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Botulism in cattle in Mato Grosso do Sul: History, current situation, and perspectives[1]

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ABSTRACT.- Lima S.C., Paula J.P.L., Guizelini C.C., Pupin R.C., Avila L.G., Gomes D.C., Barros C.S.L., & Lemos R.A.A. 2024. **Botulism in cattle in Mato Grosso do Sul: History, current situation, and perspectives**. *Pesquisa Veterinária Brasileira 44:e07477, 2024*. Laboratório de Anatomia Patológica, Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul, Av. Sen. Filinto Müller 2443, Campo Grande, MS 79070-900, Brazil. E-mail: ricardo.lemos@ufms.br

Botulism is a neuroparalytic disease caused by ingesting neurotoxins (BoNTs) produced by the anaerobic bacterium *Clostridium botulinum*. It is a significant cause of cattle mortality in Brazil. BoNTs block the release of acetylcholine at the neuromuscular synapses and cause muscle flaccidity. The diagnosis of botulism is challenging due to two main factors: the absence of macro and microscopic lesions and the difficulties inherent in laboratory tests considered the gold standard for botulism. Due to these difficulties, reviews of botulism diagnoses in Mato Grosso do Sul and extensively in the Center-West of Brazil became discrepant according to the diagnostic methodology used, making it difficult to correctly assess the disease's prevalence, epidemiology, and clinical picture. This study aimed to review the diagnoses of botulism in cattle reported in Brazil, comparing the criteria used in each case. We studied the cause, the pathogenesis, clinical signs, and all the diagnosis methods applied to botulism. Based on the subject reviewed, we propose a diagnostic protocol based on standards adequate to the Brazilian circumstances. We also reviewed the critical diseases of cattle diseases to be included in the differential diagnosis of botulism.

INDEX TERMS: Clostridial diseases, botulism, livestock, cattle, BoNTs, peripheral nervous system.

RESUMO.- [**Botulismo em bovinos no Mato Grosso do Sul: histórico, situação atual e perspectivas**]. O botulismo é uma doença neuroparalítica causada pela ingestão de neurotoxinas (BoNTs) produzidas pela bactéria anaeróbica *Clostridium botulinum*. É uma causa significativa de mortalidade de bovinos no Brasil. As BoNTs bloqueiam a liberação de acetilcolina nas sinapses neuromusculares e causam flacidez muscular. O diagnóstico do botulismo é desafiador devido a dois fatores principais: a ausência de lesões macro e microscópicas e as dificuldades inerentes aos exames laboratoriais considerados padrão ouro para o botulismo. Devido a essas dificuldades, as revisões de diagnósticos de botulismo no Mato Grosso do Sul e extensivamente no Centro-Oeste do Brasil tornaram-se discrepantes de acordo com a metodologia diagnóstica utilizada, dificultando a avaliação correta da prevalência, da epidemiologia e do quadro clínico da doença. Este estudo teve como objetivo revisar os diagnósticos de botulismo em bovinos notificados no Brasil, comparando os critérios utilizados em cada caso. Estudamos a causa, a patogênese, os sinais clínicos e todos os métodos de diagnóstico aplicados ao botulismo. Com base no assunto abordado, propomos um protocolo diagnóstico baseado em padrões adequados às circunstâncias brasileiras. Também revisamos as doenças críticas de bovinos a serem incluídas no diagnóstico diferencial de botulismo.

TERMOS DE INDEXAÇÃO: Doenças de clostrídios, botulismo, bovinos, BoNTs, sistema nervoso periférico.

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INTRODUCTION

Botulism is an important cause of cattle mortality in several Brazilian states (Tokarnia et al. 1970, Dutra et al. 2001, Costa et al. 2008, Galiza et al. 2010, Lucena et al. 2010, Ribas et al. 2013, Câmara et al. 2014, Rondelli et al. 2017, Nobre et al. 2019, Oliveira et al. 2019, Pupin et al. 2019, Santos et al. 2019). It is characterized as a neuroparalytic disease caused by the ingestion of carcasses, water and food contaminated by botulinum neurotoxins (BoNTs) produced by the bacterium *Clostridium botulinum* (Le Maréchal et al. 2016). Currently, seven types (A-G) and 40 subtypes of BoNTs are recognized (Peck et al. 2017). Regardless of the source, BoNTs act on peripheral nerve endings, blocking the release of acetylcholine at the neuromuscular synapses, which results in progressive flaccid paralysis of the muscles of locomotion, chewing, breathing and swallowing (Zachary 2018).

The main clinical sign is difficulty in locomotion, characterized by motor incoordination that progresses to flaccid paralysis of the limbs and permanent sternal decubitus. However, the mental state and skin sensitivity of affected cattle remain normal. Other commonly observed signs are hypotonia of the tongue and tail, dysphagia and dyspnea with occasional biphasic inspiration. In the final stages of the disease, cattle lie in lateral recumbency and die from respiratory failure, as the muscles that aid breathing are likely affected (Dutra et al. 2001, Câmara et al. 2014, Le Maréchal et al. 2016).

The diagnosis of botulism is challenging considering the absence of both gross and histopathological changes in affected cattle and the technical and ethical difficulties involving bioassay tests and serum neutralization in mice, considered the gold standard for detecting BoNT in serum, liver fragments and samples of ruminal and intestinal contents of sick cattle (Le Maréchal et al. 2016).

In Brazil, the criteria adopted for diagnosis are quite variable, as observed in the different reports about the disease in the country, including descriptions of outbreaks (Tokarnia et al. 1970, Dutra et al. 2001, Costa et al. 2008, Maboni et al. 2010, Câmara et al. 2014, Guizelini et al. 2019, Helayel et al. 2019, Nobre et al. 2019, Barbosa et al. 2022, Campagnolli et al. 2022) or retrospective studies of veterinary diagnostic laboratories (Galiza et al. 2010, Lucena et al. 2010, Ribas et al. 2013, Rondelli et al. 2017, Oliveira et al. 2019, Pupin et al. 2019, Santos et al. 2019). There are no defined criteria for the conclusive diagnosis of the disease, as the gold standard techniques have a low sensitivity in detecting the BoNT in cattle.

The difficulty in defining criteria for the diagnosis of botulism is mainly related to the lack of understanding, on the part of veterinarians, of fundamental points about the epidemiology and pathogenesis of the disease.

The objective of this study was to review the diagnoses of botulism in cattle reported in Brazil by comparing the criteria used for this purpose and proposing a diagnostic approach based on standards that fit Brazilian circumstances. Achieving this goal can improve the understanding of concepts about epidemiology, pathogenesis, clinicopathology, diagnosis, and more significant differential diagnoses.

MATERIALS AND METHODS

Ethical approval. Since all the data were obtained from database literature searches, this study did not perform any animal experiments. Submitting to the Ethics Committee on Animal Use (CEUA) was unnecessary.

For the literature review, we use the following (in English and Portuguese): botulism, cattle diseases, retrospective study, survey, botulism outbreak, causes of death, flaccid paralysis, and *Clostridium botulinum*. The search bases included Capes Journals, Scielo, Pubmed/Medline, and Scopus. We also obtained data on the case series from the "Laboratório de Anatomia Patológica" (Pathological Anatomy Laboratory – LAP) of the "Faculdade de Medicina Veterinária e Zootecnia" (Faculty of Veterinary Medicine and Animal Science – FMVZ) at the "Universidade Federal de Mato Grosso do Sul" (UFMS) through articles, theses, books, manuals, and proceedings of digital or printed conferences.

RESULTS AND DISCUSSION

General concepts of the disease applied to diagnosis

Clostridium botulinum is a Gram-positive, anaerobic bacterium in nature in vegetative and sporulating forms (Kriek & Odendaal 2004). The vegetative form corresponds to the active form of the bacteria, capable of multiplying and producing toxins. Spores are a form of resistance that enables *C. botulinum* to survive unfavorable conditions, such as heat, light, desiccation, chemicals, and radiation (Kriek & Odendaal 2004, Barsanti 2015, Rood 2016). As they are disseminated widely in the environment, including pastures, food, and water, the spores are constantly ingested by cattle, and if microanaerobic conditions exist in the rumen, germination occurs. The bacteria acquire the vegetative form and multiply. Consequently, cattle eliminate large amounts of bacteria through feces, further increasing environmental contamination.

This entire cycle occurs without any harm to cattle. However, when the spores encounter ideal conditions such as anaerobiosis and organic matter, the bacteria germinate and multiply (vegetative form); its maximum multiplication capacity occurs at temperatures between 15 and 45°C. The bacteria produce toxins when there is anaerobiosis, low acidity, and low solute content. When BoNTs are ingested, botulism develops (Barsanti 2015, Rood 2016, Peck et al. 2017). In Brazil, BoNTs C, D, and the C/D complex are responsible for botulism in cattle (Tokarnia et al. 1970, Lisbôa et al. 1996, Maboni et al. 2010, Guizelini et al. 2019, Soares et al. 2018, Helayel et al. 2019). BoNTs are highly toxic biological substances, resistant to chemical agents, sensitive to heat and desiccation, and quickly inactivated by sunlight (Barsanti 2015). The place where the *C. botulinum* finds suitable conditions for toxin production determines the different forms of disease that occur: (*i*) botulism associated with osteophagy, (*ii*) botulism associated with drinking water, and (*iii*) foodborne botulism (Silva et al. 2016).

The crucial predisposing factor to osteophagy-associated botulism is phosphorus deficiency, the primary clinical manifestation of which is consumption by cattle of bones from carcasses decomposing in the pasture (Tokarnia et al. 1970). Regardless of the cause of death, the spores in the gastrointestinal tract of a bovine carcass can find suitable conditions of anaerobiosis, pH, and nutrients in the decomposing process. The bacteria multiply, invade tissues throughout, and produce botulinum toxins. As BoNTs are sensitive to sunlight, most are inactivated except those shielded in the bone marrow, protected from sunlight, and may remain viable for up to 30 years. When practicing osteophagy, cattle can ingest bones containing large amounts of BoNTs.

Botulism associated with drinking water occurs when toxins are ingested while consuming contaminated water (Dutra et al. 2001). Water contamination can happen when carcasses of any species (e.g., cattle, birds, rodents, and armadillos) are caught in the water source or even through the deposit of organic matter, such as feces, food

remains, and sludge in still ponds (Dutra et al. 2001). Armadillos are particularly important in the occurrence of this form of botulism, as they are refractory to the toxin and feed on carcass remains, including removing bones from buried carcasses. Armadillos have a double role in the development of the disease, as they favor the form associated with osteophagia, and if they die in a drinking site for cattle, they become a source of BoNTs. This situation is critical when the drinking troughs are built close to the ground, or there is a grounding of the edges of the trough, facilitating armadillos' access.

Cattle becoming ill from foodborne botulism ingest food contaminated with BoNTs, especially poorly preserved hay, rations, corn, and silage (Costa et al. 2008, Maboni et al. 2010, Guizelini et al. 2019, Soares et al. 2018). As *C. botulinum* spores are disseminated in the environment, foodstuff must decompose so that anaerobic conditions, alkaline pH, and appropriate nutrients and temperature occur to promote spore germination, multiplication of the vegetative form and, consequently, the production of BoNTs. In the case of feed and hay, the main predisposing factor is the moistening of the food, which, in addition to putrefaction, causes an increase in the volume of grains or forage fibers, leading to the expulsion of air and, consequently, anaerobiosis. Silage production is already anaerobically, but failures in the fermentation that maintain the pH above 4.5 further favor the multiplication of anaerobic bacteria (Le Maréchal et al. 2016). Foodborne botulism also occurs through ingesting poultry litter, especially if mixed with chicken carcasses (Dutra et al. 2005, Lobato et al. 2008).

Once ingested, BoNTs are absorbed in the initial portions of the small intestine and spread via a hematogenous route throughout the body, reaching the peripheral motor neurons that contract skeletal muscles. Neurons absorb the toxin and prevent the exocytosis of acetylcholine to the neuromuscular junction, compromising the stimulus for muscle contraction, which results in flaccid paralysis (Zachary 2018, Santos et al. 2019). The toxin has a cumulative effect and does not induce immunity nor cause degeneration or inflammation damage to nerves or muscles, causing loss of function without loss of sensitivity.

The incubation period and clinical evolution depend on the amount of BoNTs ingested. Cattle ingesting large amounts of BoNTs can manifest the disease with a neglectable incubation period, while cattle that ingest smaller amounts can become ill up to 16 days after ingestion (Barros et al. 2006). The clinical course can be super-acute, acute, sub-acute, or chronic. Cattle that present a super-acute evolution die quickly without it being possible to observe clinical signs. In contrast, cattle that manifest the disease with a chronic evolution have a greater chance of spontaneous recovery (Lemos & Riet-Correa 2023).

Regardless of the clinical evolution, the main muscles affected are those involved in locomotion, chewing, breathing, and swallowing. The first clinical signs observed are due to partial or complete flaccid paralysis of the muscles, mainly those of the pelvic limbs. They are staggering gait or sternal recumbency, during which the bovine cannot stand up. A hallmark of botulism is that the central nervous system is not affected; in this way, the affected animals have a preserved level of consciousness. Other clinical signs include hypotonia of the tongue, observed as the animal's difficulty in retracting it immediately after being exposed; hypotonia of the tail, identified by the reduction or absence of movements (mainly when the animal, in sternal recumbency, defecates and does not shoo away the insects that approach the feces), flaccid paralysis of the neck, evidenced by the self-auscultation position, and chin resting on the ground. Bradycardia, mydriasis, and ptosis occur in some animals. Dyspnea is a common sign and can be seen as biphasic breathing during inspiration, when there is a rapid respiratory movement in an attempt to distend the chest, followed by a second, more prolonged and difficult phase with the aid of the diaphragm (Tokarnia et al. 1970, Dutra et al. 2001, Barros et al. 2006, Costa et al. 2008, Maboni et al. 2010, Câmara et al. 2014, Guizelini et al. 2019, Helayel et al. 2019, Nobre et al. 2019).

The diagnosis of botulism is challenging since there are no macro and microscopic lesions characteristic of the disease (Döbereiner & Dutra 2004). A definitive diagnosis requires laboratory analysis because clinical signs are suggestive but not specific (Le Maréchal et al. 2016). The gold standard tests for diagnosing botulism are the bioassay and serum neutralization in mice, capable of detecting botulinum toxin in biological samples collected from sick animals, water and suspected foods (Tokarnia et al. 1970, Dutra 2001, Costa et al. 2008, Maboni et al. 2010, Le Maréchal et al. 2016, Guizelini et al. 2019, Helayel et al. 2019, Nobre et al. 2019). Despite being considered more accurate for diagnosis, these tests have several disadvantages that have encouraged studies to develop other laboratory resources for diagnosis, such as complement microfixation with heating, isolation of clostridia that produce botulinum toxins, Endopep-MS (endopeptidase mass spectrometry) and enzyme-linked immunosorbent assay (ELISA) (Menegucci et al. 1998, Le Maréchal et al. 2016). However, we usually do not apply these alternative methods in routine diagnostic work in Brazil. Each of them has advantages and disadvantages that we will discuss further ahead.

For bioassay and serum neutralization in mice, one should send samples of serum, liver (250g), rumen contents (250g), and small intestine (15cm long, with contents), refrigerated or frozen. In the laboratory, the samples should be diluted and inoculated into the mice's peritoneum; if active BoNT is present, clinical signs of the disease will manifest in the mice. If the bioassay result is positive, serum neutralization with a specific antitoxin is performed to determine the group to which the BoNT belongs (Lindström & Korkeala 2006). This technique is considered one of the most sensitive for detecting and has the advantage of detecting the activity of the toxin, not just its presence. Although the bioassay is considered the most sensitive test for detecting BoNTs, false-negative results are prevalent in cattle, including in samples from the same outbreak, such as those with similar epidemiology and clinical features. Such false negatives occur for the following reasons: (*i*) the amount of toxin present in the samples must be significant to cause clinical signs and death in mice since they have less sensitivity to BoNT when compared to cattle (Döbereiner & Dutra 2004, Barros et al. 2006, Le Maréchal et al. 2016); (*ii*) BoNTs are degraded by the rumen microbiota and by autolysis, compromising the diagnosis when necropsy is performed sometime after death (Allison et al. 1976, Le Maréchal et al. 2016); (*iii*) after absorption, BoNTs quickly bind to myoneural junctions, preventing their detection in the serum (Döbereiner & Dutra 2004). There is no international standardization regarding sample preparation, interpretation of the clinical signs in mice, the number of mice needed for the test, interference related to their genetic lineages, or their age and sex. This method is also laborious and costly, requires adequate facilities, and uses animals, which generates ethical issues (Le Maréchal et al. 2016). However, it is still the only laboratory test accessible in Brazil to confirm the diagnosis of botulism.

Complement microfixation with heating is one option to avoid the use of animals and has been tested in Brazil. The technique presented high sensitivity and specificity, proving superior to bioassay and

serum neutralization in mice. However, its application in routine diagnostics is not yet available (Menegucci et al. 1998).

In other countries, the detection of *C. botulinum* in biological samples, food, water, or even in the environment close to cattle with clinical signs of botulism is one of the strategies proposed for diagnosis. The isolation of bacterium has two objectives: (*i*) to identify BoNTs produced *in vitro* and (*ii*) to detect by polymerase chain reaction (PCR) the presence of genes that encode the production of BoNTs. Regardless of the objective, the isolation of *C. botulinum* requires the presence of spores or the vegetative form of the bacterium in the samples so that samples that contain only pre-formed toxins will be falsely negative. There is also no guarantee that the isolated bacterium is the cause of the disease in cattle, as it is naturally found in the digestive tract and spreads throughout the carcass after death. Generally, the number of spores recovered from samples is low, and there are still no standardized protocols for enriching culture media that favor germination and growth (Le Maréchal et al. 2016). In Brazil, an obstacle to the cultivation of *C. botulinum* is the scarcity of laboratories with adequate facilities, such as anaerobic greenhouses, which makes this diagnostic method difficult to access.

Other methods, such as Endopep-MS and ELISA, are being improved. Endopep-MS identifies active toxins through the cleavage of synthetic peptides and subsequent detection of the cleavage products by immunological tests or mass spectrometry. However, the technique needs further studies so that different types of samples can be tested and the most important BoNTs for cattle (C and D and C/D and D/C mosaics) can be identified. ELISA only detects BoNT through specific mono or polyclonal antibodies. It is an inexpensive, fast, and sensitive method, but attention must be paid to the type of antibody used, as its quality affects the test result (Hedeland et al. 2011, Le Maréchal et al. 2016, Åberg et al. 2021).

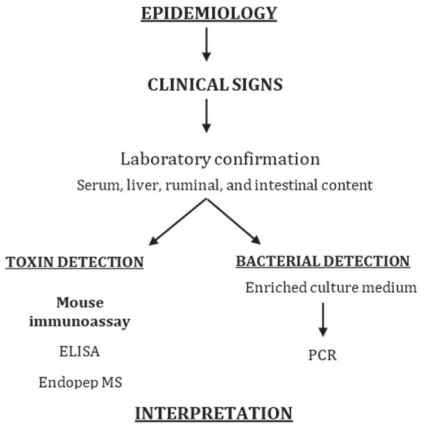
As the diagnosis of botulism in cattle is considered a challenge, some authors (Maréchal et al. 2016) suggest standardizing the criteria for diagnosing the disease, designing a flowchart with criteria for reaching the diagnosis of botulism that integrates clinical signs, laboratory tests (detection of botulinum toxin or isolation of *C. botulinum*) and exclusion of diagnoses differential tests, to determine whether the animals have botulism, do not have botulism or are suspected of having the disease (Fig.1). After eliminating differential diagnoses, we confirm botulism by observing indicative clinical signs and the presence of BoNT or *C. botulinum* in tissues, food, water, or the environment. If there are clinical signs without laboratory confirmation, the cases are considered suspicious. If another disease is detected, the sample is considered negative for botulism. These procedures suit European countries, where the disease occurs sporadically, in intensive systems, regions without much environmental contamination by *C. botulinum* spores, and with well-equipped laboratories. In Brazil, there is no standardization for diagnosing botulism in cattle.

It is our understanding that the main limitation of the use of this protocol in Brazil is that it does not consider, as a diagnostic aid, the negative laboratory diagnosis for rabies and the absence of gross and microscopic lesions; we consider it essential to evaluate spinal cord, brain stem, skeletal muscles, and peripheral nerves to ensure the absence of lesions characteristic of other diseases, which is particularly important in regions where there is high environmental contamination, and rabies, myopathies or neuropathies are prevalent, as is the case of Mato Grosso do Sul. The collection of information on the epidemiology associated with the appropriate necropsy procedures is the first step in ruling out differential diagnoses and avoiding false-positive results of botulism (Fig.2).

Diagnosis of botulism in Brazil with a focus on Mato Grosso do Sul

Botulism in cattle was first diagnosed in Brazil in 1970 in the state of Piauí and subsequently described as one of the leading causes of cattle mortality in the country (Tokarnia et al. 1970, Galiza et al. 2010, Lucena et al. 2010, Ribas et al. 2013, Rondelli et al. 2017, Oliveira et al. 2019, Pupin et al. 2019, Santos et al. 2019). The main reports of the occurrence of the disease in Brazil are listed chronologically in Table 1 and 2.

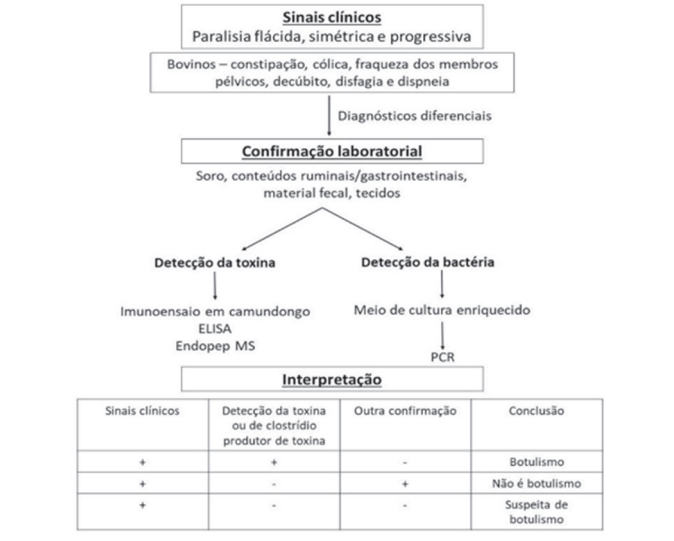
One can see that there needs to be uniformity in the criteria used to diagnose the disease in Brazil, which makes it difficult to establish comparisons between these studies. Specifically, publications referring to botulism in Mato Grosso do Sul have several variations in the criteria adopted for diagnosis over the years. A relevant point about the studies conducted in Mato Grosso do Sul by the LAP/FAMEZ/UFMS team is that the diagnosis of botulism was conclusive only in cases where well-trained laboratory professionals performed *post mortem* examinations. Tissue samples from cattle sent by veterinarians external to the Institution received a suggestive diagnosis of botulism, even though they met the criteria generally used for diagnosing the disease, such as epidemiology and indicative clinical signs, absence of macro and microscopic lesions, and direct immunofluorescence results (IFD) and intracerebral inoculation in rabies-negative mice (IIC). This criterion was adopted to establish a standard since the interpretation of clinical signs is entirely subjective. As explained later, it is still possible to identify flaws in interpreting clinical signs among the LAP/FAMEZ/UFMS team, even with this measure.



Clinical signs	Toxin or toxin-producing clostridia detected	Other confirmation	Conclusion
+	+	–	Botulism
+	–	+	It is not botulism
+	–	–	Suspected botulism

Fig.1. General botulism diagnosis workflow. Based on suspected signs of botulism, the diagnosis is confirmed by detecting botulinum toxins with *in vivo* or *in vitro* assays instead of (or after) culture. A positive diagnosis for botulinum toxins, made directly from suspected samples, confirms the diagnosis. Detecting neurotoxin or a toxin produced by clostridia after the culture stage reinforces the diagnosis of botulism but must be interpreted within the epidemiological context since the organism may be present naturally in the environment. Adapted from Le Maréchal et al. (2016).

The first study on botulism with data from LAP/FAMEZ/UFMS appears in a study that documented and analyzed necropsy reports from August 1993 to October 1995 (Lemos & Salvador 1996). On this occasion, the "Agência Estadual de Defesa Sanitária Animal e Vegetal do Mato Grosso do Sul" (State Agency for Animal and Plant Health Defense of Mato Grosso do Sul – IAGRO) introduced, into its laboratory routine, the bioassay technique in mice followed by serum neutralization, making it possible to confirm the diagnosis of the disease. During this period, 297 cattle were necropsied, of which 66 presented clinical and epidemiological data suggestive of botulism. Samples of liver, intestinal contents, and rumen contents from 21 cattle were submitted to the gold standard tests for diagnosing botulism. BoNT toxins were detected in at least one of the samples from 10 cattle: type C (four cattle), type D (four cattle), and type C/D mosaic (two cattle). Despite the negative result in the bioassay in mice, 56 cattle received a presumptive diagnosis of botulism as they presented epidemiological data and clinical signs similar to those in which the toxin was identified. Subsequently, in a retrospective study on diseases characterized by nervous



Sinais clínicos	Detecção da toxina ou de clostrídio produtor de toxina	Outra confirmação	Conclusão
+	+	-	Botulismo
+	-	+	Não é botulismo
+	-	-	Suspeita de botulismo

Sinais Clínicos	Clinical Signs
Paralisia flácida simétrica e progressiva	Symmetrical and progressive flaccid paralysis
Bovinos – Constipação, cólica fraqueza dos membros pélvicos, decúbito disfagia e dispneia	Cattle – Constipation, colic, weakness of the pelvic limbs, recumbency, dysphagia, and dyspnea
Diagnósticos diferenciais	Differential diagnoses
Confirmação Laboratorial	Laboratory Confirmation
Soro conteúdos ruminal/gastrointestinal, fezes, tecidos	Serum ruminal/gastrointestinal contents, feces, tissues
Detecção da toxina	Toxin detection
Detecção da bactéria	Detection of the bacterium
Imunoensaio em camundongo	Mouse immunoassay
Meio de cultura enriquecido	Enriched culture medium
ELISA	ELISA
Endopep Ms	Endopep Ms
PCR	PCR

INTERPRETATION

Clinical signs	Detection of the toxin or toxin-producing clostridia	Another confirmation	Conclusion
+	+	-	Botulism
+	-	+	It's not botulism
+	-	-	Suspected botulism

Fig.2. The general workflow for diagnosing botulism in Brazil. Adapted from Le Maréchal et al. (2016). "Other confirmation" means confirmation of diseases other than botulism (Guizelini & Lemos 2022).

symptoms in cattle from Mato Grosso do Sul (Lemos et al. 2002), botulism was considered the most common nervous system disease. The study was based on the review of 1,307 reports from LAP/FAMEZ/UFMS from August 1993 to November 2000, of which 298 were considered inconclusive, and 890 had a history of nervous symptoms. Of these, 250 were diagnosed as botulism based on clinical signs and epidemiological data, absence of macro and microscopic lesions that would justify the clinical picture, and negative IFD and IC for rabies (Lemos et al. 2002). A new study was conducted from January 2000 to December 2003, in which 1,431 necropsied bovine tissue samples were evaluated; among them, 530 were classified as inconclusive, 54 were diagnosed as botulism, and another 91 were considered suggestive of botulism. This study considered cases as a diagnosis of botulism whose materials were sent with an epidemiological history indicative of the disease, negative results for rabies in the IFD and IC tests, absence of significant macro and microscopic changes, and evidence of flaccid paralysis of the locomotor limbs, paralysis or hypotonia of the tail and tongue, dyspnea, preserved level of consciousness and anal and skin sensitivity reflexes present. The detection of BoNT by bioassay and serum neutralization in mice was also a diagnostic criterion; however, only five cases were positive, three for BoNT type D and two for type C. This was the first study on botulism in Mato Grosso do Sul that established clinical signs taken into consideration by the LAP/FAMEZ/UFMS team to suspect the disease (Lemos 2005).

A condition common to the three studies mentioned is the need for more standardization in collecting samples from the central nervous system (CNS), which does not allow consistent evaluation of the same anatomical parts. Several cases negative for rabies could be false negatives, as often the brain stem and spinal cord, essential for rabies diagnosis (Bassuino et al. 2016), were not sent for histopathological evaluation, IC, and IFD. Although this flaw is crucial, at the time of the studies above, the diagnoses were still suggestive of botulism when clinical signs and epidemiology were indicative of the disease, there were no microscopic lesions, and IFD and IC were negative, even in materials that did not present a trunk brain and spinal cord. In the study published by Ribas et al. (2013) on diseases of the nervous system of cattle in Mato Grosso do Sul, the necropsy reports of 1,082 cattle were reviewed from January 2008 to December 2012. Three hundred and forty-one cattle were diagnosed with diseases of the nervous system, and 247 were inconclusive. Again, botulism was the most diagnosed nervous system disease, totaling 102 cases between conclusive and suggestive diagnoses. The criteria used to classify the case as conclusive were the absence of macro and microscopic lesions, clinical signs, epidemiological data indicative of the disease, and identification of the possible source of the toxin. As in 2010 there were several cases of toxic myopathies caused by the ingestion of *Senna occidentalis* (Fig.3) and *Senna obtusifolia* in Mato Grosso do Sul (Santos et al. 2011), from December of that year onwards, the LAP/FAMEZ/UFMS team began to consider as inconclusive cases that presented a clinical picture indicative of botulism, but that did not contain skeletal muscle samples sent for histopathological examination. This measure led to a decrease in botulism cases diagnosed from 2011 to 2012. Clinical signs considered frequent and essential for diagnosis, such as flaccid paralysis of the limbs, abdominal breathing,

tail paralysis, motor incoordination, hypotonia of the tongue, and attempts to incapacity to stand, were not described or were mentioned in a few cases (Ribas et al. 2013). As clinical characterization is fundamental for the diagnosis of botulism and many signs are common to other diseases, the lack of standardized management compromises the diagnosis. In addition to clinical signs, there may be predisposing factors for botulism in cases where mortality has occurred due to other diseases. Thus, epidemiological assessment dissociated from careful clinical signs and pathology assessment can also contribute to diagnostic errors.

In a retrospective study, 5,083 results of an autopsy and histopathological examinations were carried out by the LAP/FAMEZ/UFMS team from 1995 to 2018, of which 146 were

Table 1. Reports of botulism outbreaks diagnosed in Brazilian cattle

Reference	State	Clinical signs	Possible source of toxin	Methods of diagnosis	Toxins detected
Tokarnia et al. (1970)	PI	Progressive flaccid paralysis, recumbency, anorexia, dry or diarrheic stools, ruminal hypotonia, and preserved consciousness	Osteophagy	Necropsy and histopathology, negative DFA[a] and IC[b], clinical signs, bioassay, and serum neutralization in mice	C and D
Lisbôa et al. (1996)	SP	Ruminal hypotonia, lateral or sternal decubitus, preserved consciousness, muscular hypotonia of the limbs, and decreased or absent tonus of the tongue	Bones and carcasses	Epidemiological data, clinical signs, bioassay, serum neutralization in mice, and complement microfixation	C, D, and C/D
Dutra et al. (2001)	SP and MS	Paresis and paralysis of the pelvic limbs, difficulty in locomotion, decubitus, biphasic breathing during inspiration, preserved consciousness, hypotonia of the tongue, and paralysis of the swallowing and chewing muscles	Water	Necropsy, spore detection, clinical signs, bioassay and serum neutralization in mice, and complement microfixation	C and D
Costa et al. (2008)	MG	Anorexia, permanent sternal recumbency, pedaling movement, pleurothotonus or ascending flaccid tetraparesis, dyspneic and abdominal breathing, bulging of the pelvic limbs, and hypotonia of the tongueAnorexia, permanent sternal recumbency, pedaling movement, pleurothotonus or ascending flaccid tetraparesis, dyspneic and abdominal breathing, bulging of the pelvic limbs, and hypotonia of the tongue	Silage	Clinical signs, necropsy, bioassay, serum neutralization in mice, and negative DFA[a] and IC[b]	C and D
Maboni et al. (2010)	RS	Difficulty in mobility, flaccid paralysis of the limbs, recumbency, dyspnea, sialorrhea, and abdominal breathing	Silage	Clinical signs, bioassay, serum neutralization in mice, and complement microfixation	C
Câmara et al. (2014)	RN	Apathy, hyporexia, incoordination of the pelvic limbs, decubitus, position of "self-auscultation," difficulty in locomotion, flexion of several joints of the pelvic limbs, hypotonia of the tongue, difficulty in swallowing, hypoalgesia of the tail and anus, decreased sensory reflexes in the lumbar regions, sacral and perianal, decrease in milk production, flaccid paresis of the pelvic limbs and preserved consciousness	Carcasses and Osteophagy	Epidemiological data, clinical signs, necropsy, and histopathology	Not done
Helayel et al. (2019)	TO	Hyporexia, hypodipsia, preserved consciousness, incoordination, tail paralysis, flaccid limb paralysis, and biphasic inspiration	Silage	Epidemiological data, clinical signs, necropsy and histopathology (included muscle) without alterations, bioassay, and serum neutralization in mice	C
Nobre et al. (2019)	AC	Paresis of the pelvic limbs, prostration, recumbency, bradycardia and bradypnea, dehydration, rumen hypotonia, pale mucous membranes, hypotonia of the tongue, and preserved consciousness	Osteophagy	Clinical signs, necropsy, histopathology, bioassay, serum neutralization in mice, negative DFA[a], and immunohistochemistry for BSE[c] negatives	Negative
Campagnolli et al. (2022)	RO	Flaccid paralysis, lateral decubitus, and dehydration	Water	Epidemiology, clinical signs, necropsy, and histopathology, negative DFA[a]	Not done
Barbosa et al. (2022)	PA	Lateral decubitus, sternal decubitus, level of consciousness preserved, normal skin sensitivity, and reduced palatal and lingual cranial nerve reflexes	Osteophagy	Epidemiology, clinical signs, necropsy and histopathology, identification of phosphorus deficiency, and end of cases after control	Not done

[a] Direct fluorescent antibody, [b] intracerebral inoculation in mice, [c] bovine spongiform encephalopathy

diagnosed with botulism, and 2,734 were inconclusive (Pupin et al.2019). This survey included two outbreaks of botulism in cattle described in greater detail by Guizelini et al. (2019) and Soares et al. (2018). This same study differs from other retrospective studies carried out by the team at this laboratory because, unlike previous studies, which only considered the diagnosis present in the autopsy form, all reports were reviewed. For a case to be considered botulism, the reports must contain all of the following information:

(*ii*) Epidemiological conditions necessary for the occurrence of the disease (source of toxin with evidence of ingestion by cattle).

(*ii*) Report of characteristic clinical signs (flaccid paralysis of locomotor limbs, paralysis or hypotonia of the tail and tongue, dyspnea, preserved level of consciousness, presence of anal reflex, and skin sensitivity).

(*iii*) Absence of macroscopic and histopathological lesions, including in the brain stem, spinal cord, and skeletal muscle.

(*iv*) Negative laboratory results for rabies.

Adopting these criteria, except for cases in which BoNT was detected, the authors reclassified the diagnosis of botulism that did not contain this information to the inconclusive diagnosis category, which explains why this retrospective study presented a lower number of botulism diagnoses compared to other similar studies carried out by the laboratory.

The main criteria for suspecting and diagnosing botulism are the absence of macroscopic and histopathological lesions. However, the absence of lesions is different from failure in detecting lesions. Therefore, neglecting necropsy steps, such as evaluating and collecting the spinal cord, brain stem, skeletal muscles, and peripheral nerves, compromises the differential diagnosis with other diseases that may present similar clinical signs (Barros et al. 2006). Failure to send the brain stem and spinal cord for laboratory testing is an essential cause of false-negative results for rabies (Silva et al. 2010, Bassuino et al. 2016). It may increase the chances of misdiagnosis of botulism. Diseases that cause muscle necrosis, such as *S. occidentalis* and *S. obtusifolia* toxicosis, ionophore antibiotic poisoning, and vitamin E and selenium deficiency, can cause signs that can be confused with botulism. Therefore, it is essential that, in suspected cases, during necropsies, the skeletal muscles are thoroughly examined, and samples are collected for histopathological examination (Barros et al. 2006, Ribas et al. 2013, Lemos & Riet-Correa 2023). Currently, a peripheral neurodegenerative disease (Fig.4) has emerged in cattle from Mato Grosso do Sul and should also be listed as a differential diagnosis. The disease causes damage to peripheral nerves and can be diagnosed by histopathological evaluation of the peroneal nerve (Santos et al. 2023). Cases with similar clinical signs were previously diagnosed as chronic botulism (Lemos & Katayama 2004).

Hypothermia, hypocalcemia, urea, and organophosphate toxicosis are diseases that do not cause gross and microscopic changes in affected cattle. One should rely on epidemiological characteristics to differentiate them from botulism. Cases of hypothermia cause the simultaneous death of a large number of cattle on different properties, often in other regions of the state, always associated with sudden drops in temperatures, gust winds, and rain (Santos et al. 2012). Hypocalcemia occurs in cows in the postpartum period, uncommonly in the form of outbreaks (Barros et al. 2006). Hypocalcemia associated with the consumption of plants with high levels of oxalates

Fig.3. Necropsy findings in *Senna* sp. poisoning in cattle. Extensive pallor area in skeletal muscle corresponds to myofiber necrosis under microscopy.

Table 2. Retrospective studies reporting cases of botulism in Brazil

Reference	State	Clinical signs	Possible source of toxin	Methods of diagnosis	Toxins detected
Galiza et al. (2010)	PB	Recumbency, absence of anal reflex, flaccid paralysis of the limbs and tail, sialorrhea, jaw relaxation, and ease of exposure of the tongue	Chicken litter and contaminated water	Epidemiological data, clinical signs, necropsy and histopathology	Not done
Lucena et al. (2010)	RS	Not reported	Not reported	Not reported	Not done
Rondelli et al. (2017)	MT	Recumbency, flaccid paralysis of the limbs and tail, relaxation of the jaw and flaccidity of the tongue	Osteophagy	Epidemiological data, clinical signs, necropsy and histopathology	Not done
Terra et al. (2018)	GO	Flaccid paralysis of the limbs, tail, and tongue, incoordination, recumbency, and decreased sensitivity	Osteophagy and contaminated food	Epidemiological data, clinical signs, history, necropsy and histopathology	Not done
Oliveira et al. (2019)	TO	Flaccid paralysis, hypotonia of the tongue, biphasic breathing, and recumbency	Osteophagy and contaminated water	Epidemiological data, clinical signs, necropsy, and differentials rule outs	Not done
Santos et al. (2019)	RS	Flaccid paralysis	Flooded area that contained carcasses	Not reported	Not done

Fig.4. Peripheral neurodegenerative disease in cattle. Hyperflexion of the fetlock joints, with support on the pastern of both limbs.

has also been reported. In this specific case, collecting blood from live cattle for serum calcium measurement is essential since the diagnosis is made based on the clinical picture, elimination of other causes, detection of low serum calcium levels, and response to treatment (Constable et al. 2017). Urea poisoning has an acute course and occurs after cattle consume large quantities of the product, which die quickly, often around troughs (Barros et al. 2006). Organophosphate poisoning can have an acute, subacute, or chronic course. In acute poisoning, cases occur shortly after the application of products containing organophosphates. In subacute and chronic cases, the effects of intoxication occur later, producing clinical signs that are easily confused with botulism (Barros et al. 2006). Another differential diagnosis is poisoning by *Amorimia pubiflora*, a plant that contains monofluoroacetic acid and causes sudden death. Poisoned cattle are found dead or show clinical signs and death shortly after they are moved. Gross lesions are nonspecific; however, in some cases, microscopic lesions located in the kidneys are present and are characterized by hydropic-vacuolar degeneration and karyopyknosis in epithelial cells of the uriniferous tubules (Tokarnia et al. 2012).

CONCLUSION

The activity of veterinary diagnostic laboratories in Brazil, including LAP/FAMEZ/UFMS, was and is fundamental for recognizing botulism as a significant cause of mortality in cattle. Based on their studies, it is now possible to establish efficient and scientifically based measures to control the disease. However, currently available diagnostic techniques have low sensitivity, so epidemiology, clinical signs, and eliminating other possible causes of the observed signs are fundamental for diagnosis. Establishing well-defined and standardized criteria for the diagnosis of botulism is essential to assess the actual importance of the disease in the conditions of Mato Grosso do Sul.

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