ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)
Volume 40 Number 6
June 2020

PESQUISA VETERINÁRIA BRASILEIRA

Brazilian Journal of Veterinary Research

Colégio Brasileiro de Patologia Animal
www.pvb.com.br

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Apoio:

Pesq. Vet. Bras. also has resources from the *Conselho Federal de Medicina Veterinária* (CFMV).

Pesquisa Veterinária Brasileira. - - vol.1, n.1 (1981) - . - - Rio de Janeiro: Colégio Brasileiro de Patologia Animal, 1981 -

il.; 28 cm

Mensal.

Resumos em inglês e português.

ISSN 0100-736X (*Print*).

ISSN 1678-5150 (*Online*).

Título traduzido: *Brazilian Journal of Veterinary Research*.

Continuação de: "Arquivos do Instituto de Biologia Animal" (1959-1961) e à Série Veterinária (1968-1976) da "Pesquisa Agropecuária Brasileira".

I. Colégio Brasileiro de Patologia Animal.

Tiragem: 100 exemplares.

ISSN 0100-736X (*Print*)
ISSN 1678-5150 (*Online*)

PESQUISA VETERINÁRIA BRASILEIRA

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Cover illustration: Cholangiocarcinoma with acinar pattern in the liver of a cow. (Vielmo A. et al., p.414)

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ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(6):409-416, June 2020
DOI: 10.1590/1678-5150-PVB- 6629

Original Article
Livestock Diseases

Primary hepatic neoplasms in cattle[1]

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ABSTRACT. - Vielmo A., Panziera W., Bianchi M.V., Argenta F.F., Lorenzo C., Vielmo L.A., Pavarini S.P. & Driemeier D. 2020. **Primary hepatic neoplasms in cattle.** *Pesquisa Veterinária Brasileira 40(6)409-416*. Setor de Patologia Veterinária, Departamento de Patologia Clínica Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Prédio 42505, Porto Alegre, RS 91540-000, Brazil. E-mail: andreiavielmo@yahoo.com.br

Primary hepatic neoplasms are mostly detected in cattle as incidental findings in slaughterhouses or diagnosed at the necropsy, wherein it may be related to the cause of death. A proper characterization of primary hepatic neoplasms is essential to provide an accurate diagnosis, especially at the slaughter lines, in order to reduce erroneous condemnations. This work aimed to characterize the gross, histological, and immunohistochemical features of primary liver neoplasms detected in slaughtered cattle in Southern Brazil. Nineteen primary hepatic neoplasms were identified. Grossly, these lesions were classified according to their distribution, as focal, multifocal, or diffuse. Histologically, the shape and arrangement of the cells, as well as possible malignant features were evaluated. Immunohistochemistry (IHC) was also performed for biliary epithelium (anti-CK7) and hepatocytes (anti-Hep Par-1) markers. Hepatocellular carcinoma (84.2%) was the most frequently detected hepatic neoplasm, followed by cholangiocarcinoma (15.8%), and these were only identified in adult cows. Hepatocellular carcinomas occurred as solitary masses or multifocal nodules, which on the cut surface were often green. Cholangiocarcinomas occurred as multifocal nodules, occasionally showing an umbilicated appearance. Histologically, hepatocellular carcinomas had mostly trabecular and solid patterns, while cholangiocarcinomas presented mostly a solid arrangement. Upon IHC, all hepatocellular carcinomas were immunolabeled for anti-Hep Par-1, ranging from mild (25%), moderate (31.2%) to marked (43.7%), while immunolabeling for anti-CK7 was detected only in one case of cholangiocarcinoma.

INDEX TERMS: Neoplasms, cattle diseases, abattoir study, hepatic tumors, hepatocellular carcinoma, cholangiocarcinoma, hepatic markers.

RESUMO.- [**Neoplasmas hepáticos primários de bovinos.**] Os neoplasmas hepáticos primários são detectados em bovinos principalmente como achados incidentais em matadouros ou diagnosticados na necropsia, quando podem estar relacionados à causa da morte. A caracterização adequada dos tumores hepáticos primários é essencial para obter diagnósticos precisos, especialmente nas linhas de abate, com o propósito de reduzir condenações errôneas. Este trabalho teve o objetivo de determinar as características macroscópicas, histológicas e imuno-histoquímicas dos neoplasmas primários do fígado de bovinos abatidos em um matadouro-frigorífico no Sul do Brasil. Dezenove neoplasias hepáticas primárias foram identificadas. Macroscopicamente, os tumores hepáticos foram classificados de acordo com sua distribuição, como focais, multifocais ou difusos. Histologicamente, a forma e o arranjo das células e possíveis características malignas foram avaliados. Também foi realizada imuno-histoquímica (IHQ) para marcadores de epitélio biliar (anti-CK7) e hepatócitos (anti-Hep Par-1). O carcinoma hepatocelular (84,2%) foi o neoplasma hepático mais frequentemente detectado, seguido pelo colangiocarcinoma (15,8%). Esses tumores foram identificados apenas em vacas adultas. Os carcinomas hepatocelulares eram vistos como massas solitárias ou nódulos multifocais que na superfície de corte geralmente

[1] Received on March 10, 2020.
Accepted for publication on March 24, 2020.
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eram esverdeados. Os colangiocarcinomas foram observados como nódulos multifocais, ocasionalmente com aspecto umbilicado. Histologicamente, os padrões mais observados nos carcinomas hepatocelulares foram trabeculares e sólidos, enquanto nos colangiocarcinomas o arranjo sólido foi o mais frequente. Na IHQ, todos os carcinomas hepatocelulares foram marcados por anti-Hep Par-1, com marcação que variou de leve (25%), moderada (31,2%) a acentuada (43,7%); imunomarcação para anti-CK7 foi detectada em apenas um caso de colangiocarcinoma.

TERMOS DE INDEXAÇÃO: Neoplasmas, bovinos, doenças de bovinos, estudo em abatedouro, tumores de fígado, carcinoma hepatocelular, colangiocarcinoma, marcadores hepáticos.

INTRODUCTION

Primary hepatic neoplasms (PHN) are uncommon in domestic animals, except in dogs, and are generally hepatocellular or cholangiocellular in origin (Cullen & Stalker 2016). In cattle, PHN frequency varies geographically, with incidences ranging from 3.1 to 10% in South Africa and in the United Kingdom (UK), respectively (Anderson & Sandinson 1968, Bastianello 1982). Recent data from Brazilian studies (Lucena et al. 2011, Tessele & Barros 2016, Reis et al. 2017) indicated lower frequencies of 1.88-4.6% of liver tumors when compared to other studies (Anderson & Sandinson 1968). Overall, lymphoma (enzootic bovine leucosis) and squamous cell carcinoma are the most common detected bovine neoplasms. The latter may occur in the upper alimentary tract, the eyes and adnexa, and in the vulva (Lucena et al. 2011, Rosa et al. 2012, Carvalho et al. 2014, Mello et al. 2017, Reis et al. 2017).

Primary tumors that originate from hepatocytes may include adenomas, hepatocellular carcinomas, and hepatoblastomas, while those originated from the biliary epithelium are classified as adenomas, biliary cystadenomas, cholangiocarcinomas, or biliary cystadenocarcinomas (Head et al. 2003, Cullen 2017). Mixed hepatocellular and cholangiocellular carcinomas are rarely observed and have histological characteristics of both cell origins: hepatocytes and biliary epithelium (Cullen 2017).

The diagnosis of PHN is not always obtained based solely on histological characteristics; on these instances, immunohistochemical exams are essential to establish a conclusive diagnosis. Several immunohistochemical markers may assist in the differential diagnosis of liver tumors (Chan & Yeh 2010), such as hepatocyte paraffin 1 (Hep Par-1), which is used to label neoplastic hepatocytes and, thus, identify hepatocellular carcinomas (Chu et al. 2002), and cytokeratin 7 (CK7), which labels bile duct epithelial cells (Yabushita et al. 2001), allowing a proper recognition of cholangiocarcinomas.

The characterization of PHN is important to improve the accuracy of identification in meat inspection lines. According to the Industrial Regulation of Animal Producer Inspection (Ministério da Agricultura 2017), carcasses, part of them, or organs affected by malignant tumors must be condemned, regardless of the occurrence of metastases. Thus, the difficulty in the diagnosis may lead to an incorrect destination of the carcass (Freitas 1999). In order to assist veterinary pathologists and meat inspectors, as well to improve diagnostic accuracy, this study aimed to characterize the gross, microscopic, and immunohistochemical aspects of PHN detected in slaughtered cattle in Southern Brazil.

MATERIALS AND METHODS

From 2015 to 2016, liver samples of cattle slaughtered in an abattoir of the municipality of Santa Maria, Rio Grande do Sul state (29°46'54.2"S; 53°46'38.8"W) were collected. These samples, previously fixed in 10% formalin, were sent for gross and histological evaluation after condemnation at the meat inspection line. Metastases were also sent for analysis.

The slaughtered cattle were from different regions of the state. For this study, only those livers with PHN were selected. The samples were photographed, trimmed, routinely processed for histology, and stained by hematoxylin and eosin (HE). Data regarding sex and age (estimated by dentition) (Food Safety Inspection Service 2013) of the cattle involved were obtained from the slaughterhouse files.

Grossly, PHN were classified according to their distribution into focal, multifocal, or diffuse. Focal tumors appeared as a single nodule or mass in the liver. Tumors distributed as multiple nodules or masses with a random distribution in the liver parenchyma were included in the multifocal category. Diffuse neoplasms affected all or most parts of the liver. In this latter pattern, there was an increase in the whole volume of the organ, accompanied by changes in color and consistency of the affected part.

Histological evaluation was performed according to previously established criteria (Cullen 2017). The following features were considered: (1) cellular arrangement; (2) stromal pattern; (3) cell pleomorphism; (4) vascular or lymphatic invasion; (5) necrosis, hemorrhage, and intratumoral inflammatory infiltrate; (6) intratumoral vascular spaces; and (7) mitotic index. The degree of mitoses was evaluated by three independent veterinary pathologists and considered as mild (less than or equal to one mitosis per high power field - 400x), moderate (2-4 mitosis per high power field), and marked (greater than five mitoses per high power field).

In order to assess desmoplasia levels and mucin content, histological sections of the neoplasms were stained by Masson's trichrome (MT) and Periodic Acid Schiff (PAS), respectively. Selected sections of the neoplasms were submitted to immunohistochemistry (IHC) using the universal polymer method marked with peroxidase (MACH 4 Universal HRP-Polymer - Biocare Medical) for the biliary epithelium [monoclonal antibody anti-cytokeratin-7 (anti-CK7); clone/brand: M7018/Dako; dilution 1:40; antigenic retrieval proteinase K] and hepatocytes [monoclonal antibody anti-hepatocyte paraffin 1 (anti-Hep Par-1); clone/brand: M7018/Dako; dilution 1:40; antigenic retrieval citrate buffer pH 6.0 for 40 min at 96°C]. For both protocols, revelation was obtained by using the chromogen 3-amino-9-ethylcarbazole (AEC) and counterstain with Mayer's hematoxylin. As a positive control, a section of the liver previously tested was used, and the same material was used as a negative control, by replacing the primary antibody with phosphate-buffered saline. The immunolabeling intensity was classified as absent, mild, moderate, or marked.

RESULTS

Nineteen primary hepatic neoplasms were identified during the period studied, of which 16 (84.2%) were hepatocellular carcinomas and three (15.8%) cholangiocarcinomas. All affected cattle were adults above 3-year-old. Female cattle were mostly affected, both in cases of hepatocellular carcinomas

(93.75%, 15/16), and in cases of cholangiocarcinoma (66.7%, 2/3). Grossly, hepatocellular carcinomas were focal in 43.7% (Fig.1A) of the cases, multifocal in 43.7% (Fig.1B), and diffuse in 12.5% (Fig.1C). The focal pattern occurred as nodules measuring 4-17cm in diameter, which occupied and partially effaced the affected hepatic lobe. The multifocal pattern occurred as random nodular areas, ranging from 1-20 cm in diameter. In the diffuse pattern, the entire organ was affected. The neoplastic masses were white, yellow or red, and soft or firm. The cut surface showed white, yellow, dark green (Fig.1D) and red areas. Occasionally there were extensive friable areas (necrosis) intermixed with the tumor mass.

Histologically, hepatocellular carcinomas had a solid pattern in half of the cases (Fig.2A) and trabecular pattern in the other half (Fig.2B). In the solid pattern, the hepatic architecture was disorganized by the proliferation of dense mantles of neoplastic hepatocytes. Trabeculae of varying thickness formed by neoplastic cells characterized the trabecular pattern. Neoplastic cells were polygonal, with distinct cytoplasmic boundaries, abundant and faint eosinophilic, sometimes vacuolated, cytoplasm. Nuclei were rounded, with finely stippled chromatin, containing one to five evident nucleoli. Binucleated or multinucleated neoplastic hepatocytes were observed in 56.2% of the tumors (Fig.2C). Inflammatory infiltrate of lymphocytes, plasma cells, neutrophils, and macrophages occurred in 87.5% of hepatocellular carcinomas; while in 25% of the cases, there were intratumoral vascular spaces, and in 6.25% there was discrete multifocal mineralization. Vascular invasion (blood or lymphatic) occurred in 37.5% of the cases (Fig. 2D). Among the tumors with vascular dissemination, 25% had a solid pattern and 12.5% a trabecular pattern. Extrahepatic metastasis (to mediastinal lymph node) of a solid pattern hepatocellular carcinoma occurred in one case (Fig.2E). Additional histological characteristics of hepatocellular carcinomas are summarized in Table 1.

IHC for anti-Hep Par-1 revealed an intracytoplasmic granular immunolabeling ranging from mild (25% of cases), moderate (31.2% of cases) to marked (43.7%) (Fig.2F). IHC anti-CK7 did not reveal any labeling for hepatocellular carcinomas.

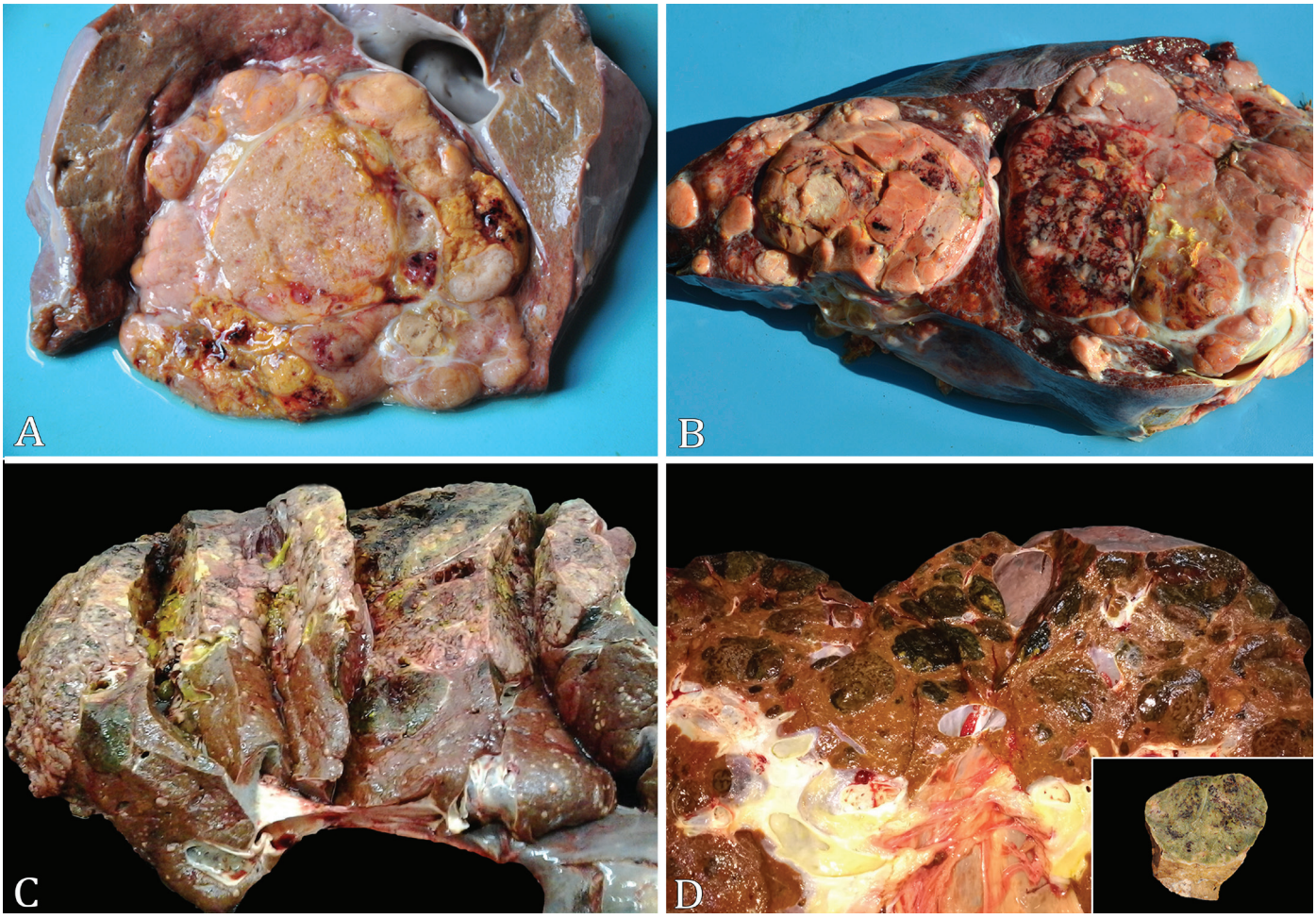

Fig.1. Gross aspects of hepatocellular carcinomas in cattle. (**A**) Focal pattern. A nodular area partially effaces the liver parenchyma. The cut surface has a predominance of white areas interspersed with yellow to red foci. (**B**) Multifocal pattern. Multiple nodular structures of different sizes are observed. The cut surface is similar to that of Figure 1A, with extensive red areas. (**C**) Diffuse pattern. The neoplasm almost completely replaces the liver parenchyma. Within the tumor parenchyma, there are friable green areas. (**D**) Cut surface. Dark-green nodular multifocal areas are distributed randomly across the parenchyma. Inset: a formalin-fixed liver fragment with a green nodular area.

Grossly, all three cholangiocarcinomas had a multifocal pattern of liver involvement, which was characterized by slightly irregular, occasionally umbilicated, and firm nodules of 1-6cm in diameter, randomly distributed within the liver parenchyma. On the cut surface, these nodules were white to yellow.

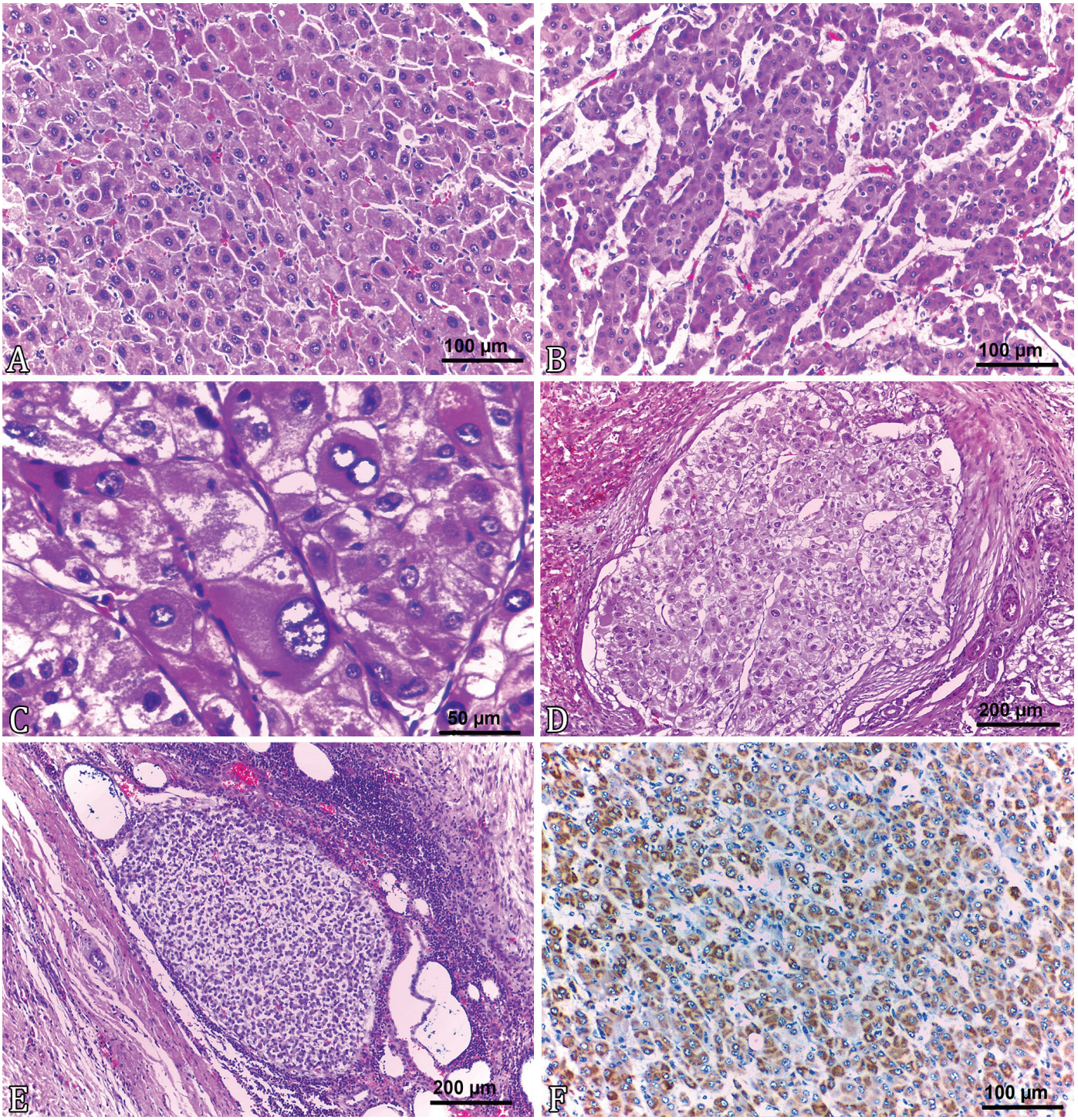

Fig.2. Histological and immunohistochemical aspects of hepatocellular carcinomas in cattle. (**A**) Solid pattern. The parenchyma is replaced by a mantle of neoplastic hepatocytes displaying marked nuclear pleomorphism. HE, bar = 100µm. (**B**) Trabecular pattern. The neoplastic hepatocytes are arranged in trabeculae of varying thickness. Unlike Figure 2A, the tumor cells are well differentiated. HE, bar = 100µm. (**C**) Cellular pleomorphism characterized by marked anisocytosis and binucleated and vacuolated neoplastic hepatocytes HE, bar = 50µm. (**D**) Embolus of neoplastic cells occluding the lumen of a blood vessel within the liver parenchyma. HE, bar = 200µm. (**E**) Metastasis of hepatocellular carcinoma in a lymph node. In the medullary sinuses, a blood vessel is obliterated by tumor cells. HE, bar = 200µm. (**F**) There is a marked diffuse granular immunolabeling in the cytoplasm of neoplastic cells. Anti-Hep Par-1 IHC, AEC chromogen, bar = 100µm.

Histologically, the solid pattern occurred in two out of three cases (Fig.3A); in the remaining case, the neoplasm was arranged in acini or irregular ducts (Fig.3B). In both histological patterns, neoplastic cells partially effaced the liver parenchyma. The cells varied from cuboidal to rounded, with indistinct cytoplasmic borders, moderate and faint eosinophilic cytoplasm. Nuclei were predominantly oval, with finely stippled chromatin and one to three evident nucleoli. Cellular pleomorphism was moderate in two cases and marked in the other, in which there was marked anisocytosis and anisokaryosis (Fig.3C). The mitotic index was mild (1/3), moderate (1/3), or marked (1/3). Fibrous connective tissue proliferation was observed in HE stained sections (Fig.3D) and evidenced by the MT technique (Fig.3E), in which it was mild in one of the tumors and marked in the other two (scirrhous cholangiocarcinomas). Mild (two cases) and moderate (one case) areas of necrosis and hemorrhage were also observed within the neoplasm parenchyma. Vascular invasion was found in two cases, and in one of the tumors, there was pulmonary metastasis. In all three cases, there was mild intratumoral inflammatory infiltrate of lymphocytes, plasma cells, neutrophils, and macrophages. A mild amount of mucin was evidenced within the neoplastic ducts in two cases by the PAS technique. Upon IHC evaluation, only one cholangiocarcinoma had marked intracytoplasmic and membranous anti-CK7 immunolabeling (Fig.3F). No case of cholangiocarcinoma was positive in the IHC for anti-Hep Par-1.

DISCUSSION

The diagnosis of all PHN cases in this study was obtained through the association of the gross, histopathological, histochemical, and immunohistochemical findings. Liver neoplasms are considered uncommon when compared to other tumors of cattle (Lucena et al. 2011, Tessele & Barros 2016, Reis et al. 2017). Usually, PHN in cattle are incidental findings of slaughterhouses (Tessele & Barros 2016) or are diagnosed at the necropsy, wherein it may be related to the cause of death (Reis et al. 2017).

All cattle affected in this study were adults, aged three years or older, similar to that reported by other authors (Anderson & Sandinson 1968, Wettimuny 1969, Braun et al. 2005, Jeong et al. 2005). Females were more affected than males, and, although few studies assessed a possible sex predilection for the development of liver tumors in cattle (Wettimuny 1969, Braun et al. 2005), in dogs and cats it is known that there is no such predisposition (Patnaik et al. 1981, Trigo et al. 1982, Lawrence et al. 1994, Liptak et al. 2004, Flores et al. 2013, Van Sprundel et al. 2014). Probably females were overrepresented in this study since these are slaughtered later, and are thus, in the so-called "cancer age", as previously postulated (Reis et al. 2017).

Table 1. Histological features observed in 16 hepatocellular carcinomas

Feature	Intensity			
	None	Mild	Moderate	Marked
Pleomorphism	-	-	75% (12/16)	25% (4/16)
Mitotic index	-	75% (12/16)	18.75% (3/16)	6.25% (1/16)
Desmoplasia	25% (4/16)	43.75% (7/16)	31.25% (5/16)	-
Mucin	100% (16/16)	-	-	-
Necrosis	12.5% (2/16)	56.25% (9/16)	31.25% (5/16)	-

In the present study, there was a predominance of hepatocellular carcinomas over cholangiocarcinomas. Most studies in cattle demonstrated similar ratios (Bastianello 1982, Bettini & Marcato 1992, Lucena et al. 2011), with occasional exceptions (Anderson & Sandinson 1968). PHN in this study had distinct gross morphological patterns. The vast majority of hepatocellular carcinomas were characterized by solitary masses or multifocal nodules, which are patterns commonly described in cattle (Anderson & Sandinson 1968, Wettimuny 1969). Observation of this pattern can assist in a presumptive diagnosis at the gross examination. The diffuse pattern was an unusual presentation for hepatocellular carcinomas and observed in only two cases, which suggests a prolonged clinical evolution, which allowed an almost complete replacement of the healthy parenchyma.

The parenchyma of hepatocellular carcinomas may present a wide range of colors caused by hemorrhages, areas of necrosis, and cholestasis, including gray-white, red, brown, yellow, and dark-green (Wettimuny 1969, Bettini & Marcato 1992). These variations occurred in the hepatocellular carcinomas of the current study. The green areas are a critical macroscopic characteristic and, combined with the pattern of distribution of the neoplasm, provide valuable clues for a gross presumptive diagnosis. Metastases from hepatocellular carcinomas are uncommon (Bettini & Marcato 1992), but when they occur, the sites commonly affected include mediastinal lymph nodes, and lungs (Anderson & Sandinson 1968). In our study, metastasis was observed by the meat inspector veterinarian in a single case involving the mediastinal lymph node.

In the cholangiocarcinomas of the current study, only the multinodular pattern was observed, similar to other cases with the same gross pattern in hepatocellular carcinomas (Ohfuji 2012, Azizi et al. 2016). In PHN, it is unclear whether multiple nodules arise from intrahepatic vascular metastases or multiple origins in different foci (Wettimuny 1969, Barros 2016). A distinctive feature that can help differentiate cholangiocarcinomas from other liver tumors is that they are frequently umbilicated. This presentation was present in one of the cases in this study and is generally attributed to intratumoral necrosis and subsequent retraction by fibrosis (Head et al. 2003, Cullen 2017). Although this does not represent a particular characteristic of cholangiocarcinomas, it is a helpful criteria for gross differentiation. Another feature of cholangiocarcinomas is desmoplasia, which gives these neoplasms a firm consistency (Cullen 2017), as observed in this study in which all three cases had this characteristic. Metastases are common in cholangiocarcinomas; the main secondary sites are the lungs, lymph nodes, and peritoneum (Anderson & Sandinson 1968).

The cellular features of hepatocellular carcinomas and cholangiocarcinomas vary according to the degree of differentiation (Head et al. 2003, Cullen 2017). The pattern of cell growth in hepatocellular carcinoma includes trabecular, solid, pseudoglandular, or squamous (Cullen 2017). We detected two patterns: trabecular and solid. As previously

stated, the trabecular pattern was the most common in cattle (Wettimuny 1969, Bundza et al. 1984, Bettini & Marcato 1992). Necrotic foci and cavities filled with red blood cells are frequent (Wettimuny 1969, Schlageter et al. 2014) and were observed, respectively in 100% and 25% of the cases here reported. These histological characteristics, associated with the polygonal shape of the tumor cells, may help in the morphological diagnosis of these tumors. In one case, there

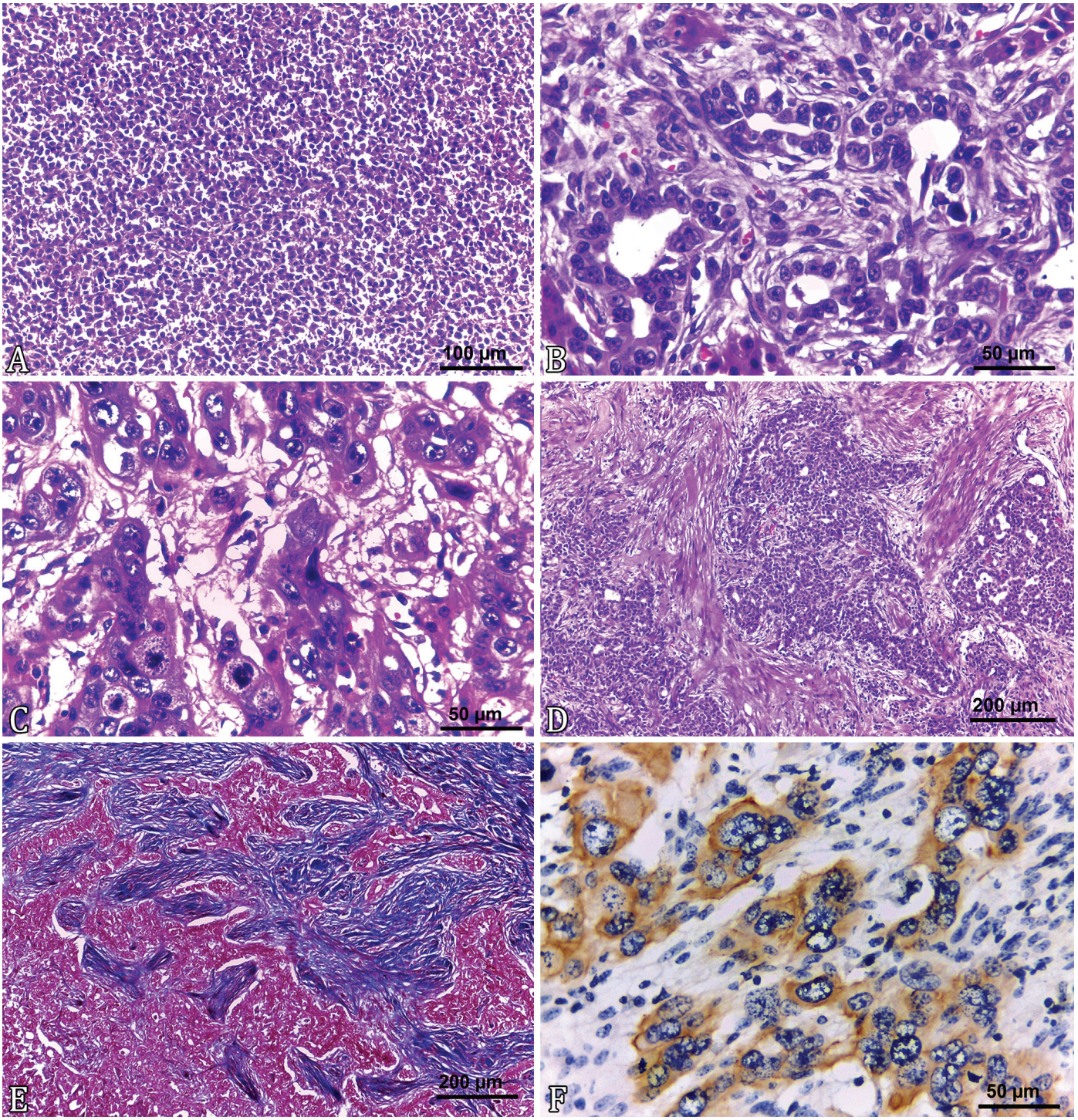

Fig.3. Histological and immunohistochemical aspects of cholangiocarcinomas in cattle. (**A**) Solid pattern. Neoplastic cells homogeneously replace the liver parenchyma. HE, bar = 100µm. (**B**) Acinar pattern characterized by ductules immersed in an abundant fibrous stroma. The cells are cuboidal to rounded, with predominantly oval nuclei and one or more prominent nucleoli. HE, bar = 50µm. (**C**) Cellular pleomorphism is characterized by marked anisocytosis, anisokaryosis, and occasional megalocytosis. Mitosis and occasional bi or multinucleated cells are evident. HE, bar = 50µm. (**D**) A marked proliferation of fibrous connective tissue dissects and isolates groups of neoplastic cells. HE, bar = 200µm. (**E**) Abundant fibrous connective tissue. MT, bar = 200µm. (**F**) There is marked immunolabeling in the cytoplasm of neoplastic epithelial cells. Anti-CK-7 IHC, AEC chromogen, bar = 50µm.

was marked intracytoplasmic vacuolation; this is mentioned as a frequent finding in hepatocellular carcinomas and mainly related to glycogen or lipids accumulation within the cytoplasm of the neoplastic cells (Cullen 2017).

Cholangiocarcinomas may have acinar or tubular histological patterns, which, when well-differentiated, resemble the normal biliary epithelium (Barros 2016), while in undifferentiated tumors a solid pattern may predominate (Head et al. 2003, Cullen 2017). The solid pattern of cholangiocarcinoma was the most frequent in this study. Although the acinar patterns indicate fairly differentiation, in our study, this was not the case, as this pattern displayed marked cellular pleomorphism, high mitotic index, and vascular invasion. Fibrous connective tissue and mucin are classical histological findings of cholangiocarcinomas and valuable features for the recognition of this tumor (Head et al. 2003, Barros 2016, Cullen & Stalker 2016, Cullen 2017). In the present cases, a pronounced desmoplasia was confirmed by MT technique, and a slight amount of mucin in the PAS staining was found in two of the cholangiocarcinoma cases.

Upon IHC technique, all hepatocellular carcinomas expressed Hep Par-1, while only one cholangiocarcinoma expressed CK7. The relative lack of immunolabeling in cholangiocarcinomas is probably due to the degree of differentiation. However, as there was also no labeling in the internal control (normal biliary epithelium), a prolonged fixation period in formaldehyde solution could be possible a factor involved. Previous studies have showed a decrease in the intensity of IHC-staining in some tissues after three days of exposure to formaldehyde and even an absence of immunostaining within seven days of fixation (Battifora & Kopinski 1986, Leong & Gilham 1989, Webster et al. 2009). However, a good sensitivity and specificity of Hep Par-1 was detected as a marker of normal and neoplastic hepatocytes in cattle, and the combined employment of anti-Hep Par-1 and anti-CK7 may contribute to improve the accuracy of PHN diagnosis in cattle.

Other neoplasms and tumor-like lesions should be considered as macroscopic differential diagnoses of PHN. Among neoplasms, metastatic carcinomas of different primary sources (squamous epithelium, pulmonary, and endometrial cells) and lymphoma should be included. Infectious or parasitic diseases such as degenerate hydatid cysts and tuberculosis (Cullen & Stalker 2016, Kamelli et al. 2016, Brown et al. 2017, Faccin et al. 2018) may be considered as possible candidates for a gross dispute over a definitive diagnosis. In some cases, histological, histochemical, and immunohistochemical evaluation should be used for differentiation.

CONCLUSIONS

Hepatocellular carcinoma is the most common primary liver tumor in cattle. Adult cows, as they are allowed to reach the "cancer age", are more often affected than males.

Grossly, hepatocellular carcinomas were characterized by solitary masses or multifocal nodules, and their greenish color is an essential clue for tumor recognition.

Histologically, solid and trabecular patterns are the most common.

Cholangiocarcinomas appear macroscopically as multiple firm nodules, occasionally umbilicated, with a solid histologic arrangement.

Immunohistochemistry was an important tool to improve accuracy in the diagnosis of PHN. Hep Par-1 is a useful immunohistochemical marker for hepatocellular carcinomas.

Acknowledgments.- The authors thank the "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq) and the "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES) for supporting this study.

Statement of conflict of interest.- The authors declare no conflict of interest with respect to the publication of this paper.

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PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(6):417-425, June 2020
DOI: 10.1590/1678-5150-PVB-6499

Original Article
Livestock Diseases

Gastric disorders of cattle in western Rio Grande do Sul State, Brazil[1]

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ABSTRACT.- Brandolt I.M.C, Maurique A.P., Damboriarena P.A., Trost M.E., Pozzobon R. & Anjos B.L. 2020. **Gastric disorders of cattle in western Rio Grande do Sul State, Brazil**. *Pesquisa Veterinária Brasileira 40(6):417-425*. Laboratório de Patologia Veterinária, Hospital Universitário Veterinário, Universidade Federal do Pampa, BR-472 Km 585, Uruguaiana, RS 97500-970, Brazil. E-mail: anjosbl@gmail.com

A retrospective study of gastric disorders in autopsied cattle in the Western region of Rio Grande do Sul State, was performed. The exam reports of bovine necropsy of the Veterinary Pathology Laboratory, Unipampa, were analyzed in the period from 2010 to 2018. All cases in which death was primarily caused by disturbance in the gastric chambers were included. During the period evaluated, 141 cattle were necropsied. Of those, 25 had gastric disorders. Of those, 53% had alterations in the rumen, followed by abomasum (17%), involvement of two chambers (13%) and reticulum (9%). Most cases corresponded to beef cattle raised in an extensive system and most them for calf production and fattening with an average age of approximately three years. The cases occurred in farms of four different municipalities. Bullous bloat by excessive *Trifolium repens* ingestion was the gastric disturbance with the highest number of dead cattle observed in this study, especially in irrigated áreas of livestock farms. Cases such as lactic acidosis, ruminal alkalosis due to excessive urea ingestion and *Baccharis coridifolia* poisoning were also important gastric disturbances in necropsied cattle, associated especially with poor management and period of scarcity of good quality fodder. Cases of *Clostridium perfringens* infection were also observed in young cattle suggesting that it is an important infectious agent in the evaluated cattle herds, also showing failures in vaccination of the herds. As observed, gastric disturbances in cattle in the western region of Rio Grande do Sul have several causes. Metabolic/toxic and infectious disturbances were important causes of mortality in the herds, inducing considerable economic losses. Based on this study, it is clear that the majority of outbreaks or isolated cases occurred due to errors in the management of the properties and the vast majority of them could have been avoided with improvements in the technical qualification of the workers and simple adjustments in the farming methods. It is also emphasized the importance of the conclusive diagnosis to control these disorders, once after the orientation to the producers, was observed significant decrease in cattle losses in the farms.

INDEX TERMS: Gastric disorders, cattle, Rio Grande do Sul, Brazil, diseases of cattle, gastric disease, veterinary pathology.

RESUMO.- [**Distúrbios gástricos de bovinos no Oeste do Rio Grande do Sul.**] Foi realizado estudo retrospectivo dos distúrbios gástricos em bovinos necropsiados na região Oeste do Rio Grande do Sul. Foram analisados os relatórios de exame de necropsia de bovinos do Laboratório de Patologia Veterinária (LPV) da Universidade Federal do Pampa (Unipampa), Rio

[1] Received on January 31, 2020.
Accepted for pulication on March 12, 2020.
Part of the Master's Thesis of the first author.

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Grande do Sul, no período de 2010 a 2018. Foram incluídos todos os casos nos quais a morte foi causada primariamente pelo distúrbio nas câmaras gástricas. De um total de 141 bovinos necropsiados, 25 corresponderam a distúrbios gástricos. Dentre esses, 53% apresentaram alterações no rúmen, seguido de abomaso 17%, acometimento concomitante de duas câmaras 13% e retículo 9%. A maioria dos casos ocorreram em bovinos de corte criados em sistema extensivo e a maioria destinados à produção de bezerros e engorda com média de idade de aproximadamente três anos. Os casos ocorreram em propriedades rurais de quatro municípios da região Oeste do estado. O timpanismo bolhoso por ingestão excessiva de *Trifolium repens* foi o distúrbio gástrico com maior número de bovinos mortos observados nesse estudo, especialmente em propriedades com criação de animais em áreas de irrigação. Casos como acidose láctica, alcalose ruminal por intoxicação por ureia e intoxicação por *Baccharis coridifolia* também foram importantes distúrbios gástricos nos bovinos necropsiados e percebeu-se sua associação a falhas no manejo e à época de escassez de forragem de boa qualidade. Foram observados ainda casos de infecção por *Clostridium perfringens* em bovinos jovens o que sugere também tratar-se de um importante agente infeccioso nos rebanhos bovinos avaliados, demonstrando ainda falhas na vacinação dos rebanhos. Conforme observado, diversos são os distúrbios gástricos em bovinos na região Oeste do Rio Grande do Sul, tendo como importantes causas de mortalidades os distúrbios metabólicos/tóxicos e infecciosos, induzindo consideráveis perdas econômicas. Com base nesse levantamento, percebe-se que a maioria dos surtos ou casos isolados estudados ocorreram por erros no manejo nas propriedades e, na sua grande maioria, poderiam ter sido evitados com especialização da mão de obra e ajustes simples. Ressalta-se ainda a importância do diagnóstico conclusivo para controle desses distúrbios, uma vez que, após a orientação aos produtores, observou-se significativa diminuição das perdas de bovinos nas propriedades.

TERMOS DE INDEXAÇÃO: Distúrbios gástricos, bovinos, Rio Grande do Sul, Brasil, doenças de bovinos, distúrbios gástricos, patologia veterinária.

INTRODUCTION

Most of the herds in the state of Rio Grande do Sul are raised in an extensive or semi-extensive manner and are affected by diseases that are directly linked to feeding (Dalto et al. 2009, Lucena et al. 2010, Kitamura et al. 2002). Conversely, in other countries where intensive management is practiced primarily, respiratory and metabolic diseases are more prevalent (Gagea et al. 2006). Factors such as a sudden change in diet (Câmara et al. 2009), an increase in protein mineral supplements (Kitamura et al. 2002), excessive grain intake (Maruta & Ortolani 2002), and fields with a predominance of legumes (Dalto et al. 2009) compose important risk factors for the occurrence of gastric disorders in ruminants.

Gastric disorders may be associated with motility, characterized by changes in ruminal contractions (Câmara et al. 2009), metabolic disturbances, mechanical alterations, infectious agents, or an undetermined origin (Júnior et al. 2008, Cleef et al. 2009, Dalto et al. 2009, Oliveira et al. 2013). This can lead to changes in the ruminal microbiota, triggering a series of changes in ruminal physiology and consequent systemic changes that can lead to death (Maruta & Ortolani 2002, Radostitis et al. 2007, Júnior et al. 2008, Dalto et al. 2009, Marques et al. 2018).

Knowledge of the regional diseases that affect cattle herds, especially those related to management, is necessary in order to establish primary differential diagnoses when a disease breaks out in a herd (Lucena et al. 2010). However, the veterinary diagnostic service in western Rio Grande do Sul is underutilized by professionals working in the region. Accurate data on the causes of mortality in cattle is extremely important, especially in border areas. There are some small- and medium-sized, but several large rural producers who, for the most part, raise cattle using extensive management and sometimes use crop-livestock integration systems, which require constant attention to the health status of the herds (Anjos 2018, personal communication).

In this context, the objective of this paper was to report on the main causes of gastric disorder-related cattle deaths in western Rio Grande do Sul diagnosed by the "Laboratório de Patologia Veterinária" (LPV) of the "Universidade Federal do Pampa" (Unipampa) from 2010 to 2017.

MATERIALS AND METHODS

Necropsy records of ruminants examined at the LPV-Unipampa from September 2010 to December 2018 were analyzed. The cattle diagnosed with gastric disorders were then sampled; only deaths associated with such disorders were considered. Information about the epidemiological aspects, clinical signs, macroscopic and microscopic findings obtained during laboratory evaluations, and those obtained from consultations in the region's rural areas were investigated.

RESULTS

Of the total 1,238 necropsies performed in the sector, 141 corresponded to necropsies of cattle. Twenty-five diagnoses were attributed to gastric disorders. These diseases caused lesions in one or more stomach chambers: the rumen was the most frequently affected (15/25), followed by the abomasum (5/25), concomitant involvement of two chambers (3/25), and the reticulum (2/25). The exclusive involvement of the omasum was not present in any case. The vast majority of cattle came from rural properties, in municipalities in western Rio Grande do Sul, especially Uruguaiana. Most of the cattle evaluated were adults (11/25), were destined for meat production (20/25), and were raised using extensive farming (21/25). The Braford breed (14/25) was the most common. All cases observed came from herds that received mineral supplementation, and those destined for meat production received urea-based protein salt at some point in the year. Cases of bullous white clover tympanism (*Trifolium repens*, 8/25), lactic acidosis (4/25), ruminal alkalosis due to urea poisoning (2/25) and poisonous plants (*Baccharis coridifolia*, 2/25), and necro-hemorrhagic abomasitis associated with *Clostridium perfringens* (2/25) were most common (Table 1).

All cases of tympanism occurred in the form of an outbreak in young to adult cattle of the Braford, Angus, and Hereford breeds. In these cases, the cattle were in a field with a large quantity of *T. repens* (Fig.1). The clinical signs were characterized by apathy, diarrhea, anorexia, prostration,

Table 1. Epidemiological aspect and diagnostisis aspects of gastric disorders that caused the death of cattle examined by the Veterinary Pathology Laboratory, Unipampa, from October 2010 to December 2018

Case	Municipality	Age	Sex	Breed/ Production system	Diagnosis
1	Uruguaiana	Adult	Female	Holstein/confined	Right displaced Abomasum and ruminal atony
2	Uruguaiana	8 years	Female	Holstein/semi-extensive	Traumatic reticulopericarditis
3	São Borja	1,5 years	Male	Braford/extensive	Rumenitis by Lactic acidosis
4	São Borja	1,5 years	Male	Braford/extensive	Rumenitis caused by Lactic acidosis
5	São Borja	1,5 years	Male	Braford/extensive	Rumenitis caused by Lactic acidosis
6	Uruguaiana	9 years	Female	Holstein/semi-extensive	Right displaced Abomasum and Ulcerative abomasitis
7	Uruguaiana	1,5 years	Male	Braford/extensive	Frothy bloat and ruminitis by *Trifolium repens* ingestion
8	Uruguaiana	1 years	Male	Braford/extensive	Frothy bloat and ruminitis by *Trifolium repens* ingestion
9	Uruguaiana	1,5 years	Male	Braford/extensive	Frothy bloat and ruminitis by *Trifolium repens* ingestion
10	Uruguaiana	1,5 years	Male	Braford/extensive	Frothy bloat and ruminitis by *Trifolium repens* ingestion
11	Uruguaiana	1,5 years	Male	Braford/extensive	Frothy bloat and ruminitis by *Trifolium repens* ingestion
12	Uruguaiana	1,5 years	Male	Braford/extensive	Frothy bloat and ruminitis by *Trifolium repens* ingestion
13	Uruguaiana	Adult	Female	Braford/extensive	Ruminal alkalosis by urea toxicosis
14	Manoel Viana	5 day	Female	Angus/extensive	Necrohemorrhagic abomasitis by *Clostridium perfringens*
15	Uruguaiana	2 years	Male	Hereford/extensive	Frothy bloat and ruminitis by *Trifolium repens* ingestion
16	Uruguaiana	7 years	Female	Braford/extensive	Necrohemorrhagic omasitis and abomasitis by *Clostridium perfringens*
17	Uruguaiana	9 years	Male	Braford/extensive	Traumatic reticulopericarditis
18	Quaraí	1 years	Female	Braford/extensive	*Baccharis coridifolia* poisonig
19	Quaraí	1 years	Female	Braford/extensive	*Baccharis coridifolia* poisonig
20	Uruguaiana	1,5 years	Male	Holstein/extensive	Ulcerative abomasitis
21	Uruguaiana	Adult	Male	Red Angus/extensive	Ruminal alkalosis by urea toxicosis
22	Uruguaiana	2 years	Male	Simmental/intensive	Necrohemorrhagic rumenitis and reticulitis caused by Lactic acidosis
23	Uruguaiana	2 years	Female	Hereford/extensive	Mild multifocal ulcerative abomasitis
24	Uruguaiana	2 years	Male	Red Angus/extensive	Frothy bloat and ruminitis by *Trifolium repens* ingestion
25	Uruguaiana	9 years	Female	Holstein/extensive	Abomasal lymphosarcoma (Bovine enzootic leukosis)

Fig.1. *Trifolium repens* in pasture cultivated under irrigation.

Fig.2. Bovine in the right lateral position with rumen sharply distended in a case of frothy bloat due to excessive ingestion of *Trifolium repens.*

recurrent tympanism, and decubitus followed by death, with an average evolution of two days. In one of the outbreaks, a bovine with an approximate one-year history of recurrent tympanism developed chronic vagal indigestion. In this case, a markedly distended rumen was observed (Fig.2) and a tympanism line was present in the esophageal mucosa (Fig.3). The ocular and oral mucous membranes were markedly congested. Areas of necrosis and focal ulcers were observed in the rumen mucosa, with diffuse peritonitis (Fig.4) and multiple abscesses in the liver. Only in the bovine with vagal indigestion was extensive focal ulcerative ruminitis observed, with vacuolization and keratinocyte necrosis. The microbiological evaluation of the abscess was positive for *Enterobacter aerogenes*.

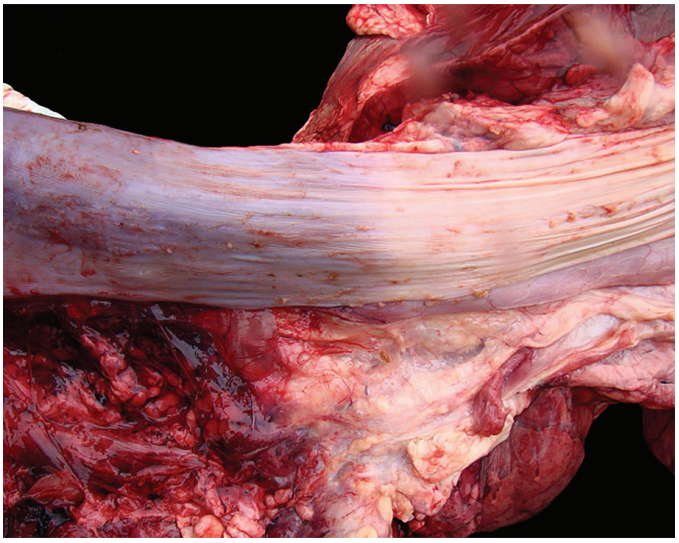

Fig.3. Esophagus of a bovine with frothy bloat with excessive intake of *Trifolium repens*. Note the congestion of the more cranial portion of the esophageal mucosa with a sudden change in color of the more caudal portion due to rumen distension (tympanism line).

The cases of lactic acidosis occurred in a Simental bull of approximately two years fed with corn silage, rice bran and oats in addition to pasture of oats and ryegrass which at macroscopic examination showed moderately hyperemic ruminal mucosa with areas of loss of epithelium and papillae (Fig.5) and in the form of an outbreak in Braford cattle with approximately two years of age. The herd consisted of 170 animals, of which 14 showed clinical signs, six died and three were necropsied. Approximately 15 days before prior to the appearance of clinical signs, husked grains of rice and sorghum grass silage were introduced into the cattle feed. The clinical signs were marked apathy, decreased food intake, goosebumps, severe dehydration, diarrhea, decubitus, and death with a clinical evolution of approximately 3 days. During necropsy, extensive focal lesions of erosion and focal areas of ulceration and necrosis in the rumen mucosa were observed, in addition to marked hyperemia of the pre-stomachs and abomasum. The contents of the stomach and pre-stomachs consisted of a large quantity of rice grains. Microscopically, areas of marked multifocal suppurative necrosis were observed in the rumen mucosa. Extensive areas of hydropic degeneration with loss of the epithelial lining associated with intense underlying neutrophilic infiltrates were also observed. In some of these areas of ruminal wall necrosis, there were thrombi associated with negative images of angioinvasive hyphae up to 15μm in diameter with positive periodic acid-Schiff and Grocott stains. The hyphae were wide, up to 30μm in diameter, were non septated, and had irregular branching compatible with fungi of the *Zygomycete* class.

The outbreak of Mio-mio (*B. coridifolia*) (Fig.6) resulted in 100% mortality, where 31 cattle died, two of which were necropsied. The cattle had been purchased from a property in the region and introduced to a farm in the municipality of Quaraí. The clinical signs observed were anorexia, severe dehydration, hyperthermia, polydipsia, weakness, motor incoordination, sternal and lateral decubitus, and death. During necropsy, there was ruminal content that was unusually liquid and fetid in the ruminal wall, mainly in the ventral sacs

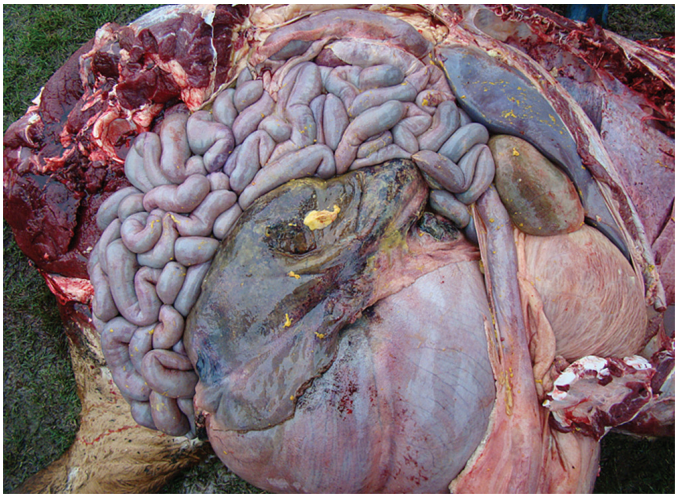

Fig.4. Bovine. Transmural ulcerative ruminitis with peritonitis in a bovine with secondary vagal indigestion relapsing chronic cases of bullous tympanism associated with excessive ingestion of *Trifolium repens*. Yellow fibrin filaments and hemorrhagic area are observed near the rupture site.

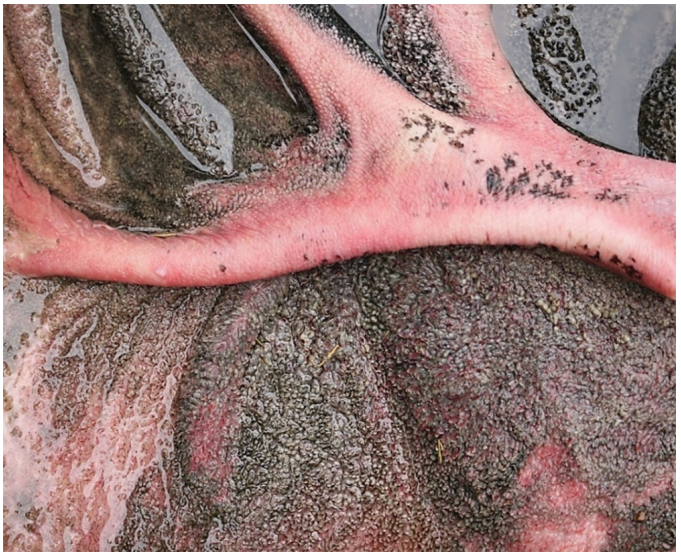

Fig.5. Bovine. Ruminitis due to lactic acidosis due to excessive ingestion of grains. The rumen mucosa is markedly hyperemic and with marked loss of ruminal papillae.

(Fig.7); hyperemia with multifocal areas of ulceration and necrosis; and loss of ruminal papillae in some areas (Fig.8). There was also moderate transmural edema in the ruminal wall. Microscopically, there was hydropic degeneration, individual necrosis of the mucosal cells associated with vascular congestion, and vessels of the lamina propria full of inflammatory cells (neutrophils). Serious injuries were characterized by a significant decrease in the thickness of the ruminal mucosa associated with large areas of degeneration and epithelial necrosis, infiltration of neutrophils, and basophilic bacillary bacteria. In addition, in some places, large cracks were identified between the mucosa and the lamina diffusely congested in the rumen.

Both cases of ruminal alkalosis caused by excess dietary urea occurred in the form of an outbreak with high morbidity and low mortality and was associated with protein salt intake. In the first case, the outbreak occurred in a herd of bulls. The owner reported that he had started a protein salt diet 20 days before the clinical signs appeared and did not acclimate the animals beforehand. The clinical signs reported were: ruminal distension, ammonia odor, and ruminal motility cessation, which progressed to motor incoordination, muscle tremors, tachypnea, sternal decubitus, and finally lateral decubitus and death. In the second case, the owner reported that during the outbreak, the deaths were rapid and no marked neurological clinical signs were observed besides cattle discommodity and slight rumen distension. During necropsy, no specific macroscopic lesions were observed, however, there was marked congestion of the ocular and oral mucosa and of organs such as the lung, liver, and kidneys. The content of the rumen was also darker.

C. perfringens caused abomasitis in two calves, one at 7 days and the other at 5 days of life. According to the owner, the clinical signs observed were apathy, weakness, pale mucous membranes, and severe dehydration. During necropsy, the abomasum was markedly distended with red-blacked focal areas (Fig.9), which were also present in the serosa of the omasum.

Fig.6. Branches of *Baccharis coridifolia* in a field with native pasture invaded by the plant.

Fig.7. *Baccharis coridifolia* poisoning in a bovine. The rumen presents a focal area of transmural hemorrhage in the region of the ventral ruminal sac.

Fig.8. Bovine rumen. Marked loss of ruminal papillae with a focal area of ulceration and hemorrhage.

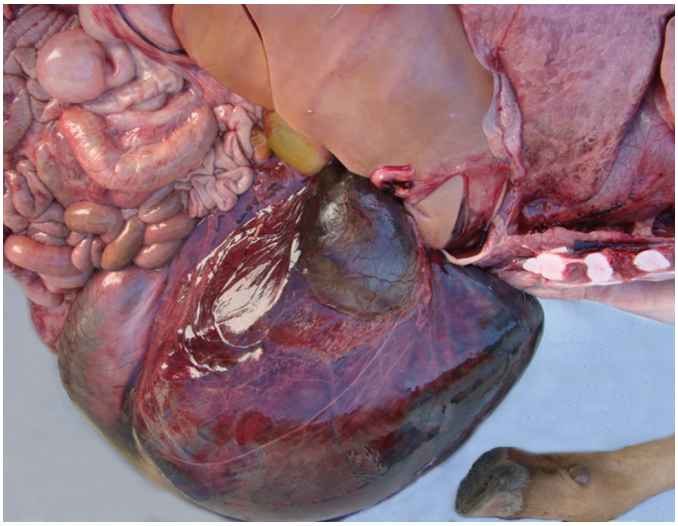

Fig.9. Bovine. Necrohemorrhagic abomasitis associated with infection by *Clostridium perfringens* in a calf. Transmural hemorrhage is noted throughout the organ.

The abomasum mucosa had multifocal areas of hemorrhage and necrosis, and the contents were markedly liquid and blackish (Fig.10). In the cranioventral portion of the lungs, focal areas of consolidation were observed. Microscopically, there were focal areas of extensive necrosis and hemorrhage in the mucosa of the abomasum with inflammatory infiltrate composed predominantly of neutrophils associated with intralesional bacillary bacterial aggregates. The diagnosis of *Clostridium* spp. was confirmed by bacterial isolation and cultivation.

The diagnosis of traumatic reticulopericarditis occurred in a Holstein cow and a Braford bull and was associated with metallic foreign bodies that compromised cardiac function. In the Holstein cow, traumatic reticulopericarditis was found in addition to peritonitis with adhesions and a large amount of fibrin, and an intrathoracic abscess close to the pericardial sac, which produced a large amount of pus when cut (Fig.11). In the Braford bull, a 15cm long metal wire was found, which had perforated the reticular wall, triggering an inflammatory reaction with the formation of an abscess 3cm in diameter, which adhered to the serosa of the pericardial sac.

DISCUSSION

The epidemiological data observed in this study revealed a high frequency of gastric disorders resulting from failures in the feeding management o of herds. This factor is preponderant in the development of diseases that affect the pre-stomachs and stomach of cattle (Radostitis et al. 2007, Afonso & Mendonça 2007, Riet-Correa 2007, Dalto et al. 2009, Lucena et al. 2010).

The epidemiological and clinicopathological findings of the tympanism cases were compatible with foamy tympanism caused by the ingestion of white clover (*Trifolium repens*) (Garry 2006, Guard 2006, Dalto et al. 2009). Several rural properties in the region use this leguminous plant intercropped with grasses, such as ryegrass, to provide greater pasture availability for cattle during the winter. One of the main triggering factors of tympanism is a cattle pasture that is more than 60% occupied by only white clover (Riet-Correa 2007). This was the case for all the outbreaks researched in this study. Similar to the literature, tympanism was characterized mainly by the presence of the tympanism line and marked rumen distension (Garry 2006, Brown et al. 2007).

For all the outbreaks, the owners reported that the main supposition of death was hematic anthrax. It is important to note that three hours after death, the body retains a large amount of gas and can seem like other diseases that cause sudden death or are hyperacute (Riet-Correa 2007). It had been recommended that all owners, if possible, reduce the grazing time of cattle in fields high in white clover.

Ruminal lactic acidosis is generally associated with lactating cows (Maruta & Ortolani 2002) and confined steers (Ogilvie 2000). The animals in this outbreak, however, were beef cattle raised in a semi-extensive manner and fed a large amount of grains that, combined with epidemiological aspects such as the presence of dominant cattle and a small amount of feeders, contributed to some cattle eating excessive quantities of grains. (Quevedo et al. 2014). There is a subclinical form of the disease, however, the acute form has clinical signs similar to those described in other studies (Maruta & Ortolani 2002, Afonso & Mendonça 2007, Júnior et al. 2008). The intralesional hyphae observed in the ruminal lesions presented a morphology similar to that of *Zygomycetes* such as *Aspergillus*, *Absidia*, *Mucor*, *Rhizopus*, or *Mortierella*. All these fungi have angioinvasive capabilities and can induce thrombosis, as observed in one of the cattle, leading to infarction in areas of the ruminal wall (Quinn et al. 2005, Ortega et al. 2010). As a form of control, the owner was instructed to temporarily suspend the supply of grains, and to gradually reintroduce it, so that the ruminal microbiota could adapt (Afonso & Mendonça 2007, Quevedo et al. 2014).

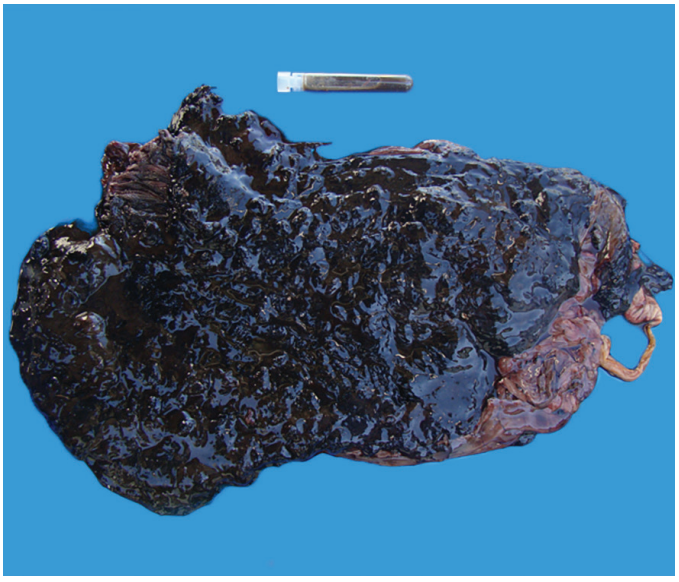

Fig.10. Bovine. Necrohemorrhagic abomasitis associated with infection by *Clostridium perfringens* in a calf. The abomasum presents necrosis and marked diffuse hemorrhage of the mucosa. Note the hemorrhagic content contained in the collection tube.

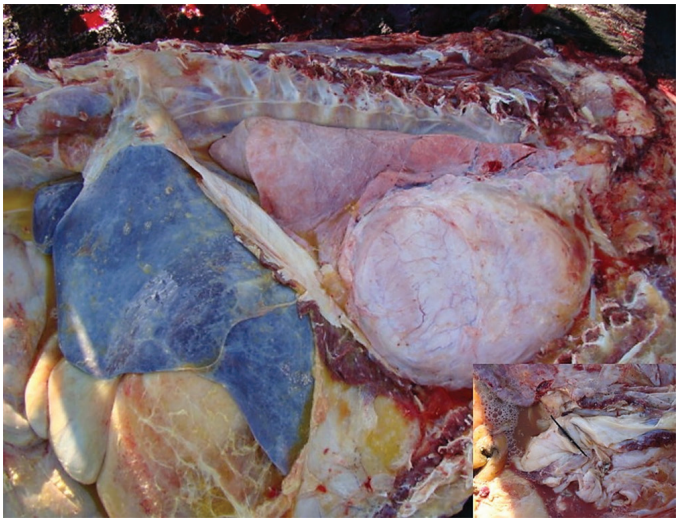

Fig.11. Bovine. Traumatic reticulopericarditis in a Holstein cow. Marked peritonitis is observed with adhesions of the pre-stomach wall and abomasum to the diaphragm, in addition to marked distension of the pericardial sac. In detail, the perforating foreign body can be seen in a fibrous path formed between the reticulum and the pericardium sac.

The outbreak of Mio-mio poisoning occurred in cattle that came from a property where there were already specimens of *Baccharis coridifolia*. Stress conditions such as fasting and thirst, and an introduction to highly-invaded pastures without prior knowledge of the plant are described as triggering factors for poisoning (Varaschin et al. 1998, Rissi et al. 2005, Almeida et al. 2009). The cattle affected by this outbreak were familiar with the plant, however, after arriving at the property they were confined for approximately two days before being introduced into the field. The animals developed clinical signs of poisoning one day after being released and deaths occurred for up to five days after that. All sick cattle died, with an index of 100% mortality. The injuries observed in this outbreak were identical to those described in the literature on natural (Tokarnia et al. 2002, Rissi et al. 2005) and experimental poisoning (Varaschin et al. 1998). Some methods have been described to induce an animal's aversion to the plant; however, they have not been entirely effective (Almeida et al. 2013). As a form of control, prophylactic measures should be adopted, such as providing adequate fodder and water to the animals before introducing them to places with the plant (Riet-Correa & Méndez 2007).

In this region, a large amount of *Baccharis megapotamica* (Anjos 2018, personal communication) has also been observed, with clinical signs and lesions similar to those seen with *B. coridifolia* poisoning (Oliveira-Filho et al. 2011, Panziera et al. 2015). However, on the properties where the outbreaks occurred in this study, this plant was not found.

The cases of ruminal alkalosis caused by an excessive intake of urea occurred in the form of an outbreak in extensively-raised beef cattle who were supplemented with protein salt. The use of non-protein nitrogen, through urea, together with mineral mixtures (protein salt) is a cost-effective alternative to providing greater amounts of protein in the diet, especially during the winter period (Kitamura et al. 2010). Alkalosis usually occurs in non-adapted animals that ingest high doses of urea in the first few days of consumption (Kitamura et al. 2002, Riet-Correa 2007), which was observed in both outbreaks in this study. According to the cattle owner in one of the outbreaks, there were dominant animals in the batch, which may have contributed to some cattle ingesting greater amounts of protein salt. A preventive measure against ruminal alkalosis is to keep the saltshakers covered, preventing them from being hit on rainy days (Riet-Correa 2007, Kitamura et al. 2010). On both properties, there were unshelled saltshakers where urea-rich salt was available. However, this factor was not associated with the poisoning in these two outbreaks. The clinical signs described in the literature are ruminal changes that can progress to incoordination, hypersensitivity, dyspnea, ruminal atony, muscle tremors, and convulsive conditions (Kitamura et al. 2002, Antonelli et al. 2004, Garry 2006), all of which were observed with more or less intensity in the cases described here, with the exception of convulsive episodes. The marked rumen distension found during necropsy in one of the cattle may be due to ruminal atony during the clinical picture. As a treatment, the rapid administration of 3 to 5 liters of weak acids (acetic acid or vinegar) orally or intra-ruminally (Kitamura et al. 2002, Riet-Correa 2007) is recommended, a measure adopted in one of the cattle committed, however, without success. As a form of prevention, urea should be introduced gradually into the diet, so that the ruminal microbiota can adapt (Riet-Correa 2007, Kitamura et al. 2010).

The abomasitis caused by *Clostridium perfringens* type A occurred in two calves at seven and five days of life. This bacterium is commonly associated with enteritis in newborn cattle (Songer & Miskimins 2005). The disease is characterized by a rapid onset of abomasal tympanism, abdominal pain, and hemorrhagic diarrhea (Schlegel et al. 2012). For the bovines of this study, no characteristic signs of the disease were observed, which may be due to the rapid death of the animal since the disease usually presents itself in the hyperacute form (Schlegel et al. 2012). Macroscopic lesions were similar to those described in the literature, characterized mainly by necrotizing and hemorrhagic inflammation of the abomasum mucosa (Songer & Miskimins 2005, Schlegel et al. 2012). In some cases, similar lesions could be seen in the rumen, reticulum, and duodenum. These macroscopic findings are characteristic of abomasitis caused by *C. perfringens* type A, and the visualization of intralesional bacillary bacteria together with the isolation of the agent through bacterial culture are sufficient to establish the disease diagnosis (Songer & Miskimins 2005, Schlegel et al. 2012). As a form of prevention, vaccination of the entire herd is recommended.

Traumatic reticulopericarditis is more frequent in dairy cattle, as they are more predisposed to risk factors such as receiving food in a trough and being managed close to fences and corrals with the risk of encountering sharp objects (Marques et al. 1990, Oliveira et al. 2013). In this study, only one bovine was of dairy ability. The Braford bull was in confinement at a semen sale center and received feed in the trough daily. The variation in the clinical picture demonstrates that it may be acute or the animal may adapt, triggering a chronic condition (Marques et al. 1990). The pathological findings in this study were characteristic of chronicity, with adhesions, the presence of varying degrees of fibrin, purulent secretions over the reticulum and adjacent organs, and a pleural effusion and abscesses in both the abdominal and thoracic cavities near the pericardial sac (Marques et al. 1990, Oliveira et al. 2013). These injuries are associated with the size and location of the sharp foreign body, which, depending on the inflammatory process, often cannot even be found (Mendes et al. 2009). In both cases, it was possible to detect the foreign body, which had pierced the wall of the reticulum and diaphragm and reached the pericardial sac. The use of a metal detector can be a good alternative for an early diagnosis, improving treatment possibilities, however the best form of control is through prophylactic measures with attention to the feeding management of cattle (Mendes et al. 2009, Marques et al. 2018).

Based on the present study, the need for joint diagnostic action to apply more effective measures to control these conditions is clear. The need for implementing diagnostic methods and frequently monitoring pathological conditions that occur in the region is also evident.

Raising and handling dairy and beef herds still requires very simple techniques with low investment in new technologies and modernization of the rearing system. Thus, the present work provides significant evidence for necessary improvements in health management, focusing on food management as a major factor in the development of gastric disorders responsible for substantial economic losses in the region. It also emphasizes

the dangerous consequences of excessive ingestion of *Trifolium repens* and urea and poisoning by *B. coridifolia* due to errors in the handling of animals resulting in high mortality rates.

Acknowledgments.- To "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES) for financial assistance to the "Programa de Pós-Graduação em Ciência Animal", and to "Universidade Federal do Pampa" (Unipampa) for financial assistance for the development of this work.

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

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PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(6):426-429, June 2020
DOI: 10.1590/1678-5150-PVB- 6636

Original Article
Livestock Diseases

PVB- 6636 LD

Arthrogryposis multiplex congenita in Aberdeen Angus cattle in Uruguay[1]

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ABSTRACT.- Romero A., Briano C. & Dutra F. 2020. **Arthrogryposis multiplex congenita in Aberdeen Angus cattle in Uruguay**. *Pesquisa Veterinária Brasileira 40(6):426-429*. División de Laboratorios Veterinarios "Miguel C. Rubino", Laboratorio Regional Este, Avelino Miranda 2045, CP 33000, Treinta y Tres, Uruguay. E-mail: aromero@mgap.gub.uy

Arthrogryposis multiplex congenita is reported for the first time in the Aberdeen Angus (AA) breed in Uruguay. In a commercial herd of 30 purebred Aberdeen Angus cows, two calves with severe musculoskeletal malformations died at birth. The cows had been inseminated using semen imported from Argentina from one elite AA sire only. At necropsy, one calf showed severe muscular atrophy, arthrogryposis affecting all four limbs and the spine, kyphoscoliosis and torticollis. Histopathology showed muscular atrophy with marked fiber size variation and abundant fibroadipose fibers. The central nervous system only showed congestion and edema due to dystocia, whereas the peripheral nerves and the number of motor neurons in the spinal appeared normal. DNA analysis confirmed arthrogryposis multiplex congenita. It is concluded that disease in Aberdeen Angus cattle is due to failure in the neuromuscular junction.

INDEX TERMS: Arthrogryposis multiplex congenita, bovine, Aberdeen Angus, cattle, Uruguay, hereditary diseases.

RESUMO.- [**Artrogripose múltipla congênita em bovinos Aberdeen Angus no Uruguai.**] Artrogripose múltipla congênita é relatada pela primeira vez em bovinos Aberdeen Angus (AA) no Uruguai. Num rebanho comercial de 30 vacas a Aberdeen Angus, dois bezerros com graves malformações musculoesqueléticas morreram logo após o nascimento. As vacas foram inseminadas utilizando sêmen importado da Argentina, de apenas um touro de elite de AA. Na necropsia, um dos bezerros apresentava atrofia muscular grave, artrogripose afetando todos os quatro membros e a coluna vertebral, cifoscoliose e torcicolo. A histopatologia demonstrou atrofia muscular com acentuadas variações no tamanho das fibras e abundantes fibras fibroadiposas. O sistema nervoso central apresentava apenas congestão e edema devido à distocia, enquanto os nervos periféricos e o número de neurônios motores na medula espinhal pareciam normais. A análise de DNA confirmou artrogripose múltipla congênita. Concluiu-se que a doença em bovinos Aberdeen Angus se deve a falha na junção neuromuscular.

TERMOS DE INDEXAÇÃO: Artrogripose múltipla congênita, bovinos, Aberdeen Angus, Uruguai, doenças hereditárias.

INTRODUCTION

Hereditary diseases in cattle are a cause of growing concern worldwide. The use of artificial insemination, multiple ovulation embryo transfer, and international trade in germplasm have led to the propagation of economically valuable productive features, but also some defective recessive genes, with significant impact on fertility and animal health (Windsor & Agerholm 2009, Windsor et al. 2011). Modern breeding in cattle has caused the simultaneous appearance of inherited disorders in many countries (Charlier et al. 2008, Windsor & Agerholm 2009, Jolly & Windsor 2010, Windsor et al. 2011), including Uruguay (Kelly et al. 2012, Dutra et al. 2017). Diseases such as osteopetrosis and neuropathic hydrocephalus in Aberdeen Angus (AA) breed and cardiomyopathy wooly haircoat and maple syrup urine disease (MSUD) in Hereford and Polled Hereford (HR) breeds are examples of this (Windsor et al. 2011, Dutra et al. 2011, 2015).

In Uruguay, commercial beef cattle production is unique because it is mostly performed using purebreds, mainly HR and AA, with limited use of crossbreeding systems (DIEA 2003). It is also common to import germplasm from a few genetic lines for use in commercial and elite herds (Dutra 2016).

Arthrogryposis (crooked joints) is a congenital malformation characterized by curvature of the extremities, multiple joint stiffness, and muscular dysplasia, and is reported in different dairy and beef cattle breeds, such as HR, AA, Charolais,

[1] Received on March 18, 2020.
Accepted for publication on April 2, 2020.
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Shorthorn, Jersey, Holstein-Friesian and Red Danish (Shupe et al. 1967, Windsor et al. 2011, Agerholm et al. 2016, Craig et al. 2016). Arthrogryposis multiplex congenita (AMC; OMIA 2020, 002135-9913, commonly known as curly calf syndrome) is a lethal autosomal recessive genetic disorder of AA, originating in the bull Rito 9J9 of B156 7T26 and distributed widely through the bull GAR Precision 1680 (Kaiser 2009, Whitlock 2010, Windsor et al. 2011). The disease in AA has been confirmed in North America (Beever & Marron 2011) and Australia (Windsor et al. 2011) only. Affected animals have severe contraction of the joints in limbs, neck, and spine and are born dead or die shortly after birth; however, there may also be embryonic and fetal losses. Calves have a significantly lower weight (15-25kg) due to poor muscular development (Windsor et al. 2011, Cooper & Valentine 2016). The forelimbs are normally in fixed flexion and hindlimbs can be fixed in flexion or extension, with deviation of the spine and facial bones. In some cases, cleft palate, mild hydrocephalus and abnormalities of the ribs and sternum have been observed (Agerholm et al. 2016), but no histological findings have been reported. The disease is due to a deletion of 23,363 bp that cover three different genes: ISG15, HES4 and AGRN (Beever & Marron 2011). The AGRN gene encodes for agrin, identified as an essential neural regulator that induces the aggregation of acetylcholine receptors (AChRs) and other postsynaptic proteins on muscle fibers and is crucial for the formation and maintenance of the neuromuscular junction (NMJ) (Burgess et al. 1999, Maselli et al. 2012).

This study reports the macroscopic and histological examination of two Aberdeen Angus calves with arthrogryposis multiplex congenita, subsequently confirmed by molecular analysis.

MATERIALS AND METHODS

The pathological diagnosis was made at the "Laboratorio Regional Este" of the "División de Laboratorios Veterinarios (DILAVE) Miguel C. Rubino", Treinta y Tres, Uruguay. A complete post-mortem examination, including the central nervous system and the skeleton, was performed in the affected calves. The entire brain and spinal cord, and samples of fore and hindlimb muscles and several organs were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5-7µm and stained with hematoxylin and eosin (HE), phosphotungstic acid hematoxylin (PTAH) and Masson's trichrome (MT).

Blood clots and spleen samples were collected and stored at -20°C until genomic DNA extraction. The extraction of genomic DNA was carried out using commercial DNA extraction kits (MagMAX™-96DNA Multi-Sample Kit). Samples were submitted to a commercial laboratory (Neogen GeneSeek, Lincoln/NE, USA) for genotyping using the AM2 test, which detects the mutation response of AMC. This test uses Illumina Infinium chemistry.

RESULTS

The disease was observed in a herd of 30 pregnant purebred AA adult cows in the Department of Rocha, Uruguay, during September 2017. Two calves with severe malformations that required cesarean section died at birth. The cows had been inseminated using semen imported from Argentina from one elite AA sire only. No cases or fetal losses were detected previously in the affected cows.

Post-mortem examination of two affected calves showed full-term animals with low body weight, arthrogryposis, kyphoscoliosis and torticollis. In both animals, the forelimbs were curled in flexion and the hindlimbs in extension (Fig.1). After opening, all distal and proximal limb joints allowed free flexion and extension motion, thus showing the absence of ankylosis. There was lateral deviation of the head and severe scoliosis, with deviation of the cervical, thoracic and lumbar vertebral column and deformation of the rib cage and sternum in Calf No. 2 (Fig.2). Internal organ lesions were mostly associated with the narrowing of the body cavities. The lung was compressed and had fetal atelectasis. Grossly, the brain was very congestive and on the cut surface the gray cortex appeared pale and the subcortical white matter hyperemic ("ribbon effect"), indicating birth asphyxia (Dutra et al. 2007). The entire spinal cord was macroscopically normal. All the muscles of the carcass were severely atrophic, pale and sticky in texture. In Calf No. 2, both hip joints were abnormally shaped and partially dislocated. No lesions were found in the bones, including growth plates, epiphysis and bone marrow.

Histologically, the skeletal muscles were largely replaced by fibrofatty connective tissue with a severe lack of myofibers. The few remnant muscle fibers exhibited variation in diameter with many atrophic fibers that appeared rounded instead of polygonal and abundant endomysial and perimysial connective tissue. MT staining showed predominantly collagen fibers and

Fig.1. Arthrogryposis multiplex congenita in Calf No. 1. Note contracted forelimbs, extended rear limbs, curved neck and the very thin appearance due to reduced muscle development.

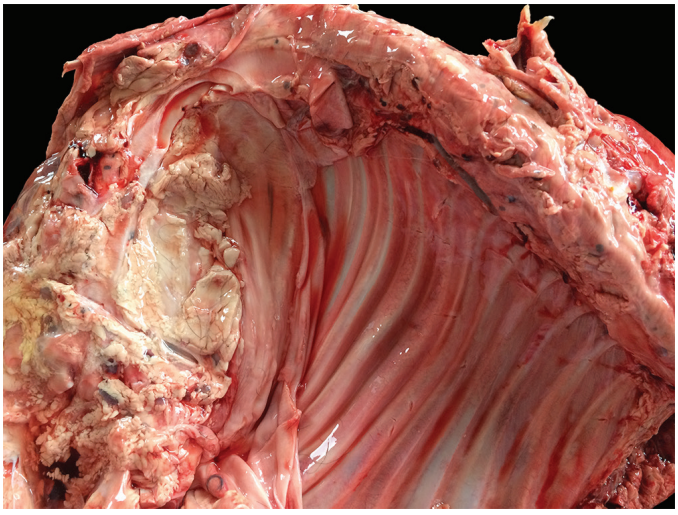

Fig.2. Arthrogryposis multiplex congenita in Calf No. 2. Note the severely curved thoracolumbar spine and deformed rib cage.

muscular hypoplasia at the skeletal muscle level (Fig.3), and with PTAH staining the fibers appeared smooth and faint, and cross striations were apparent (Fig.4). Intramuscular nerves containing myelinated and Schwann cells in normal proportions were apparent (Fig.5). The brain had marked congestion of the gray cortex and subcortical white matter, thoracic and lumbar spinal cord was normal, with the cytomorphology and number of spinal ventral motor neurons within the normal range. The rest of the organs stained with HE were normal.

The homozygous mutation responsible for AMC (AM-Affected) was confirmed by genotyping (AM2 test - NEOGEN). The SNP Call Rate was 0.9618, ensuring a high-confidence genotype.

DISCUSSION

Arthrogryposis can be caused by teratogenic viruses, toxic plants, and hereditary diseases (Agerholm et al. 2015, Craig et al. 2016). During early embryogenesis, movement is essential for the development of joints and skeletal muscles, and fetal akinesia at this stage produces joint stiffness, muscle contractures, or arthrogryposis (Hall 2014, Kowalczyk & Feluś 2016). Absence of fetal mobility may be due to fetal (neurogenic or myogenic) or maternal (infections, drugs, trauma) abnormalities (Hall 2014, Agerholm et al. 2016, Kowalczyk & Feluś 2016).

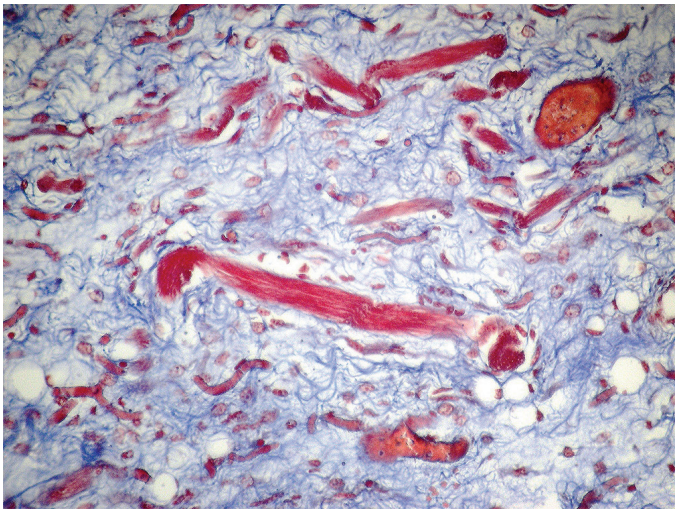

Fig.3. Skeletal muscle. Few atrophic muscle fibers (stain red) within abundant fibroadipose connective tissue (stain blue). Masson Trichrome, bar = 50µm.

In cattle, arthrogryposis with hydranencephaly, porencephaly and cerebellar hypoplasia is associated with teratogenic viral infection (Akabane virus, Bovine viral diarrhea virus, Border disease virus, Schmallenberg virus, Bluetongue virus). Viral infection was ruled out since there were no macroscopic or histological lesions in the CNS (Kessell et al. 2011, Bayrou et al. 2014, Agerholm et al. 2015, Kirkland 2015, Peperkamp et al. 2015, Cantile & Youssef 2016). A toxic origin was also rejected. *Lupinus* sp., *Conium maculatum, Nicotiana glauca*, when ingested during days 40 to 80 of the pregnancy, can cause arthrogryposis, but these plant species were not present on the farm, and no clinical signs of toxicosis were observed in pregnant cows (Green et al. 2015). Viral infections or toxins that affect the nervous system are generally epidemic, which did not happen in this case.

The presence of lesions in the muscular system, with their absence in the encephalon and spinal cord, suggest that AMC in black AA may be due to a failure in the NMJ. Similarly, Agerholm et al. (2016) described a familial arthrogryposis multiplex congenita in Red dairy cattle, with complete lipomatous muscular atrophy, an absence of lesions in the brain and a normal number of motor neurons in the spinal cord. This neuromuscular disorder is due to a deletion in the CHRNB1 gene that is of essential importance in the NMJ (Agerholm et al. 2016).

In this case, the AA herd had no previous record of births of animals with congenital malformations, and imported semen was used for the first time. So, it can be established that at least two cows, as well as imported semen from Argentina, are carriers of AMC. So, it can be established that AM-Carriers are present in the AA breed in Uruguay and Argentina and that the disease may occur in both countries.

Reporting of congenital malformations to diagnostic laboratories, along with surveillance programs to identify carriers

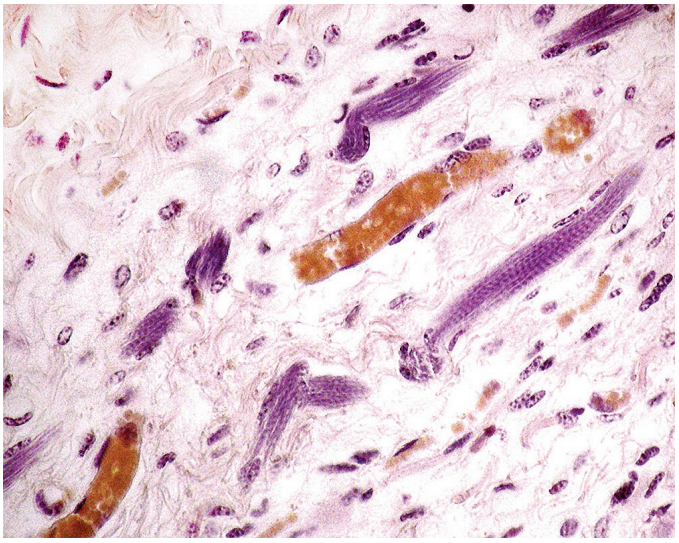

Fig.4. Sarcoplasmic cross-striations of muscle fibers (stain blue). Phosphotungstic Acid Hematoxylin, obj.40x.

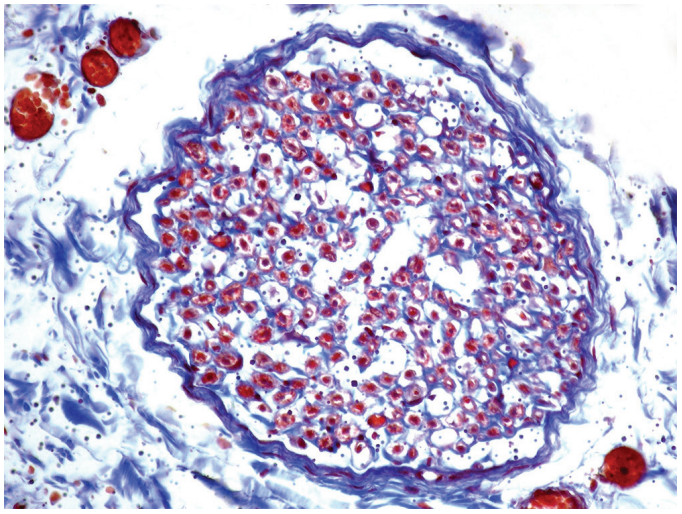

Fig.5. Light microscopy image of an intramuscular nerve. Normal appearance of myelin is seen; myelin sheaths (stain purple-red), collagen fibers (stain blue). Masson Trichrome, obj.40x.

(DNA tests) of undesirable traits, would help to prevent the spread of hereditary diseases (Jolly & Windsor 2010, Windsor et al. 2011). The use of genetic screening is much more effective when applied to all pedigree animals of the breed.

CONCLUSIONS

This seems to be the first study demonstrating the existence of arthrogryposis multiplex congenita in Aberdeen Angus cattle in Uruguay.

The presence of lesions limited to the muscular system, with the absence of pathological findings in the central nervous systems and intramuscular nerves could establish that the disease in this breed is due to a failure in the neuromuscular junction (NMJ).

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PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(6):430-437, June 2020
DOI: 10.1590/1678-5150-PVB- 6139

Original Article
Livestock Diseases

Detection of *Treponema* spp. in bovine digital dermatitis in the Amazon biome, Brazil[1]

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ABSTRACT.- Bomjardim H.A., Oliveira M.C., Cordeiro M.D., Brito M.F., Fonseca A.H., Oliveira C.M.C., Silva N.S. & Barbosa J.D. 2020. **Detection of *Treponema* spp. in bovine digital dermatitis in the Amazon biome, Brazil**. *Pesquisa Veterinária Brasileira 40(6):430-437*. Instituto de Medicina Veterinária, Campus de Castanhal, Universidade Federal do Pará, Rodovia BR-316 Km 61, Castanhal, PA 68741-740, Brazil. E-mail: henriqueanjos18@hotmail.com

Bovine digital dermatitis (BDD) is a polybacterial claw disease that is endemic to dairy cattle kept in loose house systems, and treponemas are the main bacteria implicated in this disease. The objective of this study was to report the occurrence of *Treponema* spp. in BDD from crossbred dairy cattle (Holstein x Zebu) kept in a pasture in the Brazilian Amazon biome. The diagnostic of BDD was performed by inspecting the distal extremities of cattle during milking in one or more visits comprising 15 farms. In total, it could be inspected 1,847 cows from August 2016 to July 2017, and 25 lesions of BDD were diagnosed. The feet were scored (System M: M0 = no lesion, M1 = ulcer stage <2cm, M2 = ulcer stage >2cm, M3 = healing stage, M4 = chronic stage, M4.1 = chronic stage with ulcer area). Twenty four biopsy samples were taken from feet with BDD and five biopsy samples from feet with no lesions. The histopathology of stained tissues was performed by hematoxylin and eosin and Warthin-Starry method. The samples were also tested by nested PCR for the three previously isolated BDD *Treponema* phylogroups (*T. medium*/*T. vincentii*-like, *T. phagedenis*-like and *T. putidum*/*T. denticola*-like). Spirochetes were observed in 54.2% (13/24) of the lesions, and in 91.7% (22/24) of the samples were detected the DNA of this spirochete belonging to the treponema phylogroups implicated in BDD. In 25% (6/24) of the lesions were detected all the phylogroups. Forty percent (40%, 2/5) of the M0 samples were also positive for the nested Polymerase Chain Reaction (nested-PCR), as 8.3% (2/24) of the lesions were negative in both techniques employed. *Treponema putidum*/*T. denticola*-like was the most detected bacterial in all the stages, and active lesions (M2 and M4.1) presented a greater proportion of *T. medium/T. vincentii*-like and *T. phagedenis*-like, but no statistical differences were observed (p>0.05). It could be concluded that BDD lesions in crossbred dairy cattle kept to pasture in the Amazon biome were classified as "polytreponemal" infections and the phylogroup *T. putidum*/*T. denticola*-like was the most frequent in the lesions.

INDEX TERMS: *Treponema* spp., bovine digital dermatitis, Amazon biome, Brazil, Mortellaro disease, Warthin-Starry, nested-PCR, cattle.

[1] Received on July 11, 2019.
Accepted for publication on August 13, 2019.
Part of the Doctoral Thesis of the first author.

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RESUMO.- [**Detecção de *Treponema* spp. em dermatite digital bovina no bioma amazônico, Brasil.**] Dermatite digital bovina (DDB) é uma enfermidade polibacteriana dos dígitos endêmica em vacas leiteiras criadas em estábulos e as treponemas são as principais bactérias envolvidas. Este estudo teve como objetivo relatar a ocorrência de *Treponema* spp. em DDB em bovinos leiteiros mestiços (Holandês x Zebu) criados a pasto no bioma amazônico brasileiro. O diagnóstico da DDB foi realizado pela inspeção, em uma ou mais visitas, das extremidades distais das vacas durante a ordenha em 15 propriedades. No total, foram inspecionadas 1.847 vacas de agosto de 2016 a julho de 2017 e diagnosticou-se 25 lesões de DDB. As extremidades distais inspecionadas foram classificadas em escores (M0 = sem lesão, M1 = estágio ulcerado <2cm, M2 = estágio ulcerado >2cm, M3 = estágio em cicatrização, M4 = estágio crônico, M4.1 = estágio crônico com área ulcerada) e realizada 24 biópsias de dígitos com DDB e cinco biópsias de dígitos em estágio M0. Foram realizadas a histopatologia pelas colorações de hematoxilina e eosina e pelo método de Warthin-Starry, e a *nested* de reação em cadeia de polimerase (nested-PCR) para os três filogrupos de treponemas previamente isolados de DDB (*Treponema medium/T. vincentii*-like, *T. phagedenis*-like e *T. putidum/T. denticola*-like). Espiroquetas foram observadas em 54,2% (13/24) das lesões e em 91,7% (22/24) detectou-se o DNA de, pelo menos, um dos filogrupos de treponemas pesquisados. Em 25% (6/24) das lesões foram detectados o DNA dos três filogrupos. Em 40% (2/5) das amostras em estágio M0 também foram positivas na nested-PCR, assim como 8,3% (2/24) das lesões foram negativas em ambas as técnicas empregadas. *T. putidum/T. denticola*-like foi o filogrupo mais detectado em todos os estágios e lesões ativas (M2 e M4.1) apresentaram uma maior proporção para *Treponema medium/T. vincentii*-like e *T. phagedenis*-like, mas não se obteve diferença estatística na ocorrência dos filogrupos entre os estágios das lesões (P>0,05). Conclui-se que lesões de DDB em rebanhos leiteiros mestiços criados a pasto no bioma amazônico brasileiro são "politreponemais" e o filogrupo *T. putidum/T. denticola*-like é o mais frequente nas lesões.

TERMOS DE INDEXAÇÃO: *Treponema* spp., dermatite digital bovina, Amazônia, doença de Mortellaro, Warthin-Starry, nested-PCR, Brasil, bovinos.

INTRODUCTION

Bovine digital dermatitis (BDD) is an infectious disease characterized by inflammation and ulceration of the skin of bovine digits, and also associated with different bacterial agents (Cheli & Mortellaro 1974, Santos et al. 2011, Krull et al. 2014). In the BDD lesions, the spirochetes which are bacteria of the genus *Mycoplasma*, *Fusobacterium*, *Porphyromonas*, *Bacteroides* spp., *Campylobacter* spp. have been isolated, as well as the species of *Guggenheimella bovis* and *Dichelobacter nodosus* (Döpfer et al. 1997, Schlafer et al. 2008, Rasmussen et al. 2012, Krull et al. 2014, Nielsen et al. 2016). Among these, spirochetes are the bacterial agents which are the most related to the disease, since they are detected in more significant proportions and found in deeper layers of the epidermis, these bacterial agents also have the ability to suppress the innate immune system and to induce the formation of lesions (Stamm et al. 2002, Cruz et al. 2005, Zuerner et al. 2007, Klitgaard et al. 2008, Nordhoff et al. 2008, Nielsen et al. 2016). According to molecular studies, *Treponema* is the most important genus of spirochetes isolated from BDD consisting of various strains, which characterizes a "polytreponemal" disease (Evans et al. 2008, Klitgaard et al. 2008, Krull et al. 2014, Nielsen et al. 2016).

The BDD is a common foot condition in dairy cows reared in a free-stall farming system in England, Germany, United States of America (USA), and Japan (Evans et al. 2008, Klitgaard et al. 2008, Nordhoff et al. 2008, Yano et al. 2010). In these countries, three different *Treponema* phylogroups are commonly identified in BDD lesions, such as: "*T. medium/T. vincentti*-like", "*T. phagedenis*-like" and "*T. pudidum/T. denticola*-like" (Evans et al. 2008, Yano et al. 2010, Döpfer et al. 2012, Marcatili et al. 2016). These bacterial phylogroups are also isolated from BDD in beef cattle (Sullivan et al. 2013), sheep (Sullivan et al. 2015a), goats (Sullivan et al. 2015b), and in North American elk (Clegg et al. 2015).

In Brazil, BDD is a disease that occurs in cattle herds that may be raised under three different management systems (intensive, semi-intensive, or extensive practices) in their different regions, but with different rates of occurrence. Among foot lesions diagnosed in dairy cows, BDD comprised 38.9% of this disease in the state of Goiás (GO) (Silva et al. 2001), 33% in the state of Minas Gerais (MG) (Moreira et al. 2018a), 29.9% in the state of Rio Grande do Sul (RS) (Cruz et al. 2001) and 0.92% in the state of Pará (PA) (Silveira et al. 2009). However, studies related to bacterial agents in lesions are still limited. In dairy cows and beef cattle raised in the Midwest and South regions, the presence of spirochetes in stained tissues performed by silver has already been demonstrated (Cruz et al. 2005, Castro et al. 2008), as well as different species of the *Treponema* genus by using the fluorescent *in situ* hybridization (FISH) technique and by the nested-PCR (Nascimento et al. 2015, Moreira et al. 2018b).

In the Brazilian Amazon biome (northern region), the climatic conditions and territorial extension favor cattle-breeding on pasture land all year long, and it can be noted that the BDD is reported in dairy and beef cattle (Silveira et al. 2009, Silveira et al. 2018). It is important to note, it seems that there are no studies related to the bacterial agents involved. This study aimed to demonstrate *Treponema* spp. in BDD lesions observed in histological fragments using the silver impregnation technique and complemented by nested-PCR in dairy cattle herds, raised on pasture in the Amazon biome.

MATERIALS AND METHODS

Study region, diagnosis, lesion classification, and biopsies. A search for *Treponema* spp. through silver impregnation was performed and also complemented by nested-PCR in BDD lesions in crossbred dairy cattle (Holstein x Zebu) bred on pastures of *Urochloa* (*Brachiaria*) *brizantha* in southeastern Pará and western Maranhão, situated at the Amazon biome, from August 2016 to July 2017. 1,847 cattle from 15 rural properties were inspected in one or more visits, and 25 BDD lesions were diagnosed. The lesions were classified as follows: M1 = skin in an ulcer stage, diameter <2cm; M2 = skin in an ulcer stage >2cm; M3 = skin in a healing stage, covered by a crust; M4 = skin in a chronic stage, hyperkeratotic surface; and M4.1 = chronic stage skin with an ulcer area (Döpfer et al. 1997, Berry et al. 2012). Twenty-four biopsies of BDD and five digits were performed in stage M0. From the lesions and digits in stage M0 (digit without injury),

two fragments were collected, approximately 0.5cm each, after anesthesia of the distal and by intravenous Bier block, with 20ml of 2% lidocaine. Regarding the BDD lesions, the first fragment, obtained with a scalpel blade and anatomical forceps, clean and sterile, was removed from the center of the lesions. In stage M4.1, the fragment was extracted from the ulcer area. These biopsies were stored in polyethylene tubes, previously identified, and kept at -20ºC until laboratory procedures for molecular biology were performed. The second fragment, removed from the intersection of normal skin and the center of the lesion, was fixed in 10% buffered formaldehyde. In the digits of stage M0, both biopsies were performed at the caudal border of the pelvic limbs in the interdigital commissure, following the same procedures for collecting the lesions.

Histopathology. Samples fixed in formaldehyde were processed by the usual methods for histopathology, in the "Setor de Anatomia Patológica" of the "Universidade Federal Rural do Rio de Janeiro" (UFRRJ). These samples were soaked in paraffin, cut into a microtome at 5µm thickness, and stained with hematoxylin and eosin (HE) and by the Warthin-Starry method.

DNA extraction and nested-PCR. The biopsies were thawed at room temperature, and DNA extraction was performed following the protocol based on the use of phenol/chloroform as described by McIntosh et al. (2015).

The extracted DNA was submitted to nested-PCR using specific primers for the three treponema phylogroups "*T. medium/T.vincentii*-like", "*T. phagedenis*-like" and "*T. pudidum/T. denticola*-like" according to the methodology described by Evans et al. (2008) and (2009).

Statistical analysis. To evaluate a possible association between the phylogroups (*T. medium/T. Vincentii*-like, *T. phagedenis*-like and *T. putidum/T. denticola*-like) and the BDD stages (M0, M1, M2, M3, M4 and M4.1), the Fisher's exact test was used, with a significance level (α) of 5%. All analyzes were performed after registration in spreadsheets (Microsoft Excel® 2010), and the data were analyzed using the statistical software SPSS 20.0 (IBM Corp. Released 2011, IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). The frequencies of the variables presented descriptive analyzes.

RESULTS

The BDD lesions were classified as 16.7% (4/24) in stage M1 (Fig.1), 37.5% (9/24) in M2, 12.5% (3/24) in M3, 12.5% (3/24) in M4 and 20.8% (5/24) in M4.1 (Fig.2) (Table 1). Histopathology revealed extensive ulceration (stages M1, M2, and M4.1) associated with ulcer clusters of bacterial colonies, acanthosis with hypergranulosis, and hyperkeratosis (sometimes referred to as parakeratotic, sometimes as

Fig.1. Bovine digital dermatitis. Spherical lesion, with a granular, moist surface and hypertrophied hair. Interdigital commissure of the plantar region of the left pelvic member of Bovine 9 (Table 1: stage M1, score by Döpfer et al. 1997). Amazon biome.

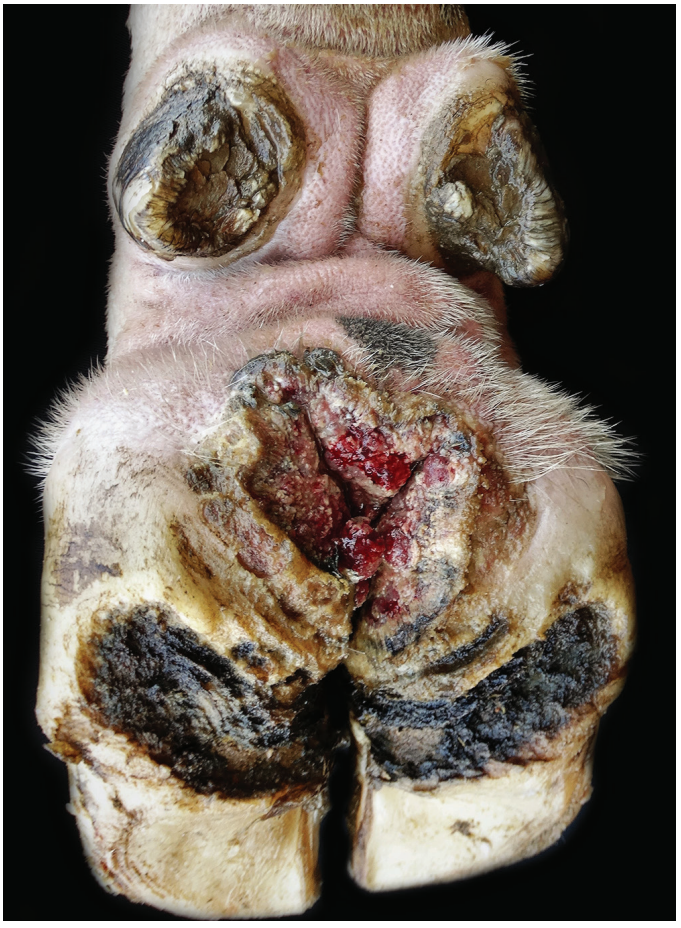

Fig.2. Bovine digital dermatitis. Ulcer lesion, irregularly shaped with a granular, reddish and moist surface, with raised and hyperkeratotic margins. Horny tissue with marked bead erosion. Plantar region of the left pelvic member of Bovine 28 (Table 1: stage M4.1, score by Döpfer et al. 1997 and Berry et al. 2012). Amazon biome.

orthokeratotic), which were accentuated in stages M3 and M4. Inflammatory cells had infiltrated the epidermis, the dermo-epidermal junction, and the perivascular region in the dermis (Fig.3 and 4). In the M0 stage, the skin presented its usual histological architecture with mild acanthosis, hypergranulosis, and a discrete perivascular infiltrate of inflammatory cells.

The silver impregnation revealed spirochetes in the epidermis superficial strata in 54.2% of the lesions (13/24) (Fig.5 and 6), and its absence in the M0 stage. Nested-PCR detected the genetic material of *Treponema* ssp. in 91.7% (22/24) of BDD injuries. The DNA of the phylogroup *T. pudidum/T. denticola*-like was the most frequently detected (83.3%, 20/24) in samples with lesions. In 25% (6/24) of the lesions, the three *Treponema* phylogroups surveyed were detected. In the M0 stage, *Treponema* genetic material was detected in 40% (2/5) of the samples, and 8.3% (2/24) of the BDD lesions were negative for spirochetes and *Treponema* spp. in both techniques employed, respectively (Table 1).

Through the distribution of the genetic material between the stages of BDD, it could be observed that the *T. pudidum/T. denticola*-like phylogroup was the most frequently detected (p=0.048). In fact, it could be detected a higher proportion of positive samples for the *T. medium/T. vincentii*-like phylogroups and *T. phagedenis*-like in stages M2 and M4.1, however, there was no statistical difference (p=0.408 and p=0.279, respectively) in the occurrence of this phylogroup and the BDD stage (Fig.7).

DISCUSSION

These results suggested the presence of *Treponema* spp. in the etiology of BDD, as observed in dairy cows kept in pastures in the Brazilian Central region (Moreira et al. (2018b), in dairy cows housed in the southern region of Brazil (Nascimento et al. 2015) and free-stalled dairy cows in the USA (Zinicola et al. 2015), Germany (Nordhoff et al. 2008), Denmark (Klitgaard et al. 2008), England (Evans et al. 2008) and Japan (Yano et al. 2010).

By the methodology used, a high frequency of *Treponema* spp. was observed in the lesions of BDD, comprising 91.7%. However, there was lesser diversity of phylogroups between the lesions, consisting of 25%. In dairy cows intensively reared in southern Brazil, Nascimento et al. (2015) detected *Treponema* spp. in 100% of the researched injuries, and 81.8%

Table 1. Detection of spirochetes in tissues impregnated with silver and Treponema spp. in skin biopsies of digits with and without lesions of bovine digital dermatitis (BDD) in crossbred dairy cattle raised on pasture in the Amazon biome

Groups	Score	Animal	SI*	*T. medium/ T. vincentii*-like	*T. phagedenis*-like	*T. putidum/ T. denticola*-like	*Treponema* sp.
Animals without BDD lesions	M0	01	-	-	-	+	+
		02	-	-	-	-	-
		03	-	-	+	-	+
		04	-	-	-	-	-
		05	-	-	-	-	-
Animals with BDD lesions	M1	06	+	-	+	+	+
		07	-	-	-	-	-
		08	+	-	+	-	+
		09	+	-	-	+	+
	M2	10	-	-	-	+	+
		11	+	-	+	+	+
		12	-	-	-	+	+
		13	-	+	+	+	+
		14	+	+	+	+	+
		15	-	+	+	+	+
		16	+	+	+	-	+
		17	+	+	+	+	+
		18	-	-	-	+	+
	M3	19	-	-	-	+	+
		20	-	-	-	-	-
		21	+	-	-	+	+
	M4	22	+	-	-	+	+
		23	+	-	-	+	+
		24	+	+	+	+	+
	M4.1	25	-	-	-	+	+
		26	-	+	-	+	+
		27	-	-	-	+	+
		28	+	-	-	+	+
		29	+	+	+	+	+
TOTAL (%)**			54,2	33,3	41,7	83,3	91,7

* SI = Silver impregnation, ** total percentage calculated only in animals with BDD lesions (M1, M2, M3, M4 e M4.1).

of the injuries showed all three phylogroups. This lower frequency, with a low variety of *Treponema* detected in the lesions, may be related to the lower environmental pressure to which these animals raised on pasture in the Amazon biome are subjected. On pasture, the animals are susceptible to low humidity (digit), especially in the non-rainy season, less contact with feces, and less contact between animals. In the etiopathogenesis, the main reservoirs of *Treponema* for healthy cattle suggested by Shibahara et al. (2002), Evans et al. (2012), Klitgaard et al. (2014), Nascimento et al. (2015) and Zinicola et al. (2015) were the digestive tract and animals with BDD. A lower prevalence (72.9%) of *Treponema* in BDD lesions, using the same molecular technique, was also obtained by Moreira et al. (2018b) in dairy cows grazing in Central region of Brazil. This lower prevalence pointed out to a breeding environment relationship influencing the frequency of *Treponema* in BDD lesions.

The histopathology of the lesions revealed extensive areas of ulceration and inflammatory changes in the epidermis and dermis. It was similar to the pathological changes in the BDD observed in dairy cows stabled by Döpfer et al. (1997) and in beef cattle by Sullivan et al. (2013). Bacterial colonies in the form of coconuts and bacilli were also observed, which suggests the presence of other bacterial agents, in addition to spirochetes, in the BDD lesions of crossbred dairy cows raised on pasture in the Amazon biome. In the BDD lesions, different phyla of bacteria were isolated and, therefore, this disease was characterized as polybacterial, according to Krull et al. (2014), Klitgaard et al. (2014) and Zinicola et al. (2015). However, spirochetes are the most prevalent bacterial agent in BDD lesions and are commonly found in deep strata of the epidermis, which points to a close relationship with the pathogenesis of this disease (Klitgaard et al. 2014, Zinicola et al. 2015, Moreira et al. 2018b).

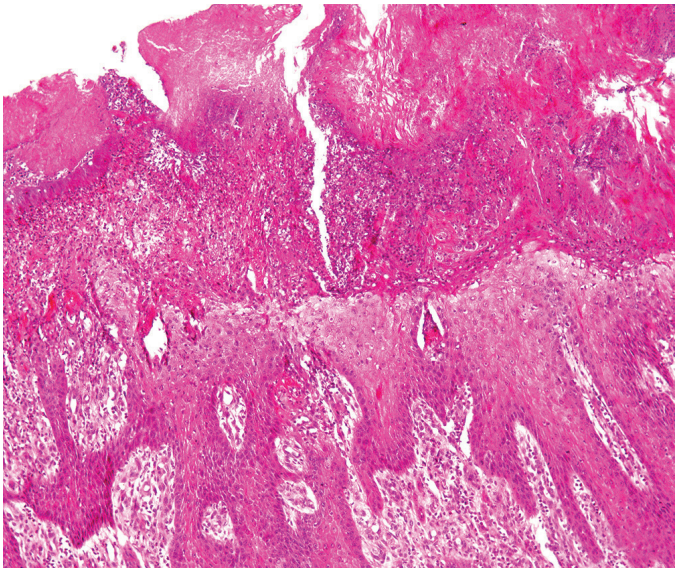

Fig.3. Bovine digital dermatitis. Skin with extensive area of ulceration and exudation of the epidermis. Dermis with mild inflammatory infiltrate at the dermo-epidermal junction (DEJ). HE, obj.10x.

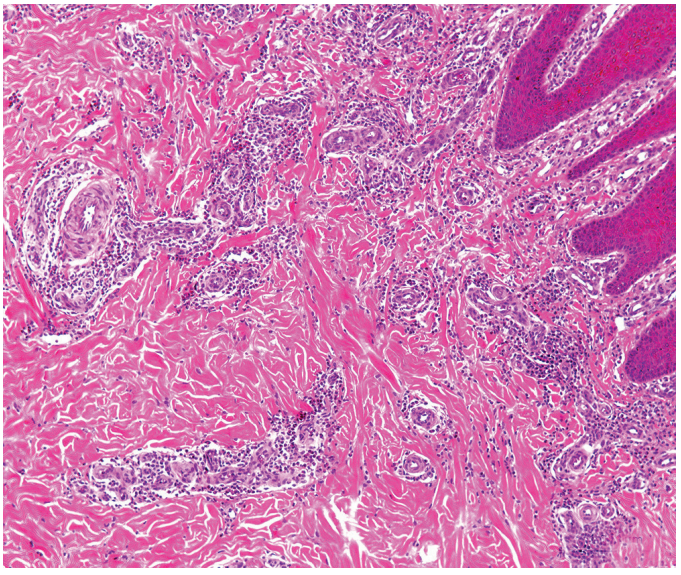

Fig.4. Bovine digital dermatitis. Skin with moderate inflammatory infiltrate, predominantly perivascular in the dermis and dermal-epidermal junction (DEJ). HE, obj.4x.

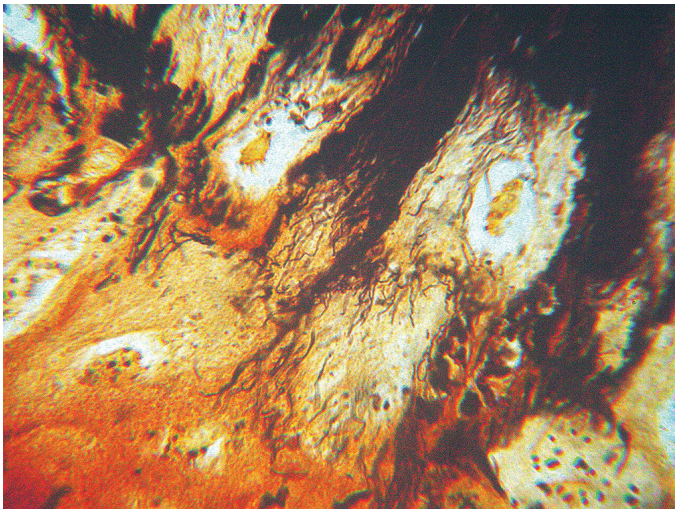

Fig.5. Bovine digital dermatitis. Spirochetes in the superficial dermis and around hair follicles. Warthin-Starry method, obj.100x.

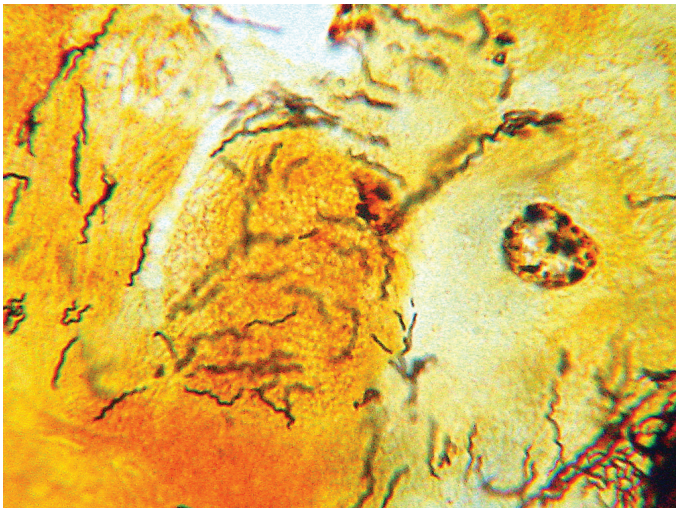

Fig.6. Bovine digital dermatitis. Spirochetes in the spinous stratum of the epidermis. Warthin-Starry method, obj.100x.

According to the frequency distribution of the surveyed phylogroups among the BDD scores, and to the M system, a high frequency of the phylogroup *T. pudidum/T. denticola*-like was observed in all stages, which suggests that this phylogroup is dominant in BDD lesions in the Amazon biome, as observed by Yano et al. (2010) in dairy cows housed in Japan. A higher proportion of the *T. medium/T. vincentii*-like phylogroups were also obtained and *T. phagedenis*-like in active lesions (stages M2 and M4.1) in relation to healthy skin (stage M0) and non-active lesions (stages M3 and M4), which indicated a change in the population of *Treponema* regarding the stage of the injury. However, no statistical association was found between the *Treponema* phylogroup and the stage of BDD lesions (p>0.05). A marked difference in the microbiota between active (M1, M2, and M4.1) and non-active (M3 and M4) lesions were observed by Krull et al. (2014) and Zinicola et al. (2015). *Treponema denticola*, *T. medium*, *T. maltophilum*, *T. paraluiscuniculi*, *T. phagedenis*, *T. putidum*, and *T. vincentii* were detected more frequently in active lesions in the study by Zinicola et al. (2015).

Moter et al. (1998) observed that *T. denticola* was distributed between cellular debris and the superficial layers of the spinous stratum of ulcerative BDD lesions. Therefore, it suggested this agent as a secondary one in the pathogenesis of the disease. Nordhoff et al. (2008) observed that *Treponema* of the *T. medium/T. vincentii*-like phylogroups and *T. phagedenis*-like were located between the interface of healthy tissue with injured one. They inferred a close relationship between these phylogroups with the etiology of BDD. Probably, humoral and cellular immune responses triggered by *Treponema* in BDD, as described by Trott et al. (2003), can change the bacterial population present in BDD and characterize the stage of the lesion. Additional studies, involving a larger number of biopsies, is necessary to assess whether there is a significant change in the *Treponema* population according to the lesion stage in the BDD of crossbred dairy cows raised on pasture in the Amazon biome.

Bacterial research involving molecular techniques of PCR and FISH pointed out that bacteria of the genus *Treponema* spp. are the main dominant agents in the BDD lesions, as observed by Moreira et al. (2018b), Nordhoff et al. (2008) and Zinicola et al. (2015). However, two samples from the present study, in stages M1 and M3, were negative for these both techniques employed. Negative results by FISH were also obtained in a sample by Moreira et al. (2018b). These authors correlated this result with the final stage of wound healing. These results indicated that BDD lesions in the early stages (M1), in addition to lesions in the final stage of healing (M3), may lack the presence of *Treponema* in the tissues. Also, they may have a low concentration of these agents, in which the techniques employed were not capable of detecting it, or may be distributed in a non-homogeneous manner in which only the biopsies did not contain the genetic material. In two samples of skin in stage M0 (without lesion), the phylogroups *T. phagedenis*-like and *T. putidum/T.denticola*-like were detected, but no spirochetes were observed in the silver impregnation. Rasmussen et al. (2012), Knappe-Poindecker et al. (2013), and Moreira et al. (2018b) also obtained positive samples for *Treponema* in PCR in apparently healthy tissues. However, they are not frequently observed in BDD lesions, according to Yano et al. (2010) and Zinicola et al. (2015).

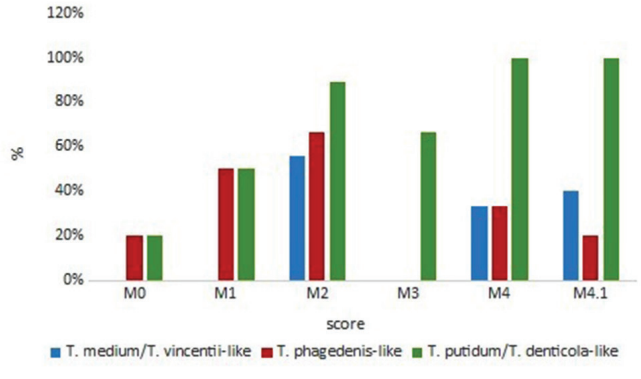

Fig.7. Distribution of frequencies (%) of *Treponema* detected using the nested-PCR technique in biopsies of BDD and skin of the digit without injury (M0) according to the stage (scored by Döpfer et al. (1997) and Berry et al. (2012) of crossbred dairy cattle raised on pasture in the Amazon biome.

CONCLUSION

The bovine digital dermatitis (BDD) in crossbred dairy cattle herds raised in a pasture in the Amazon biome was characterized as a "polytreponemal" lesion with more significant frequency from the phylogroup *T. pudidum/T. denticola*-like in all stages.

Acknowledgments.- The authors would like to thank the "Pró-Reitoria de Pesquisa e Pós-Graduação" of the "Universidade Federal do Pará" (Edital 01/2020 - PROPESP/PAPQ) and to the "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq) (process number 432100/2016-4), for the financial support; and also, to the "Coordenação de Nível de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES) for the exchange scholarship for doctoral researcher.

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

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PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(6):438-442, June 2020
DOI: 10.1590/1678-5150-PVB-6535

Original Article
Livestock Diseases

Spontaneous poisoning by *Stryphnodendron rotundifolium* var. *villosum* in cattle[1]

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ABSTRACT.- Santos I.R., Lima J.C., Oliveira F.H., Ferreira H.H., Ramos M.V.V. & Santos A.S. 2020. **Spontaneous poisoning by *Stryphnodendron rotundifolium* var. *villosum* in cattle.** *Pesquisa Veterinária Brasileira 40(6):438-442*. Laboratório de Histologia e Patologia Animal, Instituto Federal Goiano Campus Urutaí, Rodovia Geraldo Silva Nascimento Km 2,5, Zona Rural, Urutaí, GO 75790-000, Brazil. E-mail: adriana.santos@ifgoiano.edu.br

This is a report on an outbreak of cattle poisoning by *Stryphnodendron rotundifolium* var. *villosum* (Benth.) Scalon in the state of Goiás in Brazil. In a herd of 80 cattle that consumed the mature fruit of this plant that was present in their pasture, 12 animals fell ill and died. The clinical signs that they presented were apathy, progressive weight loss, reeling, bristling, and dry stools containing seeds. The main necropsy findings were jaundice, an increased lobular pattern and orange coloration in their liver, enlarged kidneys with yellowish medullae, and pre-stomachs containing fruit peels and seeds. A histopathological examination revealed vacuolar degeneration and necrosis in the liver and kidneys and vacuolar degeneration in the rumen, omasum, reticulum, and intestine. The diagnosis of poisoning by *S. rotundifolium* var. *villosum* was based on epidemiological data, clinical findings, and pathological changes. Our results can aid in the differentiation between poisoning by *S. rotundifolium* var. *villosum* and poisoning by other toxic plants that are of interest to lifestock.

INDEX TERMS: Spontaneous poisoning, *Stryphnodendron rotundifolium,* cattle, barbatimão, cattle poisoning, Goiás, poisonous plants.

RESUMO.- [**Intoxicação espontânea por *Stryphnodendron rotundifolium* var. *villosum* em bovinos**.] Relata-se um surto de intoxicação em bovinos por *Stryphnodendron rotundifolium* var. *villosum* (Benth.) Scalon no estado de Goiás, Brasil. De um rebanho composto por 80 bovinos, os quais consumiram frutos maduros da planta presente na pastagem, 12 animais adoeceram e morreram. Os sinais clínicos apresentados foram apatia, emagrecimento progressivo, andar cambaleante, pelo eriçado e fezes secas contendo sementes. Os principais achados de necropsia foram icterícia, fígado com padrão lobular e coloração alaranjada, rins aumentados e com amarelamento da medular e pré-estômagos com presença de cascas e sementes do fruto. Ao exame histopatológico, notou-se degeneração vacuolar e necrose no fígado e rins e degeneração vacuolar no rúmen, omaso, retículo e intestino. O diagnóstico da intoxicação por *S. rotundifolium* var. *villosum* baseou-se nos dados epidemiológicos, nos achados clínicos e nas alterações patológicas. Nossos resultados podem auxiliar na diferenciação entre a intoxicação por *S. rotundifolium* var. *villosum* e a intoxicação por outras plantas tóxicas de interesse pecuário.

TERMOS DE INDEXAÇÃO: Intoxicação espontânea, *Stryphnodendron rotundifolium,* bovinos, barbatimão, Goiás, intoxicação em bovinos, planta tóxica.

INTRODUCTION

Stryphnodendron rotundifolium Mart., popularly known as "barbatimão", belongs to the Fabaceae family (=Leguminosae) and Mimosoideae subfamily. The species has two varieties: *S. rotundifolium* Mart. var. *rotundifolium* (syn. *S. obovatum* Benth., *S. discolor* Benth., and *S. rotundifolium* f. *retusa* Chodat & Hassl) and *S. rotundifolium* var. *villosum* (Benth.) Scalon (syn. *Stryphnodendron goyazence* Taub. and *Stryphnodendron*

[1] Received on February 18, 2020.
Accepted for publication on March 4, 2020.
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humile E.M.O. Martins). The *S. rotundifolium* var. *rotundifolium* is found in the Midwestern, Southeastern, Northeastern, and Northern regions of Brazil and is described as being toxic for cattle (Camargo 1965, Tokarnia et al. 1998, Brito et al. 2001a, 2001b, Braga et al. 2018).

Despite the fact that veterinarians and farmers have cited that *S. rotundifolium* var. *villosum* is also toxic to cattle, so far, there have been few reports regarding poisoning due to the consumption of this plant. This variety is shrubby to arboreal, reaches up to 6m in height, and is widely distributed in the central Brazilian "cerrado" (savanna type vegetation) that is found in the Federal District, states of Goiás, Mato Grosso, and Mato Grosso do Sul (Scalon 2007).

In this study, we aimed to describe the epidemiological, clinical, and pathological characteristics associated with an outbreak of spontaneous poisoning by *S. rotundifolium* var. *villosum* in cattle.

MATERIALS AND METHODS

An outbreak of plant poisoning in cattle was investigated. Epidemiological and clinical data were obtained from a veterinarian and the cattle owner during visits to the property. Fragments of various organs were collected from the necropsied animals, following which they were fixed in 10% formaldehyde, routinely processed for histopathology, and stained with hematoxylin eosin (HE).

Samples of plants suspected of being the cause for the poisoning were collected, and the species were identified by examining their morphological characteristics and distribution areas and by consulting the herbarium of the "Instituto Federal Goiano", Campus Urutaí, where the exsiccate of *Stryphnodendron rotundifolium* var. *villosum* (Benth.) Scalon was registered under the number 758. Pluvial precipitation values that were evaluated during the intoxication period were obtained from the Instituto Nacional de Meteorologia (INMET 2018).

RESULTS

Plant identification

The plants were arboreal in size, measuring approximately 3-5m in height (Fig.1A). The leaves were bipinnate with a pubescent rachis and petiole measuring 12-21cm in length. The pinnae consisted of 9-11 pairs organized in an alternate or opposite arrangement, measuring 3-9cm in length. Each pinna contained 10-13 pairs of follicles that had a slightly asymmetric base and that were arranged alternate to or opposite each other with sessile or short petioles that were discolored, villous, and measured 8-14mm in length.

The inflorescences were arranged in axillary spikes that were 2-7cm long and were on a peduncle that was 2-3cm long. The fruit were indehiscent, erect or slightly curved, 9-10cm long, reddish brown, puberulous, and brilliant. Each fruit had 10-12 smooth, ellipsoid-shaped seeds that were 7-8mm long with a dark brown forehead and a yellowish-brown pleurogram. The species was identified as *Stryphnodendron rotundifolium* var. *villosum* (Benth.) Scalon (Fig.1B).

Epidemiological and clinical findings

The outbreak occurred on a property located in the municipality of Ipameri (latitude 17°71' S, longitude 48°16' W, and altitude 772.99m) in Southeastern Goiás during the second week of September 2018. The total rainfall in this region from August to September 2018 was 71.0mm.

The studied herd consisted of 80 Nelore cattle that were reared via an extensive system (9 between 0-12 months, 11 between 13-24 months, 15 between 25-36 months, and 45 over 36 months). The animals were confined to an enclosure of approximately 19 hectares in size covered with *Brachiaria brizantha* (Hochst. ex A. Rich.) Stapf [syn. *Urochloa brizantha* (Hochst. ex A. Rich.) R. D. Webster] and native "cerrado" vegetation among which were numerous individual *S. rotundifolium* var. *villosum* plants with ripe fruits. Further, concerning other toxic plants that may interest livestock, only *Palicourea marcgravii* St. Hil. was identified on the property. The animals were only supplemented with mineral protein.

Fig.1. (**A**) Native "Cerrado" area containing specimens of *Stryphnodendron rotundifolium* var. *villosum*, Ipameri, Goiás, October 2018. (**B**) The exsiccata including vegetative and reproductive samples from *Stryphnodendron rotundifolium* var. *villosum.* Bar = 5cm.

Twelve animals (4 between 13-24 months and 8 over 36 months) exhibited signs of poisoning and died (morbidity and mortality 9.6% and lethality 100%). The first clinical sign observed was apathy, followed by progressive weight loss (Fig.2), reeling, bristling, and dry stools containing seeds of *S. rotundifolium* var. *villosum*. The period between the onset of first clinical signs and the animals' death ranged from 10 to 15 days. The affected herd was supplemented with hay and was removed from the pasture after the first deaths.

Necropsy and histopathological findings

Two bovines were necropsied and were found to have similar macroscopic lesions. Dehydration, cachexia, and pale oral and ocular mucosa were observed. The bovines' prestomachs contained a moderate number of seeds and peels of the fruit of *S. rotundifolium* var. *villosum* (Fig.3). In the liver, an evident lobular pattern and orange coloration were noted (Fig.4A). The gallbladder was distended and thick-walled, and the kidneys were enlarged with yellowish marked medullae. Our other findings included mild to moderate yellowing of the subcutaneous tissues, tracheal mucosa, abdominal free fluid, and cerebrospinal fluid.

Histologically, there was marked diffuse hepatocellular vacuolization in the liver (Fig.4B), marked centrilobular necrosis, mild and multifocal retention of bile pigment in the bile ducts. An aggregate of random foamy macrophages was observed with negative structures, like crystals, in their cytoplasms. In the kidneys, tubular epithelium exhibited mild multifocal vacuolar degeneration and rare moderate individual necrosis. Multifocally, the mucosal epithelium of the rumen, reticulum, omasum, and intestine exhibited mild vacuolar degeneration. In the mesenteric lymph nodes, we observed multifocal hemosiderosis.

Fig.2. Cattle exhibiting cachexia. Spontaneous poisoning by *Stryphnodendron rotundifolium* var. *villosum* in cattle.

Fig.3. Ruminal content of the cattle containing peels and seeds of the fruit of *Stryphnodendron rotundifolium* var. *villosum*. Spontaneous poisoning by *Stryphnodendron rotundifolium* var. *villosum* in cattle.

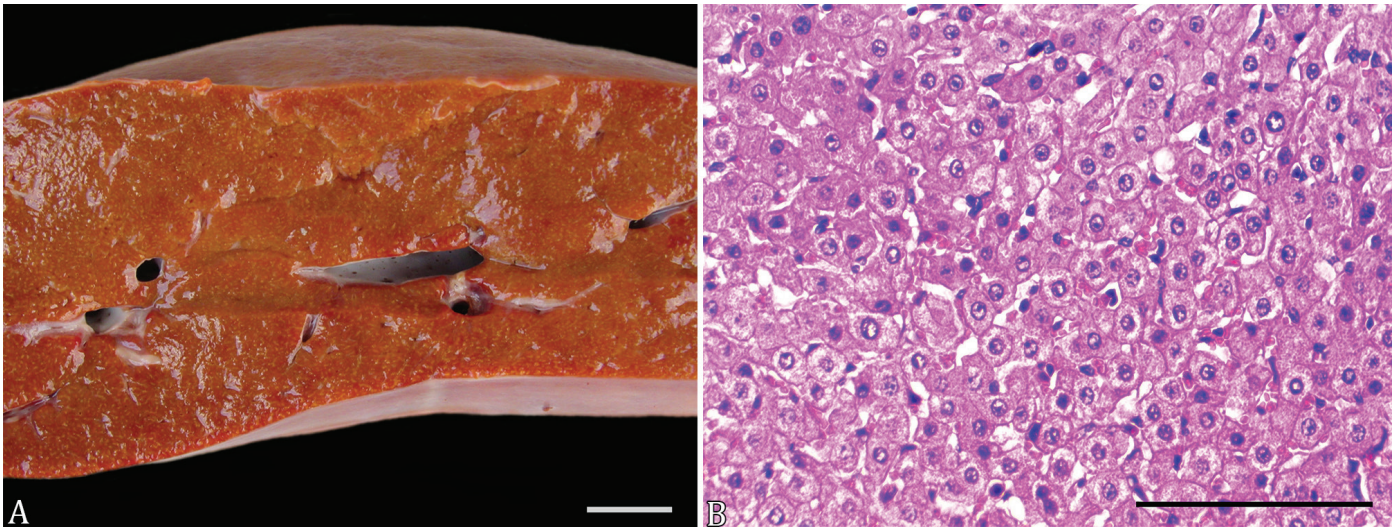

Fig.4. (**A**) Liver with a cut surface exhibiting orange coloration and evidence of a lobular pattern. Bar = 1cm. Spontaneous poisoning by *Stryphnodendron rotundifolium* var. *villosum* in cattle. (**B**) Hepatocytes with abundant, pale, and finely vacuolated cytoplasms. Degeneration, liver, spontaneous poisoning by *Stryphnodendron rotundifolium* var. *villosum* in cattle. HE, bar = 100µm.

DISCUSSION

The indications suggesting fruit intake by the cattle, epidemiological data, clinical findings, and pathological changes led to the diagnosis of poisoning by *Stryphnodendron rotundifolium* var. *villosum* (Benth.) Scalon.

Due to overlapping locations and morphological similarities among some species or among varieties of a single species of the genus *Stryphnodendron*, it can be difficult for field veterinarians to identify them. In these situations that are similar to those described in this study, it is recommended to send plant samples containing vegetative and reproductive structures for botanical analysis. In the specific case of the two varieties of *S. rotundifolium* Mart., the main morphological difference that can be observed to differentiate them is the presence of trichomes on the branches, petioles, rachis, second order petioles, rachioles, and folioles in *S. rotundifolium* var. *villosum* (Scalon 2007).

The farm where the outbreak occurred was already used for extensive cattle rearing in previous years; however, poisoning was never previously observed at the site. The extreme drought that occurred in the region may have caused forage shortages. It has been suggested that the drought situation that is associated with a high density in the population of *S. rotundifolium* var. *villosum* plants with ripe fruits may have caused the occurrence of intoxication. This type of situation has already been described in other studies on spontaneous poisoning by the *Stryphnodendron* spp. (Brito et al. 2001a, Ferreira et al. 2009).

In this study, spontaneous *S. rotundifolium* var. *villosum* poisoning in the affected cattle resulted in mild and nonspecific clinical signs that were characterized by apathy, progressive weight loss, reeling, bristling, and dry stools. The clinical picture described in previous studies that characterized the poisoning by *S. rotundifolium* Mart. var. *rotundifolium*, another variety of the same species, was not evidenced in this report; poisoning by this variety is mainly associated with digestive tract lesions (Brito et al. 2001a, Braga et al. 2018), abortions (Tokarnia et al. 1998), and severe lesions compatible with photosensitization (Camargo 1965).

Among all the necropsy findings, only jaundice (Camargo (1965) and an enlarged liver with yellowish coloration (Braga et al. 2018) were noted in the case of poisoning by *S. rotundifolium* var. *rotundifolium*. Gross lesions in the pre-stomach (papilla redness and adherence, mucosal detachment, congestion, gas tympanic fluid, and foul-smelling ruminal fluid), abomasum (congestion, erosions, and ulcerations), and intestines (congestion and Peyer's plaques evidenced) that were observed in experimentally poisoned cattle (Brito et al. 2001b, Braga et al. 2018) were also not evidenced in this study.

Marked histopathological findings that were observed in the liver of the examined cattle may potentially be related to jaundice owing to reduced bilirubin metabolism and bile duct obstruction (Werner 2011). Vacuolar degeneration and necrosis of the liver associated with photosensitization and jaundice have been described in cases of poisoning by *S. rotundifolium* var. *rotundifolium* (Camargo 1965). These histopathological findings were recently outlined by Braga et al. (2018) in an experimental study; however, their cases presented mild intensity and absence of photosensitization and jaundice. It is suggested that clinical signs variations may occur in animals due to differences in the clinical course of intoxication and extent of sun exposure.

The severe histopathological findings compatible with ruminal acidosis described in cattle experimentally poisoned by *S. rotundifolium* var. *rotundifolium* were not observed in this study. Acanthosis, spongiosis, parakeratosis, hyperkeratosis, necrosis, and hydropic-vacuolar degeneration have been reported (Brito et al. 2001b) to be present through the oral cavity to the omasum. The lesions in the intestine result in necrotizing enteritis (Braga et al. 2018), congestion, and bleeding (Brito et al. 2001b). These differences are potentially a result of the course of poisoning.

Due to the occasional presence of foamy macrophage aggregates in the liver, poisoning by *Brachiaria* spp. was considered as a differential diagnosis. However, this histopathological change can be observed in cases involving the chronic ingestion of this plant by cattle (Driemeier et al. 1998) and does not necessarily indicate intoxication (Araújo et al. 2017). Most cases of *Brachiaria* spp. poisoning occur in cattle with photosensitization and rarely occur in animals over two years of age (Souza et al. 2010); further, the clinical and epidemiological features differ from those observed in this study.

Among the toxic plants that grow in the Brazilian Central-West region and that can cause liver, kidney, and digestive tract lesions in cattle (Lemos & Lima 2017), it is necessary to consider poisoning by *S. fissuratum* E.M.O Martins as a differential diagnosis. The clinical-pathological condition observed in cattle poisoned by *S. fissuratum* (Ferreira et al. 2009) is partially similar to that observed in this study; however, it presents with severely intense findings that are compatible with those of renal and hepatic insufficiency and photosensitization cases (Lazaro et al. 2018). In addition to these differences, this plant was not identified in the region where the outbreak occurred.

So far, it is not clear which toxic principles are responsible for the clinical-pathological picture found in cattle poisoned by the *S. rotundifolium*. A recent experimental study on mice has revealed the hepatotoxicity of the ethanolic extract extracted from the bark of *S. rotundifolium* (unspecified variety) (Aquino et al. 2017). This extract contains numerous secondary metabolites including tannins, flavonoids, and alkaloids (Oliveira 2010). In addition, saponins have been cited as being potentially responsible for the poisoning due to *S. rotundifolium* var. *rotundifolium* (Brito et al. 2001a). However, there was no evidence to support that ethanolic extract and saponins were responsible for the lesions found in the cattle in this report.

The main prophylaxis measures that can be implemented to prevent cattle poisoning by the *Stryphnodendron* spp. include not leaving the animals in areas containing the plant in the dry season or eliminating all their grazing specimens (Tokarnia et al. 2012). As there is no evidence that the fruits of *S. rotundifolium* var. *villosum* are palatable to cattle, unlike in the case of *S. fissuratum* (Ferreira et al. 2009) and *S. rotundifolium* var. *rotundifolium* poisoning (Brito et al. 2001a), it is suggested that supplementation with roughage in the dry period may also be effective. This measure could prevent animals from seeking fruits in the months of low forage availability.

CONCLUSIONS

The drought situation that is associated with the presence of *Stryphnodendron rotundifolium* var. *villosum* with ripe fruits was a determining factor responsible for the poisoning.

Additionally, our results can aid in the differentiation between poisoning by *S. rotundifolium* var. *villosum* and poisoning by other toxic plants that are of interest to livestock.

Acknowledgements.- This research was partially supported by "Instituto Federal Goiano" scientific initiation scholarship program.

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

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PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(6):443-450, June 2020
DOI: 10.1590/1678-5150-PVB- 6561

Original Article
Livestock Diseases

Tremorgenic syndrome caused by *Ipomoea pes caprae* in cattle[1]

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ABSTRACT.- Graça F.A.S., d'Avila M.S., França T.N., Armién A.G., Rolim M.F., Caldas S.A., Santos A.M., Miranda I.C. & Peixoto P.V. 2020. **Tremorgenic syndrome caused by Ipomoea pes caprae in cattle.** *Pesquisa Veterinária Brasileira 40(6):443-450*. Departamento de Epidemiologia e Saúde Pública, Universidade Federal Rural do Rio de Janeiro, BR-465 Km 7, Seropédica, RJ 23890-000, Brazil. E-mail: mariana_davila@hotmail.com

Poisonous plants are a significant cause of death among adult cattle in Brazil. Plants that affect the central nervous system are widely spread throughout the Brazilian territory and comprise over 30 toxic species, including the genus *Ipomoea*, commonly associated with a lysosomal storage disease and a tremorgenic syndrome in livestock. We describe natural and experimental *Ipomoea pes caprae* poisoning in cattle from a herd in the Northside of Rio de Janeiro, Brazil. Affected cattle presented episodes of severe ataxia, abnormal posture followed by falling, muscular tremor, contraction, and spasticity, more prominent in the limbs, intensified by movement and forthcoming, and recumbence. Grossly, a substantial amount of leaves and petioles were found in the rumen. Histopathological examination showed degenerative neuronal changes, mostly in cerebellar Purkinje cells, which were confirmed with Bielschowsky silver. The characteristic clinical changes and mild histological lesion strongly suggested a tremorgenic syndrome. Lectin- immunohistochemistry evaluation reinforced this hypothesis; all lectins tested failed to react with affect neurons and Purkinje cells, which ruled out an underlying lysosomal storage disease. One calf given *I. pes caprae* leaves experimentally developed clinical signs similar to natural cases. On the 28th day of the experiment, the plant administration was suspended, and the calf recovered within four days. *I. pes caprae's* spontaneous tremorgenic syndrome in cattle is conditioned to exclusive feeding for several months. We were able to experimentally reproduce toxic clinical signs 12 days following the ingestion.

INDEX TERMS: Tremorgenic syndrome, *Ipomoea pes caprae*, cattle.

RESUMO.- [**Síndrome tremorgênica causada pela ingestão de *Ipomoea pes caprae* em bovinos.**] A intoxicação por plantas tóxicas está entre as três causas de morte mais importantes em bovinos adultos no Brasil. O grupo das plantas que causam alterações neurológicas, muito bem representada no país, encerra mais de trinta espécies tóxicas, entre as quais do gênero *Ipomoea*, amplamente distribuídas no território brasileiro. As plantas tóxicas desse gênero podem causar doenças do armazenamento ou síndrome tremorgênica. Descrevem-se a intoxicação natural e reprodução experimental por *Ipomoea*

[1] Received on January 30, 2020.
Accepted for publication on March 16, 2020.
Doctoral research with financial support from CAPES.

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pes caprae em bovinos, verificada no norte do Estado do Rio de Janeiro. Foram observados episódios de intensa ataxia locomotora, postura anormal seguida de queda, incapacidade de levantar-se, tremores, contrações, espasticidades musculares nos membros, intensificados após estimulação ou a simples aproximação e decúbito. Nos bovinos afetados há mais de 6 meses, os sinais clínicos tornavam-se permanentes. À necropsia havia apenas significativa quantidade de folhas e pecíolos da planta no rúmen. O estudo histopatológico evidenciou lesões neuronais degenerativas principalmente nos neurônios de Purkinje. A impregnação argêntica pela técnica de Bielschowsky ratificou esses achados microscópicos. As lesões histológicas sutis associadas ao quadro clínico indicam que trata-se de intoxicação tremorgênica. O fato de não haver nenhum armazenamento intracitoplasmático, confirmado pelo resultado do estudo lectino-histoquímico (não houve afinidade das lectinas Con-A, WGA e sWGA e de outras lectinas empregadas aos neurônios de Purkinje e outros neurônios afetados), é suficiente para descartar a possibilidade de tratar-se de doença do armazenamento. No bezerro intoxicado experimentalmente verificaram-se sinais clínicos semelhantes, entretanto, com a interrupção do fornecimento da planta no 28º dia, os sinais clínicos desapareceram após quatro dias. *I. pes caprae* causa síndrome tremorgênica espontânea em bovinos, quando ingerida como alimentação exclusiva durante períodos prolongados (muitos meses). Experimentalmente, os primeiros sinais clínicos da intoxicação foram reproduzidos após 12 dias de ingestão da planta.

TERMOS DE INDEXAÇÃO: Síndrome tremorgênica, *Ipomea pes caprae*, bovinos.

INTRODUCTION

According to data from diagnostic laboratories in different regions, between 7.4% and 15.83% of cattle deaths in Brazil are caused by toxic plants (Riet-Correa & Medeiros 2001, Pedroso et al. 2007, Rissi et al. 2007, Casagrande et al. 2008, Assis et al. 2010). Toxic plants of agricultural interest that cause neurological changes can be divided into five distinct groups: plants that cause storage disease; plants that cause tremorgenic syndrome; plants that cause lesions located in the central nervous system (CNS); plants that contain thiaminase and with other neurological actions (Tokarnia et al. 2012). This last group includes more than thirty species of toxic plants - including the genus *Ipomoea*, widely distributed in the Brazilian territory. Some of them are capable of inducing storage diseases, while others determine tremorgenic syndrome (Tokarnia et al. 2012).

This study aimed to describe the epidemiological and clinical-pathological aspects of spontaneous poisoning by *Ipomoea pes caprae*, a plant widely distributed on the Brazilian coast (Tokarnia et al. 2012), in cattle in the north of the State of Rio de Janeiro.

MATERIALS AND METHODS

Natural poisoning. We made six visits (4 visits in 2013 and 2 visits in 2014) to the property where cattle with neurological signs were observed. The property is located on one of the islands of the delta (Ilha da Convivência, located in the municipality of São Francisco do Itabapoana/RJ) at the meeting of the Paraíba do Sul river with the sea, latitude 21°36'0'' South and longitude 41°1'60'' West, northern Fluminense mesoregion.

Clinical follow-up. The clinical follow-up of cattle spontaneously poisoned by *Ipomoea pes caprae* occurred at the property of outbreak, except for Bovine 1, referred to the Veterinary Hospital of UENF. During the visits, we observed 12 cattle (Nelore crossbred) raised at the property. As the animals were raised on pasture, the evaluation was carried out in part by analyzing their behavior in the field, mainly the gait pattern and the presence of tremors.

The complete neurological exam (Dirksen et al. 1993), was only performed in three cattle with more evident signs: a heifer (Bovine 2), a bull (Bovine 3), and a wither (Bovine 4) crossbred Nelore. These were examined and monitored periodically, more carefully, from the first visit to the property until death. The neurological clinical examination protocol included assessment of the level of consciousness, behavior, posture, cranial nerve pairs, fundus examination, locomotion pattern, cervical-facial reflex and cutaneous muscle, muscle mass, sweating, evaluation of the forelimbs and hindquarters (hooves, proprioception, tone, sensitivity, reflexes), tail and anus (tone, reflexes, and sensitivity).

Necropsy and histopathology. Necropsy of Bovine 2, 3, and 4 was performed at the property immediately after death. Fragments of liver, gallbladder, kidneys, spleen, skin, superficial lymph nodes, in addition to salivary glands, pancreas, adrenal, bladder, small and large intestines, rumen, reticulum, omasum, abomasum, lungs, heart, testis or ovary, brain, spinal cord, costochondral junction, thyroid, pituitary, eyeball, and muscles were collected. Two of the animals (Bovine 2 and 4) were euthanized by sedation with 2% xylazine hydrochloride intramuscularly at a dose of 0.2mg/kg, followed by 50ml of 2% lidocaine hydrochloride in the atlantooccipital space. The bull (Bovine 3) drowned after a fall (due to the tremorgenic syndrome) near a stream. The necropsy of this bovine was also performed immediately after death. Bovine 1 was not necropsied, the owner chose to take it back to the property to try to recover it, but the animal died.

The collected fragments were fixed in 10% buffered formalin, except for the CNS, which was fixed in 20% buffered formalin. The samples were fixed immediately, except for muscle fragments, fixed three hours after the bovine's death. The fragments were processed routinely, the sections were stained with hematoxylin and eosin (HE), and the slides examined under an optical microscope and photographed. Silver impregnation was also performed (Bielschowsky staining).

Lectin-histochemistry. Histological sections of the central nervous system of Bovine 2, 3, and 4 were dewaxed, hydrated, and incubated in 3% hydrogen peroxide in two 15-minute steps (dilution of the peroxide at the time of the procedure) to block endogenous peroxidases. After washing the slides for two minutes with phosphate buffer (PBS), they were submerged in citrate buffer (pH 6.0) for antigenic recovery for 15 minutes in a water bath at 98 °C. After this procedure, they were cooled for 15 minutes at room temperature and washed with distilled water. The blocking of non-specific reactions was performed with 5% skimmed milk (Molico® - Indústria Brasileira). The sections were incubated "overnight" with lectins (Vector Laboratories, Burlingame, California, 94010, USA) at a dilution of 5µg/mL (except Con-A lectins with a dilution of 0.5µg/mL and RCA µg/mL with dilution 1.0) in PBS. We used the lectins *Canavalia ensiformis* agglutinin (Con A; ad-Man; ad-Glc-specific), *Dolichos biflorus* agglutinin (DBA; ad-Gal-Nac-specific), *Glycine Max* agglutinin (SBA; ad-GalNac; b-Gal- specific), *Arachis hypogaea* agglutinin (PNA; bd-Gal / (1-3) GalNAc-specific), *Ricinus communis* agglutinin-I (RCA - I; bd-Gal-specific), Ulex europaeus

agglutinin-I (UEA-1; aL-Fuc-specific), *Triticum vulgaris* agglutinin (WGA; ad-GlcNAc/NeuNAc-specific, Succinyl-WGA (sWGA), *Griffonia* (Bandeiraea) *simplicifolia* (GSL), *Sophora japonica* (SJA), *Pisum sativum* (PSA), *Phaseolus vulgaris* (PHA-L and PHA-E), *Lens culinaris* or *L. esculenta* (LCA) (Lectin Kit Biotinylated BK 1000 and 2000, Vector Laboratories Inc., Burlingame/CA, USA), subsequently incubated with the streptavidin-peroxidase complex (red drop only - Vector Laboratories Inc.) for 20 minutes. All sections were counterstained with Harris' hematoxylin and evaluated under an optical microscope. To compare the marking patterns, we used sections of the brain of a sheep affected by glycoproteinosis associated with the ingestion of *Sida carpinifolia* and a bovine poisoned by *Ipomoea asarifolia*, a plant that determines tremorgenic syndrome, and a healthy bovine.

Experimental poisoning. The experiment, submitted and approved by the Animal Ethics and Experimentation Commission (CEUA protocol 222), was conducted at the "Setor de Clínica Médica de Grandes Animais", UENF. In the experimental study, were used a male calf (Bovine 5), crossbred Girolando, weighing 110kg live weight, without alterations to the clinical examination, examined for worms and treated against ectoparasites 15 days before the start of the experiment (ivermectin at a dose of 0.2mg/kg). At first, their food consisted of roughage at will, *Pennisetum purpureum* (elephant grass) chopped, *ad libitum*, and 1kg of concentrate divided into two portions a day. The supply of *I. pes caprae* was initiated in the amount of 20 to 30g per kg/PV day, and supplementation with concentrate was maintained. The plant was collected at the site of the poisoning outbreak every four days and kept under refrigeration at 2 to 8 °C. Bovine 5 spontaneously ingested the plant in the lame.

Bovine 5 was observed every six hours, and the general physical and neurological examination performed every 12 hours. After the onset of neurological signs, the administration of *I. pes caprae* was suspended to verify the possibility of eventual recovery. The complete neurological examination was also performed (Dirksen et al. 1993). Blood samples were collected by puncture of the jugular vein and, at the "Laboratório de Patologia Clínica" of UENF, a complete blood count (12th and 20th days after the onset of clinical signs), platelet counts, biochemical profile (glucose, urea, creatinine, GGT evaluation), ALT, AST) and feces were also collected for coproparasitological examination.

Botanical identification of the plant. Samples of the plant (Fig.1) collected at the property were placed between paper sheets and pressed for 24 hours, with a subsequent change of papers and repetition of the process for 14 days. The obtained exsiccates were sent to the "Departamento de Botânica" of the "Instituto de Biologia", UENF, for taxonomic identification.

RESULTS

Botanical identification of the plant

The samples were identified as *Ipomoea pes caprae*.

Clinical signs of animals naturally poisoned

Bovine 1 was taken to the veterinary hospital at UENF in January 2013, presenting tremors, inability to stay in season, marked decrease in muscle tone, marked thirst, and lack of appetite. The response to stimuli was verified, although the ability to react was greatly diminished.

During the first visit to the island (January 10, 2013), Bovine 2 showed clinical signs characterized by head tremors, uncoordinated walking, decubitus, and paralysis. Tremors and incoordination were exacerbated when approaching and threatening movements were carried out.

In the following three visits (June 5, July 3 and September 17, 2013), Bovine 2 showed worsening of clinical signs and Bovine 3 showed slight tremors in the head region but did not present the tremorgenic crisis when stimulated. In one of the crises, Bovine 3 was unable to get up (Fig.2) and ended up drowning; necropsy was performed immediately.

In two visits (November 27 and December 11, 2014), Bovine 4 was identified with discrete neurological signs; according to the owner, he would have stayed 15 days on the island with only *I. pes caprae* as a food source. Also, Bovine 4 initially would have been without food for three days, before starting to ingest *I. pes caprae*.

In addition to those mentioned so far, other sick cattle also showed tremorgenic signs, some with episodes lasting an average of 20 minutes and subsequent partial recovery with no new stimulus. At this stage, the main findings were acute locomotor ataxia with shaking of the head, broad base (abduction mainly of the pelvic limbs), tremors in the thoracic and pelvic limbs constantly exacerbated on approach. After the intensification of stress, there were falls, inability to stand, flexed

Fig.1. *Ipomoea pes caprae* flower, Ilha da Convivência, Municipality of São Francisco de Itabapoana/RJ, Brazil.

Fig.2. Natural poisoning by *Ipomoea pes caprae*. Attempt to get up only with thoracic limbs, Bovine 3.

limbs. Atypically, attempts to get up were made on the thoracic limbs (Bovine 3, Fig.2). There were no significant variations in temperature, heart rate, and respiratory rate. The evolution of the disease was chronic, and deaths occurred due to accidents.

Macroscopic and microscopic findings

The necropsy of Bovines 2, 3, and 4 revealed only a significant amount of *I. pes caprae* leaves and petioles in the rumen content.

The histopathological study of the CNS showed chromatolysis of Purkinje cells in the cerebellum (Fig.3) with axonal spheroids in the granular layer, degenerative changes in Golgi neurons (Fig.4), proliferation and astrocytic edema in white matter. Additionally, in some neurons of the gray matter of the cerebral cortex, slight regressive neuronal changes, in the form of swelling, vacuolization, and chromatolysis with evolution to lysis accompanied by astrocyte proliferation, astrocytic edema and perineuronal proliferation of glial cells. Similar, but milder, we observed changes in several areas of the brain's base and the gray matter in the spinal cord. In the white matter, regardless of the location, there was mild astrocytic and interstitial edema. The histochemical technique by silver impregnation using the Bielschowsky method allowed better visualization of axonal spheroids of Purkinje neurons (Fig.5).

Experimental poisoning

Bovine 5 ingested the plant spontaneously in the trough with variations in the daily amount of 30g/kg of live weight and showed a predilection for the leaves, but ingested the stem mixed with them. Appetite and dipsia were maintained throughout the experiment.

The first nervous sign was the constant head tremor, observed from the 12th day after the plant started to be supplied. From the 14th day on, there was an increase in tremor, slight ataxia, and abnormal posture. On the 18th day onwards, there was intense sialorrhea, dehydration, and capillary refill time of three seconds followed by mild enophthalmos. The plant supply was maintained until the 28th day, and the symptoms disappeared four days after the suspension.

Regarding laboratory tests, the only change found was polycythemia characterized by an increase in hematocrit between 42 and 44%. The other indexes evaluated were regular. Bovine 5 recovered and was not necropsied.

Lectin-histochemistry

There was no affinity between Con-A, WGA, and sWGA lectins for Purkinje cells and Golgi neurons with degenerative changes (Fig.6), nor for other neurons, in cattle poisoned by *I. pes caprae* and *I. asarifolia* (control used for comparison). With these same lectins, there was a specific marking of the interior and the edge of the intracytoplasmic vacuoles on the Purkinje and Golgi neurons of the control poisoned by *Sida carpinifolia* (Fig.6). The lectins PSA, LCA, PHA-E, despite having lighter labeling, were not specific for the Purkinje cell vacuoles in the positive control (bovine poisoned by *S. carpinifolia*).

DISCUSSION

The cattle poisoned by *Ipomoea pes caprae* presented tremors and motor incoordination like those observed in tremorgenic poisonings of varied etiology. Considering that on the island where the *I. pes caprae* outbreak occurred was the only food source for the affected animals and that the disease was reproduced experimentally, it can be concluded that the cause of the tremorgenic disease was poisoning by *I. pes caprae*.

Similar clinical signs were also observed, in varying degrees and intensity, in cattle poisoned by different agents, including *Ipomoea asarifolia* (Döbereiner et al. 1960), *Phalaris angusta* (Gava et al. 1999, Sousa & Irigoyen 1999), *Marsdenia* spp. (Riet-Correa et al. 2004, Silva et al. 2006, Pessoa et al. 2011, Neto et al. 2013), *Ipomoea carnea* subsp. *fistulosa* (Balogh et al. 1999, Antoniassi et al. 2007, Armién et al. 2007, Oliveira et al. 2009, Tokarnia et al. 2012), *Sida carpinifolia* (Furlan et al. 2008, Pedroso et al. 2010), *Solanum fastigiatum* var. *fastigiatum* (Medeiros et al. 2004, Guaraná et al. 2011, Rego et al. 2012), *Claviceps paspali* (Riet-Correa et al. 1983a), *Neotyphodium lolii* (Munday & Mason 1967, Odriozola et al. 1993, Miyazaki et al. 2001, 2007), *Cynodon dactylon* (Odriozola et al. 2001, Rivero et al. 2011) and *Aspergillus clavatus* (Loretti et al.

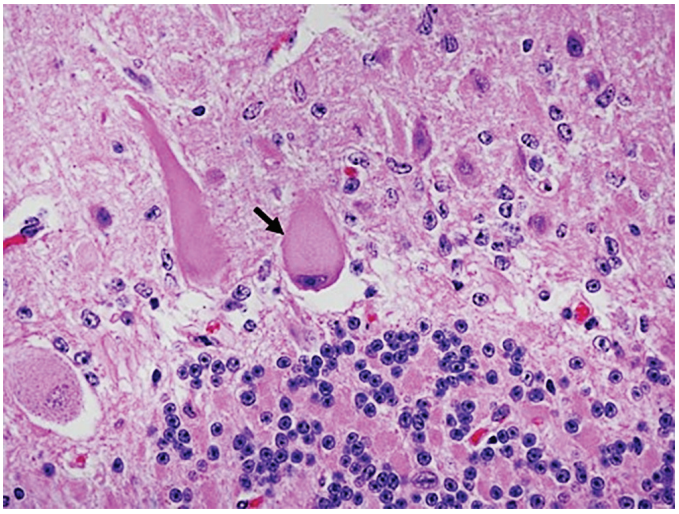

Fig.3. Poisoning by *Ipomoea pes caprae*. Purkinje neuron degeneration (eosinophilic cytoplasm and dissolution of the Nissl substance) (arrow), cerebellum, Bovine 3. HE, obj.63x.

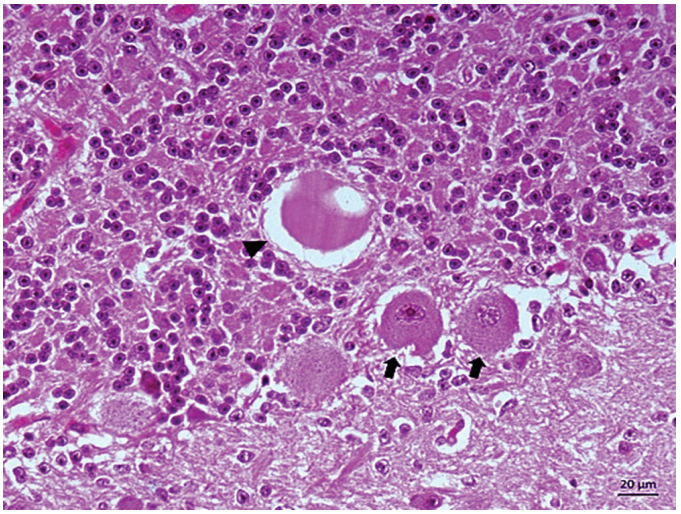

Fig.4. Poisoning by *Ipomoea pes caprae*. Purkinje neurons in chromatolysis (arrows) and Golgi neuron with vacuolated cytoplasm (arrowhead), cerebellum, Bovine 2. HE, obj.40x.

2003, McKenzie et al. 2004, Sabater-Vilar et al. 2004, Bezerra et al. 2009, Oliveira 2016). Tremors have also been described in cases of hepatic encephalopathy (Méndez & Riet-Correa 2001, Karam et al. 2004, Lucena et al. 2010), rabies (Acha & Szyfres 2003), polioencephalomalacia (Lemos & Nakazato 2001, Sant'ana et 2009a, 2009b) and meningoencephalitis due to BoHV-5 (Salvador et al. 1998, Colodel et al. 2002, Elias et al. 2004, Riet-Correa et al. 2006, Rissi et al. 2006). However, there was no sign of the agents mentioned above, nor was there any anatomopathological evidence of indicative or compatible lesions caused by them.

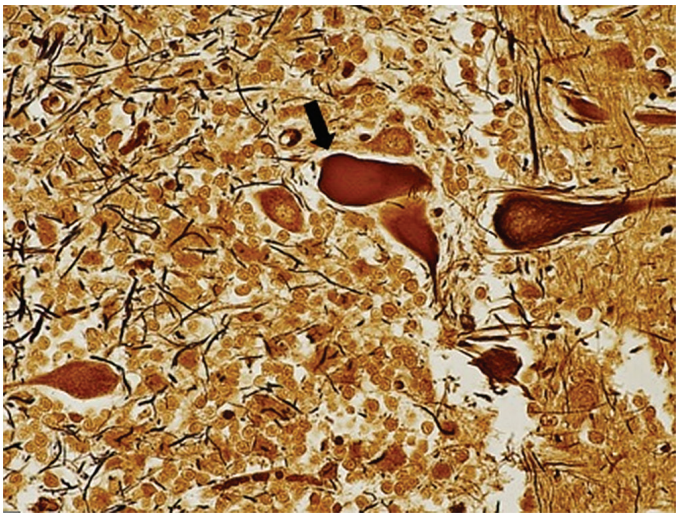

Fig.5. Poisoning by *Ipomoea pes caprae.* Degenerated Purkinje neuron with dilated axon. Axonal spheroid (torpedo), cerebellum, Bovine 3. Bielschowsky, obj.40x.

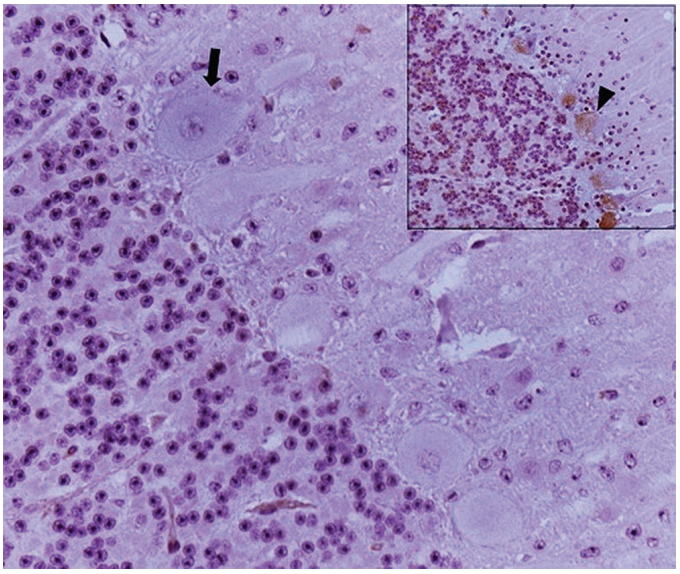

Fig.6. Poisoning by *Ipomoea pes caprae*. Purkinje neurons without lectin-histochemical marking (arrow) using sWGA lectin, cerebellum, Bovine 2. Insert: positive control, bovine poisoned by *Sida carpinifolia* with intracytoplasmic vacuoles with storage and marked edges (arrowhead) in Purkinje neurons. Lectin-histochemistry, obj.40x.

One of the fundamental points to be determined in this report was whether *I. pes caprae* causes storage disease or a condition compatible with that described in so-called tremorgenic syndromes. The absence of storage, confirmed by histopathological analysis and lectin-histochemical evaluation, indicates that *I. pes caprae* is responsible for a tremorgenic picture similar to those described in cases of *Ipomoea asarifolia* (Döbereiner et al. 1960, Riet-Correa et al. 2003, Araújo et al. 2004, Barbosa et al. 2005, Barbosa et al. 2012).

Preliminary studies, probably of short duration and with low doses, carried out in the 1960s (Tokarnia 2013) indicated that *I. pes caprae* was not toxic to cattle. However, this plant, when ingested as an exclusive food for prolonged periods (many months or even a few years), is capable of poisoning cattle, as demonstrated for buffaloes and other ruminants poisoned by *I. asarifolia* (Barbosa et al. 2005). Indole diterpenes were identified in the *I. asarifolia* and *Ipomoea muelleri* species, and associated with the tremorgenic picture in cattle poisoned by these plants (Gardner et al. 2018). This association may be the active ingredient responsible for the symptoms observed in animals poisoned by *I. pes caprae*.

As with poisoning by some other plants that affect the central nervous system (Riet-Correa et al. 1983b, Rivero et al. 2011), deaths from trauma and drowning can occur, as observed in the case of Bovine 3 that occurred during the outbreak of the tremorgenic crisis.

In the experimental case of poisoning by *I. pes caprae*, the onset of clinical signs was later than the two to four days seen in experimental poisoning in cattle by *I. asarifolia* (Barbosa et al. 2005). The daily doses ingested by the bovine submitted to experimental poisoning were like those used by Barbosa et al. (2005) in experiments with *I. asarifolia*.

The silver impregnation by the Bielschowsky method is widely used to evidence neuronal processes, including dendrites, axons, and neurofibrils (Minbay et al. 2001). In Bovine 4, this staining allowed the visualization of axonal spheroids of Purkinje cells (Fig.5). This result proves the degenerative lesions of Purkinje cells of animals poisoned by *I. pes caprae*. The Purkinje neuron is considered the "central functional unit" of the cerebellar cortex, as it receives afferent stimuli directly or indirectly and is solely responsible for the efferent stimulus (Mullen et al. 1976, Bauer-Moffett & Altman 1977). It also forms inhibitory synapses with deep cerebellar nuclei and vestibular nuclei (Damiani et al. 2016). Lesions in this cell could justify the signs described in animals poisoned by *I. pes caprae*. Besides, changes in gait have already been described in other conditions that lead to lesions in Purkinje cells (Mullen et al. 1976, Thomas et al. 1998). Some Golgi neurons were also injured, these neurons have an essential inhibitory function (Watanabe et al. 1998), and this alteration may have contributed to the pathogenesis of the tremors.

The lectin-histochemical evaluation was used, above all, to rule out the possibility of oligosaccharide storage being involved in the pathogenesis of poisoning, as occurs in poisoning by other species of *Ipomoea*, except for *I. asarifolia*. The lectins Con-A, WGA and sWGA that, in general, mark the accumulation of oligosaccharides in animals poisoned by *S. carpinifolia* (Driemeier et al. 2000, Colodel et al. 2002, Loretti et al. 2003, Pedroso et al. 2010) and *I. carnea* (Armién et al. 2007), did not mark Purkinje neurons and other neurons affected in the disease caused by *I. pes caprae*.

CONCLUSION

Ipomoea pes caprae causes tremorgenic syndrome in cattle when ingested as exclusive food for prolonged periods.

Acknowledgments.- The authors thank Professor Carlos Tokarnia (*in memorian*) for his collaboration, the "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES) and the "Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro" (FAPERJ) for the financial support (protocol 645, process No. E26/010.2595/14) for the financial support, and the laboratory technician Letícia Pinto for the assistance in the elaboration of the histological slides.

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

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PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(6):451-465, June 2020
DOI: 10.1590/1678-5150-PVB-6482

Original Article
Small Animal Diseases

Differential diagnoses in 83 dogs with icterus[1]

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ABSTRACT.- Andrade M.C., Oliveira L.B., Santos A.F., Moreira M.V.L., Pierezan F. & Ecco R. 2020. **Differential diagnoses in 83 dogs with icterus.** *Pesquisa Veterinária Brasileira 40(6):451-465.* Setor de Patologia, Departamento de Clínica e Cirurgia, Escola e Veterinária, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Belo Horizonte, MG 31270-901, Brazil. E-mail: ecco@vet.ufmg.br

Icterus (jaundice) is a yellowish pigmentation resulting from the depositing of bilirubin in tissues due to its high plasmatic concentration. The pathogenesis of icterus includes metabolic changes or obstructed bilirubin excretion and it is classified as pre-hepatic, hepatic and post-hepatic. This study aimed to evaluate and classify different causes of icterus in dogs during *post mortem* examination. These dogs were examined from 2014 to 2017, using macroscopic and histologic exams as well as ancillary tests. Eighty-three dogs were examined macroscopically and microscopically. They were separated into groups of icterus types: 24 (28.9%) dogs had pre-hepatic icterus, 45 (54.2%) had hepatic, 13 (15.7%) pre-hepatic and hepatic and one (1.2%) had post-hepatic icterus. Many factors were identified as a cause of icterus, including infectious agents (51/83), neoplasms (13/83), hepatic degeneration (11/83), chronic hepatic diseases (6/83), and obstructive causes (1/87). Among the infectious causes, leptospirosis, ehrlichiosis and disorders suggestive of septicemia were diagnosed. Neoplasms associated with icterus were cholangiocarcinoma, hemangiosarcoma and lymphoma. Other causes of icterus included degenerative diseases, such as lipidosis and glycogen degeneration. Hepatic fibrosis (cirrhosis) as a chronic disease and cholelithiasis also produced icterus. PCR was performed to confirm leptospirosis and ehrlichiosis. Samples of total DNA were used to amplify a fragment of a gene from *Leptospira interrogans* and *Ehrlichia canis*. In some dogs, co-infection of these agents was detected. The classification and identification of icterus etiologies in dogs is very important due to the number of diseases with this alteration, where *ante mortem* diagnosis is not always easily performed when some of these conditions are present.

INDEX TERMS: Diagnosis, dogs, icterus, jaundice, bilirubin, histopathology, PCR, serology, leptospirosis, ehrlichiosis, neoplasm, septicemia.

RESUMO.- [**Diagnósticos diferenciais em 83 cães com icterícia.**] Icterícia é a pigmentação amarelada decorrente da deposição de bilirrubina em tecidos devido à elevada concentração plasmática. A patogênese da icterícia inclui alterações no metabolismo ou na excreção de bilirrubina, sendo classificada em pré-hepática, hepática ou pós-hepática. O objetivo desse estudo foi identificar, avaliar e classificar as causas de icterícia em cães necropsiados de 2014 a 2017, associando as lesões macroscópicas, histológicas e exames complementares. Foram avaliados macro- e microscopicamente 83 cães com diferentes intensidades de icterícia. Os cães foram separados em grupos de acordo com o tipo de icterícia: 24 (28,9%) cães com icterícia pré-hepática, 45 (54,2%) cães com icterícia hepática, 13 (15,7%) com icterícia pré-hepática e hepática e um (1,2%) com icterícia pós-hepática. Foram identificadas várias etiologias associadas à icterícia, dentre elas pode-se destacar, agentes infecciosos (51/83), neoplasmas (13/83), processos degenerativos (11/83), crônicos (6/83) e obstrutivos (1/83). Dentre as causas infecciosas, destacam-se a leptospirose, a erliquiose e as lesões sugestivas de septicemia. Entre os neoplasmas associados com icterícia destacaram-se

[1] Received on January 22, 2020.
Accepted for publication on February 7, 2020.
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o colangiocarcinoma, hemangiossarcoma e linfoma. Outras causas de icterícia incluiriam os processos degenerativos como as degenerações gordurosa e glicogênica. Fibrose hepática (cirrose) e colelitíase foram também diagnosticados como causa de icterícia. A PCR foi utilizada para o diagnóstico confirmatório de leptospirose e erliquiose. Amostras de DNA total foram utilizadas para amplificar um fragmento dos genes de *Leptospira interrogans* e de *Ehrlichia canis.* Em alguns cães foi detectada co-infecção por estes agentes. A classificação e a identificação das causas de icterícia em cães são relevantes devido ao grande número de doenças que apresentam essa alteração, muitas vezes sem diagnóstico *ante mortem*.

TERMOS DE INDEXAÇÃO: Diagnóstico diferenciais, cães, icterícia, bilirrubina, caninos, histopatologia, PCR, sorologia, leptospirose, erliquiose, neoplasia, septicemia.

INTRODUCTION

Icterus refers to the yellow pigmentation of tissue caused by accumulated deposits of bilirubin. Bilirubin deposition is more evident in the mucous membranes and in tissues with highest proportion of elastin, such as the aorta and sclera. Icterus can be classified into pre-hepatic, hepatic and post-hepatic (Murdoch 1976, Beckingham & Ryder 2001).

Hyperbilirubinemia is characterized as unconjugated (indirect) or conjugated (direct) (Murdoch 1976). Unconjugated bilirubin (bilirubin bound to albumin) is formed during the metabolic degradation of hemoglobin as hemolysis. Unconjugated bilirubin is also created when there is insufficient bilirubin uptake or insufficient conjugation of bilirubin with glucuronic acid due to hepatocyte degeneration or necrosis (Thompson 1970, Beckingham & Ryder 2001). Conjugated bilirubin (bilirubin bound to glucuronic acid) can also be associated with liver diseases that decrease bilirubin uptake (e.g., hepatitis) or associated with reduced biliary tract drainage due to obstruction and/or compression of the bile canaliculi (Thompson 1970).

Prehepatic icterus is caused by increased bilirubin production due to hemolysis and may be caused by infectious agents such as *Babesia* spp., *Leptospira* sp., and *Rangelia vitalii* (Krauspenhar et al. 2003, Loretti & Barros 2005, Fighera et al. 2010) or immune-mediated diseases (Murdoch 1976). This excess of bilirubin overloads the liver's capacity to remove bilirubin from the plasma and secrete it into bile. Severe hemolysis may also result in hypoxia and consequently promote hepatic lesions, resulting in pre-hepatic and hepatic icterus (Cullen & Brown 2013). Hepatic icterus occurs due to insufficient bilirubin uptake as well as insufficient bilirubin conjugation and excretion. The etiology is related to acute or chronic severe hepatic disease, such as toxic liver diseases, neoplasms, liver fibrosis (cirrhosis) or infectious agents such as *Leptospira* sp. Post-hepatic icterus occurs in cases of obstruction and/or compression of bile ducts, resulting in reduced bile drainage. This leads to the accumulation of bilirubin in the blood (Murdoch 1976).

Icterus is a clinical finding common to several diseases. Therefore, the aim of this study is to classify the types of icterus and associate with the etiology, clinical, pathological and laboratory findings in 83 dogs.

MATERIALS AND METHODS

Samples. A prospective study was performed from June 2014 to June 2017 in the "Setor de Patologia" of the "Escola de Veterinária" of the "Universidade Federal de Minas Gerais" (UFMG). Eighty-three dogs with mild to marked icterus were examined. The data collected from these dogs, including medical history and clinical pathology exams, were obtained from the integrated veterinary hospital archive of UFMG. The reference values for the hemograms (45/83) and serum biochemical tests (43/83) performed in this study were interpreted according to Kaneko et al. (1997).

Serology anti-*Leptospira*. The microscopic agglutination test (MAT) was standardized and utilized according to Fields et al. (1965) for the following serovars: Icterohaemorragiae, Wolfi, Hardjo, Bratislava, Autumnalis, Canicola, Grippothyphosa, Pomona, Ballum, Bataviae, Copenhageni and Tarassovi. This test was performed on 13 out of 40 dogs suspected of leptospirosis. The results were interpreted according to WHO (2003) and Azócar-Aedo et al. (2017).

Gross and histopathology. The *post mortem* examinations were performed on all 83 dogs. The procedures in this study were performed in accordance with the recommendations by the Ethics Committee of the UFMG (Protocol 61/2017). Samples of the liver, kidneys, lymph nodes, spleen, bone marrow, lungs, heart and other organs that were found to have changes during necropsy were collected. The samples were sectioned, fixed in 10% neutral buffered formalin for 48 to 52 hours for histological evaluation. The tissues were dehydrated in increasing ethanol series, diaphanized in xylene and paraffin embedded to obtain sections of 4.0µm, stained by hematoxylin and eosin (HE) (Luna 1968) and examined using white light microscope.

Cytopathology was only performed with samples from dogs with clinical suspicion and/or macroscopic lesions suggestive of leishmaniasis or to rule out babesiosis. The smears were performed using bone marrow and spleen samples. A thin layer of tissue was spread on the glass microscopy slide, air-dried and stained with a quick stain (Diff Quick) for subsequent microscopic examination.

Polymerase chain reaction. For all dogs, samples of the liver, kidneys, lymph nodes, spleen and bone marrow were collected and stored at -20°C until PCR was performed to detect *Ehrlichia canis* and *Leptospira interrogans* DNA. PCR was only performed with samples from dogs with histologic lesions suggestive of these diseases. The total DNA extraction method was based on the use of sodium iodide and silica according to the method described by Vogelstein & Gillespie (1979). To amplify a fragment of the gene named p28 from *E. canis,* a set of primers: ECp28-F 5'-ATGAATTGCAAAAAAATTCTTATA-3' and ECp28-R 5'-TTAGAAGTTAAATCTTCCTCC-3' were used. These primers generated a product of 843 base pairs (bp) (Nakaghi et al. 2010). For positive control, DNA was extracted from a cell culture infected with *Ehrlichia* sp., provided by the parasitology laboratory of UFMG. To detect DNA of *L. interrogans,* the primer sets utilized were: LipL32-F 5'-CGCTTGTGGTGCTTTCGGTG-3' and LipL32-R 5'-GCGCTTGTCCTGGCTTTACG-3'. This reaction generated a product of 152 bp, according to the method described by Coutinho et al. (2014) with modifications. PCR conditions were 95°C for 5 min, followed by 30 cycles at 95°C for 30 seconds, annealing for 1 min at 52°C, extension at 72°C for 2 min and final extension at 72°C for 5 min. An inactivated bacterial sample of *L. interrogans* serovar Icterohaemoragiae were provided by the "Laboratório Nacional Agropecuário" (Lanagro-MG) as a positive control. As a negative control, the primers were used in conjunction with PCR Master Mix and 2µl of ultrapure water without DNA. To assess viability and quality of extracted DNA, all negative samples were subjected to PCR

to detect *β-actin* (endogenous control gene), using the previously described primers (Turchetti et al. 2015) with modifications. The following PCR conditions were used: 95°C for 10 min, 35 cycles at 95°C for 30 seconds, annealing temperature for 30 seconds at 60°C, extension at 72°C for 30 seconds and a final elongation at 72°C for 5 min. PCRs were performed using a final volume of 25µl (PCR Master Mix Promega, Madison/WI, USA) and 200-300ng of DNA template, in a thermocycler (Veriti® Thermal Cycler, Applied Biosystems, Inc., Foster City/CA, USA). The final product of each reaction was subjected to electrophoresis in 1.5% agarose gel stained with ethidium bromide along with a molecular weight marker of 100 bp (LowRanger100bp DNA Ladder Norgen®). This final product was visualized using an UV trans-illuminator.

RESULTS

From 2014 to 2017, 83 dogs that presented with icterus were necropsied. Forty-five dogs (54.2%) were male with an average age of eight years and two months. Their ages varied between seven months and 20 years. Thirty-eight dogs were female (45.8%), with an average age of seven years and eight months. Their ages varied between two months and 14 years. The most common breeds were mixed-breed (43.4%) followed by Poodles (7.2%) and Golden Retrievers (7.2%), Yorkshire Terriers (6.0%), German shepherds (4.8%) and various others (31.4%).

The type of icterus in the dogs of this study was evaluated and classified according to the diagnoses (Table 1). Twenty-four dogs had pre-hepatic icterus (28.9%), 45 (54.2%) had hepatic, 13 (15.7%) had pre-hepatic and hepatic and one (1.2%) had post-hepatic icterus. The classification of the types of icterus was based on macroscopic and histologic lesions. Dogs classified with pre-hepatic icterus had multicentric hemorrhages and/or hemoglobinuria. Dogs classified with hepatic icterus had degenerative, necrotic or compressive lesions in the hepatic parenchyma. Dogs with multiple hemorrhages and hepatocytes lesions were classified with pre-hepatic and hepatic icterus. Dogs with obstructed and/or compressed extrahepatic bile ducts were classified with post-hepatic icterus. Different diagnoses were obtained, including infectious etiologies (51/83), neoplastic diseases (13/83), degenerative diseases (11/83), chronic conditions (6/83), neoplastic associated with degenerative diseases (1/83) and biliary tract obstruction (1/83). Regarding the infectious diseases, the most frequent were leptospirosis (35/51), ehrlichiosis (7/51) or coinfectious by both agents (5/51). Septicemia was diagnosed in three dogs (3/51). One of these dogs had bacterial pancreatitis and hepatitis associated with ehrlichiosis. The neoplastic disease diagnoses were cholangiocarcinoma (5/13), hemangiosarcoma (4/13), pancreatic carcinoma (2/13) and lymphoma (3/13). The degenerative hepatic diseases included lipidosis (8/11) and glycogenic degeneration (3/11). One case of lipidosis accompanied with multicentric lymphoma was also diagnosed. Hepatic fibrosis was diagnosed in six dogs (6/6). Finally, one dog (1/83) was found with obstructive cholelithiasis. Cytopathological smear examinations from liver, spleen and bone marrow for *Babesia* sp. infection, performed during *post mortem* examination, were negative in these dogs. Similarly, no histological lesions indicative of *Rangelia vitalli* were found.

Hematology tests were performed on 45 dogs (Table 2). The majority of animals with icterus also had anemia. Most of these anemia cases were regenerative as they were associated with an increased number of reticulocytes.

Biochemical tests were performed on 43 dogs (Table 3). The values of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and

Table 1. Classification of icterus intensity and histopathologic diagnostic

Icterus % (n/total)	Histopathologic diagnosis	N	(%)
Pre-hepatic 30.1% (25/83)	Leptospirosis	11	45.8
	Ehrlichiosis	7	29.1
	Hemangiosarcoma	3	12.5
	Septicemia	2	8.3
	Septicemia and suggestive of ehrlichiosis	1	4.16
Total		24	100
Hepatic 53.0% (44/83)	Leptospirosis	15	33.34
	Lipidosis	8	17.78
	Hepatic fibrosis (cirrhosis)	6	13.34
	Cholangiocarcinoma	5	11.11
	Glycogenic degeneration	3	6.67
	Leptospirosis and ehrlichiosis	2	4.44
	Metastatic pancreatic carcinoma	2	4.44
	Lymphoma with hepatic metastases	2	4.44
	Lipidosis and multicentric lymphoma	1	2.22
	Bacterial hepatitis and pancreatitis	1	2.22
Total		45	100
Pre-hepatic and hepatic 15.7% (13/83)	Leptospirosis	9	69.2
	Leptospirosis and ehrlichiosis	3	23.1
	Hemangiosarcoma	1	7.7
Total		13	100
Post-hepatic 1.2% (1/83)	Cholelitiasis	1	100

gamaglutamiltransferase (GGT) were generally high when the icterus was classified as hepatic. In the majority of pre-hepatic icterus cases the ALP values were generally high. For animals with pre-hepatic and hepatic icterus, the AST and ALP values were generally increased. When the total protein values were low, this was mostly due to decreased albumin values. Creatinine and urea sera values were usually above the reference values, independent of icterus classification. Biochemical exams were not performed on the dog with post-hepatic icterus.

Leptospirosis

Laboratory diagnosis of leptospirosis was performed in 40 out of 83 dogs using histopathology in conjunction with serology and/or PCR. Macroscopically, mild to marked levels of icterus were found in the mucous membranes (Fig.1A), skin, subcutaneous and visceral adipose tissue, intimal layer vessels, serosae and joints. In some dogs, numerous multifocal petechial hemorrhages in the mucous membranes, subcutaneous and/or serosae were also present. Hemorrhages in the lungs (Fig.1B) were also seen in some dogs. The livers were mildly to moderately enlarged and red-yellow to red-green (Fig.1C). The kidneys were yellow-green to red-yellow with evident alterations especially in the medullar region (Fig.1D).

The histological lesions found were mild to marked multifocal or diffuse dissociation of hepatocytes (15/40), mild to marked randomly multifocal necrosis of hepatocytes (21/40) with mild to marked biliary stasis (40/40) (Fig.2A). There was also mild to moderate multifocal cholemic nephrosis (19/40), mild to moderate multifocal hemoglobinuric nephrosis (4/40) (Fig.2B and 2C). In two dogs with hemoglobinuric nephrosis, Prussian blue stains were positive, revealing iron inside the cytoplasm of the epithelium and the lumen of the tubules (Fig.2D). Mild to marked multifocal membranous glomerulonephropathy (27/40), mild multifocal glomerulosclerosis (6/40), and moderate multifocal proteinuria (8/40) were also seen. In the spleen and lymph nodes there was lymphoid depletion with moderate to marked increase differentiation of plasm cells (plasmacytosis) (12/40) and mild to moderate multifocal erythrophagocytosis (15/40). In addition, there were macrophages infected with *Leishmania* spp. amastigotes in the bone marrow (11/40).

From 40 dogs diagnosed with leptospirosis, serology to detect antibodies anti-*Leptospira* spp. were performed in 13 dogs. Ten were reagents and three were negative. Sorovars reagents were the following: Icterohaemorrhagiae (6/10), followed by Bratislava (3/10), Autumnalis (3/10), Canicola (3/10), Grippothyphosa (2/10), Pomona (1/10), Andamana (1/10), Ballum (1/10), Bataviae (1/10), Copenhageni (1/10), Pyrogenes (1/10) and Tarassovi (1/10). There were five (5/13) dogs with multiple sorovars. The antibody titers for these serovars ranged from 1:100 to 1:800. The highest titer (1:800)

Table 2. Hematological results from 45 dogs with icterus

Hematological exams		Pre-hepatic (n=11)	Hepatic (n=29)	Pre-hepatic and hepatic (n=5)
Anemia		81.8% (9/11)	79.3% (23/29)	60.0% (3/5)
Increased	Leucocytes	54.5% (6/11)	72.4% (21/29)	80.0% (4/5)
	Bastonets	36.4% (4/11)	53.6% (15/28)	75.0% (3/4)
	Segmented cells	54.5% (6/11)	75.8% (22/29)	80.0% (4/5)
	Monocytes	36.4% (4/11)	75.9% (17/29)	40.0% (2/5)
	Platelets	0	6.9% (2/29)	0
Decreased	Leucocytes	18.2% (2/11)	3.4% (1/29)	0
	Segmented cells	18.2% (2/11)	0	20.0% (1/5)
	Lymphocytes	72.2% (8/11)	48.3% (14/29)	80.0% (4/5)
	Monocytes	18.2% (2/11)	3.4% (1/29)	40.0% (2/5)
	Platelets	63.6% (7/11)	75.9% (22/29)	100.0% (5/5)

Table 3. Serum biochemical results from 43 dogs with icterus

Biochemical profile		Pre-hepatic (n=13)	Hepatic (n=25)	Pre-hepatic and hepatic (n=5)
Increased	Urea	61.5% (8/13)	76.0% (19/25)	100.0% (5/5)
	Creatinine	53.8% (7/13)	63.6% (14/22)	40.0% (2/5)
	ALT	30.8% (4/13)	60.0% (15/25)	40.0% (2/5)
	AST	23.1% (3/13)	72.0% (18/25)	60.0% (3/5)
	ALP	75.0% (9/12)	84.0% (21/25)	60.0% (3/5)
	GGT	23.1% (3/13)	56.0 (14/25)	25.0% (1/4)
	Amilase	23.1% (3/13)	44.0% (11/25)	50.0% (2/4)
	Total protein	7.7 (1/13)	4.2% (1/24)	40.0% (2/5)
	Globulin	46.2% (6/13)	20.8% (5/24)	40.0% (2/5)
Decreased	Amilase	7.7% (1/13)	8.0% (2/25)	0
	Total protein	46.2% (6/13)	41.7% (10/24)	40.0% (2/5)
	Albumin	92.3% (12/13)	79.2% (19/24)	100.0% (5/5)
	Globulin	0	25.0% (6/24)	20.0% (1/5)

ALT = alanine aminotransferase, AST = aspartate aminotransferase, ALP = alkaline phosphatase, GGT = gamaaglutamyltransferase.

was detected for serovars Icterohaemorrhagiae, Bratislava, Autumnalis, Bataviae and Pomona. Considering the clinical history (vaccinated or not vaccinated dogs), titers above 1:200 were considered positive results (Azócar-Aedo et al. 2017).

PCR for *Leptospira interrogans* resulted positive in 37/40 dogs, for one or more organs. In dogs that tested positive, DNA amplification was performed on samples from the liver (24/40), kidney (16/39) and spleen (20/39). One of the dogs that had a negative PCR result for *L. interrogans* (1/40), had a positive serological test for Bratislava, Ballum and Pyrogenes serovars. In five of these dogs there were lesions suggestive of coinfection by *Ehrlichia* sp., with four testing positive for *E. canis* using PCR.

In the hematological tests (21/40), anemia was diagnosed in 17 dogs (17/21). The types of anemia were normocytic normochromic (11/17), macrocytic normochromic (5/17) and normocytic hypochromic (1/17). There was also moderate to marked leukocytosis (16/21) by neutrophilia (17/21) with swift to the left (12/21) and monocytosis (12/21). Lymphopenia (11/21) and mild to severe thrombocytopenia (17/21) were also detected. In the biochemical exams, the most relevant changes were a mild to accentuated increase in the sera values of urea (15/18), creatinine (12/17), ALT (7/18), AST (9/18), ALP (14/17) and GGT (6/18). A mild to accentuated increase in the sera values of amylase was detected in nine (9/18) dogs. In addition, the total protein values were found to have decreased (6/18) more often than increased (3/18).

Ehrlichiosis

A diagnostic of ehrlichiosis, using histological lesions and/or PCR, was performed on 12 out of 83 animals. Dogs diagnosed with ehrlichiosis were characterized macroscopically by anemia and mild to moderate icterus (Fig.3A). There were also multifocal petechial hemorrhages in the subcutaneous tissue, serosae (Fig.3B) and mucous membrane. The lymph nodes were dark-red to brown (Fig.3C) and the kidneys light-red to yellow (Fig.3D). Histologically, the spleen and lymph nodes had diffuse lymphoid depletion (Fig.4A), marked multifocal plasmacytosis, several Mott cells and erythrophagocytosis. Hemossiderosis was also found in the lymph nodes in six out of 13 dogs. There was also mild multifocal perivascular (2/12) random (2/12) or centrilobular (1/12) lymphoplasmacytic hepatitis with mild multifocal bile stasis (3/12). In addition, there was mild multifocal hepatocytic necrosis (2/12). In the kidneys, there was multifocal moderate to marked membranous

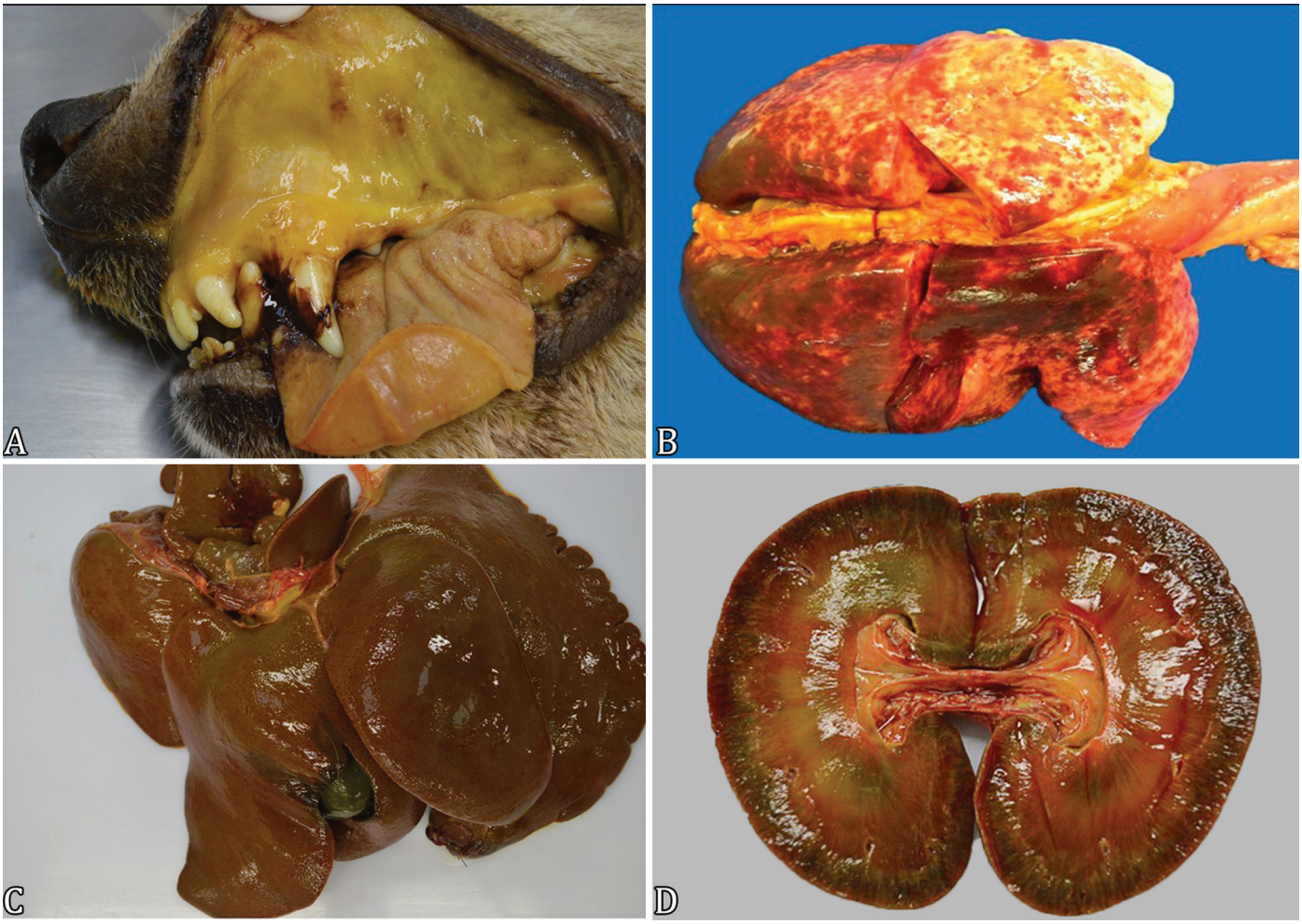

Fig.1. Adult male German Shepherd dog, diagnosed with leptospirosis. (**A**) Markedly yellow oral mucous membrane with ecchymoses. (**B**) Lung of a dog with numerous hemorrhagic areas in the visceral pleura. (**C**) Liver diffusely yellow-red and slightly green. (**D**) Kidney of dog from Figure 1A with diffusely red-brown cortical and medullar with yellow and green areas.

glomerulonephropaty (5/12), multifocal moderate fibrosis (5/12) and nephrosis (5/12), majority bilirubinuric (3/12), multifocal mild to moderate proteinuria (2/12) and moderate multifocal, interstitial nephritis, predominantly by plasm cells (4/12). In the bone marrow, varying degrees of hypocellularity (Fig.4B) and plasmacytosis were seen. In three dogs (3/12), macrophages with intracytoplasmic amastigotes compatible with *Leishmania* spp. were also found.

In the PCR for *E. canis,* five out of 12 dogs had one or more positive organ: bone marrow (2/12), lymph node (2/12) and spleen (2/12). In seven dogs (7/12), *E. canis* DNA was not amplified.

Three dogs (3/12) diagnosed with ehrlichiosis had hematologic tests. One dog presented macrocytic and normocytic anemia and another dog had normocytic and normochromic anemia. In addition, these dogs had severe leukopenia by neutropenia, lymphopenia, monocytopenia and thrombocytopenia. The biochemical exams (4/12) detected a mild increase in serum values of urea in one dog (1/4) and a marked increase in urea and creatinine values in another dog (1/4). For AST (2/4), ALT (1/4) and GGT (1/4) there were mild increases and a moderate increase for ALP (4/4).

Septicemia

Three dogs (3/83) were diagnosed with septicemia in accordance with their macroscopic and histopathological findings. Macroscopically, these dogs had mild to moderate icterus. The probable cause of septicemia in each of these cases were: fibrinous pericarditis; pyelonephritis and prostatitis; epididymitis and hepatitis (this dog also had ehrlichiosis). Multiple hemorrhagic areas were present in all three dogs (3/3).

Liver histopathology examinations revealed random multifocal histiocytic and lymphoplasmacytic hepatitis (3/3), moderate multifocal hyperemia (1/3) and mild multifocal biliary stasis (3/3). In the kidneys, there was moderate

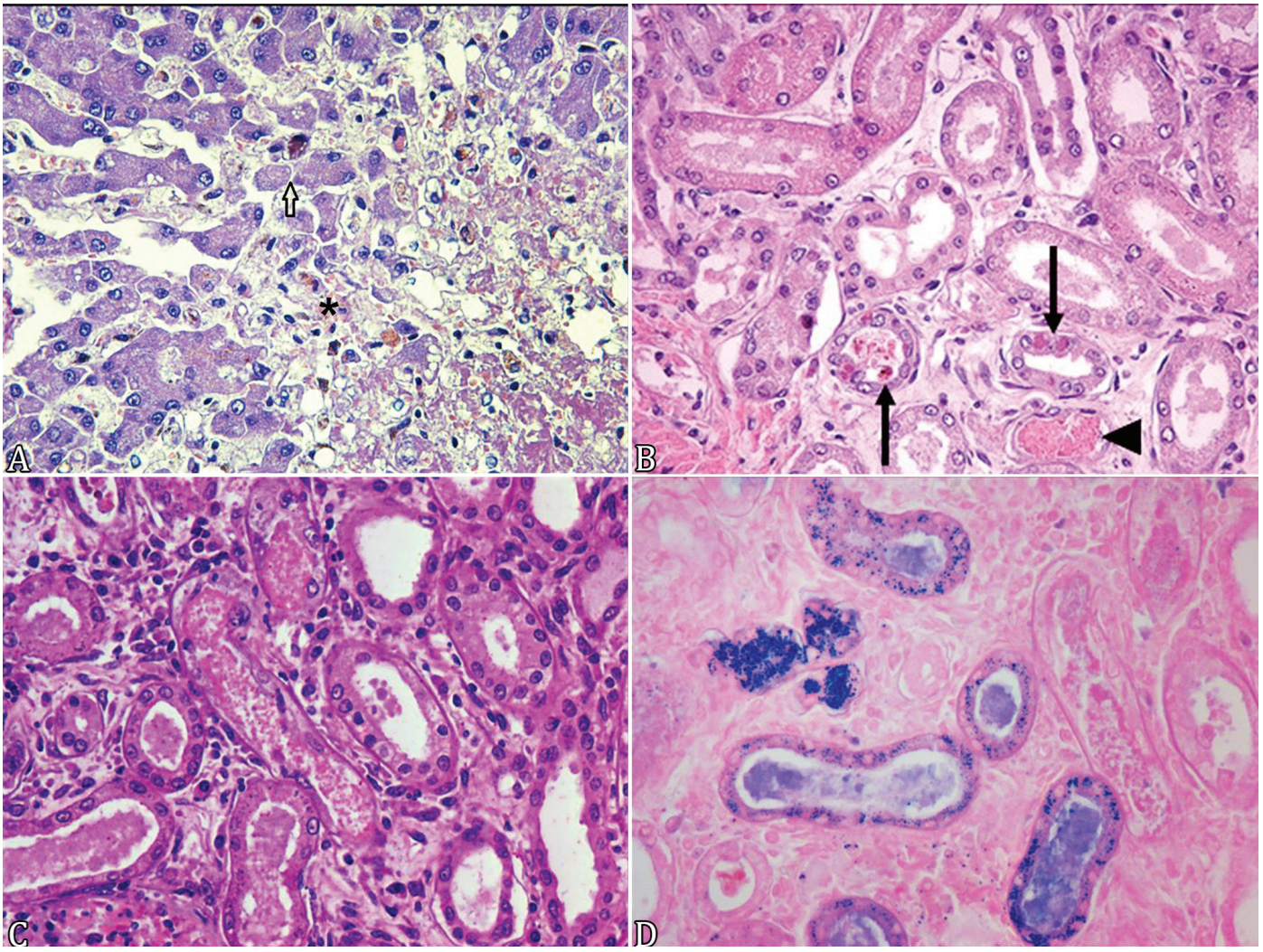

Fig.2. Histologic lesions in dogs with leptospirosis. (**A**) Adult male White Swiss Shepherd dog. Liver. Individual coagulative necrosis (*) and dissociation of hepatocytes (arrow). HE, obj.40x. (**B**) Kidney of dog from Figure 2A. Tubular lumen (arrow head) and cytoplasm of epithelial cells (arrow) containing yellow-brown pigment compatible with bilirubin (bilirubinuric nephrosis). HE, obj.40x. (**C**) Female mixed-breed dog. Kidney. Tubular lumen filled with eosinophilic to yellow casts and drops compatible with bilirubin (bilirubinuria) and brown granules within the cytoplasm of the epithelial cells compatible with hemosiderin. Necrosis of tubular epithelium can also be seen. HE, obj.40x. (**D**) Kidney from Figure 2C. Blue casts and granules indicating hemosiderin. Prussian Blue stain, obj.40x.

Fig.3. (**A**) Nine-year-old female German shepherd dog, diagnosed with ehrlichiosis caused by *Ehrlichia canis*. Moderately yellow oral mucous membrane. (**B**) Parietal pleura with numerous multifocal to coalescing petechial and ecchymoses hemorrhages. (**C**) Cervical and axillary lymph nodes, diffusely dark-red (drainage hemorrhages). (**D**) Kidneys diffusely pale-red in the cortical region and yellow in the medullar and pelvic adipose tissue.

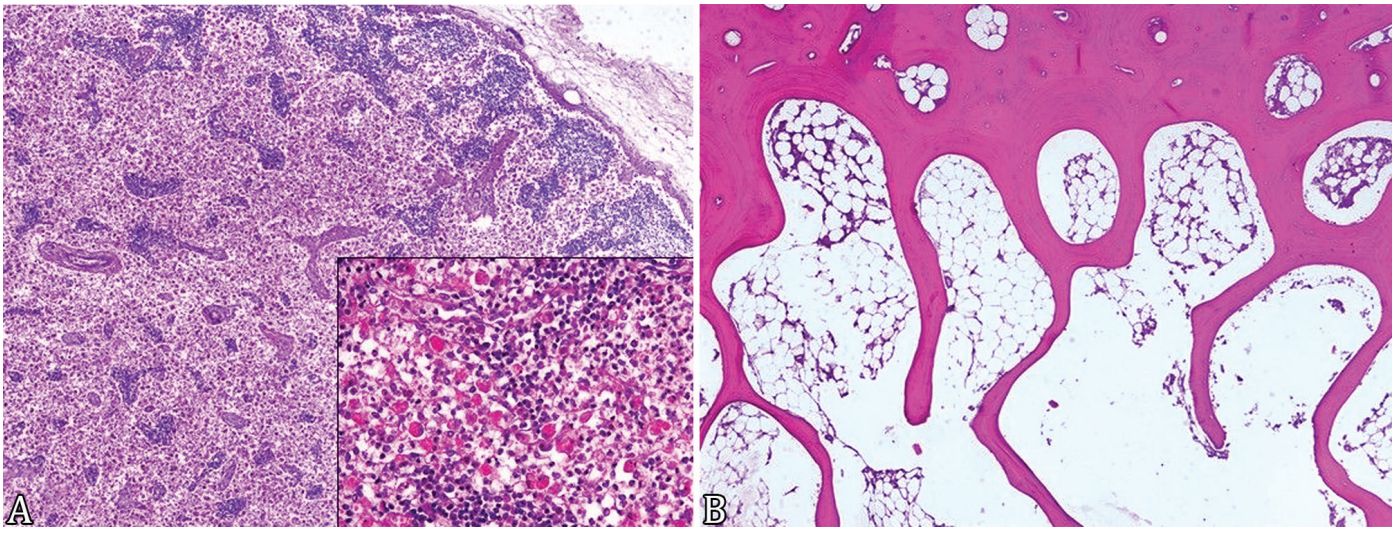

Fig.4. Histopathology of an adult male German shepherd dog with icterus diagnosed with chronic ehrlichiosis caused by *Ehrlichia canis.* (**A**) Lymph node. Marked decrease of lymphocyte density in the cortical region, and loss of follicular organization. HE, obj.50x. The inset shows the medullar area of the lymph node with marked erythrophagocytosis. HE, obj.20x. (**B**) Bone marrow. Diffuse decrease of cellularity characterizing marked hypoplasia. HE, obj.50x.

multifocal *membranous glomerulonephropathy* (1/3), mild multifocal to moderate interstitial lymphoplasmacytic nephritis (3/3), moderate multifocal tubular necrosis (2/3) and mild multifocal bilirubinuric nephrosis (3/3). There were also moderate multifocal hemorrhages (1/3), hyaline cylinders (1/3) and mild multifocal neutrophilic and lymphoplasmacytic pyelonephritis (1/3).

In the spleen and lymph nodes, there was mild to moderate lymphoid depletion (3/3). In addition, there were mild to moderate hemosiderosis (3/3), mild to moderate erythrophagocytosis (2/3) and hyperemia (1/3). The dog with concomitant ehrlichiosis also had plasmacytosis with some Mott cells. In the bone marrow and lymph nodes, there were some macrophages with intracytoplasmic amastigotes compatible with *Leishmania* spp.

Hematological tests in two dogs (2/2) revealed moderate to marked normocytic normochromic anemia (2/2), a mild to accentuated neutrophilic left shift (1/2) and thrombocytopenia (1/2). In two dogs the biochemical tests detected a mild to moderate increase in the plasma concentrations of urea and creatinine (1/2). A mild increase for AST and ALP (1/2) were also found.

Bacterial pancreatitis and hepatitis

One dog (1/83) was diagnosed with bacterial pancreatitis and hepatitis based on macroscopic and histopathological findings. Macroscopically the icterus was mild. The liver had accentuated lobular pattern and was mildly yellowish with several multifocal whitish areas (0.5cm). At histopathology, there were necrotizing and lymphoplasmacytic hepatitis and pancreatitis. In addition, thrombosis was found in the kidneys and lungs.

Hematological tests detected moderate normocytic normochromic anemia with marked neutrophilia, left shift neutrophils and monocytosis. Biochemical tests revealed increase in the sera values for AST, GGT, ALP and marked hypoproteinemia by hypoalbuminemia and hypoglobulinemia.

Cholangiocarcinoma

Five dogs (5/83) were diagnosed with primary hepatic neoplasia compatible with cholangiocarcinoma based on the macroscopic and histopatological findings. Macroscopically, these dogs had mild to moderate icterus. In the liver there were multiple well-demarcated, yellow-whitish nodules. In some prominent nodules, umbilication was present (5/5). In one dog, similar nodules were also present in the kidneys (metastases). At histopathology, neoplastic cells were arranged in tubular forms interspersed by abundant connective tissue stroma. These areas widely replaced the hepatic parenchyma (5/5). In addition, there were random multifocal necrotic areas and moderate multifocal biliary stasis (5/5). Some of these dogs had metastatic cholangiocarcinoma in the lymph nodes (2/5), pancreas and kidneys (1) and bone marrow (1/5).

Hematological and biochemical tests were performed on two dogs (2/5). The hematological tests revealed neutrophilic left shift (1/2), neutrophilia (1/2), lymphopenia (2/2) and mild to marked thrombocytopenia (2/2). The biochemical tests revealed a moderate to marked increase in the sera values of urea (2/2), a marked increase of creatinine (1/2), a marked increase of ALT and ALP (1/2), a moderate increase of GGT (2/2) and a mild to marked increase of AST (2/2).

Hemangiosarcoma

In four dogs (4/83), vascular endothelial neoplasia consistent with metastatic hemangiosarcoma was diagnosed. Macroscopically, they had mild to moderate icterus. Well-demarcated dark-red nodules were found in the liver (2/4), lungs (2/4), spleen (2/4), lymph nodes (1/4), brain (1/4) and heart (1/4).

Liver histopathology showed neoplastic mesenchymal proliferation, forming vascular spaces filled with erythrocytes and aligned with one or more poorly differentiated endothelial cells layer (2/4). The parenchyma adjacent to the metastatic areas was compressed and necrotic (1/4). In all dogs there was moderate multifocal biliary stasis. In the kidneys there were mild to marked multifocal bilirubinuric nephrosis (4/4), tubular hemosiderosis (1/4) mild to moderate multifocal *membranous glomerulonephropathy* (3/4), glomeruloesclerosis (2/4) and moderate multifocal interstitial lymphoplasmacytic nephritis (2/4). Mild lymphoid depletion (3/4), erythrophagocytosis (3/4) and hemosiderosis (4/4) were found in the lymph nodes. Hemosiderosis in the renal tubules was confirmed by Prussian blue stain method.

Hematological tests were performed in two dogs (2/4). One dog had moderate normocytic normochromic anemia with neutrophilia, left shift neutrophils and monocytosis. Another dog had neutropenia and monocytopenia. Both had anemia and thrombocytopenia. Biochemical tests (2/4) revealed a marked increase in the plasma concentrations of urea and creatinine and mild hyperglobulinemia.

Lymphoma

Three dogs (3/83) were diagnosed with lymphoma. Macroscopically, they had moderate icterus. In two dogs all lymph nodes were markedly enlarged with replacement of normal parenchyma with neoplastic cells. Microscopically, the neoplastic cells had morphologic characteristics of lymphoma. These dogs had metastatic multicentric lymphoma with extensive liver invasion and multiple neoplastic nodules in the lungs, spleen and kidneys. One dog was also diagnosed with alimentary lymphoma. The jejunum and ileum had transmural infiltration by this neoplasia with focal luminal stenosis. Metastases in the lungs, liver and kidneys were found.

Hematological and biochemical tests were performed on all three dogs. Results revealed macrocytic normochromic (1/3) or normocytic normochromic (2/3) anemia with neutrophilic left shift (3/3), lymphopenia (2/3) and thrombocytopenia (2/3). Plasma concentrations were mildly increased for urea and creatinine (1/3), GGT (1/3) had a marked increased and AST and ALP (2/3) had moderate increases. Two dogs had hypoalbuminemia.

Pancreatic carcinoma

Two dogs (2/83) were diagnosed with pancreatic carcinoma based on pathological findings. Macroscopically, there was mild to moderate icterus. One dog had a solid and single yellow mass and another dog had multiple white to yellow nodules in the pancreas. Metastases in the liver, lungs, pancreatic lymph node, kidneys and bone marrow were found in one dog. Microscopically, neoplastic cells had morphology compatible with acinar pancreatic cells. Hematological tests detected normocytic normochromic anemia (1/2), neutrophilic left shift (1/2), monocytosis (2/2) and thrombocytopenia (2/2).

These dogs also had moderate to marked increases in the plasma concentration of urea and creatinine (2/2), ALT, AST, ALP and GGT (1/2). One dog had hypoalbuminemia.

Lipidosis

Nine dogs (9/83) were diagnosed with lipidosis with moderate to marked icterus (Fig.5A). The liver was enlarged, diffusely yellow (Fig.5B) and friable. Microscopically, hepatocytes were seen markedly enlarged with well-demarcated vacuoles within the cytoplasm, displacing the nucleus. Moderate to marked biliary stasis was present in all dogs. In the kidneys, there was mild to moderate multifocal bilirubinic nephrosis. Mild or marked *membranous glomerulonephropathy* (7/9), glomeruloesclerosis (2/9), moderate multifocal interstitial lymphoplasmacytic nephritis (4/9) and mild proteinuria (4/9) were also found. The lymph nodes had moderate multifocal hemossiderosis (3/9).

The hematological tests detected normocytic normochromic (3/5) and macrocytic normochromic (2/5) anemia, neutrophilia, left shift neutrophils (4/5) and monocytosis (3/5). In four dogs (4/5) mild to moderate lymphopenia and mild to marked thrombocytopenia were detected. In five dogs (5/9), their urinary and hepatic biochemical profiles showed mild to marked plasma concentration increases in urea (5/5), creatinine (3/5), ALT (3/5), ALP (5/5) and GGT (3/5). Total protein values were mildly to moderately decreased (3/5).

Glycogenic degeneration

Three dogs (3/83) were diagnosed with marked glycogenic degeneration in the liver. Macroscopically, these dogs had mild to marked icterus. In all dogs the liver was moderately enlarged and diffusely red-orange with *accentuation* of the normal *lobular pattern. The bile was* thick and *grumous.* Histopathologically, the hepatocytes were markedly enlarged with poorly demarcated vacuoles within the cytoplasm and the nucleus was generally centrally located. There was also a moderate multifocal biliary stasis. In the kidneys there was mild multifocal bilirubinic nephrosis (2/3) and moderate glomerulonephropathy (2/3). In the spleen and lymph nodes there were mild to moderate lymphoid depletion (3/3) and hemosiderosis (2/3).

Hematological tests in three dogs (3/3) detected normocytic normochromic (2/3) and macrocytic normochromic (1/3) anemia, moderate neutrophilia (2/3), monocytosis (1/3) and thrombocytopenia (1/3). The urinary and hepatic biochemical profiles showed moderate plasma concentration increases in urea and creatinine (2/3), AST (1/3), and mild to marked increases in ALT, ALP and GGT (3/3).

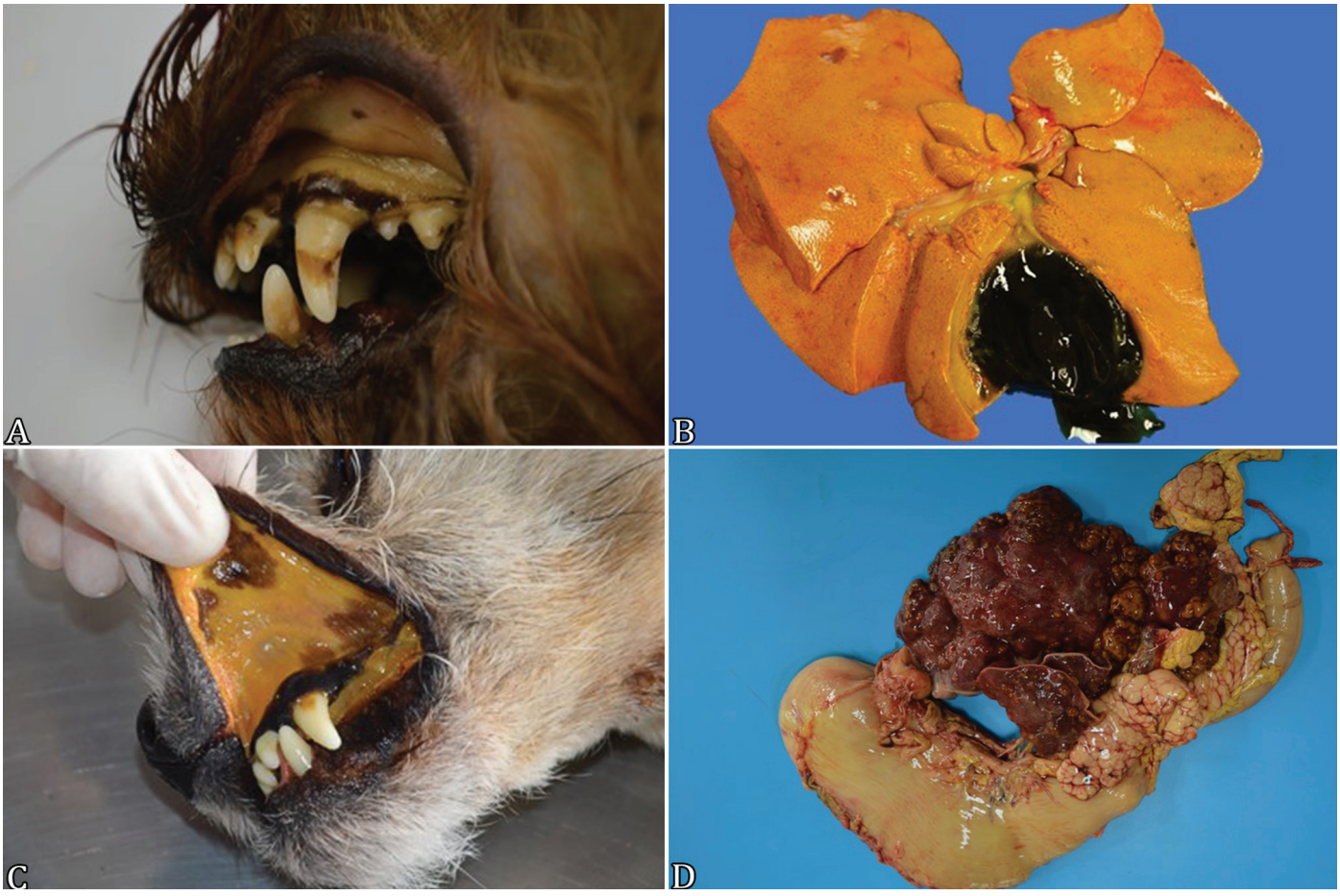

Fig.5. Liver with degenerative lesions. (**A**) Oral mucous membrane with moderate icterus. (**B**) Liver of the dog from Figure A with marked diffuse yellowish discoloration (lipidosis). (**C**) Terminal hepatic fibrosis in an adult male mixed-breed dog. (**D**) Liver of the dog from Figure C. Liver markedly decreased in size with irregular surface and yellow-greenish nodules (cirrhosis).

Hepatic fibrosis (cirrhosis)

Six dogs (6/83) had advanced hepatic fibrosis. Macroscopically, all dogs had moderate to marked icterus (Fig.5C). The liver of these dogs was moderately to markedly smaller than expected, with an irregular surface and with multiple yellow nodules (Fig.5D).

Histopathologically, there was a loss of normal hepatic architecture and replacement with portal-portal to portal-central bridging fibrosis, biliary duct proliferation and nodular areas of regenerating hepatocytes. In addition, multifocal necrosis was associated with moderate lymphoplasmacytic and neutrophilic infiltration (6/6) and biliary stasis. In the kidneys there was multifocal bilirubinic nephrosis (6/6) and a mild to moderate multifocal membranous glomerulonephropathy (3/6). In the spleen and lymph nodes there was mild lymphoid depletion (2/6) and moderate hemosiderosis (6/6). In the bone marrow of one dog (1/6) there was a mild number of macrophages with amastigotes compatible with *Leishmania* spp. within the cytoplasm.

Hematological tests were performed in three dogs. Normocytic normochromic anemia was diagnosed in three dogs (3/3). In addition, there were moderate neutrophilia, left shift neutrophils and monocytosis in two dogs. All dogs had moderate to marked thrombocytopenia. In three dogs (3/6), the biochemical profile showed a mild increase in the sera values of GGT (1/3), a moderate ALT increase (2/3), a mild to moderate AST increase (3/3) and a marked ALP increase (2/3). Two dogs had hypoalbuminemia.

Biliary duct obstruction

Complete extrahepatic biliary duct obstruction caused by cholelithiasis was diagnosed in one dog (1/83). Clinically, the animal presented anorexia and recurrent vomiting. Macroscopically, there was marked icterus (Fig.6A) and the gallbladder was markedly enlarged and distended. The lumen was filled with yellow-green thick grumous bile. Gallstones were found blocking the ductal lumen (Fig.6B). Microscopically, the gallbladder mucous membrane had moderate neutrophilic infiltration and multifocal hemorrhages. Hematological and biochemical tests were not performed.

DISCUSSION

Pre-hepatic and hepatic icterus were the most frequent types of icterus in this study. Only one was classified as post-hepatic icterus. Leptospirosis stood out as the main cause of pre-hepatic or hepatic icterus in the dogs of this study.

The pre-hepatic form can occur as a result of erythrocytes destruction inside blood vessels or erythrophagocytosis in the lymphoid organs (Valli et al. 2016). The hepatic icterus can be related with several diseases, such as *Leptospira interrogans* infections; degenerative liver diseases (lipidosis and glycogenic degeneration); primary hepatic neoplasia (cholangiocarcinoma) or metastases and hepatic fibrosis with loss of hepatocytes. This type of icterus occurs as a result of lesions in the hepatocytes and a decrease in or unconjugation of bilirubin (Murdoch 1976, Barros 2011). Toxins, medicaments and viruses can also cause hepatic icterus (Barros 2011). Post-hepatic icterus was diagnosed in a dog with biliary stones in this study. The occurrence of this condition in dogs is not common (Cipriano et al. 2016). Generally, biliary stones associated with cholecystitis can cause severe biliary obstruction of the main duct with marked icterus and subsequent rupture of the gallbladder (Cullen & Stalke 2015). Other reported causes related to duct obstruction include extrahepatic biliary tumors (Eulenberg & Lidbury 2018).

One important etiology of icterus is *Leptospira interrogans* (Murdoch 1976, Bernheimer & Bey 1986). In this study, leptospirosis mainly occurred in mixed-breed adult dogs older than one year old, similar to other studies (Batista et al. 2005). The fact that adult dogs are more affected by *Leptospira* spp., could be related to increased exposure and contact with contaminated external environments than puppies. Puppies normally receive more care from their owners (Greene et al. 2006). Another factor could be leptospirosis vaccination failure or an inadequate vaccination schedule (Batista et al. 2005, Adler & de la Pena-Moctezuma 2010).

The most frequent serovars in our study were Icterohaemorrhagiae and Bratislava, followed by Canicola and Autumnalis. Other researchers in Brazil have reported similar results, whilst others have found different results. One study detected the serovars Autumnalis, Copenhageni and Canicola as the most frequent (Batista et al. 2005). Another study revealed that predominant reactive serogroups were Icterohaemorrhagiae, Australis, Pomona, Butembo, and Castellonis (Miotto et al. 2018). A study from the United States detected antibodies against serovars Autumnalis, Grippotyphosa, Pomona, and Bratislava as the most common in dogs (Gautam

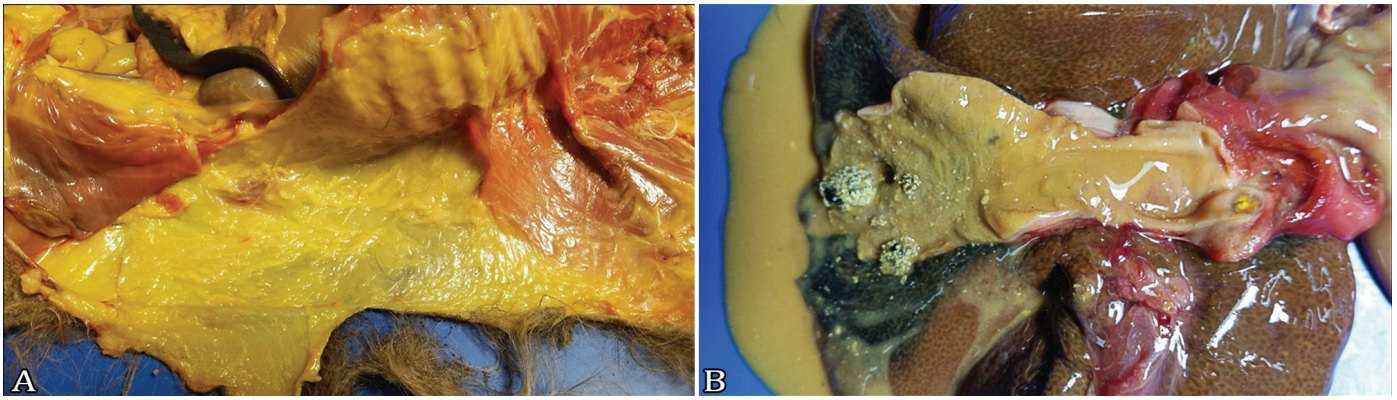

Fig.6. Five-year-old male Yorkshire Terrier dog. (**A**) Subcutaneous adipose tissue and abdominal serosa characterized by marked icterus. (**B**) Liver. Complete obstruction of biliary duct by gallstones accompanied by a viscous yellow exudate.

et al. 2010). Cross- reaction between different serovars can occur. In this case, the serovar with the highest titer (>1:800) should be interpreted as the infecting serovar (Brown et al. 1996, Adler & de la Pena-Moctezuma 2010). The serovars with the highest titers (1:800) detected in the dogs from this study were Icterohaemorrhagiae, Bratislava, Autumnalis, Pomona and Bataviae. Autumnalis and Bratislava could possibly be the serovars responsible for clinical cases, because they are not included in the vaccines against leptospirosis in Brazil.

Hemoglobinuria was not common in dogs diagnosed with leptospirosis in this study. The mechanism for hemolysis production in the leptospirosis is not completely elucidated. Some studies related the hemolysis with production of sphingomyelinases or phospholipases activity by pathogenic serovars Icterohaemorrhagiae and Pomona (Kasãrov 1970). These enzymes produce pores in the cellular membrane of erythrocytes and subsequently cell death (Kasãrov 1970, Trowbridge et al. 1981, Picardeau et al. 2008, Adler & de la Pena-Moctezuma 2010). The main types of phospholipids in the erythrocyte membrane were found to be susceptible to the action of specific leptospiral enzymes. However, the type of these phospholipids differs among animal species as well as the different types of haemolytic enzyme produced by each serovar (Kasãrov 1970). This could be related to differences in the susceptibility of an animal species to each specific serovar. Hemolytic anemia associated with leptospirosis infection has been previously reported in some animal species including lambs (Smith & Armstrong 1975) cattle (Bernheimer & Bey 1986) and horses (Delph et al. 2018). An experimental study in hamsters demonstrated hemolytic activity of the serovar Pomona (Sobroza et al. 2014). For canine leptospirosis, different results were found in some studies, which described the diseases with hemorrhagic, renal and hepatic lesions without hemolysis. The icterus manifestation in these studies was related to hepatic lesions and hyperbilirubinemia (Tochetto et al. 2012), as diagnosed in several dogs in our study. Similar clinical manifestations were reported for serovars Australis and Bratislava infections in Europe. Dogs from these reports were routinely vaccinated against serovars Canicola and Icterohaemorrhagiae (Major et al. 2014). *L. interrogans* serovars Icterohaemorrhagiae was described by Murdoch (1976) as causing icterus in dogs because haemolysis and concomitant hepato-cellular lesions. Icterus is a common manifestation of acute leptospirosis and may be the result of hemolysis or hepatocellular lesions (Cianciolo & Mohr 2016). A dog examined in the present study diagnosed with hemoglobinuria had MAT titers for serovar Icterohaemorrhagiae, another dog for Bataviae and Bratislava and another for serovar Bratislava only. The hemoglobinuria in these dogs was interpreted as chronic because of iron positive stain in the renal tubules using Prussian blue stain.

The serovar Icterohaemorragiae is especially implicated with hepatic lesions, and the serovar Canicola mainly with renal lesions. Other serovars could affect both organs, although the lesions can be less marked than both the serovars aforementioned (Freire et al. 2008, Adler & de la Pena-Moctezuma 2010). Hepatic and renal lesions found in the dogs of the present study varied among serovars and no correlation was found with a specific serovar. Hematological results from several positive leptospirosis dogs in our study included regenerative anemia, neutrophilic left deviation and thrombocytopenia, corroborating with other studies (Sykes et al. 2011, Maele et al. 2008).

L. interrogans detection could be performed by PCR using DNA extracted from blood, urine, kidney or liver (Coutinho et al. 2014, Maele et al. 2008). In the present study, this test produced confident results in approximately 90% of dogs using samples from the liver, kidney and spleen. Based on these results, a limitation detection may have occurred. *Leptospira* spp. are readily eliminated by antibiotics, particularly using doxycycline (Truccolo et al. 2002) or doxycycline and rifampicin, decreasing the PCR sensitivity (Kim & Byun 2008). In some dogs from this study, with compatible pathological diagnosis of leptospirosis and PCR negative results, we speculated about the antibiotic therapy influence.

The biochemical profile of the dogs with leptospirosis examined in this study revealed increased plasma concentrations of urea and creatinine in most of the dogs. These results are similar to other leptospirosis studies, which identified that sera values of these renal catabolic were increased but can vary with the clinical evolution of the disease (Sykes et al. 2011, Maele et al. 2008).

Increased activities of enzymes indicating hepatic dysfunction was also detected in dogs from this study. ALT, AST and ALP, which are almost always associated with azotemia, are usually described in dogs with leptospirosis (Azócar-Aedo et al. 2017). Increased sera concentrations of ALP usually occur in association with biliary tree lesions (Valli et al. 2016). Increased sera concentrations of ALT and AST can also be found in other hepatic conditions (Murdoch 1976), as described in the present study.

Chronic glomerular lesions present in several dogs from this study and not related with leptospirosis could have contributed to renal biochemical changes. These lesions were frequent and suggestive of chronic visceral leishmaniasis, which was confirmed in some dogs from this study with chronic glomerular lesions. The frequency of this infection is high in the animals from the region studied (Lima et al. 2004, Rigo et al. 2013). Glomerular alterations are commonly described in dogs with leishmaniasis and associated with functional changes (Costa el al. 2003).

Co infection with *Ehrlichia canis* and *Leptospira* sp. was found in some dogs from the present study, similar to another report (Morais et al. 2011), confirming that concurrent infections could occur for these etiologic agents. The PCR was an important ancillary test to confirm *E. canis* infections, in addition to the histopathology exam helping to confirm the disease.

Icterus associated with ehrlichiosis is a clinical manifestation (Rungsipipat et al. 2009) possibly related to hemorrhages caused by thrombocytopenia, hemorrhagic drainage by the lymph nodes and erythrophagocytosis with the consequent production of bilirubin. In this study, two dogs with icterus caused by ehrlichiosis (single infection), had hematological changes similar to those reported in other studies (Moreira et al. 2003, Greene 2006, Harrus 2015). There are some hypotheses about the pathogenesis of hematological changes of ehrlichiosis. Thrombocytopenia detected in dogs diagnosed with ehrlichiosis, could be caused by immune-mediated mechanisms (Waner et al. 2000, Rungsipipat et al. 2009, Harrus 2015). Thrombocytopenia, anemia and lymphopenia were also related to the action of *Ehrlichia* in the bone marrow

and lymphoid tissues (Mylonakis et al. 2004, Greene 2006). Recent studies about iron metabolism in animals infected with *Ehrlichia canis*, reported a marked decreased in circulating iron, suggesting that the host may be induced by the bacteria to transport iron to infected tissue for bacterial multiplication, contributing to anemia (Bottari et al. 2016). This may explain the numerous hemosiderin granules commonly found in the bone marrow, liver, lymph nodes and spleen from animals with ehrlichiosis in this study. Also, in our dogs, the histopathologic findings included plasmacytosis in the lymph nodes and spleen. In addition, lymphocytic and plasmacytic perivascular cuffing were found in numerous organs, including the liver, kidneys and bone marrow. In these dogs, bone marrow was also marked hypoplastic. These findings are similar to those described by Waner & Harrus (2013) in dogs with chronic ehrlichiosis.

Septicemia associated with icterus was also found in dogs from the present study. Some mechanisms underlying this process have been researched. Systemic bacterial endotoxins can activate inflammatory mediators, such as TNF-α and IL-6 cytokines. This could reduce biliary excretion by the canaliculi (Chand & Sanyal 2007). Biliary excretion is dependent on ATP. If septicemia is present, a decrease in cell energy is possible (ATP), as a result of hypoxia, toxins or cytokines effects. This interfers with the transport proteins of bile and its components causing bile stasis. This results in an increase in unconjugated bilirubin and consequently icterus (Nesseler et al. 2012). In addition, high amount of bacterial toxins and cytokines in the spleen could produce necrosis and inflammation (Ackermann 2013). Endothelial lesions can cause hemorrhages, erythrophagocytosis and bilirubin production, contributing to icterus (Goyette et al. 2004). A marked increase in leucocytes, especially neutrophils, could suggest the presence of septicemia (Klosterhalfen et al. 1996). This increase was detected in the dogs in this study diagnosed with septicemia. A dog with lesions compatible with septicemia had pancytopenia, with marked neutropenia. These hematological changes could occur due exhaustive production of progenitor cells (Aird 2003).

Lipidosis was the second most frequent cause of hepatic icterus in the present study, followed by primary hepatic neoplasia and advanced hepatic fibrosis. Lipidosis in dogs mainly occurs due to excessive ingestion of carbohydrates, hypoxia, abnormal hepatocyte function, decreased apoprotein synthesis, hormonal diseases and medication intoxication. The degeneration of the hepatocytes could reduce bilirubin uptake and their increased size compresses the bile canaliculi, resulting in reduced biliary tract drainage (Cullen & Brown 2013). Lipidosis was common in the dogs from the present study but the etiology was not well determined. Nevertheless, high caloric diets were inferred to be a possible cause based on the clinical history and absence of others etiologies.

Glycogenic degeneration was associated with icterus in this study. This condition is caused by endogenous or exogenous steroids. Endogenous steroids are found in high concentrations in cases of hyperadrenocorticism (functional cortical adrenal neoplasia) or functional tumors in the adeno-hypophysis (inducing cortical adrenal hyperplasia). High steroid plasma concentrations occur due to excessive or prolonged doses of exogenous glucocorticoids. The overload of glycogen in the liver could induce diffuse and marked degeneration of the hepatocytes (Cullen & Stalke 2015), as seen in the animals from the present study. Most dogs had a clinical history of therapeutic corticoid use. Its likely these lesions were caused by this medication.

Dogs with advanced hepatic fibrosis could also present icterus (Murdoch 1976, Elhiblu et al. 2015) due to loss of hepatocytes and canaliculi (Elhiblu et al. 2015). Hepatic fibrosis can be induced by lipidosis, glycogen degeneration, hepatitis and toxic hepatopathies (Cullen & Stalke 2015). In the resolution phase, depending upon the distribution, intensity and persistent of injury caused to the hepatocytes, and basal membrane destruction, fibrosis and nodular regeneration can occur (Cullen & Stalke 2015, Eulenberg & Lidbury 2018). In the present study, the lesions were markedly chronic and confirmation of the etiology responsible for the pre-fibrotic lesions was not possible.

Cholangiocarcinoma originates from hepatic biliary ducts, usually composed of small to wide nodule proliferations, replacing the normal liver parenchyma and causing icterus (Aloia et al. 2012). Regarding primary hepatic neoplasms, cholangiocarcinoma was the most frequent hepatic neoplasm is this study, similar to another study in Brazil (Aloia et al. 2012). The dogs studied here had macroscopic and microscopic changes interpreted as primary intrahepatic ductal origin and no other changes indicating other cause for the icterus were found.

Dogs with hemangiosarcoma usually have frequent hematological changes including microangiopathic hemolysis, disseminated intravascular coagulation (DIC) and hemorrhages (Hammer et al. 1991, Valli et al. 2016). These changes are mainly characterized by thrombocytopenia, an increase of fibrin degradation products and fragmented erythrocytes (Hammer et al. 1991), which are removed by splenic macrophages (Valli et al. 2016). DIC in dogs with hemangiosarcoma are frequent (Maruyama et al. 2004). Besides this, in the neoplastic vessels with turbulent blood flow, mechanical trauma can produce physical lysis to the erythrocytes and consequently icterus (Valli et al. 2016). In the present study, dogs with disseminated hemangiosarcoma had anemia, mild icterus, thrombocytopenia and histologic lesion of hemoglobinuria in absence of hepatic lesions, characterizing intravascular hemolysis and pre-hepatic icterus. Also, a marked hemosiderin deposition was found in the renal tubular cells of one dog with hemangiosarcoma of this study. Intravascular hemolysis generally is characterized by hemoglobinuria, and hemosiderosis are seen in the renal tubular cells consequent of reabsorbed filtered hemoglobin (Valli et al. 2016).

Metastatic neoplasms in the liver from the dogs of this study, such as lymphoma, were also responsible for producing icterus. In dogs, the most common metastatic hematopoietic, mesenchymal, and epithelial neoplasms are lymphoma, hemangiosarcoma, and pancreatic carcinoma, respectively. These neoplasms can cause icterus either by extrahepatic bile duct obstruction or by intrahepatic cholestasis, or both (Cullen & Stalke 2015), as diagnosed in our study.

CONCLUSIONS

Hepatic and pre-hepatic icterus were the most frequent type of icterus diagnosed in the 83 dogs examined during the 36 months of this study.

The most frequent infectious etiologies were *Leptospira interrogans* and *Ehrlichia canis*.

Degenerative hepatic diseases, end-stage hepatic fibrosis as well as primary and metastatic neoplastic diseases were also diagnosed and considered important differential etiologies of the infectious causes.

Clinical pathology tests, serology, molecular and other complementary tests, such as a liver biopsy, are important auxiliary tools for clinical presumptive diagnosis.

Acknowledgements.- Financial support for this study and fellowships were provided by the "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq), by "Programa de Pós-graduação em Ciência Animal", "Pró- Reitoria de Pesquisa" of the "Universidade Federal de Minas Gerais" supported by "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES), Brazil, Finance Code 001.

Conflict of interest statement.- The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(6):466-473, June 2020
DOI: 10.1590/1678-5150-PVB-6360

Original Article
Small Animal Diseases

Risk factors associated with mammary tumors in female dogs[1]

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ABSTRACT.- Santos T.R., Castro J.R., Andrade J.C., Silva A.C.R., Silva G.M.F., Ferreira F.A., Headley S.A. & Saut J.P.E. 2020. **Risk factors associated with mammary tumors in female dogs.** *Pesquisa Veterinária Brasileira 40(6):466-473*. Faculdade de Medicina Veterinária, Universidade Federal de Uberlândia, Campus Umuarama, Avenida Pará 1720, Bloco 2T, Bairro Umuarama, Uberlândia, MG 38400-902, Brazil. E-mail: thaisareis.vetufu@gmail.com

Mammary tumors in female dogs are the most frequent and corresponds to half of the canine tumors. The objectives of this study were to determine the risk factors associated with the occurrence of mammary tumors in female dogs and to evaluate the macroscopic characteristics of these neoformations, using 386 dogs from the "Outubro Rosa Pets" events done within the cities of Uberlândia and Patos de Minas, Minas Gerais State, Brazil, in 2015 (n=194), 2016 (n=105) and 2017 (n=87). For the determination of risk factors, the binary logistic regression test (P<0.05) was performed. The occurrence of mammary tumors was 23.6% (91/386). The significant risk factors identified were increased age (P<0.001), overweight (P=0.048) and non-castration (P<0.001) with a chance of, respectively, 1.6, 2.3 and 9.3 times for the development of mammary tumors. In dogs with mammary tumors (n=91), 153 lesions were present, of which 39 female dogs had two or more lesions (42.8%). Most of the lesions were at the caudal abdominal (M4) and inguinal (M5) mammary glands (60.13%, 92/153). Relative to the size of the lesions, it was observed that in 78% of the female dogs the lesions were determined asT1 (<3cm), 16.5% were T2 (3-5cm) and 5.5% T3 (>5cm). At least 15.4% (14/91) of the dogs had one of the regional lymph nodes increased. In conclusion, the occurrence of mammary tumors in the evaluated population was 23.6% and that age, overweight and non-realization of ovariohysterectomy are risk factors associated with the development of mammary tumors.

INDEX TERMS: Mammary tumors, female dogs, bitches, Minas Gerais, Brazil, ovariohysterectomy, overweight, prevention, risk factors, dogs.

RESUMO.- [**Fatores de risco para tumores mamários em cadelas de Uberlândia e Patos de Minas, Minas Gerais.**] Em cadelas os tumores mamários são os mais frequentes e correspondem a aproximadamente metade dos tumores em cães. Este estudo teve os objetivos de determinar os fatores de risco envolvidos na ocorrência de tumores mamários em cadelas e avaliar as características macroscópicas destas neoformações, utilizando 386 cadelas do evento "Outubro Rosa *Pets*" nos municípios de Uberlândia e Patos de Minas, Minas Gerais, Brasil, em 2015 (n=194), 2016 (n=105) e 2017 (n=87). Para a determinação dos fatores de risco utilizou-se o teste de Regressão logística binária (P<0,05). A ocorrência de tumores mamários foi de 23,6% (91/386). Os fatores de risco significativos identificados foram aumento da idade (P<0,001), sobrepeso (P=0,048) e não-castração (P<0,001) com a chance de, respectivamente, 1,6, 2,3 e 9,3 vezes de desenvolvimento de tumores mamários. Nas cadelas com tumores mamários (n=91), constatou-se a presença de 153 lesões, sendo que 39 cadelas apresentaram duas ou mais lesões (42,8%). A maioria das lesões localizaram-se nas mamas abdominais caudais (M4) e inguinais (M5) (60,13%; 92/153). Em relação ao tamanho das lesões, observou-se que 78% das cadelas

[1] Received on July 10, 2019.
Accepted for publication on July 27, 2019.
Doctoral Research with support from CAPES.

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eram T1 (<3cm), 16,5% T2 (3-5cm) e 5,5% T3 (>5cm). Pelo menos 15,4% (14/91) das cadelas apresentaram um dos linfonodos regionais aumentados. Conclui-se que a ocorrência dos tumores mamários na população avaliada foi de 23,6% e que a idade, sobrepeso e não ovariohisterectomia são fatores de risco para o desenvolvimento de tumores mamários.

TERMOS DE INDEXAÇÃO: Fatores de risco, tumores mamários, cadelas, Minas Gerais, Brasil, ovariohisterectomia, prevenção, sobrepeso, tumor de mama.

INTRODUCTION

Mammary tumors are the most frequently diagnosed in female dogs and are responsible for approximately 50% of canine tumors (Queiroga & Lopes 2002, Kumaraguruparan et al. 2006, Cassali et al. 2014). In both veterinary and human medicine, breast cancer has resulted in numerous studies aimed at prevention and early diagnosis, mainly because this alteration is associated with elevated morbidity and mortality rates in the affected patients (Humphrey et al. 2002, Cassali et al. 2014, Pascoli et al. 2017).

Canine mammary tumors serve as appropriate and valid experimental models for the study of cancer biology in humans due to the similarity of epidemiological, clinical, biological and genetic characteristics (Silva at al. 2004, Kumaraguruparan et al. 2006). Studies have shown that obesity, age, sex, nutrition, and hormonal activities are associated with the etiology of breast cancer in dogs and women (Pérez Alenza et al. 1998, Cassali et al. 2014, Takalkar et al. 2016).

Although in female dogs, breast tumors may occur in any of the mammary glands, with the presence of multiple nodules with similar or different histological patterns are frequently described (Queiroga & Lopes 2002, Oliveira et al. 2003, Hellmén 2005, Cassali et al. 2014). Careful physical examination of the mammary glands, assessment of regional lymph nodes, distant organ metastasis, and histopathological examination are essential factors to determine the diagnosis, prognosis, and treatment. In addition, it is recommended to define the severity of the disease by staging the patient based on tumor size (T), presence of metastases in the lymph node(N) and distant metastases (M), according to the TNM system established by the World Health Organization, WHO (Owen 1980).

In female dogs with mammary neoplasms pulmonary metastases are frequently observed, but dissemination can also occur in lymph nodes, myocardium, spleen, adrenals, bones, and the brain (Misdorp 2002, Cassali et al. 2014). Additionally, retarded diagnosis makes treatment difficult and reduces survival of affected animals (Silva et al. 2004, Cavalcanti & Cassali 2006, Estrela-Lima et al. 2010, Toríbio et al. 2012). Alternatively, the realization of early ovariohysterectomy (OSH), i.e., begore the third estrus cycle, positively impacts the reduction of the development of mammary neoplasms in dogs and is an important form of prevention (Sonnenschein et al. 1991, Fonseca & Daleck 2000).

Since 1990, actions have been taken during the month of October to alert women about breast cancer prevention (INCA 2016). Recently, veterinary medicine has taken advantage of this month to promote actions to provide guidance on the prevention of breast cancer and the awareness of tutors regarding clinical signs, diagnosis and treatment, as well as collecting data relative to epidemiology and risk factors for this disease (Pascoli et al. 2017). The objective of this study was to determine the risk factors associated with the occurrence of mammary tumors in female dogs and to evaluate the macroscopic characteristics of these neoformations, using the database of the "October Rosa Pets" events maintained at cities of Uberlândia and Patos de Minas, Minas Gerais (MG), from 2015 to 2017.

MATERIALS AND METHODS

The study was done according to the ethical principles for experimentation of the Ethics Committee for Animal Use (CEUA), "Centro Universitário de Patos de Minas" (Unipam), under protocol number 50/17 and with authorization of the owners of the animals, based to the terms outlined in the consent forms that were made available and signed by all participating clients.

Study design and characterization of the study population. A retrospective cross-sectional study was done by using the evaluation and analysis of the clinical records completed during the realization of the mammary cancer prevention events in female dogs. These events took place within the cities of Uberlândia and Patos de Minas/MG, during 2015, 2016, and 2017.

The "October Rosa Pets" campaign was conducted by undergraduate students in Veterinary Medicine, under the supervision of professors and veterinarians of the "Universidade Federal de Uberlândia" (UFU), "Centro Universitário do Triângulo" (Unitri), and the "Centro Universitário de Patos de Minas" (Unipam). The campaign was realized in a public place with free access to the population of both cities. All animals participated voluntarily in the event and were subjected to specific clinical examination of the mammary glands, as recommended by Feitosa (2014). The axillary and inguinal lymph node nodes of all dogs were palpated and evaluated for size, mobility, consistency, shape, surface regularity, and temperature. Additionally, the nipples of the mammary glands were massaged and drained to assess the possible presence of secretions (Cassali et al. 2014).

Inclusion criteria and epidemiological survey. The inclusion criteria established for the present study were female dogs of different weights and sizes, irrespective of breed and age, who voluntarily participated in the mammary cancer prevention campaign and were selected for participation (Fig.1).

The independent variables analyzed to establish possible predictors for the development of mammary tumors in dogs were: a) age (years); b) breed, classified as mixed and pure breeds; c) realization of ovariohysterectomy, OSH (no/yes); d) history of pseudo-pregnancy (no/yes); e) diet identification, being considered ration when the supply was exclusive of commercial rations and mixed when the diet consisted of homemade diet with or without commercial rations; f)

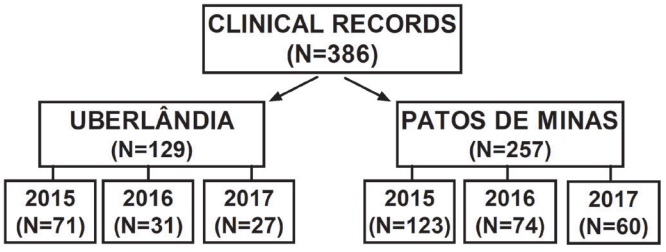

Fig.1. Flowchart of the dogs participating in the retrospective clinical study to determine risk factors and the occurrence of mammary tumors, at the "October Rosa Pets", event held in Uberlândia and Patos de Minas/MG, during 2015, 2016 and 2017.

history of contraceptive use (no/yes); g) parturition history (no/yes); h) overweight, evaluated based on the body condition score, being considered overweight female dogs presenting a score 4 or 5 on the scale of 1 to 5, as described (Edney & Smith 1986).

Information relative to the clinical evaluation of the mammary chain of all animals included: presence or absence of mammary gland enlargement, tumor characteristics (size, consistency, shape, surface, adherence, and ulceration), evaluation of regional lymph nodes (axillary and inguinal), presence or absence of secretion and location of the lesion (M1 = right or left cranial thoracic mammary gland, M2 = right or left caudal thoracic mammary gland, M3 = right or left cranial abdominal mammary gland, M4 = right or left caudal abdominal mammary gland and M5 = right or left inguinal mammary gland) (Cassali et al. 2014). To determine the size of the lesion, a pachymeter in millimetres, using the TNM clinical staging system (T = tumor, N = lymph node, M = metastasis) was used as described (Owen 1980, Cassali et al. 2014). The largest length of each lesion was measured (cm), after which the lesions were classified as T1 (when the tumor was less than 3cm in length), T2 (3 to 5cm) and T3 (>5cm). Only the largest tumor was evaluated in dogs with more than one mammary tumor.

Statistical analysis. The statistical analysis was done by using the binary logistic regression model, via the IBM SPSS 21.0 Statistical Program (IBM Corp., Armonk/NY, USA). To achieve this, the dependent variable (binary) was determined as the results (yes/no) for the presence of mammary tumors at during physical examination, and the following were considered as independent variables: a) quantitative: IN1- age (years); b) qualitative: IN2- breed (0-SRD/1-purebred), IN3- diet (0-ration/1-mixed), IN4- contraceptive usage (0-no/1-yes), IN5- ovariohysterectomy (0-yes/1-no), IN6- pseudo-pregnancy (0-no/1-yes), IN7- parturition history (0-yes/1-no), IN8- overweight (0-no/1-yes). The descriptive statistical analysis of the data was presented as the absolute and relative frequency of each of the independent variables (n=8) relative to the dependent variable (mammary tumor).

Chi-square and Fisher's exact tests were used to determine if there was any difference in the frequency of animals with mammary tumors between the evaluation period and the onset of OSH, respectively. Continuous variables were submitted to the Shapiro-Wilk test as normal distribution (P>0.05). Variables between the three years of the study were analyzed by the Mann Whitney test (nonparametric variables) and ANOVA (parametric variables). All análises were considered significant if p<0.05 and tendência if 0.05<p<0.1.

RESULTS

A total of 386 female dogs from the "October Rosa Pets" event was examined in the cities of Uberlândia and Patos de Minas/MG, during 2015 (n=194), 2016 (n=105), and 2017 (n=87). The epidemiological data of the evaluated population, based on the years of study, are provided in Table 1. Mammary tumors occurred in 23.6% (91/386) of the population evaluated. There was no difference (P=0.346) between the number of animals affected by mammary tumors lesions between 2015 (26.3%,), 2016 (22.8%), and 2017 (18.4%).

When the number of dogs (n=91) with mammary tumors was evaluated, 153 neoplastic growth were identified, with 42.8% (39/91) of dogs presented two or more mammary tumors. Most (60.1%, 92/153) of the neoplastic growths were located at the caudal (M4) and inguinal (M5) abdominal mammary glands. When the size of the mammary tumors was evaluated, in 78.0% (71/91) of the dogs evaluated contained neoplastic growths at T1, 16.5% (15/91) were located at T2, and 5.5% (5/91) at T3 (Fig.2). At least 15.4% (14/91) of the dogs evaluated had one of the regional lymph nodes enlarged on palpation.

From the total of 386 dogs used in this study, 96.4% (n=372) were eligible for inclusion in the binary logistic regression, since the clinical form of these animals contained all data of the independent variables, while dogs (n=14) with at least one of the missing information were eliminated. Eight

Table 1. Epidemiological data of female dogs treated at "October Rosa Pets" and presence of mammary gland tumors, according to the year of study, in the cities of Uberlândia and Patos de Minas/MG, during 2015, 2016 and 2017

	Evaluation year		
	2015 (n=194)	2016 (n=105)	2017 (n=87)
No. of breeds	20	21	19
Predominant breeds	Mixed breed (45.9%); Dachshund (11.8%); Shitzu (8.8%); Poodle (8.2%); Yorkshire (4.6%)	Mixed breed (43.8%); Poodle (9.5%); Shitzu (8.6%); Border Collie (6.7%); Pinscher (4.8%); Dachshund (4.8%)	Mixed Breed (45.9%); Shitzu (10.3%); Pinscher (8.0%); Yorkshire (4.6%); Border Collie (4.6%)
Predominant breeds in female dogs with mammary cancer	Mixed Breed (49.9%); Poodle (17.6%); Pinscher (13.7%); Basset Hound (5.9%); Shitzu (3.9%); Yorkshire (3.9%)	Mixed Breed (54.2%); Poodle (20.8%); Dachshund (8.3%); Boxer (4.2%), Basset Hound (4.2%), Pinscher (4.2%); Pitbull (4.2%)	Mixed Breed (81.2%); Poodle (6.2%), Chow Chow (6.2%); Cocker Spaniel (6.2%)
General age *	4a (P25%-75%=1.6-7)	4a (P25%-75%=1.5-8)	3.5a (P25%-75%=1-7)
Age - dogs with mammary tumors	8.2a (±3.24)	8.5a (±2.48)	8.79a (±4.53)
% Mammary tumors	26.3% (56/194)	22.8% (21/105)	18.4% (19/87)

Medians ($P_{25\text{-}75\%}$) for nonparametric distribution data, means (±SD) for parametric distribution data, and number (n) or percentage (%) for frequency data;
[a] similar superscript letters on the same line indicate no statistical difference (P> 0.05).

possible predictors or risk factors for the development of mammary tumors in female dogs were evaluated; the relative and absolute frequencies are given in Table 2.

The model containing all eight independent variables was significant [$\chi 2$ (G.L.8) = 163.134; p<0.001; $R2_{Nagelkerke}$ = 0.537]; the Hosmer and Lemeshow tests were equal to $\chi 2$ (G.L.8) = 7.011 (P=0.535). With this model three predictors or significant risk factors (P <0.05) for the development of mammary tumors in female dogs were identified (Table 3). The factors increase in age (years), non-realization of OSH, and overweight (score 4 and 5) were considered predictors or significant risk factors for the development of mammary tumors.

There was a tendency for the frequency (27.6%, 13/47) of mammary tumors to increase in dogs that were submitted to OSH after the third estrus cycle (P=0.0848 - Fisher's Exact Test), when compared with dogs submitted to OSH before the third estrus cycle (9.4%, 3/32).

DISCUSSION

This study reinforced the importance of mammary tumors in the canine population, using information from derived from the database of the prevention campaigns of mammary gland tumor in female dogs from the cities of Uberlândia and Patos de Minas, Minas Gerais, from 2015 to 2017. Although the animals participated voluntarily via adhesion of their tutors, this exploratory observational and case-dependent study, the relative frequency mammary tumors (23.6%) herein identified, was comparatively more elevated than 15.3% (17/111) described in survey done during a similar

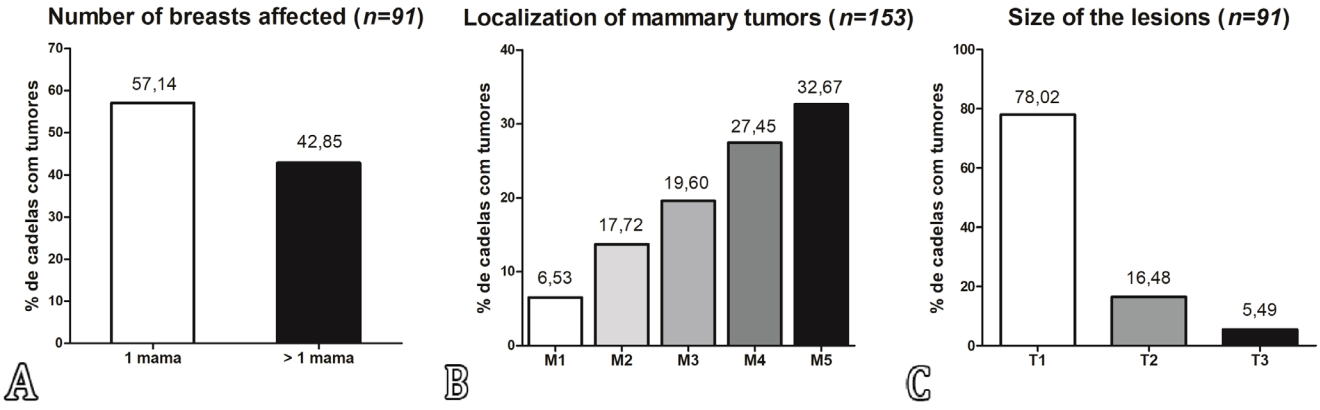

Fig.2. Female dogs with mammary tumors evaluated in the "October Rosa Pets" event, held in the city of Uberlândia and Patos de Minas/MG, during 2015, 2016 and 2017. (**A**) Number of breasts affected. (**B**) Localization of mammary tumors (M1 = right or left cranial thoracic mammary gland, M2 = right or left caudal thoracic mammary gland, M3 = right or left cranial abdominal mammary gland, M4 = right or left caudal abdominal mammary gland, and M5 = right or left inguinal mammary gland). (**C**) Size of the lesions (T1 = tumors <3cm in diameter, T2 = 3-5cm, and T3 = >5cm).

Table 2. Distribution of the relative and absolute frequencies of possible predictors or qualitative risk factors associated with the development of mammary neoplasms in female dogs during "October Rosa Pets" events in the cities of Uberlândia and Patos de Minas/MG, during 2015, 2016 and 2017

Independent variable - IV (qualitative)		Female dogs		
		Total n(%)	Tumors (n)	Frequency (%)
IV 2 - Type of breed	Mixed breed (0)	169 (45.4%)	49	28.9 %
	Pure breeds (1)*	203 (54.6%)	37	18.2 %
IV3 - Diet	Mixed (1)	117 (31.5%)	39	33.3 %
	Ration (0)	255 (68.5%)	47	18.4 %
IV4 - Contraceptive use	Yes (1)	26 (7%)	14	53.8 %
	No (0)	346 (93%)	72	20.8 %
IV 5 - Ovariohysterectomy	Yes (0)	79 (21.2%)	16	20.2 %
	No (1)	293 (78.8%)	70	23.9 %
IV 6 - History of false pregnancy	Yes (1)	101 (27.2%)	41	40.6 %
	No (0)	271 (72.8%)	45	16.6 %
IV 7 - History of parturition	Yes (0)	100 (26.9%)	39	39 %
	No (1)	272 (73.1%)	47	17.3 %
IV 8 - Overweight (4-5/5)	Yes (1)	68 (18.3%)	25	36.8 %
	No (0)	304 (81.7%)	61	20 %

The variable IV 1 = age did not enter because it is an independent quantitative variable; * (1) identifies the reference category used for the Binary Logistic Regression Test (P<0.05).

event in the city of Blumenau, Santa Catarina, in 2014 (Pascoli et al. 2017). Interestingly, the results herein described are similar to those identified in a population of 1,359 female dogs attended at the "Hospital Veterinário" of the "Universidade Federal da Bahia", where a prevalence of 24.7% was reported (Toríbio et al. 2012).

The prevalence of mammary tumors is usually based on studies derived from the general population of animals treated at Veterinary Hospitals or a specific population of dogs with cancer (De Nardi et al. 2002, Oliveira et al. 2003, Filho et al. 2010, Toríbio et al. 2012). However, studies describing the prevalence of mammary tumors in a canine population from a given region were not located when major databases were accessed, as is well established for breast tumors in humans. Consequently, the occurrence described in this paper highlights the importance of this pathology in the female canine population in the cities of Uberlândia and Patos de Minas and suggests that additional studies should be done to fill the reduced number of epidemiological studies that determined the actual prevalence of mammary tumors in the canine populations and not just in canine hospitals.

Among the risk factors evaluated, we must highlight importance of the age predictor, which increases the risk of breast tumor by 1.625 times annually; these results are in accordance with other studies that indicated a greater predisposition of elderly female dogs for the development of neoformations of the mammary gland (Rutteman et al. 2001, Egenvall et al. 2005, Stratmann et al. 2008, Sorenmo et al. 2009).

In addition to age, spaying was identified as a protective factor for the occurrence of mammary tumors, since uncastrated female dogs were 9,344 times more likely to have breast tumors compared with dogs submitted to OSH surgery. It was reported that early castration positively influences the reduction in the occurrence of mammary tumors pathology, with 99.5% reduction if performed prior to the first estrous cycle (Fonseca & Daleck 2000). However, there is no consensus on this marked reduction, since several authors (Kristiansen et al. 2016, Pascoli et al. 2017) did not identify a similar relationship. In the present study, it was observed that within the group of castrated female dogs, there was a tendency for the reduction of mammary tumors in those castrated before the third estrous cycle (9.4%), when compared to castrated female dogs after the third estrous (27.6%), indicating that early castration may be more effective in reducing the risk of mammary tumors in female dogs.

Additionally, OSH done even after the third estrous cycle in association with mastectomy, is associated with the reduced recurrence of benign breast lesions, and in preventing the occurrence of uterine and ovarian diseases (Kristiansen et al. 2013). Furthermore, the realization of OSH influences the survival rate of female dogs with mammary carcinoma (Sorenmo et al. 2000). In the present study, most (78.8%, 293/372) of the evaluated female dogs were not ovariohysterectomized, demonstrating the need for additional spaying campaigns in the canine population of the studied municipalities, considering the benefits associated with this procedure.

In this study, contraceptive usage and pseudo-gestations were not considered as risk factors for mammary carcinogenesis; similar results were described by Pascoli et al. (2017) who found no association between contraceptive use and mammary tumors, being different from the observations Fonseca & Daleck (2000) and Silva et al. (2004). Contraceptive usage was low (7%) in the study population herein described; reduced (11.6%) use of contraceptive was also described in female dogs and this tendency was more prevalent (41%) among less literate and low-income tutors (Toríbio et al. 2012).

It must be highlighted that exogenous progesterone stimulates growth hormone synthesis in the mammary gland with lobe-alveolar proliferation and consequent hyperplasia of myoepithelial and secretory elements, inducing the formation of benign or malignant nodules in young animals and may increase the number of estrogenic receptors (Silva et al. 2004). Additionally, estrogen and prolactin are involved in the growth of breast cancer (Fonseca & Daleck 2000). Receptors for estrogen, progesterone, androgens, prolactin, and epidermal growth factor have been demonstrated in female mammary tumors, with a possible relationship between the number of these receptors and the proliferative capacity of neoplastic cells being proposed (Silva et al. 2004).

During this study, overweighed animals had an increased risk (2.331x) for the development of tumor tumors, which was not influenced by the breed of the affected dog; breed was not considered as a risk factor based on the proposed grouping (mixed breed and pure breeds). These results corroborate with the proposed association between overweight and obesity with the occurrence of mammary tumors (Pascoli et al. 2017). Moreover, obesity was observed in 26.8% of the female dogs, and 80% of these obese and breast cancer patients received homemade diet (Toríbio et al. 2012). In the present study, the number of overweighed dogs with mammary tumor was

Table 3. Risk factors associated with the development of mammary neoplasms in female dogs during "October Rosa Pets" events in the cities of Uberlândia and Patos de Minas/MG, during 2015, 2016 and 2017

Risk factors	Coefficient	P-value	Exp (B) Odds ratio	95% CI
Constant	-5.758	0.000	0.003	
IV 1 - Age	0.486	0.000*	1.625	(1.448 - 1.824)
IV 2 - Breed (1)	0.370	0.284	1.447	(0.736 - 2.847)
IV 3 - Diet (1)	-0.339	0.333	0.713	(0.359 - 1.414)
IV 4 - Contraceptive use (1)	0.843	0.105	2.324	(0.838 - 6.443)
IV 5 - Ovariohysterectomy (1)	2.235	0.000*	9.344	(3.480 - 25.09)
IV 6 - False pregnancy (1)	0.029	0.936	1.029	(0.508 - 2.087)
IV 7 - Parturition (1)	-0.517	0.136	0.596	(0.302 - 1.178)
IV 8 - Overweight (>3/1-5) (1)	0.846	0.048*	2.331	(1.006 - 5.400)

CI = Confidence interval; * significant risk factor (P<0.05) for the development of mammary tumors in female dogs; (1) identifies the reference category used for the Binary Logistic Regression test (P<0.05) as described in Table 2.

higher (36.8%, 25/68) than that described by Toríbio et al. 2012. Additionally, the number of the overweighed dogs and with mammary tumor (64%, 16/25) that received a mixed diet was significantly (P=0.0261) higher than non-obese dogs and with tumors (37.7%, 23/61); these results are in accordance with the direct relationship observed between the type of food and the body condition of dogs with tumors (Toríbio et al. 2012).

Obesity in women may alter the expression of progesterone receptors, interferes with the course of the disease and may influence the survival rate of the patient (Sparano et al. 2012). Additionally, it was shown that obese women had high concentrations of oestrogen derived from transformed fatty tissue, with the transformation of androstenedione into estrone and later into oestrogen (Yoo et al. 2001). Since obesity is related to worse prognosis for breast cancer in women (Widschwendter et al. 2015, Choi et al. 2016), nutritional factors may be an important contributor towards the etiology of mammary tumors in dogs (Sonnenschein et al. 1991). According to Pérez Alenza et al. (1998), and as was demonstrated in this study, risk factors such as advanced age and obesity are associated with the development of canine mammary tumors. Furthermore, although the pathophysiological mechanisms are still poorly understood, there may be a relationship between obesity, inflammation and cancer (Argolo et al. 2015).

Relative to the size of the lesions, 78% of the dogs had smaller T1 nodules (<3cm), differing from Toríbio et al. (2012), who observed a higher prevalence (36.6%) of female dogs with stage III breast cancer; T3N0M0 (>0.5cm and no metastases in lymph nodes and distant organs). We propose that tutors from the cities studied seem concerned about the disease and joined the campaign in a positive way, showing up with their animals at an early stage of the disease.

Moreover, these results reinforce the importance of early detection of mammary tumors for a better prognosis and response to therapy, since there is a positive correlation between tumor size and malignancy, as well as the tumor size and the presence of distant metastases (Sorenmo 2003, Toríbio et al. 2012, Cassali et al. 2014).

The caudal and inguinal abdominal mammary glands were the most frequently affected (60.1%); similar results were previously described (Cassali et al. 2014, Pascoli et al. 2017), and may be related to the larger amount of parenchyma tissue within these mammary glands and, consequently, greater hormonal stimulation (Rutteman et al. 2001).

The regional lymph nodes were enlarged in 15.4% of the dogs evaluated. It must be highlighted that axillary and inguinal lymph nodes may be affected by mammary neoplasia, and are important routes for distant metastases, which occurs in 5 to 10% of dogs during the first three years after a diagnosis was established (Bloom et al. 1962, Cassali et al. 2014). Additionally, regional lymph node metastasis is an unfavorable prognostic factor (Hellmén et al. 1993, Toríbio et al. 2012). However, it cannot be confirmed in this study that lymph node enlargement was related to metastasis, since complementary evaluations such as cytology and/or histopathology were not performed.

In this study, the number of pure breed dogs was over representative (n=28), which made it difficult to evaluate each bread individually, which would have been the ideal situation. According to the division proposed in this study (mixed breed and pure breed), the breed of the affected dogs was not a risk factor for the development of mammary tumor. Although, a genetic predisposition for the development of mammary cancer in Poodles was proposed (Misdorp 2002), there may be no breed predisposition to mammary cancer (Cavalcanti & Cassali 2006), even though in certain breeds tumors are more frequently diagnosed.

The results obtained in this study reinforce the theory that age, obesity and non-realization of OSH influence the increased risk for mammary tumors in female dogs. However, the absence of studies designed directly at the canine population of a given region to determine the occurrence of mammary tumors in dogs and to verify the real impact of these factors. In human medicine, studies to determine the risks of cancer in Brazil are relevant public health issues, and there is a continuing need for research on this topic for the development of appropriate health policies aimed at cancer control in the country (Guerra et al. 2005).

Knowing the influence of the risk factors associated with the development of mammary tumors in female dogs in a given canine population, will facilitate the establishment of adequate and well designed-based strategies to prevent and reduce the occurrence of this neoplasm.

CONCLUSION

The occurrence of mammary tumors in the evaluated population was 23.6%, while age, overweight and non-realization of OSH were considered as risk factors for the development of mammary tumors in female dogs.

Acknowledgements.- The CAPES Foundation for the PhD scholarship provided to the first author (Santos T.R.). The authors thank "Liga Acadêmica de Oncologia Veterinária", "Centro Universitário de Patos de Minas", the "Programa de Educação Tutorial", "Faculdade de Medicina Veterinária", "Universidade Federal de Uberlândia", and the "Grupo de Estudos de Pequenos Animais", "Centro Universitário do Triângulo" for their commitment, participation and dedication in the realization of the Campaign "Outubro Rosa Pets".

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

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PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(6):474-478, June 2020
DOI: 10.1590/1678-5150-PVB-6512

Original Article
Small Animal Diseases

Evaluation of platelet-rich plasma gel as an angiogenesis-inducing agent in canine advancement skin flaps[1]

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ABSTRACT.- Aleixo G.A.S., Coelho M.C.O.C., Almeida T.L.A., Pereira M.F., Teixeira M.N., Andrade L.S.S., Bessa A.L.N.G. & Evêncio-Neto J. 2020. **Evaluation of platelet-rich plasma gel as an angiogenesis-inducing agent in canine advancement skin flaps.** *Pesquisa Veterinária Brasileira 40(6):474-478*. Departmento de Medicina Veterinária, Universidade Federal Rural de Pernambuco, Rua Dom Manuel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. E-mail: grazielle@yahoo.com

This work aimed to evaluate the effect of platelet-rich plasma (PRP) on advancement skin flaps in dogs regarding improvement of vascularization, with focus on increasing its viable area, since there are reports that it is a potential angiogenesis stimulator. The experimental group was composed of eight adult bitches, in which two advancement skin flaps were made in the ventral abdominal region. No product was applied in the control flap (CF), while PRP was used in the contralateral flap, called treated flap (TF). The areas were clinically evaluated every two days until the 7th postoperative day regarding skin color and presence of necrosis. At 10 days, both flaps were removed and submitted to histological examination and blood vessel morphometry. The vessels counted in each group were statistically analyzed by the F-test at 1% probability. Results showed no significant difference in macroscopic changes in the wound, or CF and TF vascularization, thus suggesting that PRP gel did not improve advancement skin flap angiogenesis in bitches under the experimental conditions in which this research was developed.

INDEX TERMS: Platelet-rich plasma gel, angiogenesis, canine, skin flap, platelet gel, growth factors, dogs.

RESUMO.- [**Avaliação do gel de plasma rico em plaquetas como agente indutor da angiogênese em flapes cutâneos de avanço em cães.**] Objetivou-se com o presente artigo avaliar a ação angiogênica do gel de plasma rico em plaquetas (PRP) em flapes cutâneos de avanço em animais da espécie canina, visando aumentar a viabilidade da pele após o procedimento, uma vez que existem relatos de que o produto é um potente estimulador da angiogênese. O grupo experimental foi composto por oito cadelas adultas, onde foram confeccionados dois flapes de avanço (de padrão subdérmico) na região abdominal ventral. Em um dos flapes, considerado controle (FC) não foi aplicado nenhum produto, enquanto que no flape contralateral, denominado tratado (FT), foi usado o gel de PRP. As áreas foram macroscopicamente avaliadas a cada dois dias até o 7º dia de pós-operatório em relação à coloração da pele e presença de área de necrose, e com 10 dias ambos os flapes foram coletados por biópsia e submetidos ao exame histológico e morfometria dos vasos sanguíneos. Os vasos contados em cada grupo foram estatisticamente analisados pelo teste de F ao nível de 1% de probabilidades. Os resultados demonstraram que não houve diferença significativa nas alterações macroscópicas das feridas e na morfometria vascular dos FC e FT, sugerindo dessa maneira que dentro das condições experimentais nas quais a pesquisa foi executada, que o gel de PRP não incrementou a angiogênese de flapes de avanço em cadelas.

TERMOS DE INDEXAÇÃO: Plasma rico em plaquetas, indução, angiogênese, flapes cutâneos, cães, retalhos cutâneos, gel de plaquetas, fatores de crescimento, neoangiogênese, caninos.

[1] Received on February 6, 2020.
Accepted for publication February 24, 2020.

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INTRODUCTION

Flaps consist of a skin segment transferred from a donor site to a recipient site to cover flaws while keeping a connection to the former through a pedicle that preserves its blood irrigation (MacPhail 2014). Since it has its own vascularization and does not depend on the vascularization of the recipient area, the blood from its pedicle is essential for its survival (Estevão et al. 2013).

Skin flaps have the advantage of providing an immediate cover for a region, which decreases the healing period (MacPhail 2014, Ober et al. 2019) and treatment expenses, also providing excellent aesthetic and functional results (Biondo-Simões et al. 2000).

Flap viability is highly dependent on its vascularization (Nardi et al. 2016); therefore, the use of substances aiming to improve its irrigation is a relatively common practice (Estevão et al. 2009).

Platelet-rich plasma (PRP) derived from autologous blood is defined as a plasma volume with platelet concentration higher than physiological levels, obtained via centrifugation (Marx 2004, Silva et al. 2007, Garcez et al. 2016). The therapeutic use of PRP has been described since 1990 (Floryan & Berghoff 2004) as contributing in soft and hard tissue healing (Arora et al. 2009).

The product is applied on the wound as a gel or solution (Martinez-Zapata et al. 2012) and at that moment, it deposits growth factors (GFs), which are important for tissue recovery, since they promote angiogenesis, mitogenesis, chemotaxis (Maia & Souza 2009), and cellular differentiation (Vendruscolo et al. 2012). Approximately seven GFs have already been identified (Marx 2004); however, the only ones proven to participate in vascular formation are: platelet-derived growth factor (PDGF), transforming growth factor beta (TGFß), epithelial growth factor (EGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF) (Vendramin et al. 2010).

Vascular endothelial growth factor also known as vascular permeability factor (VPF), is one of the most important factors for stimulation of "*in vivo*" angiogenesis through a complex process which involves endothelial cells mitosis. It also has the ability to increase the permeability of microvessels in the skin, peritoneal wall, mesentery, and diaphragm (Bao et al. 2009). Platelet-derived growth factor acts by stimulating mitosis in the vascular endothelium cells (Moreira et al. 2008) and the smooth muscle cells of blood vessels, since angiogenesis is one of its main functions. The EGF also demonstrates mitogenic potential in endothelial cells, while FGF stimulates endothelial and smooth muscle cells (Litwack 2018).

Despite many papers describing that PRP application on wounds has the potential to increase tissue recovery, it is still not widely used and there are many controversies in the literature regarding the benefits of its use (Arora et al. 2009, Vendruscolo et al. 2012), especially because there are numerous protocols available (Vendruscolo et al. 2012).

Based on such information, the present experiment aimed to evaluate the use of platelet-rich plasma gel for stimulation of angiogenesis to consequently improve the integration of experimentally produced advancement skin flaps in dogs.

MATERIALS AND METHODS

Prior to start of the research, the project was submitted to and approved by the Ethics Commission on Animal Use (CEUA) of the "Universidade Federal Rural de Pernambuco" (UFRPE) (Protocol No. 23082.005174).

In order to obtain a more homogeneous sample and, consequently, decrease variability and allow a lower number of animals to be used, only medium-sized (between 10 and 15kg), adult (two to five years old) and clinically healthy females were used.

Three weeks before the date chosen for the surgical procedure, the eight selected bitches were placed in the kennel of the "Departmento de Medicina Veterinária" (DMV), where they remained in individual stalls for environmental and handling adaptation. Their diet consisted of commercial dog food given twice daily and water *ad libitum*.

After a 12-hour food fasting and 4-hour water fasting period, and approximately three hours before the surgical procedure, blood samples were collected for producing the PRP gel which would be used in the skin flap. Blood was collected from the cephalic or saphenous veins using a vacuum collection system. Four 4.5mL glass tubes containing sodium citrate as an anti-coagulant (for PRP production) and one 2mL plastic tube with Ethylenediaminetetraacetic acid (EDTA) (for the complete blood count - CBC, and platelet count from the total blood) were filled.

The four tubes of blood used to produce PRP were centrifuged at 104.83g for 10 minutes in an ordinary laboratory centrifuge (Baby I Model 206, Fanem). After the first centrifugation, all plasma and buffy coat were pipetted from each tube using an automated precision pipette and sterile tips, which were transferred to four other sterile tubes for another centrifugation at 186.37g for 10 minutes. Later, 80% of the supernatant plasma was discarded and the rest was homogenized to resuspend the remaining platelets (platelet button), obtaining the PRP. One of the tubes was separated to perform a quantitative evaluation of the PRP. This was done by direct count and morphological evaluation of the platelets via microscopic examination of a blood smear.

From the blood collected in the tube with EDTA, complete blood count and platelet count were obtained to evaluate the patient's health for surgery and to determine the platelet count, which would serve as reference for the value found in the PRP.

The anesthetic protocol used was composed by 0.1mg/kg of acepromazine and 1mg/kg of tramadol hydrochloride administered intramuscularly (IM) as pre-anesthetic medication. Later, 0.1mg/kg of meloxicam and 5mg/kg of enrofloxacin were administered subcutaneously (SC). After moving the patient to the operating room, the anesthetic induction was carried out with 4mg/kg of propofol, intravenously (IV), followed by endotracheal intubation for anesthesia maintenance with isoflurane via a semi-closed circuit for inhalation anesthesia. The patient received sodium lactate ringer, IV, throughout the surgical procedure.

Two flaps with 4cm in length and 1cm in width were made in the ventral abdominal region: one cranial (treated flap) and one caudal. With a pair of surgical scissors, 1cm of tissue was removed from the distal extremity of each flap to create an advancement flap. Afterwards, simple interrupted stitches were done with n. 3-0 monofilament nylon thread leaving only an opening big enough to place the PRP gel between the treated flap (TF) and its receptive field.

Before placing the PRP on the surgical bed, the three remaining tubes containing the product were homogenized and deposited in sterile stainless steel bowls where thromboplastin was added in a 2:1 proportion (1mL of PRP per 0.5mL of thromboplastin) in order to activate the platelets. No product was applied between the caudal flap and its receptive field and, consequently, this flap was considered the control (CF).

Throughout the post-operative period, the dogs were prescribed 5mg/kg of enrofloxacin antibiotic once a day orally (P.O.) and 0.1mg/kg of meloxicam (anti-inflammatory) every 24 hours P.O. for seven and four days, respectively. An Elizabethan collar and surgical garment were placed on the dogs throughout the post-operative stay in order to avoid self-inflicted injuries.

On the first day after surgery (D_1), the first clinical evaluation of CF and TF was made and, afterwards, the bandage was changed and flaps were clinically examined every two days until the seventh day (D_3, D_5, and D_7).

At each time (D_1, D_3, D_5, and D_7), both flaps were photographed with a digital camera for macroscopic recording of the region and further calculation of the necrotic area using Imagelab software. The pictures were taken with the camera always at a 5cm focal distance between the lens and the region to be photographed.

On day 10 (D_{10}), biopsy of both flaps (CF and TF) were done, and they were submitted to histological analysis. Tissue characteristics, such as the presence of necrosis and angiogenesis, were observed by examination of hematoxylin-eosin (HE) stained slides. Morphometry of blood vessels was performed by adapting a 100-squares grid reticle to the microscope lens. In the histological cut, three fields were selected and, in each area, the vessels found within 10 squares of the central column of the grid reticle were counted.

The experiment was arranged in a completely randomized design as a function of the homogeneity between the animals, and the statistical analyses for the vessel count in CF and TF were carried out by the F test at 1% probability using the Systat 10 software (demo version).

RESULTS

Macroscopically, the necrotic area was evaluated by skin color, where the areas that were initially pale from the third post-operative day, were continuously evolving into a purple color and, on the seventh day, to a black color. Necrosis was identified in three patients (3/8) in both groups; however, the area was restricted to the distal extremity of the flap. The necrotic regions were calculated by the Imagelab 2000 software (Table 1).

The necrotic area identified clinically and by the Imagelab 2000 software on the distal extremity of three flaps from each group was confirmed by histology, and the absence of cell nucleus were observed, compromising the epidermis and papillary dermis.

Microscopically, the presence and intensity of new vessel formation were similar in both groups. The analysis of variance for morphometric counting, performed via F test, presented lower results than the tabulated ones, thus showing that there are no statistically significant differences between the control and treated groups (Table 2).

Table 1. Necrotic areas of the control and treated flaps, calculated using Imagelab® 2000 software

Patient	Necrotic area	
	Control flap	Treated flap
1	0.00%	0.00%
2	0.00%	0.00%
3	14.00%	17.77%
4	0.00%	0.00%
5	0.00%	0.00%
6	10.00%	14.51%
7	10.48%	11.67%
8	0.00%	0.00%

DISCUSSION

Experimental research in dogs aiming to evaluate integration of skin flaps are rare. Since most researchers used rats or other laboratory animals as an experimental model (Biondo-Simões et al. 2000, Almeida et al. 2004, Estevão et al. 2009, Pazzini et al. 2016, Kemper et al. 2018), finding scientific papers to conduct a comparative analysis with the results obtained herein was a limiting factor.

Determining the total area that would be necessary for maintaining flap irrigation, focusing on improving tissue viability, is still a great challenge for surgeons (Almeida et al. 2004). In this study, it was decided to exceed the limits recommended by most authors using a 4:1 proportion between length and height by removing 1cm from the distal margin of the skin flap, in order to evaluate the tissue viability and vascularization under the least favorable tension conditions and venous return, as described by Estevão et al. (2009).

Studies have been developed with the aim of increasing the survival rate of skin flaps by enhancing its vascularization. Since several authors (Moreira et al. 2008, De Rossi et al. 2009, Bosch et al. 2011, Pazzini et al. 2016) have already reported the angiogenesis potential of the GFs present in PRP, it was chosen to test this product's efficacy on advancement skin flaps in dogs.

After production of PRP, it was observed that platelets were well preserved, since they did not present morphological alteration upon microscopic examination, and the material acted quickly upon stimulation of the agonist, creating a consistent clot only in a few seconds, just as observed by Pazzini et al. (2016). According to Yung et al. (2017), a good platelet function is retained during the PRP processing if there is a satisfactory response from platelets after adding the clotting factor, with resulting clot formation.

Based on the platelet count obtained on total blood and PRP (Table 3), it was observed that it was possible to provide a four to five times increase on the count, obtaining the concentration

Table 2. Mean number of vessels per mm² identified in the control and treated flaps

Treatments	Means
Control flap (CF)	21.125 per mm²
Treated flap (TF)	28.125 per mm²

Table 3. Platelet count in total blood and in PRP of all patients

Patient	Platelet count	
	Total blood	PRP
1	218.000/µL	1.197.000/µL
2	304.000/µL	1.517.000/µL
3	204.000/µL	1.078.000/µL
4	241.000/µL	1.114.000/µL
5	246.000/µL	1.395.000/µL
6	237.000/µL	1.129.000/µL
7	287.000/µL	1.345.000/µL
8	217.000/µL	1.293.000/µL

PRP = platelet-rich plasma. The calculation of sample sufficiency demonstrated that the number of patients used was adequate for performing the experiment.

considered as therapeutic by many researchers, such as Marx (2004), Whitlow et al. (2008), and Pazzini et al. (2016).

Macroscopic observation of the flap for the presence of necrosis was performed continuously every two days until the 7th day from the first 24-hours evaluation, because, according to Pavletic (2007), periodical observations are necessary to determine survival of a flap on the days following transplant. Tobias (2017) describes that flap necrosis may not be evident until six days after surgery, making it necessary to wait for a week to identify viable and non-viable areas.

Factors such as infection, compression and tension may contribute to development of necrosis in a flap, though tissue irrigation is the main cause, once vessels are responsible for local nutrition (Nardi et al. 2016). The cause of necrosis in all flaps was attributed to improper vascularization, since no signs of infection were observed and, if excessive tension had been the cause, some sutures would have probably broken and areas of dehiscence would have been identified. The flaps were also not submitted to excessive compression during the post-operative period, since the wound was protected via a bandage composed by a single layer of hypoallergenic tape and non-compressive post-surgical garment. Moreover, according Estevão et al. (2013), the setting-in of ischemia and subsequent necrosis depends on the blood supply provided by the blood vessels in the pedicle.

In the control and treated groups, in all evaluations, no odor or dehiscence was observed, even on necrotic regions as previously mentioned, though dehiscence is one of the main complications related to the use of skin flaps (Nardi et al. 2016). This probably happened because the necrosis was more superficial, compromising only the epidermis and the papillary dermis. It is believed that the force exerted by the reticular dermis was enough to keep the flap and the edges of the surgical wound closed by the stitches since this layer presents thick collagen and elastic fiber bundles (Kierszenbaum & Tres 2016).

Aside from the macroscopic evaluations, which are considered more subjective because they are determined according to the evaluator's observation, microscopic analysis was conducted in order to substantiate or complement the information from clinical examinations. In the work carried out by Suaid et al. (2007), evaluating PRP action in grafts for covering gingival retraction in dogs, the evaluations were based only on clinical observations, nevertheless, the same authors mentioned that it is not enough to analyze the action of the platelet gel, requiring histological examination. In another study conducted by Kemper et al. (2018), where histopathological analyses were performed to evaluate the use of PRP in rabbit skin grafts, the results showed that there were no statistically significant differences between the treated and control groups considering the average numbers of vessels, corroborating our findings, despite the fact they evaluated skin grafts and not flaps.

In spite of the promising results cited by some researches using PRP activated by thromboplastin, no differences were found in the use of the product when compared with CF, which agrees with results found by Silva et al. (2007), Gurgel et al. (2007), Suaid et al. (2007), Martinez-Zapata et al. (2012), and Kemper et al. (2018).

An important factor to be considered regarding papers that presented favorable effects of PRP on tissue recovery, more specifically, on angiogenesis, is that most of them base their satisfactory results only on clinical analyses, especially with experiments carried out in humans. However, it is recommended that histology should be an essential part of evaluating the effectiveness of PRP, since many alterations, such as intensity of neovascularization, can only be identified via microscopic analysis.

CONCLUSION

Within the conditions in which the experiment was conducted, the results obtained made it possible to conclude that platelet-rich plasma (PRP) gel did not improve angiogenesis or, consequently, the viability of advancement skin flaps in dogs.

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

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PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(6):479-483, June 2020
DOI: 10.1590/1678-5150-PVB-6520

Original Article
Wildlife Medicine

Pestivirus spillover effect: molecular detection of bovine viral diarrhea virus in domestic and feral pigs[1]

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ABSTRACT.- Aniţă D.C., Popa E., Aniţă A., Oşlobanu L.E. & Savuţa G. 2020. **Pestivirus spillover effect: molecular detection of bovine viral diarrhea virus in domestic and feral pigs.** *Pesquisa Veterinária Brasileira 40(6):479-483.* Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Iasi, 8 Mihail Sadoveanu Alley, 700489, Iaşi, Romania. E-mail: aeanita@uaiasi.ro

Pestivirus infections are important in the livestock industries, with infection occurring in cattle, sheep and pigs. The *Pestivirus* genus of the family *Flaviviridae*, includes four recognized species: bovine viral diarrhea virus 1 (BVDV-1), bovine viral diarrhea virus 2 (BVDV-2), border disease virus (BDV), and classical swine fever virus (CSFV). All pestivirus species can infect pigs, therefore accurate and specific pestivirus detection and differentiation is of great importance to assure control measures in swine populations. The aim of the study was the molecular detection of different pestiviruses in domestic and feral pigs. A total of 527 samples (92 pigs and 435 wild boars) were tested for pestiviruses detection using molecular assays. Eleven positive samples (6 wild boars and 5 domestic pigs) were identified using panpestivirus primers targeting the 5'- UTR region of the pestivirus RNA genome. Further all the positive samples were sequentially tested for detection of CSFV, BVDV-1 and BVDV-2 using specific primers. All RNAs were identified as positives for BVDV-1 and no amplification signals were obtained from BVDV-2 and CSFV. The current detection of BVDV-1 in clinical swine specimens highlights the important risk factor of swine population as reservoir and consequently carrier for BVDV.

INDEX TERMS: Pestivirus, molecular detection, bovine viral diarrhea virus, domestic pigs, feral pigs, BVDV, pigs, wild boar, PCR, wildlife animals.

RESUMO.- [**Efeito *spillover* de pestivírus: detecção molecular do vírus da diarreia viral bovina em suínos domésticos e javalis.**] As infecções por pestivírus são importantes nas indústrias pecuárias, com infecções em bovinos, ovinos e suínos. O gênero *Pestivirus* da família *Flaviviridae* inclui quatro espécies reconhecidas: vírus da diarreia viral bovina 1 (BVDV-1), vírus da diarreia viral bovina 2 (BVDV-2), vírus da doença de fronteira (VDF) e vírus da peste suína clássica (VPSC). Todas as espécies de pestivírus podem infectar porcos, portanto a detecção e diferenciação precisas e específicas de pestivírus são de grande importância para garantir medidas de controle nas populações suínas. O objetivo do estudo foi a detecção molecular de diferentes pestivírus em suínos domésticos e javali. Um total de 527 amostras (92 porcos e 435 javalis) foram testados para detecção de pestivírus usando ensaios moleculares. Onze amostras positivas (6 javalis e 5 porcos domésticos) foram identificadas usando iniciadores de panpestivírus visando a região 5'-UTR do genoma do RNA do pestivírus. Além disso, todas as amostras positivas foram testadas sequencialmente para detecção de VPSC, BVDV-1 e BVDV-2 usando iniciadores específicos. Todos os RNAs foram identificados como positivos para BVDV-1 e nenhum sinal de amplificação foi obtido do BVDV-2 e CSFV. A detecção atual do BVDV-1 em amostras clínicas de suínos destaca o importante fator de risco da população suína como reservatório e consequentemente portador do BVDV.

TERMOS DE INDEXAÇÃO: Pestivírus, detecção molecular, vírus da diarreia viral bovina, suínos domésticos, javalis, BVDV, porco, javali, PCR, suínos, animais selvagens.

[1] Received on October 21, 2019.
Accepted for publication on February 19, 2020.
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INTRODUCTION

The genus *Pestivirus* in the family *Flaviviridae* currently comprises four species, bovine viral diarrhea virus 1 (BVDV-1), bovine viral diarrhea virus 2 (BVDV-2), border disease virus (BDV) and classical swine fever virus (CSFV) (Simmonds et al. 2017). Pestiviruses infect pigs and ruminants with significant economic impact (Moennig & Becher 2015) but have also been detected in wild ruminants and wild boar. Pestiviruses are sub-divided according to their host but serological cross-reactivity has been demonstrated between all pestiviruses (Wieringa-Jelsma et al. 2006). While CSFV is restricted to pigs, the other Pestivirus species have been recovered from hosts of a greater variety. There are less clear-cut differences in host range. BVDV-1, BVDV-2 and BDV can infect a wide range of ruminants, including cattle, sheep, goats, and a number of wild ruminants as well as pigs. A number of atypical pestiviruses have been described originating from giraffe, reindeer, pronghorn antelope and from fetal calf serum ("HoBi" virus) (Liu et al. 2009). Recently, another pestivirus, "Bungowannah", was isolated from pigs (Kirkland et al. 2007). Also, newly identified atypical porcine pestivirus (APPV) was demonstrated to be the causative agent of the neurological disorder "congenital tremor" in newborn piglets (Jin et al. 2017).

Pestiviruses are highly variable RNA viruses, their number increasing constantly. RNA viruses are characterized by high mutation rates and the lack of proofreading activity of RNA-dependent RNA polymerases is believed to be the main driving force for the generation of altered genomic sequences. Evolution of pestiviruses occurs by point mutation and by homologous recombination within species (Weber et al. 2015). A growing number of novel pestiviruses has been discovered in domestic and wild species in the last two decades (Smith et al. 2017). Members of the different Pestivirus species can be distinguished from each other by the presence of sequence motifs in the 5′-untranslated region that are involved in RNA secondary structures (Giangaspero & Harasawa 2011). The secondary structure of the 5'-UTR is divided into four domains, the last one encompassing the two thirds in the 3' region of the 5'-UTR, is responsible for translational, transcriptional and replicational events in pestiviruses. Therefore, random mutations at the 5'-UTR have a high probability of incompatibility with viral survival (Giangaspero et al. 2008).

MATERIALS AND METHODS

During 2014-2016, tissue samples from 527 pigs: 92 backyard pigs respectively 435 wild boars were collected. From each animal were sampled spleen, tonsils and lymph nodes (mesenteric and retropharyngeal), followed by automatically disruption of 100mg of pooled tissue samples suspended in 900µl DPBS 1X (Gibco, Paisley, Scotland, UK) using MagNA Lyser (Roche, Mannheim, Germany). Total RNA was extracted from 200µl supernatant using RNeasy Mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions.

Reverse transcription and PCR amplification of the viral RNA were done in one-step using the One-step RT-PCR Kit (QIAGEN GmbH, Hilden, Germany). The 5'UTR genes from the genome of pestivirus were PCR amplified. The 5'UTR genes from the pestivirus genome were amplified using the primers listed in the table below (Table 1).

At the beginning one-step RT-PCR was performed for all RNAs using described panpestivirus primers 324 and 326 (Vilcek et al. 1994). The amplification was carried out with 10µl RNA in a total volume of 50µl. Sequentially for detection of CSFV, BVDV -1 and BVDV-2 were used three sets of primers for specific identification. The amplification was carried out in a total volume of 25µl comprised of 2µl RNA, OneStep RT-PCR (QIAGEN GmbH, Hilden, Germany) mix enzyme, 0,1µM of each primer, 5x Buffer OneStep RT-PCR (QIAGEN GmbH, Hilden, Germany), 10mM dNTP (QIAGEN GmbH, Hilden, Germany), 0,1µl of 40U/µl RNAsin ribonuclease inhibitor. The thermal profile used consisted of: revers transcription 30 min at 50°C, initial PCR activation step 15 min at 95°C, 45 cycles of the following; 94°C for 45 sec, variable annealing temperature depending of the primer set used (56°C for panpestivirus primers/58°C for BVDV-1/54°C for BVDV-2 and CSFV) for 45 sec and extension at 72°C for 45 sec; and a final extension at 72°C for 7 min. Each amplification was validated by the use of three positive controls: positive RNA for CSFV, BVDV-1 and BVDV-2. The commercial live vaccines containing BVDV-1 and BVDV-2 (Bovela® Boehringer Ingelheim, Germany) and classical swine fever virus (Rompestivac® Romvac, Romania) were used as positive controls for the RT-PCR reactions. The negative control used in order to check for possible contamination of the reagents was nuclease free water. The PCR products of 5′-UTR genes were separated by gel electrophoresis in 1.5% agarose gel stained with ethidium bromide. The fragments were visualized using a UV transilluminator (GelDoc BioRad, Marnes-la-Coquette, France). Positive amplicons were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The DNA sequencing was performed at BaseClear B.V. (Leiden, The Netherlands). Nucleotide sequences were analysed and edited individually using Bioedit soft. Afterwards, the obtained sequences and sequences available in GenBank were aligned using Clustal W and the evolutionary tree was generated with MEGA 7 using the Neighbor-Joining method with a total of 1000 replications on the bootstrap.

The study was performed in accordance with the Directive 2010/63/EU regarding animal handling ethical guidelines. Animal samples (wild boar tissues samples) were collected during the

Table 1. Primer sequences and sizes of one step RT-PCR products

Name	Sequence of the primers	PCR fragment	Reference
324 (forward)	5'- ATGCCCWTAGTAGGACTAGCA - 3'	288 bp	Vilcek et al. 1994
326 (reverse)	5'- TCA ACTCCATGTGCCATGTAC - 3'		
CSFV (forward)	5' – CTAGCCATGCCCWYAGTAGG - 3	420 bp	Greiser-Wilke et al. 1998
CSFV-R (reverse)	5' - CAGCTTCARYGTTGATTGT - 3'		
BVDV-1 (forward)	5' – GGTAGCAACAGTGGTGAG-3'	211 bp	Tao et al. 2013
BVDV-1 (reverse)	5' – GTAGCAATACAGTGGGCC-3'		
BVDV-2 (forward)	5' - CGACACTCCATTAGTTGAGG -3'	117 bp	Tao et al. 2013
BVDV-2 (reverse)	5' – GTCCATAACGCCACGAATAG -3'		

hunting seasons and according with EU and national low: The Law of hunting and protection of hunting fund no. 407/2006 and amendment Law no. 149/2015.

RESULTS

The pestiviruses genome includes a single, large, open reading frame (ORF) which encodes a polyprotein of approximately 3,900 amino acids. The ORF is flanked, at each end, by untranslated regions (UTRs). The genome is uncapped at its 5'-terminus and the 5'-UTR contains an internal ribosomal entry site (IRES) which directs cap-independent translation initiation on the viral RNA (Fletcher & Jackson 2002). High conservation of 5'UTR sequences is related to IRES formation. Using panpestivirus primers targeting the 5'-UTR region of the pestivirus RNA genome 11 positive animals out of 527 tested (6 wild boars and 5 backyard pigs) were detected (Table 2).

Further all RNAs were sequentially tested for the detection of CSFV, BVDV-1 and BVDV-2 using specific primers. All panpestivirus positives RNAs were identified as positives for BVDV-1 and no amplification signals were obtained from BVDV-2 and CSFV. The 5'-UTR (360-390 bases) is highly conserved among all members within the genus *Pestivirus*, yet use of specific primers for amplification of the 5'-UTR genes can readily distinguish BVDV-1 isolates from BVDV-2, CSFV and BDV isolates. (Ridpath & Bolin 1998, Flores et al. 2002). To characterize BVDV-1 strains, the positive products were sequenced and analyzed. The characterization of viral strains circulating in swine population analyzed in this study is based on a 288bp fragment from 5'-UTR region. Two out of 11 positive samples were successfully sequenced. The sequences obtained have a 98% nucleotide sequences homology with each other and clustered together based on alignment with the best hits of our sequences in GenBank. Phylogenetic analysis showed also that the Romanian sequences formed a branch with a BVDV-1 strain from France and one isolated in Turkey (Fig.1).

DISCUSSION

Since different pestiviruses are closely related, both immunologically and genetically, the ruminant's pestivirus infections in swine can result into a false diagnosis. Bovine viral diarrhea is an important infectious disease of cattle due to the specific nature of virus epidemiology and pathogenesis together with economical losses that follows a herd infection. BVDV transmission to pigs usually requires direct contact with cattle or sheep. Other possible methods by which infection may be transmitted include exposure of swine to ruminant faeces or feeding of un-pasteurised cow's milk. The three prerequisites for animal infectious disease (the source of infection, transmission route and sensitive animals) must be well understood in order to control and eradicate pestiviruses.

The objective of this work was to evaluate the role of swine (domestic and feral) as reservoir of pestiviruses in general and bovine viral diarrhea virus in particular, persistent infection being described in pigs (Terpstra & Wensvoor 1997). Thus 527 pigs and wild boars were tested for pestivirus using one step RT-PCR reaction with 324 and 326 panpestivirus primers (Fig.2). The eleven positive RNAs were sequentially tested for detection of CSFV, BVDV-1 and BVDV-2 using specific primers. As a result, for all samples were obtained specific amplification signals (211bp PCR product) to BVDV-1 (Fig.3). No positive PCR product was detected after amplification with specific primers for CSFV and BVDV-2.

These results are supported by epidemiological data, infection with BVDV in cattle being wide spread in Romania, seroprevalence rates varying from 20% to 100% depending on the region and the size of the herd (Aniţă et al. 2008). The presence of the virus has been detected previously using direct immunofluorescence method in 55.5% (15 out of 27) young unvaccinated bovines (10-16 month of age) raised in household system (Aniţă et al. 2009). Moreover, BVDV was detected by real-time RT-PCR in 28.12% (9 out of 32) Romanian wild boars in 2010 (Turcitu et al. 2010). The

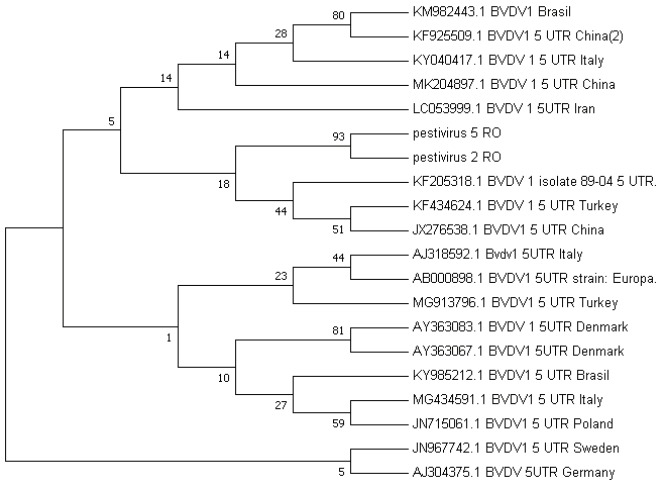

Fig.1. Phylogenetic tree of BVDV-1 sequences. Evolutionary analyses were conducted in MEGA 7 with the bootstrap consensus tree inferred from 1000 replicates. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei 1987). The evolutionary distances were computed using the Tamura 3-parameter method and are in the units of the number of base substitutions per site (Tamura 1992).

Table 2. Results of the one step RT-PCR using panpestivirus primers

County	No. of animals tested		Animals positive for pestivirus RNA	
	Wild boars	Domestic pigs	Wild boars	Domestic pigs
			% (95% CI)	% (95% CI)
Iaşi	150	25	1.33% (-0.50-3.17)	8% (-2.63-18.63)
Vaslui	85	15	0	0
Bacău	200	52	2% (0.06-3.94)	5.8% (-0.57-12.11)
Total	432	92	1.4% (0.29-2.49)	5.4% (0.80-10.07)

CI = Confidence interval.

negative results on CSFV were expected considering that since 2013, Romania was included in the disease-free countries list for classical swine fever. The last outbreak of CSF was diagnosed on October 2007 and resolved in January 2008. No new case of infection has been registered since 2007. Control and eradication programmes consisted in vaccination against CSF in domestic pigs until November 2009, respectively until December 2011 in wild boars.

Since the first report of ruminant pestivirus detection in naturally infected pigs in 1973 (Fernelius et al. 1973), cases of BVDV infection in swine were described worldwide. Higher rates of seroconversion were detected in pigs located near cattle farms (Paton et al. 1992). In the Netherlands, the BVD prevalence rate was 0.42% in finishing pigs and 2.5% for sows (Loeffen et al. 2009). In Poland, BVDV antibodies were detected in 11 (68.75%) out of the 16 provinces, and the seroprevalence varied from 0.1% to 1.04% (Lipowski 2014). Studies on BVDV-1 revealed a high prevalence (137 BVDV- positive samples out of 511) in Chinese swine herds were pigs exhibited clinical symptoms (Deng et al. 2012). In Brazil the presence of BVDV infection in domestic pigs has been reported by Almeida et al. (2017) by assessment of BVDV antibodies prevalence in pigs from rearing farms.

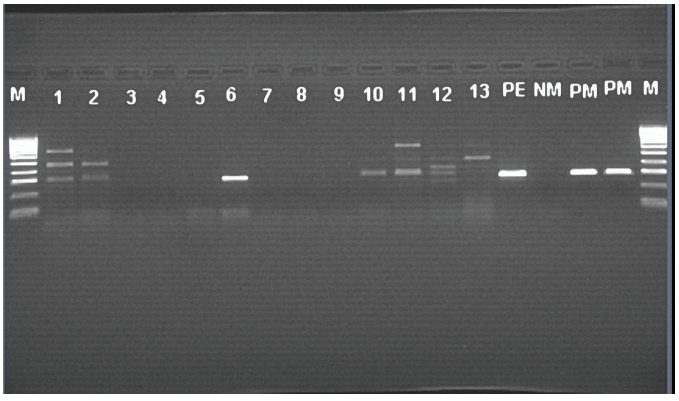

Fig.2. PCR products for one step RT-PCR amplification with panpestivirus primers of swine RNA samples from Iaşi County. M = molecular weight marker XIV (Roche), 1 to 13 = swine RNA, PE = positive control (CSFV), NM = negative control, PM = positive control (BVDV-1 and BVDV-2).

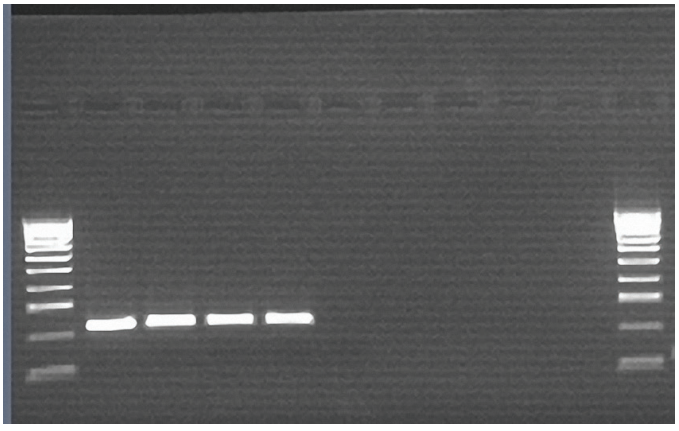

Fig.3. PCR products for one step RT-PCR amplification for BVDV-1 of the four pestivirus positive samples from Iaşi County. M = molecular weight marker XIV (Roche).

Our data together with reviewed literature data suggests that wild boars, domestic pigs and ruminants could be sharing bovine diarrhea virus due the similarity of the antigenic structures and that both were reported in the same region. Swine can potentially become sources for BVDV transmission because persistent infection can occur. Our results draw up concerns about the existence of accurate diagnostic tests and questions about risk factors involved in bovine viral diarrhea virus circulation.

CONCLUSIONS

The present study reported what seems to be the first molecular evidence of BVDV-1 in domestic pigs from Romania.

The pestivirus RNA genome was detected in eleven samples (6 wild boars and 5 backyard pigs) out of 527 tested (92 backyard pigs and 435 wild boars), using panpestivirus primers targeting the 5'-UTR region of the pestivirus genome. All eleven RNAs were identified as positives for BVDV-1 and no amplification signals were obtained from BVDV-2 and CSFV.

Our findings highlight the potential of swine (domestic and feral) as reservoir and consequently carrier for bovine diarrhoea virus thus being a signal of awareness for the health management in mixed farms.

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

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PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(6):484-492, June 2020
DOI: 10.1590/1678-5150-PVB-6437

Original Article
Animal Morphophysiology

Systematization, distribution, and territories of the caudal cerebral artery on the surface of the brain in nutria (*Myocastor coypus*)[1]

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ABSTRACT.- Goltz L.V., Azambuja R.C. & Campos R. 2020. **Systematization, distribution and territories of the caudal cerebral artery on the surface of the brain in nutria (*Myocastor coypus*).** *Pesquisa Veterinária Brasileira 40(6):484-492*. Departamento de Ciências Morfológicas, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Bairro Agronomia, Porto Alegre, RS 91540-000, Brazil. E-mail: lauragoltz@gmail.com

The nutria (*Myocastor coypus*) is a medium-sized, semi-aquatic rodent valued by the skin and meat industry. This study aimed to describe and systematize the caudal cerebral artery on the brain surface in nutria, establishing a standard model and its main variations in this species. The thirty animals used were euthanized according to animal welfare rules. The vessels were filled with latex stained with red pigment and the samples were fixed in formaldehyde. In nutria, the brain was vascularized by the vertebral basilar system. The terminal branches of the basilar artery originated the rostral cerebellar, caudal cerebral, rostral choroidal and middle cerebral arteries, and its terminal branch, the rostral cerebral artery. The terminal branch of the basilar artery projected the caudal cerebral artery, which is usually a single medium-caliber vessel, into the transverse fissure of the brain. The caudal cerebral artery was presented as a single (66.7% of the cases to the right and 76.7% to the left) and double vessel (33.3% of the cases to the right and 23.3% to the left). It originated the rostral mesencephalic artery, the proximal component, and the caudal inter-hemispheric artery. The terminal branches of the rostral and caudal tectal mesencephalic arteries formed a typical anastomotic network. The caudal inter-hemispheric artery emitted central branches, the caudal choroidal artery, hemispherical occipital arteries, rostral tectal mesencephalic branches and distal components, and anastomosed "*in osculum*" with the terminal branches of the rostral inter-hemispheric artery. The caudal choroidal artery anastomosed with the rostral choroidal artery, where it branched out on the thalamic mass, vascularizing all diencephalic structures and the hippocampus. The caudal cerebral artery and its terminal branches anastomosed with the terminal branches of the rostral and middle cerebral arteries in a restricted region of the caudal pole of the cerebral hemisphere. The vascularization area of the caudal cerebral artery and its central branches in the paleopallial of the piriform lobe is extremely restricted, caudomedially.

INDEX TERM: Caudal cerebral artery, brain, nutria, *Myocastor coypus*, arterial vascularization, anatomy, rodents.

RESUMO.-[Sistematização, distribuição e territórios da artéria cerebral caudal na superfície do cérebro em Nutria (*Myocastor coypus*).] A nutria (*Myocastor coypus*) é um roedor semi-aquático de tamanho mediano, apreciado na indústria de peles e carne. Este trabalho tem por objetivo descrever e sistematizar a artéria cerebral caudal na superfície do cérebro em nutria, estabelecendo um modelo padrão e suas principais variações e territórios nesta espécie. Os trinta animais utilizados foram eutanasiados segundo as regras de bem-estar animal, os vasos foram preenchidos com látex, corado em vermelho e as peças foram fixadas em formoldeído. O cérebro foi vascularizado exclusivamente pelo sistema vértebro-basilar. Os ramos terminais da artéria basilar originaram as artérias cerebelar rostral, cerebral

[1] Received on December 11, 2019.
Accepted for publication on February 4, 2020.
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caudal, corióidea rostral, cerebral média e seu ramo terminal, a artéria cerebral rostral. O ramo terminal da artéria basilar lançou a artéria cerebral caudal, um vaso normalmente único, de médio calibre, para o interior da fissura transversa do cérebro. A artéria cerebral caudal foi um vaso único em 66,7% à direita e em 76,7% à esquerda e mostrou-se dupla em 33,3% à direita e em 23,3% à esquerda. Ela lançou a artéria tectal mesencefálica rostral, componente proximal e a artéria inter-hemisférica caudal. Os ramos terminais das artérias tectais mesencefálicas, rostral e caudal, formavam uma rede anastomótica típica. A artéria inter-hemisférica caudal lançou ramos centrais, a artéria corióidea caudal, as artérias hemisféricas occipitais, os ramos tectais mesencefálicos rostrais, componentes distais e anastomosou-se "em ósculo" com o ramo terminal da artéria inter-hemisférica rostral. A artéria corióidea caudal anastomosava-se com a artéria corióidea rostral, onde ramificavam-se sobre a massa talâmica, vascularizando todas as estruturas do diencéfalo e hipocampo. A artéria cerebral caudal com seus ramos terminais apresenta anastomoses com os ramos terminais das artérias cerebrais rostral e média em uma região restrita do pólo caudal do hemisfério cerebral. A área de vascularização da artéria cerebral caudal com seus ramos centrais no páleo-palio do lobo piriforme é extremamente restrita, caudo-medialmente ao mesmo.

TERMOS DE INDEXAÇÃO: Artéria cerebral caudal, cérebro, nutria, *Myocastor coypus*, vascularização arterial, anatomia, roedores.

INTRODUCTION

Research on the central nervous system functions has been intensified in recent years, bringing the need to increase knowledge on cerebral vascularization. The present study was conducted with this intention.

The first studies on brain irrigation (Tandler 1898, De Vriese 1905) brought important considerations on the phylogenesis and ontogenesis of brain arterial models.

This study discusses the systematization, description, ramifications, and territory of the caudal cerebral artery on the brain surface in nutria (*Myocastor coypus*), a medium-sized, semi-aquatic rodent appreciated by the fur and meat industry, which lives in wetlands, rivers and lakes, where it digs burrows along the banks and feeds on grass, roots and aquatic plants (Baroffio et al. 1979).

Studies addressing brain irrigation have been carried out with several species such as dogs (Alcântara 1992), wild boars (Oliveira 2004), pampas foxes (Depedrini & Campos 2003), chickens (*Gallus gallus*) (Campos 1987), domestic pigs (Ferreira 1998), and opossums (Lindemann 1994). In rodents, there are reports in chinchillas (Jablonski & Brudnicki 1984, Roskosz et al. 1988, Gielecki et al. 1996, Araújo et al. 2004, Araújo & Campos 2005, Araújo & Campos 2007), capybaras (Reckziegel et al. 2001, 2004a, 2004b), guinea pigs (*Cavia porcellus*) (Bugge 1971, 1974), muskrats (*Ondatra zibethica*) (Jablonski & Brudnicki 1984), guinea pigs (*Cavia cobaya*), rats (*Mus rattus*) (Tandler 1898, De Vriese 1905), mice (*Mus muscullus* and *Rattus norvegicus*) (Lazorthes et al. 1976, Scremin 1995), guinea pigs (Majewska-Michalska 1995, 1997), gerbils (*Meriones unguiculatus*) (Kuchinka et al. 2008), Cairo spiny mice (*Acomys cahirinus*, Desmarest) (Szczurkowski et al. 2007), and nutrias (Azambuja et al. 2018, Goltz 2017). Due to the lack of information on this species, both in the classic literature and in specialized articles, our results will be compared with those reported by other authors who have discussed brain vascularization in rodents.

This study aimed to describe and systematize the caudal cerebral artery on the surface of the brain in nutria to understand the phylogenetic development of this vessel, establishing a standard model and its main variations and territories in this species.

MATERIALS AND METHODS

Thirty brains of adult nutrias (*Myocastor coypus*), 15 males and 15 females, from a commercial breeding farm, certified by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA), located in the municipality of Caxias do Sul, state of Rio Grande do Sul, Brazil, were used to in this study. The project was approved by the Committee on Animal Research and Ethics of the Federal University of Rio Grande do Sul (CEUA-UFRGS) under protocol no. 29415. The specimens provided for the experiments were animals that would be discarded by the breeder.

The animals were physically restrained and received a 10,000IU dose of heparin (Hepamax-s; Blausiegel Indústria e Comércio Ltda, Cotia/SP, Brazil), intraperitoneally. After 30 min, the animals were sedated with an association of the following pre-anesthetic medications: acepromazine (Acepran 1%; Vetnil, Louveira/SP, Brazil) 0.5mg/kg and pethidine hydrochloride (Dolosal; União Química, São Paulo/SP, Brazil) 20mg/kg, intramuscularly. After sedation, they were euthanized using sodium thiopental (Thiopentax 2.5%; Cristalia, Itapira/SP, Brazil) at a dose of 120mg/kg and lidocaine (Dorfin 1%; Hertape Calier, Juatuba/MG, Brazil), at 1% concentration, intraperitoneally.

After confirmation of death, the thoracic cavity was opened ventrally. The thoracic aorta was clamped close to the diaphragm, the cardiac apex was sectioned, and the aortic arch was cannulated through the left ventricle. The internal thoracic artery was clamped close to the xiphoid process. The arterial system was washed with 150ml per animal of refrigerated 0.9% saline solution (Sodium Chloride 0.9%; Fresenius Kabi Brasil, Barueri/SP, Brazil), and filled with latex (Latex Cola 603; Bertoncini, São Paulo/SP, Brazil) stained with red pigment (Suvinil Corante; Suvinil BASF, São Bernardo do Campo/SP, Brazil). The animals were immersed in running water for approximately 1 hour allow to polymerization of the latex. The head skin was removed and a bone window was opened in the cranial vault. The samples were then immersed in 20% formaldehyde for seven days. After this period, the encephala were removed along with a segment of the cervical spinal cord for dissection and observation of the cerebral arteries.

The material was analyzed under magnifying lens (LTS lamp, 5X magnification, and Stemi SV8 microscope - Zeiss, Goettingen, Germany) and, to illustrate the results, schematic drawings of the cerebral arteries on the surface of the brain were made in ventral, dorsal, right and left lateral, right and left medial, and dorsal brainstem views for all samples. Photographic records of the preparations were made for documentation.

The vessels were named according to Nomina Anatomica Veterinaria (2017), with some names were added, at our discretion, based on the blood supply territories of other animal species found in literature. Percentage calculations were applied to statistically analyze the results.

RESULTS

The internal carotid artery, right and left, in nutria (*Myocastor coypus*) was atrophied in both antimeres in all cases. Its terminal branch was found at the base of the skull before penetrating the foramen lacerum, not cooperating to the arterial vascularization of the brain.

The brain was vascularized exclusively by the vertebrobasilar system. The vertebral artery was a collateral branch of the subclavian artery, ascending the neck through the transverse canal of the cervical vertebrae. When it reached the atlantal fossa, it crossed the alar and lateral vertebral foramina of the tlas, reaching the interior of the vertebral canal. Its terminal branch was anastomosed with its contralateral counterpart, on the ventral surface of the medulla, forming a basilar artery of large caliber when penetrating through the foramen magnum.

The basilar artery, a large caliber rectilinear vessel, when ventrally traversing the base of the rhombencephalon, emitted pairs of caudal, medium and trigeminal cerebellar arteries dividing into its terminal branches, which diverged latero-rostrally at an approximate angle of 90°.

The terminal branches of the basilar artery originated, in sequence, the rostral cerebellar, caudal cerebral, rostral choroid, middle cerebral arteries, and its terminal branch, the rostral cerebral artery (Fig.1).

The rostral cerebellar artery originated from the terminal branch of the basilar artery, penetrated the transverse fissure of the brain, vascularized a large part of the cerebellum, and emitted the caudal tectal mesencephalic branch to the caudal surface of the caudal colliculi.

The terminal branch of the basilar artery projected the caudal cerebral artery into the transverse fissure of the brain, along with the apparent origin of the oculomotor nerve (III pair of cranial nerve).

The caudal cerebral artery was a regular, single, medium-caliber vessel. It projected lateral-dorsally into the transverse fissure of the brain, bypassing the cerebral peduncle. The rostral tectal mesencephalic artery was projected caudal-dorsally, and vascularized most of the mesencephalic tectum, except the caudal surface of the caudal colliculi, which was vascularized by the caudal tectal artery, a branch of the rostral cerebellar artery. This network also received ramifications from the two rostral mesencephalic tissue vessels, a distal component that was emitted from the inter-hemispheric artery and the caudal choroidal artery at the height of the lateral surface of the rostral colliculi (Fig.2).

The next branch of the caudal cerebral artery was the caudal inter-hemispheric artery, which emitted two to three central branches to the medial surface of the piriform lobe, caudal part. Then it emitted a well-developed caudal choroidal artery, which, before bypassing the lateral geniculate body, anastomosed with the rostral choroidal artery, where both branched out on the thalamic mass, vascularizing all the structures of the diencephalon (lateral geniculate body, pineal gland, medullary streak, and third ventricle choroid plexus), as well as the entire hippocampus. Next, the caudal inter-hemispheric artery emitted two to three occipital hemispheric arteries to the medial part (tentorialpart) of the cerebral hemisphere, small vessels that reached the occipital pole (caudal) of the cerebral hemisphere. The caudal inter-hemispheric artery then became a thin vessel that, bypassing the splenium of the corpus callosum, anastomosed "*in osculum*" with the termination of the rostral inter-hemispheric artery. Before the cerebral hemisphere, the caudal inter-hemispheric artery emitted, mid-caudally, two rostral tectal mesencephalic branches, distal components, which were incorporated into the network of the mesencephalic tectum (Fig.3).

The caudal cerebral artery was presented as a single vessel (66.7% of the cases to the right and 76.7% to the left) and double vessel (33.3% of the cases to the right and 23.3% to the left). In the samples where the caudal cerebral artery was presented as a double vessel, the first component emitted from the terminal branch of the basilar artery was the rostral tectal mesencephalic artery, its proximal component. Where the caudal cerebral artery was presented as a single vessel, the rostral tectal mesencephalic artery was its first collateral branch. It also originated at the base of the brain before penetrating its transverse fissure.

The rostral tectal mesencephalic artery, a proximal component, appeared as the first collateral branch of the caudal cerebral artery in 66.7 and 76.7% of the cases to the right and the left, respectively, and was presented as the first collateral branch of the terminal branch of the basilar

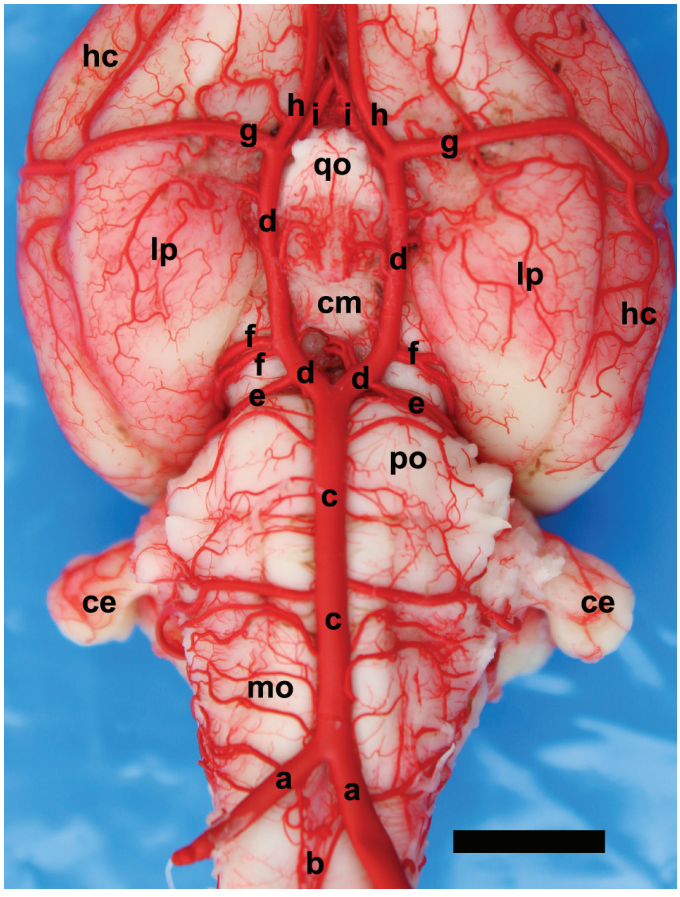

Fig.1. Ventral view of the nutria (*Myocastor coypus*) brain (Obs.18), highlighting the formation of the cerebral arterial circle and the cerebral arteries. Vertebral artery(a), ventral spine artery (b), basilar artery (c), terminal branch of "c" (d), cerebellar rostral artery (e), caudal cerebral artery (f), middle cerebral artery (g), cerebral rostral artery (h), medial branch of "h" (i), cerebral hemisphere (hc), optical chiasm (qo), nipple body (cm), piriform lobe (lp), bridge (po), cerebellum (ce), medulla oblongata (mo). Bar = 7mm.

artery in 33.3% and 23.3% of the cases to right and the left, respectively. The rostral tectal mesencephalic artery, a distal component in both antimeres, was observed as a double vessel in all preparations.

The caudal inter-hemispheric artery was the continuation of the main axis of the caudal cerebral artery, which, when penetrating the transverse fissure of the brain, emitted a sequence of two to three central branches to the paleopallial of the piriform lobe (caudomedial part). It also emitted large-caliber caudal choroidal branches to the diencephalon and two rostral tectal mesencephalic branches, distal components, to the network of the mesencephalic tectum. The caudal inter-hemispheric artery was presented as a single vessel in 100% of the samples in both antimeres.

Near the rostral colliculi, the caudal inter-hemispheric artery curved dorsally, projecting two to three occipital hemispheric arteries. Its terminal branch was a thin-caliber vessel that anastomosed "*in osculum*" with the terminal branch of the rostral inter-hemispheric artery, the terminal branch of the rostral cerebral artery, at the level of the splenium of the corpus callosum.

The central branches of the caudal inter-hemispheric artery to the right varied from two to three branches in 83.3% of the samples and from one to six branches in 16.7% of them, whereas to the left, they varied from two to three branches in 86.7% of the preparations and from one to four branches in 13.3% of them.

The caudal inter-hemispheric artery, when passing over the medial geniculate body, emitted caudal choroidal branches that projected medial-rostrally on the thalamic mass, vascularizing the diencephalic structures and the hippocampus. It anastomosed with the rostral choroidal artery, vascularize the diencephalic structures (lateral geniculate body, thalamic mass, medullary streak, pineal gland and third ventricle

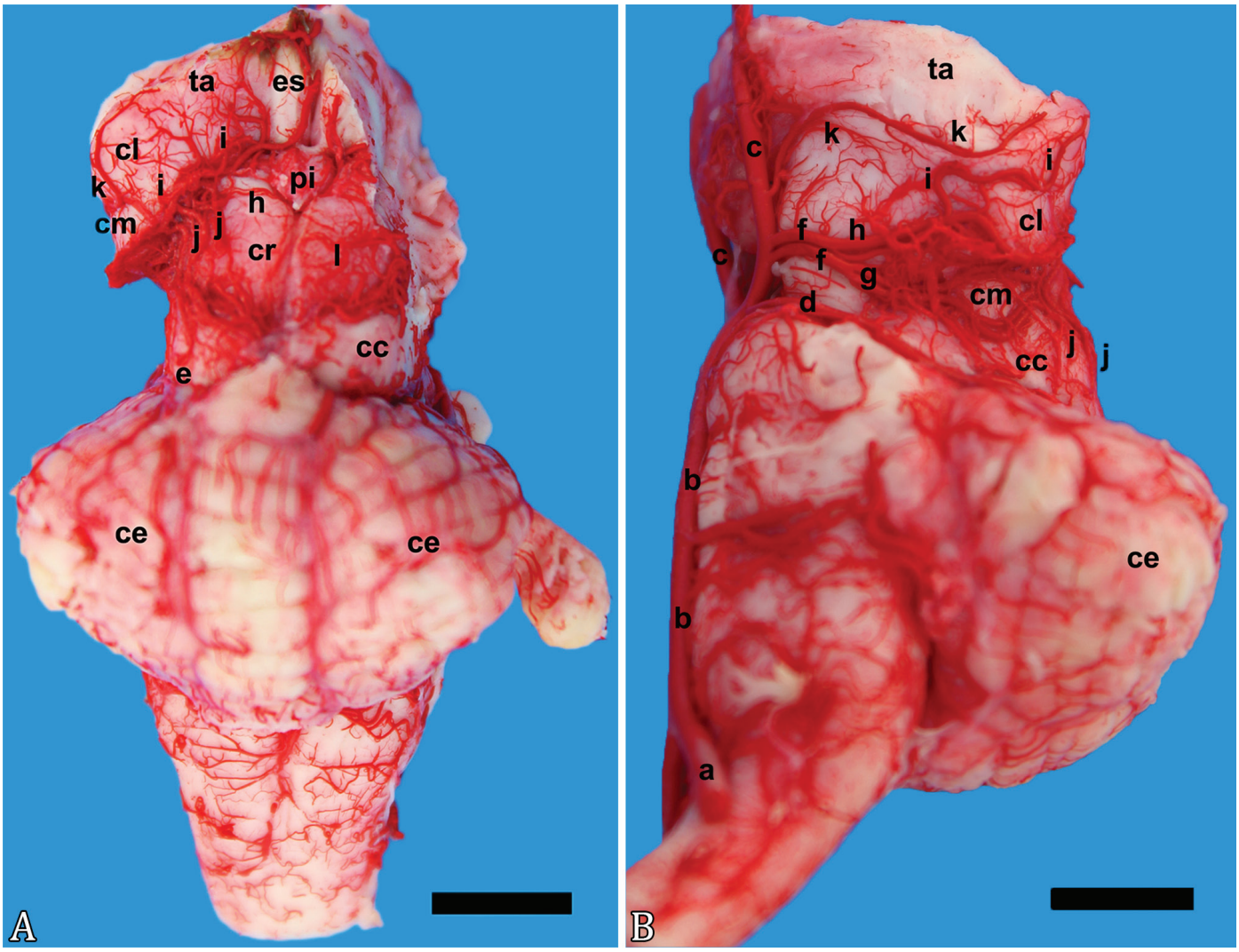

Fig.2. (**A**) Dorsal and (**B**) left lateral views of the brainstem, with cerebellum, of nutria (*Myocastor coypus*) (Obs.14). Vertebral artery (a), basilar artery (b), terminal branch of "b" (c), cerebellar rostral artery (d), caudal tectal mesencephalic artery (e), caudal cerebral artery (f), rostral tectal mesencephalic artery - proximal component (g), caudal inter-hemispheric artery (h), caudal choroid artery (i), rostral tectal mesencephalic artery - distal component (j), rostral choroid artery (k), network of the mesencephalic tectum(l), medullar streaks (es), thalamus (ta), lateral geniculate body (cl), medial geniculate body (cm), rostral colliculi (cr), caudal colliculi (cc), pineal gland (pi), cerebellum (ce). (**A**) Bar = 6.1mm. (**B**) Bar = 5mm.

choroid plexus) and the hippocampus. Most caudal branches of the caudal choroidal artery protruded over most part of the rostral colliculi, anastomosing with the network of the rostral tectal mesencephalic arteries. The caudal choroidal artery was a single vessel in both antimeres in all samples.

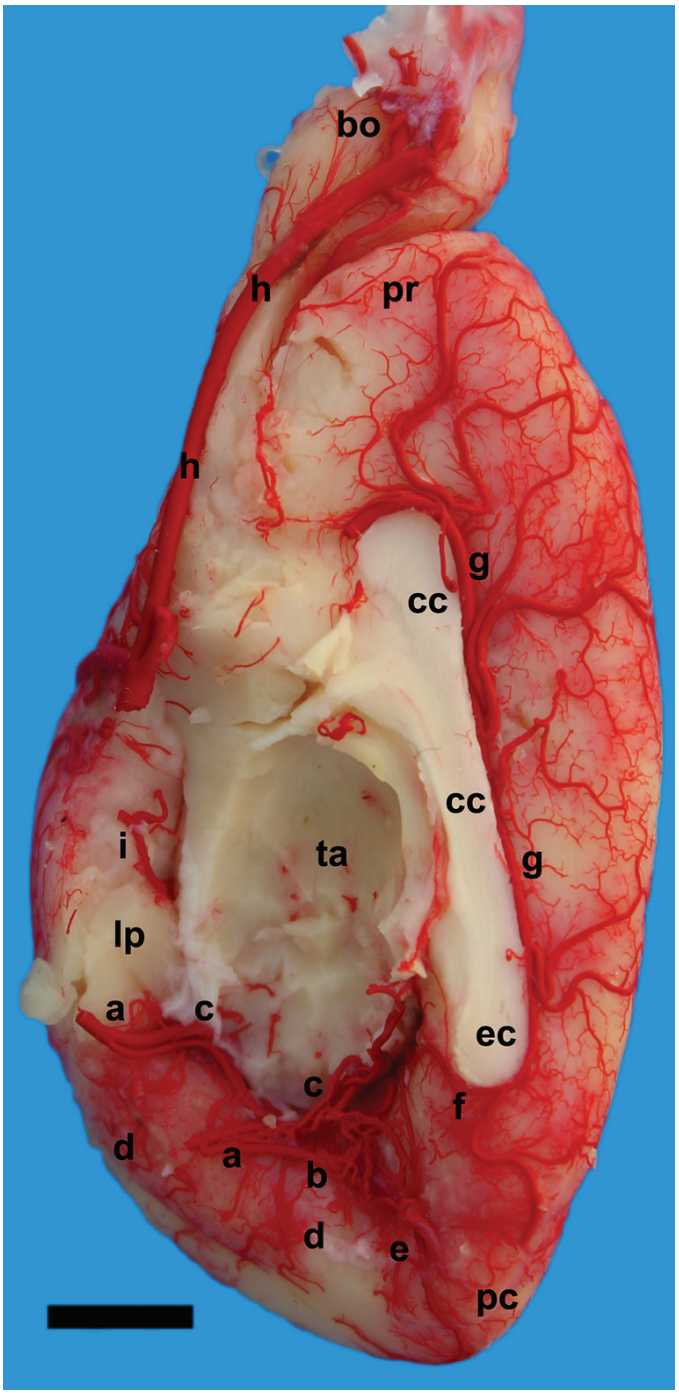

Fig.3. Right medial view of the cerebral hemisphere in nutria (*Myocastor coypus*) (Obs.18). Caudal cerebral artery (a), rostral tectal mesencephalic artery - proximal component (b), caudal inter-hemispheric artery (c), central branch of "a" (d), occipital hemispheric branch (e), anastomosis between interhemispheric rostral and caudal arteries (f), inter-hemispheric rostral artery (g), main axis of cerebral rostral artery (h), rostral choroid artery (i), olfactory bulb (bo), rostral pole of the cerebral hemisphere (pr), caudal pole of the cerebral hemisphere (pc), corpus callosum (cc), splenium of "cc" (ec), thalamus (ta), piriform lobe (lp). Bar = 4.5mm.

The caudal inter-hemispheric artery, when reaching the cerebral hemisphere, originated from one to three occipital hemispheric branches. These thin-caliber vessels, were emitted, in sequence, to the occipital pole of the cerebral hemisphere, reaching the convex surface. The progress of these ramifications was very small on this side. Its terminal branches anastomosed with the caudal convex hemispheric terminal branches of the middle cerebral artery.

The hemispherical occipital branches in 70% on the right and 43.3% on the left, were double; in 16.7% on the right and in 36.7% on the left, it was triple; in 13.3% on the right and in 20% on the left, it was shown as a single vessel (Fig.4).

The territory of the caudal cerebral artery in nutria comprised a small caudal and medial area of the piriform lobe, the tentorial surface of the cerebral hemisphere, a small caudal area of the convex surface, limiting the transverse fissure of the brain, the mesencephalic tectum, except the caudal face of the caudal colliculi, the pineal gland, the medullary streaks, the habenula, the lateral and medial geniculate body, the thalamic mass, and the third ventricle choroid plexus. It also comprised the splenium of the corpus callosum, the fornix, and the hippocampus. The structures of the diencephalic tectum presented vascular complementation by the rostral choroidal artery, which was a collateral branch of the terminal branch of the basilar artery (Fig.5).

Distribution of the caudal cerebral artery and its branches showed anastomoses with the terminal branches of the middle cerebral artery at the caudal limit of the convex surface of the cerebral hemisphere. They also presented anastomosis with the caudal tectal mesencephalic artery, a branch of the rostral cerebellar artery, at the limit of the caudal and rostral surfaces of the caudal colliculi.

DISCUSSION

The caudal cerebral artery in nutria (*Myocastor coypus*) was normally single, medium-caliber vessel originated from the terminal branches of the basilar artery at the base of the cerebral peduncle close to the oculomotor nerve (III pair of cranial nerve). This description was also found in studies on capybaras (*Hydrochoerus hydrochaeris*) (Reckziegel et al. 2004a), chinchillas (*Chinchilla lanigera*) (Araújo & Campos 2007), and nutrias (Goltz 2017, Azambuja et al. 2018). In rats (*Mus rattus*), the caudal cerebral artery originated from the initial portion of the rostral cerebellar artery, which emitted branches to the thalamus region (Scremin 1995). In rats (*Rattus norvegicus*) and gerbil (*Meriones unguiculatus*), the caudal cerebral artery was emitted from the terminal branch of the internal carotid artery, as a single, large-caliber vessel (Kuchinka et al. 2008, Esteves et al. 2013). In Cairo spiny mouse (*Acomys cahirinus*, Desmarest), the arteries at the base of the brain showed a wide range of presentations, such as open, partially open and closed arterial circles, with the caudal cerebral artery originating from the internal carotid artery in some cases, and in the basilar artery in others (Szczurkowski et al. 2007).

In nutria, the right caudal cerebral artery was presented as single in 66.7% of the preparations and double in 33.3% of them, whereas to the left it was presented as single in 76.7% of the samples and double in 23.2% of them. Unlike

in nutria, the caudal cerebral artery in capybara was mainly single, both to the right and the left, with cases of triplicity. Emergence in the right antimere was double in 56.7%, simple in 40% and triple in 3.3% of the samples, whereas to the left antimere it was single in 53.3%, double in 40% and triple in 6.7% of them (Reckziegel et al. 2001). The caudal cerebral artery in nutria was also a single vessel in 66.7% of the cases to the right, but was single in 73.3% of the case to the left (Goltz 2017, Azambuja et al. 2018). In chinchilla, the caudal cerebral artery was a single vessel in 53.3% of the samples to the right and in 63.3% of them to the left however, it was double in 46.7% to the right and 36.7% to the left, where most of the rostral artery showed large caliber and the flow rate was from the caudal choroidal artery (Araújo & Campos 2007). In chinchilla, the main caudal component was the caudal choroidal artery, whereas in nutria it was the rostral tectal mesencephalic artery. In chinchilla, the rostral tectal mesencephalic artery was always a direct branch of the terminal branch of the basilar artery, not being dependent on the caudal cerebral artery as in nutria. In *R. norvegicus*, the caudal cerebral artery was a single vessel, and duplication often occurred (Esteves et al. 2013).

In nutria, the caudal cerebral artery emerged as collateral branches of the rostral tectal mesencephalic artery (proximal component) and the caudal inter-hemispheric artery. The same description was found in nutria (Goltz 2017, Azambuja et al. 2018). The caudal cerebral artery in Guinea pig originated in the collateral branches that went to the occipital, lateral temporal and parietal areas of the cerebral hemispheres (Majewska-Michalska 1995). In capybara, on the dorsal surface of the cerebral hemisphere, the caudal cerebral artery ran to the para-hippocampal gyrus, emitting small branches, which penetrated the hippocampal groove, and small branches, which were superficially distributed along the hippocampal formation (Reckziegel et al. 2004b). In nutria, the hippocampus was vascularized by the rostral and caudal choroidal arteries, with the caudal choroidal artery being a branch of the caudal inter-hemispheric artery. In *M. rattus*, the caudal cerebral artery joined the caudal communicating artery and formed the caudal mesencephalic tectal artery, whose branches irrigated the surface of the caudal colliculi.

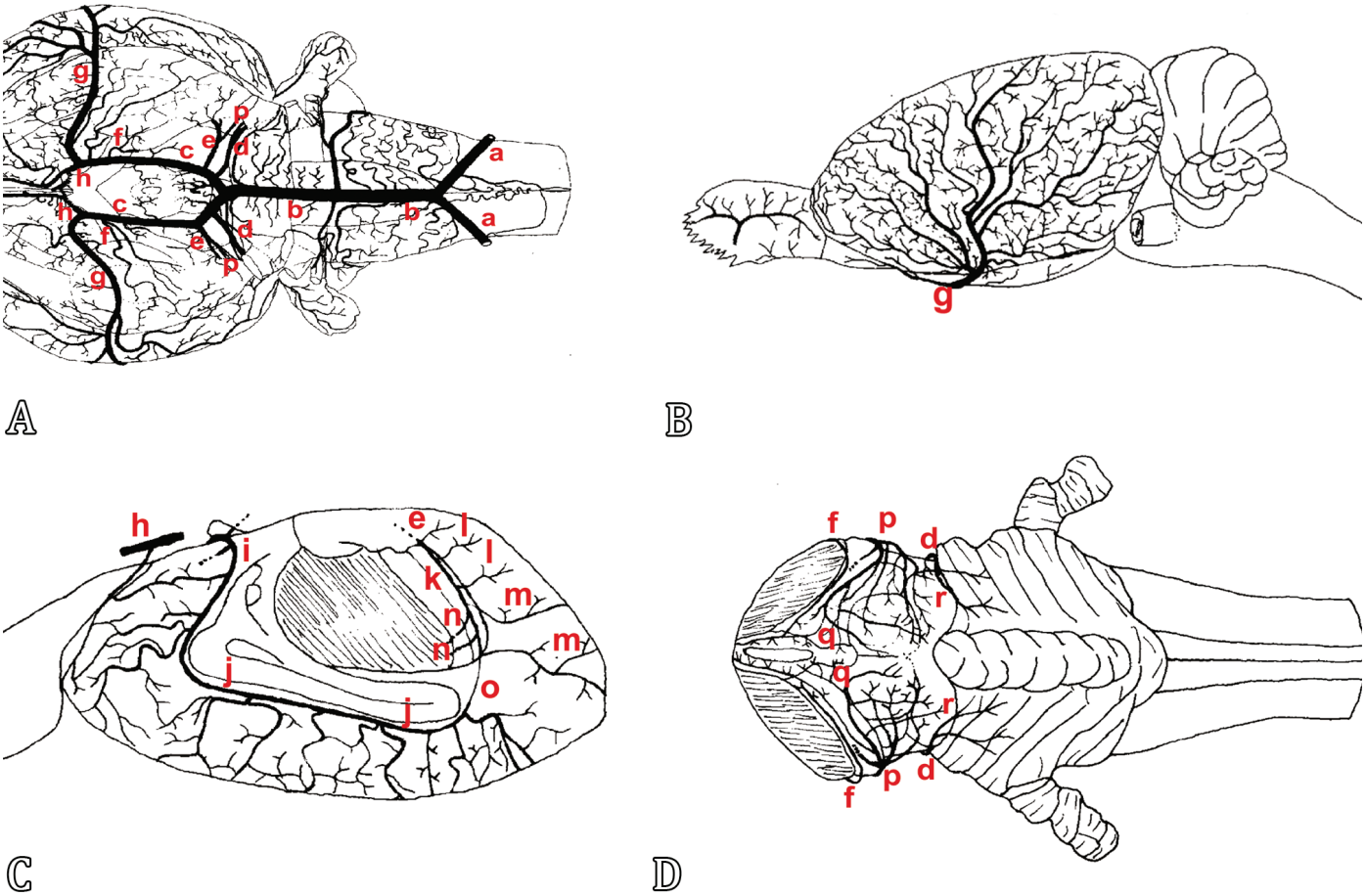

Fig.4. Schematic drawings in (**A**) ventral, (**B**) left lateral, (**C**) left medial and (**D**) dorsal views of brainstem with cerebellum in nutria (*Myocastor coypus*) (Obs.3). Vertebral artery (a), basilar artery (b), terminal branch of "b" (c), cerebellar rostral artery (d), caudal cerebral artery (e), rostral choroid artery (f), middle cerebral artery (g), cerebral rostral artery (h), medial branch of "h" (i), inter-hemispheric rostral artery (j), caudal inter-hemispheric artery (k), central branches of "e" (l), occipital hemispheric branches (m), rostral tectal mesencephalic artery - distal component (n), anastomosis between interhemispheric rostral and caudal artery (o), rostral tectal mesencephalic tectal artery - proximal component (p), caudal choroid artery (q), caudal tectal mesencephalic artery (r).

Then it emitted the longitudinal hippocampal artery, the caudal lateral choroidal artery (for the lateral ventricle and the third ventricle choroid plexus) and three to four cortical branches. The caudal cerebral artery ended in a collateral anastomotic network on the surface of the rostral and caudal colliculi (Scremin 1995).

The rostral tectal mesencephalic artery in *M. coypus* originated from the caudal cerebral artery, dorsally. In the samples where the caudal cerebral artery was a double vessel, the first component that emerged from the terminal branch of the basilar artery was the rostral tectal mesencephalic artery, its proximal component. When the caudal cerebral artery appeared as a single vessel, the rostral tectal mesencephalic artery was its first collateral branch. The rostral tectal mesencephalic artery vascularized most of the mesencephalic tectum, except the caudal surface of the caudal colliculi, which was vascularized by the caudal tectal mesencephalic artery, a branch of the rostral cerebellar artery. The same description was found in nutria (Goltz 2017, Azambuja et al. 2018). In capybara, the rostral tectal artery was distributed in the mesencephalic tectum, in the rostral colliculi, and part of the caudal colliculi, further vascularizing the para-hippocampal gyrus, the hippocampus, and the thalamus (Reckziegel et al. 2004a). In chinchilla this artery was emitted from the terminal branch of the basilar artery, between the origins of the rostral cerebellar and caudal cerebral arteries (Araújo & Campos 2007).

In nutria, the terminal branches of the rostral and caudal mesencephalic tissue arteries formed a typical anastomotic network on the surface of the rostral and caudal colliculi. In the rostral tectal mesencephalic arteries, the distal components were emitted from the inter-hemispheric artery and the caudal choroidal artery. Unlike in nutria, in capybaras, the rostral tectal artery emerged as a collateral branch of the caudal cerebral artery in 27.9% of the samples and was emitted directly from the basilar artery in 72.1% of them (Reckziegel et al. 2004a). In chinchilla, the rostral tectal artery was a single vessel in 96.7% of the samples on the right and in 90% of them to the left. However, two rostral tectal arteries were emitted from the terminal branch of the basilar artery in 3.3% of the samples to the right and 10% to the left (Araújo & Campos 2007).

The next branch of the caudal cerebral artery in nutria was the caudal inter-hemispheric artery. The latter projected two to three central branches to the medial surface of the piriform lobe the caudal part. The caudal inter-hemispheric artery was single in 100% of the encephalo both to the right and left. The chinchilla caudal inter-hemispheric artery was the continuation of the caudal cerebral artery, after the emission of the first caudal hemispheric branch to the tentorial part of the medial surface of the cerebral hemisphere, and ascended branching on the medial surface of the cerebral hemisphere,

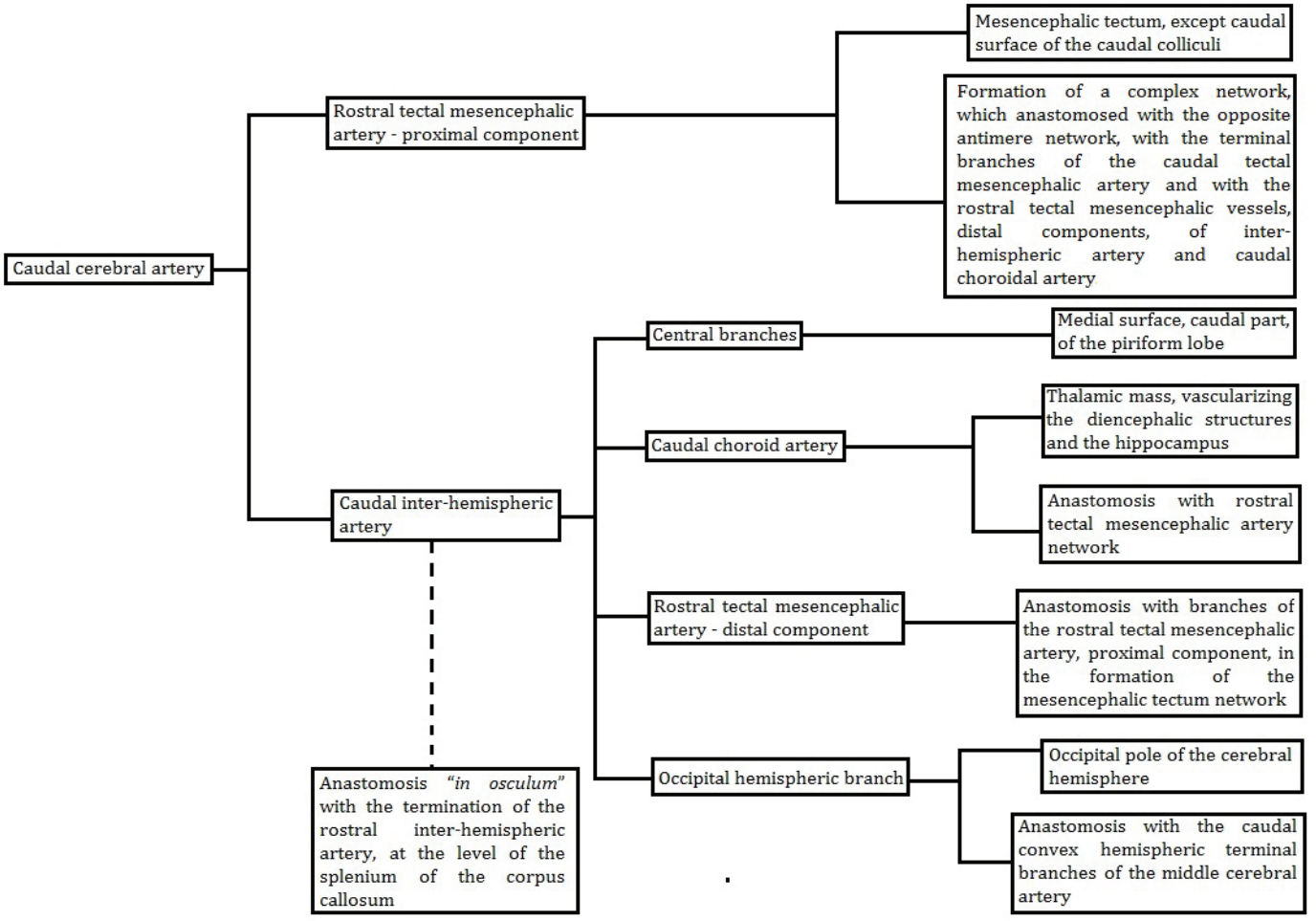

Fig.5. Organization chart showing the cerebral caudal artery in nutria (*Myocastor coypus*) and its distribution.

emitting the para-hippocampal gyrus and caudal medial hemispheric branches (Araújo & Campos 2007). In chinchilla, when the caudal cerebral artery ascended, thin-caliber central branches were emitted on the lateral surface of the cerebral peduncle, being distributed ventrally, in most of the caudal third of the piriform lobe. On the right, two central branches were emitted in 53.3% of the samples, three branches in 20%, four branches in 16.7% and one branch in 10%; on the left, they projected three branches in 40%, two branches in 26.7%, one branch in 20% and four branches in 13.3% of the preparations (Araújo & Campos 2007).

The caudal inter-hemispheric artery in nutria emitted a well-developed caudal choroidal artery, which before bypassing the lateral geniculate body anastomosed with the rostral choroidal artery, where both branched out on the thalamic mass, vascularizing all the structures of the diencephalon and hippocampus. The caudal choroidal artery, in capybara, arose from the caudal cerebral artery rostral-medially on the hippocampal gyrus, and it anastomosed with the rostral choroidal artery (Reckziegel et al. 2004b). The chinchilla caudal choroidal artery was a single or double thin-caliber vessel. It was projected dorsal-medially vascularizing the hippocampus, ventrally following the fimbria, reaching and running through the terminal medullar streak, emitting branches to the third ventricle choroid plexus and the lateral ventricle (Araújo & Campos 2007).

The caudal choroidal artery in capybara was presented anastomosed with the rostral choroidal artery, contributing to the formation of the third ventricle choroid plexus and the lateral ventricle (Reckziegel et al. 2004a). In chinchilla, the caudal choroidal artery, when double, had one component originating from the caudal cerebral artery and the other originating from the terminal branch of the basilar artery in 46.6% of the cases to the right and 36.7% on the left, and it was also double, being the only branch of the caudal cerebral artery in 26.7% of the samples to the right and in 33.3% of them to the left, and it was a single vessel, branch of the caudal cerebral artery in 26.7% to the right and 30% to the left (Araújo & Campos 2007).

The caudal inter-hemispheric artery in *M. coypus* emitted, near the medial geniculate body, the rostral mesencephalic tectal artery, a distal component, a double vessel, which anastomosed to the branches of the rostral tectal mesencephalic artery, proximal component, participating in the formation of the network of the mesencephalic tectum. This distal component has never been mentioned in any other animal species. Then the caudal inter-hemispheric artery emitted, to the medial surface (tentorial part) of the cerebral hemisphere, occipital hemispheric arteries, which were thin-caliber vessels that reached the occipital (caudal) pole of the cerebral hemisphere. The caudal inter-hemispheric artery in chinchillas emitted caudal-medial, collateral hemispheric branches to the tentorial and medial portion of the cerebral hemisphere surface. Two to seven caudal-medial, collateral hemispheric branches originated from the right and left (Araújo & Campos 2007).

In nutria, the caudal inter-hemispheric artery became a thin-caliber vessel that, bypassing the splenium of the corpus callosum, anastomosed "*in osculum*" with the termination of the rostral inter-hemispheric artery. The same description was found in chinchilla, and this anastomotic branch was present in 88.3% of the samples to the right and 76.7% of them to the left (Araújo & Campos 2007). In capybara, the terminal branch of the caudal cerebral artery emitted, on the medial side of the cerebral hemispheres, a rostral anastomotic branch to the corpus callosum artery, which is the terminal branch of the rostral cerebral artery (Reckziegel et al. 2004a).

It was concluded that the vascular territory of the caudal cerebral artery in capybara (Reckziegel et al. 2004a) included the thalamus, the rostral colliculi, part of the caudal colliculi, the caudal surface of the piriform lobe, the tentorial surface, the rostral splenium portion of the medial surface, and convex surface of the cerebral hemispheres along the longitudinal and transverse fissures of the brain. Arterial vascularization of the hippocampus in capybara (Reckziegel et al. 2004b) was supplied by branches originating from the caudal cerebral artery and the rostral choroidal artery. The territory of the caudal cerebral artery in chinchilla comprised the caudal third of the piriform lobe, the pineal gland, the medullary streak, the habenula, the dorsal surface of the thalamus, the lateral and medial geniculate bodies, the hippocampus, the third ventricle choroid plexuses and the lateral ventricle, the splenic corpus callosum, the tentorial part of the medial surface of the cerebral hemisphere, and the caudal part of the convex surface, bypassing the transverse fissure of the brain (Araújo & Campos 2007). The basic difference in the territorial area of the caudal cerebral artery between nutria and chinchilla was that the rostral colliculi was vascularized by the rostral tectal mesencephalic artery, and did not belong to the vascularization of the caudal cerebral artery, but was a branch of the terminal branch of the basilar artery.

In nutria, the distribution of the caudal cerebral artery and its branches showed anastomoses with the terminal branches of the middle cerebral artery at the caudal limit of the convex surface of the cerebral hemisphere. In nutria (Goltz 2017, Azambuja et al. 2018) and in chinchilla (Araújo & Campos 2007), it has been described that the terminal branches of the caudal cerebral artery anastomoses "*in osculum*" with the terminations of the rostral cerebral artery close to the splenium of the corpus callosum. In chinchilla (Araújo & Campos 2007), anastomoses were also observed between the terminal branches of the caudal cerebral artery and the terminal branches of the middle cerebral artery, on the convex surface of the cerebral hemisphere, surrounding the entire transverse fissure of the brain and the caudal third of the piriform lobe. In *G. pig*, in the diencephalon, the thalamus received branches from the caudal cerebral, the caudal communicating, and the rostral and middle cerebral choroid artery (Majewska-Michalska 1995, 1997). The caudal cerebral artery in capybara (Reckziegel et al. 2004a) projected to the caudal part of the piriform lobe, the para-hippocampal gyrus, and the caudoventral portion of the cerebral hemispheres and emitted cortical branches to the caudal surface of the piriform lobe and tentorial surface of the cerebral hemisphere, where it anastomosed with cortical branches of the middle cerebral artery. In the caudal region of the brain of *M. rattus*, anastomoses were observed between the azygos pericallosal artery and middle cerebral and caudal cerebral arteries (Scremin 1995).

CONCLUSIONS

The caudal cerebral artery in nutria is a branch of the terminal branch of the basilar artery whose ramifications are distributed with greater emphasis on the mesencephalic tectum

and the diencephalon, vascularizing the entire hippocampus, but extending very little onto the neopallium of the caudal pole and the medial surface of the cerebral hemisphere.

The caudal cerebral artery and its terminal branches present anastomosis with the terminal branches of the rostral and middle cerebral arteries in a restricted region of the caudal pole of the cerebral hemisphere.

In nutria, the vascularization area of the caudal cerebral artery and its central branches in the paleopallial of the piriform lobe is extremely restricted caudal-medially to it.

Acknowledgements.- The authors would like to thank "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES) for the scholarship provided.

Conflict of interest statement.- The authors report no conflicts of interest and are responsible for the contents of this manuscript.

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PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(6):493-500, June 2020
DOI: 10.1590/1678-5150-PVB-6381

Original Article
Animal Morphophysiology

Quantification of the neurons of myenteric plexus of the bat *molossus rufus*[1]

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ABSTRACT.- Serenini G.F., Beltrami J.M., Gerônimo E., Favetta P.M., Legnani N.G.E., Otutumi L.K., Martins L.A. & Germano R.M. 2020. **Quantification of the neurons of myenteric plexus of the bat *Molossus rufus.*** *Pesquisa Veterinária Brasileira 40(6):493-500*. Graduate Program in Animal Science with emphasis in Bioactive Products, Universidade Paranaense, Praça Mascarenhas de Moraes 4282, Zona III, Umuarama, PR 87502-210, Brazil. E-mail: prof.ricardogermano@gmail.com

There are no studies that characterize the enteric nervous system (ENS) bats. The organization and density of myenteric neurons may vary according to the animal species, as well as the segment of the digestive tube considered. The nitric oxide is one of the key neurotransmitters present in the myenteric neurons, acting as a mediator in the smooth muscle relaxation. These neurons are evidenced by immunohistochemistry of nitric oxide synthase (NOS) or by NADPH-diaphorase histochemistry. In this sense, this study aimed to characterize the total neuronal population and subpopulation NADPH-d$^+$ of the myenteric plexus present in the jejunum of the insectivore species Molossus rufus quantitatively. Five specimens were collected of M. rufus in a buffer area of the "Reserva Biológica das Perobas" in the microregion of Cianorte/PR. After the euthanasia, in a chamber saturated with isoflurane, segments were collected from the small intestine corresponding to the jejunum intended for two techniques for neuronal marking, Giemsa and NADPH-diaphorase, and a fragment to the histological technique of hematoxylin-eosin and Masson's trichrome. All the procedures were approved by the "Comitê de Ética no Uso de Animais Unipar" (CEUA - protocol No. 34347/2017) and the "Instituto Chico Mendes de Conservação da Biodiversidade" (ICMBio - protocol No. 60061-1) The histological sections allowed to highlight the location of the myenteric plexus between the longitudinal and circular layers of the muscular tunic. The myenteric plexus had an average of total neuronal population (neurons Giemsa$^+$) of 279.23 neurons/mm^2, being the nitrergic neurons (neurons NADPH-d$^+$) represented 20.4% of this total population, with an average of 58.14 neuron/mm^2. Therefore, the collected data are consistent with previous studies in other mammalian species concerning the location of the myenteric plexus, as well as the neural myenteric proportion NADPH-d$^+$ compared with the population of neurons Giemsa$^+$. The gaps in the knowledge of ENS of bats limits comparative intraspecific and interspecific studies.

INDEX TERMS: Neurons, myenteric plexus, bats, *Molossus rufus*, Molossidae, digestive tube, enteric nervous system, NADPH-diaphorase, morphology.

RESUMO.- [**Quantificação dos neurônios do plexo mientérico de morcegos da espécie *Molossus rufus.***] Não há estudos que caracterizem o sistema nervoso entérico (SNE) destes animais, configurando uma lacuna no conhecimento quanto à biologia destes indivíduos. A organização e densidade dos neurônios mientéricos podem variar de acordo com a espécie animal bem como o segmento do tubo digestório considerado. O óxido nítrico é um dos principais neurotransmissores

[1] Received on October 17, 2019.
Accepted for publication on November 22, 2019.
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presentes nos neurônios mientéricos, atuando como mediador no relaxamento do músculo liso gastrointestinal, de modo que estes neurônios são evidenciados igualmente pela imunohistoquímica da óxido nítrico-sintase (NOS) ou pela histoquímica da NADPH-diaforase. Neste sentido, objetivou-se caracterizar quantitativamente a população neuronal total e subpopulação NADPH-d$^+$ do plexo mientérico presente no jejuno da espécie *Molossus rufus* de hábito alimentar insetívoro. Foram coletados cinco espécimes de *M. rufus* em área de amortecimento da Reserva Biológica das Perobas na microrregião de Cianorte/PR. Após a eutanásia, em câmara saturada com isoflurano, foram coletados segmentos do intestino delgado correspondentes ao jejuno destinados a duas técnicas para marcação neuronal, Giemsa e NADPH-diaforase e, um fragmento para a técnica histológica de hematoxilina-eosina e tricômio de Masson. Todos os procedimentos realizados foram aprovados pelo Comitê de Ética no Uso de Animais da Unipar (CEUA - protocolo nº 34347/2017) e pelo Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio - protocolo nº 60061-1) Os cortes histológicos possibilitaram evidenciar a localização do plexo mientérico entre os estratos longitudinal e circular da túnica muscular. Neurônios Giemsa$^+$ apresentaram uma média de 279,23 neurônios/mm^2, já os neurônios nitrérgicos apresentaram em média 20,4% da população neuronal mientérica total, sendo evidenciados 58,14 neurônios NADPH-d$^+$/mm^2. Portanto, os dados coletados mostram-se condizentes com estudos anteriores em outras espécies de mamíferos quanto à localização do plexo mientérico, bem como, a proporção neuronal mientérica NADPH-d$^+$ comparada com a população de neurônios Giemsa$^+$. As lacunas existentes quanto ao conhecimento do SNE de morcegos limita possíveis inferências em comparativo intraespecífico e interespecífico.

TERMOS DE INDEXAÇÃO: Neurônios, plexo mientérico, morcegos, *Molossus rufus*, molossídeos, tubo digestório, sistema nervoso entérico, NADPH-diaforase, morfologia.

INTRODUCTION

The bats are animals with acknowledged importance in the dynamics of ecosystems, given the fact that the wide variety of food habits (Sazima et al. 1982, Bianconi et al. 2004). Insectivores, for example, serve as controllers of insect populations (Sipinski & Reis 1995). Medellín et al. (2000) emphasize that the bats can help as parameters for the identification of biological processes in terms of habitats, being the presence or not of the bats, an indicator of disturbed areas.

In Brazil, it is reported the occurrence of nine families, 68 genera and 178 species, of these, eight genera and 29 species are representatives of the Molossidae family, and among the different species of subfamily Molossinae, there is the *Molosus rufus* (Nogueira et al. 2014).

Molossus rufus (E. Geoffroy, 1805), insectivore, being restricted to flying insects (Wilson 1973), has as characteristic a tail beyond the interfemoral membrane, having a significant number of individuals inhabiting mainly residential buildings (Peracchi et al. 2011).

The food habits can elucidate, in addition to the characteristics already known, anatomorphological differences, and it can configure distinctions in the Enteric Nervous System (ENS). ENS is present along the digestive tube since the esophagus to the anus (Gabella 1990, Furness 2006).

Composed of small sets of nerve cells, enteric ganglia, as well as neural connections among them, the nerve fibers (Furness 2012), the submucous and myenteric plexus stand out, which act in the control of the motility, secretions and blood flow of the digestive tube, exerting influence on the absorption of nutrients (Costa et al. 2000, Schemann & Neunlist 2004, Phillips & Powley 2007), consisting of motor neurons, interneurons and sensory neurons (Furness & Costa 1980).

The myenteric plexus is located between the longitudinal and circular layers of the muscular tunic (Furness 2006), being that the organization and density of neurons may vary according to the animal species as well as the segment of the studied digestive tube (Irwin 1931).

Regarding the neurotransmitters expressed in neurons of the myenteric plexus, many have already been identified, among them the nitric oxide (NO), produced and released only when the enzyme nitric oxide synthase (NOS) becomes active (Furness 2006). It is possible to can sort the neurons of the myenteric plexus according to the neurotransmitter associated to it: catecholaminergic or adrenergic, cholinergic and non-adrenergic-non-cholinergic (NANC) (Paran et al. 2009).

NO is a neurotransmitter that acts as NANC mediator in the relaxation of the gastrointestinal smooth muscle (Brookes 1993), being reported as one of the most important inhibitory neurotransmitters of the intestine (Ekblad et al. 1994, Stebbing 1998, Chen et al. 2002) due to its presence in different biological reactions (Knowles & Moncada 1994, Rosselli et al. 1998), as participation in intestinal homeostasis and elimination of parasites (Halliez & Buret 2015), an association of inflammatory responses caused by parasites and apoptotic induction (Arantes et al. 2004, Nishikawa et al. 2007) and as a co-factor in diabetic neuropathy (Stevens et al. 1995).

Histochemical and immunohistochemical methods have already proved to be equivalent efficacy in evidencing nitregic neurons (Belai et al. 1992, Santer 1994, Saffrey 2004); those who express NOS, in a way that the NADPH-diaphorase histochemistry (Scherer-Singler et al. 1983) and the immunohistochemistry of NOS (Fabricius et al. 1996) show the same distribution of this subpopulation of neurons.

Phillips et al. (2003) highlight that 98% of the total myenteric population corresponds to the sum of the cholinergic and nitrergic population. Based on this premise and in the absence of descriptive studies regarding the ENS of bats, the objective of this study was to quantify the total population of neurons of the jejunum of myenteric bats of the species *M. rufus* as well as the subpopulation of the neurons NADPH-diaphorase$^+$.

MATERIALS AND METHODS

The collection of the specimens of *Molossus rufus* was authorized by "Instituto Chico Mendes de Conservação da Biodiversidade" (ICMBio - Protocol No. 60061-1) and the procedures were approved by the "Comitê de Ética no Uso de Animais Unipar" (CEUA - protocol number 34347/2017).

Collection of specimens. Five specimens of bats *Molossus rufus* were collected, in residence of the rural area located in the municipality of São Tomé, in the micro region of Cianorte, Paraná State, Brazil. The residence in question housed a large colony of molossidae, in view of the proximity with the urban area and riparian forest.

The selected microregion is configured as an area of buffer of the Biological Reservation of Perobas (Brasil 2012) (Fig.1), characterizing an ecological corridor for different species. The exact location of the collection area was designated via GPS (Global Positioning System), being 23°51'38.99''S and 52°60'91.70"W.

Two mist nets positioned in places of the animals' leaving, before sunset were used. The bats started their leaving around 06:00 p.m., so that the number of required specimens were obtained along a time of collection, after this period, the nets were withdrawn.

The animals were collected randomly, regardless of sex, being kept only the adult individuals. The identification of the animals captured was performed according to Vizzoto & Taddei (1973), Gregorin & Taddei (2002) and Ramos et al. (2013).

In order to minimize possible causative factors of stress, the animals were housed in cotton individual bags so that they could be sent to the Laboratory for Experimental Morphology of the Graduate Program in Animal Science with emphasis in Bioactive Products of "Universidade Paranaense", Umuarama, Paraná.

The euthanasia of each animal was carried out in camera saturated with isoflurane (1 to 3%). After confirmation of death, laparotomy was performed by the middle line of the anal region until the sternum. The collection of small intestines, corresponding to the jejunal segment was divided into two fragments of equal size, so that each one was subjected to two techniques for marking of neurons. A third fragment of jejunum, half a centimeter, was intended to routine histological processing, stained by hematoxylin-eosin (HE) and masson's trichrome (MT) in order to find the myenteric plexus.

Detection of myenteric neurons Giemsa⁺: disclosure of total neuronal population (Barbosa 1978). Fragments of the jejunum of each animal were intended to evidence the total population of myenteric neurons Giemsa⁺. The segments were washed with saline solution at 0.9%, filled and immersed in a fixing solution of Giemsa, respecting the minimum time for total fixation, 48 hours, remaining in this solution until obtaining the membrane formulations. To keep the filling, the ends of the jejunum were tied with suture.

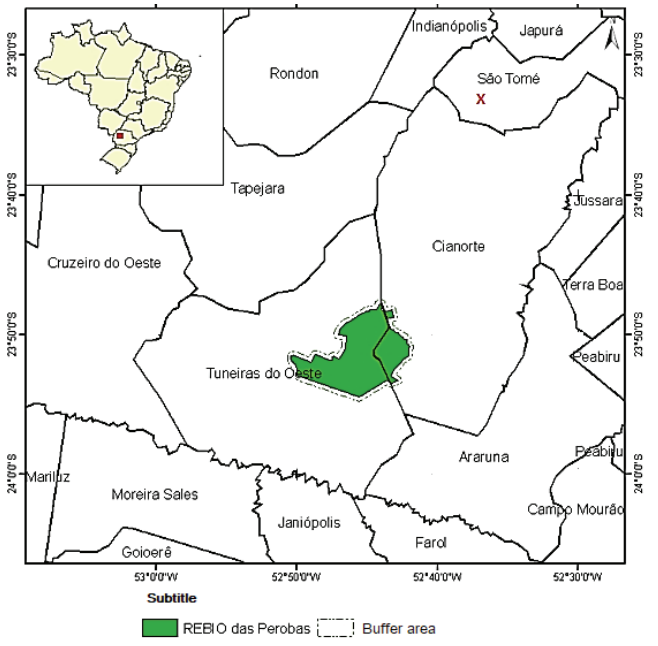

Fig.1 Location of the Biological Reservation of Petrobras, coverage area and buffer zone. The municipality of São Tomé (X), in the micro region of Cianorte, represents the site of the collection area. Adapted from: Brasil 2012.

After the period of fixation, the straps of the jejunum were removed, and this was sectioned along the longitudinal axis at the level of the insertion of the mesentery. Next, each fragment was micro dissected to the stereomicroscope with transillumination, removing the mucosal tunic as well as the submucous mesh. The muscle and serous tunics were preserved, which constituted the membrane formulations.

Each membrane was immersed in a solution containing Giemsa dye methylene blue in phosphate buffer of Sorense (pH 6.9) for 18 hours at ambient temperature. Then, they were dehydrated in sequence of alcohols (95% and Absolute I for one minute each, Absolute II for five minute) and then diaphanized in xylol (Xylol I and II for five minutes each). At the end, each membrane was placed between slide and glass plate with synthetic resin.

Detection of myenteric neurons NADPH-d⁺: disclosure of neuronal nitrergic subpopulation (Scherer-Singler et al. 1983). Fragments of the jejunum collected were subjected to histochemical technique of Nicotinamide Adenine Dinucleotide Phosphate-Diaphorase (NADPH-d). For this, the segments of jejunum were washed with phosphate buffer solution (PBS) (pH 7.4). After that, the edges were tied with suture wires so that the interior stayed filled with PBS. In the sequence, the fragments were immersed in paraformaldehyde 4% for 30 minutes and then washed in PBS containing Triton X-100 0.3% for 10 minutes.

After that, the fragments of the jejunum were again washed, for three more times (10 minutes each), in PBS and incubated for 90 minutes in the reaction medium containing 50mg of Nitro Blue Tetrazolium (NBT), 100mg of β-NADPH and 0.3% of Triton X-100 in Tris-HCl buffer (0.1M, pH 7.6). After the permeabilization, segments of jejunum were again washed in PBS, three times (five minutes each), and at the end of this period, the suture wires were removed on one end and the fragments were immersed in a solution of 4% paraformaldehyde for the interruption of the reaction, fixation and storage.

Later, the fragments of the jejunum were sectioned along the longitudinal axis at the level of the insertion of the mesentery and from these, membrane formulations were obtained, following the same procedures already described for the microdissection technique of Giemsa staining.

The obtained membrane preparations followed for dehydration in ascending sequence of alcohols (80%, 90%, Absolute I and Absolute II) followed of diaphanization in xylol (I and II). After concluding such procedures, the membrane formulations were placed between slide and glass plate with synthetic resin.

Quantification of neurons evidenced by Giemsa technique and histochemistry of NADPH-D. The delimitation of the mesenteric, intermediate and anti-mesenteric areas of the jejunum was done in accordance with Sant'Ana et al. (1997), as a way of guidance for the capture of images, in order to sample the three areas equally. The obtained material was visualized under light microscope (Nikon Eclipse E200), with a 40x objective lens, and a system of image analysis coupled to high resolution camera (Moticam 5.0 megapixels), being transferred to the computer.

Images were captured in 60 random microscopic fields per membrane being contemplated the mesenteric, intermediate and anti-mesenteric areas of the jejunum, intended for the quantitative analysis of the myenteric neurons stained by Giemsa technique and by histochemistry NADPH-d⁺. Halves neurons were considered in alternate fields.

Statistical analysis. The obtained data were subjected to descriptive analysis, using the program BioEstat 5.0 (Ayres et al. 2007). The number of total myenteric neurons (Giemsa$^+$) and the number of nitrergic myenteric ones (NADPH-d$^+$), per mm^2, were obtained for each animal, and the mean, standard deviation and coefficient of variation were obtained for both.

RESULTS

The analysis of the histological sections revealed the location of the myenteric plexus for *Molossus rufus* between the longitudinal and circular layers of muscular tunic and the presence of collagen fibers involving the myenteric ganglion.

Observed to the light microscope, neurons of the myenteric plexus of *M. rufus* were organized into ganglia mostly composed of several cell bodies, but isolated neurons were also found among the nerve fibers that interconnect the ganglia. The area sampled for each fragment of jejunum was 3.66mm^2 corresponding to 60 microscopic fields. Figure 2 presents the neuronal density per mm^2 specimen studied.

In Table 1 the following are expressed: the means ± standard deviation and the coefficient of variation of populations of total myenteric neurons (Giemsa$^+$) and subpopulation NADPH-d$^+$ of the jejunum of *M. rufus* estimated per mm^2 of each specimen studied.

For the membrane formulations stained by Giemsa technique an average was evidenced of 279.23±32.42 neurons/mm^2 and, for the histochemistry NADPH-diaphorase, average of 58.14±17.48 neurons/mm^2 (Table 1).

Table 1. Mean of total populations of myenteric neurons (Giemsa+) and subpopulation of NADPH-d + neurons of the jejunum of bat insectivore *Molossus rufus* evidenced by mm^2.

Technique	Mean	S.D.	C.V.%
Giemsa	279.23	±32.42	11.61
NADPH-d	58.14	±17.48	30.08

S.D. = Standard deviation, C.V. = coefficient of variation.

DISCUSSION

Having this study discussed the quantification of myenteric neurons of the jejunum of a species of insectivore bat, and there not being data in the literature regarding the characteristics of Enteric Nervous System (ENS) for bats in general, different studies with animal models were used, not only mammals, presuming the phylogeny occupied by different groups.

The location of the myenteric plexus of *Molossus rufus* is consistent with the described by Furness (2006), which highlights the importance of preserving the muscular tunic in the process of obtaining the membrane formulations, once that the myenteric plexus is located between the longitudinal and circular layers of muscular tunic.

Characteristics of the ganglionic arrangement of myenteric plexus are described in several studies (Sant'Ana et al. 1997, Germano et al. 2000, Stabille et al. 2000, Yang et al. 2013) that mention differences in this arrangement depending on the intestinal segment and the species studied (Irwin 1931, Furness & Costa 1980, Gabella 1990, Furness 2006) as well as in different food habits (Stabille et al. 2002, Münnich et al. 2008, Previato do Amaral et al. 2017).

Bundles of collagen fibers involved the myenteric ganglia in *M. rufus*, data also found in fish, described by Germano et al. (2000).

The specimens collected in spite of presenting different genders, four males and one female, it has been observed that for *M. rufus* the ganglionic organization of myenteric neurons does not differ among the individuals, the neurons were grouped into ganglia, having the same irregular formats and sparse distribution of isolated neurons among the fibers that interconnect the ganglia.

Assuming the phylogenetic evolution of the ENS, consider the interspecific differences such as feeding habits, region of collection of the studied intestinal fragments and body mass, as well as the intraspecific ones, which consider the morphofunctional and quantitative alterations of the myenteric plexus in animals with different ages, between the different intestinal regions, as well as the organ region sampled, are

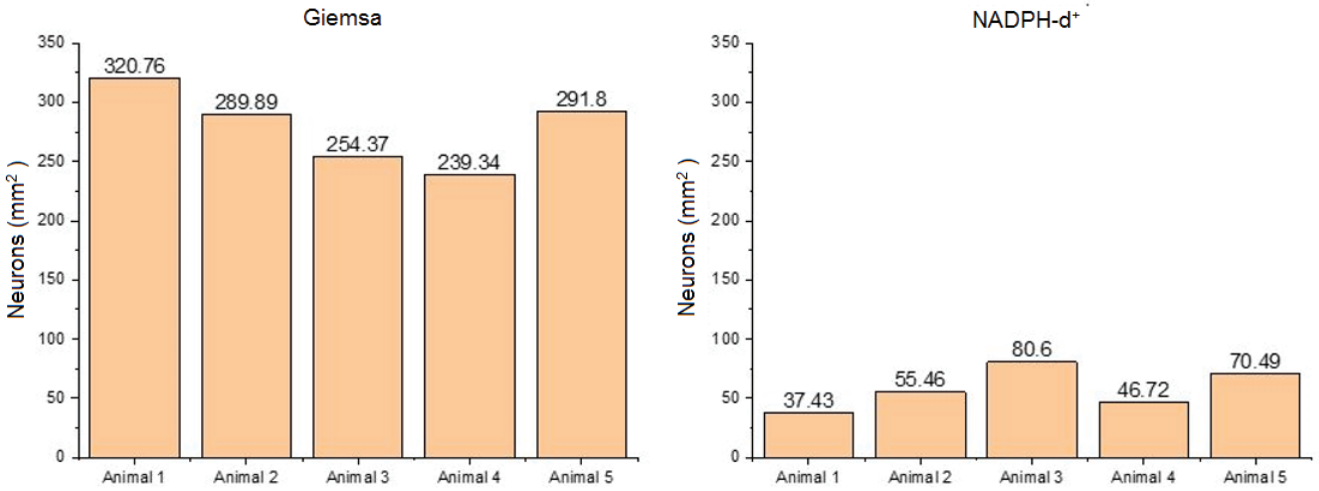

Fig.2 Population of total myenteric neurons (Giemsa+) and subpopulation of myenteric nitrergic neurons (NADPH-d+) evidenced by mm^2 per animal.

essential to the understanding of the above considerations. It is therefore conjectured that increased or decreased expression of NADPH-d$^+$ neurons correlates not only with metabolic status, but also with age, compensation for neuronal loss, and region of intestinal specimen collection.

Furlan et al. (2002) in morphoquantitative study with Wistar rats, report that the majority of myenteric neurons were found grouped in ganglia, being rare, isolated neurons. Germano et al. (2000) observed ganglia of sparse distribution, containing two or more neurons, and also isolated neurons in *Cyprinus carpio*, highlighting the location of the myenteric plexus between the longitudinal and circular layers of muscular tunic.

Previato do Amaral et al. (2017) in exploratory and descriptive study of the myenteric plexus of broilers (*Gallus gallus domesticus*) for NADPH-d$^+$ neurons in the duodenum, evidenced nitrergic neurons in both, ganglion arrangements and arranged in isolation among the fibers that interconnect the ganglia.

The data found for *M. rufus* bats in this study are similar to those described by the authors for the respective species studied, *Rattus norvegicus*, *C. carpio* and *G. gallus domesticus* (rat, fish and broiler). The data sampled by the different authors, as well as those from this study, converge to aspects of the phylogenetic evolution already described for the enteric nervous system (Furlan, 2000). Different studies reinforce this phylogeny, in fish neurons are predominantly isolated (Germano et al. 2000) and from chickens this conformation is inverted, with myenteric neurons predominantly organized in ganglia (Gabella & Halasy 1987).

Regarding the neuronal disclosure of proximal colon of adult rats, Furlan et al. (2002) showed an average of 5122 neurons Giemsa$^+$ in 17.68mm^2, these being corresponding to an average of 289.70 neurons/mm^2. Germano et al. (2000), in the intestinal bulb of carps, quantified an average of 2040 neurons Giemsa$^+$ in 6.92mm^2, equivalent to 294.79 myenteric neurons Giemsa$^+$/mm^2. Compared to the results of this study, the neuronal population Giemsa$^+$ among the different species are similar (Table 1).

Ferezin et al. (2017) reported the count of approximately 45 neurons Giemsa$^+$ in 0.249mm^2, corresponding to 180.72 neurons/mm^2 and 12 neurons NADPH-d$^+$ in 0.249mm^2, equivalent to 48.19 neurons/mm^2 for the colon of rats.

Previato do Amaral et al. (2017) found a mean ± standard deviation of 24.38±4.97 neurons NADPH-d$^+$/mm^2 in the duodenum of broilers, values which were lower than the values found in the present study, 58.14±17.48 neurons NADPH-d$^+$/mm^2.

Yang et al. (2013) found 58.88±3.16 neurons NADPH-d$^+$/mm^2 in the jejunum of broilers aged 15 days, and 45.92±17.51 neurons NADPH-d$^+$/mm^2 for broilers aged 40 days. Compared to the values of nitrergic neurons found in *M. rufus*, the results were similar although differences are expected among the species.

The mean of inhibitory neurons, as evidenced in this study, corresponds to 20.4% of the population of myenteric neurons evidenced by Giemsa technique, it is highlighted that the results fall within the expected parameters, once that they resemble those of Ekblad et al. (1994), who found 21% of neurons containing NOS in the small intestine of rats. Other studies have found a percentage next to those, 23% for guinea-pigs (Furness 2006), 29% in rats (Qu et al. 2008) and 34% in humans (Wester et al. 1999).

A smaller number of marked inhibitory neurons (nitrergic) does not necessarily indicate a lower number of nitrergic neurons, because the enzyme present in the reaction of the neuronal nitrergic marking in the NADPH-d technique, shows only those neurons with NOS activity at the time of the reaction (Scherer-Singler et al. 1983).

Considering that for the total neuronal myenteric population, the coefficient of variation presented is not expressive, 11.61% (Table 1), confirming a similarity between the samples and the studied individuals, differing only concerning the number of nitrergic neurons in activity at the time of collection.

Regarding the studies on the bats' digestive tube, they stand out for their histology (Makanya et al. 2001, Gadelha-Alves et al. 2008, Strobel et al. 2015, Zhang et al 2015) and microbiology (Ingala et al. 2018, Sens Junior et al. 2018). However, Barry-Jr (1976) already emphasized the little research about the different segments of this system, including the intestine to wild mammals, a real fact still today, once that the studies on ENS are widely disseminated to rodents (Karaosmanoglu et al. 1996, Tan et al. 2008, Luesma et al. 2013, Grundmann et al. 2015, Kulkarni et al. 2017) and more recently to primates (Noorian et al. 2011), but in terms of other groups of wild mammals, not employed in experimental models, the absence of data is notorious.

Gadelha-Alves et al. (2008), emphasizes the importance of the study of the bats' digestive system, aiming to understand the factors associated with it, such as environmental changes originated from anthropic action in their habitats.

Zhang et al. (2015), upon comparing the surface of the mucosa of the small intestine of *Tadarida brasiliensis*, also an insectivore bat, and the species *Mus musculus* discussed the high digestive capacity of bats in association with the higher density of villi and enterocytes, corresponding to a higher paracellular permeability per cm^2 in the bats. The authors also emphasize to the digestive capacity and its relation with the flight efficiency of these animals, demonstrating a digestive complex of high capacity, despite their small body mass.

In this sense it is worth mentioning that the myenteric neurons coordinate the intestinal movements, interfering not only in the intestinal transit, but also in the absorption capacity (Costa et al. 2000, Schemann & Neunlist 2004, Phillips & Powley 2007).

Considering the time of the capture of animals for this study before the start of the foraging, a lower inhibitory neuronal expression can be considered. Thus, the density of nitrergic inhibitory myenteric neurons is not related only to morphological adaptations and the diet of the animal, but also with its metabolic state at the time of marking those neurons, i.e., whether the neurons that express NOS are active or not (Scherer-Singler et al. 1983).

Gabella (1990) emphasizes that the extension of the intrinsic neuronal population of the intestine reflects the importance of the functions of the same for the survival of the animal especially in nature, in view the high level of adaptability to variation in food contents and feeding time, require a well-developed nervous control.

However, the absence of an effective blood-neuron barrier (Furness 2006) and the high paracellular absorption capacity of the bats (Fasulo et al. 2013) leave these animals very

susceptible to exposure to toxins present in environments subject to change by human occupation, and by chance, in their food, which could compromise the neuronal population. It is therefore highlighted the importance of this exploratory and quantitative study of myenteric neuronal population of bats, corroborating with a better understanding of these individuals in their entirety.

CONCLUSIONS

The myenteric plexus in bats of the species *Molossus rufus* is located between the longitudinal and circular layers of muscular tunic, while neurons organized into ganglia surrounded by collagen fibers, presenting a few isolated neurons among the nerve fibers.

Variations in the total population of myenteric neurons is expected among the different species as well as in different segments of the digestive tube. The mean of the subpopulation of active nitrergic neurons in the studied species is consistent with that described for different species of animals of different classes, assuming a variation from 20 to 35%.

The quantification of the total population of myenteric neurons and the nitrergic subpopulation contributes to know the morphology and function of the enteric nervous system (ENS) in bats, since these are distinguished from other mammals by the ability to true flight, besides helping in understanding the intestinal anatomy peculiar to these individuals, described as proportionately small, however with great digestive capacity.

These results can contribute to future research aimed at a better understanding of these animals in the context of unique health.

Acknowledgments.- The authors are grateful to "Universidade Paranaense" (Unipar) that through "Diretoria Executiva de Gestão da Pesquisa e Pós-Graduação" (DEGPP) supported the development of this research and to "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES), Brazil, for the grant of scholarship to the first author of the article.

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

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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!)

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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)

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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)

Pesquisa Veterinária Brasileira

Brazilian Journal of Veterinary Research

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Grillo Tecnologia

ISSN 0100-736X *(Print)*
ISSN 1678-5150 *(Online)*