como o diagnóstico laboratorial para confirmar a presença de BoNTs. Os resultados do bioensaio em camundongos foram negativos em todos os tecidos e nas amostras de alimentos testados; no entanto foram positivos para BoNT C nas amostras de água. A confirmação da ocorrência de botulismo em búfalos permite a implementação de estratégias preventivas nos rebanhos. O botulismo hídrico nos búfalos pode ser prevenido assegurando-se que coleções de água fossem mantidas limpas, sem a presença de animais mortos e ossadas no seu interior e não permitindo o acúmulo de matéria orgânica

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e condições de anaerobiose favoráveis à multiplicação de *Clostridium. botulinum*. Outra medida que pode ser adotada é o sombreamento das pastagens, a fim de manter o conforto térmico dos búfalos e assim evitar o excesso de sua permanência dentro das fontes de água.

TERMOS DE INDEXAÇÃO: Botulismo, búfalos, Amazônia, *Clostridium botulinum,* BoNT, bioensaio em camundongo.

INTRODUCTION

Botulism is an intoxication caused by botulinum neurotoxins (BoNTs). BoNTs are produced by *Clostridium botulinum*, a gram-positive obligatory anaerobic bacterium that survives for long periods under adverse environmental conditions in its spore form. Under specific anaerobic conditions, assumes a vegetative form, multiplies, and secretes BoNTs (Gil et al. 2013, Moreira et al. 2014, 2016). BoNTs are the most toxic biological substances known and are classified into eight serotypes (A-H) on the basis of their antigenic properties. Serotypes C and D are involved in botulism outbreaks in cattle in several countries (Dutra et al. 2001, Martin 2003, Senturk & Cihan 2007, Myllykoski et al. 2009, Brooks et al. 2011), and the disease is considered endemic in South Africa, Australia, Brazil, and Israel (Krüger et al. 2013).

In Brazil, botulism in ruminants is responsible for major economic losses and is one of the leading causes of death in adult animals. The outbreaks are primarily caused by the intake of preformed BoNTs, owing to osteophagia as consequence of mineral deficiency, particularly phosphorus, and the consumption of poorly conserved food products, decaying animal carcasses, and contaminated water sources (Döbereiner et al. 1992, Dutra et al. 2001, Myllykoski et al. 2009). Once ingested, the active toxin is absorbed in the small intestine, released into the bloodstream, and eventually reaches the peripheral cholinergic nerve endings. In the nerve endings, the toxin blocks the release of acetylcholine and produces the typical symptom of flaccid paralysis (Anniballi et al. 2013). The period of incubation and disease severity depends on the amount of toxin ingested and the animal's sensitivity to BoNTs. The course of the disease varies from a few hours to a few days, and the mortality rate is close to 100% (Moreira et al. 2014).

The initial clinical signs observed in cattle and buffaloes are impaired mobility, incoordination of the hind limbs, and a cranial progression of paralysis. In the final stage, the animal enters a pre-agonal state, followed by coma and death due to cardiac arrest. During the course of the disease, the psychological condition of the animal remains unchanged. Alterations at necropsy are negligible and limited to petechiae in the myocardium due to the respiratory distress that precedes death (Moreira et al. 2014). Definitive diagnosis is based on the detection of BoNTs using a mouse bioassay (Bano et al. 2015) which is the gold standard test for the detection and characterization of botulinum toxins (Lindström & Korkeala 2006). In ruminants, the biological samples of choice for the study of toxins are liver samples, intestinal and ruminal contents, and samples of food and water recently consumed by the affected animals (Kelch et al. 2000).

Botulism in animal production has a strong economic impact due to the high mortality rates (Moreira et al. 2014). The Food and Agriculture Organization of the United Nations (2014) reported that the population of buffaloes (*Bubalus bubalis*) worldwide is approximately 195 million heads. Brazil has a herd of an estimated 1.3 million buffaloes and is at present the largest producer of buffaloes in the Western world (FAO 2014). Most of these animals (493,000, 37.4% of the Brazilian herd) are located in the state of Pará (IBGE 2014), where they are reared within the Amazon region and are well adapted to the tropical climate and extensive grazing (Borghese & Mazzi 2005). Despite the high number of buffaloes, the real impact of botulism in buffaloes in Brazil is not known because it is not a notifiable disease and only few studies have evaluated the occurrence of this disease in buffaloes. In addition, those studies evaluated clinical signs and epidemiological data, but did not conduct diagnostic tests to confirm the presence of BoNTs (Langenegger & Döbereiner 1988).

The objective of the present study was to describe three outbreaks of botulism in buffaloes in the Brazilian Amazon region considering epidemiological and clinical data as well as laboratory diagnosis to confirm the presence of BoNTs.

MATERIALS AND METHODS

Case history. The outbreaks occurred on two farms located in the municipalities of Marituba (01° 21'19" S, 48° 20'31"W) and Tailândia (02° 56'50"S, 48° 57'11"W) in the state of Pará, in the Brazilian Amazon region. These farms did not have a history of vaccination for botulism and only performed vaccinations against foot-and-mouth disease and brucellosis and dewormed calves at birth.

First outbreak. The farm located in Marituba requested veterinary care 29 days after the first deaths of buffaloes. The farm owner reported that 21 Murrah buffaloes (one male and 20 females) aged approximately 36 months were purchased and transferred to a pasture of approximately two hectares. Three buffaloes died during the afternoon of the day of arrival. In addition, starting on the second day, the animals were supplemented with grass silage (*Pennisetum purpureum* var. Cameron) and a diet based on cornmeal and cassava chips, because of a limited food supply. Between days five and seven, three buffaloes presented with sternal recumbency and inability to stand; but the psychological state did not change, and the animals died 48 hours after the onset of clinical signs. On the 29th day after the first deaths, veterinarian care was provided by professionals from the Large Animal Unit of the Institute of Veterinary Medicine of the Federal University of Pará (Instituto de Medicina Veterinária, Universidade Federal do Pará (IMV/UFPA). The clinical examination of one buffalo found in sternal recumbency revealed inability to stand, flaccid paralysis, head turned to the side but psychological state preserved. Because of these debilitating conditions, the buffalo was euthanized, necropsied, and samples of liver, brain, spleen, kidney, lung, and heart were collected in formalin for histological examination. Liver samples, ruminal and intestinal contents were collected and refrigerated for microbiological examination. The other 14 buffaloes of the herd did not exhibit any symptoms or changes.

During inspection of the farm, no traces of bones were found on the pasture or in feeding troughs. However, the only water source for these animals was abandoned fish tanks, and the water contained mud and dead fish. The tanks did not have a water recycling system and were supplied with rainwater and waste

from a nearby pigsty. The buffaloes used these tanks for drinking and bathing. Another bufalo died in the morning of day 30. Necropsy was performed, and samples of the same clinical specimens of the sacrificed buffalo before were collected. On the same day, five water samples were collected from the water tanks and feed system and stored in refrigeration for a mouse bioassay because of the suspicion of waterborne botulism.

Second and third outbreaks. The second and third outbreaks occurred in a farm located in the municipality of Tailândia. The farm owner reported that the first outbreak occurred in December 2015, when 200 Murrah buffalo cows aged approximately 24 months were introduced into a pen, with an area of about 100 hectares, containing fodder of *Panicum maximum* cv. Mombasa and *Brachiaria brizantha* cv. Marandu. Six buffalo cows died 24 hours later. On the same day, the live bufaloes were removed from the pen, and no additional deaths occurred.

The second outbreak occurred in February 2016, when 300 Murrah buffalo cows aged approximately 24 months were introduced into the same pen. Fourteen buffaloes died the following day. The live buffaloes were removed from the pasture, and again, no additional deaths occurred. The farm owner reported that deaths occurred only in buffaloes with access to this pen. In addition, approximately 250 buffalo cows were introduced into this pen between September and October 2015, and no deaths occurred. Four days after the last outbreak, a veterinary consultation was performed by professionals from the Large Animals Unit of IMV/UFPA, who inspected the pen and did not find the presence of *Palicourea marcgravii* or *Palicourea juruana*, which are toxic plants commonly responsible for sudden deaths in cattle and buffaloes in the Amazon region. However, they found bones at the edges of a reservoir that measured about 100m^2 and which was the main water source of this pen. The farm owner reported that this reservoir was supplied by rainwater and dried out during the dry season (July to November), at which time the buffaloes used a small creek for their water supply. Five water samples from the reservoir were collected and refrigerated for the analysis of BoNTs.

Mouse bioassay. Samples collected from sacrificed buffaloes, and the feed and water samples were subjected to confirmatory diagnostic tests for botulism in the Laboratory of Microbiology of IMV/UFPA using the serum neutralization test in mice with BoNTs serotypes C and D, according to the methodology of Sebald & Petit (1997).

RESULTS

The necropsy and histopathology findings revealed no macroscopic or microscopic changes. The results of the bioassay were negative in all tissues and feed samples, but positive for BoNT C in all the water samples. Mice inoculated with water samples presented dyspnea and a wasp-like narrowed waist, with subsequent death. The mice that received the mixture of water samples and type D botulinum antitoxin also died. However, those that received the mixture of water samples and type C botulinum antitoxin or water samples heated to 100°C survived.

DISCUSSION

The symptoms observed in the affected buffaloes, including sudden death, sternal recumbency, inability to stand, and flaccid paralysis, were similar to those described in botulism outbreaks in ruminants, and the absence of macroscopic and microscopic pathological changes corroborated the results of previous studies, which reported the lack of major changes or the presence of mild changes (Senturk & Cihan 2007, Myllykoski et al. 2009, Moreira et al. 2014).

The negative bioassay results for the liver samples, intestinal and ruminal contents were not surprising since the failure to detect BoNTs using the serum neutralization test in ruminant tissues, despite clear symptoms of botulism, is not uncommon. The difficulty to detect botulinum toxin in animal tissue samples using this technique has been reported in other studies and is justified by rapid degradation of the toxins, particularly type C toxin, by the ruminal microflora and the higher sensitivity of ruminants to BoNTs compared with mice, which are less sensitive in the bioassay (Kelch et al. 2000, Myllykoski et al. 2009, Bano et al. 2015).

The waterborne spread of BoNTs in cases of botulism outbreak in buffaloes seems to play an important epidemiological role in the transmission of the disease (Langenegger & Döbereiner 1988, Silva et al. 1998); nevertheless, these studies did not conduct laboratory tests to confirm the presence of BoNTs. The detection and identification of type C BoNTs in water samples (from the main or only source of water) in the bioassay and serum neutralization tests in this study confirmed the occurrence of waterborne botulism in buffaloes. In all cases of botulism outbreaks described previously (Langenegger & Döbereiner 1988, Silva et al. 1998) was observed that the water sources used by buffaloes exhibited favorable conditions for the multiplication of *Clostridium botulinum*, including the presence of dead animals or bones. This also happened in the outbreaks described in this study where dead fish and swine waste were found in the only source of water in the first outbreak, and bones close to the main water source in second and third outbreaks; both are probably the origin of the water contamination. Sources of stagnant water can be responsible for the waterborne spread of BoNTs leading to botulism outbreaks in ruminants, particularly in the presence of carcasses, mud, and high concentrations of organic matter, combined with the development of anaerobic conditions, which favor the multiplication of the bacillus and formation of spores of *C. botulinum* (Langenegger & Döbereiner 1988, Silva et al. 1998, Dutra et al. 2001, Souza et al. 2006).

A striking characteristic of buffalo production is the need for water collections in the grazing area to serve as water sources not only for drinking but also for bathing, considering the limited thermoregulation in these animals because of the small amount of sweat glands and characteristically dark hides, which increase their sensitivity to sunlight (Damasceno et al. 2010). Therefore, the presence of water pools in the grazing area is common. However sanitary issues are ignored, including the water quality, and carcasses and bones are often present near the water sources, which foster the development of the ubiquitous *C. botulinum* microorganism and the production of BoNTs (Langenegger & Döbereiner 1988, Dutra et al. 2001, Souza et al. 2006).

CONCLUSION

The clinical-pathological findings and the epidemiological characteristics of the region such as the existence of organic matter and dead animals or bones in the water, high

morbidity and mortality in a short period of time, associated with the detection and identification of type C BoNTs in water samples, confirmed the occurrence of waterborne botulism in buffaloes in the described outbreaks.

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REFERENCES

Anniballi F., Fiore A., Löfström C., Skarin H., Auricchio B., Woudstra C., Bano L., Segerman B., Koene M., Båverud V., Hansen T., Fach P., Åberg A.T., Hedeland M., Engvall E.O. & De Medici D. 2013. Management of animal botulism outbreaks: from clinical suspicion to practical countermeasures to prevent or minimize outbreaks. Biosecur. Bioterror 11(S1):191-199. doi:10.1089/bsp.2012.0089

Bano L., Drigo I., Tonon E., Berto G., Tavella A., Woudstra C., Capello K. & Agnoletti F. 2015. Evidence for a natural humoral response in dairy cattle affected by persistent botulism sustained by non-chimeric type C strains. Anaerobe 36:25-29. doi:10.1016/j.anaerobe.2015.09.007

Borghese A. & Mazzi M. 2005. Buffalo production and research. Vol.67. FAO, REU Technical Series. Rome. 316p Available in <ftp://ftp.fao.org/docrep/fao/010/ah847e/ah847e.pdf> Accessed on Aug. 8, 2016

Brooks C.E., Clarke H.J., Graham D.A. & Ball H.J. 2011. Diagnosis of botulism types C and D in cattle by a monoclonal antibody-based sandwich ELISA. Vet. Rec. 168(17):455. doi:10.1136/vr.c7432

Damasceno F.A., Viana J.M., Tinôco I.F.F., Gomes R.C.C. & Schiassi L. 2010. Adaptação de bubalinos ao ambiente tropical. Nutritime 7(5):1370-1381.

Döbereiner J., Tokarnia C.H., Langenegger J. & Dutra I.S. 1992. Epizootic botulism of cattle in Brazil. Dtsch. Tierärztl. Wochenschr. 99(5):188-190.

Dutra I.S., Döbereiner J., Rosa I.V., Souza L.A.A. & Nonato M. 2001. Surtos de botulismo em bovinos no Brasil associados à ingestão de água contaminada. Pesq. Vet. Bras. 21(2):43-48.

FAO 2014. Food and Agriculture Organization of the United Nations. Available in <http://faostat.fao.org/> Accessed on Aug. 8, 2016

Gil L.A.F., Cunha C.E.P., Moreira G.M.S.G., Salvarani F.M., Assis R.A., Lobato F.C.F., Mendonça M., Dellagostin O.A. & Conceição F.R. 2013. Production and evaluation of a recombinant chimeric vaccine against *Clostridium botulinum* neurotoxin Types C and D. PLoS ONE 8(7):e69692. doi:10.1371/journal.pone.0069692

IBGE 2014. Produção da Pecuária Municipal 2014. Instituto Brasileiro de Geografia e Estatística.Available in <http://biblioteca.ibge.gov.br/visualizacao/periodicos/84/ppm_2014_v42_br.pdf> Accessed on Aug. 8, 2016

Kelch W.J., Kerr L.A., Pringle J.K., Rohrbach B.W. & Whitlock R.H. 2000. Fatal *Clostridium botulinum* toxicosis in eleven Holstein cattle fed round bale barley haylage. J. Vet. Diagn. Invest. 12(5):453-455. doi:10.1177/104063870001200511

Krüger M., Skau M., Shehata A.A. & Schrödl W. 2013. Efficacy of *Clostridium botulinum* types C and D toxoid vaccination in Danish cows. Anaerobe 23:97-101. doi:10.1016/j.anaerobe.2013.06.011

Langenegger J. & Döbereiner J. 1988. Botulismo enzoótico em búfalos no Maranhão. Pesq. Vet. Bras. 8(1/2):37-42.

Lindström M., & Korkeala H. 2006. Laboratory diagnostics of botulism. Clin. Microbiol. Rev., 19(2):298-314. doi:10.1128/CMR.19.2.298-314. 2006

Martin S. 2003. *Clostridium botulinum* type D intoxication in a dairy herd in Ontario. Can. Vet. J. 44(6):493-495.

Moreira C., Cunha C.E.P., Moreira G.M.S.G., Mendonça M., Salvarani F.M., Moreira A.N. & Conceição F.R. 2016. Protective potential of recombinant non-purified botulinum neurotoxin serotypes C and D. Anaerobe 40:58-62. doi:10.1016/j.anaerobe.2016.05.012

Moreira G.M.S.G., Cunha C.E.P., Salvarani F.M., Gonçalves L.A., Pires P.S., Conceição F.R. & Lobato F.C.F. 2014. Production of recombinant botulism antigens: a review of expression systems. Anaerobe 28:130-136. doi:10.1016/j.anaerobe.2014.06.003

Myllykoski J., Lindström M., Keto-Timonen R., Säderholm H., Jakala J., Kallio H., Sukura A. & Korkeala H. 2009. Type C bovine botulism outbreak due to carcass contaminated non-acidified silage. Epidemiol. Infect. 137(2):284-293. doi:doi:10.1017/S0950268808000939

Sebald M. & Petit J.C. 1997. Méthodes de laboratoire bactéries anaérobies et leur identification. 2nd ed. Institut Pasteur, Paris. 307p.

Senturk S. & Cihan H. 2007. Outbreak of botulism in a dairy herd in Turkey. Irish Vet. J. 60(8):481-484.

Silva T.M.D., Dutra I.S., Castro R.N. & Döbereiner J. 1998. Ocorrência e distribuição de esporos de *Clostridium botulinum* tipos C e D em áreas de criação de búfalos na Baixada Maranhense. Pesq. Vet. Bras. 18 (3/4):127-131. doi:dx.doi.org/10.1590/S0100-736X1998000300007

Souza A.M., Marques D.F., Döbereiner J. & Dutra I.S. 2006. Esporos e toxinas de *Clostridium botulinum* dos tipos C e D em cacimbas no Vale do Araguaia, Goiás. Pesq. Vet. Bras. 26(3):133-138. doi:10.1590/S0100--736X2006000300001