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Blackleg in cattle in the state Mato Grosso do Sul, Brazil: 59 cases[1]

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This study aimed to review cases of blackleg (*Clostridium chauvoei* infection) diagnosed in cattle from Midwestern Brazil from 1994 to 2014 considering epidemiological, clinical, necropsy and histopathological findings. Also the following laboratory tests were used for the diagnosis of some cases of blackleg: microbiological culture and identification of the agent, microbiological culture and identification of the agent by the polymerase chain reaction (PCR), and identification of the agent in formalin fixed paraffin embedded tissues (FFPE). Criteria for presumptive diagnosis of blackleg included necrohemorrhagic emphysematous myositis consisting of inflammatory infiltrate, coagulative necrosis of myofiber, interstitial edema, hemorrhage, and gas bubbles between myofibers. Fifty nine cases from 51 outbreaks of blackleg were found, which corresponded to 1.1% of 5,375 cattle deaths investigated. In five of those outbreaks, samples of affected muscles cultures for the identification of pathogenic clostridia were made. Another three samples of similar material were cultured for clostridia with subsequent identification of the isolate by PCR. Twelve samples of FFPE affected muscle fragments were submitted to PCR for identification of the etiological agent. Except for January, cases were observed in each month of the year, with higher numbers in July-October. Most affected cattle were in the age of 7-12 years, but calves younger than 6 month-old and older than 24 months were also observed. Vaccination histories were scarce. In 32 outbreaks some vaccination history was available, but only in two of those vaccination has been carried out properly. In 56 six cases the skeletal muscles were involved. Muscles of the hind limbs were the most affected. In ten cases muscles of the tongue, myocardium and diaphragm were also affected. In three of the cases the visceral form was observed. Deaths occurred after a clinical course of 6-24 hours, but in most cases cattle were found death. Sudden death was the outcome in visceral cases (cardiac) blackleg. *Clostridium chauvoei* was confirmed to be the cause by culturing in 5 cases, and by PCR and histopatology in 8 cases. Bacterial culture followed by PCR did not demonstrate *C. chauvoei*. Calculation of the economic impact indicates that blackleg is a frequent disease in the state of Mato Grosso do Sul (MS) that inflicts significant economic loss. The amount of these losses would be reduced through proper vaccination programs against the prevalent strains of *C. chauvoei* in the region.

INDEX TERMS: Blackleg, diseases of cattle, clostridial myositis, *Clostridium chauvoei*.

RESUMO.- [**Carbúnculo sintomático em bovinos em Mato Grosso do Sul: 59 casos.**] Este estudo foi realizado com o objetivo de descrever casos de carbúnculo sintomático (infecção por *Clostridium chauvoei*) diagnosticados em bovinos do Centro-Oeste brasileiro de 1994-2014, avaliando a epidemiologia, os sinais clínicos, os achados de necropsia e a his-

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topatologia; objetivou-se também avaliar os seguintes testes laboratoriais para o diagnóstico de carbúnculo sintomático: cultura microbiológica e identificação do agente, cultura microbiológica e identificação do agente por reação em cadeia de polimerase (PCR) e identificação do agente em material fixado em formol e incluído em parafina (FFIP). Os critérios para o diagnóstico presuntivo de carbúnculo sintomático incluíram miosite necro-hemorrágica enfisematosa, caracterizada por infiltrado inflamatório, necrose de coagulação de miofribras, edema intersticial, hemorragia e bolhas de gás em meio às miofribras. Cinquenta e nove casos oriundos de 51 surtos foram encontrados, o que corresponde a 1,1% das 5.375 mortes de bovinos investigadas. Em cinco desses casos, amostras do músculo afetado foram cultivadas para clostrídios patogênicos. Amostras semelhantes de outros três animais foram cultivadas para clostrídios e os isolamentos identificados subsequentes por PCR. Doze fragmentos de músculo afetado FFIP foram submetidos a PCR para identificação do agente etiológico. Com exceção de janeiro, os casos de carbúnculo sintomático foram observados em todos os meses do ano com uma maior incidência em junho-outubro. A faixa etária da maioria dos bovinos afetados era de 7-12 anos de idade, mas bovinos mais jovens que 6 meses e mais velhos que 24 meses foram também afetados. Os históricos de vacinação eram escassos nesses surtos. Em 32 surtos havia alguma informação sobre a vacinação, mas em apenas dois casos a vacinação tinha sido realizada adequadamente. Cinquenta e seis casos de carbúnculo sintomático deste estudo eram casos clássicos afetando os músculos esqueléticos. Os músculos mais afetados foram os dos membros pélvicos. Em dez casos os músculos da língua, miocárdio e diafragma estavam também afetados. Apenas três dos casos apresentaram a forma visceral (cardíaca). O curso clínico foi de 6-24 horas, mas na maioria dos casos os bovinos foram encontrados mortos. Em casos da forma visceral ocorria morte súbita. *Clostridium chauvoei* foi confirmado como o agente causal por cultura em cinco casos e por PCR em amostra FFIP em 8 casos. Cultura bacteriana seguida de PCR do isolado não demonstrou *C. chauvoei*. Carbúnculo sintomático é uma doença frequente em bovinos no Mato Grosso do Sul podendo provocar importantes prejuízos para os produtores rurais. Esses prejuízos podem ser reduzidos através de um programa de vacinação adequado usando-se vacinas eficazes contra cepas de *C. chauvoei* prevalentes na região.

TERMOS DE INDEXAÇÃO: Carbúnculo sintomático, doenças de bovinos, miosite, clostridial, *Clostridium chauvoei*.

INTRODUCTION

Blackleg refers to a condition where necrohemorrhagic emphysematous myositis is the main lesion. The condition occurs most often in cattle and sheep and rarely in other species (Cooper & Valentine 2016). Paramount for the pathogenesis of the lesions in blackleg is toxins produced through the activation of latent spores of *Clostridium chauvoei* in muscle. *C. chauvoei* might also be involved in gas gangrene; however this is a different condition where the agent gain access by wound contamination which is not the case of blackleg (Hatheway 1990, Burke & Opeskin 1999, Cooper & Valentine 2016). *C. chauvoei*, the causative agent of blackleg, is a gram-positive, anaerobic, spore-forming bacillus that occurs in soil and feces (Valentine & McGavin 2012, Barros 2016). Most affected muscles include those of the pectoral and pelvic girdle, diaphragm, tongue, and myocardium; fibrinohemorrhagic pericarditis and pleuritis also occur (Uzal et al. 2003a, Harwood et al. 2007, Barros 2016).

The detailed pathogenesis of blackleg is still somewhat uncertain, but many of the critical points in the following proposed sequence of events have been confirmed in the natural disease and in experimental infections in cattle (Cooper & Valentine 2016). The spores are ingested from soil, enter the gastrointestinal tract and, by hematogenous route, reach the muscle where the spores remain latent in cells of the mononuclear phagocytic system. The spores may remain latent in the muscle for years (Useh et al. 2003, Kriek & Odendaal 2004, Radostits et al. 2007). Transient trauma or ischemia of the muscle favors the germination of the spores and secretion of cytolytic toxins that cause necrosis of vascular endothelia (edema, hemorrhage) and myofibers. Clostridial proliferation yield gas which appears as bubbles between the muscles bundles (Barros 2016).

Usually 6-24 month-old cattle in good plane of nutrition are affected (Useh et al. 2006a, Fiss et al. 2008). The clinical manifestations of blackleg are often not observed. Due to the rapid clinical course, animals are often found dead; sudden deaths may occur (Maxie & Miller 2016) and they are usually attributed to the myocardial lesions (Uzal et al. 2003b, Casagrande et al. 2015).When affected cattle are seen while still alive, observed signs consist of lameness, swelling, and crepitation felt over the affected area (if the lesion is superficial) and fever. In those cases death invariably occurs within 24-36 hours (Barros 2016).

A bovine dead from blackleg swells and bloats rapidly. Main gross changes include localized swelling and crepitation of muscles of the quarters and chest (Barros 2016). On cut surface the affected muscle is focally extensive dark red. At the periphery the muscle is red and moist due to edema (early lesions). To the center of lesion affected muscle is dark red, dry, and friable (later lesions) (Assis et al. 2005, Radostits et al. 2007, Barros 2016). Muscle affected by blackleg often smells sweet and butyric, like rancid butter (Valentine & Cooper 2012, Cooper & Valentine 2016). Histologically, necrotic myofibers are separated by gas bubbles associated with hemorrhage and there is scarce neutrophilic infiltrate (Barros 2016). In some cases intralesional gram-positive rods can be observed (Uzal et al. 2003a, Assis et al. 2005, Casagrande et al. 2015).

The main prophylactic measure is vaccination (Schipper et al. 1978, Kriek & Odendaal 2004). Monovalent (contains only *C. chauvoei* antigen) and polyvalent (contains antigens from clostridia) vaccines are commercially available (Araújo et al. 2010).

Although anecdotal accounts indicate that blackleg is an important disease in Brazil, there is a dearth of documented reports on the disease in the country, especially those dealing with historical series, approaching the diagnostic, epidemiological, and economic impact of blackleg.

This study aims to describe of blackleg outbreaks diagnosed at the Pathologic Anatomic Diagnostic Laboratory (LAP) of the School of Veterinary Medicine and Animal Husbandry

(FAMEZ) of the University of Mato Grosso do Sul, from January 1994 to December 2014. The study tackles epidemiological aspects, necropsy findings, and histopathology. It also aims to confirm the diagnosis by laboratorial means applying techniques such as isolation of the agent by bacterial culture, isolation of the agent by bacterial culture followed by PCR identification, and identification of the of the agent by PCR carried out in formalin fixe paraffin embedded (FFPE) tissues from cases of blackleg in cattle. The economic impact to farmers in the State of Mato Grosso do Sul (MS) is also evaluated.

MATERIALS AND METHODS

A retrospective survey was carried out in the files of the LAP/FAMEZ from January 1994 to December 2014 in search of cases of blackleg in cattle. Epidemiological, clinical, gross necropsy findings , and histopathological aspects which are characteristic for this disease (Radostits et al. 2007, Riet-Correa 2007, Valentine & McGavin 2012) and the absence of penetrating wound in the skin served as the criteria for considering a case as blackleg. These criteria will be referred hereafter as "blackleg diagnosis criteria". Each blackleg case found was identified by its chronologic sequence with arabic numbers.

Table1. Outbreaks of blackleg in cattle from the state of Mato Grosso do Sul from 1994-2014 (epidemiological data)

Case number	Montth of occurrence	Age in months	Total number of cattle	Cattle at risk	Sick cattle	Dead cattle	Vaccination	Protocol of vaccination
1	March	8	NI	NI	8	8	Inadequated	A[d]
2	March	30	NI	NI	1	1	Inadequated	Vaccinated at 4-year-old
3	June	8	60	60	11	3	Yes	Protocol 1[e]
4	June	12	50	50	1	1	NI	NI
5	June	18	2000	NI	1	1	Yes	Protocol 1
6	July	4	NI	NI	NI	NI	NI	NI
7	July	6	200	200	1	1	NI	NI
8	July	2	NI	6	2	2	NI	NI
9, 10[a]	August	12	300	150	16	16	No	-
11	August	18	3500	NI	16	16	NI	NI
12	August	8	NI	NI	5	5	NI	NI
13, 14[a]	October	11	NI	78	8	8	Inadequated	A
15	November	12	NI	NI	1	1	NI	NI
16	September	11	600	600	5	5	NI	NI
17	April	12	NI	156	6	6	Yes	NI
18	June	7	16	16	1	1	NI	NI
19	September	14	26	26	1	1	NI	NI
20, 21, 22[b]	June	7	1000	940	11	11	Yes	Protocol 2[f]
23	August	8	160	NI	10	8	Inadequated	A[f]
24	May	36	100	NI	3	3	No	NI
25	September	6	NI	4	1	1	No	NI
26	September	4	400	30	1	1	NI	NI
27	October	8	100	100	7	6	Yes	NI
28	December	4	200	40	1	1	NI	NI
29	February	NI[c]	NI	NI	NI	NI	NI	NI
30	June	4	NI	NI	2	2	No	-
31	March	14	813	367	17	17	Yes	NI
32	March	18	600	NI	1	1	No	-
33	April	24	700	NI	1	1	Yes	NI
34	May	9	1943	29	2	2	Inadequated	B[g]
35	June	6	70	12	2	2	No	-
36	September	10	NI	NI	9	9	Yes	Protocol 2
37	July	NI	NI	NI	3	3	Inadequated	B
38	August	12	2000	800	7	6	No	-
39, 40[a]	September	12	693	200	5	5	Yes	Protocol 2
41	August	9	NI	NI	NI	NI	Inadequated	B
42	October	12	206	206	4	4	No	-
43	April	10	NI	NI	11	11	NI	NI
44, 45[a]	May	7	1500	NI	3	3	No	-
46	August	10	110	110	2	2	No	-
47	August	14	1053	534	13	13	Inadequated	A
48	June	36	1200	125	2	2	No	-
49	October	10	248	138	1	1	Yes	NI
50	October	8	600	NI	1	1	NI	NI
51	March	8	80	80	2	2	NI	NI
52, 53, 54[b]	Dezember	18	310	80	4	3	Yes	NI
55	September	11	480	68	2	2	NI	NI
56	October	12	115	115	5	5	NI	NI
57	November	30	700	NI	NI	NI	No	-
58	July	12	160	40	1	1	NI	NI
59	September	8	1500	NI	3	3	Yes	NI

[a] Two necropsies performed, [b] three necropsies performed, [c] NI = not informed, [d] A = vaccination 1-5 days before the onset of outbreak, [e] Protocol 1 = first vaccine dose at 4 months of age and one boost after 30 days, [f] Protocol 2 = first vaccine dose at 4 months of age and one boost after at 8-month-old, [g] B = vaccination 6-15 before the onset of the outbreak.

For epidemiological purposes, cases from the same farm, with similar blackleg diagnosis criteria, and occurring at the same time frame were grouped as only one outbreak. Several data for each outbreak were retrieved from the necropsy protocols, including total number of cattle in the farm, cattle at risk, gross and microscopic lesions.

Morbidity and lethality rates were calculated by considering the numbers of cattle at risk, defined by cattle in the same age range of the necropsied cattle and raised under similar nutritional and sanitary conditions. The numbers of dead and sick cattle occurring in the farm during the outbreak were gathered at the time of the necropsy. In five cases (Bovines 1, 2, 11, 25, and 29) muscle samples were wrapped in hot paraffin to maintain anaerobiosis during transportation to the laboratory for isolation of pathogenic clostridia. Another three samples were refrigerated at 5°C for 12 (case 59, Table 1) to 36 (cases 52 and 54, Table1) months and then submitted to identification of the agent by bacterial culture followed by PCR according to previously described technique (Ribeiro et al. 2012). PCR was also employed for identification of *Clostridium chauvoei* in FFPE samples from 12 cases (48-59, Table 1) using techniques previously described (Kojima et al. 2001).

The economic impact caused by the disease was assessed based on the numbers of dead cattle over cattle at risk. Outbreaks where this information was not available were disconsidered. Money values utilized in the calculations were obtained from the Commercial Chamber of Campo Grande, MS and were used both to estimate the animal value and the cost of vaccination. The following amounts were calculated: value of cattle stock at risk, considering the total number of cattle at risk and multiplying it by the commercial unitary value of that particular cattle category; percent of estimated loss, calculated by the following rate (total loss in R$/herd value of cattle at risk in R$) x100; estimated cost value of vaccination calculated by the total cattle at risk multiplied by the unitary value (per head of cattle) of vaccine. The impact of vaccination cost over the losses was calculated by the rate (vaccine cost in R$/total loss in R$) x100; such a rate provides an estimate of the magnitude of impact of the cost over the estimated value of losses, the smaller the rate, the higher the impact of the loss, indicating that a small expenditure with vaccines might avoid a large loss.

RESULTS

During the period of study, 59 cases of blackleg were diagnosed from a total of 5,375 necropsied cattle, corresponding to 51 outbreaks of the disease. The numerical identification of the outbreaks and their epidemiological data are on Table 1. Cases of blackleg occurred all year round and excepting for January, there were cases recorded in every month. Vaccination data were not provided in 19 outbreaks. Furthermore in most outbreaks where the vaccination was performed the utilized vaccination protocol was not informed. In two outbreaks the protocol recommended by the manufacturer was followed and in three outbreaks, for convenience of handling the herd, calves were vaccinated when 4 month-old with a boost when 8-month-old. In one outbreak (cases 9 and 10) the total cattle population was 300 heads; 150 of those were vaccinated according to one protocol indicated in the literature (first dose at 4 months after birth and a boost 30 days thereafter) and the remaining 150 were not vaccinated. The disease affected only the non-vaccinated cattle in this outbreak. This 150 were considered the group of cattle at risk.

Data on the age of affected cattle are in Table 2. There were 51 affected cattle in the range of 6-24 month-old.

Table 2. Age distribution of cases of blackleg diagnosed in cattle from the state of Mato Grosso do Sul from 1994-2014

Age in months	Total cases	%
0-6	8	13.6
>6-12	35	59.3
13-18	9	15.3
19-24	1	1.7
> 24	4	6.8
Not informed	2	3.4

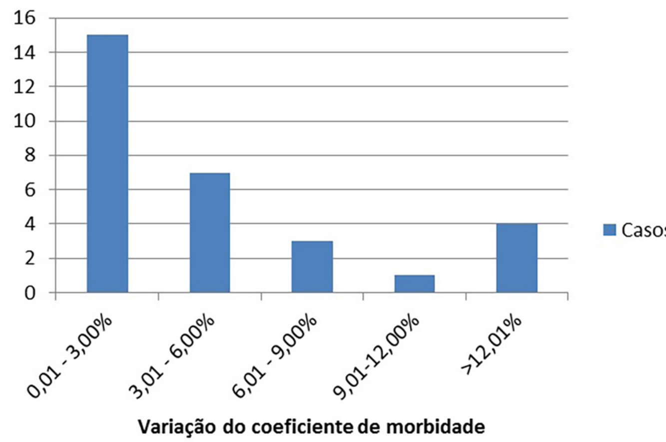

Fig.1. Frequency of cases of bovine blackleg in relation to the morbidity rate.

Morbidity and mortality rates varied from 0.5-33.33% and from 27.27-100%, respectively. However, in most of the outbreaks the morbidly rates were below 6% (Fig.1). Cattle from Outbreak 3 were treated with antibiotics every time their rectal temperatures were higher than 39°, which may explain the low lethality rate in this outbreak. Main clinical signs, most frequently affecting muscles, necropsy, and histopathological findings are in Tables 3-6. Clinical signs were observed in 41 cattle; the remaining affected cattle were either found dead (n=10) or no information were registered in their necropsy protocol (n=8). In 20 cases there were no gross description but the histopathological changes were characteristic of blackleg (Table 6) allowing for the diagnosis. Observed gross lesions in striate muscle include locally extensive hemorrhage and edema, often with crepitation of the affected area caused by gas bubbles produced by the organism multiplication. The covering hide was taut and there was a large area of hemorrhage in the subcutaneous tissue, overlying fascia and subcutaneous tissue. Necrotic muscle fibers appeared dark red to red-black (Fig.2). The lesions were wet and exudative (early lesions) or dry (later lesions). A characteristic

Table 3. Clinical signs in 41 cases of blackleg in cattle

Clinical sign	Number of cases (%)
Lameness	23 (56.10)
Prostation	14 (34.15)
Muscle swelling	12 (29.27)
Crepitation in affected muscle	7 (17.07)
Edema	7 (17.07)
Muscle tremors	4 (9.76)
Stiff gait	2 (4.88)
Anorexia	1 (2.44)
Tachypnea	1 (2.44)

Table 4. Most frequently affected muscles in cases of blackleg diagnosed in cattle from the state of Mato Grosso do Sul from 1994-2014

Affected muscle	Number of cases	%
Skeletal muscle	46/59	77.97
Right hind limb	9/46	19.56
Left hind limb	15/46	32.61
Right fore limb	5/46	10.87
Left for limb	5/46	10.87
Psoas	1/46	2.17
Anatomical site not informed	11/46	23.91
Myocardium and skeletal muscles	6/59	10.17
Myocardium	3/59	5.08
Myocardium, tongue and skeletal muscles	1/59	1.69
Tongue and skeletal muscles	1/59	1.69
Tongue, diaphragm, and skeletal muscles	1/59	1.69
Diaphragm and skeletal muscles	1/59	1.69

Table 5. Necropsy findings in 39 cattle dead from blackleg

Lesion	Number of cases	%
Dark red discoloration of affected muscle area	34	87.18
Crepitation of affected muscle area	23	58.97
Edema tinged with blood	16	41.03
Good nutritional plane	15	38.46
Butyric smell from the lesion	10	25.64
Affected muscle floating in formalin	6	15.38
Splenomegaly	6	15.38
Blood-stained froth flowing from the nose	5	12.82
Poorly circumscribed swelling in the affected area	4	10.26
Fibrinohemorrhagic pericarditis	1	0.39
Fibrinohemorrhagic pleuritis	1	0.39

Table 6. Histopathological findings in the muscle of 59 cattle dead from blackleg

Lesion	Number of cases	%
Neutrophilic infiltrate	55	93.22
Hemorrhage	52	88.14
Fibrin	37	62.71
Coagulative segmentar necrosis	32	54.24
Separation of myofibers	30	50.85
Gas bubbles between myofibers	17	28.81

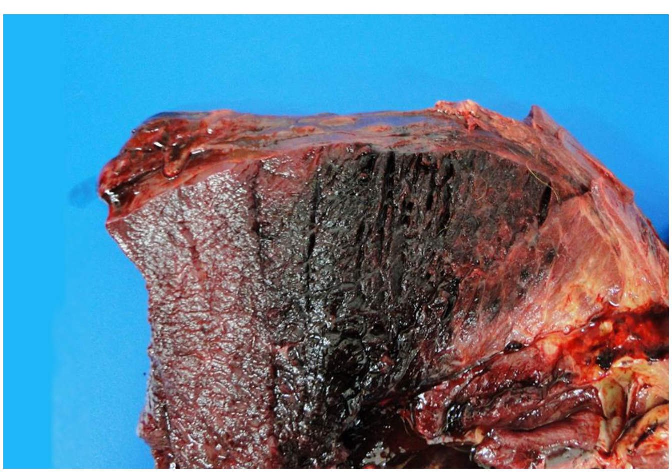

Fig.2. Gross findings in bovine blackleg. There is a dark hemorrhagic zone within the affected muscle. Necrotic muscle is dark red to red-black. A portion of the lesion to the left is dark red, wet and exudative (early lesions) or dry, red-black with muscle bundles separated by gas bubbles (later lesions).

odor of rancid butter from butyric acid was reported in some in stances. Affected muscle floated in the formalin solution (Fig.3). In case 58 cardiac involvement (Fig.4) and fibrinous pleurits (Fig.5) were observed. Histologically, locally extensive areas of muscle fibers undergoing

Fig.3. Gross findings in bovine blackleg. A fragment of an affected muscle floats in 10% formalin solution.

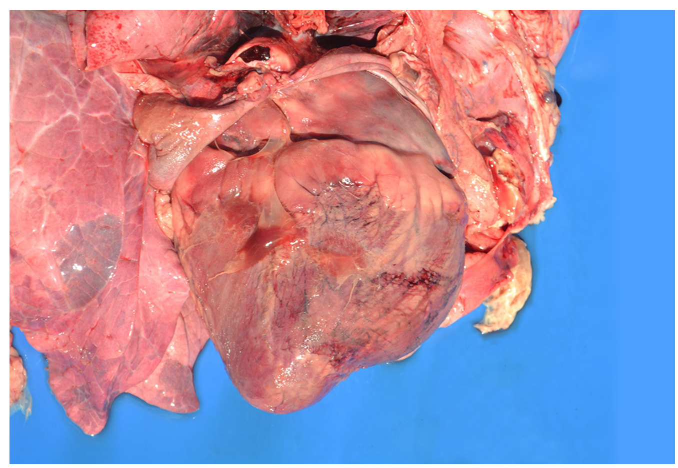

Fig.4. Gross findings in bovine blackleg. Necrohemorrhaghic myocarditis and fibrinous pericarditis.

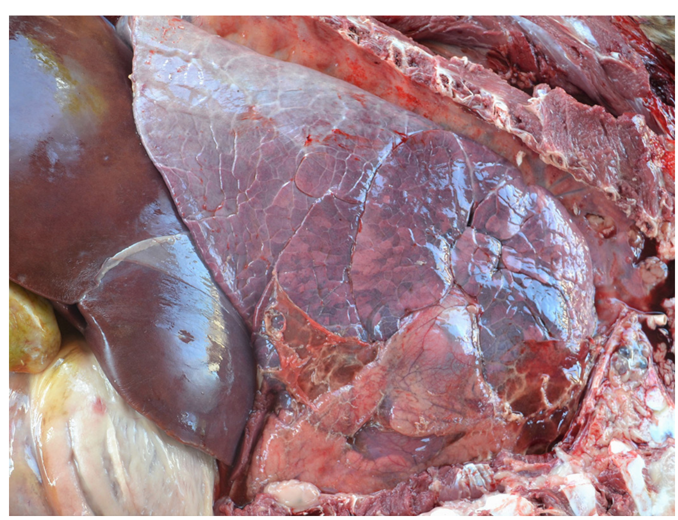

Fig.5. Gross findings in bovine blackleg. Fibrinous pleuritis adhere lung lobes to one another and to pericardial sac.

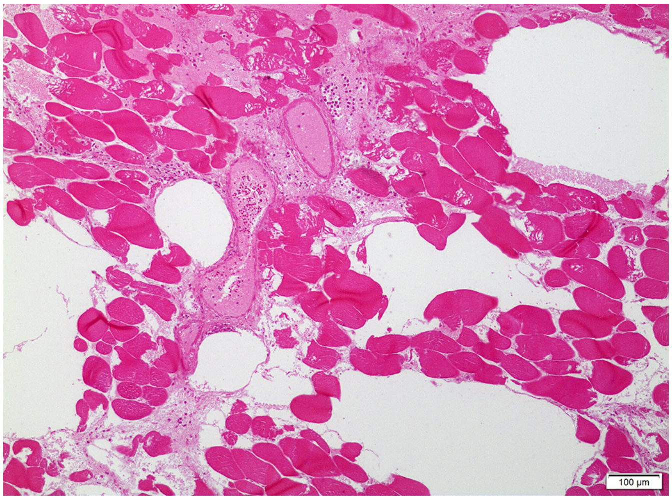

Fig.6. Histopathological findings in bovine blackleg. Coagulative necrosis of myofibers, hemorrhage and distended bundles of myofibers by gas bubbles. There is mild inflammatory infiltrate of neutrophils. HE, obj.40x.

coagulation necrosis and fragmentation, and interstitial edema and hemorrhage were seen (Fig.6).

The laboratory tests used to identify the clostridia causing the myonecrosis and their results are in Table 7. In 12 out of the 17 submitted materials *Clostridium Chauvoei* was identified as the causative agent.

In Table 8, the economic impact estimated for the outbreaks of the disease is presented. The estimated losses were 0.75 to 27.50% in those farms which did not use vaccination; there was 0.50 to 33.33% loss in farms without information about vaccination, and 0.72 to 6.00% loss in farms where the herd was vaccinated.

The vaccine costs and estimated losses was the following: 15.96 to 0.44% for farms which did not vaccinate, from 36.14 to 0.54% in farms where information on vaccination was not supplied, and from 16.52 to 2.13% in those farms where the herd was vaccinated.

Table 7. Laboratory results from bacterial culture and molecular identification of the agent in 15 cases of blackleg in cattle

Sample	Bacterial culture	Bacterial culture followed by PCR[a] of the isolate	PCR in FFPE[b]
3	*Clostridium chauvoei*	NP	NP
9	*Clostridium chauvoei*	NP	NP
12	*Clostridium chauvoei*	NP	NP
25	*Clostridium chauvoei*	NP	NP
29	*Clostridium chauvoei*	NP	NP
48	NP[c]	NP	Negative
49	NP	NP	*Clostridium chauvoei*
50	NP	NP	Negative
51	NP	NP	Negative
52	NP	*Clostridium septicum*	*Clostridium chauvoei*
53	NP	NP	*Clostridium chauvoei*
54	NP	*Clostridium septicum*	*Clostridium chauvoei*
55	NP	NP	*Clostridium chauvoei*
56	NP	NP	*Clostridium chauvoei*
57	NP	NP	Negative
58	NP	NP	*Clostridium chauvoei*
59	NP	Negative	*Clostridium chauvoei*

[a] PCR = polymerase chain reaction, [b] FFPE = formalin fixed paraffin embedded tissues, [c] NP = not performed.

Table 8. Results of the economic analyses in cases of blackleg of blackleg in cattle from the state of Mato Grosso do Sul from 1994 a 2014

Case number	Cattle at risk	Cattle dead	Vaccination	Value of the herd (R$)	Losses (R$)	Losses (%)	Cost of vaccine (R$)	Cost/loss rate
57	80	22	No	107,600	29,590	27.50%	129	0.44%
25	4	1	No	5,044	1,261	25.00%	6	0.51%
35	12	2	No	15,132	2,522	16.67%	19	0.77%
9, 10[a]	150	16	No	183,000	19,520	10.67%	242	1.24%
13, 14[a]	78	8	No	95,160	9,760	10.26%	126	1.29%
34	29	2	No	35,380	2,440	6.90%	47	1.91%
47	534	13	No	651,480	15,860	2.43%	860	5.42%
48	125	2	No	218,750	3,500	1.60%	201	5.75%
46	110	2	No	147,950	2,690	1.82%	177	6.58%
42	206	4	No	251,320	4,880	1.94%	332	6.80%
38	800	6	No	1,076,000	8,070	0.75%	1,288	15.96%
8	6	2	NI	5,346	1,782	33.33%	10	0.54%
18	16	1	NI	20,176	1,261	6.25%	26	2.04%
56	115	5	NI	140,300	6,100	4.35%	185	3.04%
19	26	1	NI	34,970	1,345	3.85%	42	3.11%
55	68	2	NI	82,960	2,440	2.94%	109	4.49%
28	40	1	NI	50,440	1,261	2.50%	64	5.11%
58	40	1	NI	48,800	1,220	2.50%	64	5.28%
26	30	1	NI	26,730	891	3.33%	48	5.42%
4	50	1	NI	64,125	1,283	2.00%	81	6.28%
51	80	2	NI	71,280	1,782	2.50%	129	7.23%
16	600	5	NI	732,000	6,100	0.83%	966	15.84%
7	200	1	NI	178,200	891	0.50%	322	36.14%
27	100	6	Yes	126,100	7,566	6.00%	161	2.13%
31	367	17	Yes	493,615	22,865	4.63%	591	2.58%
3	60	3	Yes	64,560	3,228	5.00%	97	2.99%
17	156	6	Yes	200,070	7,695	3.85%	251	3.26%
52, 53, 54[b]	80	3	Yes	97,600	3,660	3.75%	129	3.52%
39, 40*	200	5	Yes	244,000	6,100	2.50%	322	5.28%
20, 21, 22[b]	940	11	Yes	1,011,440	11,836	1.17%	1,513	12.79%
49	138	1	Yes	185,610	1,345	0.72%	222	16.52%

[a] Two necropsies performed, [b] three necropsies performed.

DISCUSSION

Although blackleg is considered an important cause of economic losses in several countries (Useh et al. 2003, Kriek & Odendaal 2004, Useh et al. 2006b) including Brazil (Riet-Correa 2007, Lobato et al. 2013), there are few documented surveys on the disease approaching historical series. Cases of blackleg occurred in every year of the time frame of the current study in different regions of the state demonstrating that the pasture contamination by *Clostridium chauvoei* is disseminated and that the disease is a frequent cause of death in cattle from Mato Grosso do Sul. Considering that *C. chauvoei* is a soil organism (Kriek & Odendaal 2004, Radostits et al. 2007) and is widely distributed in this State, it is probable that the low prevalence of cases of blackleg during the period of this study is due to vaccination, a common practice in most farms (Araújo et al. 2010). This is also true in other countries (Uzal 2012). Data from the Syndicate of National Industries of Products for Animal Health, estimates that 150 million of vaccine doses against blackleg are sold annually in Brazil (Araújo et al. 2010).

In the current study blackleg was responsible for 59 deaths out of a total of 5,375 necropsied cattle, thus accounting for 1.1% of the deaths in cattle in studied the period (1994-2014). In a similar study carried out in the South region of Rio Grande do Sul, from 1978-2007 (Fiss et al. 2008), out of a total of 5,133 cattle examined, there were 29 cases of blackleg, i.e., 0.56% of all the diagnosis in cattle. This made blackleg the most frequent clostridial disease of that part of the country. In another similar study carried out in the Central region of Rio Grande do Sul, from 1964-2008 (Lucena et al. 2010) blackleg was also the most prevalent clostridial disease of cattle, being responsible for 0.63% over the total (6,706) of examined cattle.

With the exception of January, cases of blackleg in cattle in the current study occurred in every month of the year. There was a larger concentration of cases between June--October. Those are similar data are also reported in other regions of Brazil (Fiss et al. 2008).

Reports from other countries (Kriek & Odendaal 2004, Radostits et al. 2007) mention seasonality for blackleg occurrence, with most of the cases occurring in the warmer months. These authors admit that variations could occur in the seasonality with most cases occurring during Spring and Fall. Several factors are used to explain the seasonality although none of them is proved.

Most of the cases of blackleg of the current study occurred in cattle from 7-12 months of age, although few younger and older stock were also affected which is similar to what is world widely observed (Kriek & Odendaal 2004, Assis et al. 2005, Riet-Correa 2007, Groseth et al. 2011). The occurrence of fewer cases in calves younger than 6 month-old is explained by the protection conferred by the colostrum; consequently the occurrence of cases from birth to 6-months-old are attributed to failure in the transfer of passive immunity. Although there are no data explaining the apparent resistance of cattle older than 24-month-old to blackleg, it is probably due to acquired immunity to successive subclinical infection with *C. chauvoei*. If this is true, then adult cattle may become affected when raised in *C. chauvoei*-free pastures and transfer when adults into contaminated pasture.

In the current study, in most the outbreak files reviewed, there were no detailed information on vaccination history. In 12 outbreaks calves have not been vaccinated, in 8 outbreaks vaccination has been carried out inadequately and in only two outbreaks vaccination has been done according to the specifications of the manufactures, i.e., the first dose in 4-month-old calves and a boost 30 days after. In three outbreaks calves have been vaccinated twice, when 4-month-old and then when 8-month-old, which can be considered an adequate practice of common use in many farms (Araújo et al. 2010).

One of the difficulties to evaluate whether the protocols were in conformity with the technical recommendations is that most of the farms that do not possess a definite sanitary agenda, in which cattle are set apart in lots according to their ages. It is then possible that the age at which the calves are vaccinated in many farms is markedly variable. For instance, when the first dose is applied at the same time for all the calves in a given farm, calves under and over 4-month-old can be vaccinated. This might be a risky procedure since older calves become susceptible due to the decline of the colostral protection and calves younger than 4-month-old may have lesser protection because maternal--derived antibody will interfere with the ability of young animals to respond to the majority current vaccines (Troxel et al. 1997, Radostits et al. 2007, Day 2012), although this negative effect is no confirmed by some studies (Schipper et al. 1978). Furthermore, failure due to differences between vaccinal and field strains of *C. chauvoel* are reported from Australia (Reed & Reynolds 1977) and Brazil (Santos 2003).

Although vaccination against blackleg is a worldwide disseminated practice there are few reports attesting vaccine efficacy (Uzal 2012). In the current study, the efficacy of vaccination was observed in one of the outbreaks were cattle vaccinated and unvaccinated were kept in the same conditions and only the unvaccinated cattle develop blackleg.

There is wide variation in the lower and upper values for morbidity (0.50 to 33.33%) and lethality (27.27 to 100%) rates in blackleg outbreaks in cattle. Ample variations in these rates have already been reported as 0.66 to 57.14% for morbidity and 85.71 to 100% for lethality (Uzal et al. 2003a, Assis et al. 2005, Harwood et al. 2007, Groseth et al. 2011, Casagrande et al. 2015). In the current study in 22 of 31 outbreaks, morbidly rates were lower than 6% and the higher rates occurred in small cattle herds where only one case would reflect a large percentage. As for the lethality it was less than 100% in 4 of 31 outbreaks. Cautions must be exerted in interpreting these data, because in some instances the outbreak was in progress when observed, and epidemiological data reflect only the time of necropsy since in some outbreaks there was no follow up. It is important to make this disclaimer since blackleg lethality rates are virtually 100% and even treated cattle have poor prognosis (Kriek & Odendaal 2004, Riet-Correa 2007).

Three cases reported here affected the heart (visceral form) and the remaining affected other striated muscles (classic form). In fact, the classic form usually predominates over the visceral for, the latter being sporadic (Glastonbury et al. 1988, Uzal et al. 2003a). Clinical signs, necropsy findings and histopathology observed here are typically the same reported in the literature (Uzal et al. 2003a, Gregory et al. 2006, Harwood et al. 2007, Groseth et al. 2011, Casagrande et al. 2015, Barros 2016, Cooper & Valentine 2016). In the current study the group of muscle most frequently affected was those of the pelvic girdle.

Our results demonstrate that “blackleg diagnosis criteria” used in the current study are reliable for diagnosis of blackleg in cattle. These criteria are used for working diagnosis at several diagnostic laboratories from Brazil (Riet--Correa 2007). and elsewhere (Kriek & Odendaal 2004, Useh et al. 2006a), due to difficulties in isolating *C. chauvoei* from samples of clinical cases (Uzal et al. 2003b) and due the fact that many times the samples are send to the laboratory fixed in formalin. The use of these criteria proved efficient also in the current study, as 12 of 17 cases diagnosed based “blackleg diagnosis criteria” were confirmed by bacterial culture, PCR or both (Table 7).

The microbiological method for isolation of *C. Chauvoei* was employed successful in five cases in which the samples were preserved in anaerobiosis environment. In spite of difficulties in the microbiological isolation of the causative agent, this procedure should be encouraged in cases of blackleg in cattle, because cases of vaccination failures due to vaccinal *C. chauvoei* can occur as already mentioned. Thus, keeping a bank of field strains consist in a measure of security for evaluation of the efficacy of vaccines.

Three samples of cattle fulfilling all the epidemiological and clinicopathological criteria of blackleg were negative for *C. Chauvoei* in bacterial culture. Two of these samples - from cattle involved in the same outbreak - were stored for 12 months and yield *C. septicum* in pure culture, which were subsequently confirmed by PCR of the isolates. However all three samples were positive for *C. chauvoei* DNA by the PCR done in FFPE muscle samples. It is inferred that the storage of material for long periods might favor post mortem proliferation of organisms.

The PCR technique in FFPE was efficient in the identification of *C. chauvoei*. It detected exclusively *C. chauvoei* in 8 of 12 cases examined by this technique. Similar results were described by others (Kuhnert et al. 1997, Uzal et al. 2003b). The utilization of PCR in formalin fixed and in FFPE samples is a promising option when the submission to the diagnostic laboratory for bacterial culture cannot be made with the necessary promptness. It is also worth mention that the isolation of *C. chauvoei* from samples without lesion should not be considered as diagnostic for blackleg, since *C. chauvoei* can be isolated from healthy cattle (Useh et al. 2006a). Several clostridial organisms including *C. perfringens*, *C. septicum*, *C. sordellii* are saprophytes of the intestine of cattle and can proliferate and disseminate in the body during the terminal stages or after the animal death from an unrelated disease. Thus the isolation of pathogenic clostridia from a necropsy case is of difficult interpretation and cannot be diagnostic unless the lesions compatible with the suspect disease are present or the toxin is demonstrated by tests in mice (Hjerpe 1990).

The difficult in isolation of *C. chauvoei* reinforces the importance of necropsy and histopathology for the diagnosis of blackleg. Several factors may interfere with the bacteriological diagnosis, such as time elapsed from the sampling of material and the performance of the analysis and also on conservation and non-contamination of the collected sample (Radostits et al. 2007, Riet-Correa 2007).

Clostridium septicum is a postmortem invasor promoting putrefaction of the carcass, and may be present at the time of tissue sampling not necessarily being involved in the pathogenesis of the disease investigated (Kuhnert et al 1997, Lobato et al. 2007). Furthermore *C. septicum* inhibits the growth of do *C. chauvoei* in solid culture medium. Co-infections with *C. chauvoei* and *C. septicum* are not uncommon; however, the importance of *C. septicum* as etiologic agent in bovine blackleg is controversial (Radostits et al. 2007).

In the current study *C. chauvoei* and *C. septicum* were not cultured from the same lesion; however in two cases in which only *C. septicum* was cultured, *C. chauvoei* was identified by the PCR technique in FFPE. In a study involving 176 cases of clostridial myonecrosis in cattle, *C. chauvoei* was found alone or associated with *C. septicum* in 56% of the cases and in 36% of the cases *C. novyi* or *C. novyi* and *C. septicum* were cultured from the lesion (Williams 1977). Although some authors consider that these results indicate that for the best protection of a cattle herd one should use polyvalent vaccines containing all these antigens (Radostits et al. 2007), in Brazil, field observations point to *C. chauvoei* as the sole cause, since for many years large cattle herds were successfully immunized by monovalent vaccines (Lobato et al. 2004).

The results of the current study demonstrate that blackleg is a frequent disease in cattle from Mato Grosso do Sul and that it could be responsible for significant economic losses to farmers. The estimated losses, costs and economic impact stemming from the outbreaks of blackleg demonstrate that herds not vaccinated or vaccinated incorrectly elevate the losses and impact in the farmers profit since cattle blackleg has a high lethality rates. There is scarcity of data compromising the more accurate assessment of the economic impact. The cost of these losses may be reduced by the installment of an adequate vaccination program that takes into account efficient vaccines against the *C. chauvoei* strains prevalent in the region, and abiding to the protocols indicated by the manufacturers.

CONCLUSIONS

Bovine blackleg is a frequent disease in Mato Grosso does Sul, occurring all year round. Most affected cattle are 7 to 12-month old calves, although sporadic cases might occur in cattle younger than 6 and older than 14-month old.

The classic form predominates, but a small percentage of visceral blackleg occur.

The number of blackleg cases in properly vaccinated cattle herds was lower than the one in not vaccinated cattle or in those vaccinated incorrectly.

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