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Cover illustration: Diffuse dark red kidney due to copper poisoning in cattle (Martins et al., pag. 654)

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Original Article
Livestock Diseases
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Chronic copper poisoning in beef cattle in the state of Mato Grosso, Brazil[1]

Kamila P.F. Martins[2], Vitor H.T. Padilha[3], Tércio K. Damasceno[4], Marcos A. Souza[3], Emanoelly M.S. Silva[2], Marlon Ribeiro[3], Asheley H.B. Pereira[3] and Edson M. Colodel[3]*

ABSTRACT.- Martins K.P.F., Padilha V.H.T., Damasceno T.K., Souza M.A., Silva E.M.S., Ribeiro M., Pereira A.H.B. & Colodel E.M. 2020. **Chronic copper poisoning in beef cattle in the state of Mato Grosso, Brazil**. *Pesquisa Veterinária Brasileira 40(9):651-661.* Faculdade de Medicina Veterinária, Universidade Federal do Mato Grosso, Av. Fernando Corrêa da Costa 2673, Bairro Boa Esperança, Cuiabá, MT 78068-900, Brazil. E-mail: moleta@gmail.com

Copper is an essential micromineral in animal feed; however, when consumed in excess, it can cause liver necrosis, hemolytic crisis, hemoglobinuric nephrosis and death in cattle. Although uncommon in this species, copper poisoning occurs as a result of exacerbated supplementation, deficiency of antagonist microminerals, or previous liver lesions. An outbreak of chronic copper poisoning is reported in semi-confined cattle after supplementation with 50 mg/Kg of dry matter copper. The cattle showed clinical signs characterized by anorexia, motor incoordination, loss of balance, jaundice, brownish or black urine, diarrhea and death, or were found dead, 10 to 302 days after consumption. Of the 35 cattle that died, 20 underwent necropsy, whose frequent findings were jaundice, enlarged liver with evident lobular pattern, black kidneys, and urinary bladder with brownish to blackish content. Microscopically, the liver showed vacuolar degeneration and/or zonal hepatocellular centrilobular or paracentral coagulative necrosis, in addition to cholestasis, mild periacinal fibrosis, apoptotic bodies, and mild to moderate mononuclear inflammation. Degeneration and necrosis of the tubular epithelium and intratubular hemoglobin cylinders were observed in the kidneys. Copper levels in the liver and kidneys ranged from 5,901.24 to 28,373.14 µmol/kg and from 303.72 to 14,021 µmol/kg, respectively. In conclusion, copper poisoning due to excessive nutritional supplementation is an important cause of jaundice, hemoglobinuria, and death in semi-confined cattle.

INDEX TERMS: Copper poisoning, beef cattle, Mato Grosso, Brazil, semi-confinement, mineral supplementation, cattle diseases, trace elements, jaundice.

RESUMO.- [**Intoxicação crônica por cobre em bovinos de corte no Estado de Mato Grosso, Brasil.**] Cobre é um micromineral essencial, que quando em excesso induz necrose hepática, crise hemolítica, nefrose hemoglobínurica e morte em bovinos. As intoxicações, apesar de incomuns nessa espécie, ocorrem devido a suplementação exacerbada de cobre, pela deficiência de microminerais antagonistas ou secundária a lesão hepática prévia. Relata-se um surto de intoxicação crônica por cobre em bovinos semiconfinados após suplementação com 50mg/kg de cobre em matéria seca. Os bovinos manifestaram sinais clínicos caracterizados por anorexia, incoordenação motora, perda de equilíbrio, icterícia, urina acastanhada ou negra, diarreia e morte ou foram encontrados mortos, após 10 a 302 dias do início de consumo. De 35 bovinos que morreram 20 foram submetidos à necropsia sendo achada frequente icterícia, fígado aumentado e com padrão lobular evidente, rins pretos e bexiga urinária repleta de conteúdo acastanhado a enegrecida. Microscopicamente, no fígado havia degeneração vacuolar e ou necrose coagulativa hepatocelular zonal, centrolobular ou paracentral, além de degeneração vacuolar com corpúsculos de Councilman, colestase, fibrose periascinar leve, e inflamação de discreta a moderada. Nos

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rins havia degeneração e necrose do epitélio tubular assim como cilindros de hemoglobina intratubulares. Os níveis de cobre no fígado e rim foram de 5.901,24 a 28.373,14µmol/kg e 303,72 a 14.021µmol/kg respectivamente. A suplementação nutricional excessiva com cobre pode causar doença hemolítica com icterícia, hemoglobinúria e morte de bovinos mantidos em sistema de semiconfinamento.

TERMOS DE INDEXAÇÃO: Intoxicação por cobre, bovinos de corte, Mato Grosso, Brasil, semiconfinamento, suplementação mineral, doenças de bovinos, oligoelementos, icterícia.

INTRODUCTION

Copper is an essential trace element that participates in biological processes. These processes include the activity of enzymes involved in cellular respiration, inactivation of free radicals, neurotransmission, connective tissue synthesis, production of pigments, iron cellular metabolism, and hemoglobin formation (Underwood & Suttle 1999, Festa & Thiele 2011).

Because of its extensive functionality and the finding of cases of deficiency in cattle in Brazil, copper has been widely used in mineral supplements (Malafaia et al. 2014). Supplementation, added to the levels of copper present in pastures and water, should contribute to cattle reaching the daily copper requirement of 4-8 mg/kg of dry matter (DM) (Suttle 2010). After ingestion, copper is absorbed by enterocytes in the small intestine, accesses the portal circulation, binds to albumin and transcuprein, and is then taken to the liver and stored in hepatocytes (Suttle 2010). Homeostasis maintenance between the accumulation of hepatic copper and bile excretion occurs through copper-dependent metalloenzymes such as ceruloplasmin and metallothionein (Rosa & Mattioli 2002, Tapia et al. 2004). In ruminants, this metabolism is influenced by the absorption and storage capacities of individuals, which differ according to race, animal category, and antagonism with other microminerals (Blanco-Penedo et al. 2006, Cuttance et al. 2018).

In general, cattle tolerate up to 5 g of dietary copper sulfate per day - a value considered to be higher than the daily needs (Tokarnia et al. 2010). However, when the storage capacity of the hepatocytes is exceeded, the free micromineral becomes toxic because of its potential oxidative effect (García-Vaquero et al. 2012, López-Alonso et al. 2017), and it causes hepatocellular degeneration and necrosis, hemolysis, and hemoglobinuric nephrosis (Cullen & Stalker 2016). The induction of these lesions is classified as acute when a large amount of copper is exposed in a short period, and this form is associated with cupric parenteral supplementation in calves (Mylrea & Byrne 1974, Sullivan et al. 1991, Hamar et al. 1997). In contrast, the chronic form is related to constant excessive mineral supplementation (López-Alonso et al. 2000, Hunter et al. 2013, Newcomer et al. 2016). After a subclinical period (weeks to months), cattle present acute manifestations that range from characteristic clinical signs, such as hemoglobinuria, jaundice, mild diarrhea and oliguria, to nonspecific signs such as apathy, dehydration, and weight loss (Minervino et al. 2009, Johnston et al. 2014).

Despite the intense livestock activity in Brazil (IBGE 2017), analysis of surveys addressing the causes of bovine mortality in different regions of the country from 1964 to 2014 showed no reports of deaths due to excessive copper supplementation in cattle (Lucena et al. 2010, Souza et al. 2015, Rondelli et al. 2017), except for one description by Tokarnia et al. (2000). Conversely, spontaneous cases of copper poisoning have been reported in Europe and Oceania in intensive dairy and beef cattle breeding, which suggests that confined cattle that receive nutritional supplementation with copper are part of a category susceptible to poisoning (López-Alonso et al. 2000, Bidewell et al. 2012, Hunter et al. 2013, Johnston et al. 2014, Kendall et al. 2015). This study aimed to report an outbreak of chronic poisoning caused by excessive copper supplementation in semi-confined cattle in the state of Mato Grosso, Brazil.

MATERIALS AND METHODS

Epidemiological investigation and clinical examination. This study was carried out on a farm that raises beef cattle under semi-confinement management located in the municipality of Rondonópolis, state of Mato Grosso, Brazil. Information was obtained through interviews with the owner, veterinarian and employees of the property and during visits made by technicians from the Veterinary Pathology Laboratory of Federal University of Mato Grosso (LPV-UFMT), Cuiabá Campus, who carried out surveys on the herd and housing areas. All paddocks in the property were inspected, and a clinical inspection of the herd was also performed.

Biochemical and molecular analysis. For biochemical analyses, blood samples of ten bovines were collected through venipuncture. The samples were placed in sterile tubes without EDTA. Biochemical tests were performed using a Wiener Lab CM-200 automatic biochemistry and turbidimetry analyzer.

Three blood samples from different cattle with clinical signs were also collected into a Vacuette® 6 ml tube without additive for serum copper measurement. The samples were analyzed at the Hermes Pardini Laboratory (Belo Horizonte/MG) by flame atomic absorption spectrophotometry (in-house method).

Blood smears from 20 cattle and spleen and liver impression smears from three cattle were made during necropsy and evaluated, after rapid panoptic staining, to investigate hemiparasites (Bain 2016).

Polymerase chain reaction (PCR) was used to detect *Ehrlichia* spp. and *Babesia* spp.. The tests were carried out at the Virology and Rickettsiosis Laboratory of UFMT (LVR-UFMT), Cuiabá campus, using a BIO-RAD T100™ thermocycler, and the images of the gels were captured and processed in a photo-documentation system (ChemiDoc XRS+, Bio-Rad) (Almeida et al. 2012).

Necropsy and histopathological examination. Twenty cattle were submitted to necropsy, nine of which showed clinical signs and died spontaneously, two were euthanized, and nine were found dead. Morphological changes were noted and photographic records were taken. Fragments of the organs of these animals were collected, fixed in 10% formalin, identified in sequence from B1 to B20, and sent to the LPV-UFMT (Table 1).

Seventeen samples from cattle submitted to necropsy by the property's veterinarian are identified in sequence from B1 to B7 and B11 to B20. Samples B1 to B7 were received between May 10, 2018 and May 23, 2018. Samples B11 to B20 were from cattle that died between May 27, 2018 and July 5, 2018. Three cattle, B8, B9 and B10, presenting clinical alterations, were collected between May 24 and 26, 2018 and submitted to necropsy by the LPV_UFMT team. Fragments of the organs received were cleaved, routinely processed, embedded in paraffin, and stained with hematoxylin and eosin (HE). Sections of the liver from a bovine were subjected to the Von Kossa histological technique (calcium). Liver and kidney fragments from all 20 cattle were subjected to histochemical staining with rubeanic acid for copper detection.

Analysis of feed and pasture, chemical analysis of soil, and evaluation of levels of copper (Cu), molybdenum (Mo) and sulfur (S) in offal. Pasture and soil samples were collected in two paddocks where animal deaths occurred. These were identified and sent to "Agroanálise Laboratórios Integrados" (Cuiabá/MT) to determine copper and sulfur levels by atomic absorption spectrophotometry.

Liver and kidney fragments, conserved in 10% buffered formalin solution, were sent to the Institute of Biosciences, Ecology Center of the "Federal University of Rio Grande do Sul (UFRGS) for sulfur, copper and molybdenum determination. These samples were analyzed by Atomic Absorption/ Air-Acetylene Flame spectrophotometry and microwave-assisted nitric acid digestion (EPA 3052).

RESULTS

Epidemiological investigation and clinical signs

The property contains 700 ha of pasture formed with *Brachiaria brizantha*, Ipyporã cultivar, and *Panicum maximum*, Mombaça and Massai cultivars, divided into 40 paddocks. The deaths occurred in seven different paddocks, and four of them were fertilized with monobasic phosphate, ammonium sulfate, potassium chloride, and limestone in December 2017. In January 2018, the farm hosted 3218 cattle under semi-confinement management, with daily consumption estimated at 1.56% of the diet's live weight with a high roughage to concentrate ratio. Concentrate consumption was estimated at 8.3 kg of DM/head/day for animals with average weight of 530 kg, with observed weight gain of 1.5-1.8 kg/animal/day.

In December 2017, some cattle from different paddocks presented neurological clinical signs and died. The presumed diagnosis, without morphological confirmation, was polioencephalomalacia. After this event, the formulation of the feed used on the property was changed in order to antagonize sulfur absorption and prevent copper deficiency. At the beginning of January 2018, copper was added to the feed mix, with an increase from 20 to 50 mg/kg of DM. The levels of S, Mo, and total iron (Fe) were not significantly changed.

From January 10, 2018 to July 5, 2018, 35 Nelore cattle, with predominance of males aged 18-36 months, died on this farm. However, 15 deaths occurred before April 20, when detailed clinical inspections or collections for complementary exams of these cattle began. In January 2018, herders found six (6/15) dead cattle during paddock inspection. Two of these cattle were examined by collaborators, who noticed marked jaundice, increased gallbladder volume, and bladders with brownish to blackish content. Subsequently, the veterinarian at the property reported that nine deaths (9/15) occurred in April 2018 in different paddocks. One of the animals found dead was submitted to necropsy, which showed serous and yellowish fat (jaundice). Samples were not collected for complementary exams.

From April 20 to July 5, 2018, monitoring showed 20 deaths in the property, and the first collection for further exams occurred on May 9, 2018. Nine cattle (9/20) were found dead without any clinical changes. In the other (11/20), clinical signs were noted, ranging from apathy, reluctance to move, and distancing from the herd. Motor incoordination, loss of balance, visual impairment, diarrhea, or dry stools were also observed. Four cattle (4/11) showed jaundice, and in one animal (1/4) presented blackened urine. In May, treatment for bovine parasitic sadness (Terramicina LA®, Ganaseg®, Borgal®, and Mercepton®) was started in three cattle, and no clinical response was observed.

Table 1. Identification of samples and gross characteristics of cattle with chronic copper poisoning in the municipality of Rondonópolis/MT from April to July 2018

Bovine	Age (months)	Sex	Breed	Feed intake time (days) 50 mg copper/kg/DM	Major gross changes				
					Jaundice	Liver volume	Disclosure of the lobular pattern	Urine color	Kidney color
B1	24	M	Nelore	282	++	+	N	AC	E
B2	24	M	Nelore	198	+++	+	**	AC	-
B3	18	M	Nelore	99	+	NA	+++	AC	N
B4	24	M	Nelore	205	-	-	-	-	-
B5	24	M	Nelore	201	+++	++	N	AC	E
B6	24	M	Nelore	212	++	++	+	AC	E
B7	18	M	Nelore	106	+	NA	+++	N	P
B8	24	M	Nelore	302	+++	++	-	AC	E
B9	24	M	Nelore	216	NA	NA	N	N	P
B10	36	M	Nelore	290	+++	++	+	AC	E
B11	24	M	Nelore	110	+++	++	+	AC	E
B12	24	M	Nelore	117	NA	NA	-	-	-
B13	24	M	Nelore	232	++	NA	A	AC	E
B14	36	F	Crossbred	297	NA	NA	N	-	P
B15	24	M	Nelore	211	+++	+++	+	AC	E
B16	24	M	Nelore	208	+++	+++	++	AC	E
B17	18	M	Nelore	76	NA	++	+++	N	N
B18	24	M	Nelore	100	+	+	++	N	P
B19	36	M	Crossbred	297	+++	++	+++	CC	E
B20	24	M	Nelore	211	+++	+++	++	CC	E

M = Male, F = female, NA = no alterations, - no information; Jaundice/enlarged liver/evident lobular pattern/distended gallbladder: A = autolysis, N = normal, + mild, ++ moderate, +++ severe; Urine color: N = normal (citrus yellow), AC = brownish, CC = black (Coca-cola color); Kidney color: N = normal, P = pale, E = blackish (metallic black).

In addition, in May, given the suspicion of chronic copper poisoning, it was recommended that this micromineral be suppressed in the feed. Cu level was adjusted from 50 to 20 mg/kg of DM, and later in that month the level was reduced to 11 mg/kg of DM, keeping the S and total Fe values at 0.2 and 17 mg/kg, respectively.

Necropsy findings

The main gross changes found at necropsy are shown in Table 1. In one case (1/20), no information on gross changes was obtained because collection was carried out by the farm employees. In cases which these findings were described, gross changes were similar, with variation in intensity and/or presence of jaundice (15/19), enlarged and brownish liver (13/19) (Fig.1A), and evidence of lobular pattern (11/16) (Fig.1B). In four cases, observations were related to the gallbladders, which were all distended, with liquid to pasty to lumpy content ranging from green to brown.

Gross changes were described for kidneys and urinary bladder content in 17 cases. The kidneys were predominantly black (11/17) (Fig.1C), pale (Fig.1D) (4/17), or had a normal color (2/17). Urine color was brownish (11/17), blackish (2/17), or normal (citrus yellow) (4/17).

In the thoracic cavity of B19, petechiae and ecchymosis were observed in the parietal pleura. B20 showed multifocal, irregular, brownish, 3 cm diameter areas in the myocardium. In B10, the mucosa of the omasum and small intestine had multifocal ulcers, whereas ulcers in the abomasum and rectum were red.

Histopathological results

Nineteen of the 20 liver and kidney samples presented morphological changes, and one was excluded from the evaluation due to marked *postmortem* changes. The main histopathological alterations observed in all livers (Table 2) were zonal coagulative necrosis (15/19) and/or hepatocellular vacuolar degeneration (14/19). Centrilobular necrosis associated with congestion and hemorrhage (Fig.2A) was seen in 13 cases (13/15), two (2/13) of which occasionally had a paracentral pattern, and four (4/13) extended to the mediozonal region. Necrosis was massive in two cases (2/15). Individual hepatocyte necrosis was present in 12 cases (12/19)

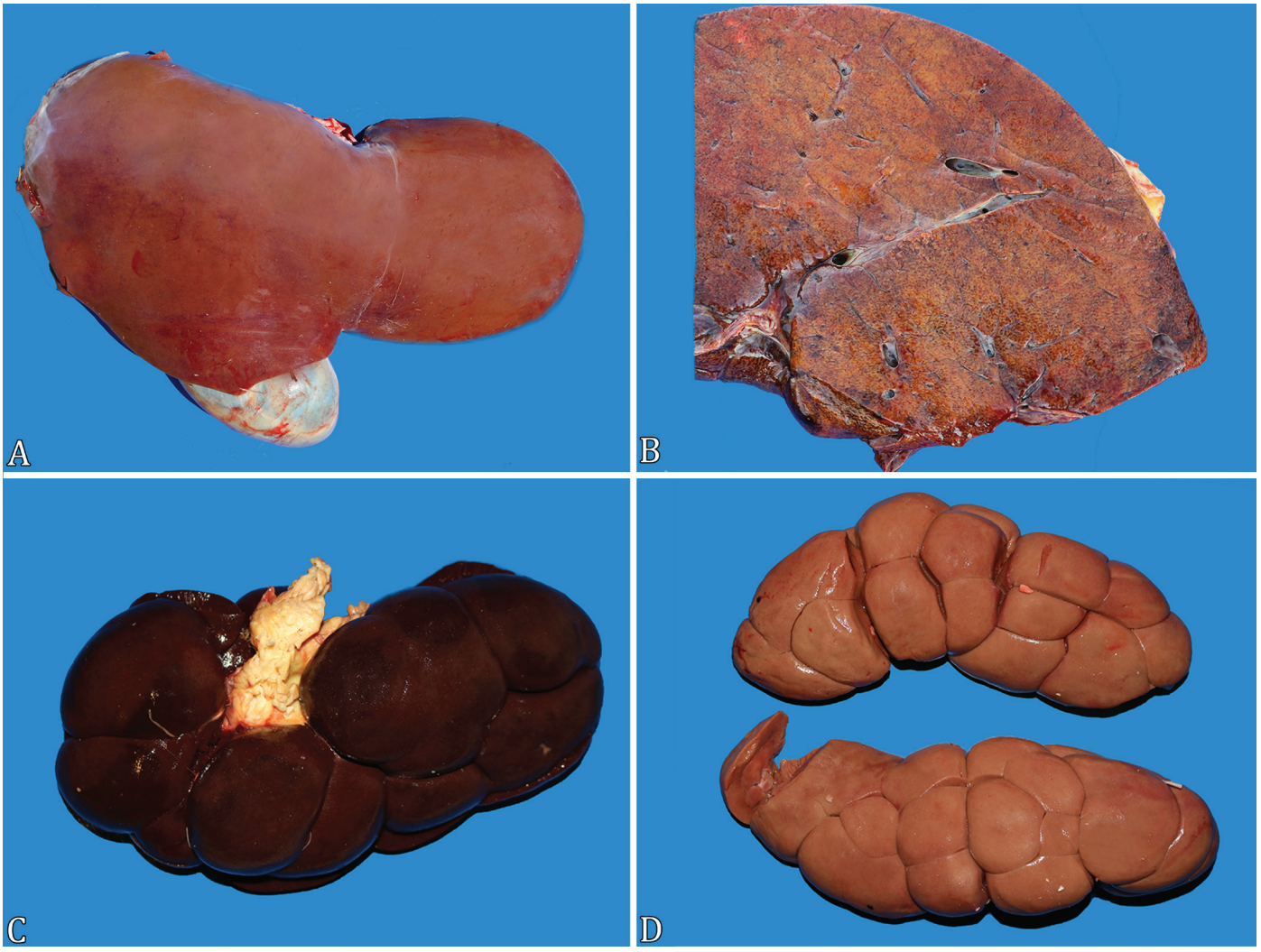


Fig.1. Gross findings in liver and kidney of cattle chronically poisoned by copper. (**A**) Liver with swelling and brownish capsular surface (13,171 µmol/kg of Cu) (B18). (**B**) On the cut surface, an evident lobular pattern (28,373 µmol/kg of Cu) is observed (B19). (**C**) Diffuse and moderately blackened kidney (13,391 µmol/kg of Cu) (B16). (**D**) Kidney with diffuse and moderate pallor (8,639 µmol/kg of Cu) (B17).

and were associated with degenerative changes and in areas adjacent to zonal necrosis (7/12), or were noted in cases where hepatocellular degeneration predominated (4/12), or only associated with the limits of areas where there was zonal necrosis (1/12).

Hepatocellular degeneration (14/19), predominantly periacinal (8/14), was classified as macrovesicular (7/8) or microvesicular (1/8). Less frequently, massive (Fig. 2B) (4/14) and centrilobular (2/14) degeneration was also observed. Proliferation of the periportal fibrous connective tissue was found in 11 samples, seven considered discrete (7/11) and four moderate (4/11). Cholestasis was present in 12 cases, six of which were mild (6/12), three moderate (3/12), and three severe (3/12). Councilman's bodies were observed in nine cases. Finally, a mild inflammatory infiltrate, adjacent to necrosis, was observed in 15 cases, 13 (13/15) neutrophilic and two (2/15) mixed.

The main renal changes (Table 3) observed in 20 cattle submitted to necropsy were coagulative necrosis and vacuolar degeneration of the tubular epithelium (Fig.2C). Tubular coagulative necrosis prevailed in nine cases (9/19), tubular degeneration was found in three cases (3/19), and they occurred with the same intensity in seven cases (7/19). Hemoglobin cylinders in the tubular lumen were verified in 14 cases (Fig.2D) (14/19). Mild to moderate glomerulonephritis was identified in nine animals. Mild to moderate interstitial inflammation was found in 11 kidneys, and interstitial fibrosis was present in four samples, three moderate and one mild.

Findings in other organs included mild vacuolization of the white matter of the brain in B2, occasional coagulative necrosis of cardiomyocytes in the myocardium in B20, and multifocal necrotizing omasitis and enterocolitis in B10.

The rubeanic acid technique was performed on all 20 samples. A positive, green to blackish, mild to moderate, intracytoplasmic marking in hepatocytes was observed in 17 samples and in the epithelium or renal tubular lumen in eight samples.

Cu, S and Mo in liver and kidney and serum Cu

Copper levels in the liver and kidneys ranged from 5,901.24 to 28,373.14 µmol/kg and from 303.72 to 14,021 µmol/kg, respectively. Of the three animals analyzed, S levels ranged from 52.55 to 217.97 µmol/kg, whereas Mo levels varied between 0.019476 and 0.45284 µmol/kg. In blood serum, Cu levels ranged from 116 to 350mcg/dL (Table 4).

Biochemical and molecular analyses

The serum activities of liver enzymes that indicate injuries, such as *Glutamic-Oxaloacetic Transaminase* (GOT) and Glutamic-Pyruvic Transaminase (GPT), were above the reference values (Table 5) in seven animals (7/7) and only GPT in six (6/7). Creatinine was measured in seven animals, with six (6/7) values overlapping the reference. In the 20 blood smears, there were no morphological structures compatible with *Babesia* spp. or *Anaplasma* spp. Serum samples from animals B10, B18, and B19 were negative for *Ehrlichia* spp. and *Babesia* spp.

Pasture and soil analyses for Cu, Mo, and S levels

Mean copper values in the pastures varied between 6.7 (*Panicum maximum* var. Mombaça) and 6.5 mg/kg (*Brachiaria brizantha*). Sulfur values averaged 1.2 mg/kg in both pastures (*Panicum maximum* var. Mombaça and *Brachiraria brizantha*). In the soil, the mean levels of Cu and S were 0.25 and 29.1 mg/dm^3, respectively.

Table 2. Histopathological characteristics of cattle livers with chronic copper poisoning

Bovine	Centrilobular necrosis	Massive necrosis	Individual necrosis	Centrilobular degeneration	Periacinal degeneration	Massive degeneration	Fibrosis	Cholestasis	Councilman body	Inflammation
B1	+++	-	-	-	++ Ma	-	-	-	+	+ N
B2	+++*	-	-	-	+Ma	-	+	+	-	+N
B3	+++	-	-		+Ma	-	-	-	-	-
B4	-	-	+	+Mi	-	-	-	-	-	-
B5	-	-	++	-	-	+++Ma	++	+	+	-
B6	++**	-	++	-	-	+++ Ma		+++	-	+N
B7	+++	-	-	-	-	-	++	-	-	+N
B8	++**	-	-	-	-	-	+	+	-	+N
B9	-	+++	-	-	-	-	-	++	-	++N
B10	++*	-	+	-	-	+++Ma	+	+++	+	+N
B11	++**	-	+	-	-	-	+	+	-	+N
B12	+++	-	+	-	+ Ma	-	-	-	+	+N
B13	A	A	A	A	A	A	A	A	A	A
B14	+++**	-	+	-	++Ma	-	+	+	-	++M
B15	-	-	+++	-	-	+++Ma	++	++	++	+N
B16	+++	-	+	-	++Ma	-	++	+++	+	+M
B17	-	+++	-	-	-	-	-	-	-	++M
B18	+++	-	+	-	++Mi	-	+	+	+	+N
B19	-	-	+	++Mi	-	-	-	-	+	-
B20	++	-	+	-	+ Ma	-	+	++	+	+N

* Centrilobular to paracentral necrosis, ** centrilobular to mediozonal necrosis; Ma macrovesicular, Mi microvesicular; N neutrophilic, M mixed; A = autolysis; + mild, ++ moderate, +++ severe.

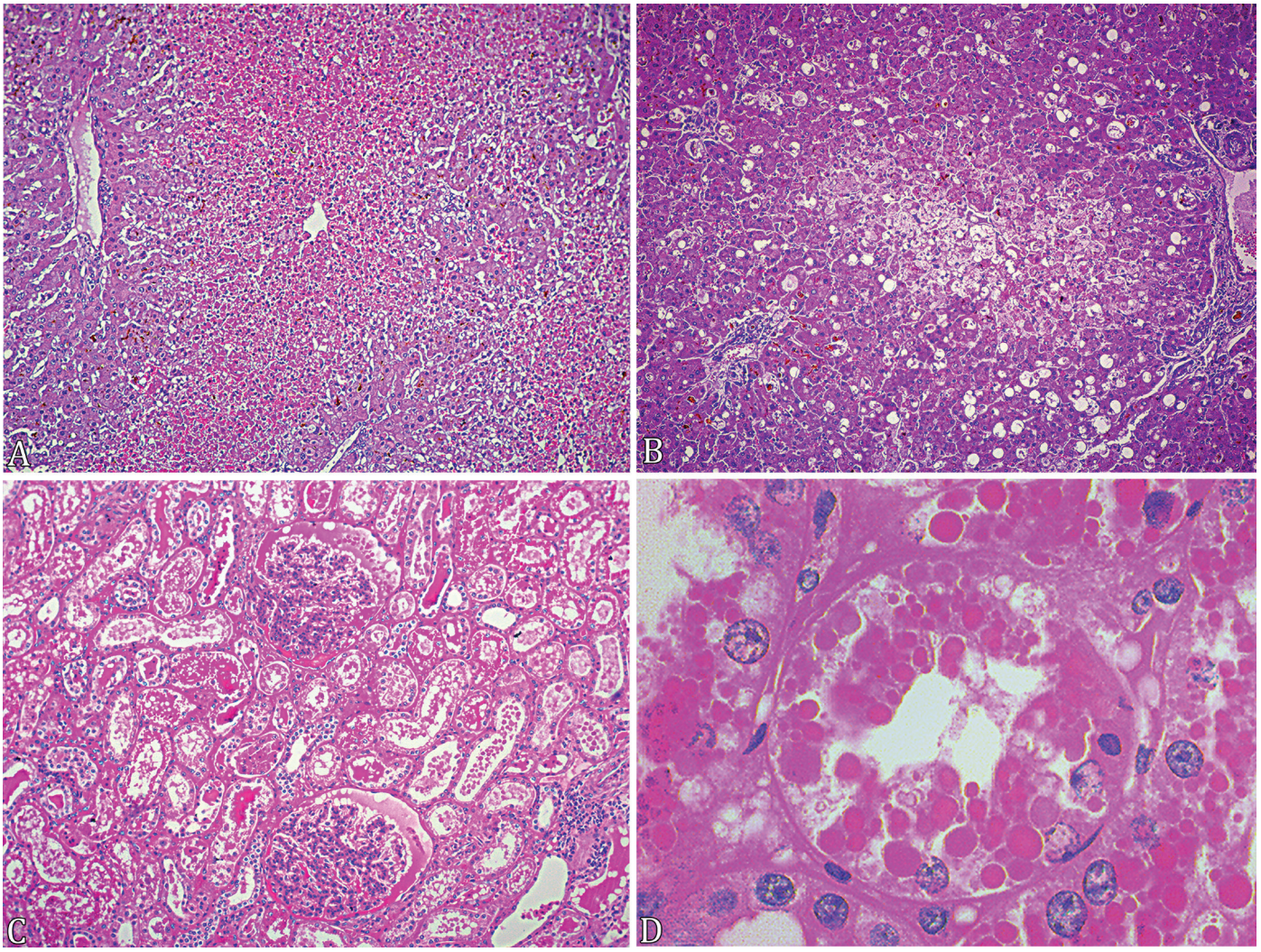


Fig.2. Histopathological findings of liver and kidney of cattle chronically poisoned by copper. (**A**) Centrilobular coagulative hepatocellular necrosis associated with congestion and hemorrhage. HE, obj.10x. (**B**) Hepatocellular, macrovesicular, massive degeneration associated with centrilobular necrosis and cholestasis. HE, obj.10x. (**C**) Kidney with multifocal areas of tubular epithelial degeneration and necrosis. In the tubular light and the Bowman space, there is amorphous and strongly eosinophilic material compatible with hemoglobin. HE, obj.10x. (**D**) There is hyaline globular material (hemoglobin) in greater magnitude in addition to cytoplasmolysis, karyorrhexis and nuclear pycnosis. HE, obj.100x.

DISCUSSION

The epidemiological, clinical and pathological aspects and the serum, hepatic and renal Cu values found in the described outbreak are consistent with chronic copper poisoning in cattle associated with excessive mineral supplementation. Copper poisoning was first investigated in Australian cattle in the 1940s (Dick & Bull 1945). From that period to the present day, cases have been reported in several countries. They are often associated with parenteral or nutritional supplementation in dairy cattle herds under intensive nutritional management (Galey et al. 1991, Bradley 1993, Steffen et al. 1997, Bidewell et al. 2012, Johnston et al. 2014). In contrast to the aforementioned description, cases of spontaneous Cu poisoning are rare in Brazil, with only one report of deaths in beef cattle after feeding with chicken litter (Tokarnia et al. 2000), and there are no reports on natural occurrence of Cu poisoning in beef cattle due to excessive supplementation.

In the present case, Cu supplementation per kg of DM was increased from 20 to 50mg/kg. Considering the last value and the daily consumption per bovine of approximately 8.3 kg of concentrate, each animal ingested an average of 415 mg Cu/day, which represents a surplus of 352 mg of Cu when considering the required value of 63.28 mg for maintenance and weight gain of male Zebu cattle (Silva et al. 2016). It is also verified that the value of 50 mg Cu/kg DM represents 20 mg Cu/kg DM in excess to the maximum limit established as safe by the European Union (EU) Commission Implementing Regulation in 2018. The EU regulation made this determination after observing that regular diets with excessive values caused poisoning (Bidewell et al. 2012, Kendall et al. 2015, Sinclair & Atkins 2015). It is believed that the gradual change of the Brazilian livestock breeding system, specifically in the state of Mato Grosso, to confinement or semi-confinement systems (IMEA 2019) allows cases of poisoning to occur due to the use of exaggerated amounts of Cu in nutrition.

Table 3. Histopathological characteristics of cattle kidneys with chronic copper poisoning

Bovine	Tubular necrosis	Tubular degeneration	Hemoglobin cylinder	Glomerulonephritis	Fibrosis	Inflammation
B1	+++	-	++	++ P	-	+ NS
B2	++	+++	+++	+MP	-	+NS
B3	++	+++	+++	++ MP	-	-
B4	-	-	-	-	++	-
B5	++	++	++	+P	-	+ M
B6	++	+	+	-	-	-
B7	++	-	-	-	-	-
B8	+++	+	++	+P	-	++NS
B9	++	+	-	++MP	++	-
B10	+	+++	+++	++P	++	-
B11	++	++	+++	+P	-	-
B12	++	+	+	++MP	-	-
B13	A	A	++	A	-	+NS
B14	++	+	++	-	-	+NS
B15	++	++	+++	-	-	+NS
B16	++	+	+++	-	-	+NS
B17	+	+	-	-	-	-
B18	+	+	-	-	-	+NS
B19	-	+	-	-	-	+NS
B20	+++	++	+++	-	+	+M

P Proliferative, MP membranoproliferative, NS nonsuppurative, M mixed; A = autolysis.

Table 4. Levels of copper, sulfur, molybdenum in the liver, kidney and serum of cattle with chronic copper poisoning

Bovine	Liver			Kidney	Blood serum
	Copper (µmol/kg)	Molybdenum (µmol/kg)	Sulfur (µmol/kg)	Copper (µmol/kg)	Copper (mcg/dL)
B2	10.606	-	-	-	-
B3	5.901	-		1.410	-
B5	10.417	-	-	-	-
B6	20.552	-		634	116
B7	18.805	-		14.021	-
B8	8.293	-	-	511	-
B9	9.536	-	-	6.168	-
B10	7.836	-		321	192
B16	13.391	0,044	217,97	303	-
B17	8.639	-	-	-	-
B18	13.171	-	-	7.427	-
B19	28.373	0,019	52,55	428	350
B20	9.992	0,045	176,38	6.341	350
Reference values	->8.000 µmol/kg*	0,191-0,383***	900-3.000****	600-800 µmol/kg **	19 -38 mcg/dl**

* Kendall et al. (2015), ** Suttle (2010), *** Underwood (1977), **** Dias et al. (2013); - unrealized.

Table 5. Serum activity of liver and kidney enzymes of cattle with chronic copper poisoning

Bovine	GGT (UI/L)	GOT (U/L)	GPT (U/L)	Creatinine (mg/dL)
B3	30.09	-	-	2.2
B6	-	633	86	-
B14	-	3166	1190	2.9
B16	-	2100	2422	26.5
B17	-	5550	1520	44.3
B18	-	182	20	1.6
B19	-	1489	40	3.2
B20	-	1150	150	6
Reference values*	≥26	45 to 110	7 to 36	0.6 to 1.8

GGT = Gamma-glutamyl transferase, GOT = glutamic-oxaloacetic transaminase, GPT = glutamic-pyruvic transaminase; * Minervino et al. (2009).

In the outbreak described in this study, the reports state that the change in feed formulation, with an increase in Cu supplementation, was made after the alleged occurrence of polioencephalomalacia assigned to S excess, and assuming that S excess could cause negative effects related to the formation of thiomolybdates (TM) in the rumen environment (Suttle 1974, Dick et al. 1975). Absorbed TM have an affinity for enzymes and other organic components that contain Cu, causing decreased enzyme activity which, consequently, can induce clinical signs of Cu deficiency. In ruminants, the signs of Cu deficiency are nonspecific or subclinical, and include a reduction in weight gain, decreased food consumption or inefficient feed conversion, altered hair pigmentation, and negative impacts on reproduction (Gould & Kendall 2011). In this occurrence, the effects related to the formation of cupro-thiomolybdates were not evaluated, and only the toxic consequences of Cu hepatocellular accumulation were observed. The antagonism that other trace elements other than S, such as Mo, Fe and zinc (Zn), have on the absorption and use of Cu is an essential factor to be considered when planning supplementation (López-Alonso et al. 2017, Sinclair et al. 2017, Skalny et al. 2019). However, the values of these microminerals were not established before changes in the Cu content were introduced in the feed.

Other less common causes of Cu poisoning in cattle include ingestion of pastures with normal Cu content and low in antagonistic microminerals (phytogenic poisoning) (López-Alonso et al. 2000) or previous liver damage (hepatogenic poisoning) (Newcomer et al. 2016). Phytogenic and hepatogenic poisoning events were ruled out due to the finding of an excessive exogenous Cu source and the absence of clinical and pathological findings that indicated previous liver damage.

The variation in the clinical course of poisoning was evident when the time of ingestion of the Cu-rich diet, the subsequent manifestation of clinical signs, or eventual death of the cattle were observed. This wide variation can be justified by the distinct individual susceptibility, which is influenced by sex, breed, and age (Littledick et al. 1995, Miranda et al. 2010, López-Alonso et al. 2017). Considering the reports of injuries made by the property's employees, it is believed that death of cattle compatible with Cu poisoning occurred 10 days after changing the feed formulation to 50 mg of copper/kg DM. Previous supplementation (20 mg copper/kg DM) possibly contributed to the hepatocellular Cu deposit and clinical manifestation of hemolysis in a shorter period after the Cu increase in the diet. However, the values of visceral Cu deposits were not measured before the onset of mortality.

Regarding the age of the bovines at the time of visceral Cu accumulation measurements, the three youngest individuals (aged 18 months) to manifest the disease were those that ingested the feed for the shortest time (76-106 days). Speed of hepatic Cu accumulation in younger animals is favored by the smaller Cu deposit in the muscles during a period of lower growth rate or when the animals are very young and receive Cu during lactation (Littledick et al. 1995, Miranda et al. 2010, López-Alonso et al. 2017). Greater breed susceptibility is observed in Holstein compared with Jersey and Angus and with Simental (Gooneratne et al. 1994, Littledick et al. 1995, Miranda et al. 2010). However, there are no studies addressing susceptibility in Nelore to establish comparison.

Of the 20 animals evaluated, nine were found dead without observation of clinical symptoms. This finding can be assigned to the type of management adopted on the farm, high stocking, and once daily inspection of herds, on average, which are facts that allow animals with shorter disease evolution to go unnoticed. This type of occurrence, added to the variation in clinical signs, demonstrate the need for *post-mortem* examinations and liver Cu measurements for diagnostic completion (Cullen & Stalker 2016). In 11 cattle, apathy, reluctance to move, and distancing from the herd were continuously observed. Although unspecific, these findings have been commonly described in cattle poisoned by copper (Galey et al. 1991, Tremblay & Baird 1991, Bradley 1993, Bidewell et al. 2012, Suttle et al. 2013). Blackened urine related to hemoglobinuria was clinically visualized in one bovine, and jaundice was observed in four cattle. These clinical signs, which are considered classic in Cu poisoning (Tokarnia et al. 2000, Minervino et al. 2009), are not always described in this disease in cattle (Galey et al. 1991, Minervino et al. 2009, Bidewell et al. 2012, Suttle et al. 2013). Minervino et al. (2009), when inducing chronic copper poisoning, obtained in most cattle, in addition to nonspecific clinical signs, a clinical condition considered atypical, such as diarrhea or dry stools, ruminal atony, and oliguria. Changes in the central nervous system, such as motor incoordination, difficulty in moving and visual impairment (Perrin et al. 1990, Bradley 1993), which were also reported in the present outbreak, have already been associated with a condition known as hepatic encephalopathy (Howell et al. 1974, Bidewell et al. 2002).

Increase in serum enzymes that indicate damage to liver or kidney function was constant in the animals evaluated in this study. The increase in transaminases such as GOT and GPT occurs mainly at the stage when hepatocellular necrosis has already been established (Minervino et al. 2009). Serum creatinine is an important marker of renal metabolism (Thrall 2014) and its elevation in these cattle is related to decreased glomerular filtration (Perrone et al. 1992) and hypoxia caused by the intravascular hemolytic crisis (Bozynski et al. 2009, Newman 2013).

Although serum Cu measurement has been considered insensitive for detection of hepatic Cu accumulation (López-Alonso et al. 2006), it can assist with diagnosing poisoning in animals that show clinical signs of the disease (Johnston et al. 2014). In the cattle assessed in this study, values higher the reference were observed in all four sera analyzed (Underwood & Suttle 1999), and in two (2/4) of these, the serum Cu level was at the upper detection limit of 350 mcg/dL.

The gross findings, characterized by jaundice and blackened urinary bladder content, are consistent with chronic copper poisoning and, in this case, were the most reported *postmortem* observations. Jaundice was the most frequent observation, and it was related to hepatocellular necrosis and hemolysis (Cullen & Stalker 2016). The visualization of blackened kidneys in 11 cases showed intravascular hemolysis, even if not clinically verified. Hemolysis occurs as a result of the direct damage that CU does to red blood cells by inducing oxidative stress in the erythrocyte membrane through inhibition of the enzyme glucose-6-phosphate (Fairbanks 1967).

Increased liver volume was a common finding related to excessive hepatocellular Cu deposit (Cullen & Stalker 2016). Associated with the event of oxidative stress, it causes

degenerative and necrotic changes (García-Vaquero et al. 2012), contributing to hepatocellular swelling and increased liver volume (Morgan et al. 2014).

Histopathological evaluation showed predominance of zonal hepatocellular necrosis, with centrilobular necrosis as the most frequent. Similar lesions have already been described in other Cu poisoning events in cattle (Minervino et al. 2009). They are related to the massive release of Cu and to the acute clinical phase of disease, when intravascular hemolysis occurs (Valli 2007). Hepatocellular degeneration has been associated with the pre-hemolytic phase of disease (Gooneratne et al. 1980). It is related to the gradual accumulation of Cu in the cytoplasm and nuclei of the hepatocytes (López-Alonso et al. 2017). It can trigger lipid peroxidation of the cell membrane, water accumulation, electrolytes, lipids and formation of autophagic vesicles capable of providing a vacuolar aspect to the cytoplasm (Gooneratne et al. 1980, García-Vaquero et al. 2012, Wang et al. 2014). In those livers, in which hepatocellular degeneration was the most important finding, individual hepatocyte necrosis occurred more frequently. These findings have already been described in other reports of Cu poisoning, but associated with the subclinical phase (Hunter et al. 2013) or the chronic phase of the disease in the absence of a hemolytic crisis (Newcomer et al. 2016). This association was not observed in the present report, since even in the only case (B19) where individual degeneration and necrosis were mild, jaundice and blackened kidneys were evident.

Tubular necrosis was the most frequently observed microscopic renal alteration. It was associated with mild intensity combined with damage to the renal tubular epithelium, caused by the oxidative potential of copper (Zhao et al. 2019). Changes are potentiated by tissue hypoxia (Newman 2013) that occurs during the hemolytic crisis and at the time when excess hemoglobin is present in the renal tubules (Gooneratne et al. 1986).

The histochemical staining of copper, from mild to moderate, did not occur only in liver samples with marked autolysis and putrefaction, but also in two cases where necrosis was massive. Intensity of copper staining in the hepatocyte cytoplasm is experimentally evident until the fifth week of excessive supplementation and decreases as poisoning progresses (Fuentealba et al. 1987). Renal copper staining in these cattle occurred in fewer cases and demonstrated less usefulness in histochemical diagnosis than liver staining (Evering et al. 1990). The smallest evidence of kidney Cu granules occurs after tubular necrosis that exfoliates cells overloaded with copper to the lumen, which may not be more evident mainly after the beginning of tubular regeneration (Haywood et al. 1985).

Regarding hepatic Cu levels, Suttle (2010) argues that, given the numerous factors that can individually influence the rate of Cu absorption, use and storage, a safety range should be used with acceptable values from 6,400 to 16,000 µmol of Cu/kg DM. According to Grace & Knowles (2015), interpretation of the term "adequate" has generated the idea that a more significant Cu nutritional supply than the necessary can provide greater productive and reproductive gains. In this expectation, excessive supplementation is recommended. However, as in this case, there are reports of deaths associated with chronic copper poisoning, in which the hepatic deposit oscillates between these reference values (Johnston et al. 2014, Morgan et al. 2014). This information reinforces that Cu rates in the liver identified as adequate may be present in cattle at risk for developing chronic poisoning. Therefore, the Animal and Plant Health Agency (APHA) of the United Kingdom established that values >8,000 µmol/g of Cu DM should be considered toxic, even in the absence of clinical signs (Kendall et al. 2015).

Levels between 600 and 800 µmol of Cu/kg DM are considered acceptable in the bovine kidney (Suttle 2010). In this case, chemical analyses of 10 samples of renal tissue revealed high values in six cases and within the normal range in four cases. Typically, urinary copper excretion is low (López-Alonso et al. 2017). Therefore, the interpretation of Cu evaluation results in this tissue should consider the dynamics between intensity of liver damage (Zischka et al. 2011) and the existing amount of the Cu carrier protein CTR1 (Han et al. 2009) in each animal (Gray et al. 2012).

CONCLUSIONS

Nutritional Cu supplementation for cattle with values from 20 to 50mg/kg of DM is potentially toxic. It can cause hepatocellular accumulation of this micromineral and, consequently, risk of degeneration and hepatocellular necrosis, hemolytic crisis, and nephrosis.

Copper poisoning should be considered a differential diagnosis mainly in confined or semi-confined cattle with jaundice and hemoglobinuria.

Conflict of interest statement.- There are no conflicts of interest.

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Glässer's disease in swine from Northeastern Brazil[1]

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ABSTRACT.- Bom H.A.S.C., Silva Filho G.B., Silva E.G., Pereira M.R., Fonseca S.M.C., Boswell R., Almeida V.M., Souza F.A.L. & Mendonça F.S. 2020. **Glässer's diseases in swine from Northeastern Brazil.** *Pesquisa Veterinária Brasileira 40(9):662-668.* Laboratório de Diagnóstico Animal, Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. E-mail: fabio.mendonca@ufrpe.br

Glässer's disease is an important infectious disorder of swine caused by *Haemophilus parasuis*. Although well recognized in most regions of Brazil, outbreaks of Glässer's disease have not been described in Northeastern region. For this reason, three municipalities of the Pernambuco State were visited in order to identify histories of high mortality in growing and finishing pigs. The main clinical signs consisted of dry cough, apathy, fever, anorexia, paresis, muscle tremors, motor incoordination, seizures leading to high mortality rates. Nine pigs were necropsied, and fragments of the nervous system, organs of the abdominal and thoracic cavities were collected for histological analysis. In addition, lung and brain fragments were used for DNA extraction and molecular testing by real-time Polymerase Chain Reaction (PCR). Grossly, the main lesions consisted of petechial hemorrhages or ecchymosis on the skin of the face, abdomen, forelimbs, and hind limbs. The main severe lesions consisted of hydropericardium, hemopericardium, fibrinous pericarditis and pleuropneumonia. Microscopically, pericarditis, epicarditis and subepicardial myocarditis, followed by a moderate to severe multifocal pleuropneumonia, fibrinosuppurative and necrotizing were the most frequent lesions observed. Real-time PCR amplified *H. parasuis inf*B gene in all samples analyzed, confirming the presence of this etiologic agent.

INDEX TERMS: Glässer's diseases, swine, Brazil, pneumonia, polyserositis, meningitis, *Haemophilus parasuis*.

RESUMO.- [**Doença de Glässer em suínos no Nordeste do Brasil.**] A doença de Glässer é uma importante enfermidade infecciosa de suínos causada pela bactéria *Haemophilus parasuis*. Embora bem reconhecida na maioria das regiões do Brasil, surtos de doença de Glässer não têm sido descritos na região Nordeste. Por este motivo, três regiões do Estado de Pernambuco foram visitadas com o objetivo de se identificar históricos de alta mortalidade em leitões e suínos em fase de terminação. Nove suínos foram necropsiados e fragmentos do sistema nervoso, órgãos das cavidades abdominal e torácica foram coletados para análise histopatológica. Além disso, fragmentos de pulmão e cérebro foram utilizados para extração de DNA e realização de teste molecular por meio da Reação em Cadeia da Polimerase (PCR) em tempo real. Os principais sinais clínicos consistiram em tosse seca, apatia, febre, anorexia, paresia, tremores musculares, incoordenação motora e convulsões levando a altas taxas de mortalidade. As lesões macroscópicas mais severas consistiam em petéquias e equimoses na pele da face, abdome, membros anteriores e posteriores, além de hidropericárdio, hemopericárdio, pericardite fibrinosa e pleuropneumonia. Microscopicamente, pericardite, epicardite e miocardite subepicárdica, seguidas de pleuropneumonia multifocal moderada a grave, fibrino-supurativa e necrosante foram as lesões mais frequentes observadas. A PCR em tempo real amplificou o gene *inf*B de

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H. parasuis em todas as amostras analisadas, confirmando a presença deste agente etiológico.

TERMOS DE INDEXAÇÃO: Doença de Glässer, suínos, Brasil, pneumonia, poliserosite, meningite, *Haemophilus parasuis*.

INTRODUCTION

Glässer's disease (GD) is an infectious disorder caused by the pleomorphic, Gram-negative bacterium of the Pasteurellaceae family, *Haemophilus* (*Glaesserella*) *parasuis* (Biberstein & White 1969, Dickerman et al. 2020), which infects members of the Suidae family, such as domestic swine and wild boars (Møller & Kilian 1990, Vengust et al. 2006, Gerveno et al. 2013). *H. parasuis* is a common inhabitant of the upper respiratory tract of conventionally raised pigs. The infection usually remains subclinical (Oliveira & Pijoan 2004) and emerge mainly in herds of piglets and juveniles pigs during stress-associated events including coinfections, moving and especially when piglets from different sources are mixed (Rafiee & Blackall 2000, Cai et al. 2005, Brockmeier et al. 2014).

GD occurs in swine populations worldwide irrespective of health status and outbreaks are economically significant because certain *H. parasuis* serovars cause high mortality rates, resulting in substantial production losses and increased costs, mainly due to the use of antibiotics (Møller et al. 1993, Nedbalcova et al. 2006). In the United States, GD is considered one of the main infectious problems in the nursery, also affecting growing pigs and sows (Holtkamp et al. 2007) and in Brazil, *H. parasuis* has been commonly isolated in swine farms at Central-Western, Southeastern and South regions of the country (Espíndola et al. 2019) being frequent, in the large, better and herds with good health status (Zanella et al. 2016).

H. parasuis strains are heterogeneous in phenotypic and genotypic traits. Fifteen variants are described and are classified according to the degree of virulence as high, moderate and non-virulent serotypes (Kielstein & Rapp-Gabrielson 1992). Most isolates of *H. parasuis* in Brazil are the virulent serotypes SV1, SV4 and SV5. But a large number of serovars consists of non-typed isolates (Espíndola et al. 2019). For this reason, the clinical picture could be variable, depending on the infecting strain. More than one serotype could be implicated in outbreaks of GD, and generally, the clinical picture is correlated with pneumonia, fibrinous polyserositis, polyarthritis and meningitis (Little 1970, Amano et al. 1994).

Clinical signs are mainly observed in 4 to 8-week-old pigs. Infected ones show high fever (41.5°C), dry cough, abdominal breathing, arthritis and nervous signs such as tremors, pedaling movements and lateral recumbency. Sporadically, GD is also observed in adult pigs. Initial diagnosis should be made by clinical and histopathological findings, however, ancillary tests are required for diagnostic confirmation and maybe performed as definitive by bacterial isolation, antibody detection, immunohistochemistry or through molecular tests such as PCR and serotyping (Nedbalcova et al. 2006, Olvera et al. 2012).

GD is still present in pig farms, and the importance of an accurate diagnosis becomes currently more importance to aiming to reduce the use of antibiotics (Aragon et al. 2019). Considering the information provided here, and the few information about the occurrence of GD in pigs at Northeastern Brazil, we aim to report the epidemiological, clinical and pathological aspects of three outbreaks of Glässer's disease in the State of Pernambuco.

MATERIALS AND METHODS

Three outbreaks of GD were accompanied from 2016 to 2018, in the state of Pernambuco, northeastern Brazil. Epidemiological, clinical and pathological data were obtained from interviews with the owners and from technical visits to those properties. Two piglets in the first outbreak, five in the second and eight in the third were separated from the herd and clinically evaluated for general condition, behavior, respiratory and cardiac rates, coordination, head posture, movement, rectal temperature and appearance of feces, urine and skin.

One swine from the first outbreak, four from the second and five from the third were necropsied and fragments of tissues from the CNS (brain, cerebellum, brainstem and medulla), thoracic cavity (lung and heart) and abdominal cavity (liver, kidney, spleen, pancreas and intestines), as well as the lymphatic system (tonsils, retropharyngeal and mesenteric lymph nodes) were collected. These fragments were fixed in 10% formalin solution were routinely processed for inclusion in paraffin blocks, cutted at 4µm and stained with hematoxylin-eosin (HE) for microscopy analysis.

Samples of lung and brain were used for DNA extraction and molecular analysis using real-time Polymerase Chain Reaction for *Haemophilus parasuis* identification. For this, approximately 1cm^3 of tissue (corresponding to 180-280mg) was diluted in sterile saline phosphate buffer (PBS, pH 7.4) and then homogenized. 200µL of DNA from the homogenized tissues were extracted using a commercial kit according to the manufacturer's protocol. A protocol of real-time Polymerase Chain Reaction previously established by Turni et al. (2009) was used. Additionally, the samples were also tested for *Mycoplasma hyopneumoniae* using the protocol previously described by Dubosson et al. (2004) and circovirus type 2 (Oh et al. 2006).

RESULTS

Three outbreaks were accompanied during two years in different regions of Pernambuco, Northeastern Brazil. The first one occurred in the municipality of Cabo de Santo Agostinho (8°17'02'' S, 35°3'54'' W), in the Coastal Region, the second one in the municipality of Paudalho (7°92'84'' S, 35°15'82'' W), at the Forest Zone and the third in the municipality of Exu (7°49'80'' S, 39°71'67'' W), at the Semiarid Region (Fig.1). All those properties were small commercial farms or raised backyard pigs (where families kept pigs at home, mostly for personal consumption and extra income). The pigs were

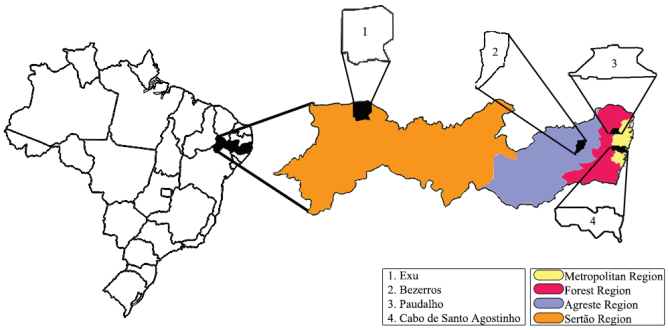


Fig.1. At the left is the map of Brazil. The state of Pernambuco and the municipalities where the outbreaks occurred are magnified.

kept in pens in groups separated by age (adults, juveniles or piglets) and category (boars, gilts, or sows), and no one of the properties had significant sanitary controls, vaccination program, or veterinary monitoring. The alimentary management were composed mainly by leftover food, and pigs also received crushed corn and commercial food intermittently.

The main history complaint obtained from the owners were similar; all of them reported tremors followed by high mortality rates, mainly in piglets aging of 2 months old on average. In the first property at municipality of Cabo de Santo Agostinho, 80 crossbred pigs were raised (2 boars, 27 sows and 51 piglets) and of these, one sow and 46 piglets died after presenting seizures, fever, and petechial hemorrhages, mainly on the skin of the ears, face, and legs.

In Paudalho, 50 crossbred Pietrain pigs were raised (3 boars, 20 sows, and 27 pigs in the growing and finishing phase). GD started after the introduction of 25 piglets from the municipality of Bezerros (8°25'72'' S, 35°74'73'' W), at the Agreste region, bought from an online classifieds' platform and without veterinary assistance. Despite noting that some of the piglets were apathetic, and coughing, no quarantine period was provided, and the owner integrated the new pigs with the present stock upon receiving them. Five days after the introduction of the new pigs, clinical signs were observed in the piglets. During the technical visit, it was observed dry cough, apathy, and fever ranging from 41°C to 42°C, muscle tremors, motor incoordination, permanent lateral recumbency, seizures and death. The piglets also remained huddled together and presented petechiae and ecchymosis on their skin. One boar presented respiratory distress and a "dog-sitting" position (Fig.2A). All those piglets (69%, 52/75), the boar and a sow died showing one or more of these clinical signs less than 12 days after the first clinical signs were observed.

In the third outbreak occurred in the municipality of Exu, 123 crossbred pigs (Landrace x Pietrain) were raised (3 boars, 20 sows, and 100 young pigs in the growing and finishing phase). From 100 young pigs, 18 (18%) presented clinical signs similar to those previously reported, and all of them died between one to seven days after the observation of the first clinical signs. Seven days later, more 20 health piglets were bought and introduced into the herd. Those pigs presented the same clinical picture and died in less than 10 days after arriving. The survivor's piglets become undeveloped, were underweight, apathetic and also presented locomotor difficulty (Fig.2B) and rough hair. The owner then decided to cull the pigs which were necropsied.



Fig.2. Clinical signs and necropsy findings of Glässer's disease. (**A**) Boar at rest in unusual "dog-sitting" position due to respiratory distress. (**B**) Swine with locomotor difficulty and dragging the clamp on the ground. (**C**) Abundant amount of fibrin covering the visceral and parietal layers of the pericardium (fibrinous pericarditis) and moderate amount of serous fluid filling the thorax cavity (hydrothorax). (**D**) Moderate amount of blood content in the abdomen and severe liver congestion.

Gross findings were similar, being variable according to the severity, and consisted of hemorrhages ranging from petechiae to ecchymosis on the skin of the face, abdomen, forelimbs and hind limbs. Five out of ten piglets (50%) had lesions associated with the heart which consisted of hydropericardium, hemopericardium and/or fibrinous pericarditis (Fig.2C). In these cases, there were also fibrinous pleuritis with adherence of the pleura and pericardial sac to the chest wall. Petechial hemorrhages in the lungs with multifocal areas of hepatization and pneumonia, mostly bilateral and accumulation of citrine fluid content, but also containing blood in the abdominal cavity were common findings (Fig.2D). The presence of other lesions was variable, but also included polyserositis characterized by fibrinopurulent exudate on serosal membranes from the abdominal cavity, mesenteric hyperemia, edema of the mesenteric lymph nodes and congestion of the liver, spleen, and kidney. In the central nervous system, mild meningeal thickening and leptomeningeal vessel congestion were noted in two necropsied piglets.

Microscopically, a moderate to severe pleuropneumonia, fibrinosuppurative and necrotizing, acute to subacute, multifocal with moderate numbers of degenerate neutrophils, fewer lymphocytes, plasma cells and macrophages were the main morphological diagnosis performed. Sometimes, hyperplasia of type II pneumocytes were also noted. Additionally, in most cases, the pleura was diffusely and moderately expanded by a mixed aggregate of inflammatory cells and fibrin (Fig.3A). Usually, the alveolar and bronchiolar lumina contained inflammatory exudate, admixed with fibrin and necrotic debris. The bronchiolar epithelial cells were multifocally necrotic, and generally, there was BALT´s hyperplasia (Fig.3B). In the heart, common lesions consisted of epicarditis and subepicardial myocarditis, mainly fibrinosuppurative and necrotizing, with infiltration of degenerated neutrophils, histiocytes and

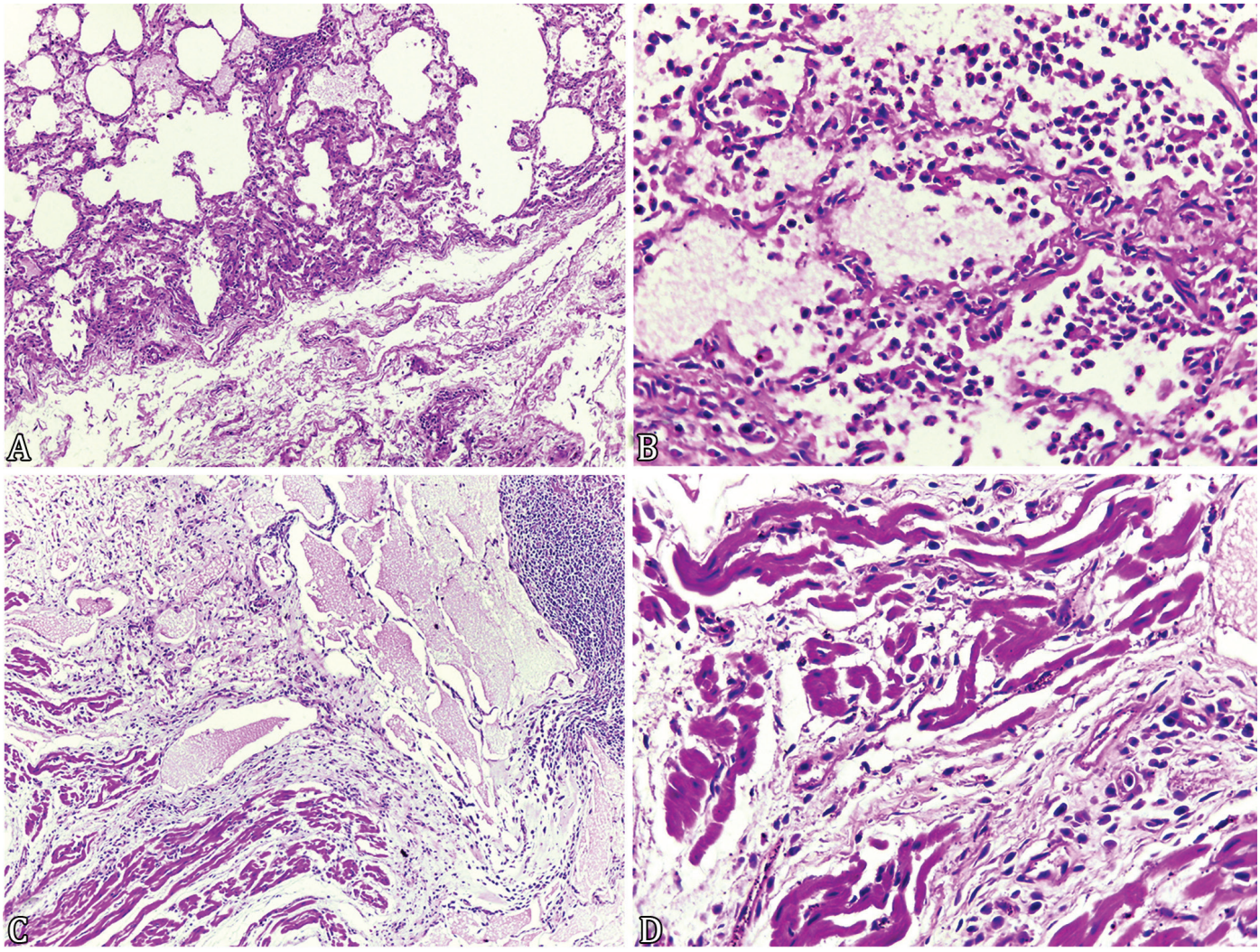


Fig.3. Histopathological aspects of Glässer's disease in swine. (**A**) Pleuropneumonia, characterized by pleural thickening with moderate presence of inflamatory fibrin-purulent infiltrate and pulmonary parenchyma with multifocal areas of intense neutrophilic infiltrate. HE, obj.10x. (**B**) Magnification of the last picture. Note in the alveolar lumen the inflammatory exudate composed by cellular debris, neutrophils, lymphocytes, and macrophages. Hyperplasia of type II pneumocytes is also observed. HE, obj.40x. (**C**) Fibrinous pericarditis. Note the high amount of fibrin and abundant inflammatory infiltration of neutrophils, histiocytes and lymphocytes replacing the subjacent cardiomyocyte layer. HE, obj.10x. (**D**) Cardiomyocytes are swollen with pyknotic nuclei and presents hypereosinophilic cytoplasm. Note the cells interspersed by fibrin and large amount of macrophages. HE, obj.40x.

lymphocytes (Fig.3C). Multifocally, subepicardial cardiac myocytes were swollen and had loss of cross striations, pyknotic nuclei and hypereosinophilic cytoplasm (Fig.3D). Aggregates of eosinophilic finely beaded to fibrillar material were also common. The heart lesions were generally moderate to severe. In the brain, a mild infiltrate of lymphocytic and plasmatic cells were observed into under the leptomeninges in two cases. In the liver, kidney, and spleen, the main lesions consisted in congestion and admixed inflammatory infiltration, mainly composed of neutrophils, lymphocytes, and plasma cells.

Real-time PCR amplified *Haemophilus parasuis inf*B gene in all samples analyzed, confirming the presence of *H. parasuis* in all samples analyzed from those three outbreaks, mainly in the lungs. In two cases, from piglets showing incoordination *H. parasuis* was identified in the brain. Additionally, in one of four samples analyzed, the genome of *Mycoplasma hyopneumoniae* was also found (Table 1).

DISCUSSION

The amplification of *inf*B of *Haemophilus parasuis* genome through the real-time PCR described herewith indicates the circulation of this bacteria in swine herds in all regions of State of Pernambuco (Coastal, Forest, Agreste, and Sertão Zones). Until to date, there were no reports concerning the molecular diagnosis and descriptions of the epidemiology and the clinical-pathological aspects of the disease in northeastern Brazil. This lack of information could be occurring because GD remains unknown or the outbreaks are far from the influence of veterinary laboratories of diagnosis. There is also a lack of information about the occurrence of GD in the North region of Brazil. However in the states of the South, Southeast and Central-Western of the country this disease is well documented (Moreno et al. 2011, Teixeira et al. 2011, Espíndola 2017).

H. parasuis strains are heterogeneous in phenotypic and genotypic traits, including virulence. Strains are classified into 15 serovars. Variants 1, 5, 10, 12, 13 and 14 are classified as high virulence; 2, 4, 8 and 15 cause polyserositis being moderately virulent and serovars 3, 6, 7, 9 and 11 are considered non-virulent (Kielstein & Rapp-Gabrielson 1992). In this study, the isolation of serovars from outbreaks of the State of Pernambuco could not be performed. However, the most common variants worldwide are 4, 5, 12 and 13 (Rapp-Gabrielson et al. 1997, Rafiee & Blackall 2000, Cai et al. 2005). And in a recent study regarding molecular serotyping of clinical strains of *H. parasuis* from outbreaks in Brazil, serovars 4, 5 and 1 were described as most important in the country, comprising 70% of the isolates. In this same study, non-typeable strains were the second most prevalent group of field strains (Espíndola et al. 2019). Considering this, additional studies in Northeastern Brazil should be made with the proposal to identify such strains. Thus, appropriate bacterin vaccines can be developed and distributed for the prevention of infection.

The clinical picture observed in the pigs from outbreaks of Pernambuco consisted mainly of petechial hemorrhages on the skin, high fever, respiratory distress, and neurological signs. In some cases, for inexperienced professionals, these clinical signs could be similar to those observed in classical swine fever and Aujeszky's disease (Brockmeier et al. 2002, Kim et al. 2002, Cai et al. 2005, Aragon et al. 2012), which are of compulsory notification to the sanitary governmental agencies. It is important to remember that the northeastern Brazil region recently had some outbreaks of classical swine fever. So, the veterinarians should be alert to the clinical and pathological differences between those diseases, mainly to make the correct decision when identifying an outbreak, because GD is not in the MAPA'S notification list (normative instruction no. 50).

The main lesions observed in GD described here, consisted in fibrinosuppurative pleuropneumonia, epicarditis and subepicardial fibrinous myocarditis. In two occasions mild meningitis was reported, and arthritis was not observed. On the other hand, although easily recognized, these typical lesions of polyserositis in nursery piglets may be caused by different bacteria (Olvera et al. 2009). For this reason, *Actinobacillus pleuropneumoniae*, *Actinobacillus suis*, *Streptococcus suis*, *Erysipelothrix rhusiopathiae*, *Pasteurella multocida* and *Mycoplasma hyorhinis* should be considered in the differential diagnosis of GD. Additionally, the occurrence of porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), and influenza virus should be investigated because those virus also cause immunosuppressive conditions and allows *H. parasuis* strains that are usually restricted to the respiratory tract to invade other systems (Brockmeier et al. 2002, Kim et al. 2002, Cai et al. 2005, Olvera et al. 2009, Aragon et al. 2012).

On those outbreaks reported in Pernambuco, *M. hyorhinis* was also identified in one sample, but considering the low sanitary *status* of the herds is reasonable to assume that other pathogens could be implicated into the onset of these outbreaks. Other ancillary exams were not performed because considering that pigs with typical clinical signs such as growth retardation and a rough coat have a poor prognosis (Nedbalcova et al. 2006) and the high mortality rate on those properties, the measure adopted was the sanitary void.

Antibiotic treatment remains an essential control measure in the face of severe outbreaks of *H. parasuis* systemic infection.

Table 1. Laboratorial real-time PCR results from swine with clinical signs of Glässer's disease in Pernambuco

Sample	Outbreak location	*H. parasuis*	Tissue	*M. hyopneumoniae*	Tissue	PCV-2*	Tissue
1	C. de Santo Agostinho	+	Lung	NP**	NP	-	Lung
2	C. de Santo Agostinho	+	Lung	NP	NP	-	Lung
3	Paudalho	+	Lung	+	Lung	NP	NP
4	Paudalho	+	Lung	-	Lung	NP	NP
5	Paudalho	+	SNC	-	NP	NP	NP
6	Exu	+	SNC	-	NP	NP	NP
7	Exu	+	Lung	-	Lung	NP	NP
8	Exu	+	Lung	-	Lung	NP	NP

*PCV-2 = Porcine circovirus type 2, **NP = not performed.

However, antibiotic susceptibility profiles are variable in different countries and reflect the election of drugs used in each region (Aragon et al. 2012). For Brazilian strains, a study showed that field isolates were highly resistant to gentamicin, bacitracin, lincomycin and tiamulin, but sensitive to ampicillin, clindamycin, neomycin, penicillin, danofloxacin and enrofloxacin. However, a susceptibility test prior to antibiotic therapy during GD outbreaks should be considered by veterinarians (Miani et al. 2017). Particularly for population-level prophylaxis, the emphasis should be on vaccination strategies to prevent systemic infection and mortality combined with good biosecurity practices (Aragon et al. 2012, Luning et al. 2015).

CONCLUSION

In this study, epidemiological data, clinical signs, macroscopic, microscopic lesions, and amplification of *inf*B gene of *Haemophilus parasuis* through real-time PCR confirms the presence of Glässer's disease in swine in the Northeast region of Brazil. Further studies should be conducted to identify the serotypes that circulate in this region.

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Conflict of interest statement.- The authors declare no conflicts of interest.

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Clinical, laboratory, ultrasonographic, and anatomopathological aspects of 30 cases of traumatic reticulosplenitis in cattle[1]

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ABSTRACT.- Silva T.V., Cajueiro J.F.P., Silva N.A.A., Souto R.J.C., Coutinho L.T., Mendonça C.L., Afonso J.A.B. & Miranda Neto E.G. 2020 **Clinical, laboratory, ultrasonographic, and anatomopathological aspects of 30 cases of traumatic reticulosplenitis in cattle.** *Pesquisa Veterinária Brasileira 40(9):669-676.* Clínica de Bovinos de Garanhuns, Universidade Federal Rural de Pernambuco, Avenida Bom Pastor s/n, Boa Vista, Garanhuns, Cx. Postal 152, Pernambuco, PE 55292-272, Brazil. E-mail: ta_ty_vitor@hotmail.com

Ingestion of metallic and/or sharp foreign bodies triggers cases of traumatic reticuloperitonitis and its sequelae in cattle. Among these sequelae, we can highlight traumatic reticulosplenitis, that has high mortality, although its frequency in the ruminant medicine is low. Therefore, based on the scarcity of information on this disease, the current study aimed to evaluate the clinical, laboratory, ultrasonographic, and pathological findings of 30 adult cattle diagnosed with traumatic reticulosplenitis. Clinical, ultrasound, and anatomopathological findings were analyzed using descriptive statistics and laboratory data were evaluated using measures of central tendency. Clinically the animals presented dehydration and alterations in behavior, appetite, and ruminal motility. Hematological findings revealed neutrophilic leukocytosis (37077.17±25004.59cell/μL) with regenerative left shift and hyperfibrinogenemia (1130±364.98mg/dL). The ultrasound examination enabled visualization of mobile and echogenic filaments that corresponded to the presence of fibrin adhesions. Displacement of the reticulum and irregularity in its contour, as well as alterations in the quantity, pattern, and amplitude of reticular contractions were also observed. Splenic alterations such as abscesses were found, characterized as circular structures of varying sizes delimited by capsules containing variable echogenicity. Splenic vein thrombosis and spleen folding were also observed. The results obtained in the current study indicated that traumatic reticulosplenitis causes nonspecific clinical signs, severe laboratory alterations and, mainly, that ultrasound is an efficient method for the diagnosis of this disease, since the anatomopathological lesions confirmed the ultrasound findings.

INDEX TERMS: Clinics, laboratory, ultrasonography, anatomopathology, traumatic reticulosplenitis, cattle, diagnostic imaging, foreign body syndrome, spleen, traumatic reticulosplenits.

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RESUMO.- [**Aspectos clínicos, laboratoriais, ultrassonográficos e anatomopatológicos de 30 casos de reticulo-esplenite traumática em bovinos.**] A ingestão de corpos estranho de origem metálica e/ou pontiagudos desencadeia em bovinos, quadros de Reticuloperitonite Traumática e suas sequelas. Dentre as quais podemos destacar a retículo esplenite traumática cuja letalidade é elevada, embora a mesma apresente uma baixa frequência na clínica de ruminantes. Portanto, baseado na escassez de informações sobre esta enfermidade, este trabalho teve por objetivo avaliar os achados clínicos, laboratoriais, ultrassonográficos

e anatomopatológicos de 30 bovinos adultos diagnosticados com retículo esplenite traumática. Os achados clínicos, ultrassonográfico e anatomopatológico foram analisados por meio de estatística descritiva, e os dados laboratoriais foram avaliados utilizando-se as medidas de tendência central. Clinicamente os animais apresentaram desidratação e alterações no comportamento, apetite e na motilidade ruminal. Os achados hematológicos revelaram leucocitose (37077.17±25004.59cell/μL) por neutrofilia com desvio à esquerda regenerativo e hiperfibrinogenemia (1130±364.98mg/dL). O exame ultrassonográfico possibilitou a visualização de filamentos móveis e ecogênicos que corresponderam à presença de aderências fibrinosas, observou-se também, deslocamento do retículo e irregularidade no seu contorno além das alterações na quantidade, padrão e amplitude das contrações reticulares. Permitiu ainda, a constatação de alterações esplênicas como abscessos que foram caracterizados como estruturas circulares de variados tamanhos delimitada por capsula contendo no seu interior conteúdo de ecogenicidade variável. Trombose da veia esplênica e dobramento do baço. Os resultados obtidos nesse trabalho, indicaram que a retículo esplenite traumática causa sinais clínicos inespecíficos, severas alterações laboratoriais e principalmente que a ultrassonografia é um método eficiente para o diagnóstico dessa enfermidade uma vez que as lesões anatomopatológicas confirmaram os achados ultrassonográficos.

TERMOS DE INDEXAÇÃO: Clínica, laboratório, ultrassonografia, anatomopatologia, bovinos, diagnóstico por imagem, síndrome do corpo estranho, baço, reticulo-esplenite traumática.

INTRODUCTION

The feeding habit of cattle combined with low sensitivity of its taste organs, nutritional deficiencies, permanence in pastures containing sharp objects and the supply of agro-industry residues as feeding in regions where food is scarce are considered the main factors responsible for ingestion of foreign bodies (Anteneh & Ramswamy 2015, Mulatu et al. 2018).

Among these, it is worth mentioning metallic and sharp objects which, when ingested, tend in most cases to stick to the reticulum mucosa where they initiate cases of traumatic reticuloperitonitis (TRP) and its sequelae (Braun et al. 2018).

These objects may remain loose in the reticulum or transfix its wall and move in various directions causing focal or diffuse peritonitis, traumatic pericarditis, diaphragmatic hernia, traumatic hepatitis and splenitis (Silva 2011, Balasundara et al. 2012, Assis 2019).

It is estimated that only 2 to 14% of animals diagnosed with TRP develop splenitis as a sequelae (Dirksen 2005). Its clinical presentation is characterized by fever, tachycardia, decreased appetite and milk production, and increased pain sensitivity to palpation of the spleen (Constable et al. 2017). However, due to the scarcity of reports of splenic disease diagnosed in live cattle, these clinical signs may be attributed to TRP.

In addition to the clinical signs above, the affected animals also present significant laboratory alterations such as expressive leukocytosis with neutrophilia, and hyperfibrinogenemia due to the formation of abscesses in the spleen (Silva et al. 2017).

Although this disease presents itself with low frequency in the ruminant clinic, its lethality is 100% and its economic losses start with the impairment of the productive life of the animal (Silva et al. 2017).

Therefore, due to the lack of information about this disease, especially regarding early diagnosis and its relevance to cattle ranching given the economic losses generated, the current study aimed to address the main clinical and laboratory findings, as well as sonographic and anatomopathological observations and to evaluate the effectiveness of ultrasonography in the diagnosis of traumatic reticulosplenitis.

MATERIALS AND METHODS

The study was conducted in the "Clínica de Bovinos de Garanhuns" (CBG) of the "Universidade Federal Rural de Pernambuco" (UFRPE) by analyzing the clinical follow-up records (including laboratory information) of the ultrasound and necropsy reports of animals attended in the period from May 2009 to May 2019 diagnosed with traumatic reticulosplenitis. Thirty purebred or crossbred dairy cattle, male and female, from dairy farms located in the Southern Agreste region of Pernambuco were studied.

All animals were clinically examined following the recommendations of Dirksen (1993). Blood samples were collected from all animals by venipuncture of the jugular, using a 21G needle, into siliconized vacutainer tubes containing EDTA anticoagulant (10%) to determine hematological variables (hemogram, total plasma protein, and plasma fibrinogen) according to the methodology proposed by Jain (1993).

Ultrasonography was performed using two Mode B devices (Logic 100 Pro, GE Medical Systems Co. Ltd., Wuxi China and Z6 Vet, Mindray Bio-Medical Eletronics Co. Ltd., Shenzhen China) and convex transducers with frequencies of 3.5MHz (Logic 100 Pro) and 5.0MHz (Z6 Vet) according to the methodology used by Braun & Götz (1994) for the reticulum examination and Braun & Sicher (2006) for the spleen evaluation.

Due to the severity of the clinical condition, some animals died or were euthanized, following the recommendations of Luna & Teixeira (2007), and submitted to anatomopathological examination.

Results obtained from the clinical, ultrasonography and anatomopathological exams were analyzed using descriptive statistics. Laboratory data were evaluated using measures of central tendency, mean, and standard deviation (Curi 1997). For evaluating the correspondence between the ultrasonographic findings and the pathological lesions, reports of 20 necropsied animals were used.

This study was approved by the Ethics Commission for Animal Use (CEUA) of UFRPE under no. 105/2018 according to the rules of the Brazilian College of Animal Experimentation (COBEA) and National Institute of Health Guide for Care and Use of Laboratory Animals.

RESULTS

Epidemiology

During the study period, 7353 cattle were treated in the CBG of the UFRPE, of which 1361 (18.50%) were diagnosed with digestive problems. Of these, 229 (16.82%) corresponded to cases of TRP and 30 (13.10%) of those presented cases of traumatic reticulosplenitis.

Of the cattle in this study, six were raised in intensive systems (20%) and 24 in semi-intensive systems (80%), and all aged between two and 15 years. Eight of the animals were Holstein, one was Girolando, and 21 were crossbred Holstein-Zebu cattle. There were two were males and 28 females,

among which six were pregnant, one was nulliparous, 12 had calved more than 100 days previously, and in nine cases the owner did not know whether the animal was pregnant.

History

The main complaints reported by the owners were that the animals had reduced appetite, weight loss and decreased milk production. In some cases, information was given on the introduction of agribusiness residues such as poultry litter and cassava bark in the animal feed.

Clinical findings

Clinically the animals exhibited alterations in appetite, varying degrees of dehydration and ruminal hipomotility. Table 1 presents the absolute (n) and relative (%) frequency of the main clinical findings of cattle with traumatic reticulosplenitis.

Laboratory findings

The results of the hematological exams demonstrated neutrophilic leukocytosis, regenerative left shift, and hyperfibrinogenemia (Table 2).

Ultrasonographic findings

The ultrasound findings in the ventral cranial region of the abdomen were characterized by the presence of heterogeneous echogenic filamentous material on the surface of the organs, suggesting fibrin and/or adhesions (Fig.1A). In addition, circular structures of varying sizes delimited by capsules with content of variable echogenicity, indicating abscesses or fibrin accumulation were also observed.

Visualization of the reticulum was possible in all cases, however, in three animals it was not clearly observed due to the presence of a large amount of inflammatory reaction. Table 3 shows the absolute and relative frequencies of the

Table 1. Absolute (n) and relative (%) frequency of clinical signs in 30 cattle with traumatic reticulosplenitis

Characteristics	Clinical findings	Absolute frequency (n)	Relative frequency (%)
Posture	Standing	27	90
	Decubitus	3	10
Appetite	Present	15	50
	Absent	6	20
	Capricious	9	30
Behavior	Calm	15	50
	Apathetic	15	50
Rectal temperature (°C)	Normal (37-39°C)	19	63.33
	Fever (>40°C)	11	36.67
Dehydration	Absent	2	6.67
	Mild (5-8%)	10	33.33
	Moderate (9-12%)	11	36.67
	Severe (>12%)	7	23.33
Heart rate	Normal (60-80)	18	60
	Low (<60)	1	3.33
	Accelerated (>80)	11	36.66
Respiratory frequency	Normal (24-36)	15	50
	Low (<24)	6	20
	Accelerated (>40)	9	30
Venous stasis	Positive	3	10
	Negative	25	83.33
	Not informed	2	6.67
Ruminal motility	Physiological	2	6.67
	Hypermotility	5	16.67
	Hypomotility	22	73.33
	Atony	1	3.33
Ruminal tympany	Present	4	13.33
	Absent	26	86.67
Ruminal stratifications	Defined extracts	20	66.67
	Undefined extracts	8	26.67
	Not informed	2	6.67
Evidence of pain	Positive (in a test)	4	13.33
	Negative	24	80
	Not informed	2	6.66
Abdominal tension	Physiological	21	70
	Increased	8	26.66
	Not informed	1	3.33

main reticulum-related ultrasound findings as well as the frequency, amplitude, and pattern of reticular contractions in cattle affected by traumatic splenitis.

The ultrasonographic examination of the splenic region revealed marked alterations in all animals. The images commonly observed in the spleen were characterized by two thin or thick hyperechoic lines delimiting a parenchyma of heterogeneous echotexture (Fig.2A). In all cases, circular and capsule-bounded structures were visualized. These alterations sometimes extended throughout the organ, while in other cases part of the splenic parenchyma maintained its normal echotexture (Fig.3A). It is worth mentioning the presence of echogenic images partially filling the lumen of the splenic vein, indicating the existence of thrombosis (Fig.4A). One case of spleen folding was characterized by the visualization of two thin and hyperechoic lines in the center of the image of the spleen (Fig.5A).

Necropsy findings

Of the 30 cattle with traumatic reticulosplenitis, 19 were euthanized and one died naturally. Thus, 20 necropsy reports were available for analysis.

The lesions found in the abdominal cavity, in all cases, were characterized by the presence of fibrin and adhesions between the organs (rumen, reticulum, diaphragm, spleen, liver) and increased peritoneal fluid. The main anatomopathological findings of the abdominal cavity, reticulum, and spleen, as well as the correspondence between these findings and the ultrasound observations of the lesions are described in Table 4 and in Figure 1-5.

As for the presence of foreign bodies, 20 metal objects with perforating characteristics (wires) were recovered from 16 animals, with lengths ranging from 4-15cm. These were free, attached to the mucosa of reticulum, rumen, and abomasum, or inside fistulas and splenic parenchyma.

Table 2. Hemogram, total plasma protein, and plasma fibrinogen of 30 cattle with traumatic reticulosplenitis

Parameters	Maximum value	Minimum value	Mean±SD	Reference[a]
Hematocrit (%)	31	9	23.10±4.51	24-46
Erythrocytes (10^6)	6.8	1.68	4.99±1.04	5-10
Hemoglobin (g/dL)	11.2	2.66	7.44±1.81	8-15
MCV (fL)	58	36.48	46.69±4.92	40-60
MCHC (%)	44	22.47	32.12±4.06	30-36
Total leukocytes (cell/µL)	123150	6900	37077.17±25004.59	4000-12000
Lymphocytes (cell/µL)	24486	1800	6022.69±4639.85	2500-7500
Neutrophils (mature) (cell/µL)	103446	4209	30137.52±22060.54	600-4000
Neutrophils (band cell) (cell/µL)	7339	0	788.52±1484.43	0-120
Eosinophils (cell/µL)	567	0	89.03±167.58	0-2400
Monocytes (cell/µL)	2735	0	353.38±541.98	25-840
Basophils (cell/µL)	253	0	25.66±69.74	0-200
TPP (g/dL)	11.6	6.3	9.33±1.19	7-8.5
PF (mg/dL)	1800	600	1130±364.98	300-700

MCV = Mean corpuscular volume, MCHC = mean corpuscular hemoglobin concentration, TPP = total plasma protein, PF = plasma fibrinogen; [a] Jain (1993).

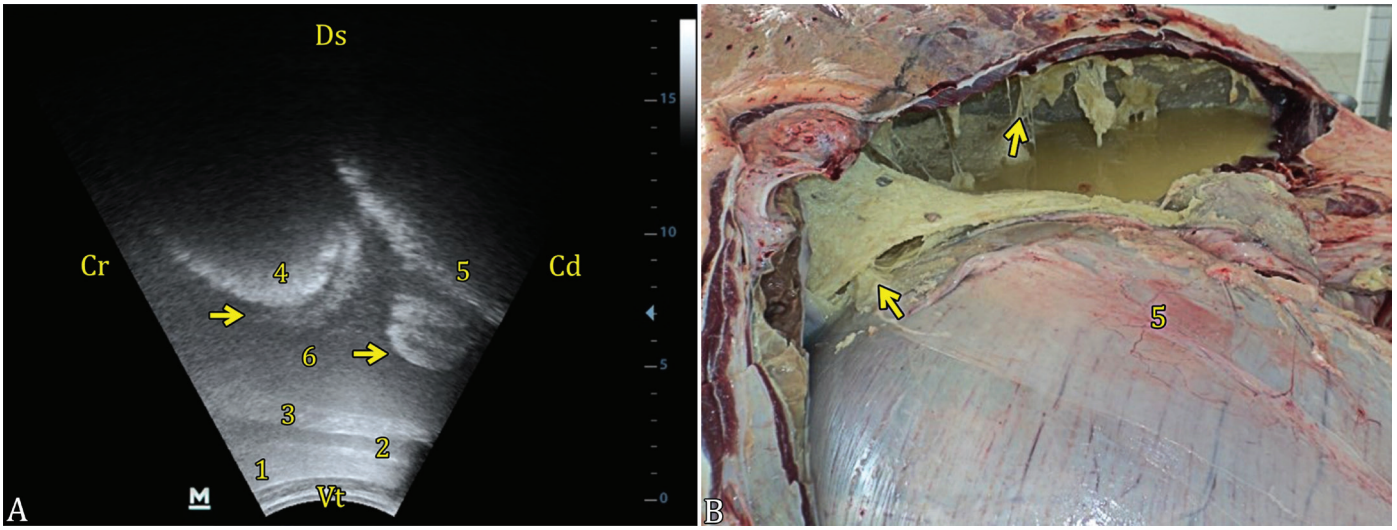


Fig.1 Traumatic reticulosplenitis in bovine. (**A**) Ultrasound image of the cranioventral abdominal region with reticulum not supported on the diaphragm, displaced dorsally by hypoechoic effusion and deposits of echogenic material, which was also adhered to the reticular and ruminal serosa. (**B**) Serofibrinous peritonitis with adhesions between the organs of the abdominal cavity (corresponding to Fig.1A). Abdominal wall (1), muscle-phrenic vein (2), diaphragm (3), reticulum (4), anterior dorsal blind sac of the rumen (5), hypoechoic effusion (6), fibrinous material (arrows), cranial (Cr), caudal (Cd), central (Vt), dorsal (Ds).

DISCUSSION

The higher occurrence of cases of traumatic reticulosplenitis in adult crossbred females observed in this study is attributed to the predominance of dairy cattle farming in the region, where the Pernambuco dairy basin is located (Silva 2011). A contributing factor to the development of this disease is the supply of feed in troughs that are highly manipulated by man in intensive and semi-intensive rearing systems, causing the accidental addition of metallic objects during the processing and/or storage stages (Fubini & Divers 2008). In the region studied, mainly on dry period season it is common to offer animals by-products from the local agro-industry, such as poultry litter and cassava bark, which in some situations may contain perforating materials that contribute to the appearance of splenitis and other sequelae of TRP (Assis 2019).

The clinical expression of this disease in pregnant and recently calved animals occurs due to the expansion of the uterus and the effort generated at calving, which causes the

Table 3. Main findings of the ultrasound examination of the reticulum of 30 cattle with traumatic reticulosplenitis

Characteristics	Ultrasound findings	Absolute frequency (n)	Relative frequency (%)
Reticular Contour	Smooth	4	13.33
	Irregular	20	66.67
	Not informed	6	20
Reticular Positioning	Supported by diaphragm	0	-
	Dorsally displaced	28	93.33
	Not informed	2	6.66
No. of contractions in 3 minutes	3-4 (normal)	7	23.33
	1-2 (reduced)	4	13.33
	5-9 (hypermotile)	1	3.33
	0 (atonic)	6	20
	Attempt at contraction[a]	10	33.33
	Not informed	2	6.66
Contraction pattern	Biphasic	7	23.33
	Triphasic	1	3.33
	Atony	6	20
	Attempt at contraction[a]	10	33.33
	Not informed	6	20
Contraction range	Normal	6	20
	Reduced	14	46.66
	Atony	6	20
	Not informed	4	13.33

[a] Contraction attempts were considered when there was a very small reticular displacement (<3cm) due to the adhesions of the organ wall.

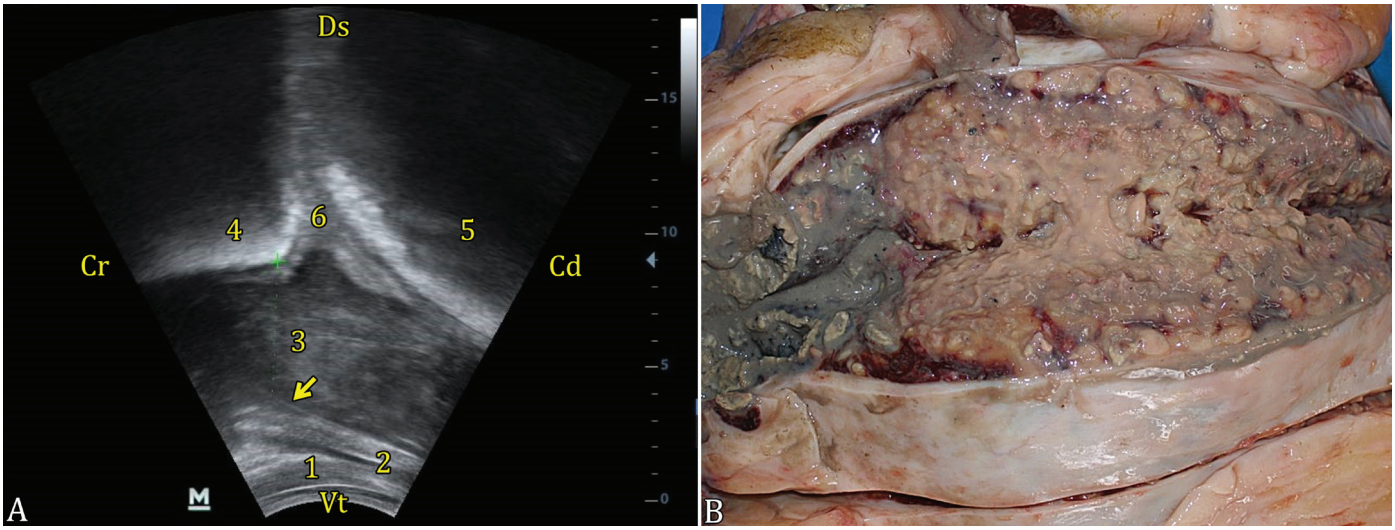


Fig.2 Traumatic reticulosplenitis in bovine. (**A**) Ultrasound image of the cranioventral abdominal region with reticulum not supported on the diaphragm, dorsally displaced by the spleen. Spleen with hypoechoic and heterogeneous parenchyma. Heterogeneous material adhered to the reticular and ruminal serosa. (**B**) Spleen with lesion (corresponding to Fig.2A). Abdominal wall (1), diaphragm (2), spleen (3), reticulum (4), anterior dorsal blind sac of the rumen (5), fibrinous material (6), splenic capsule (arrow), cranial (Cr), caudal (Cd), central (Vt), dorsal (Ds).

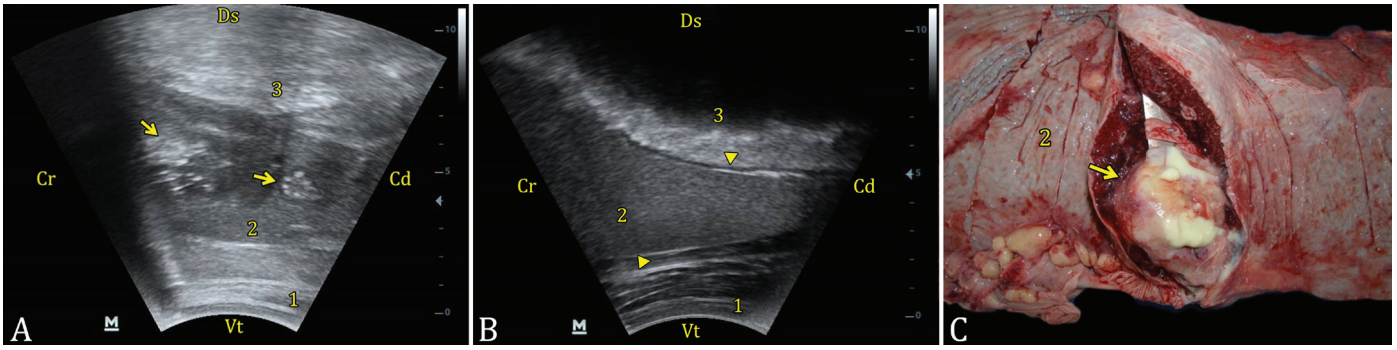


Fig.3 Traumatic reticulosplenitis in bovine. (**A**) Ultrasound image of abdominal region at eighth left intercostal space with rumen supported on the spleen whose parenchyma is heterogeneous, suggesting the presence of abscesses. (**B**) Ultrasound image of abdominal region at eighth left intercostal space with part of the spleen without alterations, with homogeneous parenchyma well delimited by a thin and hyperechoic capsule. (**C**) Spleen with a normal part and an injured part with an abscess (corresponding to Fig.3A and 3B). Abdominal wall (1), spleen (2), anterior dorsal blind sac of the rumen (3), splenic abscess (arrows), splenic capsule (arrowhead), cranial (Cr), caudal (Cd), central (Vt), dorsal (Ds).

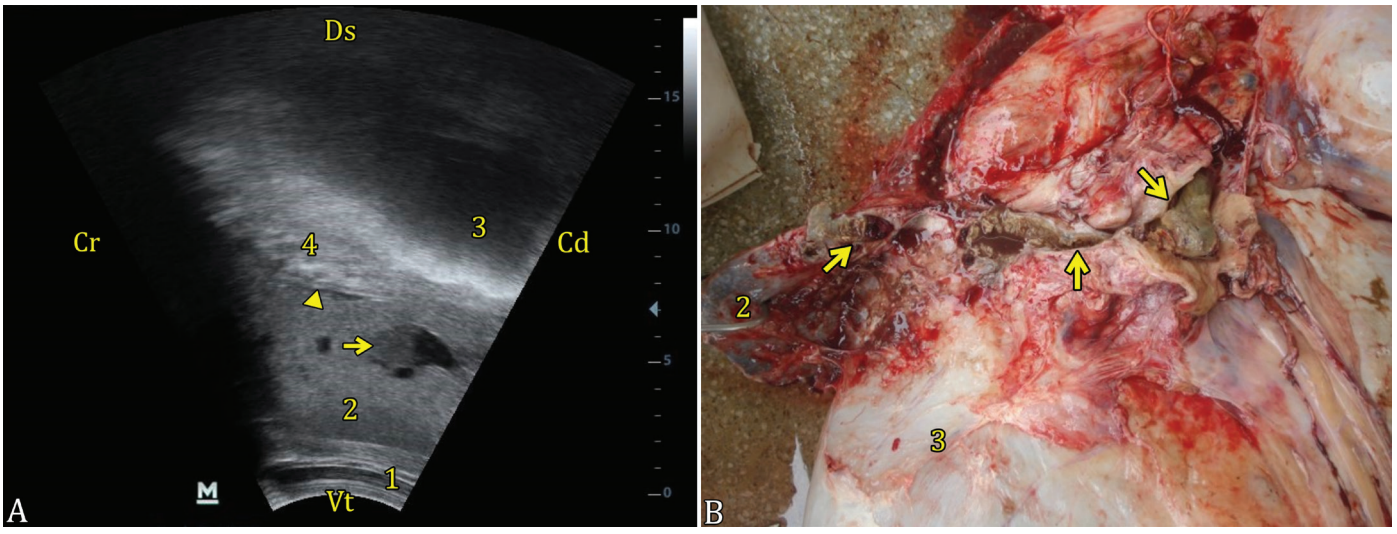


Fig.4 Traumatic reticulosplenitis in bovine. (**A**) Ultrasound image of abdominal region at eighth left intercostal space with splenic vein lumen partially filled by echogenic structure, suggesting thrombosis. Deposition of echogenic and heterogeneous material adhering the rumen serosa to the splenic capsule. (**B**) Splenic thrombosis (corresponding to Fig.4A). Abdominal wall (1), spleen (2), rumen (3), echogenic material (4), splenic vein thrombus (arrow), thickened splenic capsule (arrowhead), cranial (Cr), caudal (Cd), central (Vt), dorsal (Ds).

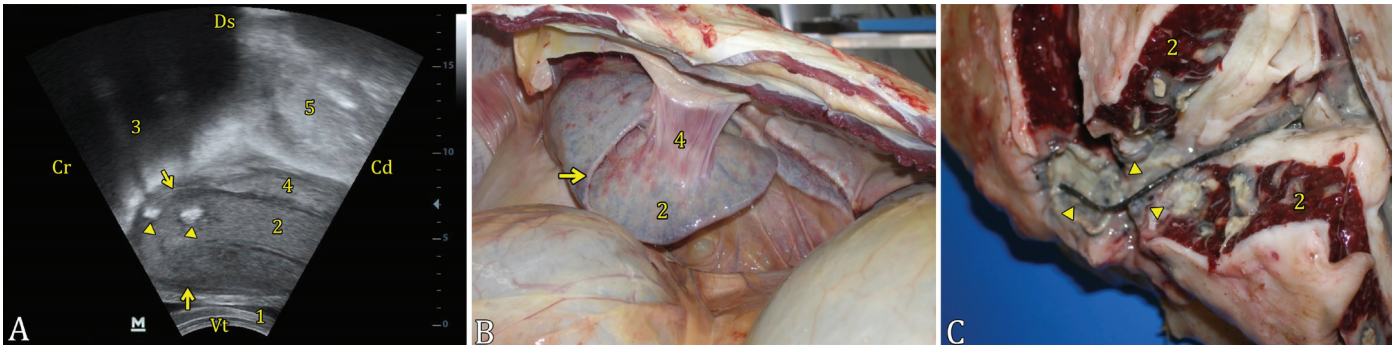


Fig.5 Traumatic reticulosplenitis in bovine. (**A**) Ultrasound image of the cranioventral abdominal region with reticulum supported on the spleen and thus displaced dorsally. Spleen folded with circular hyperechoic areas corresponding to the sites of injury by the metallic foreign body (MFB). (**B**) Spleen folded (corresponding to Fig.5A). (**C**) Abscessed splenic lesion caused by MFB (corresponding to Fig.5A). Abdominal wall (1), spleen (2), irregularly contoured reticulum (3), echogenic deposits (4), omentum (5), hyperechoic lines indicating spleen torsion (arrow), abscesses (arrowhead), cranial (Cr), caudal (Cd), central (Vt), dorsal (Ds).

uterus to exert physical pressure on the reticulum. With the existence of a metallic foreign body, perforation of this organ can occur and result in TRP sequelae (Fubini & Divers 2008, Silva 2011, Anteneh & Ramswamy 2015, Assis 2019). Clinical signs characterized by apathy, appetite alteration, varying degrees of dehydration, and ruminal hypomotility were also reported by Trecenti et al. (2015). However, due to the scarcity of reports on splenic disease diagnosed in live cattle, clinical signs resembled those observed by Assis (2019) in cases of TRP.

Regarding hematological alterations, the neutrophilic leukocytosis with regenerative left shift found in the animals of this study characterizes the severity of the inflammatory process. This condition was similar to those observed by Nuss et al. (2009) and Trecenti et al. (2015) in cattle affected by traumatic splenitis. This type of response occurs through the presence of abscesses and chronic lesions that continue to stimulate the bone marrow to produce neutrophils. On the other hand, even in prolonged inflammatory processes the acute inflammatory pattern may be present and maintain activation of neutrophil demand (Jain 1993, Weiser 2015). Hyperfibrinogenemia was also present in the current and other studies involving traumatic digestive disorders in cattle and buffaloes (Athar et al. 2010, Silva 2011, 2018, Assis 2019). Although fibrinogen is a positive acute phase protein, its concentration also remains high in chronic disease as long as liver synthesis capacity is not affected and there is an antigenic stimulus for its production (Allison 2015). The chronicity of the inflammatory process also results in an increase in total plasma protein concentration due to dehydration (Allison 2015, Braun et al. 2018, Assis 2019).

The ultrasound results demonstrated impairment in the reticular activity, which may occur due to stimuli of inhibitory mechanisms caused by pain or fever which affect the gastric center of the vagus nerve, generating a decrease in rumen-reticulum motility due to the formation of fibrin adhesions (Braun et al. 1993a). In a more recent study Braun et al. (2018) attributed this decrease in reticular motility to mechanical factors associated with peritonitis, since cattle with or without discrete lesions presented normal reticular contractions.

In addition to alterations in the motility pattern of the reticulum, due to adhesions, the organ was distanced from the diaphragm and had an irregular contour, corroborating with other authors who also observed these lesions when evaluating cattle and buffaloes affected with TRP (Braun et al. 1993a, 2018, Abdelaal et al. 2009, Khalphallah et al. 2015). This finding opposes that found in healthy animals, whose ultrasonographic aspect of the reticulum appears as a half-moon-shaped structure with a smooth contour supported by the diaphragm or ventral abdominal wall (Braun et al. 1993a, Braun 2009, Braun & Götz 1994).

The ultrasound images that defined the presence of traumatic splenitis in the animals of this study were similar to those observed by Nuss et al. (2009) and Silva et al. (2017), who reported alterations in the echogenicity pattern of the splenic parenchyma as a consequence of abscesses. These were characterized as circular structures delimited by a hyperechogenic capsule with content of variable echogenicity, similar to that described by Braun et al. (1993a). In healthy cattle, the splenic parenchyma presents a homogeneous echogenicity pattern and its capsule can be seen as a thin and echogenic line (Braun & Sicher 2006).

In addition to the alterations observed in the spleen parenchyma, the ultrasound was also efficient for identifying the splenic folding that occurred in one of the animals in this study, whose cause is not clear, but may be related to the rupture of the ligaments responsible for the spleen fixation due to the inflammatory process. This type of injury is widely reported in dogs, with the main reason being gastric dilation in this species (Ortiz et al. 2016, Gomes et al. 2017).

The majority of the ultrasound findings observed in the current study were confirmed in the *postmortem* examination of the animals, similar to that verified by Silva (2011) and Assis (2019). However, the non-visualization of adhesions by ultrasound in some cases is due to the internal location of these lesions, making it impossible to access them due to the absorption of the ultrasound waves (Braun & Götz 1994).

The inflammatory lesions of the peritoneum observed in the ventral cranial region of the abdomen indicate foreign body perforation of the reticular wall (Braun et al. 2018). However, these objects were not visualized by ultrasound, since this diagnostic method enables evaluation of the scale and location of inflammatory alterations to the peritoneum while radiography provides visualization of metallic foreign bodies (Braun et al. 1993a, 1993b, 2002, 2018, Athar et al. 2010).

The metallic foreign bodies recovered during necropsy resembled those observed by Braun et al. (2018) in radiographic surveys and by Mulatu et al. (2018) in slaughterhouse cattle. However, the oxidation process suffered by the metallic objects as well as the extent of inflammatory lesions may have contributed to the non-visualization of MFB in some of the animals, which was also observed by Braun et al. (2018).

Table 4. Correspondence between sonographic findings and anatomopathological lesions of cattle with traumatic reticulosplenitis

Lesions site	Ultrasound findings	(n)	Anatomopathological findings	(n)	Correspondence (%)
Abdominal cavity	Large quantity of anechoic content	1	Increased peritoneal fluid	1	100%
	Filamentous content of hyperechoic/hypoechoic echogenicity	11	Fibrin/adhesions between organs	17	60%
	Impaired motility	16	Adhesions	16	100%
Reticulum	Fistulas	0	Fistulas	9	0
	Presence of MFB	0	Presence of MFB	16	0
Spleen	Circular structures of varying sizes enclosed by capsules containing variable echogenicity content	20	Abscesses	20	100%
	Thin, hyperechoic lines in the center of the spleen image	1	Spleen folded	1	100%
	Echogenic image filling the lumen of the splenic vein	1	Splenic vein thrombosis	2	50%

MFB = Metallic foreign body.

CONCLUSIONS

Clinical examination alone did not allow the definitive diagnosis of traumatic reticulosplenitis since the clinical signs seen in the animals of this study resemble those observed in cattle with other sequelae of TRP. However, the complementary laboratory exams demonstrated severe alterations, which may indicate the possibility of a suppurative disease to the clinician. Laboratory findings combined with ultrasonography contributed to the establishment of definitive diagnosis and prognosis, since splenic abscesses seen through ultrasound and confirmed at necropsy ratified the significant leukocytosis of the animals.

The results of the current study indicated that ultrasonography proved to be an efficient method for the diagnosis of traumatic reticulosplenitis.

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Intention to adopt Electronic Animal Movement Permit (e-GTA) systems in Rio Grande do Sul, Brazil[1]

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ABSTRACT.- Todeschini B., Isolan L.W., Santos D.V., Maçada A.C.G. & Corbellini L.G. 2020. **Intention to adopt Electronic Animal Movement Permit (e-GTA) systems in Rio Grande do Sul, Brazil.** *Pesquisa Veterinária Brasileira 40(9):677-684.* Superintendência Federal de Agricultura no Rio Grande do Sul, Ministério da Agricultura, Pecuária e Abastecimento, Av. Loureiro da Silva 515/701, Porto Alegre, RS 90010-420, Brazil. Email: bertodeschini@gmail.com

Control of livestock and their movement has long been recognized as a crucial for the prevention and control of diseases. In Brazil, the control of livestock movement established in 1934. Since 1995 is regulated based on the Animal Movement Permit (GTA). Since then, the process has been improved and updated on its legal framework and strategies, including the use of emerging technological alternatives, which made possible the launching of the electronic Animal Movement Permit (e-GTA) in 2011. From a broader perspective, the e-GTA is inserted in a global context of the development of Information and Communication Technology (ICT) which since the early 1980s enabled governments worldwide to drive policies for the development of electronic government systems (e-government). After that, at a global level, there was an expansion and improvement of e-government services; however, discrepancies among countries persisted. Nonetheless, the levels of adoption of e-government by citizens have been lower than those expected by government authorities, which has attracted the attention of researchers in the area. In this context, studies of this nature that used theoretical antecedents related to the adoption of ICT have exposed peculiarities of the process of adoption of e-government, demanding research efforts directed to the structuring of specific models for this area. In Brazil, studies of this nature are still at an early stage, although government actions aimed at e-government date back to the 1990s. The objective of this article was to investigate, in an exploratory way, influence factors on the adoption intention of e-government related to animal health, having the e-GTA as the object of research. The conceptual framework was defined based upon the e-Government Adoption Model (GAM) For this objective, an online questionnaire oriented to intention to adopt e-GTA was applied to equine owners of Rio Grande do Sul not users of this system. Results indicated that respondents positively evaluated the quality of their computers and cellphones, as well as their efficacy to operate them, while quality of the internet connection was considered regular. In this context, the availability of computer and cellphone resources and computer self-efficacy did not influence the intention to adopt e-GTA. Moreover, aspects related to compatibility of e-GTA with users lifestyle have a positive association with the intention to adopt e-GTA. Also the study indicated that public information campaigns could contribute to the improvement of adoption of the e-GTA. Additionally, the authors suggested that further investigations considering other livestock sectors and variables, as well as the continuity of use after the adoption, could contribute to the expansion of the knowledge on this field.

INDEX TERMS: e-government, e-GTA, Animal Movement Permit, Information and Communication Technology, ICT, animal health, animal movement, public policies, Rio Grande do Sul, Brazil.

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RESUMO.- [**Intenção de adoção de Guia de Trânsito Animal Eletrônica (e-GTA) no Rio Grande do Sul, Brasil.**] O controle dos estoques e de movimentações de animais pecuários há muito tempo é reconhecido como um fator crucial para a prevenção e resposta para a introdução e disseminação de doenças animais ou zoonóticas. No Brasil, o controle de movimentação animal foi inicialmente estabelecido em 1934 e, desde 1995, é regulamentado com base na Guia de Trânsito Animal (GTA). Desde então, o processo foi aprimorado e atualizado em seu arcabouço legal e estratégias, inclusive no uso de alternativas tecnológicas emergentes, que possibilitaram o lançamento em 2011 da Guia de Trânsito Animal Eletrônica (e-GTA). Considerado desde uma perspectiva mais abrangente, o e-GTA está inserido em um contexto global de desenvolvimento da Tecnologia da Informação e Comunicação (TIC), que desde o início dos anos 80 permite que governos em todo o mundo conduzam políticas para o desenvolvimento de sistemas de governo eletrônico (governo eletrônico). Posteriormente, a disponibilidade do governo eletrônico tem estado em expansão de qualidade e de amplitude dos serviços oferecidos, embora este processo seja permeado por desigualdades entre os países. No entanto, os níveis de adoção do governo eletrônico pelos cidadãos têm sido inferiores aos esperados pelas autoridades governamentais, o que tem atraído a atenção dos pesquisadores da área. Nesse contexto, estudos dessa natureza, que utilizaram antecedentes teóricos relacionados à adoção das TIC expuseram idiossincrasias do processo de adoção do governo eletrônico, exigindo que esforços de pesquisa sejam direcionados à estruturação de modelos específicos para essa área. No Brasil, estudos dessa natureza ainda estão em estágio inicial, embora as ações governamentais direcionadas ao governo eletrônico datem dos anos 90. Dessa forma, o objetivo deste artigo foi investigar, de forma exploratória, fatores de influência na intenção de adoção do governo eletrônico relacionado à saúde animal, tendo o e-GTA como objeto de pesquisa. A estrutura conceitual foi definida com base no Modelo de Adoção de Governo Eletrônico (GAM). Para esse objetivo, um questionário online dirigido à intenção de adoção do e-GTA foi aplicado a proprietários de equinos do Rio Grande do Sul que não eram usuários deste sistema. Os resultados indicaram que os entrevistados avaliaram positivamente a qualidade de seus computadores e telefones celulares, bem como sua eficácia em operá-los, enquanto a qualidade da conexão de internet foi considerada regular. Nesse contexto, a disponibilidade de recursos informáticos e de celulares, bem como a capacidade de operação dos mesmos não influenciaram a intenção de adoção do e-GTA. Além disso, aspectos relacionados à compatibilidade do e-GTA com o estilo de vida do usuário têm uma associação positiva com a intenção de adoção do e-GTA. Além disso, o estudo indicou que as campanhas de informação pública poderiam contribuir para o incremento na adoção efetiva do e-GTA. Finalmente, o estudo identificou oportunidades para o desenvolvimento de investigações adicionais, considerando distintas variáveis, setores produtivos e sistemas de governo eletrônico dirigidos à saúde animal, o que poderia contribuir para a expansão do conhecimento nesta área.

TERMOS DE INDEXAÇÃO: Governo eletrônico, e-GTA, Guia de Trânsito Animal, Tecnologia da Informação e Comunicação, TIC, saúde animal, movimentação animal, políticas públicas, Rio Grande do Sul, Brasil.

INTRODUCTION

The control of stocks of livestock on farms and their movements has long been recognized as a crucial factor for the investigation of introduction and spread of diseases in domestic and wild animals, being even a determining factor in the creation of the World Organization for Animal Health (OIE 2017). The importance of these issues and the challenges to their implementation are widely recognized by academic institutions, governments, and non-governmental organizations dedicated to animal health (Woodford 1993, FAO 2002, Mansley et al. 2003, Fèvre et al. 2006, USDA 2014, Santos et al. 2017, Todeschini et al. 2018).

In Brazil, the control of livestock movements was initially regulated by the Decree No. 24.548/1934, which established the obligation to carry a sanitary document for the movement of animals, attesting their good health to protect national herds (MAPA 1934). For the effectiveness of movement control, further mechanisms have been established (MAPA 1977, 1986), such as the Animal Movement Permit, (GTA) implemented in 1995 (MAPA 1995), which is currently used. GTA is based on the control of cargo movements where a single GTA allows the movement of one or more animals from a same origin and moving simultaneously to a common recipient. GTA was originally conceived as a paper formulary issued by the Official Veterinary Service of each State (OVS), to which was later incorporated the legal possibility of use in electronic format, called e-GTA (MAPA 2011). Coordinated with this process, the control of holdings, producers and their of livestock has been progressively changed to digital systems, which, in some states, allow their users to consult and update their data, as well as issuing e-GTAs, as in Mato Grosso do Sul (SANIAGRO system) (IAGRO 2018), and Rio Grande do Sul (RS) (SDA system) (SEAPDR-RS 2019). In this context, presently GTAs may be issued according to three procedures: at local veterinary units of the OVS, performed by public servants and upon face-to-face producer request and issued as a paper formulary; online systems, available by state SVOs and carried out directly by the interested producers; and by accredited private veterinarians, who may issue GTA for some species and animal categories using both paper formularies or through the aforementioned online systems.

The e-GTA is inserted in a global context of development of Information and Communication Technology (ICT), which since 1980's has enabled governments to start offering online services to their citizens (Zhigang & Fengyue 2016) in a process referred as "Electronic Government", or simply "e-government" (De Róiste 2013). In Brazil, e-government initiatives were first implemented from the end of the 1990s, (Laia et al. 2011), being formally systematized by the launching of the State Electronic Government Program in 2000 (Brazil 2017). This program led to several e-government initiatives at the federal level, which presently offers around 800 services centralized in a Portal Services (Brazil 2019). Nonetheless, e-government initiatives were often misaligned with global public policies and ended by hampering their attractiveness as an alternative to be adopted by citizens (Chahin et al. 2004, Laia et al. 2011). In addition, the efforts were mainly directed to the technological structure of services, leaving a gap of understanding of the perspective of the users which finally jeopardize the adoption of e-government (Laia et al. 2011, Weerakkody et al. 2013).

Although e-government may have a positive impact on the corruption, government transparency and citizen convenience, bringing greater global efficiency to all parties (World Bank 2017), the levels of adoption of these services continue to be lower than expected (Lallmahomed et al. 2017), which after decades of investment could be considered as an "e-government paradox" (Savoldelli et al. 2014). This may be the situation of e-GTA in the RS state, as in 2016 approximately a total of 1,400,000 GTAs were issued for the movement of around 300 thousand horses, 7 million cattle, 11 million pigs and 900 million birds, and out of which less than 10% used the e-GTA system (Vergara 2017).

The ICT adoption and its continued use has interested researchers since the early 1960s, generating various theories and models, such as Diffusion of Innovation Theory - DOI (Rogers 2003), Technology Acceptance Model - TAM (Davis 1989), Task-Technology Fit (Goodhue & Thompson 1995) and Unified Theory of Acceptance and Use of Technology - UTAUT (Venkatesh et al. 2003). In general, researches on ICT adoption presupposes that potential users of a given ICT, including e-government, evaluate the potential benefits and harms that may result from the use of this technology in the final decision of adoption or rejection (Shareef et al. 2009, Al-Jabri & Roztocki 2014, Kumar et al. 2017). In this context, it must be considered that the availability and use of e-government has shown a global trend of expansion (United Nations 2016), although the adoption of these services continue at levels lower than those desired by the public managers (Savoldelli et al. 2014, Ma & Zheng 2018), resulting in a waste of taxes from society itself (Ozkan & Kanat 2011).

In a field study developed in Canada aimed at the adoption of e-government by citizens and business organizations, Shareef et al. (2011) found that most of the academic models were essentially conceptual, with a research gap on empirical studies with real users for validation and generalization of these models. Those authors tested the influence of 11 constructs on two distinct moments of adoption of e-government, reflecting the degree of interaction and exchange of information of the users with the systems. Results suggested that the adoption of e-government varies according to the maturity of their existence in a given society and led to the proposition of the e-government Adoption Model (GAM).

However, many researchers agree that there is still a lack of conceptual and empirical studies in e-government which could support the development of this area (Shareef et al. 2011, Snead et al. 2014, Alcaide-Muñoz et al. 2017, Tursunbayeva et al. 2017). Considering this aspect, the objective of this article was to investigate factors related to the adoption intention of e-government services in RS state, focusing intention to adopt e-GTA made available by the "Secretaria da Agricultura, Pecuária e Desenvolvimento Rural" of the Rio Grande do Sul State (SEAPDR-RS).

MATERIALS AND METHODS

The present study departs from the conceptual model established by Shareef et al. (2011) for the e-government Adoption Model - GAM. The decision for the alignment with the GAM was because it is specifically directed to studies related to the adoption of e-government, which is the object of the present study. In this context, it aimed to evaluate the influence of three constructs related to the initial stage of adoption of e-government in the intention to adopt animal health e-government, as declared by the respondent. The conceptual model used in the present study is presented in Figure 1, while the theoretical bases of questions addressed to each evaluated construct are presented in Table 1.

Hypothesis formulation. Several researchers on e-government have argued that the adoption of those services, being computer

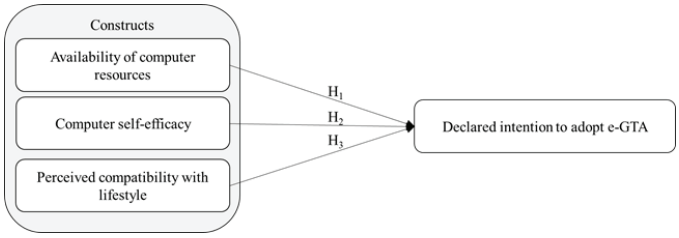


Fig.1. Conceptual model adopted in the study.

Table 1. Theoretical structure of the questionnaire

Construct	Question	Theoretical references
Perceived awareness of e-government	1. I have heard about the electronic system of GTA (e-GTA) of RS 2. I have heard of about the website of SEAPDR-RS 3. I have already visited the website of SEAPDR-RS	Anthopoulos et al. 2007, Shareef et al. 2009, Shareef et al. 2011, Lallmahomed et al. 2011.
Availability of resources	4. The internet connection of my computer is very good 5. The computer I use is very good 6. The internet connection of my cell phone is very good 7. The cell phone I use is very good	Van Dijk et al. 2008, Shareef et al. 2011, Lallmahomed et al. 2011.
Computer self-efficacy	8. I know how to use my computer and / or cell phone very well. 9. I master the use of the internet 10. I know very well how to use government systems available on the Internet (e.g. traffic authority, income and taxes, etc.)	Anthopoulos et al. 2007, Kumar et al. 2007, Shareef et al. 2009, Shareef et al. 2011.
Perceived compatibility with lifestyle	11. As far as I know, the information available on the SEAPDR-RS website about e-GTA are clear and useful 12. By the information of the enunciation, the e-GTA could be useful for me 13. By the information of the enunciation, e-GTA applies to my lifestyle	Davis 1989, Davis et al. 1989, Delone & Mclean et al. 1992, Chau & Hu 2001, Venkatesh et al. 2003, Carter & Bélanger 2005, Anthopoulos et al. 2007, Shareef et al. 2007, Wang & Liao 2008, Lallmahomed et al. 2011, Shareef et al. 2011, Al-Hujran et al. 2015, Kumar et al. 2018.
Declared adoption intention	14. I would be interested in using e-GTA	Shareef et al. 2011, Al-Hujran et al. 2015.

and internet-based, are dependent on the availability of informatics equipment and internet access, and as well as a certain degree of competence on both digital resources and internet navigation (Van Dijk et al. 2008, Shareef et al. 2011, Lallmahomed et al. 2017, Verkijika & De Wet 2018). Considering these previous researches, then we proposed hypothesis 1: the availability of computer resources positively influences the declared intention to adopt e-GTA.

Researches on ICT adoption have been proposing that the intention to adopt e-government is influenced by the ability of the user to properly perform the operational stages to go online, access, navigate on systems and achieve desired goals (Davis 1989). Persons with high computer self-efficacy are prone to have a positive perception of experiences of ICT and become users (Mensah & Mi 2019). Based on these arguments, we propose hypothesis 2: computer self-efficacy positively influences the declared intention to adopt e-GTA.

Researches on ICT adoption have demonstrated that the compatibility of the technology with the user's lifestyle is determinant on the adoption intention (Rogers 2003, Kumar et al. 2018). This construct has cultural, behavioral, and social aspects, and has been used as a predictor of e-government adoption (Carter & Bélanger 2005, Anthopoulos et al. 2007, Kumar et al. 2018). Based on this argument, we formulated the hypothesis three: perceived compatibility with lifestyle has a positive influences on the declared intention to adopt e-GTA.

Research instrument, data collection and target population. The study adopted a multi-method approach, understanding that the combination of different research methods is possible and interesting for the development of the work (Tashakkori & Teddlie 1998, Teddlie & Tashakkori 2006). In this way, it consisted of a qualitative and a quantitative phase.

In the initial qualitative phase, semi-structured interviews were conducted with four professionals with management positions in four associations of equine breeders in RS, applied in July 2017. The interview script was aimed at identifying sociodemographic, associative and habits aspects that could be relevant to be included in the subsequent quantitative phase.

Based on the results of the semi-structured interviews and on the work of Shareef et al. (2011), a preliminary questionnaire was developed using the Qualtrics® platform. The preliminary version was submitted to the evaluation of four experts, non-participating in the abovementioned interviews and selected by the authors based on their expertise in at least one of the following areas: research on ICT management; research on epidemiology applied to Official Veterinary Service; management of ICT on animal health; management of official equine health programs. Their suggestions, which were mainly related to the length of the questionnaire and the number of alternatives in sociodemographic questions, were properly addressed in the development of the final version of the closed questionnaire.

The closed questionnaire applied in the quantitative phase covered the following aspects: 1) Sociodemographic and ICT familiarity, with questions of multiple choice and selection lists; 2) Dichotomous questions (Y/N) about the awareness of the existence of e-government related to animal health in RS; 3) Availability of resources, computer self-efficacy and perceived compatibility with lifestyle, evaluated using a seven-point Likert scale, where 1= strongly disagree and 7= completely agree; and 4) Declared intention to adopt e-GTA, assessed through a seven-point Likert scale as described above.

The enunciation presented for the aspect 3) described above assumed that respondents had little or no knowledge about e-GTA, considering that this study focused on adoption intention and so did not consider those who had already adopted the system. Thus, the following text was presented as a reference for the requested responses:

> "The electronic GTA (e-GTA) is a system offered free of charge by the SEAPDR-RS which allows producers to emit their own GTA without needing to go to the Local Veterinary Unit of SEAPDR (LVU). To use the system, the producer must register at the LVU and have a computer or mobile device with internet access and printer, so it is possible to issue GTA anywhere (your house, events, etc). After issuance, the owner will be able to travel with his animal without needing to go to the LVU attend the SEAPDR-RS Veterinary Inspection, and it is necessary to carry out the same sanitary tests required for the case of GTA carried out in the province (such as glanders, equine infectious anemia, etc.). All measures for the security of the information informed are identical to those existing when a GTA is issued in the SEAPDR-RS Province."

Finally, the target population was composed by equine breeders from the state of RS that had the following characteristics: (1) were affiliated to any association of equine breeders; (2) had an active email address; and (3) were not users of the e-GTA system. In August 2017, the questionnaire was sent to the members of five different associations of equine breeders of RS by email containing a link to access the online questionnaire. A total of 387 emails were sent, being the link available for 30 days. Ninety-two replies were received (23.7% response rate), of which 21 were excluded because incompleteness, leaving 71 valid responses (77.1% of the total received). Regarding incomplete questionnaires, 16 (76.2%) were not completed in the first block of questions, while five (23.8%) were incomplete from the second block. The resulting database was analyzed using SPSS 18® software. The full questionnaire can be requested from the author, in case of interest.

Data analysis. The analysis of the questions related to sociodemographic aspects and awareness of the existence of e-Government on animal health was based on the frequency of responses, absolute and relative, which characterized the profile of respondents and their previous knowledge about the existence of these systems. The analysis of the declared intention to adopt e-GTA was based on the mean and standard deviation.

The analysis of the questions related to the construct availability of computer resources, computer self-efficacy and perceived compatibility departure from the descriptive analysis presented in Table 2. Subsequently, the reliability of the questions of each construct was analyzed using Cronbach's alpha, as shown in Table 3. The statistical adjustment of each set of questions permitted they each of these sets were grouped into three derived variables, one for each construct and whose values correspond to the arithmetic mean of their respective set of questions. The continuity of the analyses was based on these derived variables, whose descriptive statistics are presented in Table 4. The relationship between the derived variables and the declared adoption intention was measured by multiple linear regression, shown in Table 5.

RESULTS

The sociodemographic results demonstrated that most holdings (n=71 with complete responses) had only horses (57.7%), followed by those having horses and cattle or sheep (39.4%). Most holdings had up to ten horses (63.4%) of Criollo breed (85.9%) and were dedicated to both sports or leisure in general (69.7%). Regarding the owners, the majority had academic

degrees not related to agricultural areas (77.5%). They were affiliated to at least one organization of horse owners (85.9%), and had veterinary assistance only for emergencies (69.1%). Regarding animal movement, most holdings issued up to four GTA monthly (80.6%), and to move up to four horses per GTA (80.2%). None of the respondents used e-GTA and, when needed issued them either directly at SEAPDR-RS offices (88.7%) or by an accredited private veterinarian (11.3%). Regarding awareness of the existence of e-Government on animal health, most respondents (59.1%) had heard about the SEAPDR website, and 33.8% of them had already accessed it, while a minority parcel (21.1%) had heard about e-GTA.

The descriptive analysis of the declared intention to adopt e-GTA resulted in a mean (SD) of 4.56 (1.30), indicating that respondents had a positive trend to adopt this system.

Table 2. Results of the descriptive statistics of the questions related to the constructs availability of computer resources, computer self-efficacy and perceived compatibility

Question	Construct	Mean	Standard deviation
Quality computer internet connection	Availability of resources	4.58	1.46
Quality of computer	Availability of resources	5.33	0.89
Quality cellphone internet connection	Availability of resources	3.72	0.81
Quality of cellphone	Availability of resources	5.83	0.77
Computer/mobile self-efficacy	Computer self-efficacy	5.44	1.08
Internet self-efficacy	Computer self-efficacy	5.81	0.92
e-government use self-efficacy	Computer self-efficacy	4.86	0.86
Friendliness of E-GTA website	Perceived compatibility	3.22	1.07
Usefulness of e-GTA	Perceived compatibility	5.00	0.79
e-GTA *vs* lifestyle	Perceived compatibility	5.25	1.02

Table 3. Cronbach's alpha values of the questions of the constructs availability of computer resources, computer self-efficacy and perceived compatibility

Question	Construct		
	Availability of resources	Computer self-efficacy	Perceived compatibility
Quality computer internet connection	0.80		
Quality of computer	0.48		
Quality cellphone internet connection	0.48		
Quality of cellphone	0.45		
Computer/mobile self-efficacy		0.79	
Internet self-efficacy		0.69	
e-government use self-efficacy		0.77	
Friendliness of E-GTA website			0.47
Usefulness of e-GTA			0.40
e-GTA *vs* lifestyle			0.66
Cronbach's alpha	0.71	0.87	0.62

Table 4. Descriptive statistics of the variables derived from the constructs availability of computer resources, computer self-efficacy and perceived compatibility

Derived variable (construct)	Mean	Standard deviation
Availability of resources	4.86	0.75
Computer self-efficacy	5.37	0.86
Perceived compatibility	4.49	0.73

Table 5. Results of multiple linear regression having as dependent variable Y = declared adoption intention and independent variables X1 = availability of resources, X2 = computer self-efficacy and X3 = perceived compatibility

Derived variable (construct)	Coeficients		Standardized coeficients		
	B	Standard error	Beta	T	Sig.
(Constant)	0.066	1.509		0.043	
Availability of resources	-0.195	0.183	-0.113	-1.064	0.295
Computer self-efficacy	-0.225	0.170	-0.149	-1.328	0.194
Perceived compatibility	1.480	0.195	0.836	7.609	0.001

The analysis of dependent variables indicated that respondents positively evaluated the quality of their computers and cellphones, as well as their efficacy to operate them. Conversely, the quality of the internet connection of both computers and cellphone was considered regular. Regarding compatibility with lifestyle, most respondents considered the usefulness of e-GTA positively, while there is a trend to consider the friendliness of the system negatively.

Regarding the validation of the proposed hypothesis, the variable perceived compatibility presented a significance level of $p=<0.05$. Thus, based on the result obtained:

Hypothesis 1 was rejected: the availability of computer resources positively influences the declared intention to adopt e-GTA;

Hypothesis 2 was rejected: Computer self-efficacy positively influences the declared intention to adopt e-GTA;

Hypothesis 3 was validated: The perceived compatibility of e-government on animal health with lifestyle positively influences the declared intention to adopt e-GTA.

DISCUSSION

The present study aimed to explore factors that could influence the intention to adopt e-government on animal health in RS state, for which the object of research was the e-GTA in RS. Theoretical background were researches on the adoption of ICT, including seminal studies that provided the basis of current knowledge on the subject. Among these, GAM had a central role, from which the study dimension and its respective constructs were defined.

Initially, it should be noted that this is an exploratory study, and thus presents some intrinsic limitations, derived from the lack of previous studies directed to e-government on animal health public policies, and as inferential limitations arising from the sampling process. In addition, the percentage of abandonment of the questionnaire on the first and second blocks of questions (22.9%) may suggest the need to review the extension of the survey instrument.

It could be suggested future research to consider the use of multiple questionnaire delivery routes, including making them available on physical format for segments of the population not reached by electronic means. This suggestion is in line with an emerging concern in e-government research, the "digital divide" (Lin et al. 2011, Savoldelli et al. 2014, Seo & Bernsen 2016, Abu-Shanab 2017, Okunola et al. 2017), which is related to the access of rural or low-income populations to these services, and which has the been object of research, especially in developing countries.

In assessing the relationship between the three considered constructs and the declared intention to adopt e-GTA, there was a significant association between the latter and the construct "perceived compatibility". This result is in line with the theoretical foundation of the subject, considering that this construct is derived from other constructs such as perceived utility, perceived ease of use, relative advantage and expected performance, which, if verified as positive by the users, generally are positively related to adoption intention (Davis 1989, Davis et al. 1989, Delone & Mclean 1992, Chau & Hu 2001, Venkatesh et al. 2003, Carter & Bélanger 2005, Anthopoulos et al. 2007, Shareef et al. 2007, Wang & Liao 2008, Lallmahomed et al. 2011, Shareef et al. 2011, Al-Hujran et al. 2015, Kumar et al. 2018).

Regarding the awareness of e-Government and intention to adopt these systems, the present study suggested the opportunity for further studies, as while the majority of the respondents had not heard about e-GTA, most of them expressed an intention to adopt it after being presented to the enunciation containing a brief description of this system. is also aligned with reference literature, which even warns that the attention of e-government development has been excessively directed towards structural development of systems in detriment of information campaigns and digital inclusion actions (Lin et al. 2011, Weerakkody et al. 2013, Savoldelli et al. 2014, Seo & Bernsen 2016, Abu-Shanab 2017, Krishnan et al. 2017, Das et al. 2017, Okunola et al. 2017). In the present study, the fact that the majority of respondents had heard about the existence of the e-GTA, while most had not heard about the SEAPDR website, may indicate that the need of GTA is known, although the connection between the latter and its competent authority remains unclear for the users. Moreover, it may indicate that adoption campaigns could bring positive results, although further studies covering a broader spectrum of livestock producers could be necessary. Additionally, digital inclusion may enable the effective implementation of public participation on systems relevant to animal health policies, such as disease reporting systems, area on which the Brazilian government has already implemented innovative initiatives as the Brazilian Veterinary Surveillance and Emergency System - e-Sisbravet (MAPA 2020). In this context, it is important to consider the relevancy of macrostructural elements on the progress of e-government adoption. In the present study, quality of the internet connection was considered by respondents as regular, having the lowest score amongst the questions related to availability of resources, and may represent a limitation for the adoption and use of e-government. Similar consideration could also be applied to the evaluation of the friendliness of the system, as it was essentially based upon third party information rather than personal experience and may represent a message to competent authorities regarding public perception of e-government systems.

On the other hand, there was no association between the intention to adopt e-GTA and the constructs availability of resources and computer self-efficacy, which coincides with the revised literature (Kumar et al. 2007, Van Dijk et al. 2008, Shareef et al. 2011, Lallmahomed et al. 2011). It can be argued that this finding is possibly related to the fact that both constructs may refer to pre-existing capabilities rather than in attention to any specific need for e-government, which was already adopted by users to perform other activities of their interest, such as internet browsing and miscellaneous computer operations. Further studies may be necessary to appropriately investigate the relationship between these constructs and perception regarding the system, i.e., how the user's installed structural and operational capacities influence perceptions on friendliness.

Additionally, it is worth considering that the evaluation of the quality of the internet connection was unfavorable for most respondents. This aspect was an important limiting factor regarding both the adoption and continuous use of e-government (Rey-Moreno et al. 2018). It is important to consider that the quality of internet connection was embedded in a macro-environment of public infrastructure policies, and

thus the limitations found in the present study may reach multiple areas of internet-dependent e-government.

Finally, there is an opportunity for further studies on e-GTA directed to other sectors of animal production, as well as studies verifying factors influencing the continuity of use after adoption.

CONCLUSIONS

The use of e-government for animal health policies in Brazil was on early stages and presented opportunities for expansion, which was the case of e-GTA, considering both its limited current use and the clear interest of equine breeders to adopt these systems. Therefore, the use of communication strategies could contribute both in terms of the initial presentation of the systems and on what concerns the proper training of users.

The increase of the use of this system could optimize delivering of public services, as well as the possibility of developing other systems on animal health that could contribute to the implementation of public policies in this area, including those related to notification of diseases.

Moreover, the findings on the poor quality of internet connection could support public administration on the decision to implement policies aiming to improve the quality of internet services in Brazil.

Finally, the contribution of this article to the understanding of e-government adoption in Brazil, especially the ones focusing on animal health policies, was a relevant achievement, given the scarcity of researches in this area.

Conflict of interest statement.- The authors declare no conflicts of interest.

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Original Article
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High occurrence of felid alphaherpesvirus 1 and feline calicivirus in domestic cats from southern Brazil[1]

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ABSTRACT.- Becker A.S., Monteiro F.L., Scariot A.C.A., Chagas D.B., Fischer G., Lima M. & Hübner S.O. 2020. **High occurrence of felid alphaherpesvirus 1 and feline calicivirus in domestic cats from southern Brazil**. *Pesquisa Veterinária Brasileira 40(9):685-689.* Laboratório de Virologia e Imunologia, Faculdade de Veterinária, Departamento de Veterinária Preventiva, Universidade Federal de Pelotas, Campus Capão do Leão, Pelotas, RS 96010-900, Brazil. E-mail: sohubner@yahoo.com.br

Felid alphaherpesvirus 1 (FeHV-1) and feline calicivirus (FCV) affect cats worldwide. The aim of this study was to evaluate the frequency of occurrence of FeHV-1 and FCV in cats with clinical signs of respiratory, oral and/or ocular disease. Samples were collected from cats cared for in veterinary ambulatory and clinics and submitted to molecular detection and viral isolation. Of the 49 cats evaluated, 45 (92%) were positive for at least one of the viruses; 82% (40/49) were positive for FeHV-1 and 41% (20/49) for FCV. Of these, 31% (15/49) were coinfection cases. For FeHV-1, 45% (18/40) of the cats tested were positive from the collection of eye swab, and the same percentage (9/20) was obtained for the FCV by the oral swab. FeHV-1 and/or FCV were isolated in 35% (17/49) of the samples. The main clinical sign observed was ocular secretion in 71% (35/49) of cats, characterized as mild serous, purulent or serosanguineous, and in some cases associated with ocular injury and marked chemosis. Our findings demonstrate the high occurrence of FeHV-1 and FCV in domestic cats in southern Brazil and indicate that measures should be implemented to improve the diagnostic, prevention and management against of these important diseases.

INDEX TERMS: Felid alphaherpesvirus 1, feline calicivirus, domestic cats, Brazil, cat, feline respiratory disease complex, FRDC, FCV, FeHV-1.

RESUMO.- [**Alta ocorrência de alphaherpesvirus felideo e calicivirus felino em gatos domésticos no Sul do Brasil.**] Alphaherpesvírus felídeo 1 (FeHV-1) e calicivírus felino (FCV) afetam gatos mundialmente. O objetivo deste estudo foi identificar a frequência de ocorrência de FeHV-1 e FCV em gatos com sinais clínicos de doença respiratória, oral e/ou ocular. Amostras foram coletadas de gatos atendidos em ambulatório e clínicas veterinárias e submetidas à detecção molecular e isolamento viral. Dos 49 gatos avaliados, 45 (92%) foram positivos para ao menos um dos vírus; 82% (40/49) foram positivos para o FeHV-1 e 41% (20/49) para o FCV. Destes, 31% (15/49) foram casos de coinfecção. Para o FeHV-1, 45% (18/40) dos gatos foram positivos na coleta do *swab* ocular, e o mesmo percentual (9/20) foi obtido para o FCV a partir do *swab* oral. FeHV-1 e/ou FCV foram isolados em 35% (17/49) das amostras. O principal sinal clínico observado foi secreção ocular em 71% (35/49) dos gatos, caracterizada como serosa, purulenta ou serossanguinolenta e, em alguns casos, associada à lesão e quemose. Nossos resultados demonstram a alta ocorrência de FeHV-1 e FCV em gatos domésticos na região Sul do Brasil e indicam que devem ser implementadas medidas para melhorar o diagnóstico, a prevenção e o manejo contra essas importantes doenças.

TERMOS DE INDEXAÇÃO: Alphaherpesvírus felídeo, calicivírus felino, gatos domésticos, Brasil, gato, complexo respiratório felino, CRF, FCV, FeHV-1.

INTRODUCTION

Felid alphaherpesvirus 1 (FeHV-1) and feline calicivirus (FCV) are a frequent cause of diseases in the domestic feline population. Transmission occurs through nasal, ocular and oral discharges, by direct contact between cats, or even by

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fomites (Cohn 2011). Both agents are distribuited worldwide, with higher prevalence in settings where large numbers of cats coexist, including animal shelters and colonies (Radford et al. 2009, Thiry et al. 2009). FeHV-1 is a double-stranded DNA virus that belongs to the family Herpesviridae (Gaskell et al. 2007, ICTV 2018). Like other agents of the family, the significant characteristic of the virus is the establishment of latent infection, in this case occurring in the trigeminal ganglia (Gaskell et al. 2007). FeHV-1 causes clinical signs like nasal and ocular discharge, conjunctivitis and keratitis, with dendritic corneal ulcers as its pathognomonic clinical sign. Other clinical signs like pneumonia, facial dermatitis, stomatitis and meningoencephalitis are also described (Hora et al. 2013, McGregor et al. 2016, Rodriguez et al. 2016, Argenta et al. 2017, Porcelatto et al. 2018).

FCV is a single-stranded positive sense RNA virus, belonging to the family Caliciviridae (ICTV 2018). It is a non-enveloped agent, being highly resistant in the environment (Meyers et al. 1991, Radford et al. 2007, 2009). Some cats remain asymptomatic carriers after acute infection of FCV, being a source of infection for other susceptible cats (Gaskell et al. 2007). FCV is associated with different clinical patterns, including respiratory symptoms, oral ulcerations, and gingivitis/stomatitis (Afonso et al. 2017, Nakanishi et al. 2018).

Knowledge of the major viruses circulating in a region is fundamental to establish of prevention and control measures. Thus, the present study aimed at identifying the frequency of occurrence of FeHV-1 and FCV in cats from southern Brazil.

MATERIALS AND METHODS

Animals and samples. Samples were collected during January to June of 2019 from 49 cats from veterinary clinics and an ambulatory associated to the "Universidade Federal de Pelotas" (UFPel) in the city of Pelotas (Latitude: 31º46'19" S, Longitude: 52º20'33" W), located in southern Rio Grande do Sul (Brazil). Information regarding sex, age and clinical manifestations were obtained from each animal. The experiments were approved by the Institutional Committee on Animal Welfare and Ethics (UFPel - approval number 57742-2018).

The samples consisted of conjunctival, nasal and oral/oropharyngeal swabs collected from cats with clinical signs like conjunctivitis, ocular and nasal discharges, sneezing, coughing, oral ulcers and gingivitis-stomatitis complex (CGS). The swabs were immersed in 0.5mL of minimum essential medium (MEM) and immediately processed or stored in RNAlater™ Stabilization Solution (Invitrogen™, EUA) and maintained at -70°C until use. 0.2mL was used for viral isolation in cell culture and 0.15 mL for extraction of nucleic acids (DNA or RNA).

Cell culture and virus isolation. The feline kidney cell line CRFK (Crandell-Reese feline kidney) was used for isolation and amplification of the viruses. Cells were routinely maintained in MEM supplemented with 10% fetal bovine serum, containing antibiotics and antifungal (penicillin 200.000UI/L, streptomycin 200mg/L, enrofloxacin 5mg/L and amphotericin B 2.5mg/L). The swabs were subjected to vortex and the content was then transferred to tubes and centrifuged at 3.000 x g for 3 minutes. The supernatants were inoculated onto CRFK cell monolayers grown in 24-well plates and were submitted to three passages of five days each, while the cells were monitored for cytopathic effect. The strains B927 FeHV-1 and SV1425/93 FCV were used as positive controls (provided from Instituto de Pesquisas Veterinárias Desidério Finamor - IPVDF, Porto Alegre/RS and "Setor de Virologia", of the "Universidade Federal de Santa Maria", Santa Maria/RS) and cells with only MEM were used as negative control.

Nucleic acid extraction and PCR. DNA extractions were performed using ReliaPrep™ Viral Total Nucleic Acid Extraction (Promega, Wisconsin, EUA), according to the manufacturer's instructions. DNA was submitted to PCR assay for the thymidine kinase enzyme (TK) gene, using the primers Herp_F: 5'-GACGTGGTGAATTATCAGC-3' and Herp_R: 5'-CAACTAGATTTCCACCAGGA-3' (Sykes et al. 1998). The conditions were: 94°C for 5 minutes, 40 cycles of 94, 56 and 72°C for 45, 30 and 45 seconds, respectively, and 72°C for 7 minutes.

RNA extraction was performed using TRIzol® Reagent, according to the manufacturer's instructions. After RNA extraction, complementary DNA (cDNA) was synthesized using iScript™ cDNA Synthesis (Bio-Rad). Briefly, total RNA (~500ng) was mixed with 5x Reaction Mix and 1U Reverse Transcriptase. The samples were incubated for 5 minutes at 25°C, followed by incubation at 46°C for 20 minutes and 95°C for 1 minute. The synthesized cDNAs were submitted to Nested PCR assay for A-B conserved region of ORF2 (capsid protein gene), using the primers Cali1: 5'-AACCTGCGCTAACGTGCTTA-3', Cali2: 5'- CAGTGACAATACACCCAGAAG -3', and at second round, Cali3: 5'- TGGTGATGATGAATGGGCATC -3' and Cali4: 5'- ACACCAGAGCCAGAGATAGA-3' (Marsilio et al. 2005). The conditions were: 94°C for 3 minutes, 35 cycles of 94, 50 and 72°C for 1 minute, 45 seconds and 1 minute, respectively, and 72°C for 7 minutes.

The PCR reaction was performed in a 25µL volume, using 100-200ng of DNA or cDNA template, 1x GoTaq® Colorless Master Mix (Fitchburg, Wisconsin, EUA) and 0.4µM of each primer. RNA extracted from the CRFK was used as negative control and strains B927 and SV1425/93 were used as control of FeHV-1 and FCV, respectively. PCR products of FeHV-1 and RT-PCR of FCV were resolved in a 1.5% agarose gel stained (Blue Green Loading Dye I, LGC Biotecnologia, São Paulo, BR) and visualized under ultraviolet (UV) light after electrophoresis (100V, 40 minutes).

Sequencing analysis. PCR products were purified using PureLink® Quick Gel Extraction and PCR Purification Combo Kit (Life Technologies, CA, USA). Positive samples were sequenced in duplicate using the BigDye kit. Results were analyzed by the Staden program (Staden 1996) to obtain a consensus sequence, and the alignment of sequences with those from GenBank and identity matrix was performed using the BioEdit Sequence Alignment Editor Software suite 7.0.5.3. The translation of a nucleotide sequence to a protein sequence was performed using the ExPASy Bioinformatics[3].

RESULTS AND DISCUSSION

Samples of eye, oral/oropharyngeal and nasal swabs were obtained from 49 symptomatic cats located in the South of Brazil, during the period of January to June of 2019. According to age, 20 animals were classified as kittens (<1 year), nine as young (1-2 years), 15 as adults (3-7 years) and five as elders (≥8 years), and consisted of 32 males and 17 females. Regarding vaccination status, only 4% (2/49) were vaccinated, 71% (35/49) were not vaccinated and 24% (12/49) had unknown vaccination status. Besides, 67% (33/49) of the animals had outdoor access.

All samples collected were evaluated by PCR for FeHV-1 and nested RT-PCR for FCV. FeHV-1 amplified product in PCR resulted in a fragment of 287bp. FCV amplified product in

[3] Available at <https://web.expasy.org/translate/>

Nested RT-PCR resulted in a 924bp fragment in first round, and a 467bp fragment in the second round. Of the 49 cats, 45 (92%) were positive for at least one of the viruses. Among the positive animals, were 100% (20/20) of the kittens, 100% (9/9) of the young ones, 73.3% (11/15) of the adults and 100% (5/5) of the elders. Besides, 88.5% (31/35) of the not vaccinated group were positive, 100% (2/2) of the cats with no historical of previous vaccination and 100% (12/12) of the cats with unknown vaccination status. Among the animals without outdoor access, 75% (3/4) were positive, along with 100% (33/33) of the cats with outdoor access and 75% (9/12) of the animals where this information was not available.

For FeHV-1, 82% (40/49) were positive, and for FCV, 41% (20/49) of positivity was obtained. Thirty one percent (15/49) were cases of coinfection. These results demonstrate a high occurrence of these viruses in the studied population, especially regarding FeHV-1. Besides, these data are higher than what is described in Germany (Schulz et al. 2015), Spain (Fernandez et al. 2017), and Australia (Nguyen et al. 2018), where molecular detection studies involving symptomatic animals were also conducted, showing wide variations in the detection of these agents according to different geographic regions. The rate of occurrence detected in our study indicates the high circulation of these viruses in this region, which could be explained by the outdoor lifestyle and the unvaccinated status of the majority of the animals. Vaccines for FeHV-1 and FCV are essential for all cats (Day et al. 2016) and considered effective in reducing clinical signs and viral excretion, although they are not capable to completely avoid infection and transmission of the agents (Radford et al. 2009, Thiry et al. 2009). In addition, protection by vaccination against FCV may be affected by the high variability of existing strains (Radford et al. 2009). Thus, even vaccinated animals can be infected and act as carriers, spreading these agents.

In Southern Brazil, previous serological studies detected FCV and FeHV-1 in the feline population, both in vaccinated and non-vaccinated animals (Johann et al. 2009, Henzel et al. 2013). During the years from 2006 to 2009, a study was conducted to identify feline calicivirus and feline herpesvirus in southern Brazil, evaluating both symptomatic and asymptomatic cats, which makes it difficult to compare their results to the ones found in the present study. This same study found 38.2% (21/55) of positivity for FeHV-1 and 52.7% (29/55) for FCV, with co-infection in 9.1% (5/55) (Henzel et al. 2012).

Our data show a higher occurrence of FeHV-1 compared to FCV. This result can be partly explained by FeHV-1 pathogenesis. FeHV-1 remains latent in infected animals, and eventually, mainly in cases of immunosuppression or stressful conditions, returns to the lytic cycle and is reexcreted in the primary sites of infection. Possibly, the FeHV-1 detected in these cats was the cause of acute clinical signs due primary infection, but also, especially in adult cats, FeHV-1 developed the disease at the time of viral reactivation. The disease as a consequence of virus recrudescence may be consequence of immunosuppression caused by other factors or diseases that were not investigated. FCV, on the other hand, can be detected in healthy animals due to the carrier state that occurs in some animals. In these cases, the virus remains replicating and being eliminated through secretions, although no apparent symptomatology is identified (Radford et al. 2009). Anyway, the data clearly show high circulation and occurrence of FeHV-1 and FCV in the studied population.

The virus was isolated in 35% (17/49) of the cats, being 47% (8/17) from oral swabs, 29% (5/17) ocular swab, 18% (3/17) from the eye and oral swab, and 6% (1/17) from the oral and nasal swab. Figure 1 shows the FeHV-1 and FCV molecular detection according to the place of swab collection (oral/oropharyngeal, ocular or nasal regions). For FeHV-1, 45% (18/40) of the cats were positive from the collection of eye swab, and the same percentage (9/20) was obtained for the FCV from the oral swab. In 27.5% (11/40) of animals positive for FeHV-1 and 30% (6/20) positive for FCV, virus detection was possible from both oral and eye swab. These results corroborate with previous studies, where the main FCV detection location was from the oral/oropharyngeal mucosa (Schulz et al. 2015). However, in this same study, no difference was observed among the collection sites for FeHV-1, suggesting oral/oropharyngeal swab as the sample of choice for the detection of both viruses. The results obtained in the present study highlight the importance to collect samples from more than one region, in order to increase the possibility of detecting the agents. The detection of FeHV-1 and/or FCV from nasal swabs was only possible in four animals, possibly due to the greater difficulty encountered at the time of sampling of the mucosa, a fact that was also reported by Schulz et al. (2015).

Figure 2 show the clinical signs observed in animals evaluated. Ocular secretion was observed in 71% (35/49) of cats and was characterized as mild serous, purulent or

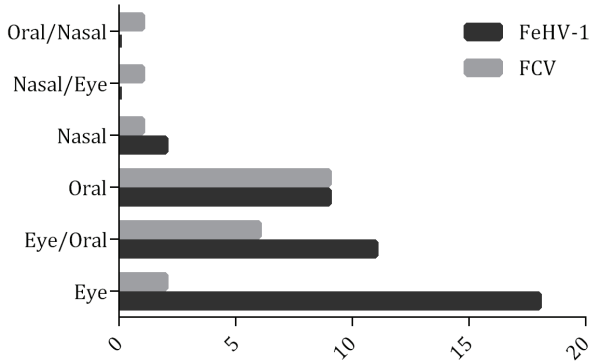


Fig.1. Felid alphaherpesvirus-1 and feline calicivirus molecular detection according to swab collection site in 49 cats from southern Brazil. Numbers refers to the number of cats that tested positive in each site.

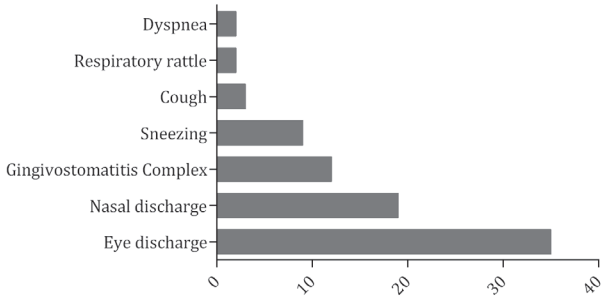


Fig.2. Clinical signs observed in 49 symptomatic cats evaluated. Numbers refers to the number of cats presenting each clinical sign.

serosanguineous, and in some cases associated with ocular injury and marked chemosis. The second most frequent clinical sign was nasal discharge in 39% (19/49) of cats, ranging from mild serous to purulent, followed by CGS in 24% (12/49), sneezing in 18% (9/49), cough in 6% (3/49), respiratory rattle in 4% (2/49) and dyspnea in 4% (2/49). The main clinical sign in FCV-positive animals was CGS, identified in 58% (7/12) of the animals. Despite CGS having multifactorial causes (Lyon 2005), it is often associated with FCV (Fernandez et al. 2017, Thomas et al. 2017, Nakanishi et al. 2018). FeHV-1, on the other hand, was detected in 50% (18/36) of animals exhibiting nasal and/or ocular secretion. To compare, Fernandez et al. (2017) detected FeHV-1 in 28% (36/127) of animals showing clinical signs related to upper respiratory tract disease and 24% (36/149) of animals with conjunctivitis. It is known that other agents may be involved in respiratory and/or ocular disease in cats, also called "feline respiratory disease complex" (FRDC), especially *Bordetella bronchiseptica* and *Clamidophyla felis* (Cohn 2011). Clinical signs in these cases are similar to FeHV-1 or FCV infection, and can cause confusion. Symptomatic animals in our study that were negative for both FeHV-1 and FCV could have an infection by these other agents. Our study shows that the most common pathogens associated with FRDC in southern Brazil are FeHV-1 and FCV.

The PCR product of FeHV-1 was sequenced and subjected to analysis, showing nucleotide (nt) identity of 99.7 to 100% with FeHV-1 sequences deposited with GenBank. Analysis about the nt and amino acid (aa) identity performed with isolated FCV detected high genetic variability in comparison with vaccine strain F9, ranging from 76.1 to 77.4 *(nt)* and 87.4 to 88.9 *(aa),* even though the more conserved regions of FCV capsid (regions A and B) were analyzed. This result may serve as an alert to possible vaccine failures in this region. In general, the level of heterologous protection will depend on the virus strains involved (Radford et al. 2009). Analysis about the *nt* and *aa* identity between the FCV sequences in this study was 88.8 to 97.4% and 99.2 to 100%, respectively.

CONCLUSIONS

Our findings demonstrate high occurrence of Felid alphaherpesvirus 1 and Feline calicivirus in domestic cats in southern Brazil. This information can be used to improve measures about diagnostic, prevention and management against these important cat diseases.

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Conflict of interest statement.- The authors declare that they have no conflict of interest.

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Original Article
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mcr-1-carrying Enterobacteriaceae isolated from companion animals in Brazil[1]

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ABSTRACT.- Kobs V.C., Valdez R.E., Medeiros F., Fernandes P.P., Deglmann R.C., Gern R.M.M. & França P.H.C. 2020. ***mcr-1*-carrying Enterobacteriaceae isolated from companion animals in Brazil.** *Pesquisa Veterinária Brasileira 40(9):690-695.* Laboratório de Microbiologia e Biologia Molecular, Universidade da Região de Joinville, Rua Paulo Malschitzki 10, Zona Industrial Norte, Joinville, SC 89219-710, Brazil. E-mail: v.kobs@univille.br

Plasmid-mediated polymyxin resistance was first described in 2015, in China, in *Escherichia coli* carrying the *mcr-1* (Mobile Colistin Resistance-1) gene. Since then, it has become a major public health challenge worldwide, representing a major threat to human and animal health. In addition, there are still few reports on the prevalence of *mcr-1* in Enterobacteriaceae isolated from humans, animals and food. Therefore, the purpose of the study was to investigate the occurrence of the *mcr-1* gene in bacterial isolates with phenotypic resistance to polymyxin B obtained from clinical specimens of companion animals. Phenotypic resistance to polymyxin B were determined by broth microdilution and the susceptibility profile to other antimicrobials (amikacin, amoxicillin/clavulanate, ampicillin, ampicillin/sulbactam, aztreonam, cefazolin, cefepime, cefotaxime, cefoxitin, ceftazidime, ceftriaxone, chloramphenicol, ciprofloxacin, doxycycline, ertapenem, gentamicin, imipenem, marbofloxacin, meropenem, phosphomycin, piperacillin/tazobactam, tetracycline, ticarcillin/clavulanate, tobramycin and trimethoprim/sulfamethoxazole) by disc-diffusion agar method. The extraction of bacterial DNA was performed via heat shock followed by spectrophotometric evaluation. To verify the presence of *mcr-1*, the Polymerase Chain Reaction was employed using specific primers, followed by agarose gel electrophoresis. The positive isolates had the corresponding amplicons sequenced. In this study, there were identified the first isolates of *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp. carrying the *mcr-1* gene derived from specimens of companion animals in Brazil. Our results suggest the dissemination of resistance to polymyxins in the community and the environment, highlighting the need for surveillance and optimized treatment guidelines.

INDEX TERMS: *mcr-1*-carrying Enterobacteriaceae, companion animals, Brazil, bacterium, polymyxin B, multiple drug resistance, MDR genes, *mcr-1* gene.

RESUMO.- [**Enterobacteriaceae portadora de *mcr-1* em isolados clínicos de animais de companhia: primeiro relato no Brasil.**] A resistência à polimixina mediada por plasmídeo teve sua primeira descrição em 2015, na China, em *Escherichia coli* portadora do gene *mcr-1* (Mobile Colistin Resistance-1) e a partir de então tornou-se um grande desafio para a saúde pública em todo o mundo, constituindo uma grande ameaça à saúde humana e animal. Além disso, ainda existem poucos relatos sobre a prevalência de *mcr-1* em Enterobacteriaceae isoladas de humanos, animais e alimentos. Sendo assim, o objetivo do estudo foi investigar a ocorrência do gene *mcr-1* em isolados bacterianos com

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resistência fenotípica à polimixina B, oriundos de materiais clínicos de animais de companhia. A resistência fenotípica à polimixina B foi determinada por microdiluição em caldo e o perfil de sensibilidade aos demais antimicrobianos (amicacina, amoxicilina/clavulanato, ampicilina, ampicilina/sulbactam, aztreonam, cefazolina, cefepime, cefotaxima, cefoxitina, ceftazidima, ceftriaxona, cloranfenicol, ciprofloxacina, doxiciclina, ertapenem, gentamicina, imipinem, marbofloxacino, meropenem, fosfomicina, piperacilina/tazobactam, tetraciclina, ticarcilina/clavulanato, tobramicina sulfametoxazol/trimetoprim) foram determinados pelo método disco difusão. A extração do DNA bacteriano foi realizada via choque térmico, seguido de avaliação espectrofotométrica. Para a verificação da presença do *mcr-1* foi utilizada a Reação em Cadeia da Polimerase com emprego de iniciadores específicos, seguida de eletroforese em gel de agarose. Os isolados positivos tiveram os correspondentes *amplicons* sequenciados. Nesse estudo foram identificados os primeiros isolados de *Escherichia coli*, *Klebsiella spp.* e *Enterobacter* spp. portadores do gene *mcr-1* derivados de espécimes de animais de companhia no Brasil. Este estudo sugere a disseminação da resistência às polimixinas na comunidade e no meio ambiente, destacando a necessidade de vigilância e diretrizes otimizadas de tratamento.

TERMOS DE INDEXAÇÃO: Enterobacteriaceae portadora de *mcr-1*, animais de companhia, Brasil, bactérias, polimixina B, resistência a múltiplos medicamentos, genes MDR, gene *mcr-1*.

INTRODUCTION

The emergence and rapid spread of resistant microorganisms is considered one of the most relevant and challenging public health problems worldwide, both for human health and for veterinary medicine, as it imposes clinical, social and economic consequences (Liu et al. 2016). On a global scale, 700,000 people die each year from infections by antimicrobial-resistant bacteria. It is estimated that these deaths could reach 10 million by 2050, which would exceed the deaths attributed to cancer, now considered the largest cause of death in the world (8.2 million deaths per year) (ANVISA 2017).

In particular, infections caused by multidrug-resistant (MDR) bacteria of the Enterobacteriaceae family have become a major concern due to significant limitations on therapeutic options. As a consequence, there was an increase in the use of colistin, a cationic peptide antibiotic from the polymyxin family considered one of the last effective drugs for the treatment of infections by Gram-negative bacterium resistant to carbapenem antimicrobials (Eiamphungporn et al. 2018). The delicate balance between the clinical need and the prevention of resistance emergence has been further compromised by the agricultural use of some human antimicrobials, being widely recognized that some countries have actively used colistin in farm animals, favoring the development of resistance and its dissemination to the environment (Liu et al. 2016).

Until recently, it was considered that the acquired resistance to polymyxins was limited to the occurrence of chromosomal mutations, essentially non-transferable (McEwen & Collignon 2018). However, in 2015, Liu et al. (2016) reported the occurrence of the first plasmid-mediated mechanism of resistance to polymyxins (*mcr-1* gene) in *Escherichia coli* isolates in China. On the other hand, in 2018, Wang et al. (2018) reported the occurrence of isolates of different bacterial species carrying the *mcr-1* gene in 31 countries, having China the highest number of reported cases, suggesting that the indiscriminate use of antimicrobials in Chinese agriculture, especially colistin, has caused the initial dissemination of the gene. In addition, other studies have shown that the reservoirs of such bacteria are increasing, not only in agriculture, but also in wild animals and the environment. In 2017, Sellera et al. (2017) reported for the first time the occurrence of the *mcr-1* gene in migratory penguins of the species *Spheniscus magellanicus*. In the same year, Fernandes et al. (2017) collected water samples from beaches in São Paulo State, Brazil, and reported the presence of international clones of *E. coli* carrying the *mcr-1* gene. Notoriously, dogs and cats also represent a possible source for the diffusion of resistance due to the indiscriminate use of antimicrobial agents in the veterinary routine and the close contact of these animals with humans. As suggested by Zhang et al. (2016) in an analysis of clonal relationships conducted in China, dissemination can also occur in pets and veterinary clinics.

Considering the accumulated data, the World Health Organization evidenced the threat to human health facing colonization and infection by bacteria carrying *mcr-1* associated with other genes encoding extended-spectrum beta-lactamases and carbapenemases in a single plasmid (Dalmolin et al. 2018). Given this world scenario, researchers emphasize that, among other actions, conducting epidemiological and molecular studies on the distribution and dissemination of the *mcr-1* gene is extremely important to protect the efficiency of the drug colistin (Liu et al. 2016). Therefore, after extensive consultation in the scientific literature on the occurrence of bacteria carrying *mcr-1* isolated from animals, it was found a scarce number of publications addressing companion animals, including the lack of data generated in Brazil. Thus, this study aimed to investigate the occurrence of the *mcr-1* gene in bacteria resistant to polymyxin B, isolated from clinical specimens of companion animals.

MATERIALS AND METHODS

Selection of clinical isolates. There were considered bacterial isolates from companion animals (dogs and cats) with resistance to polymyxin B by phenotypic screening method (Kirby-Bauer disc-diffusion) (Galani et al. 2008), identified during routine microbiological investigation from February 2017 to August 2018, together with characterizing data from the host animal and clinical specimens.

Evaluation of the antimicrobial susceptibility profile. The determination of the phenotypic profile of susceptibility to antimicrobials was performed by the Kirby-Bauer disc-diffusion test (Bauer et al. 1966). Antimicrobials recommended by the Clinical and Laboratory Standards Institute (CLSI) of the current year were employed and individually categorized for each veterinary species. In addition, in *mcr-1*-positive isolates, other antimicrobial groups were tested to define the bacterium profile as Multidrug-resistant (MDR), Extensively drug-resistant (XDR) and Pandrug-resistant (PDR), as recommended by Magiorakos et al. (2012). All discs used came from the same manufacturer (Cefar, São Paulo, Brazil). The phenotypic susceptibility to polymyxin B of isolates carrying the *mcr-1* gene was confirmed by broth microdilution to obtain the minimum inhibitory concentration (MIC), as instructed by the manufacturer of the "Policimbac" kit (Probac, Sao Paulo, Brazil).

The results were interpreted according to the CLSI guidelines of the current year, except for polymyxin B, which followed the criteria

of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for broth microdilution, with isolates showing MIC >2µg/mL being considered resistant.

***mcr-1* gene investigation.** All bacterial isolates were processed by molecular analyzes. The extraction of bacterial DNA was performed via heat shock from solid medium culture (Vaneechoutte et al. 1995). In the end, the supernatant containing the bacterial DNA was evaluated by spectrophotometry (readings at 260 and 280nm) in an Epoch (BioTek Instruments, Winooski, USA) device and stored at -20°C. The presence of the *mcr-1* gene was evaluated by Polymerase Chain Reaction (PCR), using the XP Cycler (Bioer Technology Co, Hangzhou, China) and the pair of primers CLR5-F (5'CGGTCAGTCCGTTTGTTC'3) and CLR5-R (5'CTTGGTCGTCTGTAGGGG'3), which provides the amplification of a specific segment of 309 bp of the gene (Liu et al. 2016). The thermal cycling used was composed of an initial denaturation step at 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 61.5°C for 1 min and 72°C for 30 s, besides the final extension at 72°C for 10 min. The PCR products were confirmed via electrophoresis in 1% agarose gel, followed by digitized recording (MiniBis-Pro Photodocumentation System, DNR Bio-Image Systems Ltd., Jerusalem, Israel). The standard strain (positive control for *mcr-1*), *E. coli* RM 12983, was kindly provided by Dr. Marcelo Pilonetto, "Laboratório Central do Estado do Paraná" (LACEN-PR), Curitiba, Brazil.

The *mcr-1*-positive amplicons were submitted to direct bi-directional sequencing using the Big Dye® Terminator v3.1 kit on the ABI-Prism 3500 Genetic Analyzer platform (Applied Biosystems, Carlsbad, USA) and compared to the reference nucleotide sequences of *mcr-1* (GeneBank accession no. KP347127), *mcr-1.2* (NG051170), *mcr-1.3* (NG052861), *mcr-1.4* (KY041856), *mcr-1.5* (KY283125), *mcr-1.6* (NG052893), *mcr-1.7* (KY488488), *mcr-1.8* (KY683842), *mcr-1.9* (KY780959), *mcr-2* (NG051171), *mcr-3* (KY924928), *mcr-4* (NG057470), *mcr-5* (MG241339), *mcr-6* (MF176240), *mcr-7* (NG056413) and *mcr-8* (NG061399) genes employing the ClustalW software available in the BioEdit package version 7.2.6.1.

RESULTS

There were identified a total of 64 bacterial isolates with phenotypic resistance to polymyxin B derived from 62 dogs (20 males and 42 females) and 2 cats (1 male and 1 female) in the initial screening. The species corresponding to the identified isolates are distributed according to the type of specimens in Table 1.

Bacteria with non-intrinsic resistance to polymyxins most frequently found were *Escherichia coli* (27), *Enterobacter* spp. (7), *Pseudomonas* spp. (7) and *Klebsiella* spp. (6). The genus *Proteus* was frequently isolated (17); however, it has intrinsic resistance mechanisms.

Among all investigated ones, 3 (4.7%) bacterial isolates, all derived from dogs, were positive for the *mcr-1* gene, as identified by PCR and confirmed via sequencing, specifically present in *E. coli* (urine isolate), *Enterobacter* spp. (nasal secretion isolate) and *Klebsiella* spp. (isolate of abdominal seroma).

The results of the MIC determination to polymyxin B of the positive isolates for the *mcr-1* gene and the phenotypic evaluation against the antimicrobials tested in these isolates are presented in Table 2. All *mcr-1*-positive isolates were MDR.

DISCUSSION

To date, the occurrence in Brazil of the *mcr-1* gene in *Escherichia coli* has been described in clinical isolates of human patients (Fernandes et al. 2016a, Rossi et al. 2017) and in samples from the retail trade of pork and chickens (Fernandes et al. 2016b). In addition, in *Klebsiella pneumoniae*, the presence of *mcr-1* was reported in high-risk strains concomitantly carrying bla_{KPC-2}, also derived from human clinical specimens (Dalmolin et al. 2018). In the present study, the first clinical isolates carrying the *mcr-1* gene in *E. coli*, *Klebsiella* spp. and *Enterobacter* spp., derived from clinical specimens of companion animals, are reported in Brazil.

Although data on the epidemiology of the *mcr-1* gene in veterinary medicine are still relatively scarce, other studies have reported the detection of polymyxin-resistant *mcr-1*-positive isolates from companion animals. Lei et al. (2017), in a study conducted in China, reported the presence of *E. coli*, *K. pneumoniae* and *E. aerogenes* carrying *mcr-1* isolated from companion animals and their respective owners. It was demonstrated that the bacterial isolate from one of the owners presented the same genetic group as 5 isolates from dogs and cats, highlighting the possible transmission of strains carrying this gene between animals and contacting humans. In another study, carried out in Ecuador by Loayza et al. (2018), the presence of *E. coli* carrying the *mcr-1* gene was demonstrated in companion animals sharing the same environment as a child with peritoneal infection by *mcr-1*-positive *E. coli*, suggesting a clonal dissemination, since the detected *mcr-1* gene belonged to the same plasmid in all isolates. In view of the aforementioned, it is observed that the presence of the gene in strains isolated from companion animals can be a natural reservoir, introducing another complex form of dissemination in the community (McEwen & Collignon 2018).

The identification of strains carrying the *mcr-1* gene with MIC indicative of susceptibility to polymyxin B, as observed in most cases of this study, points that the potential for manifestation of resistance provided by *mcr-1* may be difficult to define exclusively based on phenotypic tests. This aspect can contribute to the silent dissemination of strains carrying this resistance mechanism, considering that the tests to verify

Table 1. Distribution of bacterial isolates of companion animals with phenotypic resistance to polymyxin B in different clinical specimens

Clinical specimens	Isolates (n)	Bacterial identification
Urine	50	*Escherichia coli* (25), *Proteus* spp. (15), *Enterobacter* spp. (4), *Klebsiella* spp. (4) and *Pseudomonas* spp. (2)
Otological secretion	7	*Pseudomonas* spp. (4), *Enterobacter* spp. (1), *Escherichia coli* (1) and *Proteus* spp. (1)
Nasal secretion	3	*Enterobacter* spp. (1), *Klebsiella* spp. (1) and *Pseudomonas* spp. (1)
Skin swab	1	*Enterobacter* spp.
Interdigital swab	1	*Proteus* spp.
Abdominal seroma swab	1	*Klebsiella* spp.
Unidentified	1	*Escherichia coli*

the presence or absence of *mcr-1* are generally performed only in isolates with phenotypic resistance previously defined via disc-diffusion (Fernandes et al. 2016b). In fact, in a study conducted in the Netherlands based on specimens from human patients admitted to a tertiary hospital, Terveer et al. (2017) reported *E. coli* carrying the *mcr-1* gene, but susceptible to colistin (MIC=0.25µg/mL). Although the complete genome analysis showed the nucleotide sequence of the *mcr-1* gene found in this isolate was 100% homologous to the first sequence published of the gene, the open reading frame had been interrupted by a long insertion sequence (IS10R) of 1329 bp in the transposon of the *mcr-1* gene, which was probably responsible for the behavior of phenotypic susceptibility to colistin. In this sense, we cannot exclude the possibility that an equivalent phenomenon may also have occurred in the isolates described in our manuscript. In a study with clinical isolates of human patients from five cities in Brazil, Pillonetto et al. (2019) also reported *E.coli* carrying *mcr-1* exhibiting low MIC values for polymyxin B (≤2µg/mL), corroborating with the hypothesis that bacteria carrying this gene may not usually be detected and continue extending the dissemination among humans, animals and the environment.

Colistin and polymyxin B belong to the class of polymyxins, a class of antimicrobials that had its use almost suspended in the 1970s due to its high toxicity rate. In recent years, its use was reintroduced as a last resource for the treatment of infections caused by Gram-negative bacilli resistant to all other commercially available classes of drugs (McEwen & Collignon 2018). However, some mechanisms of resistance to polymyxins have already been documented, such as efflux pumps, thickening of the polysaccharide capsule, changes in the concentrations of membrane-specific proteins and, mainly, changes in the structure of the external membrane, particularly the lipopolysaccharide (LPS), modulated by two chromosomally coded regulatory components - PmrAB and PhoPQ (Eiamphungporn et al. 2018). Changes in these regulation systems cause a cationic substitution of the LPS target by the addition of positively charged molecules of 4-amino-4-deoxy-L-arabinosis (L-ara4N) and/or phosphoethanolamine (PEtN). Thus, the ionic pattern of the external membrane is modified

Table 2. List of *mcr-1*-positive isolates with corresponding minimum inhibitory concentration for polymyxin B and antimicrobial susceptibility profile

Phenotypic evaluation of antimicrobial susceptibility (Kirby-Bauer method)[a]				
Group	Antimicrobial	Interpretation		
Phosphonic acid	Phosphomycin	S	S	S
Aphenicols	Chloramphenicol	S	S	S
Aminoglycosides	Gentamicin	S	S	S
	Tobramicin	S	S	S
	Amikacin	S	S	S
β-lactams with Inhibitors	Ticarcillin/Clavulanate	R	R	R
	Piperacillin/Tazobactam	S	S	S
Carbapenems	Meropenem	S	S	S
	Ertapenem	S	S	S
	Imipenem	S	S	S
Cephalosporins - 1st and 2nd generation (not broad spectrum)	Cefazolin	R	R	S
	Cefazolin (urinary)	S	R	S
Broad spectrum Cephalosporins - 3rd and 4th generation	Cefotaxime Ceftriaxone	R	R	S
	Ceftazidime	R	R	S
	Cefepime	S	R	S
		S	R	S
Cephamycin	Cefoxitin	S	R	S
Fluoroquinolones	Ciprofloxacin	R	R	R
	Marbofloxacin	R	R	S
Folate synthesis inhibitors	Sulfa/Trimethoprim	R	S	S
Monobactamic	Aztreonam	S	S	S
Penicillin	Ampicillin	R	R	R
Penicillin + β- lactamase inhibitors	Amoxicillin+Clavulanate	S	S	S
	Ampicillin+Sulbactam	S	S	S
Polymyxins [b]	Polymyxin B	R	R	R
Tetracyclines	Tetracycline	R	R	S
	Doxycycline	R	R	S
Code		LBM 769	LBM 770	LBM 778
Microorganism		*Klebsiella* spp.	*Enterobacter* spp.	*Escherichia coli*
MIC (µg/mL)[c]		8	0.25	0.25
Interpretation (MIC) [d]		R	S	S
Resistance profile		MDR	MDR	MDR

R = Resistant, S = susceptible; [a] Clinical & Laboratory Standards Institute guidelines (CLSI), [b] Galani et al. (2008), [c] European Committee on Antimicrobial Susceptibility Testing (EUCAST), [d] Magiorakos et al. (2012).

and the total anionic load is decreased, affecting the electronic interaction of LPS with polymyxin molecules, reducing their affinity (Eiamphungporn et al. 2018). The acquisition of the plasmid-mediated *mcr-1* gene, the newest reported mechanism of resistance (Liu et al. 2016), allows the synthesis of an enzyme from the phosphoethanolamine transferase family. When expressed, it adds a phosphoethanolamine residue to lipid A, which is the portion that anchors LPS on the surface of the external membrane, resulting in a resistance mechanism very similar to the mediated by chromosomal modulations (Eiamphungporn et al. 2018).

In this study, from the isolates without intrinsic resistance to polymyxins, only 3 were confirmed as *mcr-1* gene carriers; therefore, 44 isolates should have other mechanisms not investigated, creating an additional difficulty to the predictability of the dissemination of resistance to polymyxins. However, all *mcr-1*-positive isolates were MDR, confirming the concern for human health in the face of colonization and infection by such bacteria, especially when other resistance genes occur concomitantly in the same organism, due to the great therapeutic limitation (Magiorakos et al. 2012).

CONCLUSIONS

In this study, there were identified the first isolates carrying the *mcr-1* gene derived from clinical specimens of companion animals in Brazil. This fact increases the concern about antimicrobial resistance in human and veterinary medicine, due to the close contact of companion animals with humans.

The need to improve the control of the use of polymyxins in veterinary medicine, livestock and human medicine is highlighted, in order to hinder the dissemination of resistance to other bacterial species in the community, environment and hospital facilities, as well as to prevent the association of the *mcr-1* gene to other markers of resistance in the same strains.

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Conflicts of interest statement.- There are no conflicts of interest in relation to the data presented.

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Original Article
Small Animal Diseases
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Periodontal disease and its complications in cats from a shelter in the Central Region of Rio Grande do Sul[1]

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ABSTRACT.- Maciel R.M., Mazaro R.D., Silva J.P.F., Lorenzetti D.M., Herbichi A., Paz M.C., Danesi C.C. & Fighera R.A. 2020. **Periodontal disease and its complications in cats from a shelter in the Central Region of Rio Grande do Sul.** *Pesquisa Veterinária Brasileira 40(9):696-706*. Departamento de Patologia, Universidade Federal de Santa Maria, Av. Roraima 1000, Santa Maria, RS 97105-900, Brazil. E-mail: anemiaveterinaria@yahoo.com.br

Periodontal disease is the most common infectious disease that occurs in feline patients. Although it occurs in both sexes, different age groups, and any breeds, the prevalence and severity seem much higher in cats living in animal shelters. This paper aimed to describe the clinical, radiological, cytopathological, and virological aspects of periodontal disease and its complications in cats, based on these aspects and, consequently, on the importance it brings to cat feline medicine in shelter cats. For this, nine cats with periodontal disease from a single animal shelter were evaluated. These cats demonstrated a disease characterized by halitosis, excessive salivation, and oral discomfort. Lymphadenomegaly of the mandibular and retropharyngeal lymph nodes was observed in 44.4% of the cases. Oral lesions consisted of varying degrees of gingival hyperemia, complete loss of free gingival margins, and consequently gingival retraction, dental calculus deposition, dental mobility, complete exposure of the furcation of premolars and molars, and dental roots of canines and incisors, loss of bone radiopacity due to alveolar bone resorption and tooth loss. Complications included chronic ulcerative paradental stomatitis (22.2%), faucitis (22.2%), and chronic gingivostomatitis (11.1%). None of the cats affected by periodontal disease was positive for FIV or FeLV. In 33.3% of the cases, cats were carriers of feline calicivirus, but not feline herpesvirus.

INDEX TERMS: Periodontal disease, cats, shelter, Rio Grande do Sul, Brazil, periodontitis, gingivitis, faucitis, stomatology, diseases of cats.

RESUMO.- [**Doença periodontal e suas complicações em gatos de um abrigo na Região Central do Rio Grande do Sul.**] Doença periodontal é a mais comum doença infecciosa que ocorre em pacientes felinos. Embora ocorra em gatos de ambos os sexos, diferentes faixas etárias e quaisquer raças, a prevalência e a gravidade parece muito maior em gatos que vivem em abrigos para animais. Baseado nesses aspectos e, consequentemente, na importância que ela traz para a medicina felina de gatos de abrigos, o objetivo desse artigo é descrever os aspectos clínicos, radiológicos, citopatológicos e virológicos da doença periodontal e suas complicações em gatos. Para isso, nove gatos com doença periodontal oriundos de um único abrigo de animais foram avaliados. Esses gatos demonstraram uma doença caracterizada por halitose, salivação excessiva e desconforto oral. Linfadenomegalia dos linfonodos mandibulares e retrofaríngeos foi observada em 44,4% dos casos. As lesões orais consistiam de graus variados de hiperemia gengival, perda completa das margens gengivais livres e, consequentemente, retração gengival, deposição de cálculo dental, mobilidade dentária, exposição completa da furca dos pré-molares e molares e das raízes dentárias dos caninos e incisivos, perda de radiopacidade óssea devido à

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reabsorção de osso alveolar e perda dentária. Complicações incluíram estomatite paradental ulcerativa crônica (22,2%), faucite (22,2%) e gengivoestomatite crônica (11,1%). Nenhum dos gatos afetados pela doença periodontal foi positivo para FIV ou FeLV. Em 33,3% dos casos, os gatos eram portadores do calicivírus felino, mas não do herpesvírus felino.

TERMOS DE INDEXAÇÃO: Doença periodontal, gatos, Rio Grande do Sul, Brasil, periodontite, gengivite, faucite, estomatologia, doenças de gatos.

INTRODUCTION

Periodontal disease (PD) is the inflammatory condition of the oral cavity most commonly diagnosed in feline medicine (Chaudieu & Blaizoit 1999), with a prevalence of up to 85% of adult cats older than four years of age, in a determined community (Watson 1994). PD begins with gingivitis promoted by the establishment of distinct bacterial communities (biofilm) on the dental surface and gingival cavities that progresses in the absence of adequate treatment, and inflammation of the other elements of the periodontium, that is, periodontitis (Gawor et al. 2006). In addition to soft tissue injuries, which cause oral pain and discomfort, periodontal disease compromises tooth support. It affects the periodontal ligament, cementum, and alveolar bone, into which the teeth are inserted (Whyte et al. 2014). Despite much progress in understanding periodontal disease in humans and animals in recent years, periodontal disease still has essential gaps to be clarified. Also, in the Central Region of Rio Grande do Sul (RS), periodontal disease is seen with high frequency in cats that live in animal shelters (Silva et al. 2019). A deeper understanding of the interaction between the bacteria present in the biofilm, the viruses that most commonly infect domestic cats, the inflammatory response, bone, and tissue injuries is necessary. This study aimed to evaluate the clinical, radiological, cytopathological and virological changes of cats from a shelter located in the Central Region of RS.

MATERIALS AND METHODS

The cases included in this study (n=9) occurred in mid-2018, in the municipality of Santa Maria, located in the Mesoregion of the Western Center of Rio Grande do Sul. Only cases diagnosed as periodontal disease were considered through clinicopathological criteria. The criteria used were those previously established by Ingham et al. (2002) and applied during this period from consultations to different editions of the leading textbook on veterinary dentistry (Holmstrom 2012). For comparison, three healthy cats have undergone the same assessments that will be described below.

The oral cavity's physical examination was performed according to criteria already established in the veterinary literature (Bellows 2010). It includes the extra-oral examination (skull examination), in which facial symmetry, volume of draining regional lymph nodes and presence of fistulas or any periorbital lesions that may be related to some oral condition; and the intraoral examination, in which gums are evaluated (including adherent gums, free gingival margins and gingival sulcus), cheeks, tongue, oral floor, hard and soft palates and palatoglossal and glossopharyngeal arches, as well as dental mobility, dental fractures and furcation exposure.

As for tartar (stone accumulation index), the teeth were classified into grades I to III, according to Bellows (2010). The characteristics of each degree are: I) small amount of calculus, which covers less than half of the crown; II) moderate amount of stone, which covers more than half of the crown; and III) marked amount of calculus, characterized by gingival retraction with an accumulation of calculus involving the tooth root (root calculus).

As for tooth mobility (tooth mobility index), teeth were classified into grades I to III, according to Holmstrom et al. (1992). The characteristics of each degree are: I) little mobility, so small that it cannot be measured in millimeters; II) moderate mobility, with movement of 1mm in at least one direction, other than the alveolar one; and III) marked mobility, with a movement of more than 1 mm in all directions or at least towards the tooth socket.

Regarding furcation exposure (furcation exposure index), the teeth were classified into grades I to III, according to Holmstrom et al. (1992). The characteristics of each grade are: I) observable furcation, at the level of the gum line, without penetration of a periodontal probe; II) furcation more easily observed, with penetration of a periodontal probe, but without extending to the other side; and III) completely exposed furcation, with transfixation of the periodontal probe.

The radiographic study of teeth and the bone-alveolar structure was carried out with digital X-ray equipment (Sound-Eklin Digital X-Ray), 45kV, and 35mAs, the focal distance of the film of approximately 40cm. The images were captured from the left and right dorsolateral oblique projections in the mandible and left and right ventrolateral in the maxilla, according to Debowes (2008).

The cats were anesthetized with a combination of ketamine hydrochloride (20mg/kg) and xylazine hydrochloride (2mg/kg) to perform the intraoral physical examination and radiographic study of the teeth and bone-alveolar structure. The administration was via deep intramuscular (IM) in the pelvic limb.

For cytopathological evaluation of the oral mucosa, swabs were introduced into the oral cavity, rolled firmly over the gums, and, subsequently, the material obtained was rubbed in glass slides. These slides were dried at room temperature, fixed in 1% triarylmethane, and stained with a combination of 0.1% xanthene solutions and 0.1% thiazides, according to the principle established by Romanowsky, sequentially (panoptic staining). For cats that presented lesion in the mucosa covering the palatoglossal and palatopharyngeal arches, a region described in feline medicine as fauces, another swab was used in the same collection system (bearing), fixation, and coloring of the obtained samples.

For the cytopathological evaluation of local draining lymph nodes, a non-aspirating puncture with a fine needle (capillary puncture) was performed. The material obtained was deposited on glass slides for later spreading by another glass slide (squash). These slides were treated in the same way as previously described for the oral samples.

Cats were tested for possible infections by feline leukemia virus (FeLV), feline immunodeficiency virus (FIV), feline calicivirus (FCV), and feline herpesvirus (FHV). swabs were introduced into the oral cavity to detect herpesvirus and calicivirus and rolled firmly over the mucosa of the palatoglossal and palatopharyngeal arches. The samples obtained through these swabs were stored in transport medium and, subsequently, subjected to viral isolation in Crandell-Rees Feline Kidney (CRFK) cell culture, according to the methodology described by Henzel et al. (2012). Samples that showed a cytopathic effect on the inoculated cells were subjected to screening for herpesvirus by PCR using primers for the glycoprotein E gene (Argenta et al. 2017), and feline calicivirus by RT-PCR for the ORF2 gene (Henzel et al. 2012).

Cats were tested for the presence of serum antibodies against FIV and FeLV antigen (p27 antigen) to detect infection by FIV and FeLV. About 5ml of blood was collected from the jugular vein, packed in vacutainer collection bottles containing EDTA potassium anticoagulant. The test used was the Snap FIV/FeLV Combo, according to the methodology provided by IDEXX Laboratories.

RESULTS

Clinical findings

All cats evaluated (n=9, 100%), except for the control group (n=3), demonstrated a disease characterized by halitosis, excessive salivation, and oral discomfort, demonstrated by continuous and persistent exposure of the tongue. No changes of the skull, such as facial asymmetry or the presence of fistulas and other injuries were observed. Persistent and moderate lymphadenomegaly of the mandibular and retropharyngeal lymph nodes (Fig.1) was observed in four of the nine cases (44.4%). Despite the oral disorder, all cats (n=9, 100%) had a regular nutritional status. In all patients (n=9, 100%), premolar and molar teeth were the most frequently affected, followed by canines and incisors.

Only one of the nine cats (11.1%) had mild periodontal disease, characterized by slight redness (hyperemia) of the free gingival margin surrounding the teeth, especially the right upper premolars and the lower left canine. Additionally, a yellowish material, sometimes in the form of small mineralized concretions (dental stones), resistant to removal by salivary flow, covers a small part of the crown, mainly in areas adjacent to the neck (Fig.2). None of this patient's teeth showed mobility or furcation exposure.

In two of the nine cats (22.2%), periodontal disease was classified as moderate. There was marked hyperemia in these patients and a slight increase in volume (edema) of the free gingival margins of all premolar, molar, and some canine teeth. Enlargement of the gingival sulcus was present in premolars



Fig.1. Right retropharyngeal lymphadenomegaly in a cat with severe periodontal disease complicated by bilateral symmetrical faucitis.



Fig.2. Mild periodontal disease in a cat. Marked gingival hyperemia and deposition of a small amount of calculus in the second, third, and fourth premolars of the right upper arch.



Fig.3. Moderate periodontal disease in a cat. Gingival retraction and deposition of a moderate amount of calculus cover much of the premolar quarter of the right upper arch. The ipsilateral second premolar is absent.



Fig.4. Marked periodontal disease in a cat. A giant stone concretion completely covers the third and fourth premolars of the right upper arch. In this case, the gingival retraction is evident even before the stone is removed. The canine tooth on the same side has the neck exposed and covered by a large amount of calculus.

and molars. Dental calculi, particularly in premolars and molars, were present and covered all or most of the crown (Fig.3). Moderate tooth mobility, mainly bilateral or craniocaudal, was common in both premolars and molars. Gingival retraction with exposure of the dental neck and furcation of several teeth was evident.

Most cats (6/9, 66.7%) showed severe periodontal disease. There was a complete loss of free gingival margins in the dental arch most affected areas and marked hyperemia of the remaining adherent gingiva in these cases. Often, the adhered gingiva could only be seen after removing an immense stone (Fig.4), which, after excised, allowed the rest of the physical examination. This intense gingival retraction allowed complete exposure of the furcation of premolars and molars and the dental roots of the affected canines and incisors (Fig.5). Marked mobility, both bilateral and craniocaudal, and sometimes in the alveolar direction, was frequent. In at least three cats (33.3%) with severe periodontal disease, the tooth was detached during physical examination (Fig.6).

In four of the cats with periodontal disease (44.4%), teeth were absent (Fig.7), and the tooth socket was covered with normal gingiva. In these areas, the alveolar bone thickness was noticeably thinner, increasing interdental spaces.

Other findings included cases (2/9, 22.2%) of stomatitis due to contact with the upper right fourth premolar tooth (P4), right unilateral faucitis (1/9, 11.1%) or bilaterally symmetrical (1/9, 11.1%) (Fig.8), and chronic gingivostomatitis (1/9, 11.1%). Individualized results are shown in Tables 1 and 2.

Radiological findings

All cats evaluated (n=9, 100%), except for the control group (n=3), demonstrated, in different areas of the dental arch, alveolar bone loss, characterized, in the mildest cases, by the loss of the horizontal bone line (Fig.9), and in the most severe cases, due to a marked decrease in the thickness of the



Fig.5. Marked periodontal disease in a cat. Marked gingival retraction and stone deposition in the third and fourth premolars of the left upper arch. The ipsilateral second premolar is absent. A moderate amount of calculus covers the canine tooth on the same side.



Fig.6. Marked periodontal disease in a cat. During the intraoral physical examination, the right lower canine was markedly mobile and ended up freeing itself from the tooth socket (alveolus).



Fig.7. Marked periodontal disease in a cat. All lower incisors and lower premolars on the left side are absent.



Fig.8. Marked nodular to vegetatively bilaterally symmetrical proliferation in the fauces of a cat with severe periodontal disease.

mandible body, by dissociation of the body: mandibular branch thickness and by the absence of teeth. In these more severe cases, bone rarefaction, seen with multiple radiolucencies (Fig.10), demonstrated the extent of periodontal lesions. Individualized results are shown in Table 1.

Cytopathological findings

For better observation, cytopathological findings will be subdivided into those from the oral cavity, including gingival and fauces cytology, and those from the draining local lymph nodes.

Table 1. Results of physical examinations (intraoral examination - evaluation of teeth and of periodontal tissues) and radiographs of cats with periodontal disease

	Calculus accumulation*	Gingivitis	Gingival retraction	Dental mobility*	Furcation exhibition*	Alveolar bone loss	Result
Cat 1	+++	+++	+++	++	+++	+++	PDIII
Cat 2	+++	+++	+++	++	+++	+++	PDIII
Cat 3	+++	+++	+++	++	+++	+++	PDIII
Cat 4	+	+	-	-	-	-	PDI
Cat 5	++	++	++	+	-	+	PDII
Cat 6	+++	+++	+++	+++	+++	+++	PDIII
Cat 7	+++	+++	+++	+++	+++	+++	PDIII
Cat 8	+++	+++	+++	+++	+++	+++	PDIII
Cat 9	++	+	+	+	-	+	PDII

* The calculus accumulation, furcation exposure and dental mobility represented here in crosses (+ mild, ++ moderate, +++ severe), is equivalent to grades I, II and III, previously described in the materials and methods; PD = periodontal disease (grades I, II and III).

Table 2. Other injuries found in the physical examination of cats with periodontal disease

	Toothlessness	Contact stomatitis	Chronic gingivostomatitis	Ulcerative faucitis	Persistent lymphadenopathy
Cat 1	Yes	Yes	No	No	No
Cat 2	Yes	No	Yes	No	Yes
Cat 3	Yes	No	No	Yes	Yes
Cat 4	Yes	No	No	No	No
Cat 5	Yes	No	No	Yes	Yes
Cat 6	Yes	No	No	No	No
Cat 7	Yes	Yes	No	No	Yes
Cat 8	Yes	No	No	No	No
Cat 9	Yes	No	No	No	No

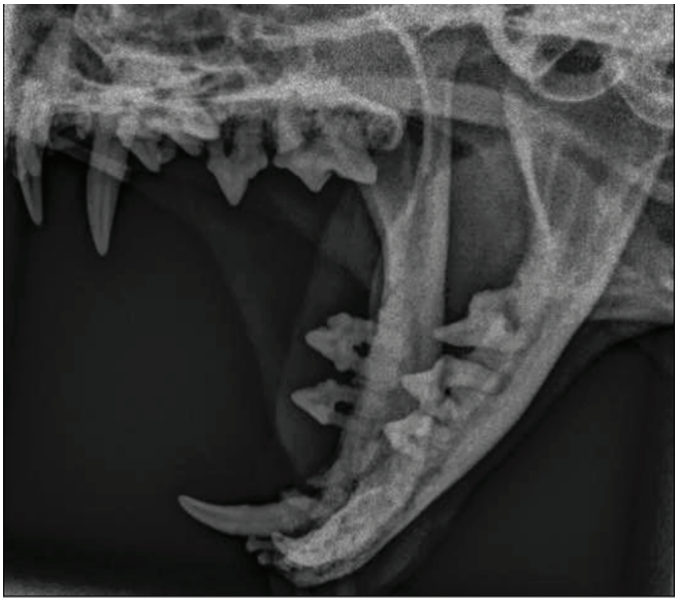


Fig.9. Radiographic image of the lower dental arch of a cat with severe periodontal disease. Note the loss of the jaw's horizontal bone line, which shows how much bone tissue has been remodeled.

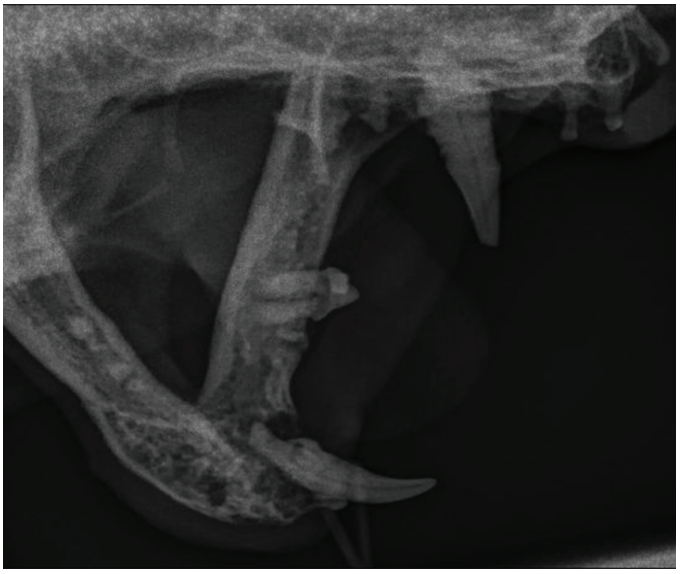


Fig.10. Radiographic image of the lower dental arch of a cat with severe periodontal disease. In addition to the absence of most teeth, we observe intense bone rarefaction, seen as a marked decrease in radiopacity.

Cytopathological findings of the oral cavity. All cats with periodontal disease and the three control cats had their oral cavity evaluated cytopathologically. In the control cats' oral cavity, there was a large number of bacteria in the form of coccus and bacilli, a small number of epithelial cells, mainly intensely keratinized and anucleate cells, but also less keratinized and anucleate cells. Inflammatory cells, basically neutrophils, were scarce (Fig.11).

All cats with periodontal disease, but none of the control cats had intense cellularity and many bacteria. Neutrophils predominated, mostly with criteria that allowed them to be identified as necrotic ("degenerate neutrophils"). Many of the healthy neutrophils had cytoplasm full of bacteria, mainly coccus. Agglomerated neutrophils surrounding bacterial aggregates were frequent (Fig.11). Macrophages with cytoplasm full of cellular debris were sparse. Other inflammatory cells were less frequent and included eosinophils, lymphocytes, and plasma cells.

Bacteria distributed among the cells included aggregates of coccus, chain coccus (Fig.11), and diplococci accumulations. Bacilli of different thickness and length, straight (Fig.12) or curved (Fig.13), small or large (Fig.14), isolated or in chains, with or without aggregated intracellular acidophilic material could be seen in abundance. A large number of spirochetes were also present (Fig.15).

Of the cats with periodontal disease, two (22.2%) and one (11.1%) had ulcerative faucitis and chronic gingivostomatitis, respectively. In addition to the same oral cytopathological results previously described, these three cats presented a large amount of lymphocytes and plasma cells (Fig.16) in the

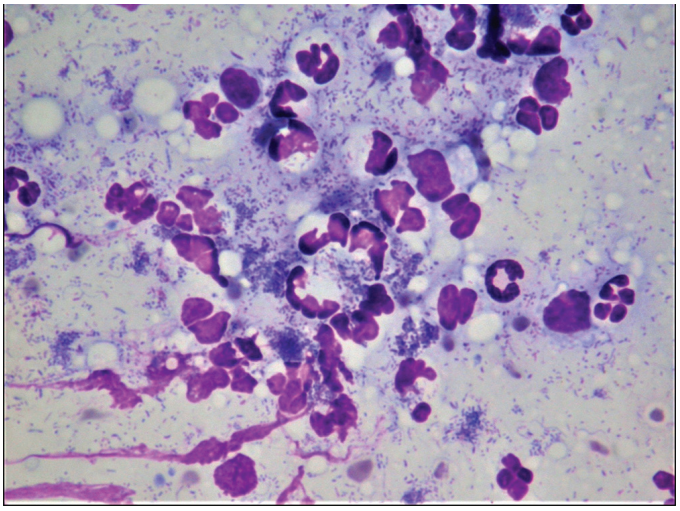


Fig.11. Gingival cytology of a cat with severe periodontal disease. Several neutrophils, mostly degenerated, amidst massive amounts of bacteria in the form of coccus. Panoptic stain, obj.40x.

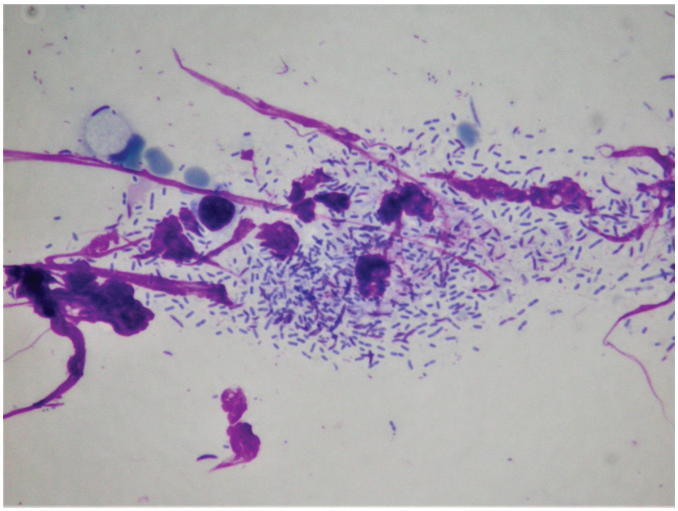


Fig.12. Gingival cytology of a cat with severe periodontal disease. Some neutrophils and chromatin filaments (nuclear remnants) amidst massive amounts of bacteria in the form of small, short bacilli and often in pairs. Panoptic stain, obj.40x.

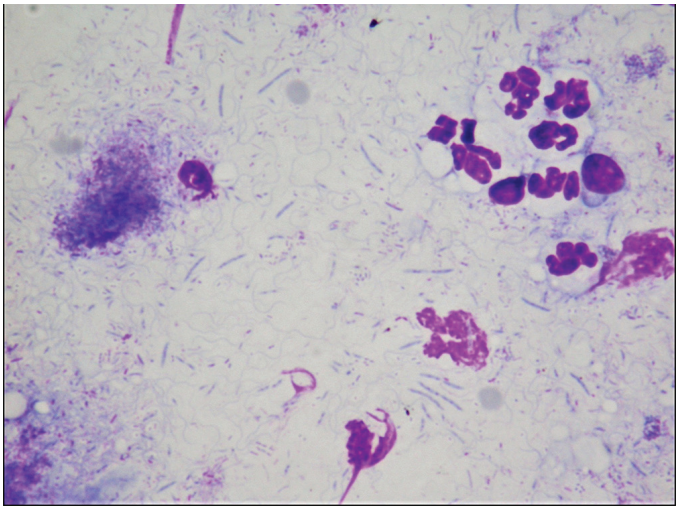


Fig.13. Gingival cytology of a cat with severe periodontal disease. Some neutrophils, mostly degenerate, and chromatin filaments (nuclear remnants) amidst massive amounts of bacteria in the form of single, curved, slightly longer bacilli than seen in Figure 12. Panoptic stain, obj.40x.

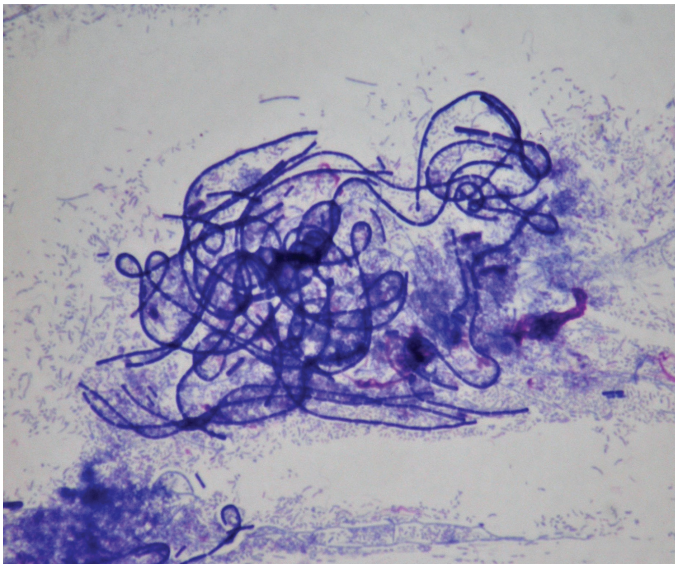


Fig.14. Gingival cytology of a cat with severe periodontal disease. Lots of bacteria in the form of large bacilli and forming chains of large extensions. Panoptic stain, obj.40x.

cytology of the fauces and affected gums, including plasma cells loaded with cytoplasmic globules (Mott cells) and plasma cells with hyaline cytoplasm ("flame cells").

Nodal cytopathological findings. All cats with periodontal disease and the three control cats had their mandibular and retropharyngeal lymph nodes evaluated cytopathologically. In the control cats' lymph nodes, there was a predominant lymphoid population with morphological characteristics that allowed them to be included as mature (small mature lymphocytes not cleaved). A second lymphoid population, represented by a smaller proportion of cells, characterized by a larger size and less maturation, was also seen in all control cases (immature lymphocytes). Fewer plasma cells with typical cell morphology completed the nodal cell findings of these patients.

Of the nine cats with periodontal disease, six (66.7%), one (11.1%) of whom had lymphadenomegaly, had a similar cytopathological pattern but was richer in immature lymphoid cells and plasma cells. In these six cases, neutrophils were seen in moderate to severe amounts amid lymphocytes, characterizing a "nodal drainage reaction".

Of the nine cats with periodontal disease, three (33.3%), who had lymphadenomegaly, had a different cytopathological pattern, not so much in cell types, but in their proportions. In these three cases, the number of immature plasma cells and lymphocytes was much greater than in other cats with periodontal disease and in control cats (Fig.17). Also, plasmacytic precursors (plasmablasts) (Fig.17), plasma cells loaded with cytoplasmic globules (Mott cells) (Fig.18), plasma cells with cytoplasmic crystals (Fig.19), and plasma

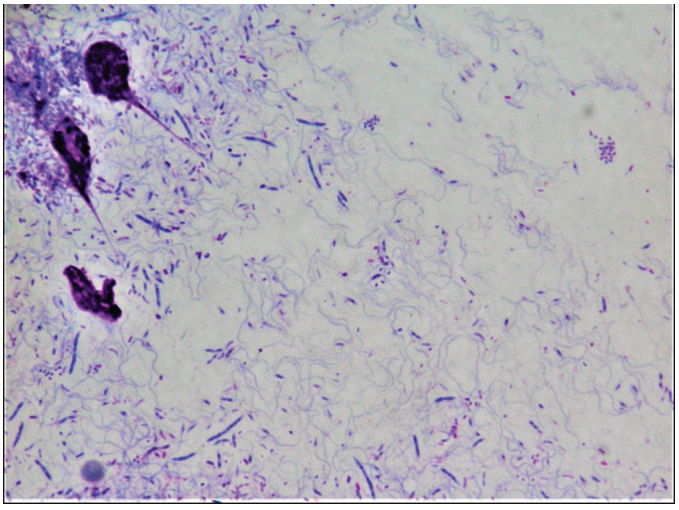


Fig.15. Gingival cytology of a cat with severe periodontal disease. Lots of bacteria in the form of spirochetes that intertwine at the bottom of the microscopic field. Bacteria similar to those seen in Figure 13 and chromatin filaments (nuclear remains) are present in lesser quantities. Panoptic stain, obj.40x.

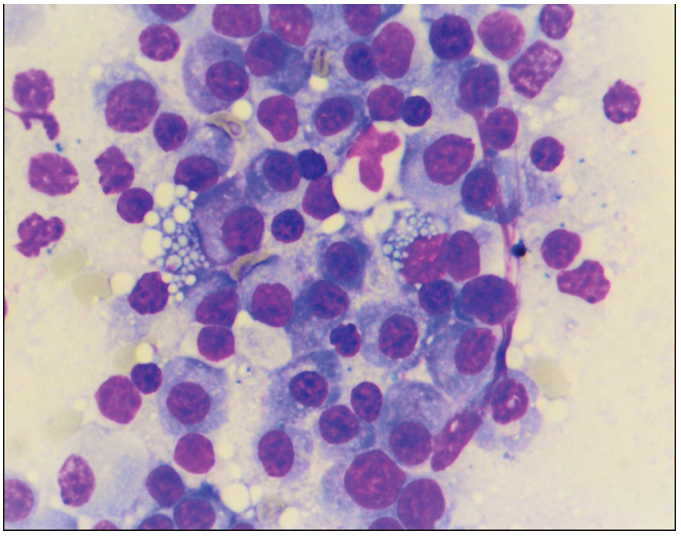


Fig.16. Cytology of the fauces of a cat with faucitis and severe periodontal disease. Observe the monomorphism of the inflammatory infiltrate, consisting almost exclusively of plasma cells, including two Mott cells. Panoptic stain, obj.40x.

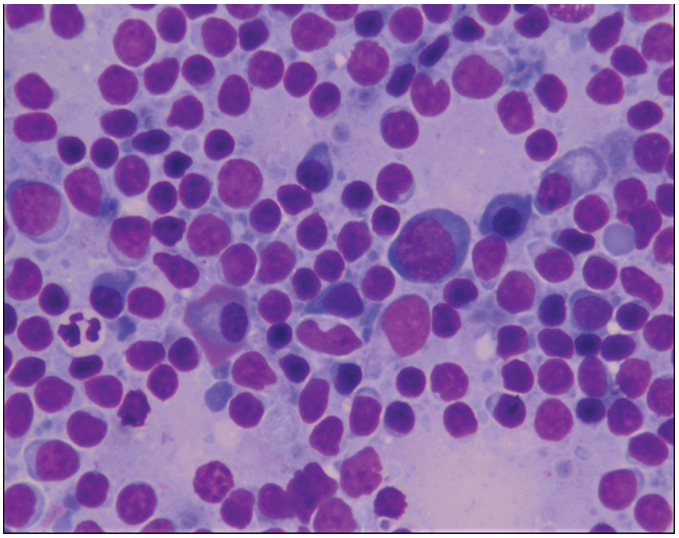


Fig.17. Nodal cytology of a cat with faucitis and severe periodontal disease. Intense lymphoid hyperplasia with plasmacytic differentiation. Panoptic stain, obj. 40x.

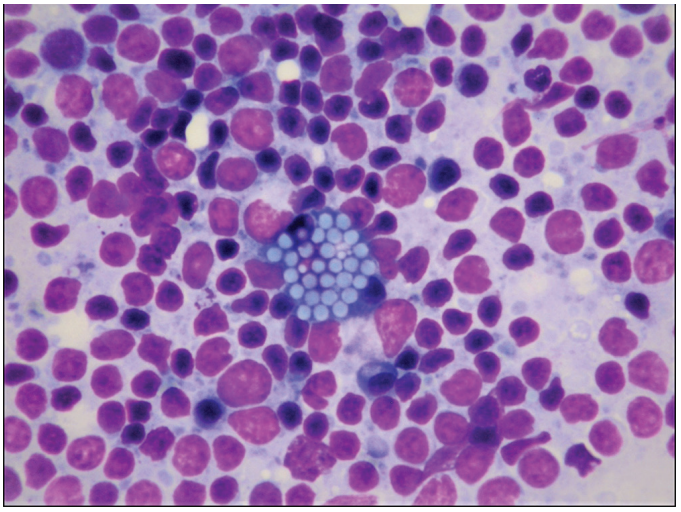


Fig.18. Nodal cytology of a cat with faucitis and severe periodontal disease. Observe the plasma cells with cytoplasm filled with large basophilic granules (Russel corpuscles) in the image (Mott cell). Panoptic stain, obj.40x.

cells with hyaline cytoplasm ("flame cells") (Fig.20) were common (nodal plasmacytosis). The number of neutrophils was similar to that of other cats with periodontal disease. The nodal patterns of each patient can be found in Table 3.

Virological findings

All cats evaluated (n=9, 100%), including patients used as a control group (n=3), tested negative for the presence of feline leukemia virus (FeLV) and antibodies against feline immunodeficiency virus (FIV) in the blood. Swabs from the oral cavity of four cats (three cats with periodontal disease (3/9, 33.3%) and a control cat (1/3, 33.3%) were positive in cell culture, as they exerted a cytopathic effect. After extracting the viral RNA and sequencing the base pairs, all were identified as feline calicivirus. Individualized results are shown in Table 4.

DISCUSSION

Behavioral changes observed in animals should not be neglected to prevent these suffering individuals from going unnoticed (Hellebrekers 2002). However, the classification of the intensity of the discomfort or pain experienced by the veterinary patient is difficult, even for species of intimate contact with humans, such as the cat (Posso & Ashmawi 2012). The persistent exposure of the tongue, observed in cats with periodontal disease, can be considered a deviation from the patient's normal behavior and be related to discomfort or pain in the oral region (Landsberg et al. 2005).

Lymph nodes, interspersed in the lymph path, are structures made up of lymphoid tissue that act as genuine filters of everything that reaches them through the afferent lymphatic vessels (Dantas & Siqueira 2000). The salivary glands, the intermandibular space, the tongue, and all other structures that comprise the "snout", are drained by the lymph nodes of the mandibular center (Dyce et al. 1997). Lymphadenomegaly means the increase in the lymph node's size, usually related to inflammatory processes or neoplasms (Fry & McGavin 2009). The persistence of lymphadenomegaly in 44.4% of cats with periodontal disease indicates the course of a chronic inflammatory process. The inflammatory process is

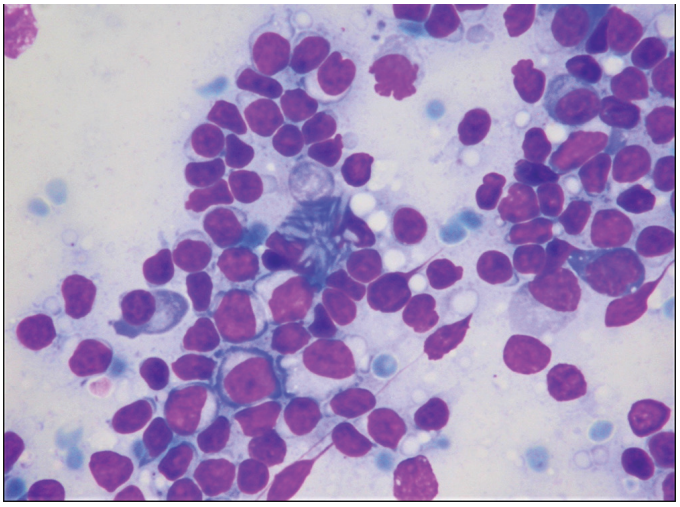


Fig.19. Nodal cytology of a cat with faucitis and severe periodontal disease. Observe the plasma cells with cytoplasm full of immunoglobulin crystals in the center of the image. Panoptic stain, obj.40x.

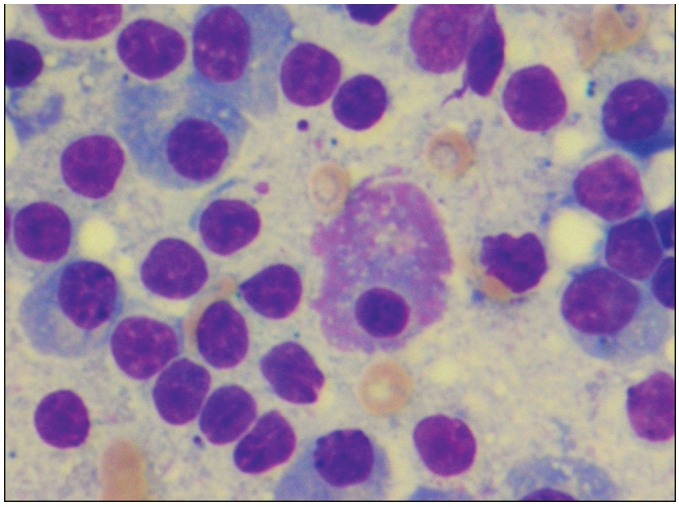


Fig.20. Nodal cytology of a cat with faucitis and severe periodontal disease. Observe the plasma cells with abundant and intensely eosinophilic cytoplasm ("flame cells"). Panoptic stain, obj.40x.

Table 3. Relationship between periodontal disease, contact stomatitis, chronic gingivostomatitis, ulcerative faucitis, persistent lymphadenopathy and the nodal cytopathological pattern

	Periodontal disease	Contact stomatitis	Chronic gingivostomatitis	Ulcerative faucitis	Persistent lymphadenopathy	Nodal cytopathological pattern
Cat 1	III	Yes	No	No	No	Drainage*
Cat 2	III	No	Yes	No	Yes	Plasmacytosis**
Cat 3	III	No	No	Yes	Yes	Plasmacytosis
Cat 4	I	No	No	No	No	Drainage
Cat 5	II	No	No	Yes	Yes	Plasmacytosis
Cat 6	III	No	No	No	No	Drainage
Cat 7	III	Yes	No	No	Yes	Drainage
Cat 8	III	No	No	No	No	Drainage
Cat 9	II	No	No	No	No	Drainage

* Drainage = short for "nodal drainage reaction", an expression widely used to describe lymph nodes with an increase in the amount of inflammatory cells, mainly neutrophils and macrophages, which were drained by local lymphatics from an inflammatory lesion; ** Plasmocytosis = term more recently used in hematopathology to describe the increase in plasma cells in places where these cells are normally present, such as lymph nodes, spleen and bone marrow; Previously plasmacytic hyperplasia or lymphoid hyperplasia with plasmacytic differentiation.

Table 4. Relationship between periodontal disease, chronic gingivostomatitis, ulcerative faucitis, persistent lymphadenopathy and infection by feline leukemia virus, feline immunodeficiency, calicivirus and feline herpesvirus

	Periodontal disease*	Chronic gingivostomatitis	Ulcerative faucitis	Persistent lymphadenopathy	FCV	FHV-1	FeLV	FIV
Cat 1	III	No	No	No	No	No	No	No
Cat 2	III	Yes	No	Yes	No	No	No	No
Cat 3	III	No	Yes	Yes	No	No	No	No
Cat 4	I	No	No	No	No	No	No	No
Cat 5	II	No	Yes	Yes	No	No	No	No
Cat 6	III	No	No	No	Yes	No	No	No
Cat 7	III	No	No	Yes	No	No	No	No
Cat 8	III	No	No	No	Yes	No	No	No
Cat 9	II	No	No	No	Yes	No	No	No

* Grades of periodontal disease (I, II and III); FCV = feline calicivirus infection, FHV-1 = feline herpesvirus type I infection, FeLV = infection with feline leukemia virus, FIV = infection by the feline immunodeficiency virus.

the organism's response to an injury, whether caused by the most varied etiological agents, microorganisms for example. The inflammation starts to be characterized as chronic when the stimulus that gave rise to the defense process extends for weeks or months (Kumar et al. 2005).

Periodontal disease is among the various diseases listed in the medical literature with a probable inflammatory course (Santos et al. 2012). With a prevalence close to 85% in adult dogs and cats, periodontal disease is a widespread disorder in the domestic cat (Reichart et al. 1984, Watson 1994). In addition to pain, caused by the variable degree of inflammation in the tissues that support the tooth, periodontal disease can lead to tooth loss and fractures in the jaw and mandible (Santos et al. 2012).

The feline oral cavity is short and wide, which facilitates physical examination, both of soft structures (vestibule lining tissues, hard and soft palates, palatoglossal and palatopharyngeal arches, palatine tonsils, frenulum, sublingual caruncle, and tongue) and hard (teeth) (Dyce et al. 1997). With 12 teeth less than the dog, felines have 30 teeth in permanent dentition, distributed between incisors, canines, premolars, and molars (West 1990). Between the third and sixth weeks of life, deciduous teeth eruption occurs when molars are not present. From the third month, permanent teeth appear, with the molars completing the feline dental arch at six months of age (Gioso 2003). Consisting of enamel (a white, calcified substance, very resistant and that covers the crown), dentin (which forms most of the tooth, presenting a central cavity, which houses the pulp) and cementum (a more flexible yellowish tissue), the teeth are inserted into the bone alveoli, both in the mandible and in the jaw (Santos 2012, Dyce et al. 1997). The set of tissues that partially cover, support, and protect teeth, we call periodontium: gingiva, alveolar bone, cementum, and periodontal ligament (Debowes 2004).

Among the various agents that can lead to PD, bacterial plaque is recognized as the primary etiologic agent (Domingues et al. 1999). Characterized as a cluster of established bacteria, both on the hard surface of the tooth and on soft tissue, plaque is also known as biofilm (Nascimento et al. 2006, Marinho & Araújo 2007). Bacteria protect themselves in the extracellular matrix, consisting of polysaccharides, exopolysaccharides, and saliva. In biofilm, they are also grouped into microcolonies that communicate through chemical signals, establishing an efficient defense system (Overman 2000, Marinho & Araújo 2007). The biofilm's oxygen concentration establishes the type of bacteria present: anaerobic in the deepest layers and aerobic in the most superficial of the bacterial plaque. The tooth's face turned to the tongue and tended to accumulate more biofilm, precisely because it exhibits lower oxygen (Marinho & Araújo 2007).

The precipitation of minerals carried by saliva, such as calcium salts, mainly calcium carbonate and phosphate, on the biofilm (Corrêa & Venturini 1996) results in dental calculus or tartar. By itself, dental calculus is inert, only causing periodontal disease associated with microorganisms in the biofilm (Gorrel et al. 2004). The accumulation of biofilm in the gingival margin and the dental groove facilitates the installation of more bacteria, which results in aggression to the gingiva and consequent inflammatory response characterizing gingivitis (Gioso 2003). As gingivitis progresses, the inflammatory process weakens the cementum's collagen fibers, leading to the detachment of the tooth's supporting ligament (Gorrel et al. 2004). Our study observed slight hyperemia in some cats' gingival margin, mainly in the premolar and canine teeth (mild periodontal disease), gum edema in premolars, molars, and possible canines (moderate periodontal disease). It was also verified that in cases where the periodontal disease was severe, the gingival retraction with exposure of the dental neck, leading to a reduction in the tooth (mobility). The formation justifies this finding and deepening of the periodontal pocket when the gingival sulcus reaches or exceeds 5mm, and the alveolar resorption responds to inflammation (Ferro et al. 2008).

The inflammation initially promoted by gingivitis and maintained by PD can extend to the oral cavity's mucous membranes, manifesting as chronic ulcerative paradental stomatitis (Gelbert 2009). These lesions occur on surfaces opposite the biofilm, causing pain and salivation, as seen in two of the cats described here. In addition to the mucous membranes, the region of fauces (the mucous structure that covers glossopalatine arches) can also be reached by inflammation, making it difficult to eat food (Niza et al. 2004). It explains persistent salivation, halitosis, and abnormal positioning of the tongue, observed in two patients. However, the animal's reasonable body condition suggests that discomfort did not prevent food intake.

In a physiological condition, the bone mass present in the body results from the balance between matrix deposition and bone resorption; this phenomenon is called bone remodeling. This process is beneficial for the organism, as it maintains the homeostasis of the serum level of calcium and phosphorus

(Cho et al. 1991). The remodeling results from a complex interaction of macrophages, osteoclasts, osteocytes, and osteoblasts, leading to the skeleton's renewal from time to time (Manolagas 2000). However, alveolar bone structures are not affected by bone remodeling, probably due to the absence of receptors sensitive to bone remodeling mediator's chemical stimuli, in the cells that colonize the root of the teeth cementoblasts (Lindskog et al. 1987, Cho et al. 1991). An erosive process is observed in the tooth structure (Girard et al. 2010). Formerly referred to as "feline caries", the tooth resorption lesion (LRD), is characterized by erosion in the hard tissues of the tooth and probably reaching the pulp region (Niemiec 2014). In our study, we observed in the PD group, a variable degree of bone loss in the alveoli, in the mildest cases, related to periapical lesions, resulting from the chronic inflammatory process (Bellows 2010), and even bone reduction in the mandible, which could lead to the occurrence of fractures.

The oral injury was already associated with the presence of FHV when Hargis and Ginn, in the late 1990s, sought to establish a possible relationship between FHV and chronic gingivostomatitis (FCGS) that affects this species (Hargis et al. 1999). Later, in the early 2000s, Lommer and Verstraete were able to isolate FHV and FCV in the saliva of 88% of cats with FCGS (Lommer & Verstraete 2003). FIV has also been found in about 50% of cats with FCGS (Cognet et al. 2001). However, the association between FeLV and FCGS has also been investigated without conclusive results (Harley 2003). In our study, the only virus found, both in cats with periodontal disease and in control cats (33.3%), was FVC.

In addition to viruses, bacteria established in the oral cavity, especially anaerobic ones, have been listed as possible causative / inciting agents for FCGS (Love et al. 1990). All cats, both those with periodontal disease and those with a healthy oral cavity (control cats), presented a significant number of bacteria, in the form of coccus and bacilli, in addition to epithelial cells. If the number of inflammatory cells was low in the control cats, the opposite was found in cats with periodontal disease, mainly neutrophils, and macrophages, all in full phagocytosis activity of the local bacteria. The degeneration observed in neutrophils is related to the synthesis and release of endotoxins by the site's bacteria. When the neutrophil becomes degenerate, its ability to control fluid hemostasis ceases, leading to cytoplasmic edema or degeneration (Meinkoth et al. 2009). Cells such as eosinophils, lymphocytes, and plasma cells were also detected, but without numerical expression. These findings are consistent with the inflammatory process present in periodontal disease.

Ulcerative faucitis and FCGS have also been observed in some cats with periodontal disease. The cytological evaluation revealed, in addition to the inflammatory cells (neutrophils and macrophages), the presence of lymphocytes and plasma cells, some of which are characterized as Mott cells and "flame cells", due to the peculiarities presented in the cytoplasm. When stimulated to produce antibodies, B lymphocytes differentiate into plasma cells, cells with an eccentric and round nucleus. When the plasma cells exhibits a cytoplasm with large vacuoles and full of immunoglobulins (Russell's corpuscles), it is called a Mott cell, reinforcing the confirmation of a scenario in which inflammation predominates (Meinkoth et al. 2009).

CONCLUSION

The presence of inflammatory cells (neutrophils, macrophages, lymphocytes, and plasma cells), collected from lesions of oral structures (mucous membranes and fauces), revealed an important inflammatory scenario. Different types of bacterial communities live in perfect harmony with each other and, protected in structures called biofilms, which mineralized (dental calculations), promote a mild inflammatory response that initially does not compromise the periodontal structure that supports the tooth in the bone alveolus. When persistent, this scenario progresses to periodontal disease, closely associated with chronic inflammation and its consequent destruction of the periodontium, tooth loss, and, ultimately, intense bone matrix loss.

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Original Article
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Macroscopic morphological aspects, ecometry and rebound tonometry of the eye bulb in sloth (*Bradypus variegatus*)[1]

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ABSTRACT.- Braz R.S., Amorim M.J.A.A.L., Sá F.B., Albuquerque P.V., Silva E.C.S., Ferri R.C., Fonsêca Filho L.B. & Andrade G.P. 2020. **Macroscopic morphological aspects, ecometry and rebound tonometry of the eye bulb in sloth (*Bradypus variegatus*).** *Pesquisa Veterinária Brasileira 40(9):707-716*. Departamento de Morfologia e Fisiologia Animal, Universidade Federal Rural de Pernambuco, Rua Dom Manuel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. E-mail: richellebraz@gmail.com

The field related to the visual system of wild animals is deeply scarce. Settling anatomical and physiological parameters for these animals is still a descriptive vision for *Bradypus variegatus* (Schinz, 1825). Thus, our research aimed to determine patterns of normal eye for this species. For this purpose, eight eye bulbs were dissected from the carcasses obtained by natural death, and then performed an overview of ocular anatomical. Rebound tonometry (RBT) and ocular B-mode ultrasonography were also applied for eight eyes in four animals from "Parque Estadual Dois Irmãos", situated in the city of Recife, state of Pernambuco (PE), to estimate the intraocular pressure and ocular ecobiometry. The ocular morphology of sloth is similar as described for other species, however, with some peculiarities. They present a third eyelid emerging in the nasal region of the inferior conjunctival sac and retina and also contain little differentiated blood vessels. Medium the intraocular pressure (IOP) was 4.25mmHg with no difference for both eyes. Ultrasonography of ocular anatomy is also similar regarding other species. Ecobiometric patterns were evaluated to determine the anterior chamber depth, lens width, vitreous chamber depth, and axial length (AL) of ocular globe and the averaged as shown 0.63±1.11mm, 3.73±0.24mm, 6.15±0.41mm, 3.70±0.27mm, and 8.48±0.22mm, respectively. There was no difference between the right and left eyes. The RBT and ocular B-mode ultrasonography are fast exams and easy for animal testing. This study contributed to the characterization of ocular anatomy as well as settling medium values of IOP and intraocular measures; however, further research on physiology and histology is necessary to better understand the visual function of the species.

INDEX TERMS: Macroscopy, morphology, ecometry, rebound tonometry, eye bulb, sloth, *Bradypus variegatus*, ophthalmology, ocular anatomy, ocular ultrasonography, tonometry, xenartros.

RESUMO.- [**Aspectos morfológicos macroscópicos, ecometria e tonometria de rebote do bulbo ocular em bicho-preguiça (*Bradypus variegatus*).**] O campo de estudo relacionado ao sistema visual de animais silvestres é muito escasso. Estabelecer parâmetros anatômicos e fisiológicos para estes animais ainda está restrito a uma visão descritiva, assim ocorre em *Bradypus variegatus* (Schinz, 1825). Diante deste fato, objetivou-se com este estudo determinar padrões de normalidade oftálmica nesta espécie. Para isto foram dissecados oito bulbos oculares de cadáveres obtidos por morte natural e realizada a descrição anatômica ocular. Além disso, foram realizadas tonometria de rebote (TonoVet®) e ultrassonografia em modo B em oito olhos de quatro animais provenientes do Parque Estadual Dois Irmãos, Recife/PE, para avaliação da pressão intraocular e realização da ecobiometria

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ocular. A anatomia ocular do bicho-preguiça é semelhante à descrita para outras espécies com algumas particularidades. Apresentam uma terceira pálpebra emergindo na região nasal do saco conjuntival inferior e retina com vasos sanguíneos pouco diferenciados. A pressão intraocular média foi de 4,25mmHg não havendo diferença entre os olhos direito e esquerdo. A anatomia ocular ultrassonográfica é semelhante à encontrada para outras espécies. Os padrões ecobiométricos obtidos foram: profundidade da câmara anterior, espessura do cristalino, diâmetro do cristalino, profundidade da câmara vítrea e comprimento axial do bubo ocular com tamanhos médios de 0,63±1,11mm, 3,73±0,24mm, 6,15±0,41mm, 3,70±0,27mm e 8,48±0,22mm, respectivamente. Não houve diferença entre os olhos direito e esquerdo. A tonometria de rebote e a ultrassonografia ocular em modo B são exames de rápida e fácil execução, sendo bem tolerados pelos animais. Este estudo contribuiu para a caracterização anatômica ocular e para o estabelecimento de valores médios da pressão intraocular e das medidas intraoculares, no entanto são necessárias outras pesquisas na área da fisiologia e histologia para melhor compreensão da função visual da espécie.

TERMOS DE INDEXAÇÃO: Morfologia, macroscopia, ecometria, tonometria de rebote, bulbo ocular, bicho-preguiça, *Bradypus variegatus*, oftalmologia, anatomia ocular, ultrassonografia ocular, xenarthros.

INTRODUCTION

The sloth is an animal that belongs to class Mammalia, superorder Xenarthra, order Pilosa (Britton, 1941), and with two genera *Bradypus* and *Choloepus* (Wetzel & Avila-Pires 1980). In the family Bradypodidae, genus *Bradypus*, includes the three-toed sloth and the species: *Bradypus torquatus*, *Bradypus tridactylus*, and *Bradypus variegatus* (Gardner 2007).

The species *B. variegatus* (Shinz, 1825). is distributed in southern Honduras, northern Argentina, and in more significant numbers in Brazil. The species can also be found in forest areas at low and medium elevations in eastern Central America (Anderson & Handley 2001). In Brazil, it can also be found in the states of Amazonas, Pará, Maranhão, Pernambuco, Alagoas, Espírito Santo, Rio de Janeiro, São Paulo, and Paraná (Gardner 2007). The three-toed sloth can be distinguished by its brown neck (Hayssen 2010). It has a variable size, but most populations weigh from 2.5 to 3.5kg (Dünner & Pastor 2017). Other authors report that the weight can vary from 3.9 to 5.5kg (Aguilar & Superina 2014) and can reach 6.2kg (Hayssen 2010). According to Goffart (1971), species from the genera *Bradypus* and *Choloepus* are incredibly shortsighted and have different senses to obtain food, make contact with other species belonging to their own species, and ensure their protection. Furthermore, the absence of a ciliary muscle and a macula, in addition to the presence of sparse nature of ganglion cells and nerve fibers, indicate a low level of visual acuity (Gilmore et al. 2000).

The eye represents the organ of vision and consists of the eyeball structures and its accessory structures (eyelids, conjunctiva, lacrimal apparatus, and extraocular muscles (EOM)). Most of these structures are located in orbit, where the eyeball is surrounded by a large amount of adipose tissue (Dyce et al. 2010, Reece & Rowe 2017). The eyeball consists of three tunics: the outer layer of the fibrous tunic is composed of the sclera and the cornea, and has the function of shaping and protecting the eye; the middle layer or vascular tunic consists of the choroid, the ciliary body, and the iris; and the inner layer (nerve) or retina is responsible for communications with the brain via the optic nerve (Eurell & Frapppier 2012).

Ultrasonography has been used in Veterinary Medicine in many cases, mainly in the evaluation of abdominal organs. On the other hand, in veterinary ophthalmology, its use is more recent. The eyes are visually accessible to the ophthalmologist; however, in those with opacity in the anterior segment, ultrasound can outline changes that would not otherwise be seen (Dziezyc et al. 1987).

For the ultrasound examination of the eyeball, high frequencies are necessary to delineate the tissues properly. Thus, the 7.5 and 10MHz transducers are used for general examination and retrobulbar portion with or without a cushion indentation (Gonçalves et al. 2009). There are two characteristics of ultrasound that can be associated with its safety and patient's absence of discomfort. It is a technique that provides reliable information in real-time, relatively inexpensive, non-invasive, and that allows the overview of ocular and retrobulbar anatomy (Gelatt-Nicholson et al. 1999).

The knowledge of the ultrasound anatomy and biometry of the ocular structures serves as a basis for ultrasound examination, as this allows the evaluation of abnormalities that cause changes in the dimensions and appearance of the ocular structures, as in cases of retinal detachment, glaucoma, and optic nerve atrophy (ONA) (Ribeiro et al. 2009, Ruiz et al. 2015). In ophthalmology, ocular A-mode and B-mode ultrasounds can be used to assess the eyeball, seeing that both modes allow the assessment of structures (Schiffer et al. 1982). B-mode is the most commonly used; being able to produces a two-dimensional image of the eye that most reflects the ocular anatomy (Moore & Lamb 2007). However, ocular B-mode ultrasonography (USS) allows the measurement of five distances from the eyeball: D1 = from cornea to the anterior lens capsule, D2 = from anterior lens capsule to the posterior lens capsule, D3 = diameter of the lens, D4 = from posterior lens capsule to the posterior wall of the eyeball, and D5 = axial length (AL) of the eyeball, which corresponds to the distance between the cornea and the posterior wall of the eyeball (Hamidzada & Osuobeni 1999, Gonzalez et al. 2001).

Tonometry is the measurement of intraocular pressure (IOP), that is, an actual diagnostic test underused in veterinary practice. Initially, this technique was only used as a diagnostic method for glaucoma, in which the IOP is increased, subsequently became to be used as a diagnostic method for anterior uveitis, where IOP is reduced, and to aid in the diagnosis of other diseases that had a clinical presentation of ocular hyperemia, such as keratitis, conjunctivitis, and scleritis, where the IOP is not compromised (Maggs et al. 2013). Tonometry can be measured indirectly through digital palpation, which is not very accurate and is not recommended, or by using specific instruments. Three techniques are used in veterinary medicine: indentation tonometry, applanation tonometry, and rebound tonometry (RBT) (Featherstone & Heinrich 2013).

For indentation, the Schiötz tonometer is used. The IOP is estimated, considering how far the cornea recedes under the action of a specific force generated by a platform supported on the anesthetized cornea. The result is converted to millimeters of mercury (mmHg) using specific tables for each species.

Flattening is considered one of the most accurate methods, consisting of estimating IOP using the pressure necessary to produce a flattening in a specific cornea area. In Veterinary Medicine, the most used tonometer is the Tonopen®, which has a contact surface of 3mm in diameter with the ceramic center of approximately 1mm that comes into contact with the cornea generating a signal, which is amplified, and sent to a microprocessor. The obtained value is shown on a small screen.

In RBT, the IOP is estimated with the help of the release of a magnetic field action against the cornea, measuring its deceleration after coming into contact with the eyeball. The TonoVet® model has calibration for dogs, cats, horses, and other species. It is necessary to displays the IOP value after obtaining an average of six consecutive measurements. (Slatter & Dietrich 2003, Jeong et al. 2007, McDonald et al. 2017). Recently, a new model has been available to the market; the Tonovet plus contains some updates aimed at improving the accuracy and being easy to use. It has specific calibration for rabbits, cats, dogs, and horses. It also presents audible and on-display indicators informing an incorrect measurement and the corresponding error (Gloe et al. 2019). Comparative IOP studies showed that the value of exam and other factors may vary according to the equipment. Data obtained from measurements with Tonovet and Tonovet Plus did not show statistical differences. However, the second one has greater accuracy in obtaining means (Zhang et al. 2014, Giannakopoulou & Williams 2019, Gloe et al. 2019). The RBT is becoming more popular in Veterinary Medicine as it provides a fast method of estimating IOP values (McDonald et al. 2017). An advantage of RBT is associated with topical anesthesia that is not necessary since contact with the cornea is very brief. In addition, the corneal flattening area is minimal (Takenaka et al. 2011).

Regarding the anatomy, IOP, and ocular ultrasound are not standardized for *B. variegatus*, limiting an adequate ophthalmic evaluation in these animals. Therefore, the need for research related to some information to fill this gap is remarkable. This study had the general objective of describing the macroscopic anatomy and the typical ultrasound aspects of the structures of animal eye bulb, specifying the average data for eye biometrics, and standardizing values for eye pressure to collaborate with scientific studies or assist in the clinical routine of this or other species of the superorder Xenarthra.

MATERIALS AND METHODS

For the ocular anatomy study, eight eye bulbs of four carcasses of the *Bradypus variegatus* species were used, from the collection of the Anatomy Area of the "Departamento de Morfologia e Fisiologia Animal" (DMFA), "Universidade Federal Rural de Pernambuco" (UFRPE), two males and two females, three adults and a young, weighing from 3 to 6kg. The carcasses were previously fixed with 20% formaldehyde and preserved in 30% saline. Subsequently, the eye bulbs were gently removed from the orbit by the transpalpebral enucleation technique proposed by Slatter & Dietrich (2003). After removing them, the eye bulbs were conserved in 70% alcohol and subsequently dissected to perform the anatomical description. Biometric data were measured using pachymetry, and measurements were obtained: AL of the eyeball, lens thickness, length of the vitreous chamber, and horizontal corneal diameter. The photographic images were obtained using a Canon Powershot Sx400is digital camera. For designation purposes, the "International Committee on Veterinary Gross Anatomical Nomenclature" determinations, Nomina Anatomica Veterinaria (2017), were used.

Four animals were assigned for *in vivo* ophthalmic analysis, three females and one male, three adults and one young weighing between 2.5 and 5kg, from the Parque Estadual Dois Irmãos, in the city of Recife. They underwent ophthalmic evaluation using slit-lamp biomicroscopy, and only those considered healthy were included in the study. All animals underwent intraocular pressure assessment of the right and left eyes using RBT (TonoVet®; Icare Finland Oy, Helsinki, Finland) calibrated for the device in P-mode, intended for different species of dogs, cats, and horses. An ultrasound examination was then performed in both eyes using the SonoSite® device, model MTurbo® (Fujifilm SonoSite, Washington, USA), with a linear transducer (6-13MHz) whose frequency was automatically determined by the device. Since these are small structures, the frequency used was close to the maximum allowed by the transducer. The images were obtained by transcorneal and transpalpebral techniques, and the scanning of the eyeball was performed in the vertical and horizontal axial plane. Each animal was placed in the arms of a technical assistant, and its head contained manually. Anesthetic eye drops of 0.5% proxymetacaine hydrochloride (Anestalcon®, Alcon, São Paulo, Brazil) and a layer of aqueous gel (Sterile Aquasonic 100®, Bio-medical instruments inc, Clinton twp, USA) were applied to the cornea for performing the exam in a comfortable way for the animal, without causing injuries. The same operator examined the eyes of all animals.

Biometrics was performed using an image obtained in horizontal section with the transducer positioned in the center of the cornea or eyelid. Positioning was considered satisfactory when it was possible to visualize the eyeball posterior wall as well as other critical intraocular structures such as the cornea, anterior and posterior lens capsules, lens, and vitreous chamber.

Five measurements were obtained from each eye: D1 = length of the anterior chamber, distance between the central point of the corneal image and the anterior lens capsule; D2 = lens thickness, distance between the anterior and posterior lens capsules; D3 = diameter of the lens; D4 = length of the vitreous chamber, distance between the posterior lens capsule and the posterior wall of the eyeball; D5 = AL of the eyeball, distance between the image of the cornea and the posterior wall of the eyeball. The measurements were obtained with the aid of an electronic cursor on the device itself.

The data of biometrics and ocular tonometry were grouped according to the eye and then analyzed. The Tukey test was applied with a significance level of 5%. The research was authorized by the Animal Use and Experimentation Ethics Committee No. 081/2016 and SISBIO No. 46665-3.

RESULTS

Eye anatomy

The sloth's eyeball has a cylindrical shape with an average axial length of 7.5±1.29mm and is fully inserted into the orbit. It is located anteriorly on the animal's head, with a level of rostral extend It is delimited anteriorly by the upper and lower eyelids and posteriorly by the retrobulbar adipose tissue and the orbital cavity; it presents a perforation in the posterior pole for the entry of the optic nerve and blood vessels.

The eyelids of the sloth are thick and have enough tensile strength of occlusion in the live animal. They are covered outside by a short hair layer and inside by the bulbar conjunctiva. Note the presence of the third eyelid, a fold of tissue located between the lower eyelid and the cornea, emerging in the nasal region of the lower conjunctival sac, being covered

by the bulbar conjunctiva. The bulbar conjunctiva near the limbus is thin and has no pigmentation.

The cornea is smooth, transparent, convex, on its anterior face, and circular, making up the anterior region of fibrous tunic. It is covered above by the eyelids, and its posterior external limit is the limbus, a place of transition between the cornea and sclera. It has an average horizontal diameter of 4±0.80mm. The sclera forms the outer layer of the eyeball; and it is variably white with shades of gray. It is located posterior to the limbus, immediately after the cornea. The sclera provides a site for the insertion of the musculature responsible for the ocular movement. Four muscles could be differentiated in this study: the rectus dorsal and rectus ventralis which were inserted in the upper and lower pole of the eyeball, respectively, and the medial rectus and lateral rectus muscle inserted in the nasal and temporal plane of the eyeball, respectively. These four muscles are inserted in the sclera posterior to the limbus through a small tendon. The identification of the dorsal and ventral oblique muscles was compromised due to limited technique and samples. They were located in some bulbs, but they could not be well differentiated at all. These are also inserted in the sclera in the region posterior to the limbus (Fig.1).

Inside the eyeball, posterior to the cornea, a small space is filled with aqueous humor, the anterior chamber that is delimited later by the iris. The iris is located between the cornea and the lens and in which is located the site of separation between the anterior and posterior chambers. It has brown pigmentation in all individuals analyzed. The sloth has a circular pupil; *in vivo*, the pupil remains circular when dilated or contracted. Immediately after the iris, the lens is surrounded by the anterior and posterior capsules and has a biconvex and circular shape with an average 4.5±0.57mm thickness.

After the posterior lens capsule, the vitreous chamber can be differentiated, filled by the vitreous body, a dense and slightly watery structure. This chamber has an average of 5.0±0.81mm in length. The retina-choroid-sclera complex (RCSC) forms the posterior wall of the eyeball. This region is

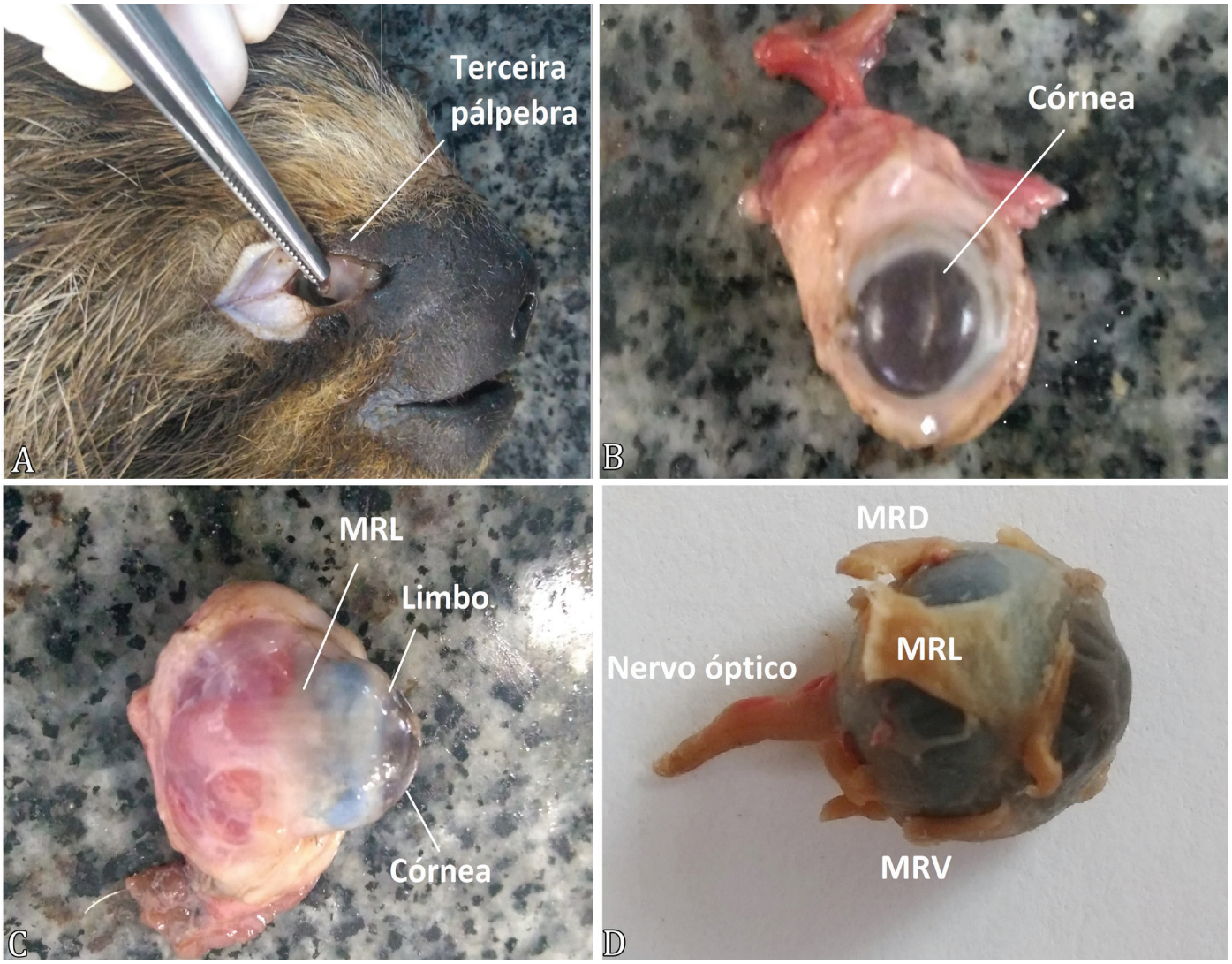


Fig.1. Sloth eye. (**A**) Traction of the third eyelid, (**B**) anterior view of the eyeball, (**C**) lateral view of the eyeball, immediately after enucleation, (**D**) side view of the eyeball. After dissection of the musculature. Lateral rectus muscle (LRM), dorsal rectus muscle (DRM), ventral rectus muscle (VRM).

completely pigmented with no apparent *tapetum lucidum*. In the macroscopic evaluation of the retina, it was not possible to identify the blood vessels in all ocular bulbs especially in some vascular fragments seen in the central region. The separation of these three segments could not be carried out, given the close communication between them and the visualization of the optical disc. At the posterior pole of the eyeball, the optic nerve enters into the orbit through the optic foramen and is accompanied by some blood vessels that provide nutrition for the eyeball.

Eye tonometry

There was no significant difference ($p>0.05$) in intraocular pressure between the right and left eyes. The mean IOP was 4.25±0.70mmHg (Table 1).

Ultrasonographic aspects and ocular biometrics

The ultrasound examination in B-mode was performed, as shown in Figure 2. According to this technique, the sloth's eyeball is characterized by a cylindrical structure, well delimited, presenting anechoic content (Fig.3) and composed of the anterior and posterior chambers and the vitreous chamber. The three chambers are presented as an anechoic structure with few reflective elements.

The structure closest to the transducer is the cornea, represented by a convex, hyperechoic, semicircular line. A space delimited previously by the cornea and posteriorly by the anterior capsule of the lens, the anterior chamber, filled with anechoic content, could be observed. The lens was visualized as an anechoic structure located between two concave and convex hyperechoic lines that corresponded to the anterior lens and posterior lens capsules, respectively. It was not possible to observe the iris and pupil by ultrasound examination. After the posterior lens capsule that extends to the posterior wall of eyeball, the vitreous chamber was located, characterized by an utterly anechoic area. The posterior wall of the eyeball was represented by a thin hyperechoic convex line, forming the scleral chorioretinal tissue layer. The optic nerve is represented by a thin hypoechoic area outlined by the retrobulbar space with moderate echogenicity. The average values of measurements of intraocular structures are shown in Table 2. Statistical analyzes using the Tukey's test at 5% significance showed no difference between the right and left eyes. The mean depth of the anterior chamber (length of the anterior chamber) was 0.63±1.11mm. The average lens thickness (lens length) was 3.73±0.24mm. The average lens diameter was 6.15±0.41mm, thus occupying 44% of the eye length. The average depth of the vitreous chamber (length of the vitreous chamber) was 3.70±0.27mm. The mean axial length of the eyeball was 8.48±0.22mm.

Table 1. Averages of intraocular pressure (IOP) in the right and left eyes in *Bradypus variegatus*

IOP	Animal A	Animal B	Animal C	Animal D	Mean	SD
RE (mmHg)	5	3	4	4	4	1.9
LE (mmHg)	5	4	4	5	4.5	0.5

IOP = Intraocular pressure, RE = right eye, LE = left eye, SD = standard deviation.



Fig.2. Performing an ocular B-mode ultrasonography in *Bradypus variegatus*.

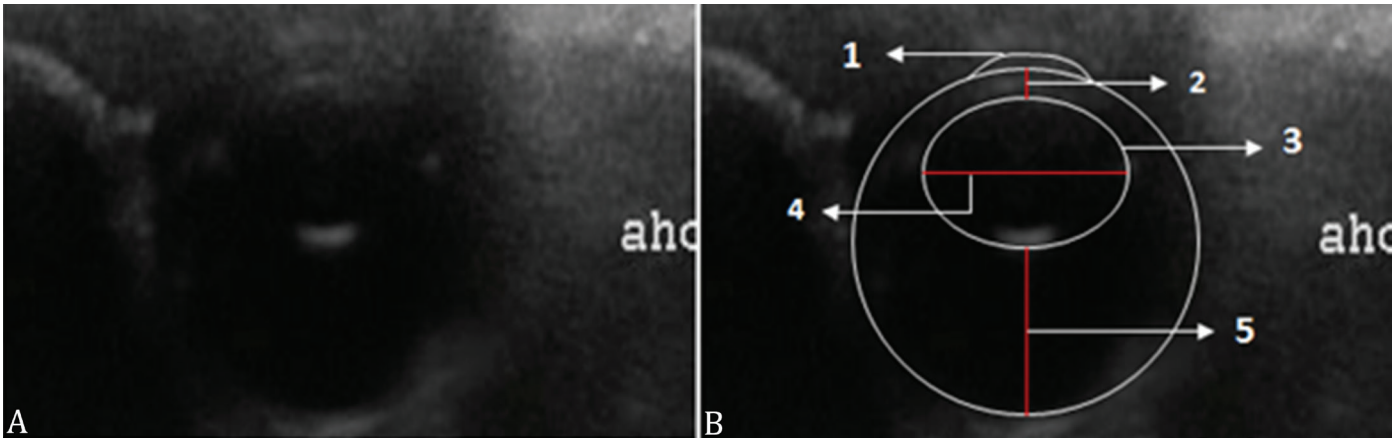


Fig.3. Image of ocular B-mode ultrasound for (**A**) normal sloth eye, and (**B**) schematic drawing of Figure 3A. Cornea (1), anterior segment (2), lens (3), lens diameter (4), glass chamber (5).

Table 2. Descriptive analysis of the results obtained by means of ocular ultrasonography in *Bradypus variegatus*

Eye data	Mean	SD	Maximum	Minimum	CV	CI (95%)
D1 (mm)	0.63	0.11	0.80	0.50	18.60	0.53 - 0.72
D2 (mm)	3.72	0.24	4.10	3.40	6.53	3.51 - 3.92
D3 (mm)	6.15	0.41	6.90	5.80	6.78	5.80 - 6.49
D4 (mm)	3.70	0.27	3.90	3.10	7.50	3.47 - 3.92
D5 (mm)	8.48	0.22	8.70	8.00	2.62	8.29 - 8.66

SD = Standard deviation, CV = coefficient of variation, CI = confidence interval, D1 = anterior chamber, D2 = lens thickness, D3 = lens diameter, D4 = glassy chamber length, D5 = axial length of eyeball.

DISCUSSION

To date, no descriptive studies on the anatomy of the ocular bulb of the sloth (*Bradypus variegatus*) have been found, so all the discussion on this subject were related from existing references to other mammals. The ocular morphology of *B. variegatus* is similar to that described for other domestic and wild animals, but with some peculiarities (Reece & Rowe 2017).

The eyeball is fully inserted into the orbit. The orbit is located on the anterior face of the skull - a similar finding was reported by Piggins & Muntz (1985) in the genus *Bradypus*. The front-facing eyes provide a wide binocular field of view that allows the animal to focus on nearby objects (Dyce et al. 2010). According to Samuelson (2013), the orbit depth contributes to the protection of the eyes and has a typical eye appearance. Its location in the skull largely influences the visual field extension and depth for certain species or breed. The position of the orbit varies between species. In cattle and horses, the eyes are located laterally, which provides a panoramic view. In dogs and cats, the eyes are located more anteriorly, emphasizing the binocular overlap between the two eyes. (Maggs et al. 2013). Piggins & Muntz (1985) reported that the angle between the diverging optical axis of each eye in *Bradypus* is estimated at 50 degrees and the monocular and binocular visual fields at 70 degrees with a binocular overlapping 35 degrees.

Four muscles inserted into the sclera could be identified. These muscles are responsible for eyeball movement in the vertical and horizontal directions (Dyce et al. 2010). As the dorsal and ventral obliques had limited identification, other muscles were associated with the quality of the samples used and the thickness of these tissues.

The third eyelid was found in the anatomical dissection. It is noted that its use could be identified in a few moments in the live animal in association with a stimulation of the cornea during the RBT performance. According to Johnson (1901), the third eyelid is absent in the family Bradypodidae, and this absence would be related to an evolutionary characteristic of the species. We consider that the non-visualization of the Johnson (1901) structure was associated with the type of procedure performed. It is used direct ophthalmoscopy in live animals for eye evaluation, however, an anatomical study on carcass was not performed and corneal stimulation was not used. The anatomical positioning of the third eyelid in the sloth can be compared to the positioning described for some domestic species such as dogs and cats, where it is also located in the medial angle of the eye, between the lower eyelid and the eyeball (Dyce et al. 2010). This structure is absent or is reported as vestigial in some species, such as the capybara (*Hydrochaeris hydrochaeris*) and chinchillas (*Chinchilla lanigera*) (Montiani-Ferreira et al. 2008, Lima et al. 2010, Montiani-Ferreira & Lima 2014).

The circular pupil in mydriasis or miosis was described by Johnson (1901) in armadillos (*Dasypus villosus*). The shape of the pupil varies between species. In primates, canines, and large cats, it has a circular shape, while in small cats, as in the domestic cat, it appears as a vertical-slit pupil when contracted. Animals with slit-shaped pupils are more sensitive to light than those with circular pupils (Samuelson 2013). According to Goffart (1971), sloths are animals with eyes that are mainly adapted for low-intensity light vision.

An anatomical description of the eyeball was performed by Piggins & Muntz (1985) in the species *Bradypus tridactylus*. According to the authors, this eye type has an average axial length of 8.4mm and a spherical shape. Moreover, it described a wide and quite convex cornea. Findings for the species *B. variegatus* are similar to the *B. tridactylus*, it has an average axial length of 7.5mm, an exposed ocular surface between the eyelids composed of the cornea that is broad and convex, but with an ocular bulb presenting a cylindrical shape. The sloth has a shallow anterior chamber when compared to the AL of the eyeball. It has a lens that occupies about 40% of the ocular volume; a similar proportion has been described in chinchillas (Lima et al. 2010). These characteristics may be related to the visual acuity of the species. A lens that occupies a large space in the eye causes the anterior chamber to approach the cornea, and its posterior segment is closer to the retina. These anatomical characteristics can be related to a balance connected with the low visual acuity of the species. According to Goffart (1971) and Hayssen (2010), animals of the genus *Bradypus* are incredibly myopic, do not have ciliary muscle or macula, their retina comprises many thin stem cells with few retinal ganglion cells (RGCs), and cones are rare or absent.

The vitreous chamber is nearly filled by the vitreous body, with its posterior wall composed of the RCSC. In the retina and choroid coat, the blood vessels are poorly differentiated macroscopically. Johnson (1901) considers this characteristic common to species of Xenartros. Goffart (1971) used the classification of pseudoangiotics.

In previous years, several investigations were carried out in order to determine the normal IOP of several species (Leiva et al. 2006, Broadwater et al. 2007, Blackwood et al. 2010, Rusanen et al. 2010, Pereira et al. 2011, Falcão et al. 2017, McDonald et al. 2017, Hibbs et al. 2018), however, there is no report describing normal values for sloths.

The RBT has been increasingly used in veterinary ophthalmology. TonoVet® was designed its use in animals with small eyes (Falcão et al. 2017). Its software is pre-programmed for six measurements, and the IOP is displayed as an average value of six consecutive central corneal rings. It is calibrated for dogs and cats as described for D-mode, horses for H-mode, and other species for P-mode (Jeong et al. 2007). As the RBT does not present an internal calibration table to measure IOP in sloths, the calibration table, that is not specific to any species, was used in these animals, that is, the P-mode.

Some factors can alter the final IOP value, such as stress, anesthesia, and external eye pressure (Pereira et al. 2011). Manual restraint was performed subtly to reduce the stress of the animals, so that, there was no interference in IOP measurement. According to Corrêa et al. (2014), the use

of mydriatic drugs can affect intraocular pressure leading to hypertension; therefore, no agent or any type of ocular medication was performed until IOP measurement. The use of TonoVet® to measure IOP did not cause discomfort to animals, considered a fast and well-tolerated technique, as has also been described by other authors (Leiva et al. 2006, Jeong et al. 2007, Rusanen et al. 2010, McDonald et al. 2017).

The measurement of IOP by using RBT has been carried out in recent years, and standards for different species have been established with the use of TonoVet®. Studies have been carried out on various domestic and wild species such as parrots (11.4mmHg), alpacas (14.2mmHg), donkey (25.5mmHg), cats (20.74mmHg), dogs (9.15mmHg), goats (7, 9mmHg), rabbits (9.51mmHg), bats (19.3mmHg) (Leiva et al. 2006, Broadwater et al. 2007, Blackwood et al. 2010, Rusanen et al. 2010, Pereira et al. 2011, Falcão et al. 2017, McDonald et al. 2017, Hibbs et al. 2018).

The values of IOP were similar to those observed in *B. variegatus* (4.25mmHg) and have also been described in chinchillas (*C. lanigera*) (2.49mmHg) and bullfrog (*Rana catesbeiana*) (4mmHg) (ChacaltanaI el al. 2016, Cannizzo et al. 2017). Cannizzo et al. (2017) related the low IOP in bullfrogs using TonoVet® in P-mode, according to this study, the P-mode tends to underestimate IOP measurements when compared to D-mode. Previous studies have shown that RBT can underestimate the IOPs less than 25mmHg in cats (Rusanen et al. 2010) and more significant than 70mgHg in horses (Knollinger et al. 2005). Löbler et al. (2011) carried out a study that aimed to calibrate the RBT using the Icare TAO1 tonometer, calibrated for humans, pigs and rabbits. They concluded that RBT tends to underestimate the true values of IOP in these animals. It can be used to determine IOP as long as the measured values are corrected with the appropriate linear function.

We consider that the mean obtained for *B. variegatus* by using TonoVet® may be linked to a physiological value of the species. However, further studies comparing the results obtained with the aid of TonoVet® with measures obtained by directly measuring IOP (manometry) are necessary to establish RBT accuracy in sloths. The discrepancies in the IOP values described significant interspecific variation. These occur due to the specific anatomical and physiological peculiarities of each species, such as the shape of the eyeball, the circadian rhythm, formation and drainage of aqueous humor flow, cornea size or the examiner's experience (Pereira et al. 2011, Rodarte-Almeida et al. 2013, Falcão et al. 2017), which makes the comparison between unfeasible or perhaps different species and emphasizes the need to establish reference standards as well as the calibration of TonoVet® for each species.

As it is a non-invasive and painless technique, the ultrasonography was a well-tolerated test in all animals. Manual restraint associated with only one drop of anesthetic eye drops of 0.5% proxymetacaine hydrochloride was sufficient to allow an adequate assessment, excluding the need for anesthetic drugs as described by Dziezyc et al. (1987) and Mattoon & Nyland (2015). According to Gonzalez et al. (2001), it is preferable to avoid the patient's sedation, so that the rotation and retraction of the eye are minimized. There were no complications, such as ocular trauma seen after the ultrasound. Such factors make ocular ultrasound a feasible diagnostic method for the routine of ophthalmic evaluation in *B. variegatus*.

According to Dziezyc et al. (1987), ocular imaging via ultrasound is easy to obtain, in which the eye is an excellent organ for the exam. In *B. variegatus*, there was some difficulty to obtain the images due to the serene membrane of the small corneal surface concerning the size effect of transducers and due to the relatively thick eyelids, thus making manual retraction difficult for adequate corneal exposure. This anatomical feature influenced the technique used to obtain the images. In some animals, it was not possible to perform the transcorneal and transpalpebral techniques, even being the most commonly used. According to Mattoon & Nyland (2015), the transpalpebral technique is easier to be performed. However, the image quality is inferior compared to the positioning of the transducer directly in the cornea. It is described in the literature that the transcorneal technique is the method of choice for evaluating intraocular structures, as it allows obtaining the most distinctive images of the eyeball and orbit and better visualization of the vitreoretinal and retrobulbar structures (Gonzalez et al. 2001). Whereas the transpalpebral technique allows an adequate assessment of the vitreous chamber, retina, and deeper orbital structures; on the other hand, the lens and the anterior chamber cannot be satisfactorily evaluated, even if a cushion indentation is used (Mattoon & Nyland 2015). In addition, it is reported that the trichotomy of the eyelid improves the quality of the ultrasound image, as it reduces the amount of air between the skin and the transducer (Mattoon & Nyland 2015). However, because these animals were in the process of rehabilitation, trichotomy was not performed. Despite these facts, in the present study, obtaining ocular images for ocular biometrics was not impaired with this technique. However, a more detailed assessment of the anterior segment was impaired with the transpalpebral technique.

Ultrasound gel was used for comfort and better assessment of intraocular structures, which allowed the transducer to be distanced a few millimeters from the ocular surface, as described by Soares et al. (1998), and served as a cushion indentation for better visualization of the cornea when a thicker layer was used.

The ocular A-mode ultrasonography is described as the method of choice for performing eye biometrics. However, the ocular B-mode ultrasonography allows a more accurate assessment of eye structures, and biometrics can be performed safely, thus being a preferred method. It is currently the most used in veterinary ophthalmology, and its use is recommended by many authors (Gonçalves et al. 2009, Merlini et al. 2011, Mattoon & Nyland 2015, Ruiz et al. 2015, Morais et al. 2019).

The use of a 6-13MHz transducer in the ocular B-mode ultrasound examination was considered adequate for a general assessment of the eyeball since it was possible to visualize and measure the most critical internal structures. A 10 or 12.5MHz transducer is ideal for a more detailed assessment of the bulb. However, orbit and bulb can be visualized with a 7.5MHz transducer, but it is impossible to differentiate the retina from the choroid or sclera with transducers less than 10MHz (Petersen-Jones & Crispin 2002).

In ocular ultrasound of animals, in general, the cornea is represented by a curvilinear hyperechogenic line in the proximal field of the screen (Dudea 2011). The anterior

chamber is then filled with anechoic material, outlined later by the iris (Mattoon & Nyland 2015). The lens is represented as an anechoic structure located between two curved echogenic lines, the anterior and posterior lens capsules (Merlini et al. 2011). The vitreous chamber is represented by the anechoic region posterior to the lens (Gonzalez et al. 2001), and the posterior wall of the eyeball is seen as a single echogenic line being called the RCSC, as the three layers cannot be identified separately (Gonzalez et al. 2001). All the above statements were compatible with those found in the ocular ultrasounds of *B. variegatus*, making the ocular ultrasound anatomy of the species similar to those described for other wild and domestic species (Gonzalez et al. 2001, Montiani-Ferreira et al. 2008, Mattoon & Nyland 2015).

The cornea and the anterior segment could not be adequately evaluated with a 6-13MHz transducer. For better image resolution and more detailed discrimination of small structures, 20MHz transducers should be used (Dietrich 2013).

Ocular biometrics can be used to determine ocular change, shape, or size as it can occur in conditions such as glaucoma. The biometric measurements obtained in this study were the same as those defined in previous studies (D1 = anterior chamber, D2 = lens thickness, D3 = lens diameter, D4 = vitreous chamber length, D5 = AL of the eyeball). However, the mean values differed, concluding that measurements vary according to each species due to their eye anatomy. Therefore, the values of such measurements are not compared between species. No study on ocular biometrics in the species *B. variegatus* was found in the literature.

In a statistical analysis at 5% of significance, no significant difference was found between the biometrics of the left and right eyes, which is in agreement with the findings of other authors for other species that used ocular B-mode ultrasound to perform eye biometry (Hernández-Guerra et al. 2007, Squarzoni et al. 2010, Toni et al. 2010). All measures of sloth eye presented a remarkable homogeneity, especially compared to ocular biometric values.

Some values obtained with ultrasound biometrics could be compared to the values obtained with the study of macroscopic anatomy. The compared parameters were the AL of the eyeball, the lens thickness, and the length of the vitreous chamber. There was a difference of ±1mm between the measurements, but all were compatible with the ultrasound examination measurements, with no statistical difference between means. However, *post mortem* changes can affect measurements due to variation in muscle tone and fluid volume that can lead to changes in the eyeball dimensions.

CONCLUSIONS

The ocular anatomy of the species *Bradypus variegatus* is similar to those features described for other species with some peculiarities. They present a retina with poorly differentiated blood vessels.

By using the RBT, it was possible to measure the IOP in this species, being easy to perform and well accepted by the animals.

The ocular B-mode ultrasound is a fast, safe, reliable, and well-tolerated procedure for sloths. Its ocular ultrasound anatomy was similar to that observed in other species. Biometric measurements such as depth of the anterior chamber, the lens thickness, the diameter of the lens, depth of the vitreous chamber, and AL of the eyeball could be performed by this method.

The techniques employed in this study can be routinely used in medical practice. The data obtained can assist in diagnosing eye diseases and favor the management and preservation of the species. Further research in histology and physiology is necessary for a better understanding of the species' visual function.

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Original Article
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Horses fed meal contaminated with mycotoxins performing short-term exercises[1]

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ABSTRACT.- Braga A.C., Almeida F.Q., Silva V.P., Almeida M.I.V., Keller K.M., Diegues V.A., Thomes F.R. & Rosa C.A.R. 2020. **Horses fed meal contaminated with mycotoxins performing short-term exercises**. *Pesquisa Veterinária Brasileira 40(9):717-725*. Programa de Pós-Graduação em Ciência, Tecnologia e Inovação em Agropecuária, Universidade Federal Rural do Rio de Janeiro, Rodovia BR-465 Km 7, Seropédica, RJ 23890-000, Brazil. E-mail: auro_vet@hotmail.com

This research was carried out to evaluate the effect of mycotoxins on the performance of horses through physiological parameters, and hematology and serum biochemistry analyses. The essay lasted 40 days, with 12 days for adaptation and 28 days of experimentation. In the experimental stage, the horses were distributed in a completely randomized design, with three treatments with four animals each. The treatments used were 0 (control), 50 ppb and 100 ppb of Aflatoxin B_1 (AFB_1) added to a concentrate in a basal diet. The basal diet contained mycotoxins from feedstuffs naturally contaminated. The exercise test was performed over the 21th day of the experimental stage. The exercise consisted in an interval training test with a warm-up of 17 mins at a trot followed by three gallops of 450m/min. The heart rate was monitored between the gallops. Before the exercise test and immediately after the third gallop, the physiological and blood parameters were evaluated, and continued up to 48 hours after the exercise. The results of the physiological, hematological and biochemical parameters were submitted to analysis of variance (ANOVA) and compared by the Tukey test at 5% of significance. The presence of AFB_1 in the diet influenced the alkaline phosphatase activity, which presented higher values in horses fed diet with inclusion of 100 ppb AFB_1, suggesting a hepatotoxic activity associated with the others mycotoxins naturally present in the feedstuffs.

INDEX TERMS: Horses, contaminated meal, short-term exercises, alkaline phosphatase, exercise, mycotoxins, morphology.

RESUMO.- [**Efeitos das micotoxinas na performance de equinos submetidos a exercícios de curta duração.**] Esta pesquisa foi conduzida para avaliar o efeito de micotoxinas no desempenho de equinos com avaliações fisiológicas e análises hematológicas e da bioquímica sérica. O ensaio durou 40 dias, com 12 dias de adaptação e 28 dias de experimentação. Na fase experimental, os equinos foram distribuídos em delineamento inteiramente casualizado em três tratamentos,

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com quatro animais cada. Os tratamentos utilizados foram 0 (controle), 50 ppb e 100 ppb de Aflatoxina B_1 (AFB_1) adicionada ao concentrado de uma dieta basal. A dieta basal continha alimentos naturalmente contaminados por micotoxinas. O teste de desempenho foi executado no 21° dia da fase experimental por meio de teste intervalado consistindo em aquecimento ao trote por 17 minutos, seguido de três galopes de 450m/min. A frequência cardíaca (FC) foi monitorada entre os galopes. Antes do exercício e imediatamente após o terceiro galope, os parâmetros fisiológicos e sanguíneos foram avaliados e continuaram sendo monitorados até 48 horas após o exercício. Os resultados dos parâmetros fisiológicos, hematológicos e bioquímicos foram submetidos à análise de variância (ANOVA) e comparados pelo teste de Tukey a 5% de significância. A presença de AFB_1 na dieta influenciou a atividade da fosfatase alcalina, que apresentou valores mais elevadas na dieta com inclusão de 100 ppb de AFB_1, sugerindo uma atividade hepatotóxica associada às outras micotoxinas naturalmente presentes nos alimentos.

TERMOS DE INDEXAÇÃO: Micotoxinas, equinos, exercícios de curta duração, fosfatase alcalina, micotoxinas, morfologia.

INTRODUCTION

Several mycotoxins have been identified in foods and diets for humans and animals in Brazil. The importance of aflatoxins (AF), fumonisins (FB), ochratoxins (OTA), zearalenone (ZEA) and trichothecenes should be emphasized due to their occurrence and also their high toxigenic potential for farm animals (Baldissera et al. 1993, Franco et al. 2019). In addition, the additive or synergistic effect between different toxins, which increase or alter their effects on animals, should be considered. Although mycotoxins have several adverse effects on animal health, little is known about their impacts in the horse nutrition (Liesener et al. 2010, Schumann et al. 2016). Raymond et al. (2005) pointed out that, especially for sport horses, chronic intake of low mycotoxin levels in diets can affect performance without animals showing typical clinical signs of mycotoxicosis.

Aflatoxins are a class of carcinogenic mycotoxins produced by *Aspergillus* fungi and are widely distributed in nature, especially in a variety of food commodities. Aflatoxin B_1 (AFB_1) is confirmed to be the most toxic of all the aflatoxins and has been well-characterized to lead to the development of hepatic cellular carcinoma (HCC) in humans and animals. The occurrence of these mycotoxins, alone and in combination, representes a real risk for human and animal health (Bertero et al. 2018, Rushing & Selim 2019).

Although the importance of mycotoxins in public and animal health is recognized, there is no uniform regulation of mycotoxin limits in foods in the different countries, particularly in Mercosur countries (Brazil/Argentina), (Mercosul/GMC 2002). The Brazilian legislation (Brasil 1988) establishes the maximum limit of 50µg/kg for aflatoxins in any feedstuffs used directly or as an ingredient in diets for animals but does not specify limits for other mycotoxins. This research was carried out to evaluate the inclusion of aflatoxin B1 (AFB_1) in the diet and to verify the effects on metabolism and performance of horses.

MATERIALS AND METHODS

The experiment was conducted at the Army Sergeant School (ESA), latitude 21°42'15.6'' South and longitude 45°15'18.9'' West, of the Brazilian Army, in Três Corações, Minas Gerais State. The assay was performed in accordance with the principles of ethics in experimentation approved by the committee on ethics in animal experimentation from the University: CEUA-UFRRJ Number 1212031017.

Experimental period. The total experimental period was 40 days, divided into two stages: one was the pre-experimental or adaption stage of 12 days and the other was the experimental stage of 28 days. A total of 12 adult Brazilian Sport Horses (9 females and 3 males), aged between 5 and 8 years old with an average body weight of 458.02±22.09kg were used. The research was conducted in a completely randomized design, with three treatments with four animals each, and the animal was the experimental unit. The experimental treatments used were: Treatment I = control diet (0µg/kg of AFB_1); Treatment II = diet with 50µg/kg of AFB_1; and Treatment III = diet with 100µg/kg AFB_1. The AFB_1 was added to the basal diet (Table 1). The experimental basal diet was composed by Tifton hay (*Cynodon* spp.) and alfafa (*Medicago sativa*), commercial concentrate (S Line -280, Royal Horse®), mineral salt and water *ad libitum*, elaborated according to the NRC (2007) for mature horses. The horses remained free in their paddocks during the day and were housed in individual stalls at night. The animals were fed according to the schedule of the Military Unit, which is 2kg of concentrate meal at 05:00 a.m. and 2kg at 17:00 p.m., alfafa hay at 10:00 a.m., and tifton hay at 13:00 p.m. and 20:00 p.m. Every day during the experimental period at the 17:00 p.m. feeding time, 1kg of concentrate feed with AFB_1 was prepared and given to the animals, according to the treatment. Before the adaptation period, all feedstuffs were analyzed, and the concentrations of mycotoxins in the basal diet were: 5.620µg/kg of DON, 708µg/kg of ZEA and 560µg/kg of FB_1.

Clinical conditions. Before, during and after the experimental period, clinical, hematological and biochemical analyzes of the horses were carried out to check the health conditions, especially regarding the presence of liver damage. Throughout the experimental period, the clinical conditions of the animals were monitored, daily, by a veterinarian.

Aflatoxin production and estimation. The aflatoxin B_1 was produced by the Mycology Laboratory of Federal University Rural of Rio de Janeiro from fermenting rice with a spore suspension of *Aspergillus parasiticus* (NRRL 2999), according to Shotwell et al. (1966). The AFB_1 content was extracted and purified through a Mycosep® Aflazon column (Romer Labs Diagnostic GmbH., Tulln,

Table 1. The average composition (g or µg /kg on dry matter basis) of the experimental diets with different inclusions of AFB_1

Diet Components	Treatments		
	T I (Control)	T II (50 ppb AFB_1)	T III (100 ppb AFB_1)
Tifton hay/Alfalfa (g)	7.400	6.900	6.600
Commercial concentrate (g)	4.000	4.000	4.000
Mycotoxin concentration (µg)			
Aflatoxin B_1 (AFB_1)	0	50	100
Zearalenone (ZEA)	2.758,1	2.687,5	2.655,2
Deoxynivalenol (DON)	21.564,7	20.689,9	20.289
Fumonisins B_1 (FB_1)	2.240	2.240	2.240
Total (g)	11.400	10.900	10.600

Austria) as per the manufacturer's instructions and an aliquot (200µl) was derivatized with 700 µl trifluoroacetic acid-acetic acid-water (20:10:70, v/v). The derivatized aflatoxin was quantified by High Performance Liquid Chromatography (HPLC), at the "Laboratório de Micologia e Micotoxinas" of "Universidade Federal de Minas Gerais", following the methodology proposed by Trucksess et al. (1994).

Aflatoxins were quantified by injecting 20µL of extract from each vial into an HPLC system consisting of a JASCO model LC-2000 pump (Tokyo, Japan) connected to a JASCO model FP-2020 programmable fluorescence detector and a data module JASCO model 6937-J041A (ChromNAV). Chromatographic separations were performed on a stainless steel, C18 reversed-phase column (Supelcosil™ LC-ABZ, 150 x 4.6mm, 5µm particle size) connected to a precolumn (Supelguard™ LC-ABZ, 20 x 4.6mm, 5µm particle size). The mobile phase was water:methanol:acetonitrile (4:1:1, v/v/v) at a flow rate of 1.0ml min^{-1}. Fluorescence of aflatoxin derivatives was recorded at excitation and emission wavelengths of λ 360nm and λ 460nm, respectively. Standard curves were constructed with different levels of AFB_1. This toxin was quantified by correlating peak heights of sample extracts with those of standard curves. The detection limit of the analytical method was 0.4ng g^{-1}.

The concentration of AFB_1 yielded was 4.25mg/kg and it was diluted daily in the concentrate feed to obtain the desired concentrations according to the equation: Ci xVi = Cf x Vf, (where Ci and Cf are the initial and final concentrations and Vi and Vf, the initial and final volumes).

Performance evaluation. The performance was assessed through an exercise test at the 21th day of the experimental stage. The average temperature during the day was 21.5°C, with a minimum of 12.2°C and maximum of 28.1°C and the relative humidity was 62.4%. The animals performed standard exercise training for 50 minutes, 5 days per week, during three weeks of the experimental stage. The training program of walking and trotting had been intercalated with flexion movements. For the exercise test, the animals were randomly split into 3 groups with 4 animals each. The test started at 9:00 a.m. and finished at 12:06 a.m. on the same day; they were performed sequentially for each group. The test consisted of a 17 min warm-up of walking and trotting followed by three sets of gallops at a speed of 450m/min for three minutes each. In the intervals between the gallops, the horses were monitored for heart rate (HR), and when the heart rate returned to 60-70bpm the animals moved on to the next gallop. In order to evaluate the performance of the animals in the test, the heart rate, the respiratory frequency was recorded. Blood samples were taken to analyze the globular volume, glucose, lactate and blood biochemistry. These variables were obtained before the exercise test, and after the third gallop and continued up to 48 hours after the test as described: M0 (Pre-exercise), M1 (Immediately after the exercise test), M2 (10min), M3 (20min), M4 (60min), M5 (3h), M6 (6h), M7 (12h), M8 (24h) and M9 (48h).

Heart rate and respiratory rate were recorded with a stethoscope (Mod LittmannMR). Blood samples were collected using a vacuum collection needle (BD®) and vacuum tubes with EDTA (BD Vacutainer®) for the complete blood count (CBC). Vacuum tubes with sodium fluoride (BD Vacutainer®) were used to determine glucose and lactate measures and vacuum tubes without anticoagulant (BD Vacutainer®) were used to obtain blood serum samples, for biochemical determination of enzymatic activity, total proteins and fractions, cholesterol, urea and creatinine measures. After collection, the samples were centrifuged at 7.000rpm for 10 minutes to separate the plasma and serum. Aliquots of both plasma and serum of 1.0mL were pipetted automatically and stored at -20°C in 1.5mL polypropylene tubes until required for analysis. Subsequently, the analyzes were performed by the enzymatic/colorimetric method in a spectrophotometer SB 190 (CELM® Cia Equipadora de Laboratórios Modernos - CELM. Brazil). Labtest® kits were used for all analyzes.

Statistical analysis. The results were evaluated for normality by Lilliefors test and the homogeneity by Cochran and Bartlett test. Data of the hematological, biochemical and physiological variables were submitted to analysis of variance (ANOVA) in a split-plot scheme with the levels of inclusions of AFB_1 in the diets evaluated in the parcels and the exercise test times evaluated in sub-parcels. The means were compared by the Tukey test, at 0.05 significance level, using the Statistical and Genetic Analysis System (SAEG 2007).

RESULTS

Occurrence of mycotoxins in feedstuffs

The mycotoxins observed in the feedstuffs of basal diet were: DON, 5.620µg/kg, in Tifton and alfalfa hay and commercial concentrate; ZEA, 708µg/kg, in alfalfa hay and commercial concentrate; and FB1, 560µg/kg, in commercial concentrate. The intake of mycotoxins by the horses according to the treatments are given in Table 2.

Physiological parameters

The intake of aflatoxin B_1 did not affect the respiratory rate (RR) and heart rate (HR) values of horses in the exercise test (P>0.05), however, there was a simple effect of the time of evaluation (P<0.05) (Table 3).

Blood and biochemical parameters

The inclusion of aflatoxin B_1 in the diets did not affected the blood parameters of the horses in the exercise test, except the enzyme alkaline phosphatase (ALP) (Table 4) (P<0.05). The globular volume (GV), total protein (TP), albumin (ALB) and cholesterol (COL) were evaluated at M0 through to M3 of the exercise test, and the results have been influenced by the exercise (P<0.05), with any effect from aflatoxin B_1 inclusion to the diets (P>0.05) (Table 5).

Table 2. Means of the mycotoxin intake per kg of dry matter (Kg DM) and per kg of body weight (kg BW), according to the treatments

Variables		T I (Control)		T II (50 ppb AFB1)		T III (100 ppb AFB1)	
		Kg DM	Kg BW	Kg DM	Kg BW	Kg DM	Kg BW
Mycotoxins intake (µg)	AFB1	0	0	4.6	0.1	9.4	0.2
	ZEA	241.1	6.1	246.7	5.7	249.3	5.9
	DON	1885.1	47.6	1899.1	43.9	1904.7	45.3
	FB1	195.8	4.9	205.6	4.8	210.3	5.0

Table 3. Respiratory rate and heart rate of horses fed diets with different inclusions of AFB_1 and submitted to an exercise test

Variable	Average	CV (%)	Interactions (P Value)		
			AFB_1	Time	AFB_1 X Time
Respiratory rate (mov/min)	13.3	21.5	NS	0.0000	NS
Heart rate (beat/min)	66.9	16.0	NS	0.0000	NS

CV = Coefficient of variation, NS = not significant.

The serum concentrations of urea and creatinine were evaluated at many times of the exercise test. The results showed that these parameters were not influenced by the aflatoxin B_1 inclusion in the diets (P>0.05). However, a simple effect of the exercise test was observed (Table 6).

The enzymes aspartate transferase (AST), lactate desidrogenase (LDH) and gama glutamil transferase (GGT) activities were evaluated from M0 to M9 of the exercise test, while the enzyme creatine quinase (CK) was evaluated from M0 to M7 of the exercise test. The activities of the enzymes were not affected by aflatoxin B_1 inclusion to the diets (P>0.05). However, the AST, LDH, GGT and CK activities were influenced by the exercise (P<0.05) (Table 7). The alkaline phosphatase (ALP) activity was affected by the inclusion of aflatoxin in the diets (P<0.05), presenting a higher value in horses fed diet with 100µg/kg of $AFB_{1,}$ (Table 8).

Table 4. Hematological and blood biochemistry of horses fed diets with different inclusions of AFB_1 and submitted to an exercise test

Variable	Average	CV (%)	Interactions (P Value)			Reference values
			AFB_1	Time	AFB_1 X Time	
Globular Volume (%)	39.8	5.5	NS	0.0000	0.1709	38-42[A]
Total protein (gdL^{-1})	6.2	16.8	NS	0.0003	NS	5.2-7.9[C]
Albumins (g dL^{-1})	2.4	17.1	NS	0.0001	0.2319	2.6-3.7[C]
Cholesterol (mg dL^{-1})	97.4	16.1	NS	0.0014	0.4034	75-150[B]
Urea (mg dL^{-1})	33.4	10.4	NS	0.0000	0.4247	24-48[A]
Creatinine (mg dL^{-1})	0.74	10.5	NS	0.0000	NS	1.1-1.8[A]
GGT (U L^{-1})	7.8	38.5	NS	0.0000	0.1161	4.3-13[E]
AST (U L^{-1})	271.3	16.3	0.0671	0.0000	0.1279	150-400[A]
CK (U L^{-1})	323.7	27.8	0.3226	0.0000	NS	100-300[A]
LDH (U L^{-1})	936.3	36.5	0.2882	0.000	NS	162-412[C]
ALP (U L^{-1})	61.4	18.5	0.0227	0.3679	0.3625	100-500[D]
Glucose (mg dL^{-1})	74.8	20.2	NS	0.0000	NS	70-140[A]
Lactate (mmol L^{-1})	1.8	19.6	0.927	0.0000	NS	1,1-1,8[F]

[A] Rose & Hodgson (1994), [B] Robinson (1997), [C] Gonzalez & Silva (2006), [D] Kerr (2002), [E] Thomassian (2005), [F] Kaneko (2008); CV = coefficient of variation, NS = not significant.

Table 5. Mean values of Globular volume (%), Total protein (g dL^{-1}), Albumins (g dL^{-1}) and Cholesterol (mg dL^{-1}) in horses fed diets with different inclusions of AFB_1 and submitted to an exercise test

Variable	Exercise test evaluation times				CV (%)
	Pre-exercise	Immediately after the exercise test	10 min	20 min	
Globular volume (%)	34.2[D]	47.6[A]	39.9[B]	37.4[C]	5.5
Total protein (g dL^{-1})	4.9[B]	7.0[A]	6.6[A]	6.4[A]	16.8
Albumins (g dL^{-1})	1.9[B]	2.8[A]	2.5[A]	2.6[A]	17.1
Cholesterol (mg dL^{-1})	81.4[B]	109.5[A]	96.7[AB]	101.8[A]	16.1

CV = Coefficient of variation; [A,B,C,D] Values followed by same letter in the line do not differ by the Tukey test (P>0.05).

Table 6. Serum concentrations of urea and creatinine (mg dL-1) in horses fed diets with different inclusions of AFB_1 and submitted to an exercise test

Variable	Exercise test evaluation times										CV%
	MO	M1	M2	M3	M4	M5	M6	M7	M8	M9	
Urea (mg dL^{-1})	37.5[AB]	36.2[BC]	28.8[EF]	30.0[DEF]	32.1[AB]	30.2[DEF]	33.4[BCD]	37.1[AB]	42.6[A]	26.2[F]	10.4
Creatinine (mg dL^{-1})	1.4[A]	0.7[B]	0.7[B]	0.7[B]	0.5[D]	0.6[CD]	0.7[B]	0.8[B]	0.7[BC]	0.5[D]	10.5

CV = Coefficient of variation; [A,B,C,D,E,F] Values followed by same letter in the line do not differ by the Tukey test (P>0.05).

Table 7. Activity of CK, AST, LDH, GGT (UL^{-1}) in horses fed diets with different inclusions of AFB_1 and submitted to an exercise test

Variable	Exercise test evaluation times										CV %
	MO	M1	M2	M3	M4	M5	M6	M7	M8	M9	
CK (UL^{-1})	328.4[BC]	288.5[C]	343.3[BC]	484.2[A]	399.9[AB]	296.5[C]	308.7[BC]	139.7[D]	-	-	27.8
AST (UL^{-1})	291.7[ABC]	326.6[A]	245.6[CD]	255.9[BCD]	268.6[ABC]	209.2[D]	292.3[ABC]	311.4[AB]	304.2[ABC]	207.5[D]	16.4
LDH (UL^{-1})	932.0[ABC]	750.2[BC]	733.9[BC]	693.9[C]	1169.8[AB]	806.2[ABC]	938.7[ABC]	1257.4[A]	1156.5[A]	823.9[ABC]	36.5
GGT (UL^{-1})	6.2[CD]	7.2[BCD]	7.2[BCD]	6.2[CD]	9.6[AB]	7.8[BC]	7.3[BCD]	9.7[AB]	11.8[A]	5.0[D]	38.5

CV = Coefficient of variation; [A,B,C,D] Values followed by same letter in a row do not differ by the Tukey test (P>0.05).

Table 8. Alkaline phosphatase activity (UL^{-1}) in horses fed diets with different inclusions of AFB_1 and submitted to an exercise test

Treatment	Exercise test evaluation times				Average	Interactions (P Value)		
	M0	M1	M2	M3		AFB_1	Time	AFB_1 time
T I (Control)	65.3	49.8	44.5	47.5	51.8^{B}			
T II (50 ppb AFB_1)	55.5	56.5	59.0	54.0	56.3^{AB}	0.0227	0.3679	0.3625
T III (100 ppb AFB_1)	75.8	82.5	75.0	72.0	76.3^{A}			

Coefficient of variation (CV) = 18,5%; ^{A,B} Values followed by same letter in a column do not differ by the Tukey test (P>0.05).

Table 9. Glucose (mg dL-1) and Lactate (mmol dL-1) concentrations in horses fed diets with different inclusions of AFB_1 and submitted to an exercise test

Variable	Exercise test evaluation times										CV %
	M0	M1	M2	M3	M4	M5	M5	M7	M8	M9	
Glucose (mg dL^{-1})	99.0^{A}	57.3^{E}	66.2^{DE}	67.83^{DE}	76.2^{ABCD}	63.6^{DE}	71.4^{BCDE}	89.6^{AB}	87.9^{ABC}	68.6^{CDE}	20.2
Lactate (mmol dL^{-1})	0.92^{C}	3.2^{A}	1.77^{B}	1.76^{B}	1.48^{B}	1.43^{B}	1.76^{B}	-	-	-	19.6

CV = Coefficient of variation; ^{A,B,C,D,E} Values followed by at same letter in a line do not differ by the Tukey test (P>0.05).

The serum concentration of glucose was evaluated from M0 to M9 of the exercise test, while the lactate concentration was evaluated from M0 to M7 of the exercise test. Serum glucose and lactate concentrations were not influenced by the inclusion of aflatoxin B_1 in the diets (P>0.05) (Table 9).

DISCUSSION

The AFB_1 was not detected in the feedstuffs of the basal diet. The AFB_1 produced in our laboratory and added to the commercial concentrate at concentrations of 50µg/kg and 100µg/kg, according to the treatments, which was the maximum amount intake by the horses, regardless of weight. Keller et al. (2007) observed in horse's feedstuffs concentrations of AFB_1 ranging from 0.01 to 99.4µg/kg. Franco et al. (2019) observed in maize food and maize-based feed from small-scale farms that median levels of total AF, total FB, ZEN, and DON were 100µg/kg, 680µg/kg, 160µg/kg, and 200µg/kg, in feed samples, respectively, and that co-occurrence of two or more mycotoxins was confirmed in 51% of feed samples.

Caloni & Cortinovis (2011) in a review of experimental AFB_1 intoxication in horses reported that, although inconclusive, aflatoxicosis can occur with AFB_1 concentrations between 0.5 and 1.0mg/kg in the diet, which induce clinical signals and hepatic damages, depending on the exposure time. The liver is a target organ in all affected animals, where the toxin induces centrilobular necrosis. Horses with aflatoxicosis show nonspecific clinical signs, such as cough, inappetence, depression, fever, tremor and ataxia. However, the blood and physiological alterations of horses in this research are resulting from the exercise test itself, with no effects from aflatoxin, except for the alkaline phosphatase activity. Dedar et al. (2019) observed growth retardation in foals, weight loss and deaths in horses ingesting millet forage contaminated with levels of 24µg of aflatoxins/kg to 70µg of aflatoxins/kg of forage. For the authors, monitoring serum/plasma GGT levels was found to be a sensitive biochemical indicator to identify liver damage caused by mycotoxins. The daily intake of horses of diet in treatment III (100µg/kg of AFB_1), corresponding a 9.4µg AFB_1 by kg DM, did not result in any clinical signs in the horses.

The simultaneous occurrence of DON, ZEA and FB_1 in the feedstuffs of basal diet is related to the 25 to 30% of corn in the diet. The concentration of mycotoxins in feedstuffs for horses is a serious problem, and the weather in Brazil is favorable to fungi multiplication and mycotoxins production (Keller et al. 2007, Schulz et al. 2015). Schumann et al. (2016) and Franco et al. (2019) pointed out that the simultaneous occurrence of Fusarium mycotoxins, such as DON and ZEA, is an important issue to be observed due to its implications on animal health and welfare.

The concentration of DON in the basal diet was 5.6mg/kg, with similar concentrations in alfafa hay and in the concentrate. Caloni & Cortinovis (2011) and Cortinovis et al. (2012) observed that horses fed naturally DON contaminated barley between 36 and 44mg/kg during 40 days they did not show any negative effects, such as refusing feed or alteration of blood biochemistry and hematology, suggesting that like ruminants, horses are relatively resistant to the adverse effects of DON.

The ZEA concentration in feedstuffs were 446.5µg/kg and 261.5µg/kg for concentrate and alfafa, respectively. Gimeno & Quintanilla (1983) reported natural outbreaks from feedstuffs containing approximately 2.7mg/kg of ZEA in horses. Intoxicated mares refused the diet, and presented vulvar edema, vaginal prolapse and internal hemorrhage, and severe sagging of the genitalia. In this research, no clinical symptoms were observed in the horses, suggesting that the amount of ZEA present in the basal diet was not enough to cause clinical alterations. It should be noted that the concentration of ZEA was lower than the European Union (2006) of 3mg/kg for corn by-products.

The FB_1 concentration in feedstuffs was 560µg/kg. Horses are the most sensitive specie to FB1 toxicity, and FB_1 concentrations of 20µg/kg and 120µg/kg in feed are already able to cause outbreak in this specie (Bertero et al. 2018). Ross et al. (1991) analyzed feedstuffs associated with an equine leukoencephalomalacea outbreaks observed contamination with FB_1 with levels ranging from 1 to 126mg/kg. Most samples presented levels above 10mg/kg. According to Reed et al. (2004) feedstuffs containing concentrations greater than

10mg/kg of fumonisin are fatal to horses. In this research, no clinical signs compatible with equine leukoencephalomalacea were observed, however the hematological and enzymatic alterations should be evaluated as they may be related to the simultaneous presence of several mycotoxins, especially aflatoxin B_1.

The physiological parameters Respiratory rate (RR) and Heart rate (HR) were evaluated at pre-exercise (M0), immediately after the exercise (M1) and at the times of 10 (M2) and 20 minutes after exercise (M3) (Table 3). There were no effects of AFB_1 inclusion in the diets in the respiratory (RR) and heart rate (HR) of the horses (P>0.05). The results showed the response of horses to exercise, with an increase of both RR and HR immediately after exercise (M1), and with a tendency to return to basal levels at 20 min (M3) after exercise. The values differed from those observed by Paludo et al. (2002) for Brazilian Sport Horses that showed mean values of heart rates of 40.8±4.7, and respiratory rate of 16.7±3.1, in the pre-exercise and of 53.7±8.2 and 37.3±11.5, for heart and respiratory rates, respectively, in the post-exercise period.

The inclusion of AFB_1 in the diets did not affected the blood parameters (Table 4), except for the alkaline phosphatase activity. The presence of aflatoxin together with other mycotoxins, especially FB, could be expected to cause changes in the blood profile during exercise due to hepatotoxicity. The intake of low doses of mycotoxins for long periods can influence the health and the performance of horses (Raymond et al. 2005, Broom 2015). Thus, hepatotoxic mycotoxins could be expected to impair hepatic metabolism, influencing the results of this research. However, the alterations observed were those resulting from the exercises on the horse, as a simple effect of time, with no effect to AFB_1 in the diets.

As expected, immediately after exercise (M1), the Globular volume presented a higher value; about 39% higher than the baseline value. The elevation of globular volume is related to spleen contraction. According to Kingston (2008) and McGowan (2008) the spleen has the capacity to store about 50% of the body's erythrocytes in horses. The release of the erythrocytes in the body occurs in response to sympathetic stimulation or increased circulation of adrenaline during exercise. The values for total protein and albumin in response to exercise were not influenced by the inclusion of AFB_1 in the diets, and only a simple effect of time was observed (P<0.05).

The increase of protein concentration immediately after exercise was observed, and its remained for 10 and 20 minutes (M3 and M4) as a consequence of blood concentration, redistribution of fluids and electrolytes from the vascular compartment to the extracellular spaces, and increased plasma viscosity (Kingston 2008). Fluid loss is related to the duration and intensity of exercise and returns to basal levels within 30 minutes of the exercise (Kowal et al. 2006). Cholesterol plasma concentration was not influenced by AFB_1 inclusion in the diets (P>0.05). The highest concentration of cholesterol immediately after exercise may be due to the increase in plasma viscosity arising from blood concentration (Kingston 2008). However, the values observed are within the reference levels for horses (Gonzalez & Silva 2006).

Plasma levels of urea and creatinine were evaluated during 48 hours post exercise (M0 to M9). As expected, the urea levels were little influenced by exercise. Snow et al. (1982) stated that the main route of energy generation used by horses is glycolytic and anaerobic, compatible with the exercise test in the experiment: warm-up followed by three sets of gallops at a speed of 450m/min for three minutes each. Also, changes in urea concentration over time may be due to the catabolism of nitrogen from the diets. On the other hand, the serum creatinine concentrations during exercise were lower than the baseline value, which was not expected. According to Fazio et al. (2011) there is an increase in plasma creatinine concentrations during exercise due to the greater use of phosphocreatine for the generation of muscular energy. Castejón et al. (2006) observed that this increase is directly proportional to the intensity of the exercise. The values of creatinine concentration in this work were below those observed by Santiago et al. (2013) evaluating eventing horses at training, and Godoi et al. (2009) who evaluated eventing horses feeding hyperlipidemic diets. The values of urea and creatinine concentrations were below the reference values for the horses according to Rose & Hodgson (1994).

The peak of CK activity was observed at 20 minutes after exercise (M3), with a tendency to return to baseline three hours after the exercise (M5). Harris et al. (1990) reported that some animals may have high physiological enzymatic levels of CK, or even that these enzymes are removed more slowly from the circulation, or some animals may be more sensitive as to the permeability of the cellular muscular membrane compared to a similar stimulus in other animals. According to Sloet van Oldruitenborgh-Oosterbaan (1998), the plasma half-life of CK is very short, around 2 hours, which is compatible with the results observed in this experiment. The mean values of CK were higher than those observed by Oliveira et al. (2014) in Brazilian Sport Horses on treadmill exercise tests, but result of CK 12 hours after exercise (M7) was similar those found by Padilha et al. (2017) in the same breed, a 48-h rest by training.

The AST serum activity shows a standard exercise profile (Thomassian et al. 2007). The peak of AST activity was observed immediately after exercise (M1) and a tendency to increase 6 hours post exercise (M6). The high concentrations of AST immediately after exercise are related to the physiological process of fluid transfer to the extravascular space during exercise, increasing the AST activity in plasma, with a return to the 30-minute-baseline (Kingston 2008). The results of AST 48 h post exercise (M9) are very similar to those found by Padilha et al. (2017) in adult Brazilian sport horses a 48-h rest after training.

Differently from AST, the LDH activity did not present a standard profile in response to exercise, and the LDH peak was observed 12 hours post exercise (M7). The results observed were higher than the values reported by Oliveira et al. (2014) in horses on treadmill exercise tests. The large variation of LDH plasma activity can be explained by the distribution of this enzyme in several different tissues. The values of LDH plasma activity refer to the sum of several isoenzymes, so the interpretation of elevated LDH values may be compromised (Harris et al. 1990). In addition, non-pathological states may also cause increase in AST activity like LDH, as well as the collection methods, processing and equipment used for the analyzes (Sloet van Oldruitenborgh-Oosterbaan M. 1998, Thomassian et al. 2007).

The GGT activity was not influenced by the inclusion of AFB_1 in the diets (P>0.05). The increase of the enzyme is

indicative of liver damage, cholestasis and proliferation of bile ducts in all animal species (Kerr 2002, Kingston 2008, Dedar et al. 2019). Horses submitted to exercise conditioning for a prolonged period may present an increase of GGT (Balarin et al. 2005). In this experiment, the GGT activity had a peak at about 60 minutes after exercise (M4), oscillating for up to 24 hours (M8). The pre-exercise physical training period in this work during three weeks, was very short, when compared with the 12 months of training cited by Balarin et al. (2005). However, the values observed are within the basal levels for horses, ranging from 4.3 to 13UL^{-1} (Thomassian 2005).

The Alkaline phosphatase (ALP) activity was affected by the AFB_1 inclusion in the diets ($P<0.05$), and ALP activity increased in horses fed diet with the 100µg/kg of AFB_1. ALP is an enzyme widely distributed in the body, and the hepatic isoform predominates in the serum, and the higher values of ALP activity is indicative of liver damage in horses (Kerr 2002). Kingston (2008) reported ALP increases in horse serum due to prolonged exercise; however, it is not clear whether this increase is from bone or hepatic origin. Hank et al. (1993) observed that about 17.9% of serum ALP in mature horses is from the bones. Trueman et al. (1983) stated that increases in ALP are often unexplained and that concentrations between 0-599IU/L can be considered normal. In this work, the horses were submitted to exercise conditioning during three weeks and the ALP activity in horses fed diet with 50µg/kg of AFB_1 was higher than ALP activity in horses fed basal diet. The ALP activity in horses fed diet with 100µg/kg of AFB_1 was higher than the ALP activity in horses feeding the other two diets. All the animals were submitted the same exercise intensity, and ALP was monitored up to 20 minutes after the test (M3).

Liver damage and gastrointestinal disorders may occur in horses feeding diets containing from 2 to 50µg/kg of AFB_1 (Reed et al. 2004). Kaneko et al. (2008) reported that chronic intoxication of pigs with fumonisins is associated with increases of ALP, AST and GGT. Thus, the potential interaction and synergism of AFB_1 with FB_1 (CAST 2003) which may have influenced the ALP results and should be considered. Thus, it appears that the results of ALP activity were from hepatic tissue and its activity is directly influenced by the increased AFB_1 concentration of the diets. However, the results observed are within the clinical values considered normal for the horses, according to Trueman et al. (1983).

The serum glucose and lactate concentrations (Table 9) were not affected by the AFB_1 inclusion in the diets ($P>0.05$). Glucose and lactate concentrations reflect the energy metabolism and performance. The mycotoxins were expected to affect the hepatic metabolism and animal response to exercise since the liver plays a central role in metabolism, synthesizing and distributing nutrients to the peripheral organs through blood circulation (Gonzalez & Silva 2006), however, did not occur. Higher glucose concentration was observed during the rest period (pre-exercise). According to Kerr (2002), the glucose concentration increases during exercises due to the adrenaline. In this work, a decrease in glucose levels was observed, which glucose levels were restored after feeding. According to Pösö et al. (2004), changes in glucose concentration depend on the type of exercise, and may increase or decrease in short-term exercises. The serum values for glucose in this work were higher than those observed by Santiago et al. (2013) in eventing horses in the initial phase of training as observed up to the moment M4. However, the glucose values reported here are within the serum levels of the horses (Rose & Hodgson 1994).

The peak in lactate concentration occurred immediately after exercise (M1). Lactate is an intermediate product of the metabolism of carbohydrates during anaerobic glycolysis. Under anaerobic conditions, such as in intense or prolonged physical activity, pyruvic acid is converted to lactic acid, in a reaction mediated by the Lactate dehydrogenase (Kaneko et al. 2008). The time to return lactate concentration to a baseline is a measure of the fitness of horses (Evans et al. 1995). The values observed in this study are similar to those observed by Santiago et al. (2013) working with horses that were beginners in eventing training.

CONCLUSION

The mycotoxins in the diets during 28 days doesn't influence the physiological, hematological and blood biochemical responses of the horses, except for the alkaline phosphatase activity, which presented higher activity in the diet with 100µg/kg of AFB_1, suggesting a hepatotoxic action and synergistic effect with the others mycotoxins naturally present in the feedstuffs.

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Craniometric geographical variation in *Hylaeamys megacephalus* populations[1]

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ABSTRACT.- Mata P.S., Ribeiro M.S.L., Faria M.B. & Lima F.C. 2020. **Craniometric geographical variation in *Hylaeamys megacephalus* populations.** *Pesquisa Veterinária Brasileira 40(9):726-732.* Programa de Biociência Animal, Universidade Federal de Jataí, Cidade Universitária, BR-364 Km 195, Prédio da Pós-Graduação, Jataí, GO 75801-615, Brazil. E-mail: paoladamata@hotmail.com

Hylaeamys megacephalus (G. Fisher, 1814) presents great genetic diversity and wide geographical distribution, and occurs in both the Amazon and Cerrado biomes. Because of its generalist aspect, this species tolerates different eating habits and habitats. It occurs in flooded and dry areas and is predominantly terrestrial, which allows greater gene flow between populations even over long distances. Studies that seek a better understanding of morphological variations resulting from differences imposed by the environment throughout this species' distribution are still lacking. This study aimed to analyze the differences between *H. megacephalus* populations based on craniometry, investigating whether the environment has an influence on morphology. We analyzed a total of 142 specimens from three scientific mammal collections: National Museum, "Universidade Federal do Rio de Janeiro" (MN-UFRJ); "Laboratório de Biologia e Parasitologia de Mamíferos Reservatórios Silvestres", "Instituto Oswaldo Cruz", "Fundação Oswaldo Cruz"(LBCE-Fiocruz); and "Laboratório de Biodiversidade", "Universidade Federal de Goiás", "Regional Jataí" (LZE-UFG), and took 20 craniometric measurements. Craniometry was explored using unweighted pair group method with arithmetic mean (UPGMA), canonical variate analysis, and principal component analysis (PCA). The results led us to conclude that there are three craniometric groups of *H. megacephalus* with a tendency to differentiate as a result of geographical influences.

INDEX TERMS: Craniometry, geographic variation, *Hylaeamys megacephalus*, anatomy, morphological characters, skull, occurrence, rodent.

RESUMO.- [**Variação geográfica craniométrica em populações de *Hylaeamys megacephalus.***] Com grande diversidade genética e distribuição geográfica, *Hylaeamys megacephalus* (G. Fisher, 1814) ocorre tanto na Amazônia quanto no Cerrado. Visto seu aspecto generalista, esta espécie tolera diversos hábitos alimentares e habitats, ocorrendo em áreas inundadas ou não, sendo predominantemente terrestre, permitindo maior fluxo de genes entre as populações, mesmo em longas distâncias. Apresenta ampla distribuição, e carece de estudos que busquem um melhor entendimento sobre as variações morfológicas resultantes das diferenças impostas pelo meio ao longo de sua distribuição. O estudo teve como objetivo, analisar as diferenças entre as populações de *H. megacephalus*, com base na craniometria investigando se o ambiente interfere na morfologia. Analisamos um total de 142 espécimes oriundos de coleções científicas de mamíferos, do Museu Nacional, Universidade Federal do Rio de Janeiro (MN-UFRJ), Laboratório de Biologia e Parasitologia de Mamíferos Reservatórios Silvestres, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz (LBCE-Fiocruz) e Laboratório de Biodiversidade, Universidade Federal de Goiás, Regional Jataí, nos quais foram tomadas 20 medidas craniométricas. A craniometria foi explorada nas análises estatísticas de agrupamento de pares não ponderados com médias aritméticas (UPGMA), variação canônica e análise dos Componentes Principais (PCA). Os resultados encontrados nos levaram a concluir a existência de três grupos craniométricos da espécie de *H. megacephalus* com tendência a se diferenciarem, por influências geográficas.

TERMOS DE INDEXAÇÃO: Variação geográfica, craniometria, *Hylaeamys megacephalus*, anatomia, caracteres morfológicos, crânio, ocorrência, roedor.

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INTRODUCTION

The evolutionary success of rodents may be attributed to their small body size, high-rate and short-cycle reproduction, and wide range of dental characteristics (Rodrigues & Naehrer 2012). This specification is surprising, and it can be found in almost all non-marine habitats. The adaptive diversity of these animals is remarkable in ecology, as they occur in most ecosystems on the planet (Hautier et al. 2011, Arregoitia et al. 2017).

Hylaeamys megacephalus (G. Fisher, 1814) (Azara's broad-headed oryzomys or large headed rice rat) presents great genetic diversity and wide geographical distribution, and occurs in both the Amazon and Cerrado biomes, forming three clades in three different regions: one in the Cerrado and two in the Amazon (north and south of the Amazonas River). Genes of the clade belonging to the south of the Amazonas River are closer to those of the clade that inhabits the Cerrado, suggesting an allopatric divergence in which historical and ecological factors have an influence on the distribution and diversification of species (Miranda et al. 2007, Machado et al. 2019).

Because of its generalist aspect, *H. megacephalus* tolerates several eating habits and habitats. It occurs in flooded and dry areas and is predominantly terrestrial, which allows greater gene flow between populations even over long distances. This rodent has a wide distribution, and studies that seek a better understanding of morphological variations resulting from differences imposed by the environments are still lacking (Emmons & Feer 1997, Bonvicino et al. 2008, Percequillo et al. 2008, Rocha et al. 2011, Paglia et al. 2012, Rocha et al. 2014, Patton et al. 2015).

Morphological variation in these animals can be explained by different ecological and/or evolutionary mechanisms, such as intraspecific and sexual selection. Size and shape are critical biological attributes of organisms, resulting from the interaction between genes and environment. Usually, a large fraction of the variability of morphometric data is related to the size of individuals, which can result in changes in the associated shape (Kardong 2014).

This study aimed to analyze the differences between *H. megacephalus* populations based on craniometry, investigating whether the environment has an influence on morphology.

MATERIALS AND METHODS

Samples. Cranial morphology was assessed in 142 specimens from three mammal scientific collections: 63 from the National Museum, "Universidade Federal do Rio de Janeiro" (MN-UFRJ), 49 from the"Laboratório de Biologia e Parasitologia de Mamíferos Reservatórios Silvestres", "Instituto Oswaldo Cruz", "Fundação Oswaldo Cruz" (LBCE-Fiocruz), and 30 from the "Laboratório de Biodiversidade", "Universidade Federal de Goiás", "Regional Jataí" (LZE-UFG) (Table 1). They are distributed over 27 Brazilian municipalities: seven in the Amazon and 20 in the Cerrado biomes. For statistical analysis, locations with fewer than five specimens were grouped into the closest geographical region (Table 2 and Fig.1).

Quantitative analysis. The samples were analyzed in January 2018 at the MN-UFRJ and LBCE-Fiocruz, and in July 2018 at the LZE-UFG. Adult individuals of both sexes were analyzed. Age was classified according to the third molar eruption criterion (Fig.2). Cranial dimensions were measured following an adaptation from Voss (1988) and Manduca (2008) using a digital precision (0.01 mm) caliper. Twenty measurements were taken (Table 3 and Fig.3-5).

Statistical analysis. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA) (Sneath & Sokal 1973). The cut-off point was established using the k-means clustering analysis to favor interpretation of score distribution patterns in the multivariate space. Subsequently, we performed canonical variate analysis, for discriminatory morphometric trends, and principal component analysis (PCA), based on the covariance matrix of the logarithmically transformed data used to determine the main independent trends in craniometric variation. The 20 craniometric variables were analyzed using the R software and descriptive statistics (mean and standard deviation). Small population samples (<5 specimens) were grouped into larger samples from the closest geographical location (Fig.1).

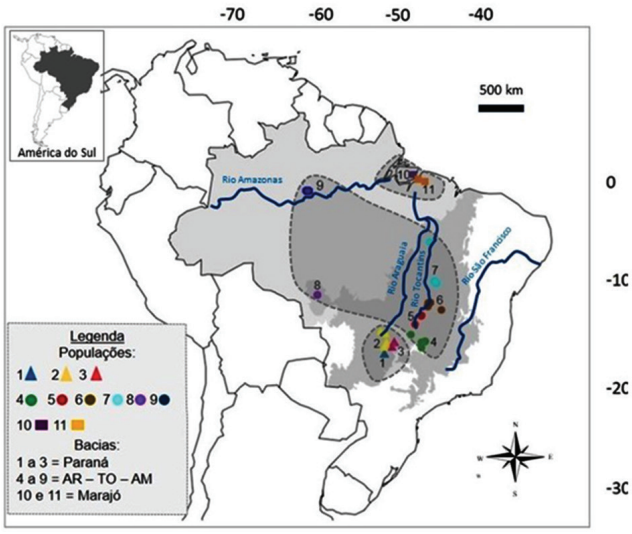


Fig.1. Distribution map of the collected *Hylaeamys megacephalus* samples, where each color and number indicates the geographically grouped populations and the symbols show the groups in the UPGMA analysis.

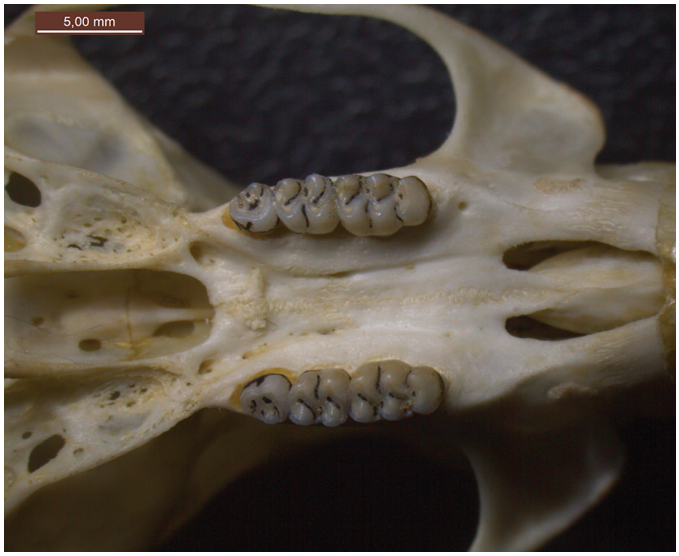


Fig.2. Microphotograph of the molar teeth showing the third molar (3M) eruption in *Hylaeamys megacephalus*, specimen 079 LZE, ventral view.

Table 1. Origin of the *Hylaeamys megacephalus* specimens analyzed

Number	Institution	Morphoclimatic domain	Latitude	Longitude	Municipality	State*
6986, 6987, 8566, 9456, 7003, 7471, 7491, 8511, 9442	LBCE	Cerrado	18º 57' 55" S	51º 55' 35" W	Aporé	GO
8754, 8751, 8752, 8755	LBCE	Cerrado	17° 37' 59'' S	47° 46' 42'' W	Campo Alegre de Goiás	GO
8718	LBCE	Cerrado	16º 57' 44" S	49º 13' 41" W	Hidrolândia	GO
8768	LBCE	Cerrado	17° 43' 19'' S	48° 09' 35''W	Ipameri	GO
13523, 13522, 13524, 13542, 13519, 13027, 13024, 13023, 13028,13031	LBCE	Cerrado	11º 49' 26" S	46º 37' 42" W	Novo Jardim	TO
13004, 13005, 13025, 13059, 13029, 13030, 13084, 13437, 13526, 13543, 13467, 13070, 13065	LBCE	Cerrado	11° 37' 40''S	46° 49' 14''W	Dianópolis	TO
18587	LBCE	Cerrado	18° 15' 47''S	48° 09' 04'' W	Cumari	GO
12194, 12152, 12158, 12172	LBCE	Cerrado	12° 59' 22'' S	58° 45' 51'' W	Sapezal	MT
54, 78, 80, 82,83, 87, 89, 90, 92, 96, 129, 144, 154,156, TX 03, 79, 141	LZE	Cerrado	17º 52' 53''S	51º 42' 52" W	Jataí	GO
165	LZE	Cerrado	18º 17' 56" S	51º 08' 43" W	Aparecida do Rio Doce	GO
167	LZE	Cerrado	16º 57' 24" S	51º 48' 37" W	Caiapônia	GO
177	LZE	Cerrado	16º 43' 13" S	52º 19' 10" W	Doverlândia	GO
221, 245, 251, 255, 212, 213, 217, 219, 256	LZE	Cerrado	17º 47' 53" S	50º 55' 41" W	Rio Verde	GO
69	LZE	Cerrado	18º 57' 55" S	51º 55' 35" W	Aporé	GO
20880, 20882, 20883, 20886, 20888, 20889, 20890, 20892, 20877, 20878, 20695, 20893, 20895, 20897, 2098, 20899, 20900, 20901, 20904, 20911, 20913, 69002, 75119	Museu Nacional -UFRJ	Amazônia	03° 06' 07"S	60º 01' 30"W	Manaus	AM
10289, 10288	LBCE	Amazônia	01° 00' 41''S	48° 57' 48''W	Cachoeira do Arari	PA
69185	Museu Nacional -UFRJ	Amazônia	00° 58' 29''S	62° 55' 27'' W	Barcelos	AM
84037	Museu Nacional -UFRJ	Amazônia	01° 40' 27''S	47° 46' 16'' W	Capim	PA
84036, 84035, 84034, 84033, 84031, 84030, 84032	Museu Nacional -UFRJ	Amazônia	01° 27' 21''S	48° 30' 16'' W	Belém	PA
46867, 46861, 46863	Museu Nacional -UFRJ	Cerrado	14° 07' 57''S	47° 30' 36''W	Alto Paraíso	GO
46864, 46870	Museu Nacional -UFRJ	Cerrado	13° 47' 51''S	47° 27' 30''W	Cavalcante	GO
43013, 43014, 43009, 43015, 43011, 43016, 43012, 43018, 43019, 43020, 43021, 46860, 81655, 81658	Museu Nacional -UFRJ	Cerrado	13° 46' 35''S	47° 15' 53''W	Teresina de Goiás	GO
81664, 81665, 81666, 81669, 81670, 81671, 81672, 81673, 81674, 81675, 81676	Museu Nacional -UFRJ	Cerrado	15° 03' 22''S	48° 09' 41''W	Mimoso de Goiás	GO
81701	Museu Nacional -UFRJ	Cerrado	15º 55' 25" S	48º 48' 31" W	Corumbá de Goiás	GO
10855	LBCE	Cerrado	14° 29' 16''S	46° 06' 47''W	Mambaí	GO
6858, 6799, 6852	LBCE	Cerrado	18º 18' 22" S	51º 57' 44" W	Serranópolis	GO

LBCE = Laboratório de Biologia e Parasitologia de Mamíferos Reservatórios Silvestres, LZE = Laboratório de Biodiversidade; *GO = Goiás, TO = Tocantins, MT = Mato Grosso, AM = Amazonas, PA = Pará.

Table 2. Number of samples, place of origin, and number of specimens analyzed

Samples	Specimen location and quantity
1	Aporé - GO (10)
2	Caiapônia - GO (01), Doverlânida - GO (01), Jataí - GO (17), Serranopólis - GO (03)
3	Aparecida do Rio Doce - GO (01), Rio Verde - GO (09)
4	Campo Alegre de Goiás - GO (04), Cumari - GO (01), Hidrolândia - GO (01), Ipameri - GO (01)
5	Corumbá de Goiás - GO (01), Mimoso de Goiás - GO (11)
6	Alto Paraíso - GO (03), Cavalcante - GO (02), Goiatins - TO (01), Mambaí - GO (01), Teresina de Goiás - GO (14)
7	Dianopólis - TO (13), Novo Jardim - TO (10)
8	Sapezal - MT (04)
9	Manaus - AM (21), Santa Isabel - AM (01)
10	Barcelos - AM (01), Cachoeira do Arari - AM (02)
11	Bélem - PA (07), Capim - PA (01)

State: GO = Goiás, TO = Tocantins, MT = Mato Grosso, AM = Amazonas, PA = Pará.

RESULTS

A total of 11 populations were observed and arranged in three cranial groups listed by hydrographic basins: Marajó Island, Paraná, and Araguaia-Tocantins/Amazonas River (Fig.1). Statistical analysis (UPGMA) showed that the populations from the Paraná and Araguaia-Tocantins/Amazonas River basins are more similar to each other compared with the those from the Marajó Island (Fig.6).

As in the UPGMA analysis, canonical variate analysis also showed discriminatory morphometric trends with formation

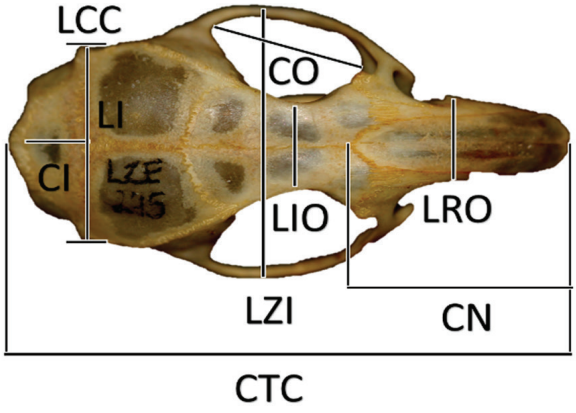


Fig.3. Dorsal view of the skull of *Hylaeamys megacephalus*, specimen 080 LZE. Total skull length (CTC), nasal length (CN), rostral width (LRO), interorbital width (LIO), zygomatic arch width (LZI), orbital length (CO), interparietal width (LI), skull width (LCC), interparietal length (CI).

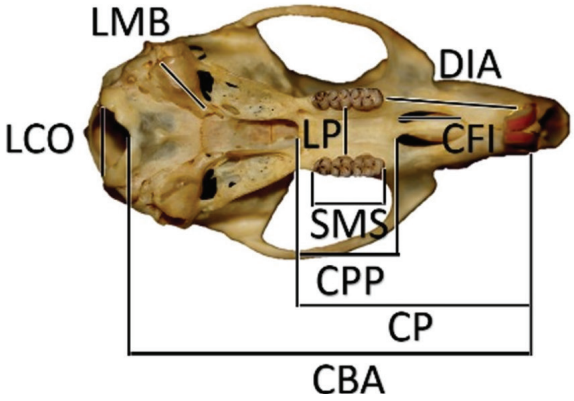


Fig.4. Ventral view of the skull of *Hylaeamys megacephalus*, specimen 080 LZE. Cranial base length (CBA), palatal length (CP), palatal bridge length (CPP), upper molar teeth series (SMS), palatal width (LP), incisive foramen length (CFI), diastema (DIA), maximum bulla width (LMB), distance between the occipital condyles (LCO).



Fig.5. Lateral view of the skull of *Hylaeamys megacephalus*, specimen 080 LZE. Zygomatic plate width (LPZ), maximum skull height (AMC).

Table 3. Cranial characters represented by their acronyms, meaning and description

Acronyms	Meaning	Description
AMC	Maximum skull height	Distance between the basisphenoid-basioccipital suture and the frontoparietal suture.
CBA	Cranial base length	Distance between the posterior border of the incisor alveolus and the anterior border of the foramen magnum.
CFI	Incisive foramen length	Distance between the anterior border and the posterior border of the incisor foramen.
CI	Interparietal length	Anteroposterior distance of the median axis of the interparietal.
CN	Nasal length	Distance between the anterior border of the nasal and the nasofrontal suture.
CO	Orbital length	Internal distance between the frontal and maxillary sutures to the root of the squamous cell present in the zygomatic arch.
CP	Palatal length	Distance between the posterior border of the incisor alveolus and the border of the palate.
CPP	Palatal bridge length	Distance between the posterior border of the incisor foramen and the border of the palate.
CTC	Total skull length	Maximum distance measured between the anterior border of the nasal and the posterior border of the occiput.
DIA	Diastema	Distance between the posterior alveoli of the incisors and the anterior alveoli of the first molar.
LCC	Skull width	Width between the anterior regions of the squamous suture and the occiput.
LCO	Width between occipital condyles	Greater distance between the outer borders of the occipital condyles.
LI	Interparietal width	Maximum distance between the lateral borders of the interparietal.
LIO	Interorbital width	Minimum interobital distance.
LMB	Maximum bulla width	Maximum distance between the junction of the basisphenoid and basioccipital sutures and the outer border of the bulla.
LP	Palatal width	Width between the molar series measured between the first and second molars.
LPZ	Zygomatic plate width	Distance measured at the base of the zygomatic plate from the anterior to the posterior limit.
LRO	Rostral width	Rostrum width measured at the height of the infraorbital foramen.
LZI	Zygomatic arch width	Greater distance between the lateral borders of the zygomatic arch.
SMS	Upper molar teeth series	Distance between the border of the anterior alveolus of the first molar and the border of the posterior alveolus of the third molar.

of three population groups: Araguaia-Tocantins/Amazonas River, Paraná, and Marajó Island. However, in the discriminatory analysis, these three groups overlapped regarding craniometry, with a tendency to segregate (Fig.7).

In PCA, the first component, axis 1 (PC1), synthesizes the dorsal metrics (CTC, IOL, CN, LZI, LCC, and CO) and corresponds to 27.7% of the variation, whereas the second component, axis 2 (PC2), synthesizes the ventral metrics (CFI, SMS, CP, LCO, and CBA) and represents 8.5% of the variation (Fig.8). The most significant morphometric differences were found in the ventral metrics, making them the main component responsible for distinction between the samples.

The structured morphological groups point to a strong relationship between the populations from the Amazonas River and Tocantins-Araguaia basins, and a less intense relationship between the Araguaia-Tocantins/Amazonas River and Paraná basins. In turn, the Araguaia-Tocantins/Amazonas River and Paraná basins alone show more significant craniometric differentiation when compared with each other. The isolated populations from the Marajó Island also proved to be different from the others. However, we emphasize that, because the data referring to the Marajó Island present a low number of analyzed specimens, they may have induced separation of the analyses, causing a deviation.

As for the craniometric characters evaluated for *Hylaeamys megacephalus*, the mean varied between a minimum of 2.62 (LP) and a maximum of 31.10 (CTC) and the standard deviation ranged from 0.29 (IOL) to 2.52 (LZI), as shown in Table 4.

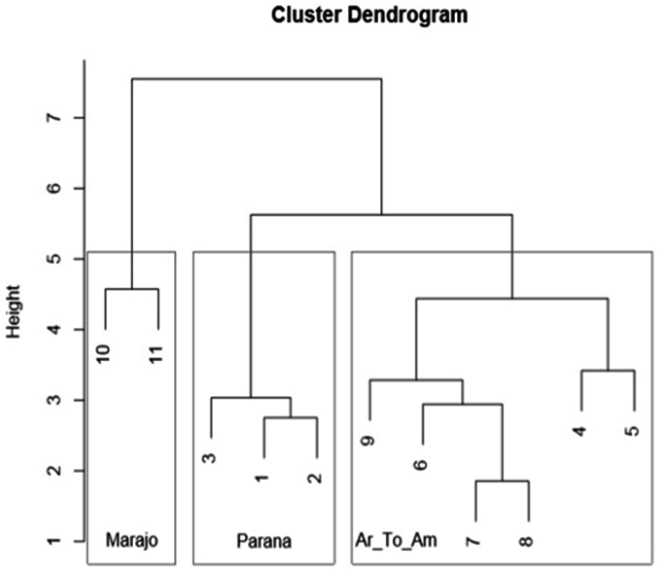


Fig.6. Cluster dendrogram of the *Hylaeamys megacephalus* populations according to craniometry by hydrographic basin.

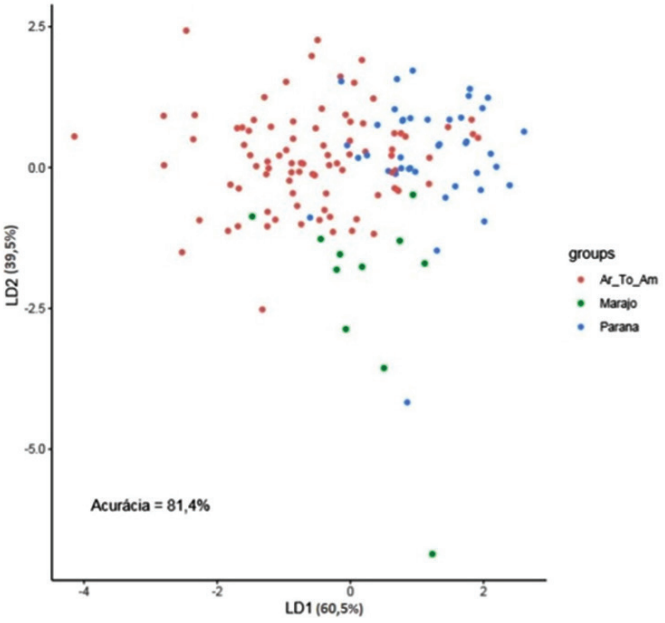


Fig.7. Canonical variate analysis discriminating the three population groups by hydrographic basin.

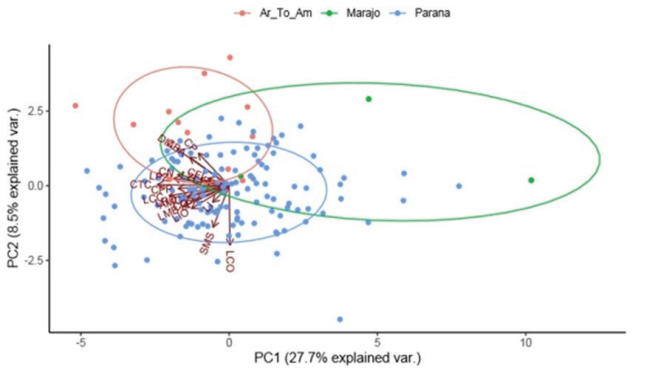


Fig.8. Principal component analysis (PCA) between the *Hylaeamys megacephalus* populations showing the craniometric characters. Principal components: axis 1 (PC1) synthesizes the dorsal metrics, representing 27.7% of the variation; axis 2 (PC2) synthesizes the ventral metrics, corresponding to 8.5% of the variation.

Table 4. Mean and standard deviation of the 20 craniometric characters of the *Hylaeamys megacephalus* samples

Craniometric characters	Mean	Standard deviation
CTC	31.10	1.34
LIO	5.18	0.29
CN	12.83	0.86
CFI	4.23	0.41
DIA	8.16	0.66
LZI	15.21	2.52
LCC	11.83	0.45
LRO	6.00	0.50
SMS	4.68	0.34
LPZ	3.64	0.32
CP	13.68	1.41
CPP	6.95	0.45
LCO	3.46	0.54
CBA	24.52	2.02
CI	4.04	0.54
LI	9.80	0.80
LP	2.62	0.29
CO	9.25	0.53
AMC	9.13	0.42
LMB	5.23	0.49

The Araguaia-Tocantins and Amazonas River basins presented higher CP, DIA and CBA values than the Paraná basin, with these characters related to internal morphology. In contrast, the Paraná basin showed higher SMS values than the Araguaia-Tocantins/Amazonas River basin, with differences also related to internal morphological variables. The Marajó Island basin population groups differ from the other two groups with respect to external variables (CPP, CTC, LPZ, LMB, LIO, AMC, and LRO), with lower values for these variables observed in the first.

DISCUSSION

According to Marroig (2007), a simple increase in skull size can lead animals to consume larger and harder food items or become more resistant to explore new environments and niches. Our results suggested that the morphological characteristic is associated with the environment where the species lives. For this reason, we correlated the craniometric results to the hydrographic basins.

Most of the variables involved in these morphometric differences are associated with the general structure of the skull. The SMS and CP measures, for example, directly contribute to the adaptation of the oral cavity structure, allowing changes in food acquisition habits. Differences in shape can mean functional differences performed by the same parts, revealing different responses when subjected to the same selective pressure (Schmidt-Nielsen 1984, Van Valkenburgh 1994).

Measurement variations should be interpreted so that the components that represent structural measures can and, usually, play an essential role in functional adaptation. Over time, such adaptations have enabled individuals to develop specializations. In this case, the skull shape is highly valuable for these interpretations, because the differences in length and width, such as in the palate, oral cavity or molar teeth series, directly represent the possibility of restructuring the diet.

Analysis of the craniometric similarity between the *Hylaeamys megacephalus* populations from the Araguaia-Tocantins and Amazonas River basins, population 9, located north of the Amazonas River, is craniometrically similar to the other populations of the Cerrado biome (Fig.1 and 6). Thus, our results compared the genetic data reported by Miranda et al. (2007) and Machado et al. (2019), who stated that genes of the clade belonging to the south of the Amazonas River are closer to those of the clade that inhabits the Cerrado, suggesting an allopatric divergence.

This difference between morphological and genetic data can be explained by the use of markers, since the changes observed in morphological characteristics occur through a relatively slower process compared with those in genetic characteristics. The contrast between these data can be justified by geographical influences, despite the strong geographical barrier exerted by the Amazonas River, which affects gene flow. This isolation directly affects two speciation components: the development and establishment of divergence between different forms of discontinuity (Mayr 1970).

Thus, differences between species are influenced by geographical variations and, whether morphological, physiological or ecological, they are potentially isolating mechanisms that reinforce the distinction between two populations (Pearson 2007).

CONCLUSION

Morphological analysis shows the existence of three craniometric groups of *Hylaeamys megacephalus* with a tendency to differentiate as a result of geographical influences.

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Conflict of interest statement.- The authors have no competing interests.

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Original Article
Animal Morphophysiology
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Anatomic study of the arterial vascularization of the brain base of paca (*Cuniculus paca*)[1]

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Paca (*Cuniculus paca* Linnaeus, 1766), rodent belong to the Cuniculidae family, has encouraged numerous scientific researches and for this reason could be an experimental model in both human and veterinary areas. And recently, the economic exploitation of the meat cuts, has being direct implication in its zootechnical importance. However, no anatomical descriptions regarding the vascularization of the base of the brain in this rodent has being found. Thus, the aim of the present study was to describe the arteries and the pattern of the vasculature and to compare it with the other species already established in the literature. For this, five pacas, donated by the Unesp Jaboticabal Wildlife Sector, were euthanized followed by the vascular arterial system was injected with red-stained-centrifuged latex by the common carotid artery. After craniectomy, the brains were removed and the arteries were identified and, in addition, compared with those described in other animal species. The presence of the right and left vertebral arteries, close to the medulla oblongata, was detected, originating the basilar artery, which divided into the terminal branches of the right and left basilar artery. Ventral to the optic tract there was the right internal carotid artery and the left, dividing the middle cerebral artery and left rostral and right; dorsal to the optic chiasm, the medial branch of the rostral cerebral arteries was identified. Based on the results, it is concluded that the vascularization of the paca brain base is supplied by the carotid and vertebrobasilar system.

INDEX TERMS: Anatomy, arterial vascularization, brain base, paca, *Cuniculus paca*, anatomy, brain, arterial circuity, rodent, central nervous system, morphology.

RESUMO.- [Estudo anatômico da vascularização arterial da base do encéfalo da paca (*Cuniculus paca*).] A paca (*Cuniculus paca* Linnaeus 1766), roedor da família Cuniculidae, tem encorajado inúmeras pesquisas científicas, tornando-a modelo experimental tanto na área humana quanto na veterinária, além da recente exploração econômica de seus cortes cárneos, que favoreceu diretamente sua importância zootécnica. No entanto, não há até o momento, descrições anatômicas referentes à padronização da vascularização da base do encéfalo neste roedor. Assim, o objetivo do presente trabalho foi realizar tal delineamento arterial nessa região do sistema nervoso central e compará-lo com as demais espécies já estabelecidas na literatura. Para isso, foram eutanasiadas cinco pacas doadas pelo setor de Animais Silvestres da Unesp Jaboticabal, as quais foram submetidas posteriormente à injeção de látex centrifugado e corante líquido xadrez vermelho, pela artéria carótida comum. Após craniectomia e segregação do encéfalo de todos os cadáveres, realizou-se a identificação das artérias presentes na base deste órgão e, ademais, comparação destas com as descritas cientificamente em outras espécies

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animais. Detectou-se a presença das artérias vertebral direita e esquerda, próximas à medula oblonga, originando a artéria basilar, que se dividiu nos ramos terminais da artéria basilar direito e esquerdo. Ventral ao trato óptico verificou-se a artéria carótida interna direita e esquerda dividindo-se na artéria cerebral média e rostral direita e esquerda; ainda, dorsal ao quiasma óptico, identificou-se o ramo medial das artérias cerebrais rostrais. Com base nos resultados obtidos, conclui-se que a vascularização da base do encéfalo da paca é suprida pelo sistema carotídeo e vertebro-basilar.

TERMOS DE INDEXAÇÃO: Anatomia, vascularização arterial, base do encéfalo, paca, *Cuniculus paca* anatomia, cérebro, circuito arterioso, roedor, sistema nervoso central, morfologia.

INTRODUCTION

Rodents belong to the order Rodentia, represented by the largest number of mammals of the Brazilian fauna, including paca (*Cuniculus paca* Linnaeus, 1766), which is found both in Brazilian territory and in Latin America (Dubost & Henry 2006).

Due to the diversity of rodent species, several studies have investigated the vascularization of the base of the brain (Reckziegel et al. 2001, Aydin et al. 2005, 2008, Araújo & Campos 2005, Azambuja 2007, Aydin 2008, Silva et al. 2016, Costa et al. 2017a); so far, there are no descriptions of this pattern in paca. Thus, the aim of this study was to describe the arterial vascularization of the brain base in this large rodent and to compare it with the other animal species, aiming at morphological standardization, especially the origin and arterial distribution, since the wild animal anatomy is essential to the clinic and surgery practices veterinary, futures researches and for conservation programs.

MATERIALS AND METHODS

The work was approved by the Animal Ethical Use Committee of the "Faculdade de Ciências Agrárias e Veterinárias" (FCAV), "Universidade Estadual Paulista" (Unesp), Jaboticabal Campus (017754/13) and by the "Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis" (IBAMA-SISBIO - 38942-1).

Five adult pacas, three males and two females, approximately 10kg, were donated by the Unesp Jaboticabal Wildlife Sector, breeding facility of specimens of the Brazilian fauna for scientific purposes (registration number 482508).

The animals were euthanized by previous sedation with 3mg/kg of meperidine hydrochloride (Cristália®, Itapira/SP) associated with 1mg/kg of midazolam (Medley®, Campinas/SP), both intramuscularly, in a single application. The animals were then anesthetized with 25mg/kg ketamine hydrochloride (Vetnil®, Louveira/SP) and 0.5mg/kg xylazine hydrochloride (Konig, Mairinque/SP) intramuscularly, in a single dose. Additionally, 19.1% intracardiac potassium chloride injection (HalexIstar Pharmaceutical Industry, Goiânia/GO), dose-effect, was performed until cardiopulmonary arrest.

Then, the left common carotid artery of all pacas was cannulated for injection of 60% centrifuged latex (Colitex, Colina/SP) pigmented with red liquid dye (Sherwin-Williams, Ribeirão Preto/SP) until filling the arterial system.

Subsequently, the bodies were frozen at -20°C for one week for complete latex polymerization and then thawed and fixed in 10% paraformoldehyde (Neon Comercial Ltda, São Paulo/SP) by intramuscular injections. The preservation was made in 30% saline solution (Eurofarma Laboratório S.A., Ribeirão Preto/SP).

Craniectomy was carefully performed by the oscillating saw (Dremel® - Racine, Wisconsin, United States) to remove the brain, avoiding damage to its vascular structures, as the meninges are closely associated with the skull. After isolation of the organ, we proceeded to identify the arteries present at the base of the brain and compare them with those already described in the literature for other animal species. The anatomical terms were based on the Nomina Anatomica Veterinaria (2017).

RESULTS

All animals in the study had the same arterial arrangement. No difference was observed when comparing males and females pacas. The arteries and branches of the base of the brain were illustrated in Figure 1 and the comparisons with the other rodents species shown in Table 1.

It was observed that the right and left vertebral artery (1), present at the level of the medulla oblongata, is anastomosed to origin the basilar artery (2), which extends over the entire length of the brainstem as a unique vessel, with rectilinear path and considerable diameter, irrigating the medulla oblongata, trapezoid body, pons and cerebellum.

In the midbrain, the basilar artery bifurcated symmetrically in terminal branches of the right and left basilar artery (3), with paths close to the mammillary body and hypophysis.

At the level of infundibulum and ventral to the optic tract, the right and left internal carotid arteries (4) were

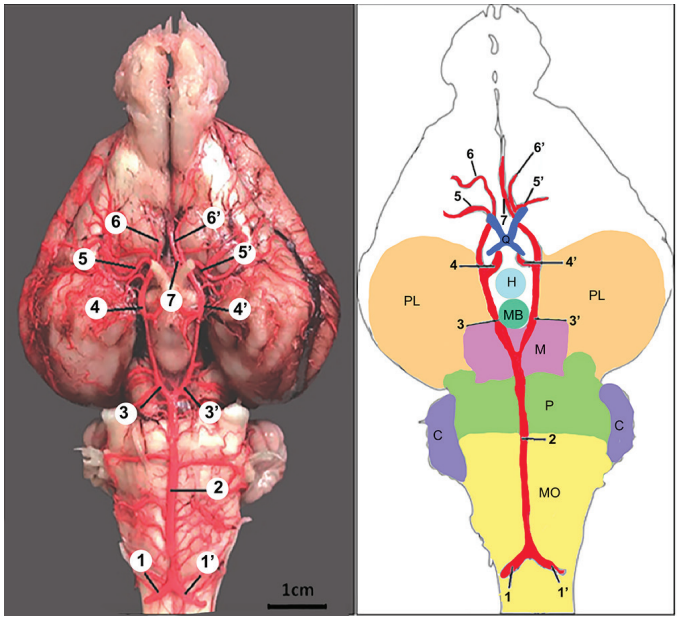


Fig.1. Photographic image (left side) and schematic (right side) of the base of the paca (*Cuniculus paca*) brain, demonstrating its arterial vascularization. Vertebral arteries right (1) and left (1'), basilar artery (2), branches of the basilar artery right (3) and left (3'), internal carotid arteries right (4) and left (4'), middle cerebral arteries right (5) and left (5'), rostral cerebral arteries right (6) and left (6'), medial branches of the rostral cerebral arteries (7), medulla oblongata (MO), cerebellum (C), pons (P), mesecephalon (M), piriform lobe (PL), mamillary body (MB), hypophysis (H), optic chiasm (Q).

Table 1. Comparasion of the arterial vascularization of the paca with the others rodents species

Animals	Arteries							
	Vertebral	Basilar	Terminal branches of the basilar artery	Internal carotid	Middle cerebral	Rostral cerebral	Medial branche of the rostral cerebral artery	Reference
Capybara (*Hydrochoerus hydrochaeris*)	x	x	x	-	x	x	x	Reckziegel & Lindemann 2001
Chincilla (*Chinchilla lanígera)*	x	x	x	x	x	x	x	Araújo & Campos 2005
Porcupine (*Hystrix cristata)*	x	x	x	-	x	x	x	Aydin et al. 2005
Paca (*Cuniculus paca)*	x	x	x	x	x	x	x	Dubost & Henry 2006
Agouti (*Dasyprocta aguti*)	x	x	x	x	x	x	x	Dubost & Henry 2006
Nutria (*Myocastor coypus)*	x	x	x	-	x	x	x	Azambuja, 2007
Red Squirrel (*Sciurus vulgaris)*	x	x	x	x/-	x	x	x	Aydin, 2008
Ground Squirrel (*Spermophilus citellus)*	x	x	x	-	x	x	x	Aydin et al. 2008
Rabbit (*Oryctolagus cuniculus*)	x	x	x	x	x	x	x	Souza & Campos 2013
Galea (*Galea spixii)*	x	x	x	x	x	x	x	Costa et al. 2017b
Gerbil (*Meriones unguiculatus)*	x	x	x	x	x	x	x	Costa et al. 2017a

identified, which, after a short course, were divided into the right and left middle cerebral arteries (5) and the right and left rostral cerebral arteries (6); the latter presenting small caliber, located between the optic nerve and piriform lobe. Dorsal to the optic chiasm, the medial branch of the rostral cerebral arteries was identified (7).

In this context, it was noted that paca presents the closed cerebral arterial circuit, as a pentagonal shape and basically vascularized by the right and left internal carotid arteries and vertebrobasilar system, being classified as type II, as established by De Vriese (1905).

DISCUSSION

The brain's arterial circuit (which surrounds mammillary body, the tuber cinereum, the hypophysis and the optic chiasm) (Ferreira & Prada 2005) is important for the vascularization of the main organ of the central nervous system, which is responsible for receiving and processing information of the whole organism (Lima et al. 2013). Thus, the identification and the pattern of the arterial structures are essential not only for taxonomy and phylogeny, but also to the care and clinical neurological surgical treatment (Kieltyka-Kurc et al. 2015).

Different morphologies in brain vascularization among animal species can be attributed to evolutionary adaptations related especially to behavioral and dietary aspects. Reckziegel et al. (2001) reported that the largest differences related to the types of brain vascularization in mammals were found in rodents and that, regardless of the type of vascularization, the vessels that carry blood to this region of the nervous system are small in diameter.

Comparisons of the arteries and branches of the brain base of pacas with the other rodents species, as well as the types of the base brain vascularization according to the classification of De Vriese (1905) are shown in Table 1 and Table 2, respectively.

In the present study, it was observed that paca presents the vertebral artery at the base of the brain in accordance with Bugge (1970) in muroiods, Bugge (1971a) in rats, bamboo rats, mices and jerboas, Bugge (1971c) in mole-rat, Alcântara & Prada (1996) in dogs, Reckziegel et al. (2001) in capybaras, Araújo & Campos (2005) in chinchillas, Aydin et al. (2005) in guinea pigs, Bugge (1971b) and Aydin (2008) in red squirrel, Aydin et al. (2009) in ground squirrels, Barreiro et al. (2012) in coatis, Lima et al. (2013) in anteater (*Tamandua tetradactyla*), Souza & Campos (2013) in rabbits, Silva et al. (2016) in agouti, Costa et al. (2017a) in gerbils and Costa et al. (2017b) in preá (*Cavia aperea*).

Similar to described in cutias by Silva et al. (2016), the vertebral artery in pacas has larger caliber when compared to other vessels. Vertebral arteries are branches of the

Table 2. Type of vascularization of the brain base of different rodents, according to De Vriese's classification* (1905)

Carotid and vertebrobasilar system	Vertebral-basilar system
Paca	Chincilla
Agouti	Galea
Capybara	Nutria
Gerbil	Porcupine
	Red Squirrel

* De Vriese (1905).

subclavian arteries (Araújo & Campos 2005, Lima et al. 2013) and close to the intervertebral foramina, emit the muscular and spinal branches, which unite and enter the spinal canal to form the ventral spinal artery (Alcântara & Prada 1996, Reckziegel et al. 2001).

In this sense, the basilar artery, which is the direct continuation of the ventral spinal artery (Alcântara & Prada 1996, Silva et al. 2016), was identified in the pacas as well as in other species listed in Table 1. In contrast, the terminal branches of the basilar artery (caudal cerebellar artery, middle cerebellar and rostral cerebellar) were not mentioned in coatis (Barreiro et al. 2012). These branches originate the right and left pituitary arteries (Araújo & Campos 2005). Lima et al. (2013) reported that the anteater's middle cerebral artery arose from the internal carotid artery in contrast to that in the agouti (Silva et al. 2016). In a systematic analysis of the arteries at the base of the capybara's brain, Reckziegel et al. (2001) observed the lack of the rostral cerebral artery in 6.7% of cases on the right and in 3.3% of cases on the left and Araújo and & Campos (2005) reported that the rostral cerebral artery was lacking in 3.3% of cases in the chinchilla on the right and left; the author also remarked that a thin vestigial vessel might be found where the rostral cerebral artery originated.

Still regarding the basilar artery, the rectilinear course of this vessel was noted in the five pacas, disagreeing with Alcântara & Prada (1996) when reporting the winding course in 70% of the dogs evaluated by them. The authors related this evident tortuosity to the phase of the arterial vascularization model proposed by De Vriese (1905) in this species, characterized by the union of the carotid and vertebro-basilar systems, with opposite flows, and with the angulation established by the collateral branches of this artery. The sinuous path of the basilar artery has also been described by Lima et al. (2006) in cats. In addition, Souza & Campos (2013) described that in rabbits, the basilar artery may originate solely from the left or right vertebral artery.

The intracranial segment of the internal carotid artery as well as its rostral and caudal branches were found in paca, similarly in castor (Frąckowiak & Śmiełowski 1998), dogs (Alcântara & Prada 1996), chinchillas (Araújo & Campos 2005), swine (Ferreira & Prada 2005), yaks (Ding et al. 2007), coatis (Barreiro et al. 2012), anteater (Lima et al. 2013), rabbits (Souza & Campos 2013), hares (Brudnicki et al. 2015), deer (Kieltyka-Kurc et al. 2015), agouti (Silva et al. 2016), gerbil (Costa et al. 2017a) and caves (Costa et al. 2017b). In contrast, it has not been described in guinea pigs (Aydin et al. 2005), adult capybaras (Reckziegel et al. 2001, Steele et al. 2006), nutria (Azambuja 2007) and ground squirrels (Aydin et al. 2009). In red squirrels, the intracranial segment of the internal carotid artery wasn´t found in four animals of the ten evaluated by Aydin (2008).

Reckziegel et al. (2001) and Steele et al. (2006) described in adult capybara the presence of fibrous cords in the common carotid arteries, where the origin of the extracranial segment internal carotid artery would normally be located, indicates that carotid brain irrigation existed during the embryonic phase and that the vertebrobasilar system invaded gradually the site, with consequent internal carotid atrophy. They also pointed out that partial or total atrophy of the extracranial segment of the internal carotid artery is present in other rodents, probably due to the strong jaw and adapted to the temporomandibular joint modified with rostro-caudal movements and well-developed musculature. In these animals, the vertebral system invades this area to compensate for the reduction in brain irrigation.

The middle cerebral artery was identified in the pacas, as well as in the other species in Table 1, providing collateral branches reaching the rostral choroid artery and the piriform lobe (Alcântara & Prada 1996, Ding et al. 2007). As well as in cutias (Silva et al. 2016), the middle cerebral artery it reaches the dorsal surface and distributes itself tree-wise; however, these authors cited the origin of the right and left middle cerebral arteries from the communicating rostral branch of the cerebral carotid at the level of the optic tract.

A rostral cerebral artery, also called the anterior cerebral artery (Alcântara & Prada 1996), associated with its middle branches, running medially through the dorsal region of the optic chiasm, reaching a longitudinal fissure, similar to Ding et al. (2007) in yaks. Unlike the current research in pacas, the rostral cerebral artery in the nutrias originates from the terminal branches of the basilar artery, according to Azambuja (2007). Araújo & Campos (2005) and Silva et al. (2016) reported that the rostral cerebral artery may be absent in chinchillas and agoutis, respectively.

Regarding the pattern of the arteries that supply the base of the paca brain, it can be classified as type II, according to De Vriese (1905), because it is performed equally by the carotid and vertebrobasilar system (responsibles for brain irrigation of the rostral and caudal regions, respectively) (Silva et al. 2016, Costa et al. 2017a, 2017b). Thus, as described in gerbils by Costa et al. (2017a), these systems emit vessels that are distributed in the regions of spinal cord, medulla oblongata, trapezoid body, pons, cerebral peduncle, cerebellum, piriform lobe, mamillary body, optic chiasm, optic nerve olfactory trigone and olfactory bulb. On the other hand, Reckziegel et al. (2001), Araújo and& Campos (2005), Aydin et al. (2005) and Azambuja (2007) described that in capybara, chinchilla, porcupine and nutria, respectively, cerebral vascularization depends solely or almost exclusively on the vertebrobasilar system (type III brain vascularization).

CONCLUSION

The vascularization of the base of the paca brain is performed by the carotid and vertebrobasilar system, similar pattern found in most rodents. It is believed that the absence of vascular anatomical variations in this region, observed among individuals of the same species and distinct gender, can be attributed to the reduced number of specimens analyzed in the present research.

Conflict of interest statement.- The authors declare no conflict of interest.

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Os autores podem submeter seus artigos em **Inglês** ou em **Português**, mas sempre com um Resumo em português. No caso de artigos aceitos escritos em **Português**, estes serão traduzidos para o **Inglês** pela Editora Cubo; pois todos os artigos publicados na PVB serão em inglês. Para os artigos já submetidos em **Inglês**, os autores devem apresentar via ScholarOne um Certificado de Tradução de uma empresa habilitada ou de um Tradutor Nativo. **Essa regra vale para artigos submetidos a partir de 1 de janeiro de 2018.**

Os pagamentos da taxa de publicação (*Paper Charge*) serão cobrados na ocasião do envio da comunicação de aceite por e-mail:

(1)Artigos submetidos em inglês, R$ 1.500,00 (*US$ 480.00*) por artigo;

(2)Artigos submetidos em português, R$ 1.500,00 (*US$ 800.00*) mais R$ 0,32 por palavra (o valor total cobrado por será enviado por e-mail pela tesouraria do CBPA).

A partir de 2019, **todos os autores deverão criar um registro no ORCID** (Open Researcher and Contributor ID - https://orcid.org/register) e vinculá-lo ao seu perfil no ScholarONE. O vínculo pode ser feito editando o perfil do usuário no ScholarONE na opção **Associate your existing ORCID iD**. Os identificadores ORCID contribuem para a identificação única dos autores e, portanto, para os processos interoperacionais e bibliométricos que envolvem autores. Usando um ORCID, os pesquisadores são fácil e corretamente conectados com seus resultados de pesquisa, publicações e afiliações.

O **texto** deve ser formatado, em todos os pormenores, de acordo com as normas de apresentação da revista (www.pvb.com.br).

Se o artigo for submetido fora das normas de apresentação da PVB, a tramitação somente ocorrerá após as devidas correções feitas pelo autor.

A PVB publica Artigos Originais, Artigos de Revisão Crítica e Tópicos de Interesse Geral; não publica artigos com a denominação de *Short Communications*.

Relatos de Caso serão aceitos somente em artigos classificados como pertencentes à área de Animais Selvagens (*Wildlife Medicine*).

Os Artigos Originais devem conter resultados de pesquisa ainda não publicados ou submetidos para outros periódicos.

Artigos de Revisão de Literatura, submetidos a convite, devem constituir-se de análise crítica, de assuntos na área de experiência dos autores, isto é, quando os autores já tiverem publicado anteriormente artigos sobre o assunto.

Os raros Tópicos de Interesse Geral devem constituir-se de assuntos de grande importância atual baseado na vasta experiência dos autores.

As opiniões e conceitos emitidos nos artigos submetidos são de responsabilidade dos autores. O Conselho Editorial da PVB, com a assistência da Assessoria Científica, pode sugerir ou solicitar modificações. Os artigos submetidos são avaliados pelos pares (*peer review*) e, aceitos para publicação com dois pareceres favoráveis, ou rejeitados por dois pareceres desfavoráveis.

Os direitos autorais dos artigos aceitos para publicação permanecem com os autores.

1. **Os artigos devem ser organizados em TÍTULO, ABSTRACT, RESUMO, INTRODUÇÃO, MATERIAL E MÉTODOS, RESULTADOS, DISCUSSÃO, CONCLUSÕES (de preferência os últimos três separadamente), Agradecimentos, Declaração de conflito de interesse e REFERÊNCIAS:**

a) O **TÍTULO** deve ser conciso e indicar o conteúdo do artigo; pormenores de identificação científica devem ser colocados em MATERIAL E MÉTODOS.

b) O(s) Autor(es) com numerosos primeiros nomes e sobrenomes, deve(m) padronizar o seu "nome para publicações científicas", como por exemplo: Cláudio Severo Lombardo de Barros, escreve Cláudio S.L. Barros ou Barros C.S.L.; Franklin Riet-Correa Amaral escreve Franklin Riet-Correa ou Riet-Correa F. **Os artigos devem ter no máximo 8 (oito) autores**. O autor para correspondência deve ser um autor que garanta o contato com o Conselho Editorial da PVB. Asteriscos de chamadas para o rodapé não devem ser sobrescritos.

c) O **Cabeçalho do ABSTRACT** deve conter além dos nomes dos autores abreviados invertido, o ano, o TÍTULO, o endereço postal do laboratório (inclusive o CEP) ou instituição principal onde foi desenvolvida a pesquisa. Endereços postais brasileiros

não devem ser traduzidos para o inglês, mesmo em artigos escritos na língua inglesa, a fim de evitar dificuldade na postagem. Deve-se conferir os nomes dos autores do artigo e do Cabeçalho do Abstract para evitar discrepâncias.

d) O **Rodapé da primeira página** deve conter os endereços profissionais postais completos dos autores (evitando-se traços horizontais), na língua do país do respectivo autor (em português, espanhol, inglês) e seus e-mails; o e-mail do autor para correspondência deve ser sublinhado. Os sinais de chamada para os nomes dos autores devem ser números arábicos, colocados em sobrescrito, sem o uso automático de "Inserir nota de fim", do Word (essas chamadas devem ser contínuas por todo artigo, isto é, em todas as notas de rodapé das outras páginas).

e) O **ABSTRACT** deve ser uma versão do RESUMO, mas pode ser mais explicativo, seguido de "INDEX TERMS" que devem incluir termos do título, por não se tratar somente de "ADDITIONAL INDEX TERMS".

f) O **RESUMO** deve conter o que foi feito e estudado, indicando a metodologia e dando os mais importantes resultados e conclusões, seguido dos "TERMOS DE INDEXAÇÃO" que incluem têrmos do título, por não se tratar somente de "TERMOS DE INDEXAÇÃO ADICIONAIS".

g) A **INTRODUÇÃO** deve ser breve, com citação bibliográfica específica sem que a mesma assuma importância principal e deve finalizar com a indicação do objetivo do artigo.

h) MATERIAL E MÉTODOS deve reunir a totalidade dos dados que permitam o desenvolvimento de trabalho semelhante por outros pesquisadores.

i) Em **RESULTADOS** devem ser apresentados concisamente os dados obtidos.

j) Na **DISCUSSÃO** devem ser confrontados os resultados diante da literatura. Não convém mencionar artigos em desenvolvimento ou planos futuros, de modo a evitar uma obrigação do autor e da revista de publicá-los.

k) CONCLUSÕES devem basear-se somente nos resultados obtidos e devem ser apresentados em diferentes parágrafos (uma Conclusão somente deve ser apresentada em parágrafo único).

l) Os **Agradecimentos** não devem aparecer no texto ou em notas de rodapé; devem ser sucintos e colocados antes da Declaração de conflito de interesse e da Lista de Referências.

m) A **Declaração de conflito de interesse** é obrigatória e deve ser mencionada nos casos positivos ou negativos; deve ser sucinta e colocada imediatamente antes da Lista de Referências.

n) A Lista de **REFERÊNCIAS** deve incluir todas as citações apresentadas no texto e que tenham servido como fonte para consulta. A Lista deve ser ordenada alfabética e cronologicamente, pelo sobrenome do primeiro autor, seguido de todos os demais autores (em caixa alta e baixa), do ano, do título da publicação citada, e abreviado (por extenso em casos de dúvida) o nome do periódico. Sugerimos consultar exemplos dos últimos fascículos (www.pvb.com.br).

(Notem: (1) As Referências citadas no texto devem ser colocadas em ordem cronológica, mas alfabética tratando-se de referências do mesmo ano; (2) Quando utilizados programas de formatação (p.ex. Endnote X7), remover o fundo automático cinzento antes da submissão, para não dificultar eventuais correções.

2. **Na elaboração do texto devem ser atendidas as seguintes normas:**

a) Fonte **Cambria, corpo 10, entrelinha simples; página formato A4, com 2cm de margens** (superior, inferior, esquerda e direita), texto corrido em uma coluna justificada, com as Legendas das Figuras no final (logo após a Lista de REFERÊNCIAS) sem repetir as legendas junto com as Figuras.

b) ABSTRACT e RESUMO serão escritos em um só parágrafo corrente e não devem conter citações bibliográficas.

c) A redação dos artigos deve ser concisa, com a linguagem, tanto quanto possível, no passado e impessoal.

d) Os nomes científicos usados no manuscrito devem ser apresentados por extenso (p.ex. *Palicourea marcgravii*), no início de cada capítulo (**TÍTULO, ABSTRACT, RESUMO, INTRODUÇÃO, etc.**), quando aparecem pela primeira vez, seguido da abreviação do gênero (p.ex. *P. marcgravii*).

e) Nos títulos dos Quadros e nas Legendas das Figuras os nomes científicos devem ser apresentados por extenso, já que estes são independentes do texto.

f) No texto, os sinais de chamada para notas de rodapé devem ser números arábicos colocados em sobrescrito após a palavra ou frase que motivou a nota. Essa numeração será contínua por todo o artigo; as notas deverão ser lançadas ao pé da página em que estiver o respectivo número de chamada, sem o uso do "Inserir nota de fim", do Word.

Notem: para evitar a separação em duas linhas, os numerais devem ser apresentados junto com suas unidades, ou seja, sem espaçamento, por exemplo: 100ppm, 10mm, 50cm, 18x10cm, (P<0,05), 15h. A abreviação de número é "nº" e não "nº"; grau Celsius é "ºC" e não "ºC".

g) Os Quadros (não usar o termo Tabela) e as Figuras devem ser citados no texto, pelos respectivos números, em ordem crescente e devem ser submetidos separadamente do texto!

h) Siglas e abreviações das instituições, ao aparecerem pela primeira vez, deverão ser colocadas entre parênteses, após o nome da instituição por extenso;

i) Citações bibliográficas serão feitas pelo sistema "autor e ano", p.ex. (Caldas 2005); artigos de até dois autores serão citados pelos nomes dos dois (Pedroso & Pimentel 2013); e com mais de dois, pelo nome do primeiro, seguido de "et al.", mais o ano (Brito et al. 2015); se dois artigos não se distinguirem, a diferenciação será feita através do acréscimo de letra minúscula ao ano (Barros 2017a, 2017b). A ordem de citação deve ser cronológica (Barbosa et al. 2003, Armién et al. 2004).

j) Recomenda-se consultar na íntegra todos os artigos citados; se isto não for possível, deve-se colocar no texto a referência original (não consultada na íntegra) seguida do ano, p.ex. (Bancroft 1921); na Lista de Referências deve ser incluída a referência original como: Bancroft 1921. ..... título. ... periódico. (Apud Suvarna & Layton 2013). A referência consultada também deve ser incluída na Lista de Referências.

k) O uso de "comunicação pessoal" e de "dados não publicados" deve ser feito apenas em casos excepcionais; no texto com citação de Nome e Ano, e na Lista de Referências como: Barbosa 2016. Comunicação pessoal (Universidade Federal do Pará, campus Castanhal).

l) As **Legendas das Figuras** devem conter informações suficientes para sua compreensão (independente do texto); e devem ser precedidas de "Fig." seguida do número sem espaço, p.ex. "Fig.8. ...". Para elaboração das legendas sugerimos consultar exemplos nos últimos fascículos (www.pvb.com.br).

(Notem: Na legenda de Figuras compostas deve-se colocar a letra de cada "subfigura" em **negrito** com parênteses claros antes do texto correspondente e devem ser mencionados letras ou sinais, que estão dentro de cada "subfigura", em parêntees e claros após o respectivo texto da legenda.)

m) O Título dos **Quadros** devem ser em **negrito**, sem ponto, e a "garganta" (título das colunas) deve ser escrita em claro e separada por dois traços longos horizontais; o Título dos Quadros e da "garganta" devem ser escritas em caixa alta e baixa. Os Quadros (não usar o termo Tabela) devem conter os resultados mais relevantes. Não há traços verticais, nem fundos cinzentos; excepcionalmente pode conter traços horizontais. Os sinais de chamada serão alfabéticos, recomeçando, com "a" em cada Quadro. As chamadas de rodapé deverão ser lançadas logo abaixo do Quadro respectivo, do qual serão separadas por um traço curto à esquerda; e devem evitar números arábicos. Os títulos não têm ponto no final, ao passo que as legendas terminam com um ponto. Os Quadros devem ser apresentados em Word e ser editáveis, a fim de inserirmos eventuais alterações de apresentação, dentro das normas da revista.

n) Dados complexos devem ser expressos por Gráficos (devem ser chamados de **Figuras**). Os gráficos devem ser produzidos em 2D, sem fundo e sem linhas horizontais. Em gráficos contendo texto a fonte deve ser Cambria tamanho 10.

3. **Apresentação das Figuras:**

a) As figuras devem ser salvas em 300dpi, arquivo TIF.

b) Enviar cada figura separadamente.

c) Identificar as figuras em ordem conforme a menção no texto.

d) As figuras solitárias devem ter seus arquivos identificados como (Fig.1, Fig.2 ...)

e) As figuras que serão destinadas a formar uma prancha devem ter seus arquivos identificados como (Fig.1A, Fig.1B ...). As pranchas devem ser compostas por múltiplas subfiguras. Imagens destinadas a uma prancha devem ser de mesmo tamanho.

f) Para micrografias usar, de preferência, barras de escala para indicar o aumento; apresentar na legenda sempre o método de coloração e a objetiva, p. ex.: HE, obj.40x.

g) As legendas de figuras devem conter inicialmente o que se observa na imagem, seguida das informações adicionais (Formato típico da legenda: Fig.1. (**A**) Descrição da imagem. Diagnóstico, órgão ou tecido, espécie animal, número do caso. Método de coloração e objetiva.).

h) As legendas de figuras devem ser apresentadas junto com o texto do artigo, após as Referências.

4. **Todas as referências citadas no texto devem ser incluídas na Lista de Referências e vice-versa**; **na revisão final do artigo pelos autores,** antes da submissão, **isto deve ser conferido criteriosamente, para evitar discrepâncias (o sistema ScholarOne bloqueia automaticamente artigos com discrepâncias).**

Exemplos de Referências

➢ Artigos publicados em periódicos:

Martins K.P.F., Fonseca T.R.S., Silva E.S., Munhoz T.C.P., Dias G.H.S., Galiza G.J.N., Oliveira L.G.S. & Boabaid F.M. 2018. Bócio em bovinos. Pesq. Vet. Bras. 38(6):1030-1037.

Rondelli L.A.S., Silva G.S., Bezerra K.S., Rondelli A.L.H., Lima S.R., Furlan F.H., Pescador C.A. & Colodel E.M. 2017. Doenças de bovinos no Estado de Mato Grosso diagnosticadas no Laboratório de Patologia Veterinária da UFMT (2005-2014). Pesq. Vet. Bras. 37(5):432440.

Hooiveld M., Smit L.A., Wouters I.M., Van Dijk C.E., Spreeuwenberg P., Heederik D.J. & Yzermans C.J. 2016. Doctor-diagnosed health problems in a region with a high density of concentrated animal feeding operations: a cross-sectional study. Environ. Health 17:15-24.

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(Notem: Os iniciais dos autores devem ser colocados sem espaço. O sinal "&" é usado para separar o penúltimo do último autor. As primeiras letras das palavras do título de artigos publicados em periódicos científicos devem ser de preferência minúsculas. A palavra "Revista" deve ser abreviada como "Revta" em diferença a "Rev.", do inglês "Review". Deve-se indicar o número do respectivo volume do periódico e, se possível, também do fascículo. Somente abreviações tem um ponto, exceto as que terminam com a última letra da palavra em extenso. O traço entre as páginas é curto (-) e não comprido. Não devem ser usados "pontovírgulas" (;) em lugar de vírgulas.

- Livros:

Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Döbereiner J. 2012. Plantas Tóxicas do Brasil para Animais de Produção. 2ª ed. Helianthus, Rio de Janeiro, p.305-348.

Marsh P. & Martin M. 1992. Oral Microbiology. 3rd ed. Chapman and Hall, London, p.167-196.

(Notem: A primeira letra de termos do título de livros deve ser maiúscula. Devem ser mencionadas as páginas que foram consultadas, em vez do total de páginas do livro.

- Capítulos de livros:

Barros C.S.L. 2007. Doenças víricas: leucose bovina, p.159-169. In: Riet-Correa F., Schild A.L., Lemos R.A.A. & Borges J.R.J. (Eds), Doenças de Ruminantes e Equídeos. Vol.1. 3ª ed. Pallotti, Santa Maria.

Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Döbereiner J. 2012. Plantas que afetam o funcionamento do coração, p.27-94. In: Ibid. (Eds), Plantas Tóxicas do Brasil para Animais de Produção. 2ª ed. Helianthus, Rio de Janeiro.

(Notem: As primeiras letras das palavras do título de capítulos de livros são minúsculas, mas as de livros são maiúsculas.)

- Dissertações e Teses:

Rech R.R. 2007. Alterações no encéfalo de bovinos submetidos à vigilância das encefalopatias espongiformes transmissíveis. Tese de Doutorado, Universidade Federal de Santa Maria, Santa Maria. 228p.

(Notem: (1) Deve-se evitar citações de Dissertações ou Teses; deve-se preferir citar artigos baseados nas mesmas e publicados em periódicos científicos que são de mais fácil acesso. (2) Não deve-se tentar de publicar o texto de Dissertação ou Tese praticamente na íntegra sem escrever um artigo conciso de seus resultados.

- Resumos publicados em eventos:

Mendonça F.S., Almeida V.M., Albuquerque R.F., Chaves H.A.S., Silva Filho G.B., Braga T.C., Lemos B.O. & Riet Correa F. 2016. Paralisia laríngea associada à deficiência de cobre em caprinos no semiárido de Pernambuco (IX Endivet, Salvador, BA). Pesq. Vet. Bras. 36(Supl.2):50-51. (Resumo)

Pierezan F., Lemos R.A.A., Rech R.R., Rissi D.R., Kommers G.D., Cortada V.C.L.M., Mori A.E. & Barros C.S.L. 2007. Raiva em equinos. Anais XIII Encontro Nacional de Patologia Veterinária, Campo Grande, MS, p.145-146. (Resumo)

(Note: Evitar na consulta o uso de Resumos ao invés de artigos na íntegra!)

Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

GUIDE FOR AUTHORS

Papers to "Pesquisa Veterinária Brasileira" (PVB), a Brazilian Journal of Veterinary Research, are submitted in Word online through ScholarOne, link <https://mc04.manuscriptcentral.com/pvb-scielo>

The authors should submit their papers in English, with a Portuguese Summary. To prove the quality of the English, a certificate of the English language is required, with exception of authors native in English.

With the communication of acceptance of the paper, the author for correspondence will be asked for payment of a Paper Charge of US$ 480.00 (*R$ 1.500,00*) for each article submitted in English.

As of 2019, **all authors should register in the ORCID** (Open Researcher and Contributor ID https://orcid.org/register) and link it to their ScholarONE profile. The link can be done by editing the user profile on ScholarONE in the option **Associate your existing ORCID iD**. The ORCID identifiers contribute to the singular identification of the authors and to the interoperational and bibliometric processes. Using an ORCID, researchers are easily and correctly connected with their research results, publications and affiliations.

Papers should be prepared in all details according to the style of the journal (www.pvb.com.br), in order to be peer reviewed. Tables and Figures should be submitted separately from the text.

PVB publishes Original Articles, but also Critical Literature Reviews and Topics of General Interest; no Short Communications are accepted.

Case Reports will be accepted only in articles classified as Wildlife Medicine.

The Original Papers should contain research results not yet published and not submitted to other journals.

Literature Reviews should be critical and consist of subjects of the author's research line.

Topics of General Interest should be of great importance and based on large experience of the authors.

The opinions and concepts emitted are of the responsibility of the authors. The Editorial Board of the journal, assisted by the peer review, may suggest or ask for modification of the text.

The author rights of the accepted papers are preserved.

1. **The submitted article should be organized in TITLE, ABSTRACT, RESUMO (the last when authors are from a Portuguese speaking country), INTRODUCTION, MATERIALS AND METHODS, RESULTS, DISCUSSION, CONCLUSION(S) (the last three preferably as separate chapters), Acknowledgements, Conflict of interest statement and REFERENCES:**

a) The **TITLE** should be concise and indicate the content of the article; details of scientific identification should be put into MATERIALS AND METHODS.

b) Authors with several first and family names should shorten their names for scientific publication, as for example: Cláudio Severo Lombardo de Barros writes Cláudio S.L. Barros or Barros C.S.L., and Franklin Riet-Correa Amaral writes Franklin Riet-Correa or Riet-Correa F. **The papers should not have more than 8 (eight) authors.** Corresponding author should be one who guarantees the contact with the Editorial Board of PVB. Asterisks for call to the footnotes should be elevated once more, in order to appear larger.

c) The **heading of the ABSTRACT** should contain the shortened and inverted names of the authors, the year, the Title (in brakes when translated), and the postal address of the laboratory or institution where the main part of the research was done (Always compare the authors of the paper and their shortened and inverted in the heading of the Abstract to avoid discrepancies).

d) The **footnote of the first page** should contain the complete professional address of each author (in the language of the author's country where to correspondence could be posted, Portuguese, Spanish, English, etc.) as well as the underlined e-mail of the corresponding author.

e) The **ABSTRACT** should be a well explained version of the Portuguese RESUMO, followed by "INDEX TERMS" which should include terms of the title, as they are not only Additional Index Terms.

f) The **RESUMO** should contain (1) what have been investigated, indicating (2) materials and methods used, (3) the most important results, and (4) the conclusion, followed by "TERMOS DE INDEXAÇÃO" (which include also words of the title, as they are not only Additional Index Terms).

g) The **INTRODUCTION** should be short, with citation of the specific literature without assuming main importance, followed by the objective of the research.

h) In **MATERIALS AND METHODS** should be given all data necessary for other research workers to repeat the research.

i) In **RESULTS** are presented the data obtained in a concise form.

j) In **DISCUSSION** the results should be confronted with the literature. Research in development or future planning should not be mentioned, to avoid the obligation for the journal to publish the results.

k) The **CONCLUSIONS** should be based only on the results obtained.

l) Acknowledgements should not be mentioned in the text or in footnotes.

m) Conflict of interest or none should be mentioned.

n) The **REFERENCES** include all citations consulted and presented chronologically in the text. The List of References should be written in alphabetical and chronological order, beginning with the family name of the first author, followed by the names of all other authors of the respective reference, in capital and small letters, and each author divided only by a comma, followed by year, title and the data of the publication (extensively in case of doubt about abbreviation) according to www.pvb.com.br.

2. During the elaboration of the paper, **the style of the journal has to be attended**, as follows:

a) Font **Cambria at 10 pitch, simple space between lines;** page **format A4, with 2cm margins** (superior, inferior, left and right), text in one column justified, with Figure captions below the list of References; without repeating the captions with the images of the Figures. Figures and Tables should be separately submitted.

b) ABSTRACT and **RESUMO** are written in only one paragraph and should not contain references.

c) The articles should be concise, always when possible in past tense and impersonal.

d) The scientific names should be presented in full (p.ex. *Palicourea marcgravii)* at the beginning of each chapter (Title, Abstract, Resumo, Introduction, etc.) when they appear for the first time, followed with abbreviation of the genus (p.ex. *P. marcgravii*).

e) In the Title of Tables and in Figure captions the scientific names are written in full.

f) In the text, calls to footnotes are given in Arabic numbers, in crescent order through the whole paper, without use of "Insert final note" of Word.

Note: To avoid separation in two lines, numbers should be presented without space to their units (p.ex.: 100ppm, 10mm, 50cm, 18x10cm, P<0.05.

The abbreviation for number is "nº" and not "nº"; for degree Celsius "ºC" and not "ºC".

g) Tables and Figures should be cited in the text with their respective numbers in crescent order.

h) Abbreviations of institutions when presented in the first place should be put within parentheses, after the full name of the institution.

i) Citations of the literature in the text are given by "author and year" (p.ex. Caldas 2005); papers with two authors are cited with the two names (p.ex. Pedroso & Pimentel 2013); citations with more than two authors are cited in the text by the name of the first author followed by "et al." and the year (p.ex. Brito et al. 2015). If two articles are not to distinguish, the differentiation is obtained through the addition of small letters after the year (p.ex. Barros 2017a, 2017b). The order of citation in the text should be chronological (p.ex. Barbosa et al. 2003, Armién et al. 2004).

j) All cited articles should be consulted in full text; if not possible, the original reference is put into the text as p.ex. Bancroft (1921); but in the List of References this should appear as: Bancroft 1921. ..... title. ... journal .... (Apud Suvarna & Layton 2013). The consulted reference should be also included in full in the List.

k) The use of "personal communication" and "non-published data" should be exceptional and cited in the text as Author and Year, and in the List of References as p.ex. Barbosa 2016. Personal Communication (Universidade Federal do Pará, campus Castanhal, Brazil).

l) Figure captions (p.ex. "Fig.3. ……..") should be sufficiently informative for understanding (because Figures are independent from the text).

m) The **Title of Tables** should be written in **bold** and the **Heading** (titles of the columns) should be in clear (not bold), written in capital and small letter and separated by two long horizontal lines. There are no vertical lines and no grey bottom; exceptionally can exist horizontal lines. The calls for footnotes should be in small letters or other signs, but not in Arabic numbers. Tables should be submitted in Word (not as images) to allow corrections according to the style of the journal.

n) Complex data should be presented as **graphics (but named Figures)** in 2D without grey bottom and horizontal lines. Graphics including text should be written with Cambria at 10 pitch.

3. **Figure presentation:**

a) Save images at 300 dpi, TIF files.

b) Send each figure separately.

c) Identify figures in the order in which they are mentioned in the text.

d) Individual figures must have their files named as (Fig.1, Fig.2, …).

e) Images that will compose a plate must have their files identified as (Fig.1A, Fig.1B,). Plates should be comprised by multiple images, and all images must have the same dimensions.

f) Use preferably scale bars for micrographs. For optical micrographs indicate at the legend finally the staining method and the objective used, for example: HE, obj.40x.

g) Figure legends should contain initially what is seen on the image, followed by additional information (Legend example: Fig.1. (**A**) Sentence description. Diagnosis, organ or tissue, animal species, case number. Staining method and objective used.).

h) Figure legends should be presented in the main document, after the **References**.

4. **All references cited in the text should be included in the List of References**; before the submission of the paper, discrepancies have to be corrected by the author (as the system ScholarOne blocks automatically if such discrepancies exist).

Exemples for References:

➢ Articles published in scientific journals:

Ubiali D.G., Cruz R.A., De Paula D.A., Silva M.C., Mendonça F.S., Dutra V., Nakazato L., Colodel E.M. & Pescador C.A. 2013. Pathology of nasal infection caused by *Conidiobolus lamprauges* and *Pythium insidiosum* in sheep. J. Comp. Pathol. 149(2/3):137-145.

Hooiveld M., Smit L.A., Wouters I.M., Van Dijk C.E., Spreeuwenberg P., Heederik D.J. & Yzermans C.J. 2016. Doctor-diagnosed health problems in a region with a high density of concentrated animal feeding operations: a cross-sectional study. Environ. Health 17:15-24.

(Note: The first letters of the words in the title of papers published in journals are small. It is preferable to indicate the number of the respective issue.)

➢ Books:

Marsh P. & Martin M. 1992. Oral Microbiology. 3rd ed. Chapman and Hall, London, p.167-196.

Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Döbereiner J. 2012. Plantas Tóxicas do Brasil para Animais de Produção. 2ª ed. Helianthus, Rio de Janeiro, p.305-348.

(Note: The first letter in the words of the title of books should be capital.)

➢ Chapters of books:

Uzal F.A., Plattner B.L. & Hostetter J.M. 2016. Alimentary system, p.1-257. In: Maxie M.G. (Ed.), Jubb, Kennedy and Palmer´s Pathology of Domestic Animals. Vol.2. 6th ed. Elsevier, St Louis, Missouri.

Barros C.S.L. 2007. Doenças víricas: leucose bovina, p.159-169. In: Riet-Correa F., Schild A.L., Lemos R.A.A. & Borges J.R.J. (Eds), Doenças de Ruminantes e Equídeos. Vol.1. 3ª ed. Pallotti, Santa Maria, RS.

Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Döbereiner J. 2012. Plantas que afetam o funcionamento do coração, p.27-94. In: Ibid. (Eds), Plantas Tóxicas do Brasil para Animais de Produção. 2ª ed. Helianthus, Rio de Janeiro.

➢ Dissertations and Theses:

Rech R.R. 2007. Alterações no encéfalo de bovinos submetidos à vigilância das encefalopatias espongiformes transmissíveis. Tese de Doutorado, Universidade Federal de Santa Maria, Santa Maria. 228p.

(Note: Use articles which originated from dissertations or theses instead of these).

➢ Abstracts published in Events:

Massa A.T., Potter K.A. & Bradway D. 2016. Epizootic bovine abortion outbreak in Eastern Nevada cattle. Annual Meeting American College of Veterinary Pathologist (ACVP), New Orleans, Lousiana. (Abstract D-50)

Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

Mendonça F.S., Almeida V.M., Albuquerque R.F., Chaves H.A.S., Silva Filho G.B., Braga T.C., Lemos B.O. & Riet Correa F. 2016. Paralisia laríngea associada à deficiência de cobre em caprinos no semiárido de Pernambuco (IX Endivet, Salvador, BA). Pesq. Vet. Bras. 36(Supl.2):50-51. (Resumo)

Pierezan F., Lemos R.A.A., Rech R.R., Rissi D.R., Kommers G.D., Cortada V.C.L.M., Mori A.E. & Barros C.S.L. 2007. Raiva em equinos. Anais XIII Encontro Nacional de Patologia Veterinária, Campo Grande, MS, p.145-146. (Resumo)

(Note: Consult entire papers instead of only Abstracts)

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