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Cover illustration: Diffuse seminoma in a dog testis. (Nascimento et al., 528)

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Original Article
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Epidemiological aspects of natural poisoning by *Prosopis juliflora* in ruminants in semiarid areas of the state of Bahia, Brazil, invaded by the plant[1]

Múcio F.F. Mendonça[2], Pedro M.O. Pedroso[3], Luciano A. Pimentel[4], Karina M. Madureira[2], Juliana T.S.A. Macêdo[3], Charmila S. D'Soares[6], Antonio W.O. Silva[2] and Tiago C. Peixoto[2,5]*

ABSTRACT.- Mendonça M.F.F., Pedroso P.M.O., Pimentel L.A., Madureira K.M., Macêdo J.T.S.A., D'Soares C.S., Silva A.W.O. & Peixoto T.C. 2020. **Epidemiological aspects of natural poisoning by *Prosopis juliflora* in ruminants in semiarid areas of the state of Bahia, Brazil, invaded by the plant**. *Pesquisa Veterinária Brasileira 40(7):501-513.* Laboratório de Patologia Veterinária, Escola de Medicina Veterinária e Zootecnia, Universidade Federal da Bahia, Av. Adhemar de Barros 500, Salvador, BA 40170-110, Brazil. E-mail: tcpeixoto@ufba.br

Poisoning by *Prosopis juliflora* (mesquite) leads to neurological signs, cachexia and death, mainly in cattle and goats. Although the uncontrolled spread of mesquite in the Caatinga biome (biological invasion), which alters the epidemiological conditions of intoxication by this plant, has been proved for approximately 20 years, strategies for its control and prophylaxis still remain out of date. These new epidemiological conditions have allowed the uncontrolled consumption of large amounts of *in natura* mesquite pods by ruminants for long periods in invaded pastures, thus resulting in increased history of poisoning. This study aimed to describe the new epidemiological aspects of *P. juliflora* poisoning in cattle and goats, 78 years after the introduction of this plant in the country, with emphasis on its degree of invasion, and to update the control and prophylaxis measures of this intoxication and the mapping of areas of outbreak occurrence in the semiarid region of the state of Bahia, Brazil. Seven outbreaks of natural mesquite poisoning, two in goats (OB 1 and OB 2) and five in cattle (OB 3, OB 4, OB 5, OB 6, and OB 7), were studied *in loco* in the municipalities of Juazeiro, Iaçu, Tucano, Santa Teresinha, Barra do Mendes, Barra and Tabocas do Brejo Velho. In the studied outbreaks, clinical-epidemiological (OB 1 to OB 7) and histopathological (OB 1, OB 2, OB 3, and OB 5) findings were compatible with mesquite poisoning, and this was the first anatomopathological proof of poisoning by this plant in this state. In addition, in the state of Bahia, disease occurs in an area approximately three times larger than previously known. On the farms investigated, mesquite introduction occurred between 1980 and 2005, through the single planting of an average of 33 seedlings. Since then, propagation of this plant has occurred progressively, with gradual invasion of native pastures, which enabled the evaluation of plant spread (biological invasion) on these farms 15 (OB 2), 25 (OB 7), 30 (OB 5 and OB 6), 35 (OB 3) and 40 (OB 1 and OB 4) years after its introduction. Historical information on the introduction and spread of mesquite in the state of Bahia had never been analyzed. In 2020, a degree of mesquite invasion an average 59.57% was verified in the pastures of

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the seven farms where the outbreaks occurred. The great biological invasion capacity of this plant drew attention, especially in OB 5. The high degree of mesquite invasion observed (new epidemiological conditions) justifies the increased number of cases of poisoning observed in this study. Processing of *P. juliflora* pods (grinding) was not carried out on any farm (OB 1 to OB 7), and ruminants uncontrollably consumed large amounts of pods *in natura* for long periods in invaded pastures, which allowed massive dispersal of seeds through feces over decades. The main factors responsible for the gradual invasion of pastures by mesquite over time were absence of crop management plans (silvicultural treatments) and lack of knowledge by producers on disease etiology. Given the new epidemiological conditions, restriction of animal access to *in natura* pods in pastures and supply of mesquite bran are crucial for the control and prophylaxis of this poisoning, as consumption of *in natura* pods had a strong correlation with the high degree of invasion in the pastures where the seven outbreaks occurred. Additionally, although the commercialization of pods and exploitation of wood of mesquite trees can provide livestock farmers with extra income – being the correct management for areas invaded by this plant, such practice is either not yet known or not carried out technically or satisfactorily by farmers. In addition to being a threat to the Caatinga's biodiversity, the increasing invasion of semiarid areas by mesquite 78 years after its introduction in the Northeast region of Brazil, is a risk for herds, since the occurrence of poisoning outbreaks may become more frequent. Therefore, it is highly advisable that effective measures be adopted to control *P. juliflora* propagation.

INDEX TERMS: Epidemiology, natural poisoning, *Prosopis juliflora*, ruminants, state of Bahia, Brazil, neurotoxic plant, invasive plant, Caatinga biome regeneration, mesquite beans, neuronal degeneration.

RESUMO.- [**Aspectos epidemiológicos da intoxicação natural por *Prosopis juliflora* em ruminantes em áreas do semiárido baiano invadidas pela planta.**] A intoxicação por *Prosopis juliflora* (algaroba) cursa com sinais neurológicos, caquexia e morte, principalmente em bovinos e caprinos. Embora a disseminação descontrolada da algaroba na Caatinga (invasão biológica) tenha sido comprovada há cerca de 20 anos, o que altera as condições epidemiológicas dessa intoxicação, as estratégias de controle e profilaxia permanecem desatualizadas. Essas novas condições epidemiológicas permitem o consumo descontrolado de grande quantidade de vagens *in natura* de algaroba, por longos períodos, nas pastagens invadidas, o que tem resultado em aumento dos históricos de intoxicação. Objetivou-se com esse estudo descrever os novos aspectos epidemiológicos da intoxicação por *P. juliflora* em bovinos e caprinos, 78 anos após a introdução da planta no país, com ênfase no grau de invasão da planta, bem como atualizar as medidas de controle e profilaxia dessa intoxicação e das áreas de ocorrência dos surtos no semiárido baiano. Foram estudados *in loco* sete surtos (S1 a S7) de intoxicação natural por algaroba, sendo dois em caprinos (S1 e S2) e cinco em bovinos (S3, S4, S5, S6 e S7) no semiárido baiano (Juazeiro, Iaçu, Tucano, Santa Teresinha, Barra do Mendes, Barra e Tabocas do Brejo Velho). Nos surtos estudados, os achados clínico-epidemiológicos (S1 a S7) e histopatológicos (S1, S2, S3 e S5) foram compatíveis com intoxicação por algaroba, sendo essa intoxicação comprovada, pela primeira vez, com exames anatomopatológicos na Bahia. Ademais, na Bahia a doença ocorre em uma área cerca de três vezes maior do que a, até então, conhecida. Nas fazendas estudadas a introdução da algaroba ocorreu entre 1980 e 2005, através do plantio único de, em média, 33 mudas. Desde então, a disseminação da planta ocorreu de forma progressiva, invadindo gradativamente as pastagens nativas, o que permitiu avaliar a disseminação da planta (invasão biológica) nessas fazendas cerca de 15 (S2), 25 (S7), 30 (S5 e S6), 35 (S3) e 40 (S1 e S4) anos, após sua introdução. Informações históricas acerca da introdução da algaroba e sua disseminação na Bahia nunca haviam sido estudadas. Em 2020, verificou-se que nas pastagens das sete fazendas onde os surtos ocorreram, o grau de invasão por algaroba alcançou, em média, 59,57%. Chamou a atenção a grande capacidade de invasão biológica da planta, sobretudo, no S5. O alto grau de invasão da algaroba observado (novas condições epidemiológicas) justificou o aumento dos casos de intoxicação verificados nesse estudo. O beneficiamento das vagens (moagem) não era realizado em nenhuma fazenda (S1 a S7) e os ruminantes consumiam de forma descontrolada (livre) grande quantidade de vagens *in natura*, por longos períodos, nas pastagens invadidas, o que possibilitou a dispersão massiva das sementes da planta pelas fezes ao longo das décadas. O que aliado à inexistência de um plano de manejo do cultivo (tratamentos silviculturais) e ao desconhecimento da etiologia da doença pelos produtores foram os principais fatores responsáveis pela invasão gradativa da planta nas pastagens, ao longo dos anos, o que justifica o aumento dos casos de intoxicação observados na Bahia. Frente às novas condições epidemiológicas, a restrição do acesso dos animais as vagens *in natura* nas pastagens e o fornecimento do farelo de algaroba são cruciais para o controle e profilaxia dessa intoxicação, pois o consumo das vagens *in natura* teve forte correlação com o alto grau de invasão das pastagens onde os sete surtos ocorreram. Adicionalmente, embora a comercialização das vagens e a exploração da madeira da algaroba possam proporcionar renda extra aos pecuaristas e serem boas formas de manejo de áreas invadidas por algaroba, tais práticas ainda não são conhecidas ou não são realizadas de forma técnica ou a contento. A crescente invasão da algaroba no semiárido, 78 anos após a sua introdução no Nordeste, além de ser uma ameaça à biodiversidade da Caatinga é um risco para os rebanhos, visto que, a ocorrência de surtos de intoxicação podem se tornar mais frequentes. Desta maneira alerta-se para adoção de medidas efetivas de controle da propagação descontrolada da algaroba.

TERMOS DE INDEXAÇÃO: Epidemiologia, intoxicação natural, *Prosopis juliflora*, ruminantes, Bahia, Brasil, planta neurotóxica, planta invasora, regeneração da Caatinga, algaroba, degeneração neuronal.

INTRODUCTION

Poisoning by plants results in significant economic loss in national livestock due, in part, to the large number of herds raised in native pastures or under extensive or semi-extensive management (Riet-Correa & Medeiros 2001, Tokarnia et al. 2012, Pessoa et al. 2013). A study showed that the highest number of deaths of animals poisoned by plants occurs in the North, Northeast and Center-West regions of Brazil (Riet-Correa & Medeiros 2001); however, there is still a lack of information on some of these poisoning occurrences in the state of Bahia. In this context, *Prosopis juliflora* poisoning stands out, presenting neurological signs, cachexia and death, mainly in cattle (Figueiredo 1993, Dantas & Menezes 1994, Silva et al. 2006, Câmara et al. 2009, Galiza et al. 2010) and goats (Lima et al. 2004, Lemos et al. 2016) and, to a lesser extent, in sheep (Almeida et al. 2017), associated with neurological injuries, especially in the trigeminal nerve motor nucleus (Lima et al. 2004).

P. juliflora is a xerophilic plant of the Fabaceae family, subfamily Mimosoideae, popularly known as mesquite (Ribaski et al. 2009). It is native to Mexico, Central America and northern South America (Peru, Ecuador, Colombia, and Venezuela) and has been used as food by humans since prehistory. In the 1940s, *P. juliflora* was introduced in northeastern Brazil - in the municipalities of Serra Talhada (PE, 1942), Angicos (RN, 1946) and Seridó (RN and PB, 1948) - through the planting seeds from Peru and Sudan as a technological alternative to cope with drought (Azevedo 1961, SPA 1989). It aimed to provide palatable forage with high nutritional value and low cost for animal feed (SPA 1989) and be used as firewood and in the production of stakes and charcoal. In addition, it was believed that its cultivation would promote regional development because of its adaptation to high temperatures and poor soils, high productivity, resistance to drought, multiple uses of its wood, source of food for herds and resource for beekeeping, as well as a solution for reforestation (Cunha & Silva 2012).

In 1951, the Brazilian Ministry of Agriculture began to supply technicians, rural producers, and city halls in the state of Rio Grande do Norte with mesquite seeds. In the following years, the plant spread to other northeastern states (Azevedo 1961, SPA 1989). By 1979, mesquite planting in the region occurred in an estimated area of more than 90,000 ha, encouraged by public institutions such as the Brazilian Institute for Forestry Development (IBDF) (Reis 1985, SPA 1989). In this context, the only program that supported the development of semiarid areas in the Brazilian Northeast region ("Project Sertanejo") deserves special mention. This project financed the planting mesquite in properties ranging from 20 to 500 ha as part of a strategy to strengthen agricultural production units (Cunha & Silva 2012). At that time, *P. juliflora* was already considered the most successful xerophilous plant introduced in the semiarid region (SPA 1989), and the Brazilian Agricultural Research Corporation (Embrapa), through its unit responsible for the semiarid region – Embrapa Semiarid Agriculture, introduced mesquite specimens from Argentina, Chile, Peru, Mexico, the United States, Honduras, Pakistan, Cape Verde and Senegal in order to genetically improve the mesquite trees in the Northeast region (Lima 1999).

In the 1990s, the area covered by mesquite, planted or of natural expansion, in the northeast semi-arid region was estimated in over 500,000 ha (Tabosa et al. 2004), with higher concentration in the states of Paraíba, Pernambuco and Rio Grande Norte, especially, in areas with greater anthropic activities, close to dams, streams and highways. These axes are located along the semiarid hinterland depression, in wilderness areas, along the São Francisco River, around the Araripe plateau, and along the coast of Ceará state (Lima 2005). Since then, mesquite spread has occurred in the different agroecological areas of the Northeast region, with millions of scattered specimens with no precise estimate on planted or regenerated areas (Lima 1999). Cunha and Silva (2012) emphasized the inexistence of an economic analysis of the contribution of mesquite to the semiarid economy and affirmed that, for over 60 years, the coexistence of individuals and groups with this species has been marked by enthusiasm, controversy, and criticism.

Although *P. juliflora* was also introduced in the state of Bahia decades ago, there was no historical information on its expansion. Only in 2005, the study conducted by Lima and collaborators, with the support of Embrapa Semiarid Agriculture, followed the expansion process of this plant in the region of the municipality of Juazeiro, state of Bahia, through the analysis of satellite images, and precisely confirmed the evolution of its biological invasion in the Caatinga biome. This study found that mesquite shows exceptional spontaneous regeneration, with a gradual annual increase in its coverage area. In 10 years, the areas invaded by mesquite increased from 19.93 ha in 1986 to 33.03 ha in 1996 (increase of 65.68%), and the area regenerated with the plant showed an increase of 37.85% in two study locations. This difference was attributed to the existence of water in the site. All areas of invasion studied in the Northeast region were located close to watercourses (riverbanks or alluvial areas) (Lima 2005).

Due to the natural advance of *P. juliflora* in the Caatinga biome, a series of plant structure analyses were carried out in invaded areas to measure the frequency, abundance, dominance and regeneration of the plant in some areas of the Northeast region. In the state of Bahia, phytosociological surveys of areas invaded by mesquite were conducted in Juazeiro (Lima et al. 2003, Barbosa & Lima 2005) and in the riparian forest of Rio de Contas in Manoel Vitorino (Lima & Kiill 2003). Among the trees and/or shrubs studied, *P. juliflora* was the most frequent found in Juazeiro and Manoel Vitorino (95-100% and 88%, respectively), with higher abundance in Serra Branca (654 ind./ha), followed by Salitre (575 ind./ha), Massaroca (440 ind./ha), Manoel Vitorino (370 ind./ha), and Juremal (313 ind./ha). As for regeneration, mesquite presented 1,255 ind./ha, which corresponded to 60.8% of the seedlings regenerated in Manoel Vitorino, and in the two location of the study conducted in Juazeiro, it corresponded to 47.3 and 78.53%, respectively (Lima et al. 2003, Barbosa & Lima, 2005).

Similar findings were reported in areas of native vegetation invaded by the plant in the municipalities of Taperoá (Marques et al. 2003) and Monteiro in the state of Paraíba (Marques et al. 2004) and of Santa Maria da Boa Vista (Lima et al. 2004a), Iguaraci (Lima et al. 2004b) and Inajá (Lima et al. 2004c) in the state of Pernambuco. In this series of studies, there were a large number of mesquite plants in relation to that of native species, confirming the excessive proliferation of the exotic species (biological invasion). This excess alerts to the need to identify the agents that facilitate the plant

propagation in order to control its invasion in the Caatinga biome (Lima et al. 2003, Lima & Kiill 2003, Marques et al. 2003, Lima et al. 2004a, 2004b, 2004c, Marques et al. 2004, Barbosa & Lima 2005, Lima 2005). However, the possibility of increased frequency of mesquite poisoning outbreaks in ruminants under these new epidemiological conditions (biological invasion) has not been considered or discussed. In 2009, the impacts caused by the mesquite invasion on the phytodiversity and the shrub-tree component structure of the Caatinga in the state of Rio Grande do Norte were studied, showing that the plant threatens the native biodiversity of this biome (Andrade et al. 2009).

Prosopis juliflora has been highlighted in international debates on the ecological impact of invasive species (Gallaher & Merlin 2010, Tessema 2012, Pegado et al. 2006, Perera et al. 2005). Biological invasion is the process of occupying a space where the invasive plant does not originate (outside its geographic distribution) in a disorderly manner, with subsequent alteration in the structure of native biological communities and/or in the functionality of ecosystems (ecological imbalance) (Pegado et al. 2006). The management of these species was defined as one of the millennium goals, as they are identified as one of the leading causes of the loss of biodiversity worldwide (Tessema 2012).

Approximately 80 years after its introduction in Brazil, thousands of *P. juliflora* specimens are spread across all agroecological zones in the Northeast region. The expansion of mesquite as an invasive species has been associated with absence of natural enemies, high resistance to drought and salinity (Lima 2005) and seed dormancy in soil for up to 10 years, as well as with massive production of seeds and their efficient dispersion (GISP 2005). Additionally, it has been proposed that the uncontrolled and massive seed dispersal through the feces of extensively created cattle, goats, and mules (Souza et al. 1999) is the main factor associated with the process of mesquite invasion in northeastern semiarid areas (GISP 2005). In addition, the eco-sociological characteristics of the Northeast region and the lack of forestry tradition resulted in neglected plant management and, consequently, in the invasion of extensive areas of Caatinga (Andrade 2013).

Another current problem is the unregulated exploitation of mesquite wood, one of the effects of its invasive plant status. There is no regulation and control of the cutting and marketing of wood in the northeastern semiarid region, with wood used in industrial ovens (bakery and pottery) and in charcoal production (Cunha & Silva 2012). Thus, the trajectory of mesquite in the northeastern semiarid region was marked by two phases: expansion (stimulated by public financing) and, recently, by disordered exploitation (use of pods and wood).

These new epidemiological conditions resulting from the biological invasion of mesquite in the semiarid region in many regions of the state of Bahia provide ruminants with free access to plant pods and their uncontrolled consumption in pastures for long periods, which has resulted in an increased history of intoxication. However, the real economic importance of this poisoning in regions invaded by the plant and the adoption of possible alternatives for the control and prophylaxis of this poisoning are currently not discussed.

Outbreaks of natural poisoning by *P. juliflora* have already been described in ruminants in several states in the Northeast region: in cattle, in Paraíba, Rio Grande do Norte and Pernambuco (Figueiredo 1993, Dantas & Menezes 1994, Silva et al. 2006, Câmara et al. 2009, Galiza et al. 2010); in goats, in Paraíba (Lima et al. 2004) and Pernambuco (Lemos et al. 2016); and, recently, in sheep, in Pernambuco (Almeida et al. 2017). However, no cases of poisoning by *P. juliflora* in the state of Bahia have been reported in the literature. It is likely that this intoxication, even requiring special conditions (ingestion of a large amounts of pods, that is, more than 50% of the diet, for prolonged periods) (Tokarnia et al. 2012), has been underdiagnosed and/or mistaken for other neurological and cachectic diseases in this state. This study aimed to describe the new epidemiological aspects of *P. juliflora* poisoning in cattle and goats, 78 years after the introduction of this plant in the country, with emphasis on its degree of invasion, and to update the control and prophylaxis measures of this intoxication and the mapping areas of outbreak occurrence in the semiarid region of the state of Bahia, Brazil.

MATERIALS AND METHODS

Seven outbreaks (OB 1 to OB 7) of spontaneous poisoning by *Prosopis juliflora* in goats and cattle in different municipalities of the state of Bahia were studied *in loco*. Data on disease epidemiology over decades, in particular, on the introduction and propagation (biological invasion) of the plant, the use of its wood and pods, and the history and clinical signs of poisoning were obtained from farm owners and/or veterinarians during visits.

In January 2020, the areas in hectares (ha) invaded by mesquite (areas where the plant was not planted) were estimated on each farm based on their respective total pasture areas. This estimate was made visually by the same appraiser based on the observation of predominance of regenerated trees and seedlings (germinated seeds, existence of seedlings, and regrowth of previously explored stumps or roots) of mesquite (exotic species) over native plants. According to Lima (2005), this indicates environmental imbalance. Such data enabled estimation of the current degree of plant invasion (%) on the farms (uncontrolled dissemination) in different periods after its introduction (15 to 40 years), through the single planting of seedlings, as well as verification of possible correlations between biological invasion and 1) outbreaks of poisoning in cattle and goats, 2) access to pods (free or controlled); 3) use of pods (fresh or ground), and 4) management of trees (thinning and use of wood in cuttings, posts, or production of firewood and/or charcoal) aiming at updating the control and prophylaxis measures of poisoning in the face of new epidemiological conditions.

Necropsies and histopathological evaluations of OB 1 and OB 3 were performed by the "Setor de Patologia Veterinária" of the "Universidade Federal do Recôncavo da Bahia" (SPV-UFRB), whereas those of OB 2 and OB 5 were conducted by the "Laboratório de Patologia Veterinária" of the "Universidade Federal da Bahia" (LPV-UFBA). At necropsy of the animals during OB 1, OB 2, OB 3 and OB 5, fragments of the central nervous system, masseter and biceps femoris muscles, and abdominal and thoracic organs were collected. This material was fixed in 10% buffered formaldehyde, processed routinely for histopathology, and stained with hematoxylin-eosin (HE).

RESULTS

Epidemiology. Outbreak one (OB 1) occurred in the municipality of Juazeiro (360 m asl; 9º24'42" S; 40°29'55" W) in 2011 and outbreak two (OB 2) occurred in Iaçú (242

m asl; 12°46'02" S; 40°12'42" W) in 2015, both affecting only goats. In outbreaks three (OB 3), four (OB 4) five (OB 5), six (OB 6), and seven (OB 7), only cattle were poisoned. OB 3 occurred in Tucano (209 m asl; 10°57'47" S; 38°47'12" W) in 2014, OB 4 in Santa Terezinha (227 m asl; 12°46'19" S; 39°31'24" W) in 2016, OB 5 in Barra do Mendes (700 m asl; 11°38'27" S; 42°00'39" W) in 2019, OB 6 in Barra (406 m asl; 11°05'20" S; 43°08'31" W) in 2017, and OB 7 in Tabocas do Brejo Velho (541 m asl; 12°42'21" S; 44°00'25" W) in 2018. All outbreaks occurred in regions of semiarid climate, with high temperatures, and scarce and poorly distributed rainfall. In the seven outbreaks studied, the etiology of the disease was unknown to the producers. The main epidemiological data on the seven outbreaks of natural mesquite poisoning in the state of Bahia are summarized in Table 1.

Outbreaks in goats. OB 1 occurred on a farm with an area of 61.8 ha. According to the farm's mesquite was introduced in this property in 1980, through the single planting of 40 seedlings. During the technical visit, it was found that the property currently has thousands of mesquite specimens, with approximately 38.6 ha of invaded native vegetation (uncontrolled natural spread). The herd assessed consisted of 150 goats and 50 sheep under semi-extensive rearing, with feed and common salt in troughs, two to three times a week. According to the farm owner, from 2006 to 2010, 22 goats kept in areas heavily invaded by mesquite showed a similar clinical condition, with evolution of six months until death. During a technical visit in May 2011, two 2.5-year-old SRD goats with clinical signs compatible with mesquite poisoning were observed in the herd. Due to the unfavorable prognosis, the farm owner opted for euthanasia and necropsy aiming diagnosis.

OB 2 occurred on a goat farm with an area of 300 ha, where mesquite was introduced through the single planting of 30 seedlings in 2005. During the inspection of pastures, there were hundreds of *Prosopis juliflora* adult trees with many pods in the pastures, lowland, and close to the shed where the animals were kept, with approximately 50 ha invaded by the plant. The herd consisted of 20 Boer goats under semi-extensive management. The pastures were formed by Buffel grass (*Cenchrus ciliares*) and native pasture with mineral salt was provided in troughs. According to the property owner, in October 2014, four goats with mean age of 4.5 years presented a similar clinical condition, and two of them died five months after onset of clinical signs. In February 2015, the other two goats were sent to the "Centro de Desenvolvimento da Pecuária" (CDP) at UFBA, where they remained hospitalized for ten days with clinical signs compatible with mesquite poisoning. Due to the unfavorable prognosis, both animals were euthanized and necropsied for diagnostic confirmation.

Outbreaks in cattle. OB 3 occurred on a 55-ha property, in which the plant was introduced in 1985, through the single planting of 50 seedlings. There was approximately 30 ha of native vegetation heavily invaded by thousands of mesquite trees during the technical visit. The herd consisted of 30 cattle, 15 buffaloes, and 70 sheep raised under extensive system on native pastures and mineralized salt in troughs. According to the farm owner, three Girolando cows aged >10 years showed chronic clinical signs (six months). The first case occurred in 2007 and the other two were clinically evaluated during technical visits in July and September 2014.

Table 1. Epidemiological data on outbreaks of natural poisoning by *Prosopis juliflora* in ruminants in the state of Bahia

Outbreak (OB)	Affected species and time of outbreaks	Municipality and management system	Introduction of the plant		Farm area (ha)		Plant management[a]		Intoxicated animals			Mortality[c]
			Planting year and time elapsed until 2020	Number of seedlings planted	Total	Invaded by mesquite in 2020	Degree of invasion	Thinning and use of wood[b]	Storage, trough supply and marketing of pods	Herd size	Total cases[c]	Average age (years) and sex[d]	
1	Goats (G) 2006-2011	Juazeiro Semi-extensive	1980 40 years	40	61.8	38.6	62.45%	No	No	150	24[e]	2.5 F	16%
2	Goats 2015	Iaçu Semi-extensive	2005 15 years	30	300	50	16.66%	No	No	20	4[e]	4.5 F	20%
3	Cattle (C) 2014	Tucano Extensive	1985 35 years	50	55	30	54.54%	Yes	Yes	30	3[e]	>10 F	10%
4	Cattle 2006-2016	Santa Terezinha Semi-extensive	1982 38 years	60	390	310	79.48%	Yes	Yes	110	21	8 F	19%
5	Cattle 2016-2019	Barra do Mendes Extensive	1989 31 years	4	280	266	95%	Yes	No	20	7[f]	>4 F and M	35%
6	Cattle 2018	Barra Extensive	1990 30 years	30	50	20	40%	No	No	40	12	10 F	30%
7	Cattle 2018-2019	Tabocas Extensive	1995 25 years	20	266	170	63.90%	No	No	50	10	>7 F	20%
Average	-	-	29.85 years	33	200.5	126.37	59.57%	-	-	60	12 (G) 10.6 (C)	3.5 years (G) >7.8 years (C)	18% (G) 22.8% (C)

[a] On the seven farms, ruminants had free access to areas intensely invaded (without fences) by mesquite with uncontrolled consumption of large amounts of fresh pods in pastures for long periods; [b] Use of wood from the pruning of mesquite trees in the property (stakes, posts, fences, or production of firewood and/or charcoal) or its commercialization; [c] The period of time considered varied in each outbreak according to the history of the poisoning occurrence; [d] F = female, M = male; [e] Two animals were euthanized; [f] One bovine was euthanized.

On these occasions, clinical signs compatible with mesquite poisoning were observed, and the two cows were euthanized and necropsied for diagnostic clarification.

OB 4 occurred on a 390-ha farm where *P. juliflora* was introduced through the single planting of 40 seedlings in 1982. During the technical visit, it was observed that approximately 310 ha had been slowly invaded by thousands of trees, forming a real "mesquite forest". The herd consisted of 110 cattle with varied age range. The animals were raised semi-extensively on native pasture and fed forage palm (*Opuntia cochenillifera*) and mineral salt in troughs. According to the property owner, from 2006 to 2016, 20 cattle showed a similar clinical condition, followed by death approximately six months after the onset of signs. In August 2016, during the technical visit, an 8-year-old SRD cow with the disease was examined, and a clinical condition compatible with mesquite poisoning was verified. One month after the visit, the animal died, but the owner did not authorize the necropsy.

OB 5 occurred on a 280-ha farm. In this property, mesquite was introduced through the planting of only four seedlings in 1989. The herd consisted of 20 SRD cattle of varying age, raised extensively on pasture formed almost exclusively by mesquite trees (thousands) - the plant had heavily invaded an area of approximately 266 ha. According to the farm owner, from 2016 to 2018, six cattle (both male and female) had a similar chronic clinical condition for six months, followed by death. In January 2019, a 4-year-old male bovine presenting clinical signs of the disease was examined during the technical visit. Due to the unfavorable prognosis, the property owner opted for euthanasia followed by necropsy for diagnostic confirmation.

OB 6 occurred on a 50-ha property where *P. juliflora* was introduced through the single planting of 30 seedlings in 1990. On this farm, 40 cattle (Nellore and mestizo) were raised extensively on native pastures, some of them intensely invaded by mesquite (approximately 20 ha) and fed mineral salt in troughs. During the technical visit of January 2019, the farm owner reported that 12 cows with mean age of 10 years died after presenting a similar clinical condition for five months in 2018. The epidemiological conditions observed at the site, associated with the history and reported clinical signs, were compatible with intoxication by mesquite.

OB 7 occurred on a 266-ha farm where the plant was introduced through the single planting of 20 seedlings in 1995. In this property, the herd consisted of 50 Nellore and SRD cattle raised extensively on native pastures with common salt offered in troughs. Mesquite had invaded approximately 170 ha of the property. According to the owner, in 12 months (from 2018 to 2019), ten cows aged >7 years presented a similar clinical condition and died in June 2019, six months after the onset of signs. The epidemiological conditions observed *in loco*, associated with the history and reported clinical condition, were compatible with mesquite poisoning.

Mapping. In this study, outbreaks of natural poisoning by *P. juliflora* were mapped in four mesoregions of the state of Bahia: São Francisco Valley (OB 1 and OB 6), North-central (O2, O4 and O5), Northeast (O3) and Far West (O7), which included seven microregions of the state [Juazeiro (OB 1), Itaberaba (OB 2), Euclides da Cunha (OB 3), Feira de Santana (OB 4), Irecê (OB 5), Barra (OB 6) and Cotegipe (OB 7)] (Fig.1).

Introduction and propagation of the plant. On the seven farms where the outbreaks occurred, mesquite introduction occurred through the single planting of an average of 33 seedlings (minimum of four and maximum of 60) from 1980 to 2005 [1980 (OB 1), 1982 (OB 4), 1985 (OB 3), 1989 (OB 5), 1990 (OB 6), 1995 (OB 7) and 2005 (OB 2)]. The single planting of mesquite seedlings enabled evaluation the plant spread (biological invasion) on these farms approximately 15 (OB 2), 25 (OB 7), 30 (OB 5 and OB 6), 35 (OB 3) and 40 (OB 1 and OB 4) years after its introduction. Planting was carried out on the farmers' initiative. Since then, no seedlings have been planted on the farms, and the spread has occurred naturally and progressively, gradually invading native pastures. In 2020, the degree of mesquite invasion in pastures where the seven outbreaks occurred was, on average, 59.57%, with a range of 40 to 95% between the seven properties studied (Table 1). There was also an absence of herbaceous species in the community's lower strata in most invaded pastures (Fig.2).

Clinical signs and pathology (outbreaks 1 to 7). Clinically, the alteration that most drew the attention of producers was the classic lateral tilting of the head ("twisted face") (Figs. 3 and 4), as this can be easily observed by inspecting the herd at a distance. Loss of facial sensation, protrusion and decreased lingual tone, dysphagia, and head tremors were also observed.

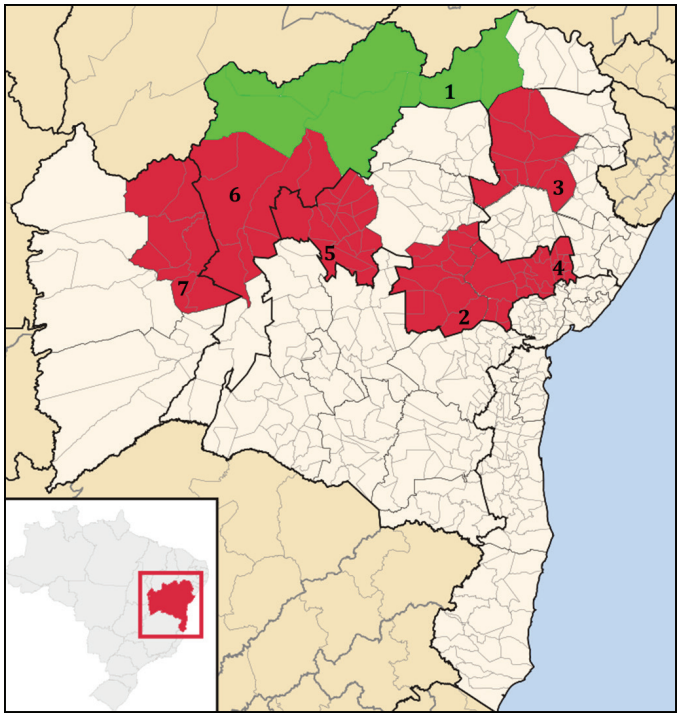


Fig.1. Update of the geographic distribution of *Prosopis juliflora* in the state of Bahia, Northeast region of Brazil (microregions in which the plant presents livestock importance in the semiarid areas of the state). Numbers 1 to 7 correspond to the municipalities where the outbreaks occurred: Juazeiro (1), Iaçu (2), Tucano (3), Santa Terezinha (4), Barra do Mendes (5), Barra (6), and Tabocas do Brejo Velho (7). The six microregions highlighted in red (Itaberaba, Euclides da Cunha, Feira de Santana, Irecê, Barra, and Cotegipe) were mapped in this study. The microregion highlighted in green (Juazeiro), where OB 1 occurred, was the only previously mapped (known) area in the state of Bahia.

In addition, cachexia was commonly observed - poisoned animals showed marked weight loss, with up to 50% reduction in body weight (chronic, lethal and cachectic condition). The other animals in the herd, raised under the same conditions, showed no poisoning, and exhibited adequate nutritional status. It was also found that, in general, the mandible becomes pendulous, which can be better evidenced during the manual movement of the head and when walking. This alteration is responsible for the popular denomination "dropped jaw", attributed to mesquite poisoning in the studied semiarid region of the state of Bahia.



Fig.2. *Prosopis juliflora* wood on the farm where the OB 4 occurred. Santa Terezinha/BA. Note the absence of herbaceous species in the lower stratum of the community and the free access of cattle to areas with large amounts of mesquite.



Fig.3. Natural poisoning by *Prosopis juliflora* in cattle. Note the cachexia and lateral deviation of the head ("twisted face") during chewing. OB 3. Tucano/BA.

At necropsy, there was, in particular, marked atrophy and pallor of the masseter muscles, and a large number of recognizable fragments of mesquite pods and seeds were observed amid the dry contents of the rumen and reticulum (Fig.5 and 6), which confirmed the ingestion of the plant. Histopathological evaluation showed degeneration (vacuolization) of neurons in the trigeminal nerve (Fig.7), which enabled diagnostic confirmation.

DISCUSSION

The diagnosis of the seven outbreaks of natural poisoning by *Prosopis juliflora* in cattle and goats in the semiarid region of the state of Bahia was established by the association of the history of uncontrolled consumption of *in natura* pods of this plant for long periods with the clinical-epidemiological data and inspection of pastures, which showed that the ruminants had free access to areas heavily invaded by mesquite. This diagnosis was confirmed, for the first time in the state of Bahia, based on anatomopathological findings (OB 1, OB 2, OB 3, and OB 5), which were similar to those described in goats (Tabosa et al. 2000, Lima et al. 2004, Lemos et al. 2016) and cattle (Dollahite & Anthony 1957, Figueiredo 1993, Figueiredo et al. 1995/96, Câmara et al. 2009, Galiza et al. 2010) intoxicated with mesquite.



Fig.4. Natural poisoning by *Prosopis juliflora* in goats. Lateral deviation of the head ("twisted face") during hospitalization at the CDP-UFBA. OB 2. Iaçú/BA.

Interestingly, in some of these outbreaks, producers and veterinarians mistakenly stated that the animals adopted the "twisted face" position as a result of tooth wear (caused by the firm consistency of pods) to keep the food in the oral cavity or of a probable contagious disease. Such an erroneous association demonstrates that, although the pathogenesis of this intoxication has already been elucidated (Tokarnia et al. 2012), there are still great misconceptions about mesquite poisoning. This fact reinforces the importance of the extension service (continuing education) offered by CDP-UFBA and SPV-UFRB to producers. The present study confirmed that the signs of "twisted face" and muscle atrophy are due to neurological injuries in the motor nucleus of the trigeminal nerve, corroborating the descriptions by Tabosa et al. (2006). This poisoning has a peculiar pathogenesis, as it is characterized by primary, selective, chronic and progressive neurological damage caused by plant alkaloids (Tabosa et al. 2004).

It is noteworthy that, in six (OB 2 - OB 7) of the seven outbreaks, poisoning occurred in microregions of the state whose livestock importance of mesquite was, until then, unknown (Itaberaba, Euclides da Cunha, Feira de Santana, Irecê, Barra, and Cotegipe). It was possible to update the geographical distribution of *P. juliflora* in the state of Bahia (Fig.1) previously described by Riet-Correa et al. (2011) and Tokarnia et al. (2012). Thus, it was found that, currently, poisoning occurs in an area approximately three times larger than the hitherto known in the state. This finding demonstrates that invasion of this plant in the Caatinga biome (Andrade et al. 2009) has expanded the areas with outbreaks of poisoning in this state's semiarid region. Recent estimates point out that mesquite has already invaded one million hectares in the northeastern semiarid region (Fabricante & Siqueira Filho 2013). This fact, associated with the occurrence of poisoning in ruminants from other states in the Northeast region (PB, RN, and PE) (Figueiredo 1993, Dantas 1996, Riet-Correa et al. 2002, Lima et al. 2004, Tabosa et al. 2004, Câmara et al. 2009, Galiza et al. 2010, Lemos et al. 2016, Almeida et al. 2017), highlights the importance of discussing the current economic impacts and perspectives on northeastern livestock resulting from the uncontrolled spread of mesquite (biological invasion) proved in the 2000s (Lima et al. 2003, Lima & Kiill 2003, Barbosa & Lima 2005, Lima 2005, Marques et al. 2003, Marques et al. 2004, Lima et al. 2004a, Lima et al. 2004b, Lima et al. 2004c). It is worth mentioning that, today, approximately 20 years after such a proof, there are still no updates on the strategies for the control and prophylaxis of this poisoning, given the new epidemiological conditions. It is recommended that the administration of mesquite pods be immediately suspended after observation of the first clinical signs or that pods be administered at safe concentrations for each species (Riet-Correa et al. 2011).

There are no estimates of the economic impact caused to farmers in the state of Bahia by the indiscriminate use of mesquite, and they are scarce in other states. However, in some properties of the states of Rio Grande do Norte and Paraíba in 1981 and 1992, respectively, mesquite poisoning



Fig.5. Natural poisoning by *Prosopis juliflora* in goats in the state of Bahia. Whole mesquite seeds amid the dried ruminal content. OB 2. Iaçú/BA.



Fig.6. Natural poisoning by *Prosopis juliflora* in cattle in the state of Bahia. Rumen content with recognizable fragments and intact mesquite pods. OB 3. Tucano/BA.

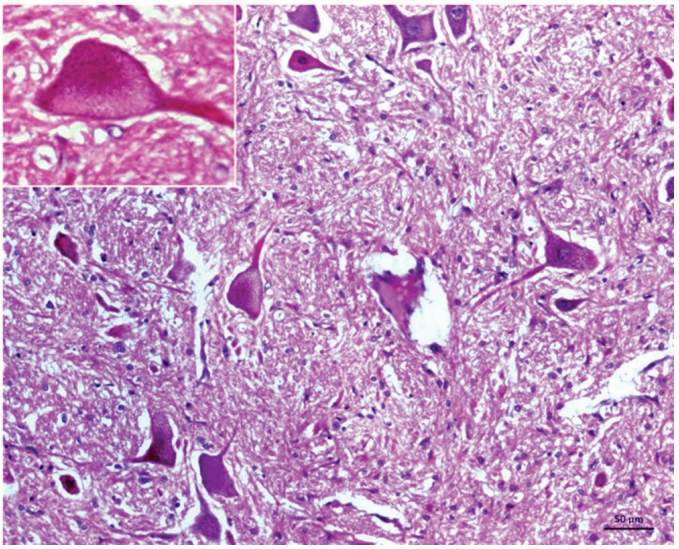


Fig.7. Natural poisoning by *Prosopis juliflora* in goats. OB 2. Iaçu/BA. Trigeminal nerve nucleus. Neurons with marked cytoplasmic vacuolization and others with hypereosinophilic cytoplasm and shriveled nuclei. HE, obj.20x. Insert: accentuated neuronal vacuolization.

was responsible for the death of more than 50% of the bovine herd (Dantas 1996). In the state of Pernambuco, from 2006 to 2007, prevalence of cattle poisoning was low in two farms [1.72% (1/58) and 2.63% (1/38)] and, reached 9.28% (112/1,206) of the herd in one farm (Câmara et al. 2009). In the present study, the prevalence of *P. juliflora* poisoning in cattle varied, being considered sporadic in OB 3 (three cases in 2014), OB 4 (21 cases in 10 years, 2006-2016) and OB 5 (seven cases in three years, 2016 -2019), since only two to three animals were affected per year. In OB 6 and OB 7, it was considered occasional, with an average of 11 cases per year in each property (22 cases in 12 months, 2018-2019). In the five poisoning outbreaks in cattle (OB 3 to OB 7), approximately 11 cattle were affected, with mortality of 23% of the herd. Among these, OB 6 and OB 7 stand out, with mortality rates of 30 and 20%, respectively, in only 12 months from 2018 to 2019.

Poisoning of cattle by mesquite has become more frequent since 2018, with increased biological invasion in the semiarid region of the state of Bahia. It is noteworthy that Dantas (1996) had already called attention to the sharp increase in cases of mesquite poisoning since 1992 in the state of Paraíba and Rio Grande do Norte, where until then, they were considered sporadic. Perception of increased occurrence of poisoning outbreaks in the state of Bahia approximately 26 years after this observation in the states of Paraíba and Rio Grande do Norte can be justified by the fact that mesquite was introduced in the latter at least four decades before in it was introduced in the first; therefore, the pasture invasion occurred later in the semiarid of region of Bahia. It is also believed that *P. juliflora* poisoning in ruminants in Bahia has been underdiagnosed and/or mistaken for other neurological and cachectic diseases, especially tuberculosis, which affect the herds in this state.

Concerning goats, it was found that in OB 1, 24 (16%) animals died from poisoning by the plant in six years (2006-2011). In OB 2, in one year (from 2014 to 2015), there was mortality of four animals in a batch of 20 goats (20%), which reinforces the hypothesis that mesquite poisoning has become more frequent as a result of pasture invasion by the plant and absence of control and prophylaxis measures. Lima et al. (2004) suggested that cases of mesquite poisoning may be more frequent than has been mentioned in the literature. These authors observed the death of 31 goats in a herd of 200 animals (15.5%) during three years in the state of Paraíba. Lemos et al. (2016) found poisoning in six goats in a batch of 25 animals (24%) in the state of Pernambuco.

Despite the fact that goats and sheep were raised together on the farm where OB 1 occurred, no cases of poisoning were observed in sheep and in goats kept in mesquite-free pastures. Similarly, in OB 3, sheep and buffaloes raised concomitantly with poisoned cattle also showed no poisoning signs. However, a study recently conducted by Almeida et al. (2017) found that, in addition to cattle and goats, natural poisoning by *P. juliflora* can also affect sheep. These authors reported the poisoning of four sheep in a herd of 500 animals (0.8%); however, these animals received feed based on mesquite pods at concentrations of up to 80% for 21 months. Although sheep are considered the least susceptible to poisoning compared with goats and cattle (the most sensitive species), natural intoxication can occur under certain feed management conditions. Nevertheless, given the new epidemiological conditions, which denote biological invasion associated with the failure to adopt control and prophylaxis measures, observed on the farms assessed, this poisoning in sheep may become more frequent.

On the farms where the outbreaks occurred, there was significant regional importance of mesquite in livestock, with all producers reporting that the use of pods in animal feed was essential and the only solution for keeping them during drought periods. It is noteworthy that disease etiology was still unknown to producers in the seven outbreaks studied and that, even in OB 1, OB 2, OB 4, OB 5, OB 6 and OB 7, convincing the farmers of the real cause of the disease was a difficult task. This difficulty can be explained by the fact that the press, especially the Northeast region, played an essential role in disseminating the culture of mesquite in Brazil, as it emphasized the advantages of using this plant as forage (Spa 1989). It is interesting to remember that, at the beginning of the 1950s, this plant was almost unknown to most of the northeasterners and that the Brazilian Society of Zootechnics played an essential role in publicizing this plant's potential as forage. They attributed it to the recovery of livestock in the state of Rio Grande do Norte with the discourse that "the herds would stop starving" [*sic*]. Other salvation speeches have also been reaffirmed by rural associations: "no plant is given characteristics such as drought and salinity resistant, rapid development, and production in times of drought like mesquite" (Cunha & Silva 2012), which stimulated the use of the plant in the Northeast region (Spa 1989). In Brazil, histories of tax and media incentives of this magnitude and nature aiming at the dissemination of other exotic plants that, later on, were classified as toxic to livestock, are rare. Among these, exceptions are *Panicum* spp., *Stylosanthes* spp. and *Brachiaria* spp., whose cultivation and dissemination has been recommended by Embrapa and have resulted in severe economic losses (Tokarnia et al. 2012).

Currently, mesquite is widely known by the population and always associated with its benefits to livestock, making it difficult for producers to recognize that, under certain conditions, it can cause poisoning and death of animals. This observation also makes it challenging to adopt measures for the control and prophylaxis of poisoning and favors the plant's uncontrolled spread (invasion). However, at the end of the 1980s, mesquite was responsible for environmental damage (drying up springs and causing the poisoning and death of cattle) (Cunha & Silva 2012) that did not have the same repercussion observed in the period of introduction of the plant. Studies discussing the positive and negative aspects of mesquite planting/use are not common in the literature, and will be discussed ahead.

Historical information on the introduction and dissemination of mesquite in the state of Bahia, as described in the present study, had never been analyzed. The excellent propagation capacity of this plant (biological invasion) drew attention especially in OB 5. On this farm, where only four seedlings were planted, 95% of the pastures (266 ha) had been invaded by the plant 30 years later. In contrast, in OB 2, where the lowest degree of invasion (16.66%) was observed, some considerations can be made. In this property, although the plant was introduced through the planting of 30 seedlings, it was carried out more recently (15 years ago) compared with the other studied farms, where the plant was introduced 10

to 25 years before. In addition, the farm where OB 2 occurred was large (300 ha) and the herd was small (only 20 animals) when compared with the average area (200 ha) and herd size (60 heads) of all farms. Moreover, it is known that among animals considered natural disseminators of mesquite seeds through feces, goats eliminate a smaller amount of seeds suitable for germination than cattle and mules (Souza et al. 1999). Such factors may justify the lower degree of mesquite invasion on this farm.

Concerning the absence of herbaceous species observed in the lower strata of the community in most invaded pastures, it is likely that competition for nutrients/water or allelopathy justify this "association problem" between mesquite and herbaceous or native trees (Lima 2005).

On the farms where OB 3 and OB 4 occurred, the producers harvested, dried (Fig.8) and stored mesquite pods, and then administered them *in natura* to the cattle. The surplus of pods was sold at regional free markets or mediated by agents, who sold it directly to feed factories with values ranging from BRL 0.30 to 0.70 per kg. These producers associated the sale of pods with the economic importance of the plant. In fact, in some municipalities in the states of Bahia and Pernambuco, the largest mesquite pod sales centers are concentrated in the São Francisco River basin. Animal feed factories are the biggest buyers, which has encouraged producers to adopt this practice (Lima 2005). Additionally, in these two properties (OB 3 and OB 4), the animals also had free access to areas intensely invaded by mesquite, which allowed the uncontrolled consumption of a large amount of fresh pods in the pastures for long periods, similarly to what occurred in the other five properties studied (OB 1, OB 2, OB 5, OB 6, and OB 7). This observation evidences that these are essential factors in the origin of this poisoning outbreaks, since they occurred in a way similar to that described in other cases of poisoning in ruminants (Figueiredo 1993, Dantas & Menezes 1994, Lima et al. 2004, Câmara et al. 2009, Galiza et al. 2010, Lemos et al. 2016, Almeida et al. 2017). In the studied outbreaks, another factor that drew attention was the total absence of fences in areas invaded by mesquite in the seven properties, thus evidencing the lack of adequate management in the availability of mesquite for animal consumption.

Whether the consumption of fresh mesquite pods occurs in troughs or pastures in a controlled or uncontrolled way, the uncontrolled dispersal of its through feces is maintained. This spread contributes to the invasion of pastures (Fig.9) and risk of poisoning, as verified in the present study. It should be noted that the average percentage of seeds suitable for germination eliminated in manure reaches 9.3% in goats, 14.8% in cattle, and 37.3% in mules (Souza et al. 1999). These high germination rates are likely to occur as a result of the reduced drying of seeds provided by the humid environment of the feces (Ribaski et al. 2009). On the other hand, sheep partially destroy the ingested pods, while the pigs were found to destroy all seeds (Pasiecznik et al. 2001).

Some considerations should be made regarding the processing of mesquite pods. Although toxicity is maintained in the mesquite bran obtained by grinding the pods (Tabosa et al. 2006), the seeds are destroyed in this preparation, thus preventing the uncontrolled propagation of the plant. Grinding should always be recommended for preparation of animal feed, as it is a good alternative (rational use) for controlling plant spread (biological invasion). In addition, this process allows the control of the amount of feed eaten by animals in the trough according to the non-toxic concentrations known for each species (Table 2), thus preventing cases of poisoning. However, mesquite pods were not processed (grinding) in any of the farms investigated, which justifies the massive pasture invasion (59.57%, on average) over the years, the appearance of the so-called "mesquite forests" observed in OB 3, OB 4, OB 5, OB 6 and OB 7 (Fig.2), and the increased occurrence of poisoning cases.

Installation of a micro-plant to process mesquite pods in the properties and/or at a regional level should be encouraged. According to Lima (2005), a mesquite pod processing unit located in the municipality of Monteiro, state of Paraíba, mills approximately 30 tons of pods a year, providing farmers in the region with mesquite bran for animal feed. Riocon® (Fazendas



Fig.8. Pods of *Prosopis juliflora* in the sun drying process after harvesting on the farm where OB 3 occurred. Tucano/BA. Process carried out prior to the storage of pods that are subsequently supplied to animals or sold in the region.



Fig.9. Uncontrolled dispersal of *Prosopis juliflora* seeds in feces. Note the germination of several seeds of the plant, amid feces, found in the pasture of the farm where OB 4 occurred. Santa Terezinha/BA.

Reunidas Rio de Contas Ltda) started its activities in 1993 in the municipality of Manoel Vitorino, located in the semiarid region of southwestern Bahia. They invested in the installation of mesquite bran production units to promote integration between local family producers to supply pods and expand the production of mesquite bran (Riocon 2019), which demonstrates the feasibility and benefits of this processing. As for intensive rearing, in which the pods are not crushed, another strategy to avoid the spread of seeds in the property would be manure composting or appropriate discard, noting that indiscriminate use of manure in crops or pastures can be compromising.

On the farms where OB 3, OB 4, and OB 5 occurred, thinning of mesquite trees for the use of wood (stakes, posts, fences, or production of firewood and/or charcoal) or their commercialization have already been modestly practiced (Fig.10); however, this activity has no longer been carried out for approximately 15 years. According to Lima (2005), mesquite wood is sold for BRL 30-40 per m^3, which can generate extra income for producers. The absence of a management plan for mesquite cultivation in the seven farms studied since the plant's introduction has also contributed to its high degree of pasture invasion. In this context, it is essential to note that, in 2005, a management plan for areas invaded by mesquite in the Northeast region was prepared to convert natural "mesquite forests" into sustainable production systems, reducing biological invasion through the rational use of their products (feed, firewood, and charcoal) and conserving the Caatinga biome (Lima 2005). Adoption of these silvicultural treatments in pastures invaded by mesquite in the semiarid region of the state of Bahia should be encouraged to prevent future cases of poisoning resulting from the uncontrolled spread of the plant and conserve the Caatinga biome. Riocon® also stood out in the production of ecological charcoal using industrial automation and raw material based on reforested mesquite trees (Riocon 2019). Such strategies were recommended to producers in order to use the potential of *P. juliflora*, adding economic and social value to these regions.

Table 2. Recommended (non-toxic) concentrations of mesquite in the diet of ruminants

Species	Concentration in the diet	Period	References
Cattle	30%	12 months	Medeiros et al. (2012)
Goats	50 to 70%	6 to 12 months	Riet-Correa et al. (2012)
Sheep	50/60 to 90%	Without restriction/12 months	Sharma (1997), Riet-Correa et al. (2012)



Fig.10. Strategy for the control of *Prosopis juliflora* trees. Extraction of wood for the production of posts, fences, and use as firewood on the farm where OB 3 occurred. Tucano/BA.

It is important to note that Africa and Asia (India and Pakistan) also face problems caused by mesquite invasion. Governmental actions have been taken to reduce the damage caused by encouraging the exploitation of fruits and wood (Franco 2008). In South Africa, Australia, India and Sudan, mesquite occupies millions of hectares. In these countries, invasions that occurred along extensive riverbanks and in degraded regions have resulted in a high population densities (Ribaski et al. 2009). In the state of Bahia, during the visits to the properties where the outbreaks occurred, a growing dispersion of the plant was verified along the margins of highways and rivers (Fig.11), particularly in the municipality of Itapicuru, contributing to increased silting. These occurrences corroborate the findings of a study conducted by Lima (2005) that monitored, through satellite images, the expansion of areas invaded by mesquite in the Juremal region, Juazeiro (BA) for ten years (1986-1996). This author found that this arboreal species has played an exceptional role in the regeneration of the semiarid region, with increasing propagation areas over these years.



Fig.11. Uncontrolled propagation (invasion) of *Prosopis juliflora* in riparian forest on the farm where OB 3 occurred. Tucano/BA.

It is essential to highlight that some countries do not recommend the planting of invasive species and have been advocating the eradication of mesquite. It has even been verified that the use of herbicides or mechanical removal cannot eradicate this species because of its high regrowth power (Lima 2005). Assessment of the Brazilian reality shows that *P. juliflora* is the most successful xerophilic plant among those introduced in the northeastern semiarid region. It plays a fundamental economic role in agriculture (Spa 1989), which was confirmed in the present study. In this context, its eradication is not justified; however, its propagation should be controlled in the Northeast region, which can be done based on the sustainable forest management techniques discussed here.

CONCLUSIONS

This seems to be the first study addressing pathological outbreaks of natural poisoning by *Prosopis juliflora* in cattle and goats in the state of Bahia with emphasis on six microregions of the state (Itaberaba, Euclides da Cunha, Feira de Santana, Irecê, Barra and Cotegipe). The marked biological invasion of the Caatinga biome by mesquite changes the epidemiological conditions of this intoxication, expanding the areas outbreak occurrence and making them more frequent in the semiarid region of the state of Bahia.

The uncontrolled consumption of mesquite pods without processing (*in natura*/not grinding) for long periods has allowed massive dispersal of plant seeds through animal feces. This consumption, combined with the lack of a cultivation management plan (silvicultural treatments), and the lack of knowledge by producers on disease etiology are the main factors responsible for the gradual invasion of this plant in pastures. Over the years, this justifies the increased number of cases of poisoning observed in the state of Bahia.

Given the new epidemiological conditions, the restriction of animal access to fresh *P. juliflora* pods in pastures and the supply of mesquite bran are crucial factors for the control and prophylaxis of this intoxication. The consumption of fresh pods has a strong correlation with the high degree of pasture invasion, consequently increasing the risk of poisoning.

The growing invasion of mesquite in semiarid areas, 78 years after its introduction in the Northeast region, is a threat to the biodiversity of Caatinga and a risk for herds, since the occurrence of poisoning outbreaks may become more frequent. Thus, it is highly advisable that effective measures be adopted to control *P. juliflora* propagation.

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DNA extraction methods for molecular detection of *Eimeria* spp. in cattle and sheep[1]

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ABSTRACT.- Reginato C.Z., Bräunig P., Portella L.P., Mortari A.P.G., Minuzzi C.E., Sangioni L.A. & Vogel F.S.F. 2020. **DNA extraction methods for molecular detection of *Eimeria* spp. in cattle and sheep.** *Pesquisa Veterinária Brasileira 40(7):514-518*. Universidade Federal de Santa Maria, Av. Roraima 1000, Cidade Universitária, Camobi, Santa Maria, RS 97105-900, Brazil. E-mail: pbraunig@gmail.com

Molecular detection of *Eimeria* species in fecal samples can be useful for experimental and diagnostic purposes. However, the parasite quantity presence in feces and the oocyst wall are an obstacle in DNA extraction protocols. Therefore, adequate sampling and effective disruption of the oocysts are essential to improve the accuracy of DNA detection by PCR. The aims of this study were to evaluate the suitability of six protocols for DNA extraction from *Eimeria* spp. present in bovine and sheep. Twenty pools of fecal samples from cattle (10 pools) and sheep (10 pools) were distributed to six DNA extraction protocols: commercial kit, commercial kit with modification, DNAzol, cetyl-trimethyl ammonium bromide (CTAB), glass beads and commercial kit for fecal samples. Fecal samples were submitted to DNA extraction and PCR. Among the protocols tested, CTAB was determined to be most suitable for DNA extraction from oocysts (90% of DNA detection by PCR); DNAzol and CTAB resulted in higher DNA detection from bovine samples (80%). CTAB and commercial kit with modification improved PCR detection of *Eimeria* spp. in sheep samples, with positive amplification of DNA in all tested samples.

INDEX TERMS: DNA extraction, molecular detection, *Eimeria* spp., cattle, sheep, PCR, fecal samples.

RESUMO.- [**Métodos de extração de DNA para detecção molecular de *Eimeria* spp. em bovinos e ovinos.**] A detecção molecular de espécies de *Eimeria* em amostras fecais pode ser útil para fins experimentais e de diagnóstico. No entanto, a quantidade de parasitas nas fezes e a parede do oocisto são um obstáculo nos protocolos de extração de DNA. Portanto, uma amostragem adequada e a ruptura efetiva dos oocistos são essenciais para melhorar a precisão da detecção de DNA por PCR. Os objetivos deste estudo foram avaliar seis protocolos para extração de DNA de *Eimeria* spp. em amostras de bovinos e ovinos. Foram distribuídos 20 grupos de amostras fecais de bovinos (10 grupos) e ovinos (10 grupos) em seis protocolos de extração de DNA: kit comercial, kit comercial com modificação, DNAzol, brometo de cetil-trimetil amônio (CTAB), pérolas de vidro e kit comercial para amostras fecais. As amostras fecais foram submetidas à extração de DNA e PCR. Entre os protocolos testados, CTAB foi considerado o mais adequado para extração de DNA de oocistos (90% de detecção de DNA por PCR); DNAzol e CTAB resultaram em maior detecção de DNA em amostras de bovinos (80%). CTAB e kit comercial com modificação melhoraram a detecção por PCR de *Eimeria* spp. em amostras de ovinos, amplificação positiva de DNA em todas as amostras testadas.

TERMOS DE INDEXAÇÃO: Extração de DNA, detecção molecular, *Eimeria*, bovinos, ovinos, DNA, PCR, amostras fecais.

INTRODUCTION

Coccidiosis is an infection caused by protozoa of the genus *Eimeria* of great economic importance because of the losses due to clinical disease (diarrhea) and subclinical infections (poor weight gain) (Chartier & Paraud 2012). *Eimeria* spp. develop in the small and large intestines of the hosts, is specie

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specific and several species of *Eimeria* were identified infecting cattle and sheep affecting mainly young animals (Daugschies & Najdrowski 2005, Chartier & Paraud 2012).

Eimeria bovis and *Eimeria zuernii* are most commonly pathogenic species in calves worldwide causing morbidity and mortality by disturbing intestinal absorption and often associated with diarrheic feces (Daugschies & Najdrowski 2005). The most pathogenic *Eimeria* spp. in sheep are *Eimeria ovinoidalis* which develops in the large intestine and *Eimeria crandallis* (Chartier & Paraud 2012).

Subclinical form of coccidiosis is difficult to diagnose and low rates of fatality are associated with this disease, however *Eimeria* spp. infection negatively impact ruminants mainly on decreasing growth and increasing susceptibility to diseases (Vieira et al. 1999). Diagnosis of eimeriosis infections should be based on epidemiological aspects, clinical signs and laboratory test results, including: light microscopy traditional fecal examination and molecular techniques, especially polymerase chain reaction (PCR) (Carvalho et al. 2011, Chartier & Paraud 2012).

Molecular assays for the diagnosis of *Eimeria* spp. infection are known to have greatly improved through more effective DNA extraction techniques and more sensitive PCR techniques for identifying protozoa (Kawahara et al. 2010, Carvalho et al. 2011, Malek & Kuraa 2018). Previous studies have revealed that one of the steps that can influence PCR results is the DNA extraction methods used (Zhao et al. 2001, Kaya et al. 2007, Tang et al. 2018).

DNA extraction process is essential for the success of the PCR technique, favoring a more sensitive and specific diagnosis. Thus, the present work aimed to compare different DNA extraction methods of *Eimeria* spp. oocysts from sheep and cattle.

MATERIALS AND METHODS

Samples and parasitological examination

Cattle and sheep feces were collected directly from the rectum using sterile gloves and placed in clean plastic bottles. The fecal samples were transferred under refrigeration to the laboratory and kept at 4°C until processing.

Eggs and oocysts per gram of feces (EPG) counting was performed using a modified McMaster technique, with a sensitivity of 50 EPG. Samples that had an oocysts count ≥500 were selected for both cattle and sheep.

Eimeria spp. oocysts examination

Positive fecal samples (oocysts count≥500) were separated according animal species (cattle or sheep) and 10 pools of feces from each experimental group was formed. Therefore, 2 to 4g from a pool of feces were homogenized, dissolved in distilled water and filtered through sieves (60µm). Several washes were performed according Hoffman et al. (1934) and when the supernatant became clear, flotation centrifugation technique (Ueno & Gonçalves 1998) was used to separate and concentrate the oocysts. Finally, the recovered oocysts were stored in 2% potassium dichromate solution, quantified in Newbauer chamber and stored at 4°C until DNA extraction.

DNA extraction protocols

Each sample was twice washed with distilled water, centrifuged for 10 min at 14,000 × g to remove the potassium dichromate solution. The pellet with a number of approximately 5000 oocysts/pool was subsequently submitted to DNA extraction protocols.

Protocol 1 (commercial kit). Genomic DNA was extracted using a commercial kit (Wizard Genomic DNA Purification Kit, Promega, USA) according to manufacturer´s instructions. Briefly, 600µL of Nuclei Lysis Solution was added to the pellet of oocysts, homogenized, and incubated at 65°C for 30 min. After incubation, 3µL of RNase Solution was added, incubated for 30 min at 37°C, followed by addition of 200µL of Protein Precipitation Solution, vortexed, chilled on ice for 5 min, and centrifuged at 13000 × g for 4 min. The supernatant was transferred to a fresh tube containing 600µL of isopropanol, mixed and centrifuged at 13000 × g for 1 min. After centrifugation, the supernatant was removed, and 600µL of 70% ethanol was added to the pellet and centrifuged at the conditions described above. The ethanol was aspirated, the pellet was air-dried, and the DNA rehydrated in 50µL of DNA Rehydration Solution for 1 hour at 65°C.

Protocol 2 (commercial kit with modification). Genomic DNA was extracted using the commercial kit (Wizard Genomic DNA Purification Kit, Promega, USA) with a modification: after RNase addition, the lysis step was carried out at 55°C and kept overnight (16h) (adapted from Moré et al. 2011).

Protocol 3 (DNAzol reagent). Genomic DNA was extracted using a specific reagent (DNAzol Reagent, Invitrogen, USA) according to manufacturer´s instructions. Briefly, 1ml DNAzol was added to the pellet of oocysts followed by homogenization and centrifugation (5 min at 10,000 × g at 4°C). Each resulting viscous supernatant was transferred to a new tube. Genomic DNA precipitation was carried out by adding 0.5ml of cold 100% ethanol, homogenized, incubated for 3 min at room temperature and centrifuged at the conditions described above. Finally, genomic DNA was washed with 1ml of cold 75% ethanol and centrifuged (same conditions described above), after centrifugation, supernatant was discarded and the resulting pellet suspended in 50µL of MilliQ water.

Protocol 4 (CTAB). The pellets of oocysts were incubated for 10 min with 100µL of lysis buffer (lysozyme 10mg/ml, SDS 10% and proteinase K 10mg/ml) at 37°C. This was followed by addition of 100µL cetyl-trimethyl ammonium bromide (CTAB) and 100µL of 5M NaCl into the solution and incubation at 65°C for 10 min. Finally, genomic DNA was isolated from the lysate by the phenol-chloroform method (Sambrook & Russel 2001), precipitated by cold ethanol, and solubilized in 50µL MilliQ water.

Protocol 5 (glass beads). To perform the protocol with glass beads, in to the oocysts pellet was added 100µL of STES buffer (0.2 M Tris-HCl, 0.1% SDS (w/v), EDTA 0.01M, pH 7.6), 50µL of 0.5mm glass beads and 100µL phenol-chloroform, vortexed for 1 minute. After that, the solution was centrifuged at 13000g for 5 min, the supernatant was collected and transferred to a new, sterile tube and precipitated by adding cold 2V (volume) absolute ethanol and 0.1V 5M NaCl. After 30 minutes, the solution was centrifuged again at 1200g for 10 min. The pellet was washed twice with 70% ethanol and after that, DNA was eluted in 50µL MilliQ water.

Protocol 6 (commercial kit for fecal samples). A specific kit for extraction of genomic DNA from fecal samples was also used according to manufacturer's instructions (PureLink® Genomic DNA Mini Kits). Basically, kit consists in specific digestion buffers, purification column for DNA, washing buffers and an elution buffer, resulting in a final volume of 50µL.

The concentration and quality of DNA extracted from each sample were analyzed using spectrophotometer NanoDrop 1000 (absorbance of 260/280nm ratio for purity evaluation) (Thermo Scientific, USA). After that, the DNA samples were stored at -20°C until use in PCR.

PCR and electrophoretic analysis

DNA extracted by the different protocols described above was submitted to the polymerase chain reaction (PCR) under the same conditions, using a set of primers (F: 5´-GCA AAA GTC GTA ACA CGG TTT CCG -3' and R: 5´-CTG CAA TTC ACA ATG CGT ATC GC-3') (Malek & Kuraa 2018) for amplification of a 348-546 bp fragment from the internal transcribed spacer 1 (ITS-1). Each PCR was performed in a total volume of 25µL, containing 10X buffer (Promega, USA); 10mM dNTPs (Ludwig Biotec, Brazil); 50µm of each primer (Sigma-Aldrich, Brazil); 1U Taq DNA polymerase (Promega, USA); and 50ng of DNA as template. DNA extracted from a pool of sporulated oocysts using the commercial kit was used as positive control and MilliQ water was used as negative control. The PCR was carried out using a T100 thermal cycler (BioRad, USA) under the following conditions: 30 seconds at 94°C for the initial hot denaturation step, followed by 35 cycles of 10 seconds at 94°C, 20 seconds at 55°C, 20 seconds at 72°C, and a final extension step of 2 min at 72°C. The PCR products were visualized by UV illumination after electrophoresis at 1% agarose gel stained with Gel Red Nucleic Acid Stain (Biotium, USA).

Statistical analysis

Software IBM SPSS Statistics 23 was used to calculate the kappa coefficient to estimate the concordance rate among the extraction protocols. The frequencies of positive and negative samples obtained using each DNA extraction protocol were compared by the Fisher's exact test at a 99% confidence level.

RESULTS

All the extraction protocols have resulted in DNA amplification by PCR (Table 1). CTAB (Protocol 4) showed better results with regard to DNA extraction from pools of oocysts (Table 1), with the *Eimeria* spp DNA detected from 90% (18/20) of the samples. This frequency was higher than the 70% (14/20) detection obtained using either the commercial kit for fecal samples (Protocol 6) or DNAzol (Protocol 3). Commercial kit (Protocol 1) showed a lower detection frequency (45, 9/20). Kappa coefficient was obtained using Protocol 4 as standard therefore, Protocols 1 (commercial kit), 3 (DNAzol) and 6 (commercial kit for fecal samples) demonstrated slight concordance (kappa 0.167 and 0.118) with Protocol 4 (CTAB). However, Protocol 5 (glass beads) showed moderate (kappa 0.48) and Protocol 2 (commercial kit with modification) fair concordance (kappa 0.318) with CTAB (Protocol 4).

In cattle fecal samples, Protocols 3 (DNAzol) and 4 (CTAB) showed better results (80%), 8/10 samples were positive

Table 1. PCR results from DNA extracted by six extraction protocols from cattle and sheep isolated oocysts

Sample	PCR results from the six DNA extraction protocols tested					
	*P1	P2	P3	P4	P5	P6
	Kit	Kit with modification	DNAzol	CTAB	Glass beads	Kit for fecal samples
**C1	+	+	+	+	+	+
C2	+	+	+	+	+	+
C3	+	+	+	+	+	+
C4	-	+	+	+	+	+
C5	-	+	+	+	-	+
C6	-	+	+	+	-	+
C7	-	+	+	+	-	-
C8	-	-	+	+	-	-
C9	-	-	-	-	-	-
C10	-	-	-	-	-	-
Total bovine samples	3/10	7/10	8/10	8/10	4/10	6/10
***S1	+	+	+	+	+	+
S2	+	+	+	+	+	+
S3	+	+	+	+	+	+
S4	+	+	+	+	+	+
S5	+	+	+	+	+	+
S6	+	+	+	+	+	+
S7	-	+	-	+	+	+
S8	-	+	-	+	+	+
S9	-	+	-	+	-	-
S10	-	+	-	+	-	-
Total sheep samples	6/10	10/10	6/10	10/10	8/10	8/10
Total fecal samples	9/20	17/20	14/20	18/20	12/20	14/20

*P = protocol, **C = pool of cattle fecal samples, ***S = pool of sheep fecal samples.

in PCR when these protocols were used for DNA extraction. Interestingly, in fecal samples from sheep, Protocols 2 (commercial kit with modification) and 4 (CTAB) resulted in 100% (10/10) of DNA amplification.

DISCUSSION

Coccidiosis causes economic losses in livestock as a result of decrease in feed efficiency which leads to slow weight gain and increased predisposition to other diseases (Malek & Kuraa 2018). Therefore, accurate diagnosis of coccidiosis is important to promote animal health and consequently to reduce losses. There is different diagnosis of *Eimeria* spp. using different methods with different specificity and sensitivity (Carvalho et al. 2011) and PCR which is known as a specific and sensitive method of diagnosis showed a high frequency of *Eimeria* spp. detection in the present study, reinforcing that coccidia are widely distributed across bovine and ovine.

Haug et al. (2007) observed that there is a variation in the detection of each *Eimeria* spp., depending on the number of oocysts used and the technique adopted for DNA extraction. However, Fernandez et al. (2003) identified *Eimeria* spp. in samples containing from two to eight oocysts proving that PCR correctly detect and discriminate species from small quantities of oocysts. Therefore, PCR is much sensitive compared to oocyst-counting using McMaster's method that can quantify as much as 100 oocysts in 1g of faeces (Kawahara et al. 2010).

In field samples, many factors may interfere in the success of diagnosis by PCR, especially the presence of contamination (Carvalho et al. 2011). According to Haug et al. (2007) the DNA extraction process in stool samples is influenced by the formation of inhibitors of Taq DNA polymerase that affect the reaction. We compared results obtained from six DNA extraction methodologies and found that CTAB was more efficient. Glass bead grinding is based on frequent physical contact between oocysts to break the wall, but the frequency of contact decreases with the small number of oocysts, resulting in less oocyst breakage (Tang et al. 2018). In addition, the amount of DNA from ruptured sporozoites that remains adhered to the glass beads and/or the container wall also reduces the total yield of DNA (Zhao et al. 2001, Tang et al. 2018). In contrast, Protocol 3 (DNAzol) and 4 (CTAB) are performed in microtubes resulting in minimal loss of DNA. In addition, since the commercial DNA kit can adsorb substances that could degrade DNA and inhibit downstream enzymatic reactions, it also dramatically reduced PCR sensitivity (Kumar et al. 2014, Tang et al. 2018).

Eimeria's oocyst walls contain two or more shell layers (Scholtyseck et al. 1971, Hammond & Long 1973, Duszynski et al. 1981, Long 1982). The thick and elastic outer skeletal layer is made of a chitin-like material (Wilson & Fairbairn 1961) and contains quinone-tanned proteins that can be dissolved in hypochlorite (Hammond & Long 1973). Some DNA extraction protocols use sodium hypochlorite to promote oocysts lysis (Carvalho et al. 2011, Tang et al. 2018) and although none of the six protocols tested used hypochlorite, they showed to be efficient in oocysts lysis resulting in positive PCRs.

Results demonstrated that the PCR assay targeting the ITS-1 region of *Eimeria* spp. in cattle and sheep can be used for detection of this parasite. The ITS-1 regions are flexible corresponding with species variation, as compared with whole rRNA genes, showing a pattern of low intra-specific and high inter-specific variations in the DNA sequence (Kawahara et al. 2010). Accurate identification of *Eimeria* spp. is important not only for the diagnosis of disease and management of subclinical infections but also for epidemiological studies (Sun et al. 2009, Lee et al. 2010). PCR facilitates the detection of parasites including at subclinical levels, at concentrations that may be missed by routine microscopy (Tang et al. 2018) and in this study we tested six DNA extraction protocols in which oocysts were ruptured, DNA released and extracted in concentration and purity appropriately for PCR detection.

CONCLUSIONS

All the extraction protocols tested have promoted DNA releasing from *Eimeria* spp. oocysts.

CTAB protocol showed to be better in DNA extraction from pools of *Eimeria* oocysts. Therefore, CTAB should be considered as DNA extraction method in molecular studies involving *Eimeria* oocysts from sheep and cattle.

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

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Original Article
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Quinolones resistance in *Salmonella* spp. isolated from broilers and chickens' carcasses under federal inspection[1]

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We analyzed 77 *Salmonella* spp. strains, from which 20 were isolated from broilers (cloacal swabs) and 57 from chickens from slaughterhouses under federal inspection. The following serotypes were identified: *Salmonella* Saint Paul (29), *Salmonella* Heidelberg (27), *Salmonella* Anatum (9), *Salmonella* Cerro (5), *Salmonella* Senftenberg (5), *Salmonella enterica* (O: 4,5) (1) and *Salmonella enterica* (O: 9.12) (1). Fifteen strains (19.5%) were resistant to enrofloxacin, six (7.8%) to ciprofloxacin, and 26 (33.8%) to nalidixic acid in the Disk Diffusion Test. The fifteen enrofloxacin resistant strains were selected for the PCR to detect the genes *gyrA, gyrB, parC,* and *parE,* and genetic sequencing to identify mutations in these genes. Five strains (33.3%) had point mutations in the *gyrA* gene, and one (6.7%) presented a point mutation in the *parC* gene. None of the 15 strains had mutations in the *gyrB* and *parE* genes, and none had more than one mutation in the *gyrA* gene or the other genes. The presence of point mutations in the strains studied corroborates with the phenotypic resistance observed to nalidixic acid. However, it did not explain the resistance to fluoroquinolones found in the 15 strains. Other mechanisms may be related to the fluoroquinolones resistance, highlighting the need for additional mutation screening.

INDEX TERMS: Quinolones, *Salmonella* spp., broilers, chicken carcass, fluoroquinolones, antimicrobial resistance.

RESUMO.- [**Resistência à quinolonas em *Salmonella* spp. isoladas de frangos vivos e carcaças sob inspeção federal.**] Foram analisadas neste estudo 77 estirpes de *Salmonella* spp., 20 isoladas de frangos vivos (suabes de cloaca) e 57 isoladas de carcaças, provenientes de abatedouros frigoríficos sob Inspeção Federal. Foram identificados os seguintes sorotipos: *Salmonella* Saint Paul (29), *Salmonella* Heidelberg (27), *Salmonella* Anatum (9), *Salmonella*C erro (5), *Salmonella* Senftenberg (5), *Salmonella* enterica (O: 4,5) (1) e *Salmonella* enterica (O: 9,12) (1). Do total de estirpes estudadas, 15 (19,5%) se mostraram resistentes à enrofloxacina, seis (7,8%) à ciprofloxacina e 26 (33,8%) ao ácido nalidíxico no Teste de Difusão em Disco. Foram selecionadas as 15 estirpes resistentes à enrofloxacina para a realização da PCR para detecção dos genes *gyrA, gyrB, parC e parEe* para sequenciamento genético do produto da PCR para identificação de mutações nesses genes. Cinco estirpes (33,3%) apresentaram mutações pontuais no gene *gyrA* e uma (6,7%) apresentou mutação pontual no gene *parC*. Nenhuma das 15 estirpes apresentou mutações nos genes *gyrB* e *parE* e nenhuma apresentou mais de uma mutação no gene *gyrA* ou nos outros genes. A existência apenas de mutações pontuais em alguns genes das estirpes analisadas está de acordo com a resistência fenotípica observada ao ácido nalidíxico, mas não explica a resistência às fluoroquinolonas encontrada nas

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15 estirpes. Outros mecanismos de resistência podem estar relacionados à resistência encontrada às fluoroquinolonas e estudos adicionais são necessários para investigar sua presença.

TERMOS DE INDEXAÇÃO: Quinolonas, *Salmonella* spp., frangos vivos, carcaças de frango, fluoroquinolonas, resistência antimicrobiana.

INTRODUCTION

Bacteria of the genus *Salmonella* are essential pathogens for causing diseases in animals and man, and are considered of great significance for both public and animal health.

Some serotypes, known as paratypical, may not cause clinical disease in birds. However, most of them can multiply and remain in their digestive tract for some time, spreading in the farm environment. By contaminating poultry origin products to human consumption, they may be responsible for human outbreaks of food infection (Berchieri Jr. & Freitas Neto 2009, Andreatti Filho 2009, Gast 2013). The paratypical serotypes most commonly described in poultry environments, birds and their products, are the serotypes Enteritidis, Albany, Heidelberg, Mbandaka and Newport (Brasil 2008, Dutil et al. 2010, FDA 2012, Robinson 2013, Cardoso et al. 2015, Pandini et al. 2015, PHAC 2017).

Salmonella infection leads to different clinical signs in humans, with gastrointestinal infection being the most common and generally self-limiting manifestation (Rezende et al. 2016, Draper et al. 2017).

Another important fact related to *Salmonella* spp. is the constant description of resistance to various antimicrobials, such as those of the quinolone class (Duarte et al. 2009, Mion et al. 2014, Casas et al. 2016, Neves et al. 2016, Rodrigues et al. 2017). Resistance in *Salmonella* spp. quinolones have been recorded in countries such as China, Hong Kong, Italy, the United Kingdom and Brazil (Eaves et al. 2004, Duarte et al. 2009, Lai et al. 2014, Lin et al. 2015,García-Fernández et al. 2015, Casas et al. 2016). Some countries showed a significant increase in resistance, such as China, which observed an increase from 4.4% to 41.5% of ciprofloxacin resistance, from 2009 to 2012 (Lai et al. 2014). Several studies have linked the therapeutic and prophylactic use of quinolones in animals with the emergence and spread of resistance from these pathogens (Yan et al. 2011, Cheng et al. 2012, Finley et al. 2013).

Quinolones, antimicrobials with a full application for the treatment of diseases in both humans and animals, were developed to identify nalidixic acid in the 1960s. In the 1980s, fluoroquinolones were developed and considered second-generation quinolones, with a spectrum of action on Gram-negative and Gram-positive bacteria. Enrofloxacin, a fluoroquinolone developed exclusively for use in animals, has broad antibacterial activity and is commonly used in Brazil's poultry production. Ciprofloxacin, in addition to its use in poultry production, is also used to treat human salmonellosis (Ito et al. 2005, Górniak 2011).

Salmonella spp. may develop chromosome and plasmid resistance to quinolones. Chromosome-mediated resistance can cause overexpression of efflux pumps or changes in porins present in the outer bacterial membrane, reducing the accumulation of antimicrobials in the bacteria. Another mechanism mediated by chromosomes is the conformational alteration of enzymes responsible for the replication of bacterial DNA (García-Fernández et al. 2015). This type of mutation is the most frequent in *Salmonella* (Sinwat et al. 2018). It occurs by specific mutations that result in amino acid substitutions in the coded enzymes DNA Gyrase and Topoisomerase IV in the subunits encoded by the *gyrA*, *gyrB*, *parC*, or *parE* genes. These enzymes are involved in the bacterial DNA replication process, which is essential for bacterial survival. Mutations in *gyrA*, *gyrB*, *parC*, or *parE* in the regions that are part of the quinolone binding site are called Quinolone Resistance Determining Region (QRDR). The mutations alter these enzymes' structure, preventing quinolones from becoming connected to this site, reducing bacterial susceptibility to quinolones (Gouvêa et al. 2015, Thong et al. 2015, Sinwat et al. 2018). In *Salmonella* spp. mutations in *gyrA* are the most frequent, which can be explained due to the positioning of the enzyme DNA Gyrase in the bacterial DNA replication fork, which makes the inhibitory action of quinolone more effective than the enzyme Topoisomerase IV. Consequently, this may have created a selective pressure that caused the DNA Gyrase genes, especially the *gyrA* gene, to mutate more frequently to avoid quinolones' inhibitory action (Thong et al. 2015). Mutations in the *parC* gene for Topoisomerases IV occur less frequently, and mutations in the *gyrB* and *parE* genes have been considered rare in *Salmonella* (Kim et al. 2011, Yang et al. 2012, Thong et al. 2015, Lin et al. 2015).

Although antimicrobials are not indicated in most cases of salmonellosis in humans, its use becomes necessary in severe cases of systemic infections (Boxstael et al. 2012, WHO 2018). However, the antimicrobials' lack of susceptibility increases the risk of treatment failures (Park et al. 2019).

This study aimed to verify resistance to quinolones, enrofloxacin, ciprofloxacin, and nalidixic acid by mutating the genes *gyrA*, *gyrB*, *parC* and *parE* in strains of *Salmonella* spp., isolated from live chickens and carcasses from slaughterhouses with Federal Inspection Service.

MATERIALS AND METHODS

Obtaining strains of *Salmonella* spp. In this study, we used 77 strains of *Salmonella* spp., 20 of which were isolated from live chickens (cloaca swabs), and 57 from carcasses from slaughterhouses under federal inspection. The samples were sent to the "Laboratório de SanidadeAvícola", "Faculdade de Veterinária", of the "Universidade Federal Fluminense" (UFF), included in nutrient Agar (VWR Chemicals).

Strains serotyping. The strains were reactivated in Brain Heart Infusion (BHI) broth, included in nutrient Agar and sent to the "Laboratório de Enterobactérias" of the "Instituto Oswaldo Cruz"/"FundaçãoInstituto Oswaldo Cruz" (Fiocruz) for serotyping.

Antimicrobial susceptibility test. The 77 strains studied were subjected to the Disc-Diffusion test (CLSI 2018) to verify resistance to nalidixic acid, ciprofloxacin, and enrofloxacin.

Polymerase chain reaction (PCR) of the *gyrA*, *gyrB*, *parC*, and *parE* genes. PCR was performed at the "Laboratório de Epidemiologia Molecular" of UFF. For the extraction of bacterial DNA, strains of *Salmonella* spp. resistant to enrofloxacin by the Disc-Diffusion test were reactivated in BHI broth and subjected to the thermal method (AndreattiFilho et al. 2011). To detect chromosomal mutations in the DNA Gyrase and Topoisomerase IV enzymes of the strains of *Salmonella* spp., The *gyrA*, *gyrB*, *parC*, and *parE* genes were amplified by PCR, in a thermocycler (Thermo Electron Corporation), according to Kim et al. (2011). Primers were used to amplify the quinolone resistance region (Quinolone Resistance Determining Region - QRDR)

in the *gyrA*, *gyrB*, *parC*, and *parE* genes, producing fragments of 610, 660, 950 and 897 base pairs (bp), respectively (Table 1).

Purification, preparation, and genetic sequencing of samples. The samples were purified using the GFX™ PCR DNA and Gel Band Purification Kit according to the manufacturer's instructions and subsequently sent to Subunit RPT01A - DNA Sequencing, RJ from Fiocruz and sequenced in the DNA sequencer ABI 3730 (AppliedBiosystems/USA).

Detection of mutation in QRDR. The files containing the nucleotide sequences of the quinolone resistance region of the *gyrA*, *gyrB*, *parC*, and *parE* genes of the *Salmonella* spp. Samples were read in the BioEdit program (BioEdit Sequence Alignment Editor) to check the sequences' quality and generate new files in the "Fasta" format. All sequences obtained were compared with those of GenBank using the Blast algorithm[4].For the detection of chromosomal mutations in the QRDR region of the *gyrA*, *gyrB*, *parC*, and *parE* genes, the files in "Fasta" format were read by the ResFinder program available on the Center for Genomic Epidemiology website[5], according to Zankari et al. (2012).

RESULTS

The most frequent serotypes of *Salmonella enterica* were Saint Paul (29/77, 37.7%), Heidelberg (27/77, 35.1%) and Anatum (9/77, 11.7%). Cerro and Senftenberg had a frequency of 6.5% (5/77), and serotypes O: 4.5 and O: 9.12 had a frequency of 1.3% (1/77).

After undergoing the Disc-Diffusion test, for the evaluation of resistance to the antimicrobials enrofloxacin, ciprofloxacin and nalidixic acid, 19.5% (15/77) of the strains showed resistance to enrofloxacin, 7.8% (6/77) ciprofloxacin and 33.8% (26/77) to nalidixic acid (Table 2).

The Heidelberg serotypes, O: 4.5 and O: 9.12, were resistant to the three antimicrobials tested. Of these, the Heidelberg serotype showed the highest frequency of resistance to enrofloxacin (11/15, 73.3%), Ciprofloxacin (4/6, 66.7%) and nalidixic acid (20/26, 76.9%). The Senftenberg serotype did not show resistance to any of the three antimicrobials tested. The complete result of the resistance profile is shown in Table 2.

Of the 15 strains resistant to enrofloxacin, five (33.3%) had mutations in the *gyrA* gene. These mutations were isolated. The five mutations found occurred at codon 83, leading to a change from the amino acid Serine to Phenylalanine (TCC → TTC). One of the 15 strains (6.7%) showed a mutation in the *parC* gene. The mutation found occurred at codon 84, leading to a change from the amino acid Glutamic Acid to Lysine (GAA → AAG). None of the 15 strains had mutations in the *gyrB* and *parE* genes or more than one mutation in the *gyrA* gene or the other genes (Table 3).

DISCUSSION

The *Salmonella* serotypes identified in this study may cause mild gastrointestinal diseases (Rezende et al. 2016, Draper et al. 2017) to more severe extraintestinalconditions, as verified by the Heidelberg serotype, responsible for cases of septicemia, myocarditis, and death (Dutil et al. 2010). This fact makes production birds and their derived products essential vehicles for the dissemination of this agent. The Heidelberg serotype was one of the most isolated in this study and in the work of Pandini et al. (2015). They detected *Salmonella* Heidelberg in 12.82% of isolates from swine trawlers from broiler farms in the state of Paraná. These same authors detected serotypes different from those isolated in this study, such as the Mbandaka and Newport serotypes, with frequencies of 10.25% and *S.* Schwarzengrund, *S.* Enteritidis, *S.* Livingstone, and *S.* Orion, with 7.70% frequency each. Cardoso et al. (2015) also detected serotypes different from those isolated in this study in chicken carcasses from slaughterhouses in the state of São Paulo/SP and the most frequent serotype was Enteritidis (49.4%) followed by *S.* Albany serotypes (15.7%), *S.*Infantiles (11.2%), *S.* Agona (5.6%), *S.* Tennessee (4.5%), *Salmonella* spp. (3.4%), *S.* Kentucky (2.3%), *S.* Montevideo (1.1%), and *S.* Newport (1.1%). The Heidelberg and O: 4.5 serotypes, also isolated in the present study, were the least frequent, with an isolation frequency of 3.4% and 2.3%, respectively. The serotype most frequently isolated in this study was Saint Paul. In 2014, this serotype was the third most reported in Australia, accounting for 11% of all salmonellosis reports in this country (Draper et al. 2017).

Salmonella resistance to quinolones has been detected worldwide (Eaves et al. 2004, Lai et al. 2014, Lin et al. 2015, García-Fernández et al. 2015). In a study carried out in Recife, Brazil, Duarte et al. (2009) analyzed the susceptibility to antimicrobials of 19 strains of *Salmonella* spp., isolated from chicken carcasses. They found a percentage of 5.2% resistance for the three quinolones tested (enrofloxacin, ciprofloxacin, and norfloxacin). Another study, carried out in São Paulo, detected 16 strains resistant (17.5%) to ciprofloxacin in 91 strains of *Salmonella* spp. (Casas et al. 2016). These percentages were like those found in this study. The presence of *Salmonella* strains resistant to quinolones in foods, especially those used for treatment in humans, such as nalidixic acid and ciprofloxacin, is worrying because when its use becomes necessary for the treatment of serious infections it can increase the risk of failure (Park et al. 2019).

The existence of point mutations in some genes of the strains analyzed corroborates with the phenotypic resistance observed to nalidixic acid. However, it does not explain the resistance to fluoroquinolones found in the 15 strains. In this study, we only found point mutations in *gyrA*, and *parC* and, therefore, resistance to fluoroquinolones in strains of *Salmonella* detected by the phenotypic test may be due to other resistance mechanisms than the mutation in the

Table 1. Primers, nucleotide sequences and size of amplicons used for PCR amplification of the*gyrA*, *gyrB*, *parC* and *parE* genes in strains of *Salmonella* spp.

Primer	Sequence (5' to 3')	pb
gyrA - F	CGA GAG AAA TTA CAC CGG TCA	610
gyrA - R	AGC CCT TCA ATG CTG ATG TC	
gyrB - F	CTG CTT TAC CAA CAA CAT TCC	660
gyrB - R	TTG TCC GGG TTG TAC TCG TC	
parC - F	ATG AGC GAT ATG GCA GAG C	950
parC - R	GCG AAC AGA TGG TTC ATC AC	
parE - F	GCG GAA GAT ATC TGG GAT CG	897
parE - R	CAG CAG CAT ATC CAT CAT CG	

[4] Available at <available at https://blast.ncbi.nlm.nih.gov/Blast.cgi>

[5] Available at <http://www.genomicepidemiology.org/>

Table 2. Frequency of isolation and resistance assessment against the antimicrobials enrofloxacin, ciprofloxacin and nalidixic acid in strains of *Salmonella* spp. (n=77) isolated from live chickens and carcasses in slaughterhouses with Federal Inspection Service

Serotype	Frequency (%)		Resistance					
			Enrofloxacin (%)		Ciprofloxacin (%)		Nalidixic acid (%)	
Saint Paul	29	37.7	1	6.7	0	0.0	2	7.7
Heidelberg	27	35.1	11	73.3	4	66.7	20	76.9
Anatum	9	11.7	0	0.0	0	0.0	1	3.8
Cerro	5	6.5	1	6.7	0	0.0	1	3.8
O: 4.5	1	1.3	1	6.7	1	16.7	1	3.8
O: 9.12	1	1.3	1	6.7	1	16.7	1	3.8
Senftenberg	5	6.5	0	0.0	0	0.0	0	0.0
Total	77	100.0	15	100.0	6	100.0	26	100.0
	100%		19.5%		7.8%		33.8%	

Table 3. Mutations in the *gyrA*, *gyrB*, *parC* and *parE* genes found in the 15 strains of *Salmonella* spp. resistant to enrofloxacin, ciprofloxacin and nalidixic acid

Serotype	Phenotypic susceptibility			Mutation in (position)	Nucleotide change	Amino Acid change
	ENO	CIP	NAL. AC.			
Saint Paul	R	I	R	-	-	-
Heidelberg	R	I	R	*gyrA* (S83F)	TCC to TTC	Serine to Phenylalanine
Heidelberg	R	I	R	-	-	-
Heidelberg	R	R	I	*gyrA* (S83F)	TCC to TTC	Serine to Phenylalanine
Heidelberg	R	I	R	-	-	-
Heidelberg	R	I	R	-	-	-
Heidelberg	R	I	R	*gyrA* (S83F)	TCC to TTC	Serine to Phenylalanine
Heidelberg	R	I	R	-	-	-
Heidelberg	R	R	R	*parC* (E84K)	GAA to AAG	Glutamic acid to Lysine
Heidelberg	R	R	R	*gyrA* (S83F)	TCC to TTC	Serine to Phenylalanine
Heidelberg	R	R	R	-	-	-
Heidelberg	R	I	R	-	-	-
Cerro	R	I	R	-	-	-
O: 9.12	R	R	R	-	-	-
O: 4.5	R	R	R	*gyrA* (S83F)	TCC to TTC	Serine to Phenylalanine

ENO = Enrofloxacin, CIP = ciprofloxacin, NAL. AC. = nalidixic acid, R = resistant, I = intermediate.

studied genes. The quinolones' targets are the enzymes DNA Gyrase and Topoisomerase IV, which subunits are encoded by the genes *gyrA* and *gyrB* (DNA Gyrase) and *parC* and *parE* (Topoisomerase IV). One of the main resistance mechanisms developed by *Salmonella* is the mutation in these genes, preventing the binding of the antimicrobial molecule to enzymes and thereby ensuring the survival of these bacteria (Thong et al. 2015, Sinwat et al., 2018). Point mutations in the *gyrA*, *gyrB*, *parC*, or *parE* genes lead to resistance to first-generation quinolones. Resistance to fluoroquinolones in Enterobacteriaceae generally results from two or more mutations in the quinolone resistance genes determining region of DNA Gyrase and Topoisomerase IV (Thong et al. 2015 & Campioni et al. 2017).

CONCLUSIONS

Saint Paul and Heidelberg's serotypes were the most frequently identified in this study. The Heidelberg serotype was the one with the highest percentage of resistance to enrofloxacin, ciprofloxacin, and nalidixic acid.

The presence of point mutations in the *gyrA* and *parC* genes was predominant in strains of *Salmonella* spp. isolated from live chickens and carcasses but did not explain the phenotype of these strains' resistance to the antimicrobials enrofloxacin and ciprofloxacin.

The presence of strains resistant to these antimicrobials in birds and their products is worrying, and the results obtained indicated the presence of other resistance mechanisms that should be investigated.

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

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Testicular tumors in 190 dogs: clinical, macroscopic and histopathological aspects[1]

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ABSTRACT. - Nascimento H.H.L., Santos A., Prante A.L., Lamego E.C., Tondo L.A.S., Flores M.M., Fighera R.A. & Kommers G.D. 2020. **Testicular tumors in 190 dogs: clinical, macroscopic and histopathogical aspects.** *Pesquisa Veterinária Brasileira 40(7):525-535.* Laboratório de Patologia Veterinária, Departamento de Patologia, Universidade Federal de Santa Maria, Camobi, Santa Maria, RS 97105-900, Brazil. E-mail: glaukommers@yahoo.com

This study aimed to characterize the prevalence and clinical, macroscopic and histopathological aspects of dogs affected by testicular tumors based on biopsy specimens from the *Laboratório de Patologia Veterinária* of the *Universidade Federal de Santa Maria* (LPV-UFSM) over 19 years. Parameters regarding the age, size, and breed of the affected dogs were also established. Of all dogs with some type of neoplasm submitted to histopathological analysis at the LPV over these 19 years (n=1,900), 213 (11.2%) had at least one testicular neoplasm. The tissues of 190 dogs (with 220 neoplasms) were available for histological reassessment. The dogs in this study had different types of testicular tumors with relatively similar frequencies. In descending order, the most frequent testicular neoplasms were seminomas (88/220), Leydig (interstitial) cell tumor (LCT; 64/220), Sertoli cell tumor (SCT; 61/220), and mixed germ cell-sex cord stromal tumor (MGSCT) (07/220). Among the dogs of defined breed (119 cases), large breeds had the largest number of cases (50/119), followed by small (47/119) and medium-sized (22/119) breeds. The ages of dogs affected by testicular tumors ranged from 10 months to 18 years. Increased testicular volume was the most common clinical manifestation. Eleven dogs presented information about clinical signs suggestive of hyperestrogenism syndrome (feminization). In seminomas, the diffuse pattern predominated over the intratubular pattern. Two sites (luminal and basal compartments) suggestive of the onset of neoplastic transformations in germ cells were observed in intratubular seminomas. They corroborate the hypothesis that canine seminomas possibly have pathogenesis similar to that observed in human spermatocytic seminomas. The SCTs and LCTs presented high cell morphology variation. SCTs had neoplastic cells organized in five different histological arrangements. As for LCT, solid-diffuse and cystic-vascular histological patterns were the most commonly observed. Through this study, it was possible to establish some of the leading clinical, macroscopic, and histopathological aspects of testicular neoplasms diagnosed over 19 years in the area covered by the LPV-UFSM.

INDEX TERMS: Testicular tumors, dogs, clinics, macroscopy, histopathology, testicular neoplasms, clinical aspects, morphology.

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RESUMO.- [**Neoplasmas testiculares em 190 cães: aspectos clínicos, macroscópicos e histopatológicos.**] Este estudo teve por objetivo caracterizar a prevalência, aspectos clínicos, macroscópicos e histopatológicos dos cães acometidos por neoplasmas testiculares, a partir dos espécimes de biópsias do Laboratório de Patologia Veterinária da Universidade Federal de Santa Maria (LPV-UFSM) em 19 anos. Parâmetros quanto à idade, porte, raça dos cães acometidos também foram estabelecidos. De todos os cães com algum tipo de neoplasma submetido à análise histopatológica no LPV nesses 19 anos (n=1.900), 213 (11,2%) tinham ao menos um neoplasma testicular. Os tecidos de 190 cães (com 220 neoplasmas) estavam disponíveis para reavaliação histológica. Os cães deste estudo apresentaram diferentes tipos de neoplasmas testiculares com frequências relativamente semelhantes. Em ordem decrescente, os neoplasmas testiculares mais frequentes foram: seminomas (88/220), leydigomas (64/220), sertoliomas (61/220) e o tumor misto de células germinativas e do estroma do cordão sexual (MGSCT; 07/220). Dentre os cães com raça definida (119 casos), as raças de grande porte tiveram o maior número de casos (50/119), seguido das raças de pequeno (47/119) e médio porte (22/119). As idades dos cães acometidos por neoplasmas testiculares variaram de 10 meses a 18 anos. Aumento de volume testicular foi a manifestação clínica mais comum. Onze cães tinham informações sobre sinais clínicos sugestivos da síndrome da feminilização. Nos seminomas, houve o predomínio do padrão difuso sobre o intratubular. Dois locais (compartimentos luminal e basal) sugestivos de início das transformações neoplásicas nas células germinativas foram observados nos seminomas intratubulares, corroborando com a hipótese de que os seminomas caninos possivelmente tem patogênese semelhante à observada nos seminomas espermatocíticos humanos. Sertoliomas e leydigomas foram neoplasmas com alta variação na morfologia celular. Os sertoliomas tinham células neoplásicas dispostas em cinco arranjos histológicos distintos. Quanto aos leydigomas, os padrões histológicos sólido-difuso e cístico-vascular foram os mais comumente observados. Através deste estudo foi possível estabelecer alguns dos principais aspectos clínicos, macroscópicos e histopatológicos dos neoplasmas testiculares diagnosticados em 19 anos na área de abrangência do LPV-UFSM.

TERMOS DE INDEXAÇÃO: Tumores testiculares, cães, clínica, macroscopia, histopatologia, caninos, neoplasmas testiculares, aspectos clínicos, morfologia.

INTRODUCTION

Epidemiological studies point to testicular neoplasms as the most common tumors of the genital tract of male dogs (Cotchin 1960, Hayes & Pendergrass, 1976, Sapierzyński et al. 2007). In these studies, the most commonly diagnosed neoplasm types were germ cell (seminomas) and sex cord stromal (Sertoli cell and Leydig cell) tumors. The most relevant associated clinical aspects include expansive growth with marked distension of the affected testicles and clinical syndromes. Some authors point to cryptorchidism as a risk factor for these neoplasms (Hayes & Pendergrass 1976, Madewell 1981, Hayes et al. 1985, Yates et al. 2003, Ortega-Pacheco et al. 2006). However, other described factors less provenly associated include breed predisposition, inguinal hernias, and action of therapeutic agents and herbicides (Hayes et al. 1985, 1990, Kesley et al. 1998).

The histopathological patterns of these neoplasms have long been known (Nielsen & Lein 1974, Maclachlan & Kennedy 2002); however, some tumors are infrequent, difficult to diagnose, and have poorly described histological peculiarities (Patnaik & Mostofi 1993, Reis-Filho et al. 2004). Thus, retrospective studies including the reevaluation of cases can be useful in establishing prevalence in dogs, characterizing factors associated with the occurrence of these tumors, and describing unusual histological peculiarities that may present diagnostic relevance in understanding the pathogenesis of testicular neoplasms. The present study aimed to characterize the breeds, breed sizes and ages of the affected dogs, as well as prevalence and clinical, macroscopic and histopathological aspects of canine testicular neoplasms through a retrospective survey of the biopsy protocols of the *Laboratório de Patologia Veterinária* of the *Universidade Federal de Santa Maria* (LPV-UFSM) in 19 years (2000-2018).

MATERIALS AND METHODS

Data on the breeds, ages, and clinical signs of the affected dogs and on the gross aspects of the neoplasms were obtained from the report files of the LPV-UFSM over 19 years (2000-2018). These data were accessed from biopsy specimens of male dogs received by the laboratory during this period. Of the total cases, dogs with testicular neoplasms were counted, as well as the total number of neoplasms diagnosed in different organs in these dogs.

Breeds were grouped according to size: small (breeds up to 10kg), medium-sized (between 11-20kg) and large (over 20kg). Giant breeds were included in the last category (adapted from Folge 2009). Dogs of defined breed size were divided into four age groups (young, adults, seniors, and geriatric), shown in Table 1. Due to lack of information on weight in the biopsy protocols, mixed-breed dogs with no established breed size were not grouped in the size categories previously described. Consequently, they were not subdivided into age groups.

As for clinical manifestations, dogs with a history of increased testicular or scrotal volume, pain on palpation, cryptorchidism, inguinal hernias, and occurrence of clinical syndromes (feminization) were counted. The gross aspects were reviewed based on presentation of the neoplasms (nodules or masses), in the distribution (single/focal or multiple/multifocal neoplasms), and regarding consistency, color, and secondary aspects (presence of hemorrhage, necrosis, and cysts). The concepts of nodule and mass were adapted as already described (Gould et al. 2007). Tumors ≤3.0cm in diameter were considered as nodules, whereas those >3.0cm were considered as masses.

For histopathological reassessment, new slides of previously diagnosed testicular neoplasms were prepared. The tissue samples were cut into 3.0 µm sections, stained with hematoxylin and eosin (HE), reassessed, and reclassified according to the histological

Table 1. Distribution of dogs of defined breed according to age group

Breed size	Young	Adults	Seniors	Geriatric
Small	1-3 years	4-8 years	9-13 years	>13 years
Medium	1-2 years	3-8 years	9-11 years	>11 years
Large	1-2 years	3-5 years	6-9 years	>9 years

Adapted from Folge (2009) and Gardner (2017).

classification of genital tract tumors in domestic animals of the World Health Organization (WHO) (Kennedy et al. 1998). When applicable, tumors with morphological peculiarities suggestive of specific types were assessed by histochemical techniques in order to improve the analysis of these aspects.

RESULTS

Prevalence of affected dogs and frequency of testicular neoplasms

During the study period (2000-2018), 3,323 cases of biopsies in male dogs were recorded. Of these dogs submitted to biopsies, 1,900 had one or more neoplasm diagnoses, accounting for 2,118 neoplasms in different organs. The prevalence of dogs with testicular neoplasms was 213/1,900 cases, corresponding to 11.2% of all dogs affected by neoplasms. Of these 213 dogs, 190 had paraffin blocks available for histopathological reassessment. Considering that these 190 dogs had more than one testicular neoplasm, 220 neoplasms were diagnosed. In descending order, the most common testicular neoplasms in the diagnostic routine were seminomas (88/220, 40%), Leydig cell tumors (LCT; 64/220, 29.1%), Sertoli cell tumors (SCT; 61/220, 27.7%), and mixed germ cell-sex cord stromal tumors (MGSCT; 07/220, 3.2%).

More than one type of testicular neoplasm was diagnosed in 30 of the 190 dogs (15.8%). Sixteen had different neoplasms confined to the same testis. In 10 dogs, both testicles were affected. In the other four cases, different tumors affected both testicles. The most common combination of neoplasms was seminoma and LCT, observed in 16 dogs, followed by seminoma and SCT (12/190), and SCT and LCT (2/190).

Breed and age characterization

Of the 190 reassessed cases, 119 dogs were distributed in 25 breeds, 60/190 were mixed-breed, and information on breed was not obtained in 11/190. Large breeds accounted for the largest number of cases (50/119), followed by small (47/119) and medium-sized (22/119) breeds. In descending order, the most affected breeds were Poodle (22/119), Cocker Spaniel (16/119), Boxer (12/119), German Shepherd (11/119), Pinscher (09/119), Dachshund (7/119), Labrador Retriever (7/119), Collie (5/119), and Yorkshire Terrier (4/119).

Age of the 190 dogs affected by testicular neoplasms ranged from 10 months to 18 years. Among the dogs of defined breed size, seniors and geriatric accounted for most cases, while a smaller proportion were adult and young animals (Fig.1). Only small dogs had neoplasms distributed throughout the four age groups. In this category, senior dogs were the most affected. In medium-sized breeds, most tumors were observed in the age group >8 years, including senior and geriatric dogs. Among large dogs, the geriatric group was the most affected, and presented a significant number of cases compared with those of other age groups. Figure 2 shows the number of dogs affected by testicular neoplasms in the four age groups according to the three categories of breed size. In 7/190 dogs, including three defined breed dogs, information about age was not available. Mean age by tumor type was 10.8 years for seminomas (range from 10 months to 15 years), 10.6 years for SCT (4 to 18 years), 11.2 for LCT (2 to 17 years), and 12.4 years for MGSCT (6 to 15 years). In dogs diagnosed with more than one type of neoplasm, the mean age was 10.5 years (range from 5 to 15 years).

Clinical aspects

Increased testicular volume was the most common clinical manifestation, reported in 92 (48.4%) of the 190 dogs. Increased scrotal volume (thickening of the skin) and pain on palpation were reported in 14/190 (7.4%) and 6/190 (3.2%) cases, respectively. In eleven dogs (5.8%), information in the biopsy protocols showed manifestation of clinical signs suggestive of feminization (hyperestrogenism syndrome). Of these, 4/11 had gynecomastia and 4/11 developed skin lesions characterized by alopecia, thickening and hyperpigmentation, being generally bilaterally symmetrical in the regions of the abdomen, thorax, and limbs. In the other 3/11, both changes previously described (gynecomastia and skin lesions) were reported. SCT were diagnosed in 7/11 dogs, three were affected by seminoma-SCT combination, and one had MGSCT.

Fifty-one (23.2%) of the 220 neoplasms were observed in 43 (22.6%) cryptorchid dogs. Seminoma was the most frequent tumor among cryptorchid dogs (15/43), followed by SCT with 13/43 cases. A smaller proportion of dogs with cryptorchidism had combinations of neoplasms (8/43), as well as LCT (4/43) and MGSCT (3/43). Table 2 shows the location of the ectopic testicles according to neoplasm type. Hernias were reported in three dogs, with two perineal cases and one inguinoscrotal case.

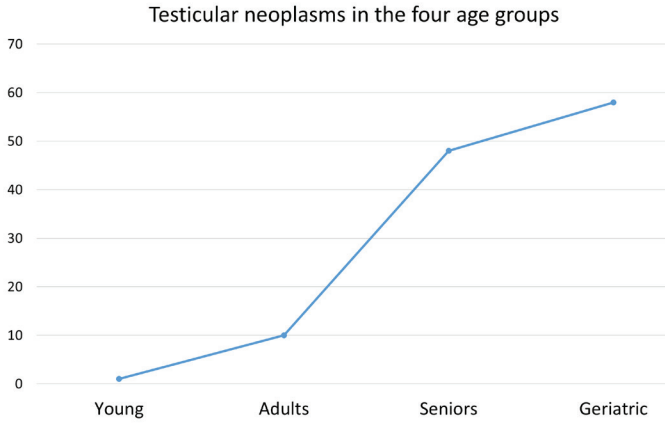


Fig.1. Distribution of testicular neoplasms in the four age groups.

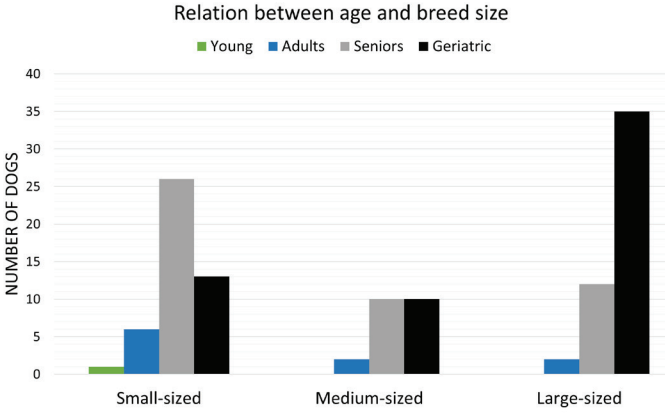


Fig.2. Dogs affected by testicular neoplasms in the four age groups according to the three breed size categories.

Table 2. Location of cryptorchidic testicles by type of neoplasm

Tumor type or combination	Inguinal cryptorchid	Abdominal cryptorchid	Uninformed	Number of dogs
Seminoma	4	3	8	15
SCT	4	-	9	13
LCT	2	2	*	4
MGSCT	2	-	1	3
Sem-SCT	2	-	5	7
Sem-LCT	1	*	*	1
TOTAL	15	5	23	43

- Missing information; * without occurrence; SCT = Sertoli cell tumor; LCT = Leydig cell tumor; Sem-SCT = Seminoma-SCT combination, Sem-LCT = Seminoma-LCT combination, MGSCT = mixed germ cell-sex cord stromal tumor.

Macroscopic and histopathological aspects

Macroscopic and histopathological aspects are described according to decreasing sequence in dogs affected by a single type of neoplasm (n=160).

Seminomas (n=60)

Most neoplasms (18/60) occurred as masses that were 5.0 to 10.0cm long, soft or firm, reddish-white, with areas of hemorrhage and, sometimes, completely obliterating the testicular parenchyma (Fig.3). Single or multiple nodules corresponded to 16/60 of the neoplasm cases, ranged from 0.4 to 3.0cm in length, and were generally soft, white or yellow, with areas of hemorrhage. In 9/60 dogs, no gross changes were found, and the neoplasms were diagnosed histologically. Fourteen of the 60 seminomas presented an intratubular pattern, 41/60 were diffuse, and both patterns were observed in 5/60 cases. Diffuse tumors were composed of sheets of neoplastic germ cells delineated by scarce fibrovascular stroma (Fig.4). The cells were round or polygonal and had moderate and eosinophilic cytoplasm. Their nuclei had vesicular or finely dotted chromatin and conspicuous nucleoli. Mitoses were frequent. Perivascular lymphocyte infiltrates, individual cell necrosis, and macrophages with vacuolated cytoplasm were common findings in diffuse seminomas.

As for intratubular seminomas, most tumors completely obliterated the lumens of the seminiferous tubules. However, in some cases, it was possible to detect two sites suggestive of neoplastic transformations. In the first and most common, the tumor cells were located in the center (luminal compartment) of the seminiferous tubules and were generally peripherally delineated by germ cells (round spermatids, spermatocytes, and spermatogonia) and stromal cells (Sertoli), with no evidence of neoplastic transformations (Fig.5). In the second pattern of localization of possible neoplastic transformations, one or two layers of neoplastic cells were observed in juxtaposition to the basement membrane (basal compartment). They had no remnants of germ or stromal components in the tubular lumen (Fig.6). Neoplastic cells with a syncytial aspect were an uncommon finding in some intratubular seminomas (Fig.7). These were fused, had more than ten nuclei, and were juxtaposed to the basement membrane. Six of the 60 seminomas were malignant. Infiltration of neoplastic cells into the lumen of blood and lymphatic vessels was observed

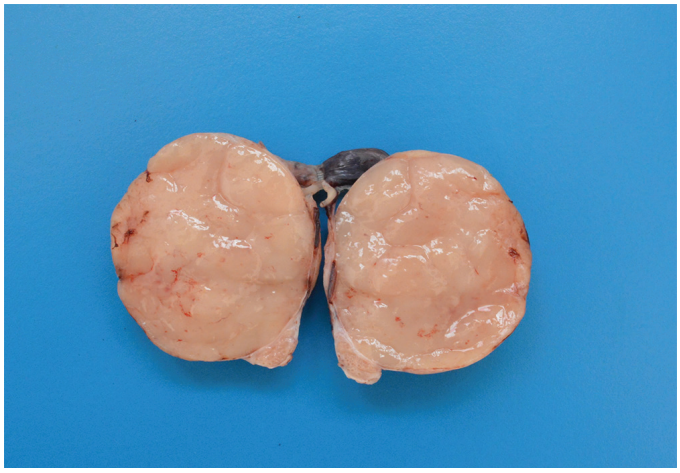


Fig.3. Seminoma. Testicular parenchyma obliterated by a white, soft mass with small areas of hemorrhage.

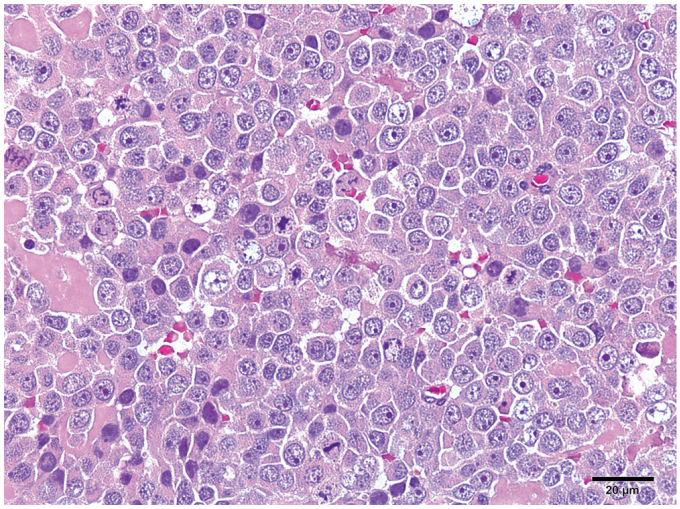


Fig.4. Diffuse seminoma. Marked anisocytosis, anisocariosis, and a high mitotic index were a marked aspect in diffuse seminoma. HE, obj.40x.

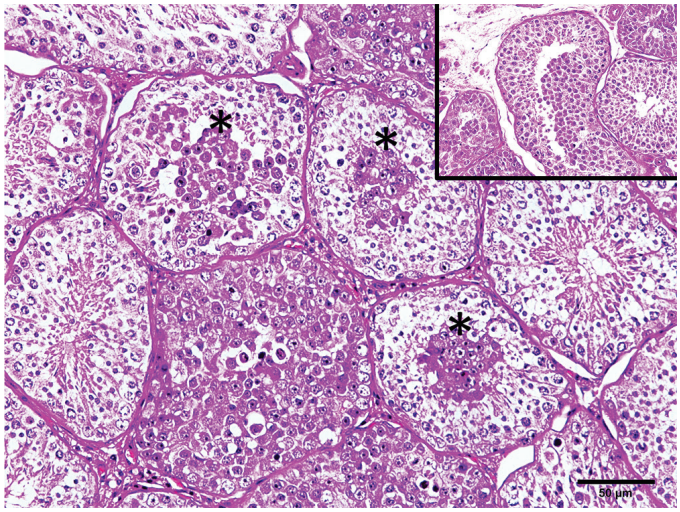


Fig.5. Intratubular seminoma. Neoplastic germ cells in the center of seminiferous tubules (asterisks), outlined by germinal epithelium with no evidence of neoplastic transformations (insert). HE, obj.20x.

in 3/60 dogs. Three of the 60 dogs had metastases to regional lymph nodes and infiltration of adjacent testicular structures (tunica albuginea and epididymis).

Sertoli cell tumors (SCT, n=47)

Most SCT (16/47) occurred as nodules. These were 0.4 to 3.0 cm in diameter. In most cases they were firm and white. The larger neoplasms (n=12) usually formed single masses ranging from 4.0 to 11 cm in length (Fig.8). When cut, they were firm or soft and had a white, multilobed, friable cut surface with areas of hemorrhage. Presence of cystic formations was a relatively frequent finding, regardless of the gross presentation and size of SCTs. These formations were single or multiple and filled with brown gelatinous content or blood.

Under microscopy, 24/47 sertoliomas had an intratubular pattern, 9/47 presented a diffuse pattern, and both patterns were observed in 14/47 cases. Among the neoplasms reassessed in this survey, SCTs presented the most significant heterogeneity regarding cell pleomorphism. Moderate to severe desmoplastic reaction and hyalinization of seminiferous tubules were striking and characteristic aspects in the vast majority of SCTs. Presence of central areas of necrosis outlined by neoplastic Sertoli cells was another characteristic finding, especially in higher cell density proliferations. Neoplastic cells were organized in five distinct histological arrangements and some tumors presented more than one cellular arrangement at times. The five histological arrangements observed in SCTs are as follows:

a) Small cell tumors, usually polyhedral, with little distinct cell boundaries, and small, hyperchromatic nuclei. In this arrangement, SCTs had expansive growth, with distention and rupture of the tubular membrane, as well as areas of hemorrhage and necrosis contiguous with the neoplastic formations (Fig.9);

b) SCTs with neoplastic cells grouped in tubules of different sizes, sometimes with disruption of the

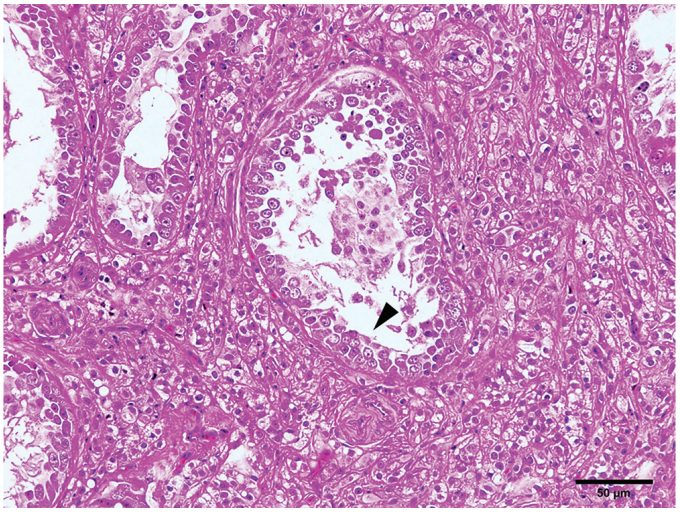


Fig.6. Intratubular seminoma. Germ cells juxtaposed to the basement membrane (arrowhead), with no stromal cell remnants (Sertoli) in the tubular lumen. HE, obj.20x.

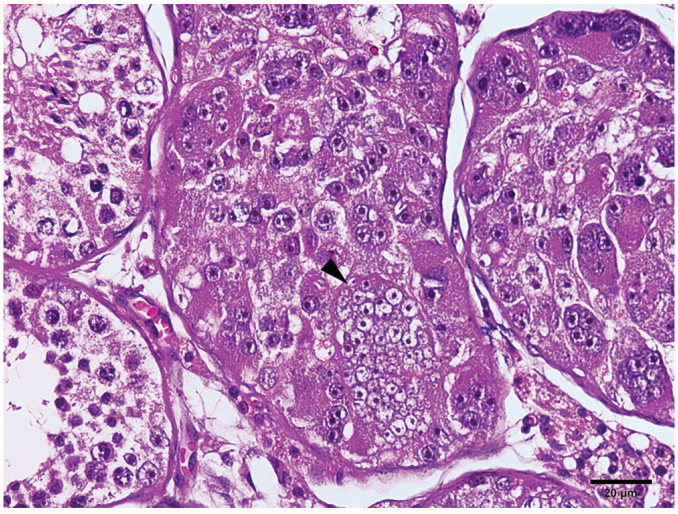


Fig.7. Intratubular seminoma. Seminiferous tubule obliterated by neoplastic germ cells. Syncytial cell juxtaposed to the basal membrane (arrowhead). HE, obj.40x.



Fig.8. Sertoli cell tumor. White, firm mass, partially obliterating the testicular parenchyma. Cystic formation filled with brown gelatinous content is observed adjacent to the neoplastic mass.

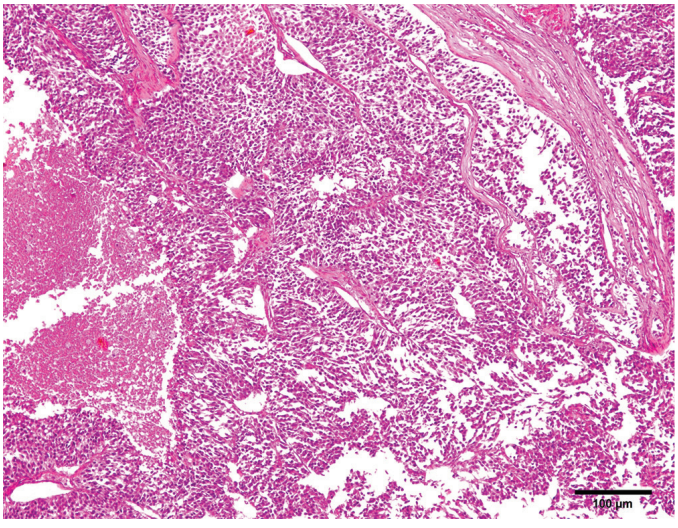


Fig.9. Diffuse Sertoli cell tumor. Neoplastic cells with expansive growth and rupture of the tubular basement membrane. HE, obj.10x.

basement membrane. The cells had scarce cytoplasm, indistinct boundaries, and round nuclei with sparse and hypochromatic chromatin (Fig.10). Hemorrhage and necrosis of Sertoli and germ cells in the center of these formations were frequent, especially in larger proliferations;

c) Neoplasms with columnar cells of indistinct cell boundaries, slightly vacuolated, with nuclei in different positions were observed in palisades and free in the lumens of the tubules (Fig.11). In this arrangement, cells were confined to tubules of different sizes, sometimes with disruption of the basement membrane;

d) SCTs with entirely fusiform cells had abundant eosinophilic cytoplasm and moderately distinct cell limits (Fig.12). Their nuclei were spindle-shaped with sparse and hypochromatic chromatin. In this arrangement, the tumors presented expansive growth, few tubule remnants, and a moderate desmoplastic reaction. Cells oriented in juxtaposition to the basement membrane were observed in some cases;

e) Neoplasms comprising vacuolated cells, with scarce eosinophilic cytoplasm, and generally intratubular pattern, with nuclei flattened by the compression of the vacuoles (Fig.13). In this arrangement, the tumors had a mild to moderate desmoplastic reaction. No malignant SCTs were observed.

Leydig cell tumors (LCTs, n=46)

Most tumors (32/46) were described as nodules, single or multiple, 0.4 to 2.5cm in diameter, soft, yellowish-white or

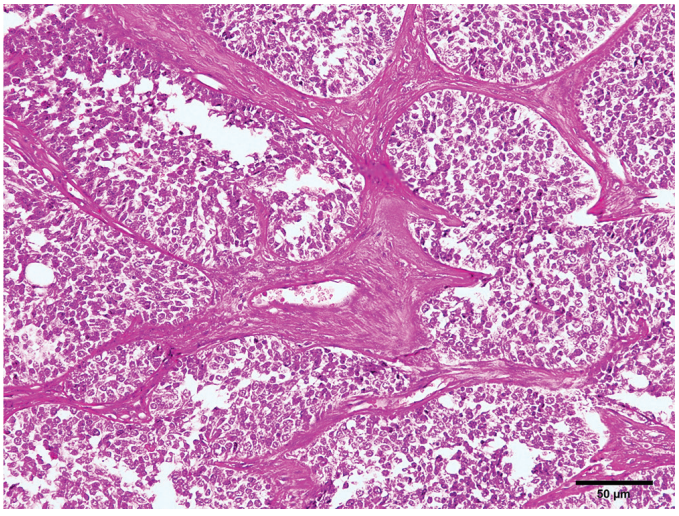


Fig.10. Intratubular and diffuse Sertoli cell tumor. Tubules of different sizes obliterated by neoplastic Sertoli cells, with rupture of the tubular basement membrane. HE, obj.20x.

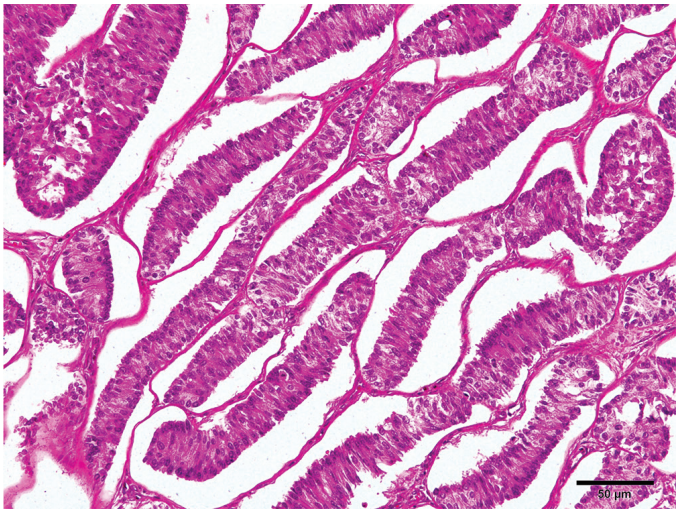


Fig.11. Intratubular Sertoli cell tumor. Columnar cells with nuclei in different positions arranged in palisades, confined to tubules of different sizes. HE, obj.20x.

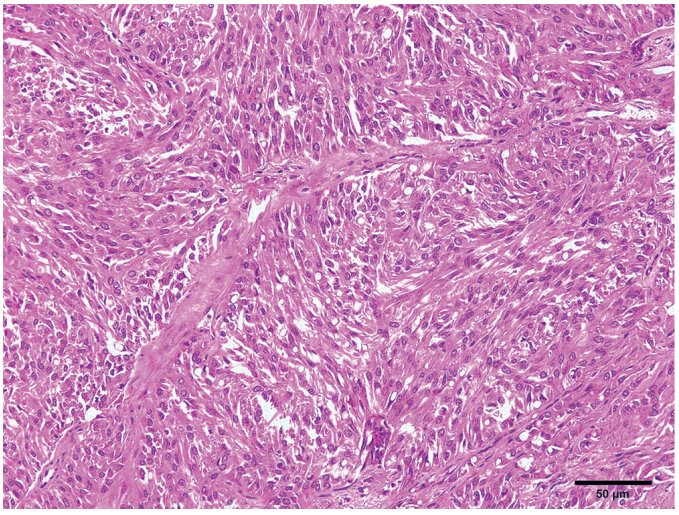


Fig.12. Diffuse Sertoli cell tumor. Entirely fusiform cell tumor. Expansive growth neoplasm with moderate desmoplastic reaction. HE, obj.20x.

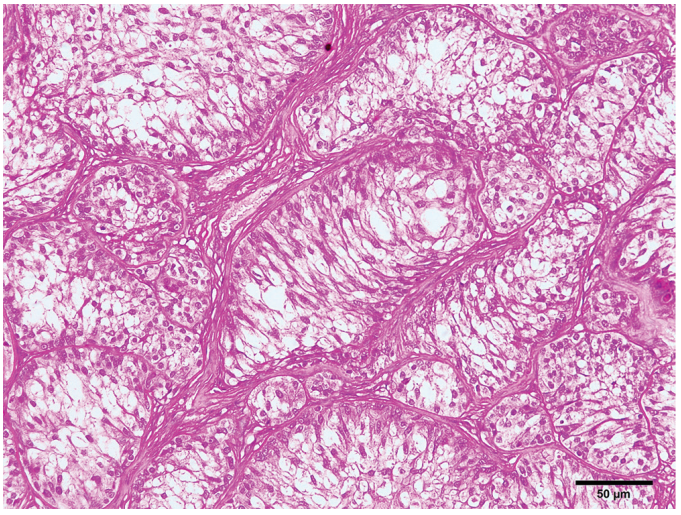


Fig.13. Intratubular Sertoli cell tumor. Neoplastic cells with intensely vacuolated cytoplasm confined to tubules of different sizes. HE, obj.20x.

red, well delimited, and sometimes with areas of hemorrhage (Fig.14). Three LCTs formed masses ranging from 3.0 to 10cm in length, with moderate to marked obliteration of the testicular parenchyma.

Under microscopy, the solid-diffuse and cystic-vascular patterns were the most commonly found, with 13/46 and 12/46 cases, respectively (Fig.15). Only one dog presented a pseudoadenomatous pattern (Fig.16). In the other 20/46 animals, two or more histological patterns were observed simultaneously. Most tumors presented solid-diffuse and cystic-vascular patterns, while a smaller proportion was formed by one of these patterns together with the pseudoadenomatous pattern.

Morphology of the neoplastic interstitial cells was somewhat variable. However, most tumors had polygonal cells, with eosinophilic cytoplasm, finely vacuolated, and round nuclei with condensed and hyperchromatic chromatin. Some neoplasms had cells with macrovacuoles that flattened and displaced the nuclei peripherally, as well as cells devoid of vacuoles, usually large, polyhedral, or columnar. A high proportion of the tumors were encapsulated and had scarce fibrovascular stroma. Accumulations of hematoidin and palisade-oriented columnar cell nests surrounding blood vessels were also relatively frequent findings. One LCT of this survey was malignant. In this case, the neoplasm obliterated the testicular parenchyma and had a high mitotic index, pleomorphism, and areas of necrosis. Multiple skin and other organs metastases were observed.

Mixed germ cell-sex cord stromal tumors (MGSCT, n=7)

The gross presentation of the MGSCTs varied between nodules and masses. They were single or multiple, firm, and white, 2.0 to 9.0cm in length, and sometimes obliterating the testicular parenchyma (Fig.17). Microscopically, a variable degree of neoplastic germ cells interposed or surrounded by neoplastic Sertoli cells was the most characteristic



Fig.14. Leydig cell tumor. Well defined, reddish-white, soft nodule with little distortion of the testicular parenchyma.

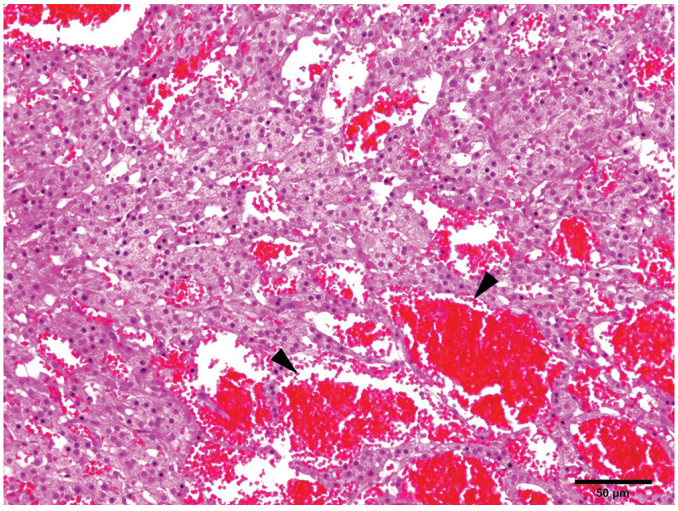


Fig.15. Cystic-vascular Leydig cell tumor. Neoplastic interstitial cells surrounding spaces filled with erythrocytes (arrowhead). HE, obj.20x.

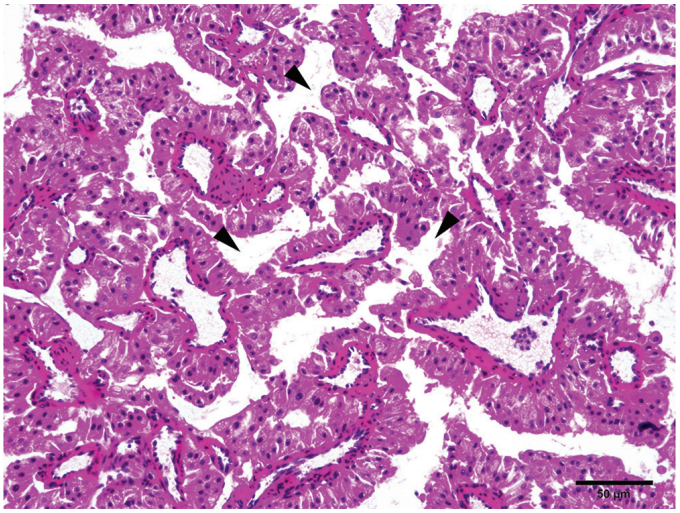


Fig.16. Pseudoadenomatous Leydig cell tumor. Neoplastic interstitial cells arranged in lobes, surrounding spaces filled with amorphous and eosinophilic fluid (arrowhead). HE, obj.20x.



Fig.17. Mixed germ cell-sex cord stromal tumor. Focal, white, soft nodule in the center of the testicular parenchyma.

finding in MGSCTs (Fig.18). Tubular hyalinization, moderate desmoplastic reaction, and predominance of one of the cellular components were also frequent findings. In 5/7 cases, Sertoli cells were more abundant, while 2/7 cases had predominance of germ cells. In the germinative component, the cells were round or polygonal, with scarce, eosinophilic cytoplasm, and presented vesicular nuclei or finely dotted and hyperchromatic chromatin. Sertoli cells were generally fusiform, with eosinophilic cytoplasm, and oval or columnar nuclei. Mitoses were infrequent and more common in the germ component. In two cases, circular spaces with homogeneous, amorphous, hyaline and eosinophilic material, strongly positive to Periodic Acid-Schiff (PAS) staining, surrounded by Sertoli cells (Call-Exner bodies) were observed in the neoplastic formations (Fig.19). In a dog affected by malignant MGSCT, infiltration of neoplastic germ cells was observed in the lumen of blood and lymphatic vessels.

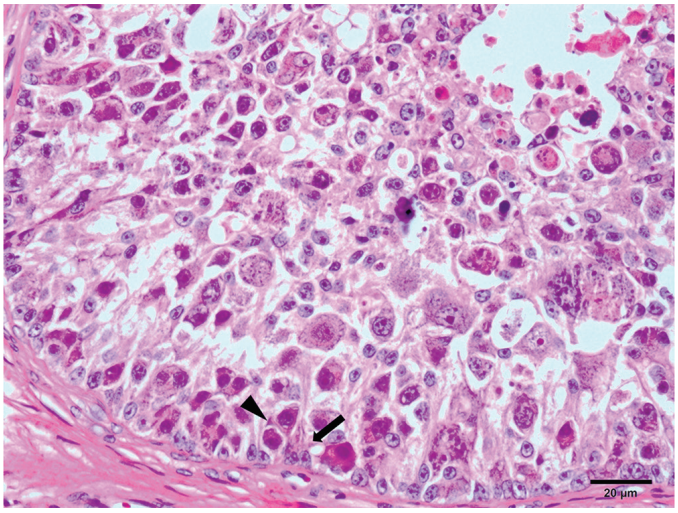


Fig.18. Mixed germ cell-sex cord stromal tumor. Neoplastic germ cells (arrowhead) interposed by neoplastic Sertoli cells (arrow). HE, obj.40x.

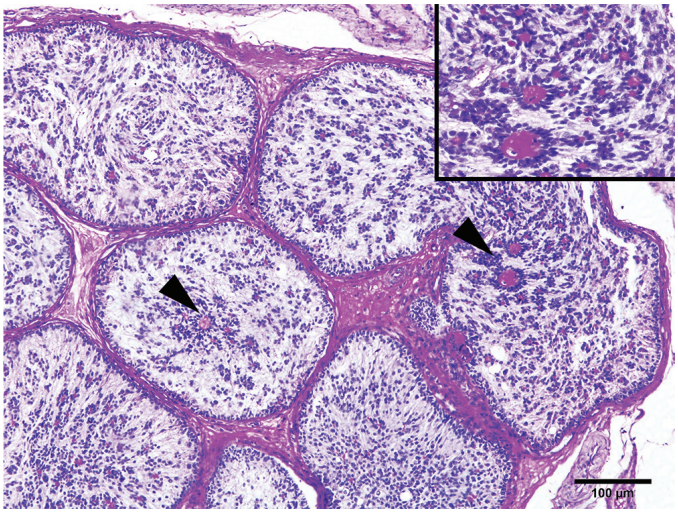


Fig.19. Mixed germ cell-sex cord stromal tumor. Call-Exner bodies (arrowhead and insert) intensely stained in pink. Periodic Acid-Schiff (PAS), obj.10x.

DISCUSSION

The present study reviewed 220 (10.4%) testicular tumors diagnosed among 2,118 neoplasms of male dogs. Corroborating the findings of other authors (Sapierzyński et al. 2007, Liao et al. 2009), seminomas were the most frequent neoplasms, followed by approximate proportions between LCTs and SCTs. In a study conducted in Slovenia, 206 (10.4%) testicular tumors were diagnosed in a total of 1,975 neoplasms affecting male dogs; seminoma was the most frequent, while MGSCT was observed in a small number of dogs (Svara et al. 2014), similarly to what was observed in the present survey. However, in other studies, tumors originating from stromal elements (SCTs and/or LCTs) have been more commonly diagnosed than those from germ cells (seminomas) (Hayes & Pendergrass 1976, Grieco et al. 2008, Argenta et al. 2016). It is worth mentioning that LCTs were the most frequently observed neoplasms in a research addressing this theme based on dog necropsies carried out in Brazil (Santos et al. 2000). Unlike what is observed in humans, where most testicular tumors originate from the germ component (Bosl & Motzer 1997), studies conducted with dogs show variations in the frequency of different testicular neoplasms. Possible factors that would influence this variation include the origin of the samples (necropsies and/or biopsies), whether they include only clinical cases or also derived from castration (for convenience) samples, among other non-established factors.

This study allowed characterizing the 190 cases and showed that the vast majority of dogs were of defined breed (distributed in 25 breeds) and that large breeds had the most significant number of neoplasms, followed by small and medium-sized breeds. Geriatric dogs were the most affected among dogs of defined breed, followed by a high proportion of seniors. These results are only demonstrative of the group of 190 analyzed dogs, thus not allowing inference about the overall predisposition of the canine population. Some studies have described specific breeds (within small and large ones) as being at high risk for testicular neoplasms (Liao et al. 2009, Nodtvedt et al. 2010). The literature also points out that elderly dogs are prone to the development of degenerative changes in the seminiferous tubules and testicular neoplasms (James & Heywood 1979, Lowseth et al. 1990, Santos et al. 2000).

SCT was the most frequent neoplasm, among the dogs of this study, presenting clinical signs of hyperestrogenism These findings corroborate those described by other authors, who reported that in 25 to 50% of the dogs affected by SCTs, this syndrome was characterized by symmetrical non-pruritic alopecia, hyperpigmentation, gynecomastia, galactorrhea, pendulous prepuce, and attractiveness to other male dogs (Siegel et al. 1967, Sanpera et al. 2002, Paepe et al. 2016). In one study, 65 of 318 dogs (20%) with testicular neoplasms developed clinical signs of hormonal dysfunction (hyperestrogenism) (Cotchin 1960). It is important to note that dogs affected by the seminoma-SCT combination or by MGSCTs also manifested hyperestrogenism syndrome (feminization) in this survey, though less frequently.

Cryptorchid dogs can be up to 14.3 times more likely to develop testicular neoplasms (Ortega-Pacheco et al. 2006). More than half of the diagnosed testicular neoplasms (52/96, 54.2%) were observed in 45 dogs with cryptorchidism (Liao et al. 2009). These results differ from those found in the present study, in which just under a quarter (51/220, 23.2%)

of the neoplasms occurred in 43 cryptorchid dogs. Smaller proportions of testicular neoplasms in cryptorchid dogs have also been observed by other authors (Hayes & Pendergrass 1976). Seminomas and SCTs were the most frequent neoplasms in the 43 cryptorchid dogs assessed, corroborating some previous reports (Hayes et al. 1985, Argenta et al. 2016). The gross characteristics observed in the seminomas of this study were similar to those described in the literature (Agnew & Maclachlan 2017).

Among dogs with seminoma, most were affected by seminomas of diffuse pattern. Conversely, studies have shown that intratubular seminomas correspond to most diagnosed seminomas (Dow 1962, Svara et al. 2014). A possible reason for this difference may lie in the type of samples analyzed (biopsy specimens and/or necropsy cases). In one of these studies (Dow 1962), many tumors had no visible lesions during necropsy, being only diagnosed under microscopy.

Two seminoma entities in humans (classic and spermatocytic) are recognized based on their histogenesis (Mostofi & Sesterhenn 2004, Bahrami et al. 2007). In dogs, histomorphological and immunohistochemical studies show that most canine seminomas have pathogenesis similar to that of human spermatocytic seminomas (Bush et al. 2011, Thorvaldsen et al. 2012). Corroborating this hypothesis, in this study, it was possible to suggest two probable sites of neoplastic transformations based on the detailed morphological evaluation of intratubular seminomas. In these cases, most of the neoplastic transformations were observed in the center of the seminiferous tubules, originating from more differentiated germ cells, mainly spermatids. Some tumors had a layer of neoplastic cells juxtaposed to the basement membrane. In these cases, it was impossible to infer whether the neoplastic transformations originate from spermatogonia or atypical germ cells equivalent to the carcinoma *in situ* cells, as demonstrated by other authors (Grieco et al. 2007).

Forty-seven dogs were affected only by SCTs. High heterogeneity in cell morphology and arrangement was a frequent finding in the SCTs assessed here, regardless of the histopathological pattern (intratubular or diffuse). However, the literature presents few in-depth studies on the histological peculiarities of this neoplasm to allow its subclassification according to these cellular characteristics (Cotchin 1960, Dow 1962). Comparative studies between histomorphological characteristics such as those described here and immunohistochemical markers could be useful determining possible prognostic relevance according to the different cell types and arrangements observed in these neoplasms.

The reassessment of the LCTs in this study showed that the histological patterns (solid-diffuse, cystic-vascular, and pseudoadenomatous) recommended by the WHO (Kennedy et al. 1998) are frequently observed, and that these tumors also have some heterogeneity in cell morphology. Corroborating these findings, other authors (Cotchin 1960, Dow 1962) have observed that the cellular arrangement of some LCTs may be similar, and even confused, with those observed in SCTs. Therefore, it is crucial to bear in mind the possible variation in cell morphology and its different histological patterns, since unusual cases can be misdiagnosed. Although some immunohistochemical panels are being used to assist with diagnosing testicular neoplasms, the absence of specific immunomarkers for LCTs (Owston & Ramos-Vara 2007) reinforces the importance of detailed morphological observation for the diagnostic accuracy of this neoplasm. In the present study, the solid-diffuse and cystic-vascular patterns were the most frequently observed, followed by a few pseudoadenomatous pattern cases. Malignant forms of LCTs have been rarely described in the literature, and are the subject of recent publications (Togni et al. 2015, Canadas et al. 2016, Kudo et al. 2019) that highlight the occurrence of metastasis, including multiple skin metastases (Canadas et al. 2016, Kudo et al. 2019). The only malignant LCT in this survey was reported in another publication (Nascimento et al. 2019).

Seven dogs were diagnosed with MGSCT in this study. Corroborating the findings by other authors, who showed that, in general, this neoplasm is uncommon in retrospective studies of canine testicular tumors (Patnaik & Mostofi 1993, Svara et al. 2014). According to Kennedy et al. 1998, it is not known whether this neoplasm is, in fact, rare or sufficiently analyzed to be diagnosed. Only one study described some histological criteria indicative of malignancy, characterized by infiltration of germ cells in adjacent testicular structures (Patnaik & Mostofi 1993). Some studies have used several cellular markers in the immunohistochemical characterization of these tumors. This technique has proved to be an excellent tool in diagnosing MGSCT because of its high diagnostic complexity (Owston & Ramos-Vara 2007, Ramos-Vara & Miller 2009, Banco et al. 2015). In the dogs reassessed in this survey, this neoplasm was characterized by varying degrees of association between neoplastic germ cells and Sertoli cells, which makes them real diagnostic challenges.

CONCLUSIONS

Through this study, it was possible to establish the prevalence (11.2%) of dogs affected by testicular neoplasms in the region covered by the LPV-UFSM, the profile of these dogs regarding breed, breed size and age, and the prevalence (10.4 %) of testicular neoplasms among the other neoplasms affecting male dogs.

Increased testicular volume was the primary clinical sign observed and the frequency of affected cryptorchid dogs was relatively low (23.2%).

Prevalence of different histological subtypes was established, and it was observed that malignancy characteristics were uncommon. Seminoma was the most frequently found tumor subtype, which had unusual predominance of the diffuse pattern over the intratubular pattern. Characteristics suggestive of transformation sites of neoplastic germ cells in intratubular seminomas were also observed, corroborating the hypothesis that canine seminomas possibly present pathogenesis similar to that of human spermatocytic seminomas.

LCTs and, mainly, SCTs were neoplasms with high cell morphology variation, emphasizing the importance of knowing their histomorphological variations, especially in cases with histological arrangements similar to those of these two tumors. MGSCT was an uncommon neoplasm, noting that its morphology is difficult to diagnose and that this neoplasm is characterized by varying degrees of association between neoplastic germ cells and Sertoli cells.

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Conflict of interest statement.- The authors declare having no conflicting interests.

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Relationship of obesity with clinical and laboratory changes in female dogs with mammary neoplasia[1]

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ABSTRACT.- Magalhães J.R., Guimarães J.B., Bonfim L.S., Carvalho C.F., Luz L.C., Cagnini D.Q., Regalin D. & Amaral A.V.C. 2020. **Relationship of obesity with clinical and laboratory changes in female dogs with mammary neoplasia.** *Pesquisa Veterinária Brasileira 40(7):536-545.* Rodovia BR-364 Km 195, Setor Parque Industrial 3800, Jataí, GO 75801-615, Brazil. E-mail: jessicarmedvet@gmail.com

Obesity may be associated with the onset of mammary tumors in women. In companion animals, these data are still scarce, mainly associating the clinic of the patient with laboratory and histological findings. This study aimed to evaluate the clinical and laboratory aspects of female dogs with mammary neoplasia, investigating whether they are related to obesity. Four groups of spayed or non-spayed female dogs were studied, divided into (G1) female dogs without mammary tumor and normal body condition score (BCS), (G2) female dogs without mammary tumor and obese, (G3) female dogs with mammary tumor and normal BCS and (G4) female dogs with mammary tumor and obese. BCS, canine body mass index (CBMI), blood count, glucose, total cholesterol, triglycerides and cytopathological and histopathological examinations were evaluated. Mixed-breed animals were the most prevalent in this study (67.5%). There was a limitation regarding the use of CBMI related to body proportionality. Considering the 28 tumors evaluated, carcinoma in mixed tumor was the most prevalent malignant histological type, while adenomyoepithelioma was the most prevalent benign histological type. It was possible to conclude that obesity was not related to clinical and laboratory changes in the female dogs affected with mammary neoplasias. In addition, no relationship was noted between BCS and CBMI with the histological type or malignancy of mammary neoplasia, since the malignant histological types were the most prevalent tumors in both the ideal score group and the overweight to obese group.

INDEX TERMS: Obesity, clinical change, laboratory change, female dogs, mammary neoplasia, dogs, bitches, cholesterol, glucose, triglyceride, mammary tumors.

RESUMO.- [**Relação da obesidade com alterações clínicas e laboratoriais em cadelas com neoplasia mamária.**] A obesidade pode estar associada ao aparecimento de tumores mamários em mulheres. Em animais de companhia, esse dado ainda é escasso, principalmente associando a clínica da paciente aos achados laboratoriais e histológicos. Este estudo objetivou avaliar os aspectos clínicos e laboratoriais de cadelas com neoplasia mamária, investigando se estão relacionadas à obesidade. Foram estudados quatro grupos de cadelas castradas ou não castradas, divididas em (G1) cadelas sem tumor de mama e Escore de Condição Corporal normal (BCS), (G2) cadelas sem tumor de mama e obesa, (G3) cadelas com tumor de mama e ECC normal e (G4) cadelas com tumor de mama e obesa. Foram avaliados o Escore de Condição Corporal (ECC), Índice de Massa Corporal Canina (IMC), hemograma, glicose, colesterol total, triglicerídeos e exames citopatológicos e histopatológicos. Os animais de raça mista foram os mais prevalentes neste estudo (67,5%). Houve uma limitação quanto ao uso do IMC relacionado à proporcionalidade corporal. Considerando os 28 tumores avaliados, o carcinoma em tumor misto foi o tipo histológico maligno mais prevalente, enquanto o adenomioepitelioma foi o tipo histológico benigno mais prevalente. Foi possível concluir que a obesidade não esteve relacionada a alterações clínicas e laboratoriais nas cadelas acometidas por neoplasias

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mamárias. Além disso, não foi observada relação entre BCS e CBMI com o tipo histológico ou malignidade das neoplasias mamárias, uma vez que os tipos histológicos malignos foram os tumores mais prevalentes no grupo escore ideal e no grupo com sobrepeso e obesidade.

TERMOS DE INDEXAÇÃO: Obesidade, alterações clínicas, alterações laboratoriais, cadelas, neoplasia mamária, cães, caninos, colesterol, glicose, triglicerídeos, tumores mamários.

INTRODUCTION

Mammary tumors are the most common types of neoplasia in female dogs, representing more than 50% of the cases, being 50% to 70% diagnosed as malignant, with metastatization capability for regional lymph nodes and lung. Consequently, the occurrence of mammary neoplasia in domestic animals is a subject addressed worldwide (Lana et al. 2007, Szczubial et al. 2012, Gundim et al. 2016, Dolka et al. 2018, Marconato et al. 2019). In addition, cancer is the leading cause of death in elderly dogs and the second largest cause in companion animals (Horta et al. 2012).

The awareness of health care for pets on the part of their guardians, along with the progress of Veterinary Medicine, has provided increased life expectancy, resulting in improved prevention of diseases, nutrition and therapeutics, in addition to increasing the need for the search for a better and more reliable prognosis (Horta et al. 2012, Matos et al. 2012, Silva et al. 2014). Nevertheless, concomitantly with the achievement of greater longevity, the cases of neoplasia diagnosed have increased (Portilho et al. 2015) because the more animals live, the greater their exposure to the carcinogenic factors that predispose to molecular changes with consequent disordered cell multiplication (Moreira et al. 2018).

Studies have shown a possible relationship between obesity and mammary neoplasia in female dogs (Lim et al. 2015, Chandler et al. 2017). According to German et al. (2006), the high ingestion of red meat had a positive correlation for the development of canine mammary tumors. For Lana et al. (2007), animals fed with home food had a higher risk of tumor development. For Cleary et al. (2010), obesity in female dogs decreases the latency period for detecting mammary tumors and increases their incidence. Kamiguchi et al. (2016) reported that obese animals fed with fatty diets have a higher risk of developing cancer.

Obese and oncological patients have a variety of systemic changes and, in oncological patients, such changes may result from the direct effect of tumor growth or paraneoplastic syndrome (Childress 2012). In obese patients, the increased pro-inflammatory cytokines may be associated with iron sequestration, with consequent reduction in the half-life of red blood cells and erythropoietin secretion, resulting in anemia (Mangieri 2009). Therefore, laboratory tests, such as blood count, are indispensable for detecting possible changes and assisting in the determination of prognosis and treatment, in order to provide better quality of life and survival (Garrido et al. 2015).

This study aimed to evaluate whether obesity triggered clinical and laboratory changes in the female dogs affected with mammary cancer treated at the "Hospital Veterinário" of the "Universidade Federal de Goiás" - Jataí Regional Campus (HV-UFG-REJ).

MATERIALS AND METHODS

Animals. Forty female dogs with or without mammary neoplasia treated at the "Hospital Veterinário" of the "Universidade Federal de Goiás" - Jataí Regional Campus (HV-UFG-REJ) were selected.

Four groups were created, primarily selecting ten female dogs for each group based on physical examination, according to the following characteristics:

Group 1 (G1): ten female dogs with ideal body condition score (BCS), without mammary neoplasia;

Group 2 (G2): ten overweight to obese female dogs, without mammary neoplasia;

Group 3 (G3): ten female dogs with ideal BCS, with palpable mammary neoplasia;

Group 4 (G4): ten overweight to obese female dogs, with palpable mammary neoplasia.

The criteria to include animals of Groups 1 and 2, in this study, were: spayed or non-spayed female dogs, over 1 year old, of the canine species, without palpable mammary nodules and without predilection for breed. For Groups 3 and 4, inclusion criteria were: spayed or non-spayed female dogs, over 1 year old, of the canine species, with palpable mammary nodules. The exclusion criteria, regardless of the group, considered the presence of concomitant disease and, specifically for Groups 3 and 4, the non-approval, by the guardian, of the mastectomy surgical protocol as treatment. The guardians were informed about the project and signed a consent term for participation in the experiment. The project was approved by the Ethics Committee on the Use of Animals (CEUA-UFG) nr 017/17.

Anamnesis and physical evaluation. In the anamnesis, the guardian was asked about nutritional life (type of food provided and daily number of meals when the animal was a puppy and when adult), reproductive life (number and frequency of estrous cycle, number of pregnancies, history of contraceptive use and castration) and tumors, when applicable (onset and evolution time, aspect and location, occurrence and location of previous tumors).

Physical evaluation consisted of physical examination (heart rate, respiratory rate, rectal temperature and evaluation of visible mucosal coloration, and inspection and palpation of lymph nodes and mammary glands) and determination of body condition score BCS and canine body mass index (CBMI). The Body Condition Score (BCS) was performed as proposed by Laflamme (1997), using the scale from one to nine points. The CBMI was determined as proposed by Muller et al. (2008), which considers as anatomical reference points the length between the base of the atlanto-occipital joint and the area immediately behind hind limbs, leaning the measuring tape on the back of the animal and on the base of the tail. Measurement was obtained and calculated by the formula: CBMI = body weight (Kg)/(height in meter)^2.

Laboratory evaluation. In the laboratory evaluation, performed after the puncture of 10mL of blood from the jugular vein, the material intended for blood count was placed in a tube with ethylenediaminetetraacetic acid (EDTA) anticoagulant and, for glucose dosage, a tube with fluoride was used. For cholesterol and triglyceride evaluation, a tube without anticoagulant was used. Previous 12-hour fasting was requested for glucose, cholesterol and triglyceride evaluation.

Cytopathology. The cytopathological evaluation was performed before surgical procedure, by a trained professional, with the puncture of the nodules and reactive lymph nodes. The material was then stained with fast stain derived from Romanowsky (Fast Panoptic) and analyzed in an optical microscope. The criteria for sample classification were as described by Grandi (2017), including,

for example, cellularity, mitotic index, nuclear and cytoplasmic morphology, nucleus: cytoplasm ratio, presence of evident nucleoli, macronucleoli, chromatin pattern, anisocytosis, anisocariasis, presence of dysplasia, anaplasia and ratio of these findings in the population of cells evaluated. The cytopathological evaluation was not performed only in the female dogs who had already been treated by another Veterinary Physician.

Staging. The female dogs evaluated with mammary neoplasia underwent staging according to the TNM system (tumor size, infiltration in regional lymph nodes and distant metastasis), proposed by the World Health Organization and created by Owen (1980). Stages one, two and three are related to tumor size, while stage four is related to lymph node involvement and stage five to distant metastases. Thoracic radiography and abdominal ultrasonography were requested to determine the distance metastasis study. When the presence of multiple tumors was observed, the tumor with the highest classification according to Owen (1980) was considered for staging.

Treatment. Patients with mammary neoplasia were submitted to surgical treatment. The choice of the surgical technique varied according to each case, considering tumor size, location, lymphatic drainage and cytopathological result. Moreover, regardless of the surgical technique, the removal with a two-to-three-centimeter safety margin of normal tissue was recommended. All nodules removed were sent for histopathological evaluation, including safety margin tissues and inguinal lymph nodes. The axillary lymph nodes were only removed if reactive.

Histopathology. For histopathological evaluation, fragments were fixed in 10% buffered formaldehyde solution, with formaldehyde volume in the 10:1 ratio as of the size of the surgical specimen. After fixation and cleavage, surgical specimens were subjected to dehydration in alcoholic solution and diaphanization in xylol, and subsequently included in histological paraffin. A mechanical rotating microtome (LEICA RM 2245) performed 4µm cuts which were arranged in matte-end slides and subsequently stained with hematoxylin and eosin. The slides were evaluated in light microscopy (LEICA DM 750) and classified according to Goldschmidt et al. (2011), Cassali et al. (2014) and Cassali et al. (2017).

Statistical analysis. Statistical analyses were performed using the Sigma Plot version 12 software (Systat Software Inc., USA). After evaluating data distribution by the Shapiro-Wilk test, parametric data were analyzed by T Test, shown as mean (± standard error of the mean) and considered significant when $P<0.05$. The non-parametric data were analyzed by the Rank Sum Test, as median (first percentile and third percentile) and considered significant when $P<0.05$.

RESULTS

Population studied

Most of the animals studied were mixed-breed, followed by the Dachshund breed, as shown in Table 1. When analyzing groups G1xG3 and G3xG4 as to breed, there was a statistical difference, since only two animals in G3 were mixed-breed. In addition, in the groups with mammary tumors, half of the patients were pure-breed.

When analyzing the age of the groups without mammary tumors, the mean observed was 4.9±2.9 for G1 (ideal BCS) and 6.8±1.3 for G2 (overweight to obese), while the mean of the groups with mammary tumors was 8.1±2.8 for G3 (ideal BCS) and 9.6±1.8 for G4 (overweight to obese).

Anamnesis and physical examination

When analyzing feeding frequency, as a puppy and then as an adult, there was a statistical difference between groups G2xG4, between the two stages of the patient´s life (Table 2). It was also observed that 87.5% (35/40) of the patients were fed with ration and 12.5% (5/40) were fed only with homemade food.

When analyzing the use of contraceptives in patients, it was observed that 25% (10/40) had already received application of the medication. Of those, three from G1, three from G3 and four from G4. When comparing them statistically, there was a difference between groups G2xG4 (Table 3), and G4

Table 1. Distribution according to breed of spayed or non-spayed female dogs, over 1 year old, with or without palpable mammary nodules

Breed	Total amount	%
Mixed breed	27	67.5%
Dachshund	5	12.5%
Shih Tzu	2	5
Blue Hiller	2	5
Pinscher	2	5
Siberian Husky	1	2.5%
Collie	1	2.5%
TOTAL	40	100%

Table 2. Feeding frequency when puppies and when adults*, of spayed or non-spayed female dogs, over 1 year old, with or without palpable mammary nodules, distributed in female dogs without mammary tumor and normal BCS (G1), female dogs without mammary tumor and overweight to obese (G2), female dogs with mammary tumor and normal BCS (G3) and female dogs with mammary tumor and overweight to obese (G4)

Groups	Puppy feeding frequency	Adult feeding frequency
G1	1.8 ± 0.6A	1.6 ± 0.5A
G3	1.3 ± 0.4A	1.3 ± 0.5A
G2	2.3 ± 0.6A,B	2.0 ± 0.7A.B
G4	1.3 ± 0.4A,B	1.6 ± 0.5A,B

Mean ± standard error of the mean; ^{A,B} different letters differ from each other ($P<0.05$) by T-Test; * according to information from guardians, obtained through the questionnaire.

Table 3. Use of contraceptives for spayed or non-spayed female dogs*, over 1 year old, with or without palpable mammary nodules, distributed in female dogs without mammary tumor and normal BCS (G1), female dogs without mammary tumor and overweight to obese (G2), female dogs with mammary tumor and normal BCS (G3) and female dogs with mammary tumor and overweight to obese (G4)

Groups	Use of contraceptives
G1	0 (0;1)
G3	0 (0;1)
G2	0 (0;0)A,B
G4	0 (0;1)A,B

Median (first percentile; third percentile); ^{A,B} different letters differ from each other ($P<0.05$) by the Rank Sum Test; * according to information from guardians, obtained through the questionnaire.

received a higher number of applications. As to the history of pseudocyesis, there was no statistical difference among groups, however 17.5% (7/40) of the female dogs showed the change at least once throughout life, being one from G2, two from G3 and four from G4.

All patients showed good general condition at physical examination, as heart rate, respiratory rate, rectal temperature and evaluation of visible mucosal coloration, and inspection and palpation of lymph nodes and mammary glands. In the BCS evaluation, comparing G1xG3 and G2xG4, no statistical differences were observed (Table 4), as expected. When CBMI was considered, there was a statistical difference between groups G2 and G4 (Table 4). When comparing BCS and CBMI evaluations, it was observed that 42.5% (17/40) of the female dogs had the same classification.

In the groups with mammary tumors, a total of 28 nodules were evaluated revealing that 65% (13/20) of the female dogs had only one palpable nodule, of which seven were from G3 and six from G4, while 35% (7/20) had two or more palpable mammary nodules, of which three were from G3 and four from G4.

When analyzing the location of mammary tumors, inguinal mammary glands constituted the most affected sites (42.86%), followed by caudal abdominal mammary glands (17.86%), cranial abdominal mammary glands (18.75%), caudal thoracic mammary glands (10.71%) and cranial thoracic mammary glands (12.5%). Among G3 patients, two had nodules in the cranial thoracic mammary gland, two in the caudal thoracic mammary gland, two in the cranial abdominal mammary gland, three patients in the caudal abdominal mammary gland and six in the inguinal mammary gland. Among G4 patients, one had nodules in the cranial thoracic mammary gland, two in the caudal thoracic mammary gland, three in the cranial abdominal mammary gland, two in the caudal abdominal mammary gland and six in the inguinal mammary gland.

Still in the macroscopic analysis of mammary tumors, it was observed that 40% (8/20) had tumor diameter smaller than three centimeters and, of those, three patients were from G3 and five from G4, 35% (7/20) between three and five centimeters, being four patients from G3 and three from G4 and 25% (5/20) larger than five centimeters, with three patients from G3 and two from G4. Two patients had ulcerated tumors (both from G3), three had inflamed tumors (two patients from G3 and one from G4), one had mammary secretion (G3) and there were two in which the development of the tumor already involved fascia and muscle (one patient from each group).

Laboratory evaluation

The evaluation of hematological parameters (blood count/leukogram) showed no statistical difference among groups (Table 5). No patient had anemia. However, 25% (10/40) had thrombocytosis, being two from G1, four from G2, one from G3 and three from G4. Other 7.5% (3/40) had lymphopenia, being one patient from G2, one from G3 and one from G4, sequentially. Another 10% (4/40) had leukopenia, corresponding to one patient from G1, two from G2 and one from G3. And 10% (4/40) had leukocytosis, being one patient from each group.

Regarding biochemical assessments, triglycerides and total cholesterol did not show statistical difference. However, glucose differed when comparing groups G1xG3 and G2xG4 (Table 6). In the individual evaluation of each group, 25% (10/40) showed increased triglycerides, being three patients from G1, two from G2, two from G3 and three from G4. Other 10% (4/40) showed increased total cholesterol, corresponding

Table 4. Determination of body condition score (bcs) and canine body mass index of spayed or non-spayed female dogs, over 1 year old, with or without palpable mammary nodules, distributed in female dogs without mammary tumor and normal BCS (G1), female dogs without mammary tumor and overweight to obese (G2), female dogs with mammary tumor and normal BCS (G3) and female dogs with mammary tumor and overweight to obese (G4)

Groups	BCS	CBMI
G1	5.0 ± 0.0^{A}	15.0 ± 3.3^{A}
G3	5.0 ± 0.0^{A}	14.3 ± 1.9^{A}
G2	7.6 ± 1.0^{A}	18.1 ± 3.0^{A,B}
G4	7.3 ± 0.7^{A}	14.2 ± 4.8^{A,B}

Mean ± standard error of the mean; ^{A,B} different letters differ from each other (P<0.05) by T-Test.

Table 5. Hematological Parameters (hematocrit, platelet, leukocyte, neutrophil and lymphocyte) of spayed or non-spayed female dogs, over 1 year old, with or without palpable mammary nodules, distributed in female dogs without mammary tumor and normal BCS (G1), female dogs without mammary tumor and overweight to obese (G2), female dogs with mammary tumor and normal BCS (G3) and female dogs with mammary tumor and overweight to obese (G4)

Groups	Hematocrit (%)	Platelet (/µL)	Leukocyte (/µL)	Neutrophil (%)	Lymphocyte (%)
G1	44.8±6.2^{A}	402.0±150.0^{A}	11.6±4.3^{A}	6.5±0.9^{A}	3.4±0.8^{A}
G3	47.5±5.0^{A}	433.6±99.0^{A}	12.5±5.2^{A}	10.6±2.1^{A}	2.6±0.5^{A}
G2	47.7±7.6^{A}	551.2±236.3^{A}	13.4± .6^{A}	10.1±1.2^{A}	3.3±0.7^{A}
G4	48.1±6.4^{A}	441.2±166.9^{A}	13.9±10.4^{A}	6.7±1.5^{A}	2.6±0.7^{A}

Mean ± standard error of the mean; ^{A,B} different letters differ from each other (P<0.05) by T-Test.

Table 6. Biochemical Parameters (triglycerides, total cholesterol, glucose) of spayed or non-spayed female dogs, over 1 year old, with or without palpable mammary nodules, distributed in female dogs without mammary tumor and normal BCS (G1), female dogs without mammary tumor and overweight to obese (G2), female dogs with mammary tumor and normal BCS (G3) and female dogs with mammary tumor and overweight to obese (G4)

Groups	Triglycerides (mg/dl)	Total Cholesterol (mg/dl)	Glucose (mg/dl)
G1	95.9 ± 88.5^{A}	172.4 ± 42.7^{A}	88.7 ± 7.0^{A,B}
G3	58.4 ± 34.5^{A}	194.7 ± 104.1^{A}	108.0 ± 26.4^{A,B}
G2	67.4 ± 56.9^{A}	210.4 ± 100.8^{A}	86.4 ± 31.5^{A,B}
G4	101.7 ± 74.7^{A}	183.4 ± 47.4^{A}	110.0 ± 12.8^{A,B}

Mean ± standard error of the mean; ^{A,B} different letters differ from each other (P<0.05) by T-Test.

to one patient from G2, two from G3 and one from G4. Another 7.5% (3/40) had increased glucose, being one patient from G2, one from G3 and one from G4, respectively.

Cytopathology

The cytopathological examination was performed in 13 patients, however two examinations had inadequate or insufficient cellularity. Of the patients who had the cytopathological examination performed, malignancy criteria attributed compatible with the histopathological examination were attributed to nine and the benign criteria to one, both in cytology and histology, determining the cytohistopathologic correlation of 90.9% (10/11). Only one patient had confronting cytological and histological results.

Staging

Of the 20 patients with mammary tumors, eight were classified as staging 1, six as staging 2, four as staging 3 and two as staging 4. No patient had distant metastasis, that is, there was no staging 5 classification. The two patients classified as staging 4 had metastasis in a regional inguinal lymph node, being one with squamous cell carcinoma, from G4 (Fig.1A and B) and the other with papillary carcinoma, from G3. No difference was observed between the groups when staging was considered.

Treatment

All patients with mammary neoplasia received surgical treatment. Of the 20 patients, 19 underwent the unilateral mastectomy technique, while the regional mastectomy technique was used in the patient with the benign cytological type. Twenty inguinal lymph nodes corresponding to the surgical excision side were evaluated. No axillary lymph node was evaluated, since they were not reactive at the time of surgery.

Histopathology

All mammary tumors found in the female dogs were histologically analyzed. Of the malignant mammary tumors, cases of carcinoma in mixed tumor, papillary carcinoma, tubular carcinoma, complex carcinoma, "in situ" carcinoma, intraductal papilloma and carcinoma, sclerosing carcinoma, carcinoma, malignant myoepithelioma and carcinosarcoma were observed (Fig.2A and B). As to malignancy, eight patients from G3 and nine patients from G4 had malignant histological types. The benign histological types were adenomyoepithelioma (Fig.3A and B), sclerosing adenoma, lactation adenoma and benign mixed tumor. Only one case showed another histological type not coming from the mammary gland, which was the squamous cell carcinoma, because a connection with cutaneous epidermis was found. One patient from G4 with a benign mixed tumor had bone and cartilaginous metaplasia and another patient from G3 with carcinoma in a mixed tumor had metaplasia in cartilage (Fig.4A and B). In total, seven patients had more than one histological type of mammary neoplasia, three from G3 and four from G4. Of the seven patients, three had more than one histological type in the same mammary gland evaluated, one from G3 and two from G4.

Histological types are shown in Table 7. No difference was observed between the groups when tumor malignancy was considered.

DISCUSSION

The fact that the patients were in good general condition at physical examination corroborates Petrov et al. (2014), in which most of the female dogs are clinically healthy when physically evaluated. The age mean in this study is within the range considered as of higher incidence to the onset of mammary neoplasia according to Munkhtuul et al. (2014) and Ariyarathna et al. (2018).

Kim et al. (2016), when analyzing the characteristics of mammary tumors according to age groups, found that the histological types of worse prognosis tended to develop in dogs with advanced age. In this study, only three elderly animals had tumors with benign histological features, revealing that most of female dogs in this age group had tumors with malignant features, corroborating the authors.

It was observed that most of the patients evaluated were mixed-breed, followed by the Dachshund, Shih Tzu, Blue Hiller,

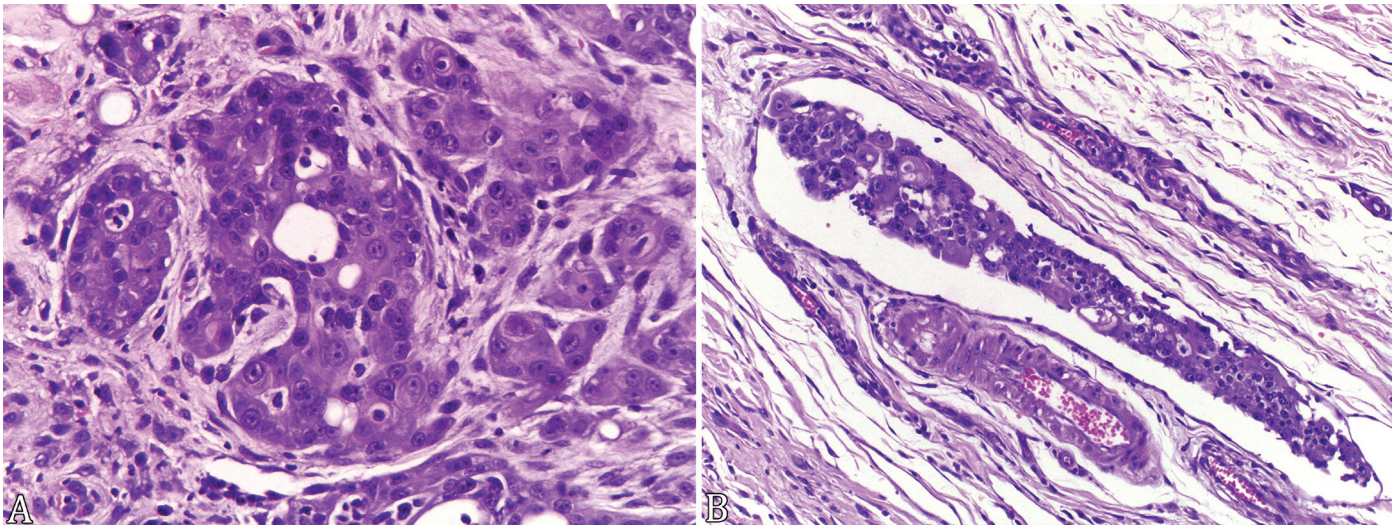


Fig.1. Canine, mixed-breed, 10 years old, Squamous cell carcinoma with inguinal lymph node metastasis. (**A**) Tissue invasion area of the neoplastic squamous epithelium (black arrow). HE, obj.40x. (**B**) Invasion of lymphatic vessels by neoplastic cells (black arrow). HE, obj.20x.

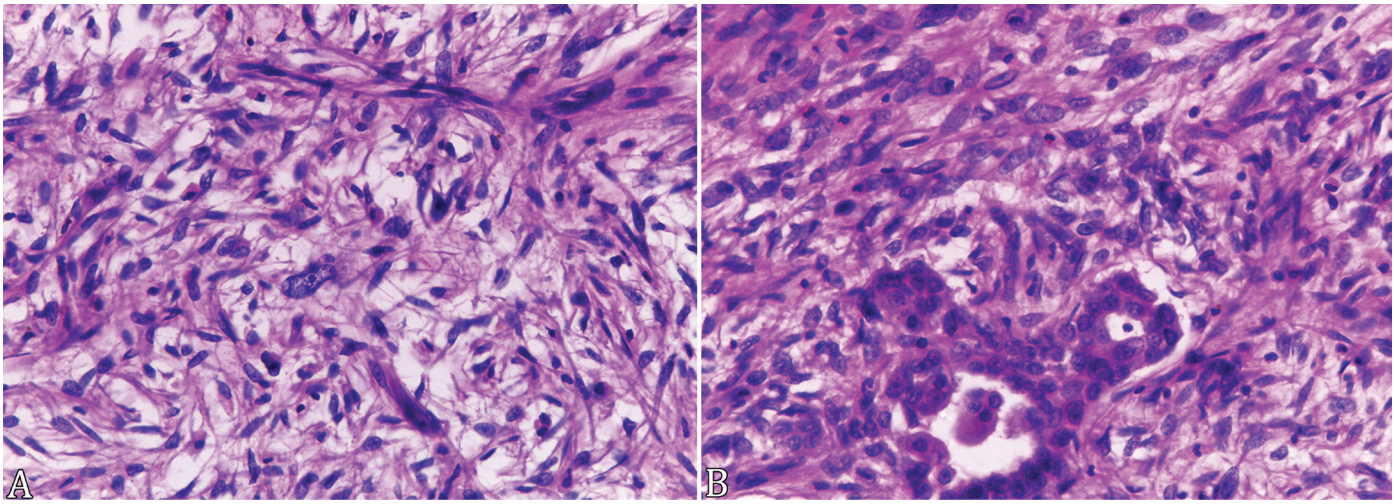


Fig.2. Canine, Dachshund, 6 years old, Carcinosarcoma. (**A**) Malignant mesenchymal proliferation, with high cellularity, showing a macronucleus with irregular borders and evident nucleolus in the black circle. (**B**) Epithelial component (black arrow) amid the highly cellular sarcomatous area. HE, obj.40x.

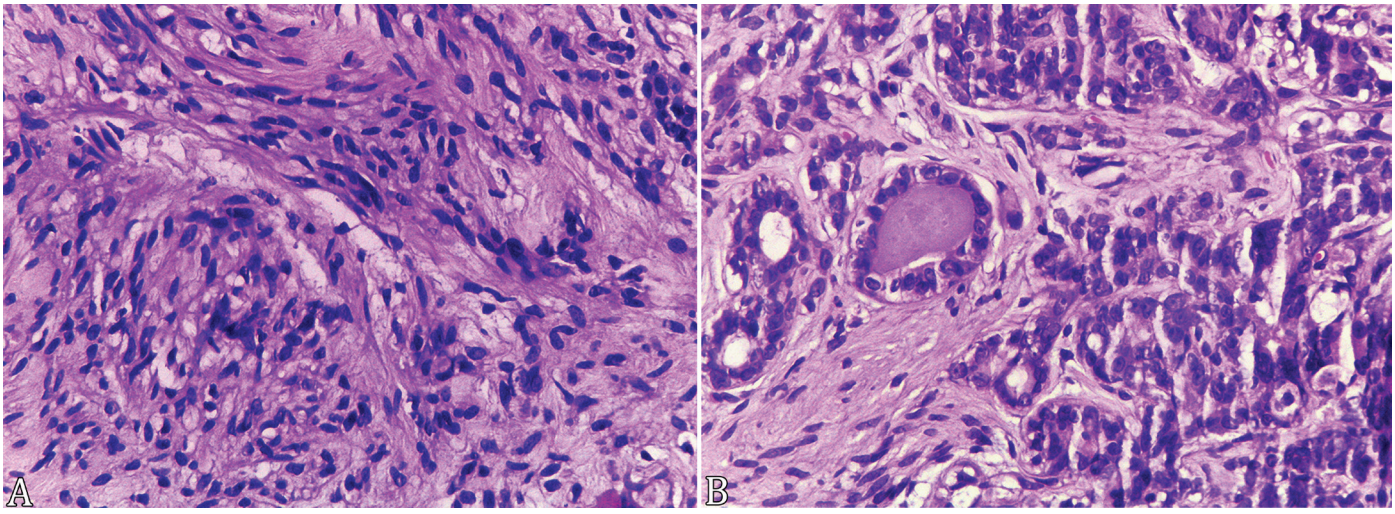


Fig.3. Canine, mixed-breed, 11 years old, Adenomyoepithelioma. (**A**) Mesenchymal cell proliferation with few morphological changes. (**B**) Area of proliferation of well-differentiated epithelial cells. HE, obj.40x.

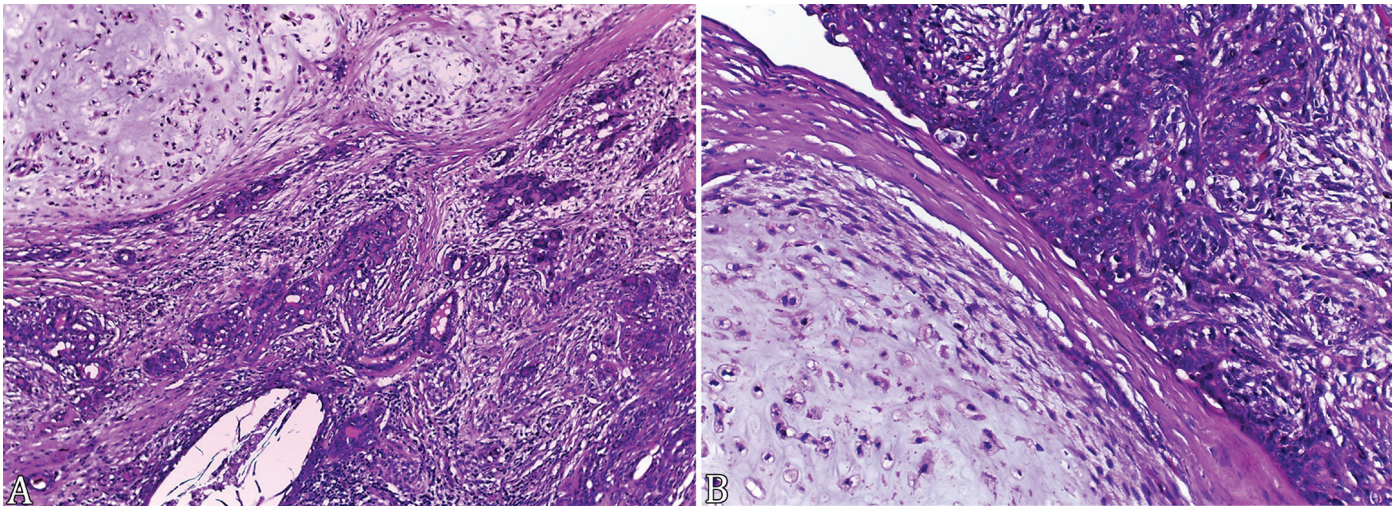


Fig.4. Canine, Dachshund, 5 years old, Carcinoma in mixed tumor with cartilaginous metaplasia. (**A**) Presence of neoplastic epithelial (black arrow) and mesenchymal (red arrow) components. HE, obj.10x obj. (**B**) Detail showing the transition between the area of metaplasia (black arrow) and the carcinoma (red arrow). HE, obj.20x.

Pinscher, Siberian Husky and Collie breeds. Ariyarathna et al. (2018) evaluated 74 female dogs and, of those, 39 were mixed-breed, followed by German Shepherd and Dachshund as the most prevalent breeds. Marconato et al. (2019) studied 32 female dogs and, of those, 21 were pure-breed (Bichon Frisé, Poodle and Golden Retriever) and 11 were mixed-breed. This breed pattern variation is possibly due to regional and country variations. For Sorenmo et at. (2011), mammary tumors in female dogs can occur in any breed.

Among overweight or obese female dogs, the group with mammary neoplasia (G4) was fed less frequently throughout the day when compared to the group without mammary neoplasia (G2). However, no relationship between feeding frequency and tumor onset was found in the literature. It is known that weight management and nutrition should receive attention from Veterinary Physicians and be included in the routine of preventive examinations and care (Bartges et al. 2017). In addition, the etiology of obesity involves genetic, biological, environmental and behavioral interactions, which also predisposes animals to organic dysfunctions (Chandler et al. 2017).

Regarding physical evaluation, Gama et al. (2016) noted that out of 30 dogs evaluated by BCS, 23.3% of the animals were classified as obese, 43.3% as overweight, 23.3% as ideal and 10% as lean. In this study, no lean female dog was observed according to BCS.

When comparing BCS and CBMI evaluations, 42.5% (17/40) of female dogs had the same classification, however, it was observed that three female dogs from G4 received different classifications, and one female dog was classified as lean in CBMI and as obese in BCS and two other female dogs pointed out as ideal in CBMI were also classified as obese in BCS. This is possibly because such female dogs were small, being one mixed-breed and other two of the Pinscher breed. Moreover, CBMI does not consider body proportionality and body fat distribution (Muller et al. 2008, Gama et al. 2016).

Table 7. Results of histological types of mammary nodules of spayed or non-spayed female dogs, over 1 year old, distributed according to histological classification

Histological type (benign)	Amount (total 28 nodules)	%
Adenomyoepithelioma	4	14.29%
* Sclerosing adenoma and Lactation adenoma	1	3.57%
Benign mixed tumor	1	3.57%
Histological type (malignant)	**Amount (Total 28 nodules)**	**%**
Carcinoma in mixed tumor	6	21.43%
Papilliferous carcinoma	3	10.72%
Tubular carcinoma	3	10.72%
Complex carcinoma	2	7.14%
In situ carcinoma	2	7.14%
*Papilloma and intraductal carcinoma	2	7.14%
Sclerosing carcinoma	1	3.57%
*Carcinoma and malignant myoepithelioma	1	3.57%
Carcinosarcoma	1	3.57%
Histological type (other)	**Amount (total 28 nodules)**	**%**
Squamous cell carcinoma	1	3.57%

* Patients with different histological types in the same mammary gland.

According to Muller et al. (2008), of the 246 dogs evaluated, most of them had ideal body condition (53.4%) and 39.5% were overweight to obese. Gama et al. (2016), when evaluating the body condition of dogs using BCS and CBMI, observed that 53.3% of the animals evaluated had the same classification. In this study, BCS and CBMI were not related to the histological type of mammary neoplasia, because both in the group with ideal score and in the overweight to obese group, the malignant histological types were more prevalent.

The hematological parameters did not differ statistically between the groups, however, some of the patients had laboratory changes. In the groups with mammary tumors, two patients had lymphopenia and leukocytosis, one with ideal score and another obese. Four patients had thrombocytosis. Garrido et al. (2015) also did not find significant changes in blood cell counts beyond the reference interval in female dogs, especially those with mammary tumors.

Silva et al. (2014) evaluated 161 female dogs with mammary tumors and found that 24.2% (39/161) of the patients had changes in blood count. They also observed a positive correlation between anemia and the progress of the disease as well as a positive correlation between leukocytosis and the progression of the oncological disease.

For Childress (2012), hematological changes are frequent in oncological patients, which may be a direct result of the tumor or secondary to the paraneoplastic syndrome. However, in this experiment, there were no female dogs with distant metastasis, patients in which most of the hematological changes would probably be observed.

There was no statistical difference in the laboratory evaluation of cholesterol and triglycerides. However, it would be opportune to develop other studies with larger sample sizes, in order to verify if there is any trend of parameter change involving obesity and the presence of mammary neoplasia. Hyperglycemia triggers the activation of TNF-α, a proinflammatory cytokine also present in obese patients, consequently increasing the survival of neoplastic cells in hypoxic conditions (Bail et al. 2016). In the biochemical parameters, there was a statistical difference among groups regarding glucose, and higher values were analyzed in the groups with mammary tumors, especially in G4, in which the patients were also obese.

Pro-inflammatory cytokines increase with inflammation, a process triggered by the tumor itself, favoring the proliferation and survival of tumor cells (Szczubial et al. 2018). In patients with increased glycemic level, no peritumoral inflammatory process was observed macroscopically, however, cytokines stimulate endothelial migration and proliferation since the onset of the tumor in order that it develops and, as this tumor grows, the vascular supply also develops (Carvalho et al. 2016).

The use of contraceptives is associated with cases of benign hyperplasia and neoplasia. Such changes may predispose the mammary tissue to malignant mutations and trigger the onset of malignant neoplasia. The regular use of these drugs increases in three times the risk of carcinoma onset (Ferreira & Amorim 2003, Oliveira Filho et al. 2010, Petrov et al. 2014).

In this study, it was observed that the groups with mammary neoplasia received more contraceptive applications (17.5%), corroborating the literature.

Feliciano et al. (2012) evaluated 60 female dogs, of which 13.37% had received contraceptives. Kamiguchi et al. (2016), when conducting the study with 20 female dogs, noted that 10% of the patients had a history of hormone administration for contraceptive purposes, which could indicate a low correlation with the development of mammary tumors. Ariyarathna et al. (2018) studied 74 female dogs and none of them had used contraceptives to prevent pregnancy. In the evaluation of 134 female dogs performed by Canadas et al. (2018), only 3% (4/134) received contraceptive medication.

In the oncological evaluation of the patients, the mean time of tumor progression until the appointment was 210 days (Ribas et al. 2012). In this study, 55% of the patients had already developed mammary tumors for more than 180 days when brought to the appointment. The guardians of nine animals from the study by Ariyarathna et al. (2018) admitted that, although they had observed mammary nodules in the female dogs before the appointment, they did not seek specific care because they thought the tumors were harmless. The delay in seeking for specialized veterinary assistance is related to the development of malignancy and worsening in the prognosis (Kamiguchi et al. 2016).

Coleto et al. (2018) evaluated 59 female dogs and 56% of them had a single nodule, 27.1% had two nodules, 11.9% had three nodules, 3.4% had four nodules and 11.9% had seven nodules. As for location, the inguinal mammary gland was also the most affected, corresponding to 37.1% of the cases. Medeiros (2017) states that the last pairs of mammary glands are more affected than the first pairs, however, the location of the tumors does not seem to affect the prognosis, results similar to those observed in this study.

It is known that tumor size is variable when it comes to mammary neoplasia. Dias et al. (2016) noticed that almost half of the animals evaluated had tumors smaller than three centimeters (48.95%), 22.38% had tumors between three and five centimeters and 28.67% had tumors larger than five centimeters. This study corroborates that, since 40% (8/20) of the patients had tumor size smaller than three centimeters. Coleto et al. (2018) showed that the size of the tumor influenced the occurrence of metastasis, strengthening the importance of diagnosis and early treatment of mammary tumors.

It is also common for mammary neoplasia to be associated with skin ulcers and local inflammatory reaction, with secretion in mammary chains and involvement of fascia and musculature (Cassali et al. 2014). Peritumoral skin ulcers were observed in 27% of the dogs studied by Ariyarathna et al. (2018) and 20.3% had adherences in the underlying tissues. In this study, these occurrences were lower.

The cytopathological examination is a safe and inexpensive method with satisfactory sensitivity and specificity for malignancy differentiation in mammary tumors, in addition to being considered an ideal method for identification of metastasis in lymph nodes (Cassali et al. 2014, Munkhtuul et al. 2014). In this study, 84.61% of the samples analyzed had adequate cellularity, which corroborated the detection of malignant and/or benign features in cytology, and 90.9% had the diagnosis of tumor behavior compatible with histopathology. Dolka et al. (2018) found correspondence between the two methods in 86.3%, which differed little from the data found in this study.

The final diagnosis of mammary tumors is also achieved by histopathological examination, considered gold standard also to classify histological types (Sorenmo et al. 2011). Malignant neoplasias were more prevalent in this study, as well as in the study by Horta et al. (2014) when evaluating 391 lesions, of which 56% were malignant neoplasias and 31% were benign neoplasias. Dolka et al. (2018) analyzed 18 tumors and, of those, 75.3% were malignant and 24.7% benign. O diagnóstico de tumores malignos e benignos, neste estudo, foi levado em consideração devido ao baixo número amostral, porém considera-se relevante o desenvolvimento de outras pesquisas para avaliar as características de cada tipo histológico.

Horta et al. (2014) pointed out in the study that benign types corresponded to 23.1% as papilloma and 17.4% as adenoma. Dias et al. (2016) considered the benign mixed tumor (16.78%), followed by adenoma (5.59%) and fibroadenoma (0.7%). This study differs from the previous two, in which 14.29% were adenomyoepithelioma, 3.57% benign mixed tumor and 3.57% sclerosing adenoma along with lactating adenoma. Benign mixed tumors are usually characterized by the presence of benign epithelial, myoepithelial and mesenchymal elements, with cartilage and/or bone formation (Cassali et al. 2017). This finding was observed in the female dog with this histological type, which had the two types of bone and cartilaginous metaplasia.

As of malignant tumors, Kamiguchi et al. (2016) found the papilliferous carcinoma histological type in 90% of the samples. Gundim et al. (2016) found tubular carcinoma, followed by carcinoma in a mixed tumor. Canadas et al. (2018) found that, of 144 tumors, the most frequent type was complex carcinoma (49/144). For Nunes et al. (2019), 75% (682/904) of the cases were carcinoma in a mixed tumor, which corroborates this study. In addition, other histological types were found, however in lesser number when compared to carcinoma in a mixed tumor.

Carcinoma in mixed tumor is the most common neoplasia of the mammary gland in female dogs with complex histological pattern, as it has components of epithelial, myoepithelial and mesenchymal origin (Cassali et al. 2014, Nunes et al. 2019). Microscopically, carcinomas in mixed tumor develop through a malignant epithelial component and a benign mesenchymal component, which may be of cartilaginous, bony or adipose tissue (Goldschmidt et al. 2011, Cassali et al. 2017). Greater cellularity and considerable pleomorphism of the epithelial part, with high mitotic index, necrotic points and infiltrative pattern should be observed to differentiate this type from mixed benign tumors (Goldschmidt et al. 2011). In this study, one of the patients with carcinoma in mixed tumor developed metaplasia in cartilage.

The two patients had inguinal lymph node metastasis confirmed by histopathological examination, one from G4 with squamous cell carcinoma and the other from G3 with papillary carcinoma. This finding influences clinical staging, conferring worse prognosis of patients, since patients with metastases in regional lymph nodes have decreased survival (Santos et al. 2015, Medeiros, 2017).

Moreover, in the patient in which squamous cell carcinoma was found, a connection with the cutaneous epidermis was

confirmed. There is a special type of squamous cell carcinoma that affects the mammary glands, which is histologically similar to the one that occurs in the skin, however there is no connection with the cutaneous epidermis. The neoplasia that affects the mammary gland is uncommon in the canine species, being usually infiltrative and disseminated by lymphatic route (Goldschmidt et al. 2011, Cassali et al. 2014). In this case, we noticed the importance of histopathological diagnosis, because the neoplasia, by macroscopic evaluation, consisted of a mass with involvement of the left inguinal mammary gland, with firm consistency, irregular surface and mobility.

In this study, one patient from G3 had the carcinosarcoma histological type. It is a mixed neoplasia with epithelial and mesenchymal malignant components, uncommon for mammary gland, with poor prognosis, since the metastasis of the epithelial component occurs by lymphatic route to regional lymph nodes and the lung, and the metastasis of the mesenchymal component occurs by hematogenous route to the lung (Goldschmidt et al. 2011, Cassali et al. 2017). The patient had already had the tumor for three years and the guardian reported slow growth. No presence of neoplastic cells was observed in the histological evaluation of the inguinal lymph node.

CONCLUSIONS

Obesity did not trigger clinical and laboratory changes in female dogs with mammary cancer.

However, it was observed that the female dogs in the overweight to obese groups had a higher number of changes in hematological and biochemical parameters, especially the female dogs of the group that also had mammary tumors, observing thrombocytosis, lymphopenia, leukocytosis, leukopenia and hyperglycemia, even at 12-hour fasting.

BCS and CBMI were not related to the malignancy of the mammary tumor, since, both in the group of overweight and obese female dogs and in the group of lean to ideal female dogs, the most observed histological types were those with malignant features.

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

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Development of a lumbosacral intervertebral cage prototype for dogs[1]

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ABSTRACT.- Dias R.C., Tsatsakis A.C. & Bahr Arias M.V. 2020. **Development of a lumbosacral intervertebral cage prototype for dogs.** *Pesquisa Veterinária Brasileira 40(7):546-553.* Departamento de Clínicas Veterinárias, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid Km 380, Londrina, PR 86057-970, Brazil. E-mail: vicky@uel.br

Several surgical procedures aim to decompress and/or stabilize the lumbosacral (LS) joint of dogs; however, the lumbar interbody fusion technique, by using a cage combined with a bone graft, is the most indicated and used in human medicine. No specific implant is available for application to the canine lumbosacral joint. Thus, this study measured lumbosacral discs in large dogs, determined whether a human cage model could fit the dogs' L7-S1 intervertebral space, and developed a LS cage prototype for dogs. Ten cadaveric lumbosacral spines from adult dog weighing 20-35kg were used. The dogs had died for reasons unrelated to this study. The vertebral body dimensions and the L7-S1 intervertebral space occupied by the intervertebral disc were measured by lateral and ventrodorsal radiographs and by computed tomography in the dorsal, sagittal, and transverse views. Measurements were also taken of the anatomical specimens in the sagittal and transverse planes. After measuring the intervertebral discs, the following mean measures were obtained for L7-S1 discs: height 12.23mm, dorsal thickness 3.3mm, central thickness 4mm, ventral thickness 5.5mm, and width 24.74mm. The human lumbar cage models from brands LDR, Baumer Orthopedics, Stryker, Synthes, and Vertebral Technologies, Inc. and cervical stabilization cages from the brands B-Braun and Stryker were evaluated and were found to be unsuitable for large dogs. Cervical human cages had measurements similar to those found in this study; however, due to their quadrangular shape, the possibility of being introduced surgically through the surgical accesses available for the articulation between L7-S1 in dogs without injuring the cauda equina or the L7 root is small. A cage model was then developed using 3D modelling software. It was designed for insertion via dorsal laminectomy in the lateral portions of the intervertebral space. To avoid cauda equina lesion, the implant model was developed to be placed laterally to the midline. The cage surface is serrated to prevent using the locking screw to fix it, thus avoiding further injury to nerve structures. The serrated surfaces are also designed to avoid cage migration and promote stability. The prototype allows graft placement in the surrounding intervertebral space, which is fundamental for fusion through integration between the cage and the endplates as well as for bone growth between and around the cage. It was also considered studies on humans showing that the lateral regions of the endplates support a more considerable load. Biomechanical and *in vivo* studies on the developed model are necessary to evaluate the actual degree of distraction, mobility and the long-term rate of fusion between L7 and S1 and its possible impact on the adjacent motor units, combined or not with dorsal fixation techniques.

INDEX TERMS: Lumbosacral, intervertebral cage, prototype, degenerative diseases, surgery, dogs.

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RESUMO.- [**Desenvolvimento de um protótipo de cage intervertebral lombossacro para cães**.] Vários procedimentos cirúrgicos visam descomprimir e/ou estabilizar a articulação lombossacra (LS) de cães; no entanto, a técnica de fusão lombar, usando um *cage* intersomático combinado com um enxerto ósseo, é a mais indicada e utilizada na medicina humana. Não há implante específico disponível para aplicação

na articulação lombossacra canina. Assim, neste estudo foi realizada a mensuração do espaço do disco intervertebral lombossacro de cães de raças grandes, para verificar se algum modelo de *cage* usado na medicina humana poderia ser usado no espaço intervertebral L7-S1 de cães. O segundo objetivo foi desenvolver um protótipo de *cage* lombossacro para cães. Foram utilizadas dez colunas lombossacras provenientes de cadáveres de cães adultos com peso entre 20 e 35kg. Os cães vieram a óbito por razões não relacionadas a este estudo. As dimensões do corpo vertebral e o espaço intervertebral L7-S1 ocupado pelo disco intervertebral foram medidos por radiografias laterais e ventrodorsais e por tomografia computadorizada nos cortes dorsal, sagital e transversal. Também foram realizadas mensurações das peças anatômicas nos planos sagital e transversal. Após a mensuração dos discos intervertebrais, foram obtidas as seguintes medidas médias dos discos L7-S1: altura 12,23mm, espessura dorsal 3,3mm, espessura central 4mm, espessura ventral 5,5mm e largura 24,74mm. Os modelos de *cage* lombar humano das marcas LDR, Baumer Orthopaedics, Stryker, Synthes e Vertebral Technologies, Inc. não possuíam dimensões adequadas para os cães. C*ages* de estabilização cervical das marcas B-Braun e Stryker também foram avaliados e apresentaram medidas semelhantes às encontradas neste estudo; no entanto, devido à sua forma quadrangular, a possibilidade de serem introduzidos cirurgicamente através das abordagens disponíveis para a articulação entre L7-S1 em cães sem lesionar a cauda equina ou a raiz L7 é pequena. Um modelo de *cage* foi então desenvolvido usando-se o *software* de modelagem 3D. Foi projetado para inserção via laminectomia dorsal nas porções laterais do espaço intervertebral. Para evitar a lesão da cauda equina, o modelo de implante foi desenvolvido para ser colocado lateralmente à linha média. A superfície do *cage* é serrilhada para evitar o uso do parafuso de travamento, evitando-se lesões adicionais às estruturas nervosas. As superfícies serrilhadas também foram projetadas para evitar a migração do *cage* e promover estabilidade. O protótipo permite a colocação do enxerto no espaço intervertebral circundante, fundamental para a fusão através da integração entre o *cage* e as placas vertebrais terminais, bem como para o crescimento ósseo entre e ao redor do implante. Também foram considerados estudos em seres humanos que mostraram que as regiões laterais das placas vertebrais terminais suportam uma carga maior. São necessários estudos biomecânicos e *in vivo* do modelo desenvolvido para avaliar o grau real de distração, mobilidade e a taxa de fusão a longo prazo entre L7 e S1 e seu possível impacto nas unidades motoras adjacentes, quando combinado ou não com técnicas de fixação dorsal.

TERMOS DE INDEXAÇÃO: Lombossacro, dispositivo intersomático intervertebral, protótipo, doenças degenerativas, cirurgia, cão, caninos.

INTRODUCTION

The canine lumbosacral junction (L7-S1) is a joint with great mobility that can be affected by several conditions. Among these, degenerative lumbosacral stenosis (DLS) is the most common cause of canine lumbosacral disease. A combination of anatomical and biomechanical changes in the joint can cause compression of the cauda equina and nerve roots (Meij & Bergknut 2010, Jeffery et al. 2013). DLS shares some similarities with lumbar degenerative spine disease (LDSD), as they both affect the cauda equina and can cause lameness, lower back pain, and neurological deficits (Jeffery et al. 2014, Bebchuck 2017). Studies on LDSD may contribute to better understanding DLS, and dogs can be used as natural models for studying the disease in humans (Meij & Bergknut 2010).

The main surgical techniques described for dogs with DLS are dorsal decompressive laminectomy, combined or not with disc fenestration, lateral foraminotomy, dorsal fixation, and in some cases facetectomy, which promotes decompression of the L7 nerve root (Gomes et al. 2018). However, there are variable results with these surgical techniques, as they do not eliminate all causes of compression and do not promote fusion (Jeffery et al. 2014, Bebchuck 2017).

Surgical procedures to promote lumbar interbody fusion are well-established treatments in human medicine. Among the several described techniques, posterior lumbar fusion using cages or lumbar spacers combined with autologous cancellous bone graft placement is a surgery with the best biomechanical results (Kim et al. 2012). These cages were developed to restore standard disc height, distract the neuroforamina and increase biomechanical rigidity, thus reducing the pedicle fixation technique failures observed in the spinal stabilization technique in humans (Polly et al. 2000, Cho et al. 2008).

In veterinary medicine, few studies exist about cage implantation between the L7 and S1 vertebrae. Recently, the Fitz intervertebral traction screw (FITS), a conical and threaded titanium spacer screw, was developed for use in several intervertebral spaces (Solano et al. 2015). In dogs with DLS, combining this screw with a stabilization technique using pins and bone cement allowed indirect decompression of the L7 foramen and long-term vertebral stability (Farrel & Fitzpatrick 2015). In cadaveric canine lumbosacral spines, a polyaxial screw-clamp fixation system was used in combination with an intervertebral distraction bolt demonstrating that the new implant system restores stability to the lumbosacral junction following destabilization (Zindl et al. 2018). In an *ex vivo* study, a cage used in human medicine to promote cervical intervertebral fusion was inserted into the lumbosacral joint of nine canine lumbosacral segments (Teunissen et al. 2017). The biomechanical properties were assessed with the cage alone and combined with pedicle screw-rod fixation (PSRF), resulting in increased disc height, reduced range of motion, and improved joint stabilization. The authors concluded that cage insertion is an alternative to dorsal fixation; however, the cage was more easily inserted into the vertebral segments of larger dogs, emphasizing the need to develop a specific intervertebral device for dogs (Teunissen et al. 2017).

Due to the limited studies evaluating intervertebral spacers for dogs, this study performed an anatomical, radiographic and tomographic evaluation of the lumbosacral joint in large dogs, with and without radiographic or tomographic signs of DLS, to determine whether a human cage model had adequate dimensions for this joint and to develop a cage prototype for dogs.

MATERIALS AND METHODS

Ethics statement. All procedures were approved by the Institutional Ethics Committee on Animal Use, under protocol number 9360.2016.64.

Phase one. Ten lumbosacral vertebral segments were collected from adult dog cadavers with and without radiographic and tomographic signs of DLS, with weights ranging from 20 to 35kg (median 27.95) and having died due to conditions unrelated to this study. Spines were collected between the L7 and the sacrum with the surrounding musculature and pelvis to maintain the lumbosacral and sacroiliac joints intact. Each specimen was wrapped in compresses moistened with saline, placed in a plastic bag, stored at -20°C, and then kept in a refrigerator at 4°C for 24 hours to perform the procedures described below. The spines were radiographed in the laterolateral (LL) and ventrodorsal (VD) projections to exclude any specimens with anatomical abnormalities, including fractures, neoplasms, or other congenital malformations. Specimens with degenerative changes compatible with DLS were used in the study. The spines were then subjected to helical computed tomography (CT), with 150 Ma and 120 kV, in 3mm slices in the bone window, using a GE HiSpeed Fxi CT scanner.

The specimens were divided into two groups to measure the vertebral bodies dimensions and intervertebral disc (IVD). Group I was represented by five lumbosacral spines sectioned in the sagittal plane to measure the height of the space occupied by the intervertebral disc and the L7-S1 disc thickness at three levels. Group II was represented by the other spines, which were sectioned on the transverse axis at the level of the intervertebral space, to evaluate the width and height of the L7-S1 disc. The sections were made with a medical electrical saw drill (BJJ-l/Bojin®) with a blade 90mm long and 0.8mm thick. All specimen measurements were made with a calliper.

To compare whether the radiographic and tomographic measurements were similar to those measured directly on the anatomical specimens, four measurements were made in a sagittal section, as follows: T1, thickness of the dorsal portion of the intervertebral disc; T2, thickness of the central portion of the disc; T3, thickness of the ventral portion of the disc; and DH, the disc height at its centre. These measurements were then compared with those from the radiographs and CT scans (Fig.1).

In all transverse specimens, the greatest intervertebral disc width was measured at its central point (W), and the heights of the vertebral endplates of the L7 vertebral body were measured at the central point (H2) and laterally (H1 and H3). The same measurements were performed on the CT scans (Fig.2). The intervertebral space between L7 and S1 on all CT scans was measured at three levels

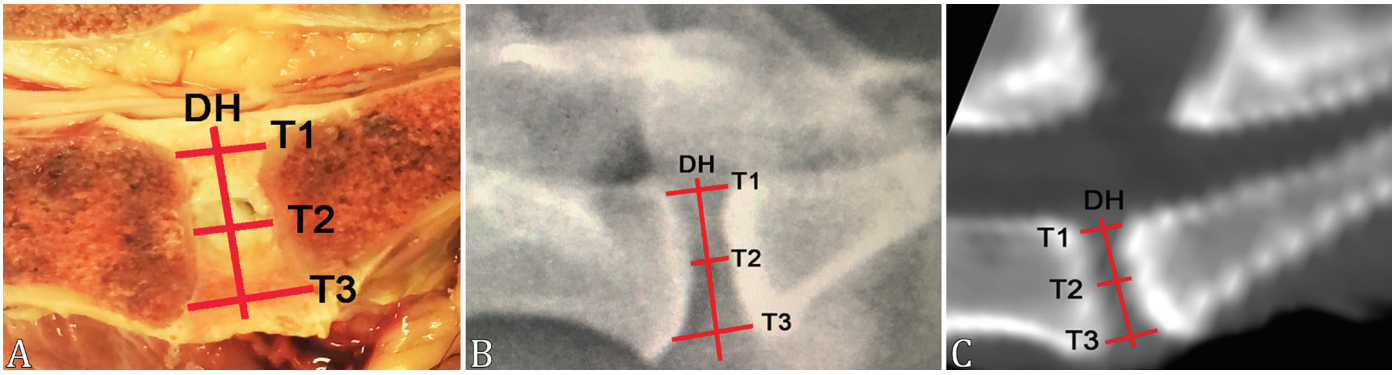


Fig.1. L7-S1 intervertebral disc measurements of the canine lumbosacral vertebrae in the sagittal section (Group I). (**A**) Anatomical specimen, (**B**) radiography , (**C**) computed tomography. Disc dorsal thickness (T1), disc medial thickness (T2), disc ventral thickness (T3), disc height (DH).

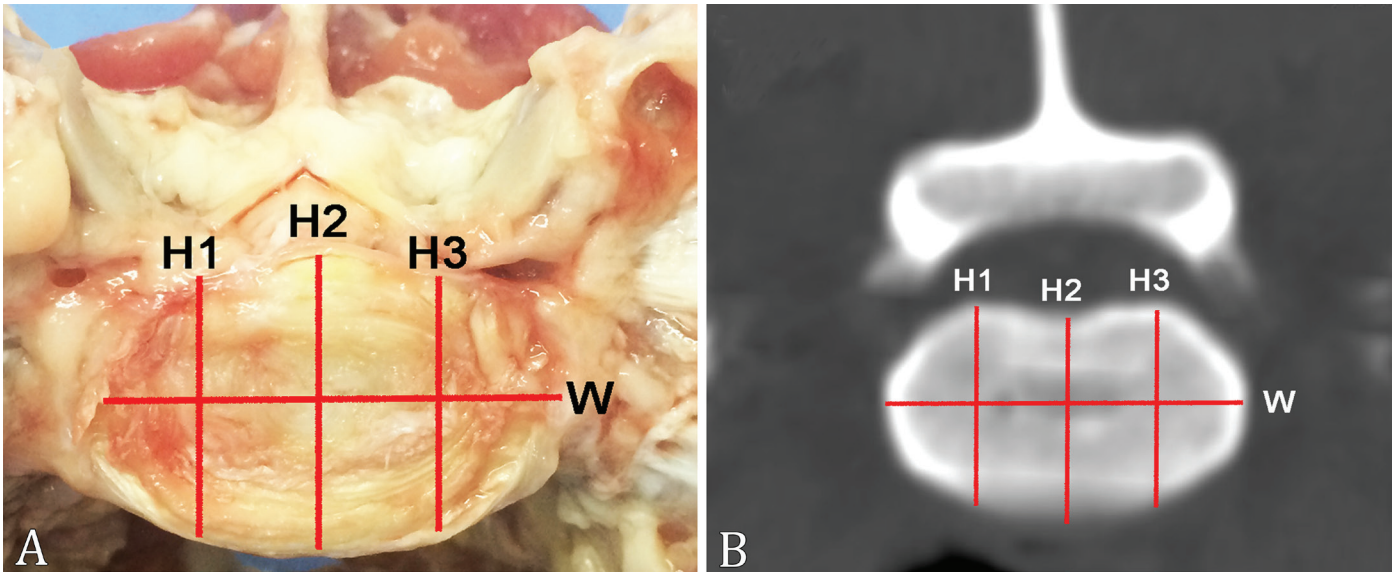


Fig.2. L7-S1 intervertebral disc measurements of the canine lumbosacral vertebrae in the transverse section. (**A**) Anatomical specimen, (**B**) Tomography. Height lateral measurements (H1 and H3), height central measurement (H1 and H3), width (W).

and two lateral points to define the prosthesis thickness: V1, the lateral thickness of the intervertebral space in its dorsal portion; V2, the lateral thickness of the intervertebral space in its central portion; and V3, the lateral thickness of the intervertebral space in its ventral portion (Fig.3). Disc thickness was also measured at two lateral points in the ventrodorsal plane by radiography and CT scan.

Phase two. After obtaining the disc thickness, height and width, the dimensions from cages used in humans for intervertebral fusion were evaluated to verify compatibility with the measurements obtained for the specimens. Cages used in the lumbosacral region were evaluated for the brands LDR, Baumer Orthopedics, Stryker, Synthes, and Vertebral Technologies, Inc. In addition, cervical stabilization cages from the brands B-Braun and Stryker were also evaluated.

Phase three: prosthesis development. A prosthesis model was developed using 3D MAX 2015, modelling software used for product design, based on width, thickness, height and the analysed geometries.

Statistical analysis. Measurements of the anatomical specimens' sagittal sections were compared with their respective radiographs and CT scans for the ten specimens, independently of the section plan group. Next, the measurements obtained from the anatomical specimens, CT scans and radiographs were compared within groups I (sagittal section) and II (transversal section). The data were analysed for normality using the arithmetic mean and simple standard deviation. The parametric t-test was used to compare measurements between the two groups. Finally, the parametric Tukey test was used for comparing three groups, and the nonparametric Mann-Whitney test was used for analysis between two groups.

RESULTS

Phase one. Breed, age, weight, and sex data on the dogs used in the study are presented in Table 1. Mean cadaver weight was 27.05kg and mean age was 6.9 years, with the same proportion of males and females. The following mean measures were obtained for L7-S1 discs: height 12.23mm, dorsal thickness 3.3mm, central thickness 4mm, ventral thickness 5.5mm, and width 24.74mm (Table 2 and 3). Radiographic and tomographic signs of degeneration were found in the lumbosacral joints of four dogs (Table 1), two males and two females. These changes included spondylosis deformans, vertebral end plate sclerosis and intervertebral foramen stenosis. None of these dogs presented clinical signs of DLS or any other condition associated with the spine or spinal cord in segments other than L7-S1 in the *ante mortem* period, according to the history reported by the owners and previously performed clinical examination.

The mean measurements of the L7-S1 disc are shown in Tables 2 and 3. In comparing the measurements of the anatomical specimens in sagittal section with the radiographs (radiography x specimen) and the CT scans (CT scan x specimen), no significant differences were observed ($p>0.05$) for the T1, T2 and T3 measurements. Further, no significant difference ($p>0.05$) was found for the height of the disc (DH) ($p=0.906$), nor were any significant differences found ($p>0.05$) in comparing the H1, H2, H3 measurements and W transverse sections (measured only in the anatomical specimens and on the CT scans due to the impossibility of obtaining transverse radiographs). The thickness measurements obtained by radiographs (V1) in the ventrodorsal plane and the CT scans

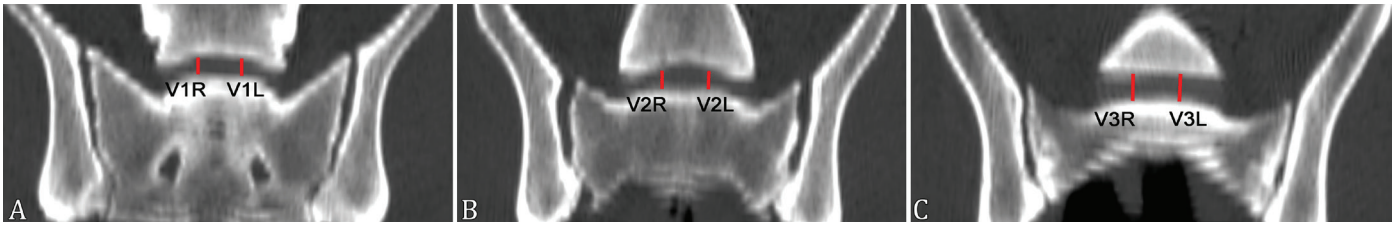


Fig.3. L7-S1 intervertebral disc measurements of canine lumbosacral specimens performed on computed tomography in the dorsal view. (**A**) Thickness of the lateral portion of the intervertebral space in the dorsal portion, (**B**) thickness of the lateral portion of the intervertebral space in the medial portion, (**C**) thickness of the lateral portion of the intervertebral space in the ventral portion.

Table 1. Epidemiological aspects of dogs used in the study, including breed, weight, sex, age, and presence or absence of degenerative signs between L7 and S1. Group I represented by spines sectioned in the sagittal plane and Group II represented by spines sectioned on the transverse axis

Group	Breed	Weight (kg)	Sex	Age (years)	Presence of degenerative signs between L7 and S1
G I	Undefined	20	Male	Adult	Yes
G II	Akita	25	Female	13	Yes
G I	Rottweiler	31.8	Female	Adult	No
G II	German Shepherd	28	Male	9	No
G I	Labrador Retriever	26.3	Female	7	Yes
G I	Labrador Retriever	29.8	Male	11	No
G II	Pitbull	31.7	Female	9	Yes
G II	Golden Retriever	30	Female	10	No
G I	Bull Terrier	27.9	Male	Adult	No
G II	Sharpei	20	Male	10	No
Mean		27.05		6.9	

Table 2. Mean and standard deviation of the lumbosacral intervertebral disc measurements obtained from anatomical specimens, radiographs, and computed tomography in the transverse and sagittal section, from Groups I and II

Measurement site	Mean and standard deviation (mm)			
	Sagittal		Transverse	
Anatomical specimens	T1	6.312 ± 1.11	H1	12.18 (10.3 - 13) *
	T2	6.218 ± 1.135	H2	13.29 ± 1.384
	T3	6.853 ± 1.131	H3	12.178 ± 1.674
	DH	13.992 ± 1.704	W	24.73 ± 6.251
Radiography	T1	7.28 ± 0.879	-	-
	T2	7.28 ± 0.719	-	-
	T3	7.68 ± 1.308	-	-
	DH	14.3 ± 1.219	-	-
Computed tomography	T1	4.92 ± 1.604	H1	11.8 (5.4 - 15) *
	T2	5.61 ± 1.758	H2	12.64 ± 1.662
	T3	5.69 ± 1.832	H3	12.6 ± 1.710
	DH	14.4 ± 1.557	W	24.74 ± 3.177

* Median values (minimum value - maximum value obtained), nonparametric statistical test; T1 = thickness dorsal portion IVD, T2 = thickness central portion IVD, T3 = thickness ventral portion IVD, DH = IVD height, W = IVD width, H = heights at central point (H2) and laterally (H1 and H3).

Table 3. Mean and standard deviation of the lumbosacral intervertebral disc measurements obtained from radiographs and computed tomography in the transverse section or ventrodorsal view, from Groups I and II

		Mean and standard deviation (mm)	p values
Radiography	V1R	2.36 ± 1.177	
	V1L	1.9 (1.1 - 5.2) *	
	V2R	-	
	V2L	-	
	V3R	-	
	V3L	-	V1R 0.007
Tomography	V1R	4.385 ± 1.743	V1L 0.003
	V1L	3.83 (2.31 - 7.11) *	
	V2R	3.976 ± 1.137	
	V2L	3.997 ± 1.479	
	V3R	5.323 ± 1.702	
	V3L	5.592 ± 2.415	

* Median values (minimum value - maximum value obtained), nonparametric statistical test; V = disc thickness, V1 = dorsal portion, V2 = medial portion, V3 = ventral portion, R = right, L= left.

(V1) in dorsal view were significantly different ($p<0.05$) (Table 3). For each measure in both groups, it was compared the specimens with and without L7-S1 degenerative signs (Table 4), and no significant differences were found between the measurements ($p>0.05$).

Phase two. The Striker, Baumer Orthopedics, and LDR cages used in humans had higher, thicker and wider values than those of the anatomical specimens. The Synthes and Vertebral Technologies, Inc. models had thickness values near those of the specimens; however, the width and height values were higher. Thus, no specific lumbosacral cage model for human use fit the measurements obtained for the large dogs in this study. The cervical cages evaluated had measurements near those of the specimens, but their shapes made it impossible to implant them using the surgical dorsal laminectomy technique without injuring the cauda equina or the L7 nerve root.

Phase three. A model (Fig.4) was designed and shaped per the obtained measurements, considering the anatomical characteristics of the dogs' L7-S1 intervertebral space and presence of the cauda equina. It was followed the procedure recommended in human medicine for using two implants, one on each side of the intervertebral space (Fig.5).

DISCUSSION

Surgeries in the lumbosacral region in dogs are unsuccessful in up to 30% of cases, and the reasons may be the weak vertebral stability promoted by existing surgical techniques after dorsal laminectomy, lack of neuroforaminal decompression and an

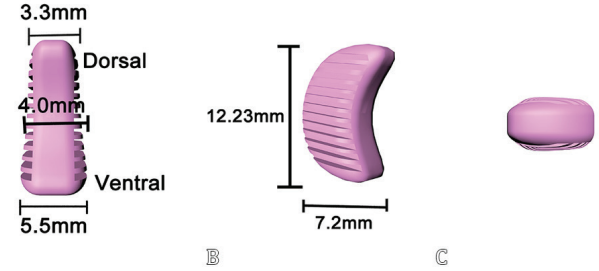


Fig.4. Intervertebral lumbosacral cage model developed for dogs. Perspective and measurements of the cage. (**A**) Medial view with dorsal, medial and ventral thickness, (**B**) caudal view with height and length, (**C**) dorsal view.

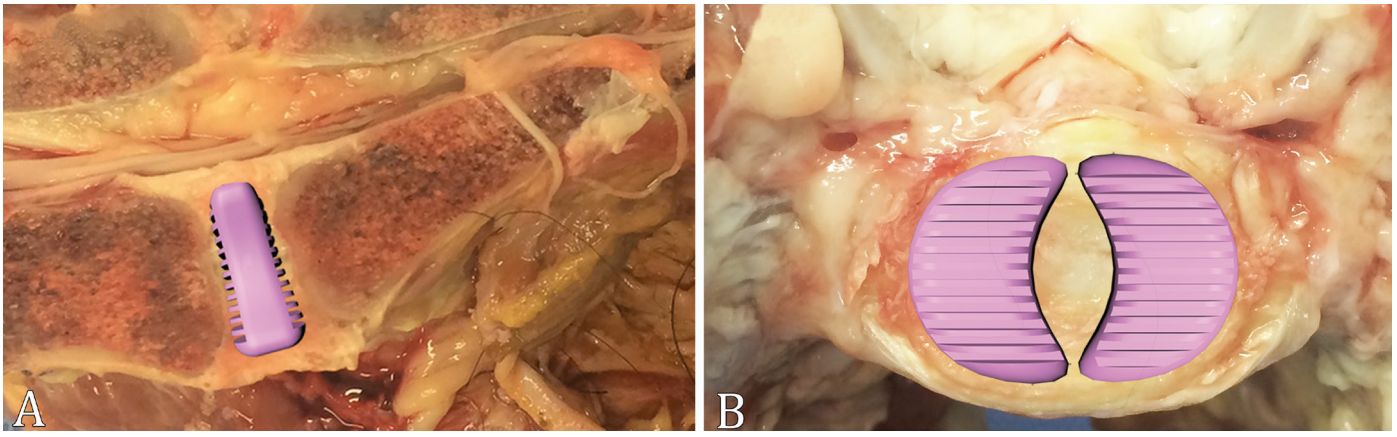


Fig.5. Positioning of the canine intervertebral cage model developed in the intervertebral disc space. (**A**) Sagittal view, (**B**) caudal view.

absence of intervertebral bony fusion. In a cadaveric study it was obtained satisfactory biomechanical results and restauration of the intervertebral disc height after implanting a cervical intervertebral cage used in humans between the L7-S1 vertebrae in dogs, but the authors reported difficulty in inserting the cage in smaller dogs (Teunissen et al. 2017), evidencing the need to develop adequately sized lumbosacral cages for different sized dogs to promote vertebral distraction/fusion.

In this study, the canine lumbosacral spine of large breed dogs was evaluated because they are more affected by DLS. Changes compatible with lumbosacral degeneration were found in four dogs, which apparently caused no clinical signs of DLS. These changes may occur in animals with no clinical evidence of the disease and changes in imaging tests had little correlation with clinical findings of DLS (Rossi et al. 2004, Mattoon & Koblik 2013). Thus, it was chosen to use the specimens from these dogs because the cage to be developed should also adapt to the disc space of dogs with lumbosacral degeneration. The degenerative changes did not alter the articulation between L7 and S1 to the point of interfering in obtaining the measurements.

The intervertebral space was measured directly on anatomical specimens, which were then analysed using radiography and CT. The anatomical specimens could not be used simultaneously for transverse and sagittal sections; thus, the same number of specimens could not be compared by the two imaging techniques. Because radiographic examination is the most common method for surgical planning in orthopaedics, its contribution to this study was evaluated. Obtaining transverse planes using radiography is impossible; thus, its use was limited to developing the cage model. The radiographic measurements were unreliable, probably due to the bone overlap in the ventrodorsal projection, while CT allowed several views and measurements. CT is a non-invasive method for visualizing tissues, without overlapping structures. It is considered superior to radiography for detecting DLS (Jones & Inzana 2000, Axlund & Hudson 2003, Higgins et al. 2011), and it allowed the specimens in several views to be accurately measured.

Cages specific for the human lumbar region are larger than that measured here, especially for the model developed to be implanted dorsolaterally. Human cages used for cervical stabilization had measurements similar to the model found in this study; however, due to their quadrangular shape, the possibility of being introduced surgically through L7-S1 dorsal laminectomy in dogs, without injuring the cauda equina or the L7 root is small. The smallest available cage for the human cervical spine was inserted into the canine lumbosacral spine in a cadaveric study, but the authors reported difficulties surgically implanting it in smaller dogs (Teunissen et al. 2017).

The intervertebral screw developed for dogs (FITS) provided adequate spacing between the L7 and S1 endplates and a satisfactory increase of the intervertebral foramen when inserted via dorsal laminectomy and central annulectomy (Farrel & Fitzpatrick 2015, Fitzpatrick 2017, Zindl et al. 2018). However, the cauda equina had to be retracted to insert and fix the spacer with a screw, which is a risky intervention and caused temporary tail flaccidity in 11.1% of dogs, that resolved within six weeks (Fitzpatrick 2017). In the present study, the implant models were also developed to be inserted via dorsal laminectomy but placed laterally to the midline, and this reduces the likelihood of nerve root damage. The cage surface was developed to be serrated, to prevent using the locking screw to fix it, thus avoiding further injury to nerve structures. To develop our model, we also considered studies on humans, that showed that the lateral regions of the endplates support a more considerable load, and that the center of the bone, where implants are currently placed, is the weakest part of the lumbar endplate (Grant et al. 2001). Unfortunately, similar studies have not been performed in dogs.

Our cage was designed to allow graft placement in the surrounding intervertebral space, which is fundamental for fusion through integration between the cage and the endplates as well as for bone growth between and around the cage (Cho et al. 2008, Abbushi et al. 2009). The serrated surfaces are also designed to avoid cage migration and promote stability (Cho et al. 2008). Combining cage use with pedicled dorsal stabilization may increase the contact between the endplates and the serrated surface. In dogs, pedicle screw-rod fixation alone, without the use of a cage, does not result in interbody vertebral bone fusion between L7 and S1 (Tellegen et al. 2015). On the other side, when FITS is placed together with PSRF in dogs, the vertebrae are distracted first, the screw is then implanted, the distraction is decreased, and the device is compressed, increasing the chances of bone growth and intervertebral fusion. This was seen by means of computed tomography in 35 dogs submitted to PSRF, and it was observed that with the placement of the interbody spacer the neuroforamen was restored and it was provided a conduit for fibro-osseous fusion of the interbody space (Fitzpatrick 2017).

Some studies in humans have shown the importance of cage size and contact area to avoid cage migration (Krammer et al. 2001, Tan et al. 2005). Larger cages tend to have larger contact surface areas at the endplate/cage interface and thus lower stress concentration at a single point. We considered these factors in our study; thus, the developed models followed the increased intervertebral space in the dorsoventral direction, increasing their contact with the L7 and S1 bone surfaces. The threaded intervertebral bolt developed by Fitzbionics Ltd® is 19mm long, with a diameter of 7.5mm proximally and 4.4mm distally and was tested in cadaveric canine lumbosacral spines of dogs weighing between 25.0-39.5kg, but the bolt format and thickness is greater in the dorsal part of the disc, and smaller in the ventral part (Zindl et al. 2018), which is inverse to the measurements obtained in our study. Further studies should be performed to verify if the screw configurations in relation to the disk measurements can affect the biomechanics of the intervertebral space after surgical stabilization with this kind of implant system.

The prosthesis design was also based on conceptions in human medicine. We considered the adequacy to the anatomy and dimensions of the canine lumbosacral region, the load that the cages must bear, the material for implant manufacturing, its positioning within the intervertebral space, and the apparatus biomechanics to prevent cage migration (Spruit et al. 2005, Cutler et al. 2006). However, it was observed in human experimental studies that cage geometry, including the shape (curved or straight), length, and surface (biconvex or flat), did not affect the implants' stability when used in combination with posterior fixation (Cho et al. 2008).

Table 4. Measurements (mm) obtained via computed tomography, radiography, and at the anatomical specimens from all dogs. Group I represented by spines sectioned in the sagittal plane and Group II represented by spines sectioned on the transverse axis

	Tomography													Radiography						Anatomical specimens					
	DH	W	T1	T2	T3	H1	H2	H3	V1		V2		V3		DH	T1	T2	T3	V1		DH	W	T1	T2	T3	H1	H2	H3
									R	L	R	L	R	L					D	E								
G II	16	29	5.31	8.42	6.49	15	14.8	15.2	4.54	4.54	5.07	4.94	7.21	6.81	18.6	7.7	8.4	7.5	5.4	5.2	-	30				13	14.82	14.51
G I	15.2	29.9	7.37	8.18	8.18	14.9	14.8	15.20	7.6	7.11	5.98	6.79	6.63	8.89	15.5	7.6	8.2	9.6	2.2	1.6	15.93	-	7	7.2	7.9	-	-	-
G II	12.7	23.4	3.42	4.57	5.32	11.5	11.9	12.3	3.86	3.86	4.54	4.46	3.58	3.86	11.6	6.1	5.3	6.3	1.6	1.5	-	23.39	-	-	-	10.3	11.2	10.27
G II	13	25.2	2.95	3.23	3.89	11.8	14	12.5	3.17	3.17	2.48	2.34	4.27	4.13	12.9	6.6	6.2	6.3	2.8	3	-	24.3	-	-	-	10.48	12.83	11.12
G I	12.5	23.5	3.04	3.46	3.25	12.9	11.7	12	2.69	2.83	2.97	2.83	3.68	2.97	12.7	5.8	6.2	6	2.2	2	11.44	-	5	5.1	5.4	-	-	-
G I	16.6	31.2	5.33	4.63	6.31	15.4	14.4	14.8	5.99	5.79	4.48	4.15	5.31	5.81	15.5	7.2	7.2	7.6	2.4	2	14.96	-	7	7	7.6	-	-	-
G II*	12.6	25.6	2.54	5.08	4.23	12.2	11.5	12.6	1.93	2.31	2.7	2.31	3.81	3.4	15.4	6	6.5	6.5	2	1.8	-	25.22	-	-	-	12.18	14.13	11.86
G II*	16.2	20.5	2.9	4.39	4.57	11.2	11	10.4	3.65	3.78	4.04	3.97	8.49	8.89	16.1	4.8	7.1	8.5	2.4	2.3	-	30.74	-	-	-	12.28	13.47	13.13
G I*	13.8	28.7	4.66	5.81	4.78	11.8	13.8	12.7	4.25	2.89	3.09	2.71	4.24	3.09	13.6	8	7.3	7.2	1.2	1.1	14.23	-	5	4.9	5.9	-	-	-
G I*	13.9	25.8	4.2	5.97	5.93	12.5	12.4	11.6	6.17	6.94	4.41	5.47	6.01	8.07	14.2	7.8	7.5	8	1.4	1.8	13.4	-	8	6.9	7.5	-	-	-

T1 = Thickness dorsal portion IVD, T2 = thickness central portion IVD, T3 = thickness ventral portion IVD, DH = IVD height, W = IVD width, H = heights vertebral at central point (H2) and laterally (H1 and H3), V = disc thickness, V1 = dorsal portion, V2 = medial portion, V3 = ventral portion, R = right, L = left.

The 3DS MAX 2015 software allowed designing two cage models according to the contour of the bone surfaces and the intervertebral space size. In the future, these cage models can be adapted and reshaped to better fit the L7-S1 segment, since 3D technology is widely used in human medicine and is promising in veterinary medicine for facilitating surgical planning and improving research (Hespel et al. 2014, Joffe et al. 2019). To measure disc space, radiography should be used with caution, as CT better delineates the contact areas between the endplate and the cage, with more reliable measurements.

CONCLUSIONS

Computed tomography (CT) was superior to radiography for determining the intervertebral space measurements used to develop the implants.

Human lumbosacral cages did not fit the average measurements of the L7-S1 space; thus, no models were found for the lumbosacral region that could be used in dogs.

We developed a cage prototype for dogs via 3D modelling that could be applied dorsolaterally to the L7 intervertebral space to promote lumbosacral intervertebral fusion/distraction, considering the intervertebral anatomy of the species and the CT measurements of the disc space. The degenerative changes observed in four spines did not alter the articulation between L7 and S1 to the point of interfering in obtaining the measurements.

Ex vivo and *in vivo* studies with the developed cage models are necessary to evaluate the actual degree of distraction, mobility and the long-term rate of fusion between L7 and S1 and its possible impact on the adjacent motor units, combined or not with dorsal fixation techniques.

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Bilateral pyelonephritis due to *Escherichia coli* infection in a captive jaguar (*Panthera onca*)[1]

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ABSTRACT.- Wronski J.G., Argenta F.F., Raiter J., Ehlers L.P., Sala R.D.V., Siqueira F.M., Cardoso D.F., Sonne L. & Pavarini S.P. 2020. **Bilateral pyelonephritis due to *Escherichia coli* infection in a captive jaguar (*Panthera onca*).** *Pesquisa Veterinária Brasileira 40(7):554-558*. 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: juliawronski@gmail.com

Extraintestinal pathogenic *Escherichia coli* (ExPEC) is a highly diverse pathotype of *E. coli* which colonizes the intestine, and it is considered an important etiological agent associated with bacteremia and other systemic infections, among them urinary tract infection. Retrospective studies evaluating morbidity and mortality of nondomestic felids have demonstrated that urinary tract diseases are among the main causes of death for geriatric animals. Also, mesenchymal neoplasms of the uterus are common in wild felids, and they possess variable morphologic characteristics related to invasiveness and malignancy. This report describes a case of bilateral pyelonephritis due to extraintestinal uropathogenic *E. coli* infection in a captive jaguar (*Panthera onca*). The diagnosis was confirmed through pathological, bacterial and immunohistochemical findings. According to molecular analysis, this *E. coli* strain was classified in the phylogroup F, possessing the following virulence-associated genes: *usp, cnf-1, hlyA, papC* and *sfa.* Additionally, this *E. coli* was highly resistant to β-lactams and first-generation cephalosporin. This jaguar also presented a uterine leiomyoma with distinct distribution, and severe degenerative articular disease, both of them described as frequently seen lesions in geriatric animals from the *Panthera* genus.

INDEX TERMS: Bilateral pyelonephritis, *Escherichia coli*, infection, captive jaguar, *Panthera onca,* extraintestinal pathogenic *E. coli*, uropathogenic *E. coli*, pyelonephritis, uterine leiomyoma, wildlife animals.

RESUMO.- [**Pielonefrite bilateral por *Escherichia coli* em uma onça-pintada (*Panthera onca*).**] *Escherichia coli* extraintestinal patogênica (ExPEC) é um patotipo altamente diverso de *E. coli* que coloniza o intestino e é considerada um agente etiológico importante, associado com bacteremia e outras infecções sistêmicas, dentre elas infecções do trato urinário. Estudos retrospectivos avaliando morbidade e mortalidade de felídeos não domésticos demostram que doenças do trato urinário estão entre as principais causas de morte de animais geriátricos. Ainda, neoplasias mesenquimais uterinas são comuns em felídeos de cativeiro e possuem características morfológicas variáveis relacionadas a invasividade e malignidade. Neste relato é descrito um caso de pielonefrite bilateral por *E. coli* extraintestinal uropatogênica em uma onça-pintada de cativeiro (*Panthera onca*). O diagnóstico foi confirmado através dos achados patológicos, bacteriológicos e imuno-histoquímicos. A partir da análise molecular, esta cepa de *E. coli* foi classificada no filogrupo F, possuindo os seguintes genes associados a virulência: *usp, cnf-1, hlyA, papC* and *sfa.* Adicionalmente, a bactéria isolada foi altamente resistente a β-lactâmicos e cefalosporinas de primeira geração. Foi observado ainda um leiomioma uterino com distribuição distinta e doença articular degenerativa severa, ambas

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descritas na literatura como comumente observadas em animais geriátricos do gênero *Panthera.*

TERMOS DE INDEXAÇÃO: Pielonefrite bilateral, *Escherichia coli*, onça-pintada, *Panthera onca*, *E. coli* patogênica extraintestinal, *E. coli* uropatogênica, pielonefrite, leiomioma uterino, animais selvagens.

INTRODUCTION

Extraintestinal pathogenic *Escherichia coli* (ExPEC) is a highly diverse pathotype of *E. coli* which colonizes the intestine, and it is considered an important etiological agent associated with bacteremia and other systemic infections, among them urinary tract infection (Johnson & Russo 2005, Smith et al. 2007). Frequently, ExPEC are classified into groups according to the anatomical site of infection, with uropathogenic *E. coli* (UPEC) being associated with urinary tract infection (UTI); these are considered the most common bacteria identified in UTI from animals and humans (Johnson & Russo 2005, Liu et al. 2015).

The capability of UPEC to lead to symptomatic UTI is related to expression of numerous virulence-associated genes (Liu et al. 2015). Among the main virulence factors (VF) of UPEC isolated from dogs and cats, studies highlight type 1 fimbriae (*fim*), pilus associated with pyelonephritis (*pap*), S fimbriae (*sfa*), afimbrial adhesion (*afa*), α-hemolysin (*hly*), aerobactin, cytotoxic necrotic factor 1 (*cnf1*), and cytolethal distengin toxin (*cdt*) as frequently responsible for acute UTI (Tramuta et al. 2011, Liu et al. 2015). Additionally, phylogenetic studies show that *E. coli* strains can be assigned to different phylogenetic groups (A, B1, B2, C, D, E, F and *E. coli* cryptic clade I), and the profile of virulence associated-genes are extremely diverse between each group and among isolates from the same group (Clermont et al. 2013, Liu et al. 2015). The predominant group in several studies is B2, presenting isolates with the most robust virulence factors and causing clinical sings considered severe (Johnson & Russo 2005, Smith et al. 2007, Tramuta et al. 2011, Clermont et al. 2013, Liu et al. 2015).

Retrospective studies evaluating morbidity and mortality of nondomestic felids have demonstrated that urinary tract diseases are among the main causes of death for geriatric animals (Hope & Deem 2006, Junginger et al. 2015). Also, felids from the genus *Panthera* are prone to the development of uterine neoplasia, among them, leiomyoma (Chassy et al. 2002, Junginger et al. 2015).

The aim of this study is to report a case of bilateral pyelonephritis due to UPEC strain infection in a 19-year-old captive jaguar and to characterize a uterine leiomyoma with atypical presentation in the same animal.

MATERIALS AND METHODS

Clinical and epidemiological data from the jaguar were obtained directly with the veterinary responsible for the zoo. During the necropsy procedure, multiple sections of organs were collected, fixed in a 10% neutral buffered formalin solution, routinely processed for histology, and stained with hematoxylin and eosin (HE). Samples of the main organs were refrigerated and submitted to microbiological evaluation.

Fragments of the kidney were incubated for 24h at 37°C in blood agar 5% and MacConkey agar and biochemistry characterized according to MacFaddin (2000). Subsequently, the isolated bacterium was tested for antibiotic susceptibility against fifteen antimicrobials by disc diffusion method: amoxicillin (30mcg), ampicillin (10mcg), amikacin (30mcg), ceftazidime (30mcg), cephalothin (10mcg), ciprofloxacin (5mcg), enrofloxacin (5mcg), flumequine (30mcg), florfenicol (30mcg), gentamicin (30mcg), imipenem (10mcg), nalidixic acid (30mcg), nitrofurantoin (300mcg), penicillin (30mcg), and sulfazotrim (25mcg). Bacterial DNA was extracted by boiling and PCR assays were performed to detect ExPEC classical virulence genes, following the phylogenetical classification as previously described by Clermont et al. (2013) as a quadriplex PCR assay.

Sections of kidney and uterus were submitted to immunohistochemistry (IHC) for smooth muscle actin (SMA), vimentin, and *E. coli*. Fragments of uterus were incubated with SMA monoclonal antibody (Dako, 1A4 clone, dilution of 1:100, antigen retrieval with Tris EDTA pH 9.0 on pressure pan) and vimentin monoclonal antibody (Zimed, V9 clone, dilution of 1:200, antigen retrieval with citrate buffer pH 6.0 on pressure pan). Fragments of kidney were incubated with *E. coli* polyclonal antibody (Virostat, Portland/ME, USA, dilution of 1:200, antigen retrieval with citrate buffer pH 6.0 on microwave oven). The amplification signal for all three IHC was achieved by using the peroxidase-labeled universal polymer method (MACH 4 Universal HRP-Polymer, Biocare Medical, Pacheco/CA 94553, USA). The reaction was revealed with 3-amino-9-ethylcarbazole (AEC, Dako North America, Carpinteria/CA 93013, USA), followed by counterstaining with Harris hematoxylin. Positive and negative controls were used in all IHC tests.

RESULTS

A 19-year-old jaguar (*Panthera onca*) was submitted for necropsy. The animal was born and remained its whole life in captivity, having three pregnancies during this period. For approximately 15 days before death, the jaguar presented clinical signs of apathy, dyspnea, diarrhea, lameness, prostration, and refusing movement. Antibiotic therapy with benzathine penicillin was applied (1.200.000IU each 48h for eight days), however the animal evolved to recumbency and death.

At necropsy, alterations were observed in multiple organ systems. At external examination, the jaguar was obese, and presented multiple swollen joints. The pelvis of both kidneys was distended due to marked accumulation of green purulent material (Fig.1A). Multiple white striations extending from the cortex to the medulla and reaching the pelvis were noted. The ureters and urinary bladder were unremarkable. Moreover, the uterus was markedly enlarged, measuring 12.0 x 4.0 x 4.0cm (Fig.1B), with multiple firm nodules disseminated in the organ, expanding the uterine wall and reducing almost completely the lumen at both uterine horns and uterine body. On cut surface, these nodules were nondelimitated, firm, and white (Fig.1B inset). The ovaries presented several cysts measuring up to 2.0cm in diameter. Additionally, multiple joints presented severe erosion of articular surfaces, with associated exposition of subchondral bone and deposition of fibrin, as well as marked osseous proliferations (osteophytes), and severe proliferation of articular capsule. In the lungs, multiple red to gray areas were noted in the caudal lobes.

Histologically, located mainly in the renal pelvis but extending also into the adjacent medullary parenchyma, there was marked inflammatory infiltrate of neutrophils, lymphocytes and macrophages, associated with multifocal areas of moderate necrosis, fibrin deposition, cellular debris

and coccobacilli bacterial aggregates (Fig.1C). At the renal cortex, there were multifocal to coalescing areas of interstitial fibrosis, mild inflammatory infiltrate of lymphocytes and plasma cells, mild multifocal glomerulosclerosis and hyaline cylinders inside tubules. The urinary bladder presented mild mononuclear inflammatory infiltrate associated with multifocal areas of hemorrhage in the submucosa.

The uterus had a neoplastic proliferation of mesenchymal cells expanding the myometrium and markedly reducing the uterine lumen, constituted by spindle-shaped cells with abundant eosinophilic cytoplasm. The cells were arranged in interlacing bundles mixed with an abundant stream of connective tissue. There was mild pleomorphism and no mitotic figures. In the ovaries, multiple cystic structures were located in the periovaric region.

Additional histological findings included severe proliferation of the articular capsule due to proliferation of synoviocytes and fibrous connective tissue (degenerative joint disease) with associated chondrocyte loss and myelofibrosis, mild keratitis and anterior uveitis, and moderate parathyroid hyperplasia. No relevant microscopic alterations were seen in the other organs.

The immunohistochemical technique demonstrated mild intracytoplasmic immunostaining for vimentin and SMA in the uterine neoplasia, and marked multifocal granular labeling for *E. coli* predominantly inside macrophages in the renal pelvis as well as associated with cellular debris, free in the renal pelvis (Fig.1D).

Pure *E. coli* colonies were isolated and identified through biochemical and molecular analysis. The strain was characterized as uropathogenic *E. coli* (UPEC) belonging to the phylogroup F and the following virulence factors (VF) were detected: *usp, cnf-1, hlyA, papC* and *sfa.* The strain was resistant to penicillin, cephalothin, and amoxicillin, and showed intermediary susceptibility to ampicillin.

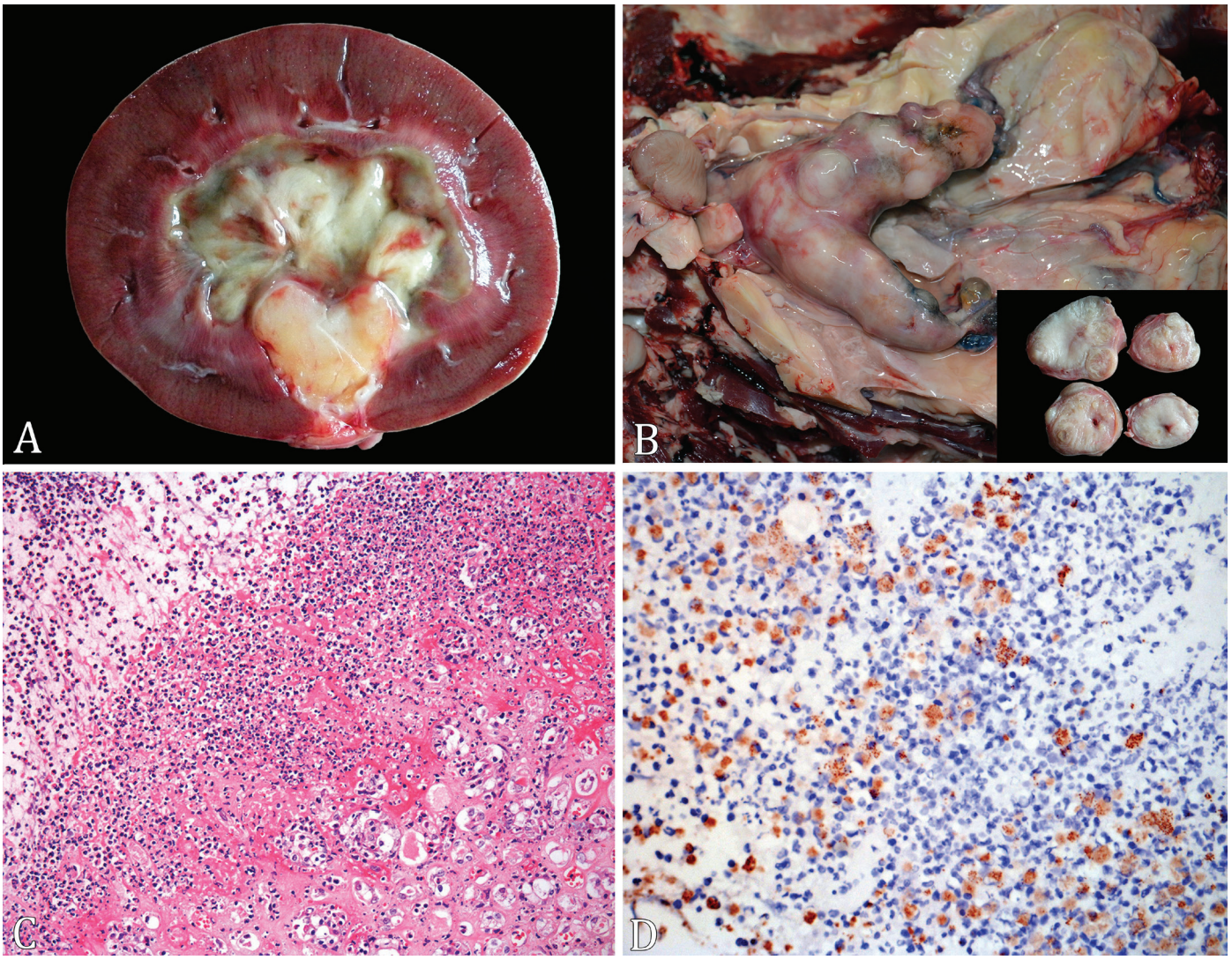


Fig.1. Bilateral pyelonephritis due to Escherichia coli infection and uterine leiomyoma in a jaguar. (**A**) Longitudinal section of kidney showing marked accumulation of green purulent material in the distended pelvis. (**B**) The uterus is markedly enlarged due to multiple coalescing nodules expanding the uterine wall. Inset: transverse sections of the uterus, with multiple nondelimitated white nodules reducing the uterine lumen. (**C**) Renal pelvis showing marked mixed inflammatory infiltrate, associated with necrosis, fibrin deposition, and cellular debris. HE, obj.10x. (**D**) Marked multifocal granular immunostaining for E. coli predominantly inside macrophages in the renal pelvis. Immunohistochemistry anti-E. coli, 3-amino-9 ethylcarbazole, Harris hematoxylin counterstain, obj.20x.

DISCUSSION AND CONCLUSION

The diagnosis of bilateral bacterial pyelonephritis due to uropathogenic *Escherichia coli* was based on pathologic, bacteriological and immunohistochemical findings. The causes of death in captive nondomestic felids vary in the literature, including chronic renal disease (Junginger et al. 2015), trauma (Metz et al. 2017), and stillbirths or neonatal (Hope & Deem 2006) as the most important. The neoplastic disease was considered an important cause in most studies, mainly for geriatric animals (Hope & Deem 2006, Junginger et al. 2015, Metz et al. 2017).

Extraintestinal infections due to ExPEC are described for several species, although reports of it causing disease in wild felids are scarce (Carvallo et al. 2010, Carvalho et al. 2012). In general, they are considered opportunistic pathogens who colonize the intestine. When it infects immunocompromised animals or in the presence of specific risk factors, bacteria can reach the urinary tract and cause UTI (Foxman 2014, Singer 2015). Among predisposing factors listed in the literature (Foxman 2014), obesity, female sex, anatomic abnormalities, and trauma/manipulation were identified in the case here described. Another important risk factor is the pathogenicity of the isolate, mainly related to its VF, since some can cause disease even in immunocompetent individuals (Johnson 2003, Hutton et al. 2018).

Isolates of ExPEC, in general, have multiple VF, and extensive profiles promote more invasive infections; inversely, no single VF is sufficient for the pathogenesis of UTI (Johnson 2003). Different studies performed by several researchers state that VF such as *papA, papC, sfa/sfc, afa/draBC, iutA, hylA Cnf-1,* and *kpsMT II* are related to acute infections of the urinary tract for dogs, cats, and humans, although the virulence profile is highly diverse according to the strain (Smith et al. 2007, Tramuta et al. 2011, Liu et al. 2015, Hutton et al. 2018). Additionally, the combination of *papC, sfa, hlyA,* and *cnf1* appears frequently (Tramuta et al. 2011), as observed in the jaguar from this case, which presented all four, in addition to *usp*.

Although single virulence genes cannot cause disease, as mentioned above, some VF can aggravate the disease. Adhesin-associated genes, such as *papC* and *sfa,* may be critical for illness severity (Hutton et al. 2018). P fimbriae, or pilus associated with pyelonephritis (*pap* type *C* in the described *E. coli*), show strong epidemiologic association with pyelonephritis, sepsis, prostatitis (Johnson 2003, Liu et al. 2015), which may explain the pyelonephritis without evidence of infection in other sites, mainly urinary bladder. Additionally, the expression of toxin genes, such as *cnf1, hlyA* and *usp* identified in this *E. coli*, may lead to more extensive tissue damage in the host, causing release of host nutrients, disabling of immune effector cells and, consequently, facilitating bacterial dissemination (Paniagua-Contreras et al. 2017).

The *E. coli* isolated was classified as phylogroup F. The predominant phylogenetic group described to UPEC strains is the B2, being the phylogroup F usually the least observed (Liu et al. 2015, Hutton et al. 2018). Group F was firstly described in a phylogenetic study performed in human samples (Jaureguy et al. 2008), and further differentiation from the other groups was achieved through a quadriplex PCR proposed by Clermont et al. (2013). Due to genetic similarities and because they are genetically closely related, phylogroup F is referred as a sister of B2 by some authors (Jaureguy et al. 2008, Clermont et al. 2013). Notably, studies performed in samples from dogs and cats have shown both phylogroups presenting a high number of virulence associated-genes (Liu et al. 2015) and the presence of extensive profiles tend to cause more aggressive infections (Johnson 2003).

Antibiotic resistance is a widely discussed topic in ExPEC literature, mainly in UPEC strains. A study performed in the United States demonstrated most strains from urinary tract of domestic felids were resistant to at least one antibiotic, being 98% and 62% of strains resistant to cephalothin and ampicillin, respectively (Liu et al. 2015). In the case presented here, the isolated *E. coli* was resistant to β-lactams and first-generation cephalosporin, explaining the negative response of the jaguar to the treatment adopted by veterinarians of the zoo. Moreover, these findings are similar to the antibiotic resistance profile of some *E. coli* strains from domestic and wild felids, as well as for humans (Smith et al. 2007, Carvalho et al. 2012, Liu et al. 2015).

In addition to the renal lesions, the jaguar from this case presented also a widespread uterine leiomyoma, periovaric cysts, degenerative joint disease in multiple joints, keratitis and anterior uveitis, and hyperplasia of parathyroid glands. Most of these lesions were reported for geriatric animals in previous studies of pathology of wild felids (Hope & Deem 2006, Junginger et al. 2015, Metz et al. 2017).

Reproductive tumors are among the most common types of neoplasia in captive *Panthera* species (Junginger et al. 2015, Kloft et al. 2019). Smooth muscle neoplasms are commonly reported in zoo felids, they involve mainly the uterus, and leiomyomas are considered the most common among all reproductive neoplasia (aside from mammary tumors) in most studies (Chassy et al. 2002, Owston et al. 2008, Junginger et al. 2015, Kloft et al. 2019). However, they are almost always not related to the cause of death, as they are common spontaneous tumors of felids (Junginger et al. 2015, Kloft et al. 2019), similarly to what is observed in the case here described. Despite, there seems to be no reports in the veterinary literature of leiomyomas with such widespread distribution as noted in this case.

Considering the characteristics of the isolated strain of *E. coli* identified in the jaguar from this study and the severity of the reported case, in addition to the predisposition of felids from the genus *Panthera* to develop uterine neoplasia, we highlight also the importance of the characterization of conditions as in this case to improve clinical approaches for captive wild felids, mainly for endangered animals.

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

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Original Article
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Fibroblastic osteosarcoma in a white-eared opossum (*Didelphis albiventris*)[1]

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ABSTRACT.- França R.T., Schulz É.T., Oliveira E.C., Damarem W.W., Giordani C., Pletsch J.A. & Sonne L. 2020. **Fibroblastic osteosarcoma in a white-eared opossum (*Didelphis albiventris*)**. *Pesquisa Veterinária Brasileira 40(7):559-563* Faculdade de Veterinária, Universidade Federal de Pelotas, Av. Eliseu Maciel s/n, Capão do Leão, RS 96160-000, Brazil. E-mail: raquelifranca@gmail.com

Osteosarcoma is characterized by the production of osteoid or woven bone, using neoplastic osteoblasts. It is the most common primary bone neoplasm in canines and humans. This neoplasm was previously reported in all vertebrate classes, including a wide variety of mammals. However, there is no case report describing this neoplasm in *Didelphis albiventris.* Therefore, the objective of this manuscript is to describe the clinical-pathological aspects of fibroblastic osteosarcoma in *D. albiventris*. A wild adult male white-eared opossum (*D. albiventris*) arrived at the zoological park of the "Universidade de Caxias do Sul" with a swollen left thoracic limb. After a general clinical examination, the animal was transferred to the Veterinary Clinic of "Universidade de Caxias do Sul" for radiographic examination of the left thoracic limb and thorax. Additionally, some material was collected through fine needle aspiration (FNA) for cytologic evaluation. The radiographic findings and cytologic evaluation indicated osteosarcoma. The animal was euthanized due to severe clinical conditions and guarded prognosis. During necropsy, macroscopic analysis of the viscera was performed, fragments of various organs were collected and fixed in 10% neutral buffered formalin. All fragments were processed following routine histological techniques. The histopathological evaluation confirmed osteosarcoma, which was classified as a fibroblastic subtype. Case reports are crucial for the knowledge of incidence, prevalence, and behavior of the current mentioned disease, as well as other diseases, in species with such limited information. In order to obtain a decisive diagnosis, a few different examination methods were associated. Although the observations presented are based on a single case, this neoplasm had a similar clinical presentation to that described in other species.

INDEX TERMS: Fibroblastic osteosarcoma, white-eared opossum, *Didelphis albiventris*, radiology, cytology, osteosarcoma, wildlife animals.

RESUMO.- [**Osteossarcoma fibroblástico em gambá-de-orelha-branca (*Didelphis albiventris*).**] O osteossarcoma é caracterizado pela produção de osteoide ou osso imaturo, por osteoblastos neoplásicos. É a neoplasia óssea primária mais comum em caninos e humanos. Essa neoplasia já foi relatada em todas as classes de vertebrados, incluindo uma grande variedade de mamíferos. Não havendo descrição dessa neoplasia até o momento em *Didelphis albiventris.* O objetivo deste trabalho é descrever aspectos clínico-patológicos de um caso de osteossarcoma fibroblástico em *D. albiventris*. Chegou para atendimento no Zoológico da Universidade de Caxias do Sul um gambá-de-orelha-branca (*D. albiventris*), macho, adulto, de vida livre com aumento de volume no membro torácico esquerdo. Após avaliação clínica geral, o animal foi encaminhado para a Clínica Veterinária da Universidade de Caxias do Sul para realização de radiografia do membro torácico esquerdo e de tórax, sendo também realizada coleta de material

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por punção aspirativa por agulha fina (PAAF) para avaliação citológica. Os achados radiográficos e da avaliação citológica foram sugestivos de osteossarcoma. Devido ao estado clínico grave e prognóstico reservado optou-se pela eutanásia. Durante a necropsia realizou-se a análise macroscópica das vísceras, foram coletados fragmentos de diversos órgãos, fixados em formalina 10%, processados pelas técnicas histológicas de rotina. Na avaliação histopatológica confirmou-se a suspeita de osteossarcoma sendo classificado no subtipo fibroblástico. A descrição de relatos é fundamental para conhecimento da incidência, prevalência e comportamento desta e de outras doenças em espécies que as informações são limitadas. A associação de diferentes métodos de exames foram necessários para a obtenção de um diagnóstico definitivo. Embora as observações apresentadas se baseiem em um caso único, esta neoplasia possuiu apresentação clínica semelhante a descrita em outras espécies.

TERMOS DE INDEXAÇÃO: Osteossarcoma fibroblástico, gambá-de-orelha-branca, *Didelphis albiventris*, radiologia, citologia, osteosarcoma, animais selvagens.

INTRODUCTION

Opossums are marsupials belonging to the family Didelphidae, order Didelphimorphia, infraclass Methateria, subclass Theria, and class Mammalia. Distribution varies widely across South America, while presenting nocturnal habits, having omnivorous feed preferences, being opportunistic, and living in peri-urban regions. Altogether, there are 65 species of the family Didelphidae described, classified within 17 different genera (Costa et al. 2015). In Brazil, three species are found: *Didelphis marsupialis, Didelphis aurita,* and *Didelphis albiventris* (Abreu 2013).

These animals are affected by infectious and neoplastic disorders (Baker et al. 1995). There is limited up-to-date scientific literature about neoplasm in opossums, being restricted to cases in the *Didelphis virginiana* species (Pope & Donnell 2017). There is also a scarcity of information about the incidence, prevalence, predisponent factors, and behavior of neoplasms in wild animals (Johnson et al. 2014, Madsen et al. 2017).

Osteosarcoma is characterized by the production of osteoid or woven bone, using neoplastic osteoblasts, presenting itself into three different subtypes: osteoblastic, chondroblastic, and fibroblastic (Thompson & Dittmer 2017). This neoplasm was reported in all classes of vertebrate animals, including a wide variety of mammals such as felines (Heldmann et al. 2000), canines (Anfinsen et al. 2011), equines (Bush et al. 2007), bovines (Nagamine et al. 2014), four-toed hedgehogs (*Atelerix albiventris*) (Reyes-Matute et al. 2017), baboons (Mezzles et al. 2015), guinea pigs (*Cavia porcellus*) (Cojean et al. 2018), rabbits (Ishikawa et al. 2012), birds (*Anthropoides paradiseus*) (Churgin et al. 2013), and reptiles (*Aspidites ramsayi*) (Cowan et al. 2011). The objective of this manuscript is to describe the clinical-pathological aspects of fibroblastic osteosarcoma in *Didelphis albiventris*.

MATERIALS AND METHODS

A wild adult male white-eared opossum (*Didelphis albiventris*) arrived at the zoological park of the "Universidade de Caxias do Sul" with a swollen left thoracic limb. After a general clinical examination, the animal was sent to a radiographic examination of the left thoracic limb and thorax. Since a neoplastic lesion was suspected, a fine needle aspiration (FNA) was performed. Consequently, a squash smear cytology was completed and colored with Rapid Panoptic.

The animal was euthanized due to the severe clinical conditions, the radiographic results, and the cytological analysis. During necropsy, macroscopic analysis of the viscera was performed, fragments of various organs were collected and fixed in 10% neutral buffered formalin. All tissue fragments were processed following routine histological techniques, stained with hematoxylin and eosin (HE), and the slides were analyzed by optical microscopy.

RESULTS

At the clinical examination, the animal was identified as anorexic, dehydrated, and unsteady on walking. It was observed an increased volume and intense superficial emaciation at the left humerus-radio-ulnar joint. The radiographic evaluation of the left thoracic limb revealed an increased volume and higher radiodensity of the soft tissue around the left humerus-radio-ulnar joint. There was a loss of the trabecular shape pattern and bone lysis at the proximal ulna and radius, and at the humeral epicondyles (more intense at the lateral region). The distal humerus presented an interrupted periosteal reaction (Codman triangle), olecranon presented a pathological fracture, and soft tissues contained discrete radiopacity areas (mineralization points), all signs suggesting a bone neoplasm (Fig.1).

The sample used in the cytological evaluation presented a moderate number of cells. The majority of cells were multinucleated (osteoclasts), in addition to a smaller number of mesenchymal stem cells, manifesting either isolated or in small groups surrounded by eosinophilic extracellular matrix. Additionally, the sample was characterized by anisocytosis, anisokaryosis, and sporadic mitotic figures. The cytological findings suggested osteosarcoma diagnosis (Fig.2). All other organs did not present any macroscopic alterations.

In the necropsy, a mass measuring 15.0 x 5.0 x 6.0cm was located around the left humerus-radio-ulnar joint. (Fig.3A). The surface cut was soft and compact, presenting white coloration, with yellowish and brownish spots, besides the bone area was easily bisected (Fig.3B). All other organs did not present any alterations during the necropsy.

The histopathological evaluation performed with the material collected from the left humerus-radio-ulnar joint showed a mesenchymal cell neoplastic proliferation, not delimited and non-encapsulated, formed by cells organized as a mantle shape, and facing different directions. These cells varied from fusiform to polygon shapes and contained eosinophilic cytoplasm, elongated nuclei, dotted chromatin, and inconspicuous nucleoli. Histopathological examination also showed moderated anisocytosis, anisokaryosis, sporadic mitotic figures, and a large number of multinucleated cells, all findings compatible with fibroblastic osteosarcoma (Fig.4).

DISCUSSION

Osteosarcoma is the most common primary bone neoplasm diagnosed in dogs and humans (Anfinsen et al. 2011). The antemortem diagnosis of osteosarcoma in wild animals has been a challenge for veterinarians due to the scarcity of published case reports in the scientific literature and to the common accelerated growth of the disease. In order to obtain a primary bone neoplasm definitive diagnosis, one needs to associate clinical data, complementary exams, and histopathological evaluations (Garcia et al. 2007). In this report, the suggested antemortem diagnosis was performed by linking clinical data, radiographic findings, and cytologic evaluations.



Fig.1. Simple mediolateral radiograph of left humerus-radio-ulnar joint showing increased volume and higher radiodensity in soft tissues. The trabecular shape pattern in the distal humerus is lost, in addition to bone lysis in the proximal third of ulna and radius, and humeral epicondyles. The distal humerus presents an interrupted periosteal reaction (Codman triangle), olecranon presents pathological fracture, and soft tissues contain discrete radiopacity areas (sparse mineralization).

It is common knowledge that osteosarcoma in dogs is most commonly diagnosed during adulthood (Anfinsen et al. 2011). In this case, the white-eared opossum was clearly an adult, even though it was not possible to define its age precisely, as a consequence of being a wild animal. Osteosarcoma habitually originates from the skeletal system, affecting both appendicular and axial skeletons, though the most common is found on long bone extremities of dogs and humans (Longhi et al. 2005, Anfinsen et al. 2011, Leeper et al. 2017). In the present case, the neoplasm was located at the appendicular skeleton, in the bone structure area forming the left humerus-radio-ulnar joint, likewise suggesting an initial injury at the bone extremity.

Current searches within scientific database platforms showed no evidence of bone neoplasm descriptions in *Didelphis albiventris*. Pope & Donnell (2017) observed 17 neoplasm cases in *Didelphis virginiana*, 14 of those categorized as malignant and 3 benign. In the same study, the neoplasm was identified as the reason for the death and euthanasia of 6 animals. The most common diagnosed neoplasm was pulmonary adenocarcinoma, followed by hemangiosarcoma, acute myeloid leukemia, undifferentiated sarcoma, trichoblastoma, mastocytoma, squamous cell carcinoma, adenoma, and osteoma.

There are a few radiographic patterns characterizing bone disorders, including mixed radiographic patterns, irregular bone contours, adjacent proliferative periosteal reaction, and adjacent soft tissue swelling (Farrow 2003). Regarding the pattern of bone destruction, the permeative pattern is considered the most aggressive, observed in cases of rapid progression tumors (Kirberger & McEvoy 2016), as well as the irregular or interrupted periosteal reactions, which suggest a neoplasm with a severe malignity (Kealy et al. 2012). In this case report, it was possible to observe the alterations previously mentioned in the left humerus-radio-ulnar joint. These patterns support the suspected neoplasm, however, it is not possible to differentiate from other bone diseases, such as osteomyelitis, since both disorders have similar

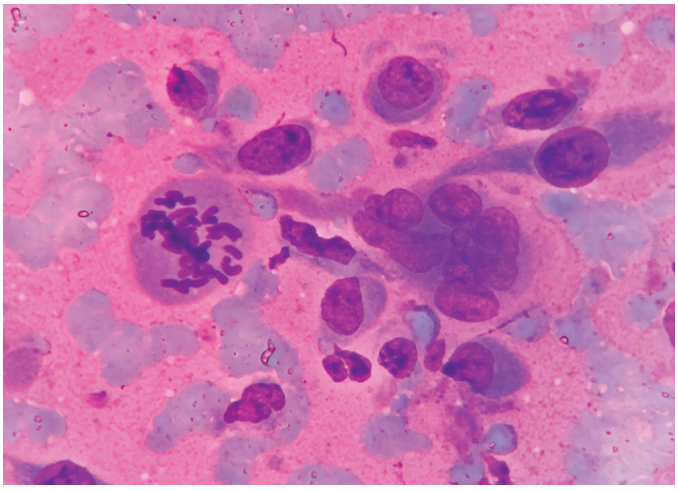


Fig.2. Cytological findings of fibroblastic osteosarcoma in *Didelphis albiventris*. There is a moderate quantity of mesenchymal stem cells and multinucleated (osteoclasts) cells, also characterized by anisocytosis, anisokaryosis and mitotic figures. Romanowksy, obj.10x.

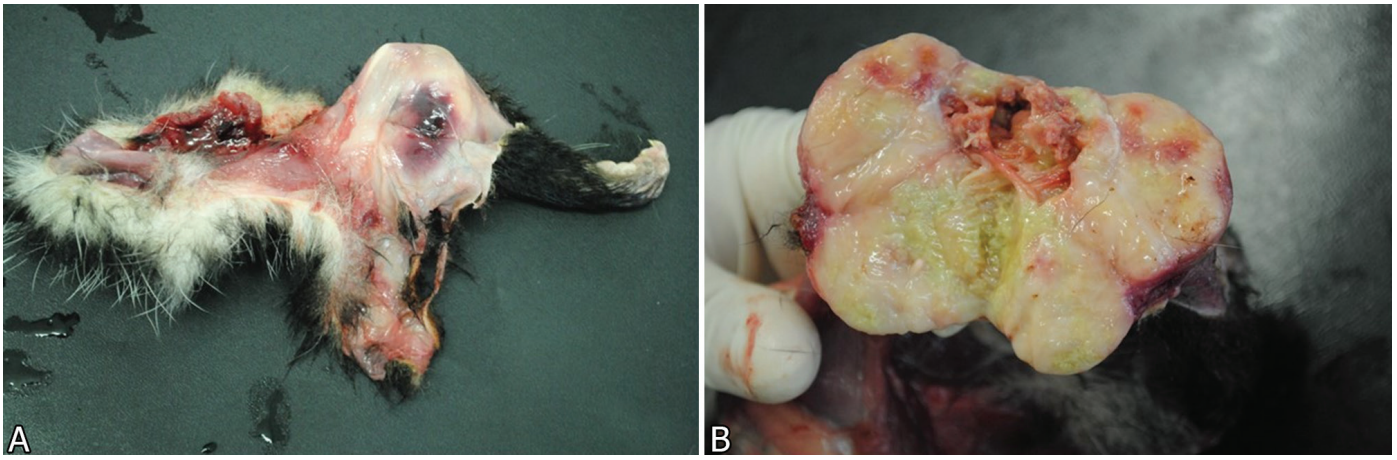


Fig.3. Macroscopic findings of osteosarcoma in *Didelphis albiventris*: (**A**) Swollen left humerus-radio-ulnar joint. (**B**) The cut surface of the tumoral area presents white coloration with yellowish spots.

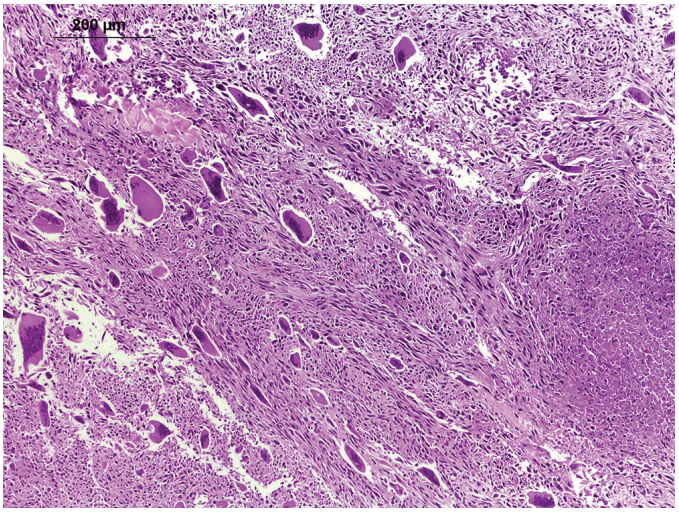


Fig.4. Fibroblastic osteosarcoma in *Didelphis albiventris*. Histopathological image with the proliferation of fusiform cells organized in different directions, and a high number of giant multinucleated cells. HE, bar = 200µm.

radiographic alterations, and possibly happen simultaneously, which requires a cytological or histopathological evaluation, in order to obtain a definitive diagnosis (Boston et al. 2010, Kealy et al. 2012). Pathological fractures can happen due to the bone fragility found in osteosarcoma, caused by bone density reduction and necrotic areas. The occurrence of metastasis mostly occurs in the skeleton and lungs of dogs and humans, and with less frequency in the liver, kidneys, spleen, heart, and regional lymph nodes (Farrow, 2003, Boerman et al. 2012). It was not possible to observe any metastasis in either the radiographic or histopathological evaluations, in the current case report.

Osteosarcoma can be divided into three different subtypes according to the histopathological evaluation, being classified as osteoblastic, chondroblastic, and fibroblastic. The fibroblastic osteosarcoma usually begins as a lytic lesion, but approximately 50% advances to a mixed pattern when fusiform neoplastic cells increase the ability to form a bone matrix (Thompson & Dittmer 2017). In this case, the histopathological evaluation showed pieces of evidence of an osteosarcoma subtype fibroblastic. Even though histopathological evaluation is considered the most accurate form of neoplasm diagnosis in animals, the cytology has seemed to be a precise form of first evaluate bone injuries, turning into a quite important tool in the clinical routine of veterinary and human medicine, in patients with suspected bone neoplasms. The cytology presents a high accuracy, especially when evaluated together with radiographic images (Thompson & Dittmer 2017). In this particular case, the presumptive diagnosis was established using cytology in conjunction with the radiographic evaluation. Osteosarcoma cytological samples frequently present a high number of cells, but in some cases, it does not represents the lesion precisely. The material can be collected from either lytic areas or medullary cavity. In some cases, osteoclasts may be abundant, but in others, they may not be present. The quality and quantity of the osteoid matrix produced by osteoclasts may vary significantly between and within neoplasms. The osteoid production may be focal, and it is frequently marked by a chondroid area and/or fibrous tissue differentiation. In most cases, this is not a diagnosis problem, though it may be a significant challenge when cytology or histopathology samples are not available. The cytology is useful for the osteosarcoma diagnosis, but not convenient for classifying osteosarcoma withing its three different subtypes (Thompson & Dittmer 2017).

CONCLUSIONS

There is a scarcity of case reports in the scientific literature mentioning neoplasm in free wild animals. Therefore, the description of case reports is extremely important for the knowledge of specific disorders in species with such limited information.

The association between different examinations was essential in order to obtain a specific diagnosis.

Although the observations showed were based solely on one case, this neoplasm has a clinical presentation similar to those described in other species.

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

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Original Article
Animal Morphophysiology
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Comparison of right and left common carotid arteries of horses and mules by two-dimensional ultrasound and Doppler flow[1]

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ABSTRACT.- Fogaça J.L., Castiglioni M.C.R., Vettorato M.C., Silva P.J., Bueno L.M.C., Filadelpho A.L., Puoli-Filho J.N.P. & Machado V.M.V. 2020. **Comparison of right and left common carotid arteries of horses and mules by two-dimensional ultrasound and Doppler flow.** *Pesquisa Veterinária Brasileira 40(7):564-570*. Departamento de Reprodução Animal e Radiologia Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rua Prof. Dr. Walter Mauricio Correra s/n, Rubião Junior, Botucatu, SP 18618-970, Brazil. E-mail: vaniamvm@fmvz.unesp.br

In ultrasonography, the bi-dimensional mode (B-mode) allows the morphological and morphometric evaluation of several types of organs and tissues, while the Doppler mode allows the hemodynamic evaluation. In humans, the Doppler evaluation is routinely used in the assessment of important arteries and veins, such as the carotid arteries and jugular veins, with significant differences between genres and sides. However, in veterinary medicine, this diagnostic method is not yet well established in the evaluation of the carotid arteries, with only few reports in domestic horses. This study aims to compare the right and left common carotid arteries of domestic horses and mules using bi-dimensional and Doppler ultrasound evaluation. The common carotid arteries from 10 domestic horses (five males and five females) and 10 mules (five males and five females) were evaluated. The following variables were measured at three different portions (cranial, middle and caudal): diameter, intima-media thickness (IMT), resistivity index (RI), pulsatility index (PI), peak of systolic velocity (pSV) and final diastolic velocity (fDV). No significant differences were observed in the bi-dimensional variables (diameter and IMT) between the common carotid arteries of horses and mules, regardless of gender ($p>0.05$). In Doppler mode, there were no significant differences between carotid values in male and female horses ($p> 0.05$). In the mules, it was only possible to observe differences between the RI and PI values ($p<0.05$), being higher on the left side (0.81 and 2.04 respectively), and the fDV ($p<0.05$) higher, on the right side (14.35) in males. As for females, there was only in fDV ($p<0.05$), with the upper right side (23.16). Diameters and IMT do not differ between sides in horses and mules in B-mode ultrasound. Spectral Doppler in horses does not differ between sides, regardless of gender. As for mules, males differ in RI, PI and fDV between sides, while females differ only in fDV.

INDEX TERMS: Carotid arteries, horses, mules, two-dimensional ultrasound, Doppler flow, vases, diagnostic image, blood flow.

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RESUMO.- [Comparação das artérias carótidas comuns direita e esquerda dos gêneros de equinos e muares por ultrassonografia bidimensional e Dopplerfluxométrico.] Na ultrassonografia, o modo bidimensional (modo B) permite a avaliação morfológica e morfométrica de vários tipos de órgãos e tecidos, enquanto o modo Doppler permite a avaliação hemodinâmica. Em humanos, a avaliação com Doppler é usada rotineiramente na avaliação de artérias e

veias importantes, como as artérias carótidas e veias jugulares, com diferenças significativas entre gêneros e lados. No entanto, em medicina veterinária, este método diagnóstico ainda não está bem estabelecido na avaliação das artérias carótidas, com apenas poucos relatos em equinos domésticos. Este estudo tem como objetivo comparar as artérias carótidas comuns direita e esquerda de equinos e mulas domésticos, utilizando a avaliação bidimensional (modo B) e por ultrassonografia Doppler espectral. Avaliaram-se as artérias carótidas comuns de 10 equinos domésticos (cinco machos e cinco fêmeas) e 10 mulas (cinco machos e cinco fêmeas). As seguintes variáveis foram medidas em três porções diferentes (cranial, médio e caudal): diâmetro, espessura íntima-média (IMT), índice de resistividade (RI), índice de pulsatilidade (PI), pico de velocidade sistólica (pSV) e velocidade diastólica final (fDV). Não foram observadas diferenças significativas nas variáveis bidimensionais (diâmetro e IMT) entre as artérias carótidas comuns de cavalos e muares, independentemente do gênero (p>0,05). No modo Doppler, não houve diferenças significativas entre os valores para carótidas em equinos machos e fêmeas (p>0,05). Nos muares, só foi possível observar diferenças entre os valores de RI e PI (p<0,05), sendo maiores no lado esquerdo (0,81 e 2,04 respectivamente), e o fDV (p<0,05) superior no lado direito (14,35) nos machos. Quanto as fêmeas, houve apenas no fDV (p<0,05), sendo o lado direito superior (23,16). Os diâmetros e IMT não diferem entre os lados em equinos e muares na ultrassonografia modo B. Já o Doppler espectral nos equinos não difere entre os lados, independentemente do gênero. Quanto aos muares, os machos diferenciam no IR, IP e fDV entre os lados, enquanto as fêmeas apenas para fDV.

TERMOS DE INDEXAÇÃO: Artérias carótidas comuns, equinos, muares, ultrassonografia bidimensional, Dopplerfluxométrico, vasos, diagnóstico por imagem, fluxo sanguíneo.

INTRODUCTION

Doppler ultrasonography is a tool combined with B-mode ultrasonography that allows the hemodynamic evaluation of the vessels (Loizou 2014, Barlinn et al. 2018, Boyko et al. 2018, Mckenna et al. 2018, Oglat et al. 2018). The common carotid artery originates three important arteries in horses and mules: the internal carotid artery, the external carotid artery and the occipital artery. These vessels are responsible for irrigating the face and the central nervous system (Getty 1975, König & Liebich 2013). Hemodynamic changes in the carotid artery and its branches may result in a compromise of the brain's function, resulting in damage, coma or death (Walker & Naylor 2006, Abdelhamid et al. 2009, Mendis et al. 2011, Kandiah et al. 2014, Birmpili et al. 2018).

There are several types of studies on the common carotid artery in domestic horses (Cipone et al. 1997, Schmucker et al. 2000), as well as other animals such as dogs (Hess et al. 2003, Calderon-Arnulphi et al. 2011), sheep (Khamas et al. 1984, Valentine et al. 2011), cows (Braun & Föhn 2005, Gregory et al. 2012), camels (Abdel-Magied & Drommer 1989, Kiełtyka-Kurc et al. 2014) and even buffaloes (Prakash & Rao 1976, Gregory et al. 2008, Sangwan et al. 2015). However, studies on carotid artery ultrasound are few in the literature, especially in mules.

Unlike horses, mules are hybrid animals, resulting from the crossbreeding between a jack and a mare (Salles et al. 2013, Gastal et al. 2014). Without further studies, it is not possible to use the horse's parameters for mules, due to a possible paternal influence. Therefore, this study aims to compare the sides of the common carotid arteries by conventional ultrasonography and spectral Doppler for the genera of horses and mules.

MATERIALS AND METHODS

Twenty healthy animals were used in the present study: ten crossbreed horses (5 males and 5 females), with mean age of 10 years and mean body mass of 394.6kg; and 10 mules (5 males and 5 females), with mean age of 9 years and mean body mass of 403.9 kg. The inclusion criteria of the animals were: clinical (heart rate evaluation) and laboratory (blood count and biochemical) tests.

This study is in accordance with the ethical principles adopted by the National Council of Animal Control and Experimentation and the Ethical Commission in Animal Use of this faculty (Protocol No. 0100/2017).

The ultrasound examination was performed using a mobile ultrasound equipment (Healthcare model Logiq V2, Chicago/IL, USA) and high-frequency linear transducer (8-12mHz). The animals were manually restrained and no chemical restraint was required. The screening windows were located, clipped and the skin was soaked with 70% isopropyl alcohol and acoustic gel. Bi-dimensional and spectral Doppler images of three different regions of the right and left common carotid arteries were obtained: caudal, middle and cranial.

The anatomical spots in these regions were classified as caudal, middle and cranial measurement points. The caudal point was established by dorsoventral line tangent caudally to the seventh cervical vertebra. The middle point was considered as a dorsalventral line tangente caudally to the articular fovea of the fourth cervical vertebra. The cranial point was identified as a line tangent dorsally to the condyles of the occipital bone and ventrally to the angle of the jaw.

For spectral Doppler images, the following variables were acquired: resistivity index (RI), pulsatility index (PI), systolic velocity (pSV) and diastolic velocity (fDV) (Fig.1). Bi-dimensional images were obtained in the transverse and longitudinal planes, measuring vessel diameter and the intima-media thickness (IMT) (Fig.2).

The statistical analysis was performed using the GraphPad Prism software v.7.00 for Windows (GraphPad Software, San Diego/CA) with statistical significance set at p<0.05. The variables RI, PI, pSV, fDV, IMT and vessel diameter were obtained for each region (cranial, mean and caudal) and analyzed jointly.

Subsequently, the variables were submitted to the Wilcoxon test for non-parametric data to compare the performance of each subject, in the sense of verifying if there are significant differences between the sides of the common carotid arteries with gender.

RESULTS

In domestic horses, regardless of gender, there were no significant difference between the right and left sides of the common carotid arteries (Table 1-4).

In the male mules, there were no significant differences in the B-mode variables between sides (Table 5), but there was a difference in the RI (p<0.0001) and PI (p=0.0002) values, which were higher on the left side, and in the fDV (p=0.0005) value, which was higher on the right side (Table 6).

As for female mules, there were no significant differences in the B-mode variables (Table 7), but there was a difference in the Doppler variable fDV between sides (p=0.0358), which was greater on the right common carotid (Table 8).

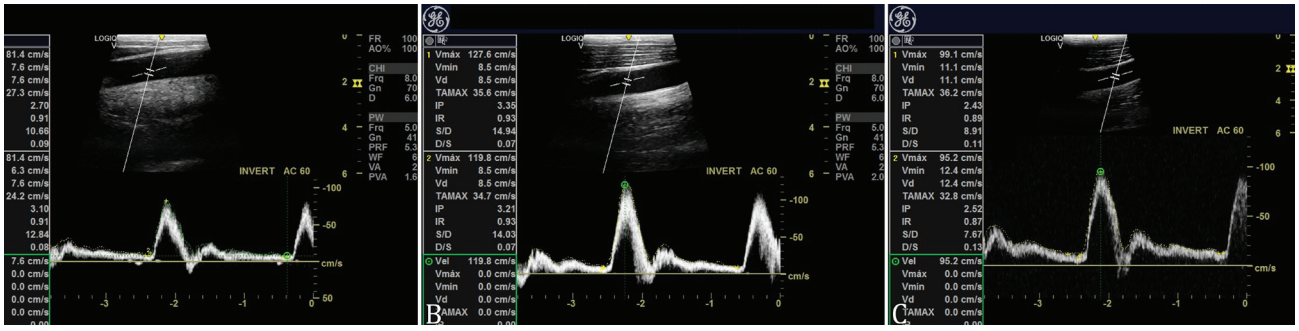


Fig.1. Doppler image acquisition in three anatomical regions: (**A**) caudal, (**B**) middle, and (**C**) cranial.

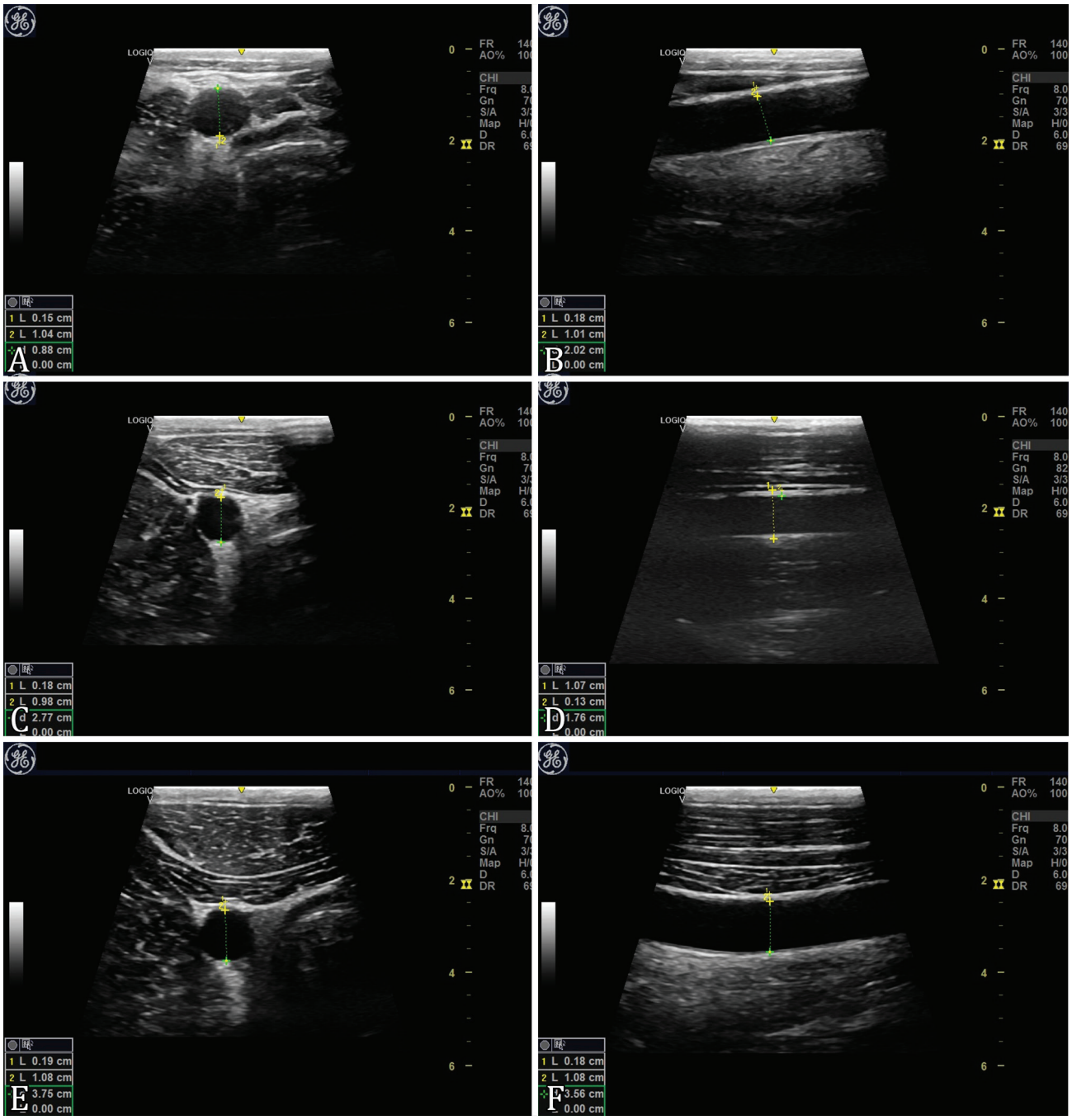


Fig.2. B-mode image acquisition in two planes for the three anatomical regions: (**A**) caudal in transversal and (**B**) longitudinal planes. Middle in (**C**) transversal and (**D**) longitudinal planes. (**E**) Cranial transversal and (**F**) longitudinal planes.

Table 1. Descriptive analysis of bi-dimensional values for the common carotids in male horses

Variables	Right			Left			P-value*
	Mean	Median	Standard deviation	Mean	Median	Standard deviation	
Longitudinal diameter (cm)	1.16	1.13	0.14	1.18	1.13	0.16	0.2992
Transverse diameter (cm)	1.26	1.29	0.18	1.27	1.26	0.21	0.4510
Longitudinal wall (cm)	0.14	0.13	0.03	0.13	0.12	0.03	0.1567
Transverse wall (cm)	0.14	0.13	0.02	0.14	0.13	0.03	0.5301

* P-value <0.05

Table 2. Descriptive analysis of Doppler values for the common carotids in male horses

Variables	Right			Left			P-value*
	Mean	Median	Standard deviation	Mean	Median	Standard deviation	
RI	0.81	0.83	0.07	0.83	0.83	0.07	0.5989
PI	2.06	2.11	0.56	2.09	2.09	0.45	0.5296
pSV (cm/s)	98.69	97.67	23.69	105.70	99.11	26.37	0.9635
fDV (cm/s)	16.06	13.98	8.12	16.00	15.40	7.58	0.8884

RI = Resistivity index, PI = pulsatility index, pSV = peak of systolic velocity, fDV = final diastolic velocity; * P-value <0.05.

Table 3. Descriptive analysis of bi-dimensional values for the common carotids in female horses

Variables	Right			Left			P-value*
	Mean	Median	Standard deviation	Mean	Median	Standard deviation	
Longitudinal diameter (cm)	1.10	0.08	0.07	1.12	1.11	0.06	0.4100
Transverse diameter (cm)	1.15	1.16	0.06	1.14	1.12	0.10	0.2928
Longitudinal wall (cm)	0.14	0.14	0.03	0.12	0.13	0.02	0.0779
Transverse wall (cm)	0.14	0.15	0.02	0.15	0.19	0.03	0.7059

* P-value <0.05.

Table 4. Descriptive analysis of Doppler values for the common carotids in female horses

Variables	Right			Left			P-value*
	Mean	Median	Standard deviation	Mean	Median	Standard deviation	
RI	0.83	0.83	0.05	0.82	0.83	0.06	0.3240
PI	2.15	2.11	0.38	2.09	2.06	0.44	0.2373
pSV (cm/s)	104.90	102.10	11.99	108.50	108.00	16.84	0.2702
fDV (cm/s)	11.78	15.99	5.13	16.97	17.55	5.04	0.0649

RI = Resistivity index, PI = pulsatility index, pSV = peak of systolic velocity, fDV = final diastolic velocity; *P-value <0.05.

Table 5. Descriptive analysis of bi-dimensional values for the common carotids in male mules

Variables	Right			Left			P-value*
	Mean	Median	Standard deviation	Mean	Median	Standard deviation	
Longitudinal diameter (cm)	1.31	1.31	0.08	1.29	1.26	0.11	0.5300
Transverse diameter (cm)	1.29	1.30	0.08	1.30	1.29	0.07	0.8012
Longitudinal wall (cm)	0.15	0.15	0.03	0.15	0.15	0.03	0.8886
Transverse wall (cm)	0.14	0.15	0.03	0.14	0.13	0.03	0.8505

* P-value <0.05.

Table 6. Descriptive analysis of Doppler values for the common carotids in male mules

Variables	Right			Left			P-value
	Mean	Median	Standard deviation	Mean	Median	Standard deviation	
RI	0.74	0.73	0.11	0.79	0.81	0.08	<0.0001*
PI	1.76	1.64	0.71	2.00	2.04	0.52	0.0002*
pSV (cm/s)	63.91	61.98	8.04	62.76	61.92	11.71	0.7329
fDV (cm/s)	14.35	11.63	7.91	11.48	10.22	6.2	0.0005*

RI = Resistivity index, PI = pulsatility index, pSV = peak of systolic velocity, fDV = final diastolic velocity; * P-value <0.05.

Table 7. Descriptive analysis of bi-dimensional values for the common carotids in female mules

Variables	Right			Left			P-value*
	Mean	Median	Standard deviation	Mean	Median	Standard deviation	
Longitudinal diameter (cm)	0.14	0.15	0.03	1.15	1.13	0.12	0.9250
Transverse diameter (cm)	1.16	1.14	0.16	1.19	1.16	0.13	0.5719
Longitudinal wall (cm)	0.14	0.14	0.04	0.14	0.15	0.03	0.7265
Transverse wall (cm)	0.14	0.15	0.03	0.16	0.16	0.04	0.2340

* P-value <0.05.

Table 8. Descriptive analysis of Doppler values for the common carotids in female mules

Variables	Right			Left			P-value
	Mean	Median	Standard deviation	Mean	Median	Standard deviation	
RI	0.71	0.71	0.09	0.72	0.72	0.08	0.0678
PI	1.48	1.38	0.41	1.56	1.42	0.52	0.3099
pSV (cm/s)	83.00	83.34	14.61	83.58	84.24	9.89	0.9284
fDV (cm/s)	24.06	23.16	9.43	21.67	22.52	8.11	0.0358*

RI = Resistivity index, PI = pulsatility index, pSV = peak of systolic velocity, fDV = final diastolic velocity; * P-value <0.05.

DISCUSSION

There are studies regarding B-mode and Spectral Doppler ultrasonography in the common carotid arteries of horses (Cipone et al. 1997, Schmucker et al. 2000, Aguiar 2015), but a review of the literature revealed no such studies involving mules. Unlike the results reported in the literature, this study did not observe differences between each side of the carotids in horses. According to Aguiar (2015), American Quarter Horses have common carotid arteries that are larger on the right side than on the left, but this study did not observe this difference in the diameter measurements obtained through B-mode ultrasound. This discrepancy may be related to the fact that this study only used animals with similar ages (adults), which could make it impossible to observe the asymmetry.

In human medicine, it is possible to observe the influence of age and even sex on the sonographic characteristics. The right carotid artery differentiates anatomically from the left and this may be related to hemodynamics, age, sex, hormone levels and other factors that may predispose this difference (Luo et al. 2011). A study has demonstrated that women had larger right-side diameter than the left side (Krejza et al. 2006). However, this difference in diameter was not observed in female horses and mules. As for men, Denarié et al. (2000) reported that diameters of the right common carotid arteries were larger than the those of the left. A study conducted by Krejza et al. (2006) did not verify this difference between the sides in men, and this study also did not observe such difference in male horses and mules.

For the IMT of the common carotid arteries of horses and mules, the difference between the sides was not significant, similar to the findings observed by Aguiar (2015), in which the IMT was not associated with the genus and sides of the animals evaluated. However, in a study by Rosfors et al. (1998), the carotid artery on the left side of men presented higher IMT values than the right side, and this may be related to the faster development of the atherosclerotic plaques in the carotid artery on the left side. According to Loizou et al. (2015), people who have not undergone cardiovascular changes do not present differences between left and right IMT. Atherosclerotic plaques deposited in the common carotid arteries are not as frequent in animals as they are in humans, where considered one of major causes of mortality in the world (Mendis et al. 2011, Ricotta et al. 2011, Barlinn et al. 2018, Boyko et al. 2018, Evans et al. 2018, Mckenna et al. 2018, Oglat et al. 2018).

In the Doppler ultrasonography, there were differences in male mules, with RI and PI having the highest values on the left side, while fDV was higher on the right side. According to Aguiar (2015), RI did not change according to gender and side in American Quarter Horses, which is in line with the results observed in horses (undefined breed) in this study. However, Doppler variations were observed in male and female mules.

The pSV variable was not significantly different between the sides for the mules. The measurement of pSV and fDV is an important tool in the detection of stenosis (Tortoli et al. 2015). In addition, RI and PI provide information about blood flow and vessel impedance (Oglat et al. 2018). The blood flow in these vessels becomes more resistant as horses age (Aguiar 2015). In healthy elderly humans, pSV and fDV were 50% lower than in younger individuals, which can be explained by vascular compliance (PI) (Wolff et al. 2007).

In healthy humans, there may be differences in the blood flow velocity of common carotid arteries between the sides (Holdsworth et al. 1999), as was the case in mules in this study. These differences may be related to the fact that they are hybrid animals and present anatomical and physiological differences in comparison to horses (Burnham 2002, Alsafy et al. 2008, Smith 2009).

An ultrasound evaluation by spectral Doppler in dogs did not demonstrate differences between the sides of the common carotid arteries (Svicero et al. 2013) similar to male and females horses in this study. According to Aguiar (2015), there may be differences according to breed or influence of sex hormones on the blood flow and vessel structures.

Schmucker et al. (2000) evaluated the common carotid arteries of anesthetized and sedated horses, which altered the blood flow velocity and vessel diameters, respectively. For this reason, this study avoided adequate chemical containment and adopted handling procedures avoiding animal stress to inhibit possible hemodynamic and structural changes.

The results observed in this study highlighted the possibility of inequalities between the variables obtained by spectral Doppler ultrasonography in the right and left common carotid arteries, so that these data can guide the professionals in the evaluation of this vessel in horses and mules, either within the respective genera or in comparison with other species. Above all, due to these differences, we recommend that, during investigations of the common carotid arteries of horses and mules by spectral Doppler ultrasonography, the vessels should be evaluated on both sides.

CONCLUSIONS

The diameters and IMT of the common carotid arteries do not differ between the sides in horses and mules during B-mode ultrasonography.

Spectral Doppler in horses does not differ between sides, regardless of gender.

As for the mules, the males present differences between the sides for the variables RI, PI and fDV, while females so only for fDV.

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

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(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)

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