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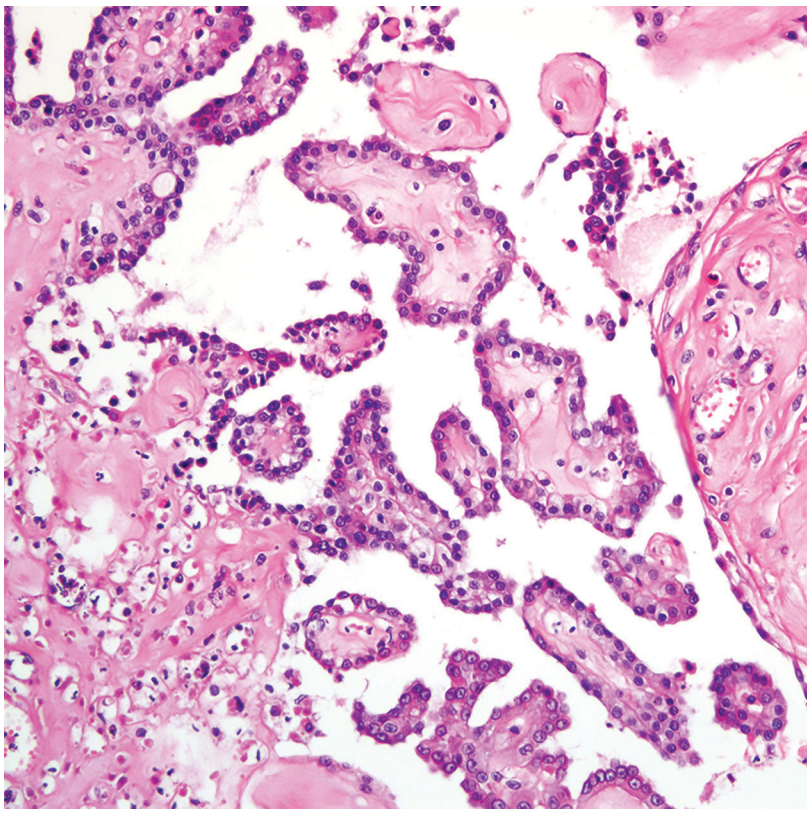

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Cover illustration: Papillary mass, composed by one layer of cubic mesothelial cells, supported by a low cellular fibrovascular stroma in lungs of a lion with mesothelioma. HE, bar = 200μm (Rocha et al., p. 417).

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Original Article
Livestock Diseases

Experimental reproduction of congenital anomalies in the progeny of cows fed apple pomace during pregnancy[1]

Nathalia S. Wicpolt[2], Raissa M. Morais[2], Francieli Adriane Molossi[2], Daiane Ogliari[2], Joana Mezzalira[3], Osmar D. Prestes[4], Renato Zanella[4] and Aldo Gava[2]*

ABSTRACT.- Wicpolt N.S., Morais R.M., Molossi F.A., Ogliari D., Mezzallira J., Prestes O.D., Zanella R. & Gava A. 2019. **Experimental reproduction of congenital anomalies in the progeny of cows fed apple pomace during pregnancy.** *Pesquisa Veterinária Brasileira 39(6):371-375.* Laboratório de Patologia Animal, Departamento de Medicina Veterinária, Centro de Ciências Agroveterinárias, Universidade do Estado de Santa Catarina, Av. Luiz de Camões 2090, Lages, SC 88520-000, Brazil. E-mail: aldo.gava@udesc.br

This study aimed to describe and discuss the results of an experiment carried out in two stages with pregnant cows fed 25kg/apple pomace/day. The first stage involved 16 pregnant Holstein Friesian cows divided into four groups: Group 0 - Control (5 cows); Group I - 1 month-gestation (4 cows); Group II - 3 month-gestation (4 cows); Group III - 6 month-gestation (3 cows) and was performed from September to December 2015. The second stage comprised 12 pregnant Holstein Friesian cows divided into three groups: Group 0 - Control (6 cows), Group I - 1 month-gestation (3 cows), and Group II - 3 month-gestation (3 cows) and was conducted from April 2016 to February 2017. All study animals received apple pomace at a dose of 25kg/day. As for the first experiment stage, a cow in Group III bred a calf with complete absence of the coccygeal vertebrae and tail, slight bending of the hind limbs, scoliosis in the thoracic spine, and limited mobility. At 30 days, it presented with diarrhea and underdevelopment, and was euthanized for necropsy. At gross examination, malformations were observed in the thoracic spine, coxofemural joint, and genitourinary tract. Regarding the second experiment stage, a cow in Group I gave birth to a calf with curved pelvic and thoracic limbs with thick joints and flattening hooves. Microscopic examination of the femur showed disorganized, irregular hypertrophic zone and scarce growth zone, in addition to primary spongy zone with short, slightly mineralized trabeculae. Samples of the apple pomace used in this study were frozen and sent for laboratory evaluation of pesticide residues, which showed a positive result for the fungicide carbendazim.

INDEX TERMS: Experimental reproduction, congenital anomalies, progeny, cows, apple pomace, pregnancy, calf, cattle, pathology.

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RESUMO.- [Reprodução experimental de anomalias congênitas na progênie de vacas alimentadas com bagaço de maçã durante a gestação.] O presente trabalho tem por finalidade descrever e discutir os resultados do experimento realizado em vacas prenhes que foram alimentadas com 25kg/dia de bagaço de maçã. Experimentos foram conduzidos em duas etapas, a primeira no ano de 2015, de setembro a dezembro onde foram utilizadas 16 vacas prenhes da raça holandês. Estas foram divididas em quatro grupos: Grupo 0, Controle (5 vacas); Grupo I, 1 mês gestação (4 vacas); Grupo II, 3 meses

gestação (4 vacas); Grupo III, 6 meses gestação (3 vacas). A segunda etapa foi realizada em abril de 2016 a fevereiro de 2017. Foram utilizadas 12 vacas prenhes da raça holandês, divididas em três grupos: Grupo 0, Controle (6 vacas); Grupo I, 1 mês gestação (3 vacas); Grupo II, 3 meses gestação (3 vacas). Todas as vacas receberam bagaço de maçã na dose de 25kg/dia/vaca. Para o primeiro experimento, uma vaca do Grupo III pariu uma bezerra, com ausência completa das vértebras coccígeas e cauda, encurvamento leve dos membros posteriores, escoliose na coluna torácica e dificuldade de locomoção. Decorridos 30 dias do nascimento, manifestou diarreia e pouco desenvolvimento, sendo eutanasiada para necropsia. Na macroscopia, havia malformações na coluna torácica, articulação coxofemoral e no aparelho urogenital. Em relação ao segundo experimento uma vaca do Grupo I pariu uma bezerra com membros pélvicos e torácicos, curvos e com articulações consideravelmente grossas e "achinelamento" de cascos. Na microscopia do fêmur foi observado na placa epifisária, zona hipertrófica desorganizada, irregular e zona de crescimento escassa. Na zona esponjosa primária observou-se trabéculas curtas e pouco mineralizadas. Amostras do bagaço de maçã utilizado na experimentação foram congeladas e enviadas para avaliação de resíduos agrotóxicos, onde foi encontrado resultado positivo para o fungicida carbendazim.

TERMOS DE INDEXAÇÃO: Reprodução experimental, anomalias congênitas, progênie, vacas, bagaço de maçã, gestação, bezerro, bovinos, patologia.

INTRODUCTION

Apple is a crop of great economic importance in the South Region of Brazil, which is responsible for 98% of the national production of this fruit. Pomace is the main by-product of this fruit, corresponding to 25% of its weight. Apple pomace is composed of 18% dry matter (DM), 6.5% crude protein (CP), 42% neutral detergent fiber (NDF), 3.2% ethereal extract (EE), 4.2% mineral matter (MM), and 62.4% total digestible nutrient (TDN). It is characterized by presenting low DM and CP contents; however, it shows medium NDF content and can be an alternative energy source in the feeding of ruminants (Ribeiro Filho et al. 2012). This by-product is often used to feed bovines and has been shown to cause osseous malformation in calves born of cows that ingested it during pregnancy (Wicpolt 2018).

According to Rumsey et al. (1977), these malformations may be associated with pesticides frequently used in apple culture, which may remain as residue in the bagasse. In the states of Santa Catarina and Rio Grande do Sul, a variety of agrochemicals are used for pest control in apple crops. According to the National Agency of Sanitary Surveillance (Anvisa 2017), over 60 different types of pesticides are used for this purpose, including carbamates and organophosphates. Exposure to these pesticides may harm the development of newborn calves (Grecco et al. 2009, Oliveira-Filho et al. 2010). Another product frequently used in apple trees is abamectin, which can cause neurological changes and death in cattle if applied at doses higher than the recommended (Seixas et al. 2006).

This study aimed to confirm the presence of osseous malformation in calves born of cows fed apple pomace during pregnancy and identify pesticides and other chemicals present in this by-product that could be the cause of these malformations.

MATERIALS AND METHODS

This study was carried out in two stages: the first stage involved 16 pregnant Holstein Friesian cows divided into four groups: Group 0 - Control (5 cows); Group I - 1 month-gestation (4 cows); Group II - 3 month-gestation (4 cows); Group III - 6 month-gestation (3 cows) and was performed from September to December 2015. The second stage comprised 12 pregnant Holstein Friesian cows divided into three groups: Group 0 - Control (6 cows), Group I - 1 month-gestation (3 cows), and Group II - 3 month-gestation (3 cows) and was conducted from April 2016 to February 2017. In both experiment stages, for each group, except for the Control groups, the pregnant cows were offered apple pomace from the *Planalto Catarinense* region (silage) at the dose of 25kg/animal/day. All study animals also grazed on *Trifolium repens* (white clover) and *Pennisetum clandestinum* (kikuyu grass) pasture. In the first stage, animals were offered apple pomace for three months, whereas in the second stage, cows in Group I received apple pomace for eight months and cows in Group II received it for six months (Table 1). Both experiment stages used cows after gestation was confirmed by rectal palpation and ultrasound (US) examination. Two calves were born with malformation: one in each of the experiment stages. At necropsy, tissue samples were collected from the heart, lung, liver, kidney, omasum, abomasum, intestine, spleen, lymph node, skeletal muscle, bone and central nervous system for histological examination. Samples were fixed in 10% formalin, dehydrated in alcohol, clarified with xylene, and embedded in 10% paraffin. 3µm-thick sections of the material were stained using the hematoxylin-eosin (HE) technique (Prophet et al. 1992) and examined under optical light microscopy. Samples of the apple pomace used in the experiment were sent to the Laboratory of Pesticide Residue Analysis (LARP) of the "Universidade Federal de Santa Maria" (UFSM) for residue identification of the pesticides most commonly used in apple crops. Residue analysis was conducted using the modified QuEChERS method; quantification was performed by liquid chromatography coupled with serial mass spectrometry (LC-MS/MS) as described by Kemmerich et al. (2018). Seventy-seven chemical substances were analyzed. The experiments were performed at the Dairy Cattle Sector of the Agronomic Sciences Center located in the municipality of Lages, Santa Catarina state, Brazil

Table 1. Experimental design with apple pomace offered to pregnant cows at the dose of 25kg/animal/day

Year	Group	Bovine (n)	Gestation time (months)	Ingestion time (months)
2015	0 (control)	5	1 to 6	-
	I	4	1	3
	II	4	3	3
	III	3	6	3
2016-2017	0 (control)	6	1 to 6	-
	I	3	1	8
	II	3	3	6

RESULTS

Of the 16 cows in experiment stage 1, one in Group III gave birth to a calf with malformations characterized by complete absence of the coccygeal vertebrae, curved posterior limbs (Fig.1), and scoliosis in the thoracic spine. Thirty days after birth, it presented with diarrhea, developmental delay and limited mobility, and was euthanized. Necropsy revealed lateral deviation of the thoracic spine (Fig.2) and thickened chondral articulations.

The urogenital tract showed deviation of the right ureter, which had a blind ending and extended from the kidney to the cervix; the left uterine horn and ovary were inside the bladder neck (Fig.3). In experiment stage 2, eight cows gave birth to normal calves, and one cow in Group I bred a calf with curved posterior limbs, moderate thickening of the articulations, and flattened hooves, which impaired mobility and balance (Fig.4).

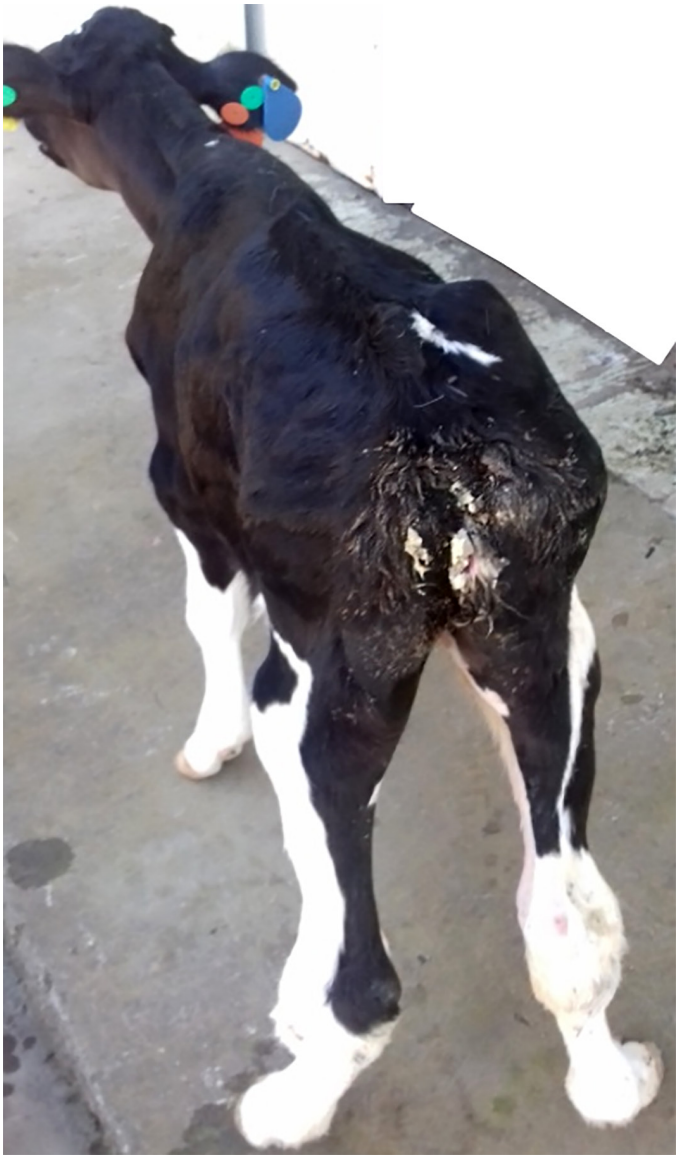

Fig.1. Experimental reproduction (Experiment stage 1). Calf with curved posterior limbs, thickened articulations, and absence of the coccygeal vertebrae.

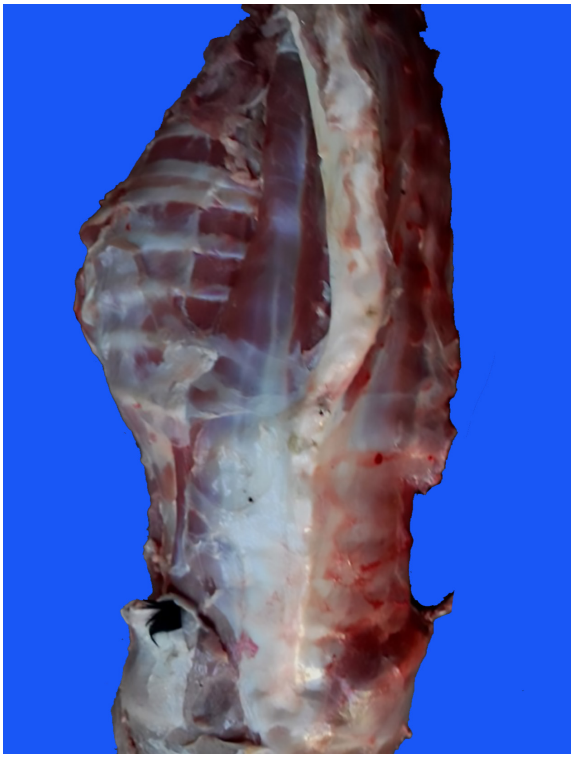

Fig.2. Experimental reproduction (Experiment stage 1). Scoliosis in the thoracic spine of a calf.

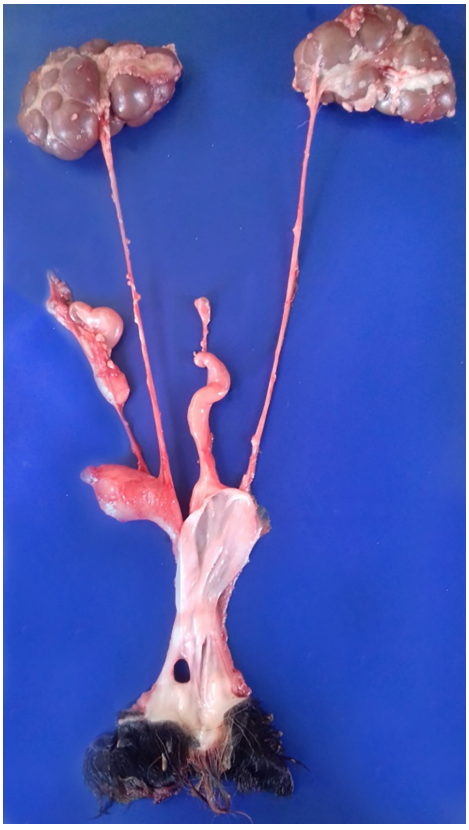

Fig.3. Experimental reproduction (Experiment stage 1). Urogenital tract of a calf showing deviation of the right ureter with blind ending and extending from the kidney to the cervix; the left uterine horn and ovary were inside the bladder neck.

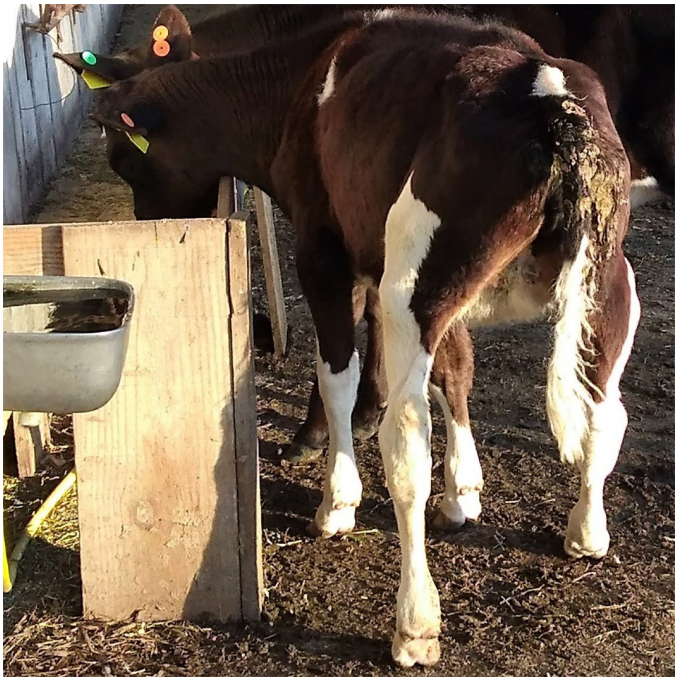

Fig.4. Experimental reproduction (Experiment stage 2). Calf with angular deformity of the pelvic limbs and moderate thickening of the joints.

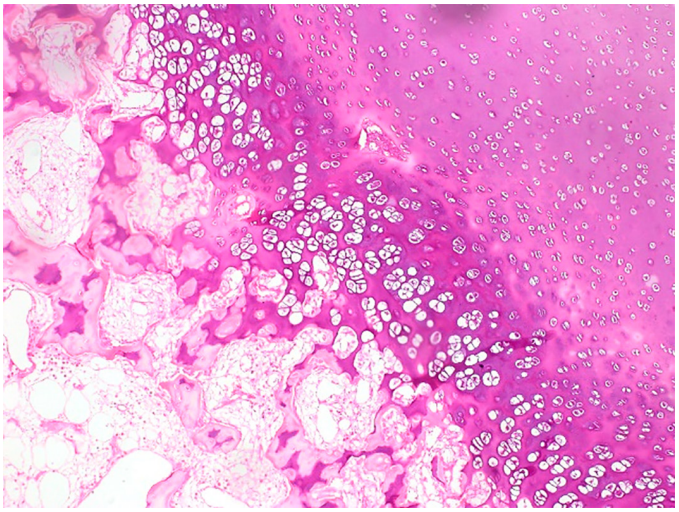

Fig.5. Femur (Experiment stage 1). Disorganized, irregular hypertrophic zone, scarce growth zone, and primary spongy zone with short, slightly mineralized trabeculae. HE, obj.10x.

Microscopically, the femur epiphysis showed disorganized, irregular hypertrophic zone and scarce growth zone. Short, slightly mineralized trabeculae were observed in the primary spongy zone (Fig.5). Analysis of chemicals present in the apple pomace was positive only for the fungicide carbendazim, belonging to the benznidazole chemical group, at a concentration of 0.006mg/kg^{-1}.

DISCUSSION

Experimentally, ingestion of apple pomace by 17 pregnant cows at the dose of 25kg/animal/day for 3 and 8 months, respectively, resulted in malformation in two calves, characterized mainly by curved limbs, especially the posterior quarters. One of the calves showed lateral deviation of the thoracic spine, absence of coccygeal vertebrae, and malformation of the urogenital tract. Bending of the limbs was similar, but less severe than that observed in calves born of cows with natural intoxication with apple pomace (Wicpolt 2018). It is worth noting that in cases of natural intoxication, the apple pomace was of unknown origin and from different regions of the states of Santa Catarina and Rio Grande do Sul. The apple pomace used in this study was from an apple juice extraction plant and, according to information obtained later, the criteria for use of pesticides were very strict.

Assessment of malformation in calves associated with ingestion of apple pomace by the pregnant cows should consider the amount ingested. According to Wicpolt (2018), in cases of natural poisoning, the osseous malformations were observed in properties where the cows ingested amounts greater than 20 kg/animal/day for a period longer than three months.

Malformation due to ingestion of apple pomace by pregnant cows should be assessed by differential diagnosis of the malformations caused by plants. In the Northeast Region of Brazil, ingestion of "jurema preta" (*Mimosa tenuiflora*) and "catingueira" (*Poincianella pyramidalis*) leads to craniofacial osseous anomalies, ocular malformation, and arthrogryposis (Pimentel et al. 2007, Medeiros et al. 2008, Souza et al. 2018, Dantas et al. 2010, Santos et al. 2012). Intoxication with *Lupinus angustifolius*, *Veratrum californicum*, *Nicotiana glauca* (Kellerman et al. 1990), and *Conium maculatum* (Tokarnia et al. 1985) experimentally affect not only bovines, but also ovine, swine and goats, which in addition to malformations, also present signs of salivation, staggering gait, tremors, convulsions, and dyspnea. Of these plants, *Conium maculatum* (poison hemlock) is the only one growing in the South Region; however, this plant was not found in any of the properties were the disease occurred.

Malformations in cattle are also caused by the Bovine Viral Diarrhea (BVD) (Flores & Schuch 2007, Quincozes et al. 2007) and the Bluetongue (Riet-Correa. 2007, Antoniassi et al. 2010) viruses. The aforementioned authors have reported that these pathogens are highly infectious and also affect ovine, goats, and free-living ruminants. In addition to causing erosive lesions and ulcers in the mucosa, affected animals also show respiratory symptoms such as fever, mucopurulent or bloody nasal discharge, embryonic death, and abortion. These clinical signs were not observed in the cows and calves in which the spontaneous disease occurred, or in the cows used in the experiment. Many factors can be involved in malformation in calves born of cows fed apple pomace. The present study was performed based on the hypothesis of association with pesticides present in the bagasse as residues, namely, organophosphates, carbamates (Oliveira-Filho et al. 2010), pyrethroids (Fenster et al. 2006, Wolansky & Harrill 2008), and abamectin (Seixas et al. 2006). Analysis of the apple pomace used in this study detected only residues of carbendazim. According to specifications from Anvisa (2017) and the U.S. Environmental Protection Agency (EPA 2017), carbendazim is a fungicide of toxicological class III, showing medium toxicity. Animal experiments performed by Silva et al. (2014), confirmed the mutagenic and/or carcinogenic potential of carbendazim. According to them, the compound can cause chromosomal anomalies, with change in the number of chromosomes in the cells, leading to DNA damage (mutagenic effects) and affecting embryonic or fetal development (teratogenic/embryotoxicity effects). Janardhan et al. (1984),

verified that doses of 4 to 80mg/kg/day of carbendazim, administered between 6 and 15 days of gestation, increased the incidence of fetal reabsorption. Embryonic death, growth delay, and development of anomalies were also observed by Cummings et al. (1992) with the use of carbendazim in pregnant mice, which was also responsible for causing malformation in fetus with hydrocephalus, deformation of skull and limbs, fusion of vertebrae, ribs and sternum, and anophthalmia (Alvarez 1987).

CONCLUSIONS

Experimentally, apple pomace provided to pregnant cows at the dose of 25kg/animal/day for a period longer than three months cause malformation in calves characterized by osseous deformities, curved hind quarters, thickened joints, absence of coccygeal vertebrae, and malformation of the urogenital tract.

Fungicide carbendazim remains in apple pomace in its residual form, and can be responsible for the malformations observed in the calves of this study.

Conflict of interest statement. - The authors declare that there are no conflicts of interest.

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Original Article
Livestock Diseases

Polioencephalomalacia (PEM) in calves associated with excess sulfur intake[1]

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ABSTRACT.- Withoeft J.A., Bonatto G.R., Melo I.C., Hemckmeier D., Costa L.S., Cristo T.G., Pisetta N.L. & Casagrande R.A. 2019. **Polioencephalomalacia (PEM) in calves associated with excess sulfur intake**. *Pesquisa Veterinária Brasileira 39(6):376-381.* Laboratório de Patologia Animal, Centro de Ciências Agroveterinárias, Universidade do Estado de Santa Catarina, Av. Luís de Camões 2090, Conta Dinheiro, Lages, SC 88520-000, Brazil. E-mail: renata.casagrande@udesc.br

Polioencephalomalacia (PEM) is the morphological characterization for softening of brain gray matter, and excess sulfur intake is one of its main causes. This study describes an outbreak of this disease in 1-to-3-month-old calves in a farm located in Santa Catarina state, Brazil. The herd consisted of 27 Jersey male calves whose diet was composed of initial feed, ground whole corn, and mineral salt. From this herd, 10 animals became ill, showing signs of apathy, anorexia and blindness, evolving to generalized weakness and death. Necropsy was performed in three of these animals, which showed flattening of the cerebral convolutions in addition to softened, yellowish areas in the cerebral cortex. Histopathological examination revealed deep laminar necrosis associated with perineuronal and perivascular edema, as well as neurons with wrinkled, eosinophilic, or vacuolated cytoplasm. The following sulfur doses were observed: 8,010mg/kg in corn, 6,385mg/kg in initial feed, 1,060mg/kg in mineral salt and 2.3mg/L in water, reaching dose values far above the accepted, totaling a daily intake of approximately 6,533.5mg sulfur/animal/day. As differential diagnosis, lead was dosed in the kidneys and liver of the three calves, with negative results. Also, the calf that sickened last was treated with 20mg/kg thiamin and 0.2mg/kg dexamethasone (IM; QID) for three days and eventually recovered. According to anatomopathological findings, excess sulfur intake and therapeutic diagnosis, sulfur poisoning was suggested as the cause of PEM in these 1-to-3-month-old calves. Occurrence of PEM is rare in calves at such a young age.

INDEX TERMS: Polioencephalomalacia, calves, excess sulfur intake, sulfur, neurological diseases, thiamin, ruminants, cattle, pathology.

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RESUMO.- [**Polioencefalomalacia em bezerros associada à ingestão de alimento com excesso de enxofre.**] A polioencefalomalacia (PEM) é a caracterização morfológica para o amolecimento da substância cinzenta encefálica, e uma de suas principais etiologias é a ingestão excessiva de enxofre. Este trabalho descreve um surto desta enfermidade em bezerros de um a três meses de idade em uma propriedade de Santa Catarina. O lote era composto por 27 bezerros machos da raça Jersey, com alimentação composta por ração inicial, milho inteiro triturado e sal mineral. Deste lote, 10 animais adoeceram, apresentando sinais de apatia, anorexia e cegueira, com evolução para fraqueza generalizada.

Nove bezerros morreram e três foram submetidos a necropsia, que demonstraram achatamento das circunvoluções cerebrais além de áreas de amolecimento e coloração amarelada no córtex cerebral. Realizou-se exame histopatológico que evidenciou necrose laminar profunda associada a edema perineuronal e perivascular, além de neurônios com citoplasma enrugado, eosinofílico ou vacuolizado. A dosagem de enxofre resultou em 8010mg/Kg no milho, 6385mg/Kg na ração, 1060mg/Kg no sal mineral e 2,3mg/L na água, atingindo valores muito acima do tolerado, totalizando a ingestão diária de cerca de 6533,5mg de enxofre/animal/dia. Como diagnóstico diferencial realizou-se dosagem de chumbo de amostras de rim e fígado dos três bezerros com resultado negativo. Ainda, o último bovino a adoecer foi tratado com 20mg/Kg de tiamina e 0,2mg/Kg de dexametasona IM, QID, durante três dias e recuperou-se. De acordo com os achados anatomopatológicos e o excesso de enxofre na dieta, sugere-se que a intoxicação por enxofre seja a causa de PEM nestes bezerros de um a três meses de idade, sendo essa enfermidade rara em bovinos tão jovens.

TERMOS DE INDEXAÇÃO: Polioencefalomalacia, bezerros, enxofre, doenças neurológicas, tiamina, ruminantes, bovinos, patologia.

INTRODUCTION

Polioencephalomalacia (PEM) is the morphological characterization for softening (malacia) of brain gray matter (Cunha et al. 2010). Until 1970, this term was believed to represent not only a lesion, but also a disease specific of ruminants associated with thiamine deficiency, characterized by necrosis of the brain cortex (Adams et al. 1956). However, many studies have reported multiple etiologies for cerebrocortical necrosis, such as poisoning with sodium chloride associated with water deprivation (Scarratt et al. 1985), lead poisoning (Traverso et al. 2004), infection with bovine herpesvirus type 5 (BoHV-5) (Cagnini et al. 2015), ingestion of thiamine-rich plants (Ramos et al. 2005), and amprolium (Nogueira et al. 2010) and sulfur (Gould 2000, Kul et al. 2006) poisonings.

Sulfur poisoning is an important cause of PEM, in addition to being common in bovines (Gould 2000, Niles et al. 2000, Traverso et al. 2001, Haydock 2003, Kul et al. 2006, McKenzie et al. 2009). Approximately 0.2% of sulfur can be found in the animal body, which is originated mainly from sulfur-containing amino acids (cystine, cysteine and methionine) and vitamins (biotin and thymine), and ruminal fermentation is the main form of sulfur transformation (Ortolani 2001). Thus, PEM outbreaks related to this etiology are associated with high levels of sulfate, sulfite or sulfide sulfur in feed (Traverso et al. 2001) or water (Gould 2000). Sources of these compounds are variable, including feed additives and pastures rich or contaminated with this element; however, the most common source is water (Olkowski 1997).

PEM progresses with clinical signs that include neurological symptoms such as ataxia, blindness, dysphagia, depression and decubitus (Hamlen et al. 1993), and calves aged 6-9 months can die in 24-48 h (Radostits et al. 2002). In calves, this disease has been reproduced with corn gluten-based diets with high sulfur concentration (Niles et al. 2002), and it has been reported as a natural form in calves that ingested ryegrass (*Lolium multiflorum*) pasture with high contents of this element (Cunha et al. 2010). The present study aims to report a PEM outbreak in calves aged 1-3 months in Santa Catarina state and describe the clinical and anatomopathological changes, as well as the results of the total dose of sulfur in the diet of the investigated animals.

MATERIALS AND METHODS

Epidemiological and clinical data on PEM were obtained with the producer and veterinarian during the visit to the property. Three calves died of natural cause and were submitted to necropsy by the veterinary in charge. Samples of the following organs were collected: encephalon, spinal cord, trigeminal ganglion, pituitary gland, rete mirabile, heart, lung, pre-stomach, abomasum, kidneys, bladder, liver, spleen, lymph node, small and large intestines and fixed in 10% buffered formalin for routine histopathological processing and hematoxylin and eosin (HE) staining. Liver and kidneys were also sent to laboratory lead dosing using the atomic absorption spectrophotometry (AAS) method. Special Ziehl-Neelsen stain was performed in the kidneys to detect eosinophilic intra-nuclear inclusion bodies compatible with lead poisoning. Samples of feed, water, minerals and corn were collected for sulfur dosing using the inductively coupled plasma atomic emission spectrophotometry (ICP-AES) method.

RESULTS

The farm was located in the municipality of Pouso Redondo, region of the *Alto Vale do Itajaí*, Santa Catarina state, Brazil (27°15'29" S; 49°56'02" W). The herd was composed of 27 male Jersey calves aged 15-80 days with weight below the breed average (20 to 90kg). The animals were acquired as newborns from several properties, raised in a wooden shed of approximately 30m^2 with corn shavings and straw bedding. All animals lived together and had access to feed consisting of 15kg of initial feed and 10kg of ground whole corn (straw and cob), totalizing 25kg of feed offered daily. 5x0.4m square feeder troughs were used, not allowing access of all animals at the same time. The property had four cows as dairy nurses, whose milk production varied from 8 to 10L/day, and only the youngest (10 to 12 animals) nursed once a day, whereas the remaining had only solid feed. In addition, mineral salt was freely available from 15 to 20 days of age. The corn used in the animal feed was produced in the property, with use of 250kg of fertilizer and 150kg of urea per hectare.

The producer reported that 10 out of the 27 animals sickened and 9 died between 1 and 3 months of age. Of the calves that died, four were nursing and the remainder were in exclusively solid feed. All animals showed clinical symptoms, including apathy, anorexia and blindness, and evolved to generalized weakness, decubitus, and death in 3 to 4 days. The last animal to sicken was treated with 20mg/kg thiamine (Monovin B1®) and 0.2mg/kg dexamethasone (Cortvet®) (IM; QID) for 3 days and eventually recovered. Necropsy of the three calves revealed flattening of the brain convolutions and diffuse, softened, yellowish areas in the cerebral telencephalic cortex (Fig.1).

Cerebral microscopic lesions in the frontal cortex were characterized by deep laminar necrosis (Fig.2A), associated with perineuronal and perivascular edema, in addition to neurons with intensely eosinophilic cytoplasm, wrinkled or vacuolated (Fig.2B). The remaining organs did not show significant histological changes. Special Ziehl-Neelsen stain was performed, which was negative for the presence of intra-nuclear lead inclusion bodies in the renal tubules.

Liver and kidney samples submitted to lead dosing also revealed negative results. Sulfur dosing for corn, initial feed, mineral salts, and water showed values of 8,010mg/kg, 6,385mg/kg, 1,060mg/kg, and 2.3mg/L, respectively. The final sulfur contents in diet were 95,775mg in initial feed, 466.4mg in mineral salt (considering ingestion of 0.8% bodyweight), 80,100mg in ground corn, and 62.1mg in water (considering ingestion of 1L of water/animal/day, expected for nursing calves up to 60 days of age in the bay). Thus, the total daily sulfur ingestion per animal was approximately 6,533.5mg.

DISCUSSION

According to Klasing et al. (2005), diet sulfur content >3,000 mg/kg in dry matter may cause disease. In this study, the sulfur content was much higher than the maximum limit and contributed to evolution of the acute symptoms (Sant'Ana et al. 2009b, Cunha et al. 2010). In addition, poisoning occurred in very young calves that did not have the ruminal microbiota completely developed and fed exclusively on feed. Moreover, the use of ground corn with high sulfur content was the main trigger for PEM because high starch content diets favor decreased ruminal pH due to changes in the microbiota. Predominance of amidolytic bacteria with faster carbohydrate fermentation causes accumulation of organic acids in the liver, which becomes a favorable environment for production of sulfuric gas, surpassing the liver oxidation capacity, thus contributing to the action of this compound in the central nervous system (CNS) (Sager et al. 1990, Gould et al. 1991). PEM associated with excess sulfur intake can display two clinical forms: in the acute form, animals evolve to death in most cases, preceded by blindness, convulsions, opisthotonus, head pressure against objects and decubitus (Gould 2000), lasting usually two to four days (Sant'Ana et al. 2009b), whereas in the subacute form, animals usually evolve to recovery with mild neurological deficit (Gould 2000). A chronic form of PEM has been reported in cattle with up to 25 days of clinical course (Gonçalves et al. 2001).

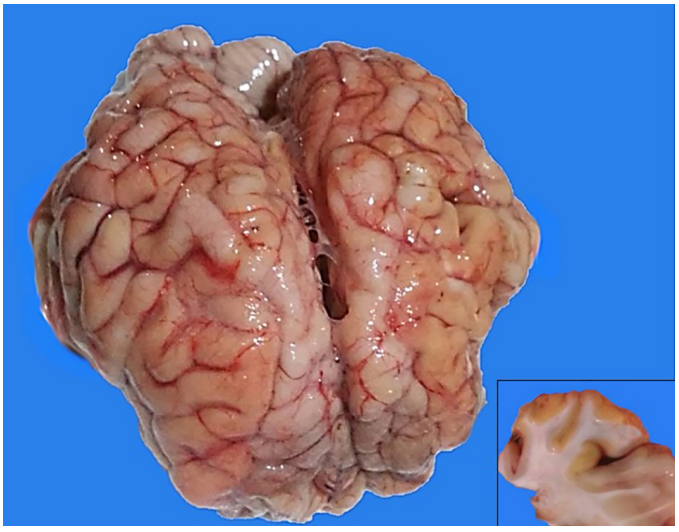

Fig.1. Polioencephalomalacia due to excess sulfur intake in calves. Brain with flattening of the convolutions and focally extensive yellowish areas in the cerebral cortex. Insert: cross section of the frontal cortex showing multiple yellowish areas in the gray matter.

The gross and microscopic lesions observed in the brain of the three investigated calves were similar to those described in a different PEM outbreak caused by excess sulfur intake, with swelling and flattening of the cerebral convolutions, microscopically characterized by laminar necrosis of the cerebral cortex associated with perivascular and perineuronal edema and vacuolated, wrinkled and eosinophilic cytoplasmic neurons (Cunha et al. 2010). Other microscopic lesions commonly observed in bovines with PEM include proliferation of blood vessels with swelling of endothelial cells in the brain, in addition to mononuclear inflammatory infiltrate and presence of Gitter cells (Nakazato et al. 2000). Gitter cells are more commonly found in subacute or chronic cases, and may also show loss of the telencephalic cortex (Moro et al. 1994). However, these changes were not evidenced in this study probably due to the short clinical course. Cases in

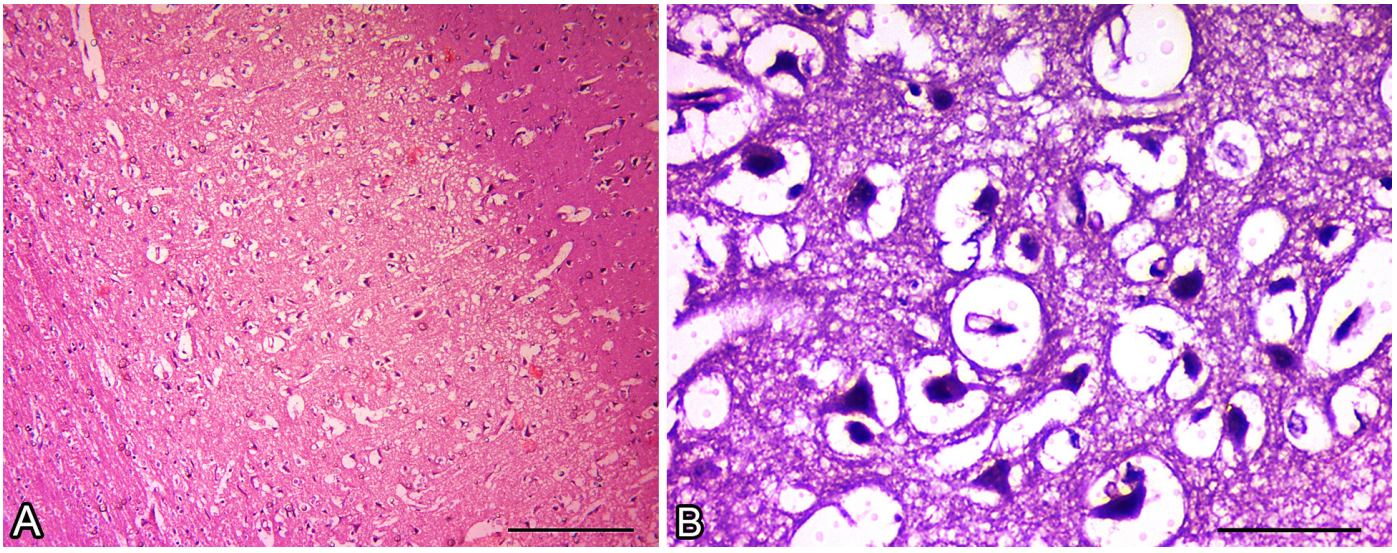

Fig.2. Polioencephalomalacia due to excess sulfur intake in calves. (**A**) Brain with deep laminar cortical necrosis associated with perineuronal, perivascular and neuropilic edema. HE, bar = 100µm. (**B**) Neurons with wrinkled cytoplasm and perineuronal edema. HE, bar = 20µm.

which the disease evolves quickly may present edema only, leading to brain swelling, or even showing no characteristic findings (Nakazato et al. 2000). In some cases, brain edema is accentuated to the point that it leads to cerebellar and bulb herniation through the foramen magnum (Moro et al. 1994). Focal hemorrhages of the thalamus and midbrain were also observed in bovines naturally poisoned with sulfur (Loneragan et al. 1998). Lesion pattern characterized by cortical neuronal necrosis in addition to lesions in the ventral structures of the brain suggest that PEM was caused by sulfur poisoning (Jeffrey et al. 1994); however, the pattern suggested in previous reports was not verified in the present study because, due to the short clinical course, there was not enough time for the development of those lesions.

It is important to take different aspects into account in order to exclude other neurological diseases and causes of PEM, such as thiamine deficiency, which occurs mainly in animals submitted to carbohydrate-rich and low-fiber diets, or changes from poor to good quality pasture (Adams et al. 1956). This occurs because carbohydrate-rich diets ferment quickly and reduce the ruminal pH, leading to a case of ruminal lactic acidosis, which impairs the growth of thiamine-synthetizing microorganisms and favors multiplication of thiaminase-producing bacteria (Haven et al. 1983). Even though the diet of the animals involved in this outbreak already contained concentrate, it also showed high fiber content; therefore, signs of ruminal lactic acidosis were not observed at necropsy and in histological analysis of the rumen. Thus, in young animals, the most common manifestation is primary thiamine deficiency, when not present in enough quantity in the diet. This occurs because animals are not yet able to synthesize this vitamin (Ferreira et al. 1986), and primary deficiency should not be excluded as a PEM-related factor in addition to elemental sulfur toxicosis. Epidemiologically, sulfur poisoning can occur in young or adult animals, depending on the age of the animal exposed to excess of this element (Jeffrey et al. 1994). This aspect is also observed in cases of sodium chloride poisoning (Pearson & Kallfelz 1982); however, in this case, in addition to the microscopic lesions characteristic of PEM, sub-meningeal eosinophilic infiltrate is commonly observed in the Virchow-Robin spaces, or even in the neuropile (Lemos et al. 1997). Cases of lead poisoning usually occur when herds are exposed to pastures containing industrial waste with this metal. The bovine species is the most affected due to lower selectivity of eating habits (Traverso et al. 2004). Bovines with lead poisoning show, in addition to concentrations of approximately 10 ppm in the liver and kidneys (Riet-Correa et al. 2007), degeneration of the kidney tubular epithelium associated with eosinophilic intra-nuclear inclusion bodies evidenced by special Ziehl-Neelsen stain (Traverso et al. 2004). Young bovines submitted to stressful and immunosuppressant situations can develop PEM due to BoHV-5 meningoencephalitis, and normally exhibit perivascular cuffs composed of mononuclear cells, as well as basophilic intra-nuclear inclusion bodies in astrocytes and neurons (Rissi et al. 2006). Cattle experimentally submitted to amprolium poisoning, a thiamine antagonist, showed hemorrhages in the gastrointestinal tract, skin, trachea, subcutis, endocardium and bladder, also visualized microscopically; in addition to the PEM characteristic lesion in the brain, they showed neutrophilic infiltrate in vessels of the leptomeninge in the parietal telencephalon (Nogueira et al. 2010).

Water, mineral salt, pasture and concentrate are important sources of sulfur. Sulfur concentrations >0.4% in the diet may result in cerebrocortical necrosis. Thus, sulfur must be quantified in all diet components as one of the diagnostic tools for excess sulfur intake PEM (Sant'Ana et al. 2009a), as performed during this outbreak. Bacteria of the genera *Desulfotomaculum* sp. and *Desulfovibrio* sp. are essential to sulfur metabolism. They are responsible for its degradation into sulfuric gas, which is a normal sulfur metabolite absorbed or eructed by the animal (Burgess 2008). However, when present at high levels, the ruminal mucosa absorbs it in excess and inhibits the oxidative process of adenosine triphosphate (ATP) production by blocking carbonic anhydrase, catalase, peroxidase, and dehydrogenase. Furthermore, sulfides also act by forming sulfahemoglobin, which has low oxygen transport ability (Bulgin et al. 1996). Nervous tissue is formed by a large amount of lipids, increasing the susceptibility to damage due to intense oxidative metabolism. Thus, sulfur poisoning creates necrotic lesion by producing sulfate-derived radicals (Gould et al. 1991), compatible with the brain lesions visualized in these animals.

Dosing of ruminal hydrogen sulfide (sulfuric gas) assists with the diagnosis of PEM caused by excess sulfur intake, reaching values of 2,300 to 13,500 ppm in ingestion of 0.9% sulfur (Loneragan et al. 1998) and 1,000 to 2,500ppm in ingestion of 0.38% of this element (Cunha et al. 2010) in a dry matter basis. In this outbreak, however, dosing was not possible because no animals with the clinical symptoms were alive by the time the farm was visited.

According to Cunha et al. (2011), dosing of this gas is also important in healthy animals exposed to diet with high sulfur contents, since animals already presenting clinical symptoms can show low indexes of hydrogen sulfide owing to anorexia. Considering the importance of sulfuric gas dosing for the diagnosis of PEM, its absence in the present study does not allow confirmation of sulfur toxicosis, but only suggests it. Water is an important source of sulfur poisoning, mainly in the hot seasons of the year, when there is increased consumption and evaporation, resulting in higher sulfate levels (Cunha et al. 2010). However, water was not the source of poisoning in this outbreak, as only levels >200mg/L can trigger PEM (Klasing et al. 2005). Milk has high levels of calcium and phosphorus and lower levels of the remaining minerals, such as sulfur (González 2001), and this concentration could not contribute to the disease. In the treatment of the animals, in addition to eliminating the source of feed containing excess sulfur, the therapeutic protocol with thiamine and dexamethasone was proven efficient, mainly in animals presenting initial symptoms of the disease. Nevertheless, although this combination was efficient in the present study, it has been shown to be inefficient in previous reports on excess sulfur intake involving ovine (Bulgin et al. 1996). Thiamine administration is important not only in cases of suspected deficiency of this vitamin, but also in those of sulfur toxicosis, since thiamine assists with eliminating the free radicals formed during the metabolism of sulfides and sulfites, protecting the brain tissue (Olkowski et al. 1992).

High doses of sulfur in animal feeding, the therapeutic protocol used in the surviving animal, the negative result for lead dosing in the kidneys and liver, and the absence of additional lesions suggestive of other diseases such as ruminal

lactic acidosis, suggest excess sulfur intake as the cause of PEM in this study. This is an extremely important disease because it is a differential diagnosis for infectious neurological diseases such as rabies and BoHV-5 meningoencephalitis. It also leads to economic losses, requiring controlled handling of animal diet and supplementation.

CONCLUSION

Neurological clinical signs associated with the anatomopathological changes, excess sulfur intake, and the therapeutic diagnosis of the animals show that sulfur poisoning is the cause of polioencephalomalacia (PEM) in calves aged 1 to 3 months; this is a rare disease in calves at such a young age.

Conflict of interest statement. The authors declare no competing interests.

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Original Article
Livestock Diseases

Obstructive urolithiasis in growing-finishing pigs[1]

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ABSTRACT.- Lorenzett M.P., Cruz R.A.S., Cecco B.S., Schwertz C.I., Hammerschmitt M.E., Schu D.T., Driemeier D. & Pavarini S.P. 2019. **Obstructive urolithiasis in growing-finishing pigs**. *Pesquisa Veterinária Brasileira 39(6):382-387*. 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: sauloppvet@yahoo.com.br

Obstructive urolithiasis is a disease characterized by the presence of uroliths in the urinary tract, with consequent obstruction of excretion pathways. This paper described the epidemiological and clinical-pathological findings of 22 outbreaks of urolithiasis in growing-finishing pigs in Southern Brazil. All affected pigs were male and clinical presentation consisted of lethargy, dysuria, rectal prolapse, abdominal distention, peripheral cyanosis and reluctance to move. Clinical progression course ranged from 12 hours to one week, and the lethality rate was 100%. Gross changes were characterized by urinary bladder rupture associated with marked amount of yellowish liquid with ammoniacal odor (urine) in the abdominal cavity (uroperitoneum), as well as mild fibrin deposition on the surface of abdominal organs and hydronephrosis. Urinary uroliths ranging from 0.3 to 1cm in diameter were often observed obstructing the lumen of the penile urethra and sometimes those were free in the abdominal cavity. Histopathological findings included diffuse and marked urinary bladder edema and hemorrhage associated with inflammatory infiltrate of lymphocytes, plasma cells, and macrophages. Diffuse and marked necrosis of the mucosal epithelium was observed in the penile urethra. Intense fibrin deposition and inflammatory infiltrate of neutrophils were noted in the peritoneum, as well as in the serosa of the organs in the abdominal cavity. Uroliths were submitted to the method of qualitative determination of the mineral components, and were compatible with calcium carbonate and magnesium ammonium phosphate. Growing pigs ration analysis revealed low levels of calcium in relation to phosphorus, resulting in a Ca:P ratio of approximately 0.35:1. Histological findings and mineral analysis suggest that outbreaks of urolithiasis were related to a nutritional imbalance in the proportions of dietary calcium and phosphorus. The main cause of mortality was related to dehydration and uroperitoneum.

INDEX TERMS: Swine, urinary system, uroliths, uroperitoneum, mineral imbalance, calcium, phosphorus.

RESUMO.- [Urolitíase obstrutiva em suínos de crescimento e terminação.] Urolitíase obstrutiva é uma enfermidade caracterizada pela presença de urólitos no trato urinário, com consequente obstrução das vias de excreção. Este artigo descreve os achados epidemiológicos e clínico-patológicos de 22 surtos de urolitíase em suínos de crescimento e terminação no Sul do Brasil. Os suínos afetados eram machos e clinicamente apresentavam letargia, disúria, prolapso retal, abaulamento do abdômen, extremidades cianóticas e relutância em movimentar-se. A duração dos sinais clínicos variou de 12 horas a uma semana, e a letalidade foi de 100%. As alterações macroscópicas caracterizaram-se por ruptura da bexiga com acentuada quantidade de líquido de coloração amarelada e odor amoniacal (urina) livre na cavidade abdominal

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(uroperitônio), além de discreta deposição de fibrina sobre os órgãos e hidronefrose. Frequentemente obstruindo o lúmen da uretra peniana e por vezes livre na cavidade abdominal, era possível observar urólitos urinários que variavam de 0,3 a 1cm de diâmetro. Os achados histopatológicos incluíram edema e hemorragia difusos e acentuados na bexiga, associado a infiltrado inflamatório predominante de linfócitos, plasmócitos e macrófagos. Na uretra peniana havia necrose difusa e acentuada do epitélio da mucosa. No peritônio e nas serosas dos órgãos da cavidade abdominal havia intensa deposição de fibrina e infiltrado neutrofílico. Os urólitos foram submetidos ao método de determinação qualitativa dos componentes minerais, os quais foram compatíveis com carbonato de cálcio e fosfato de amônio magnesiano. A análise da ração de crescimento revelou baixos níveis de cálcio, em relação ao fósforo, perfazendo uma relação Ca:P de aproximadamente 0,35:1. Os achados histológicos e as dosagens minerais sugerem que os surtos de urolitíase foram relacionados a um desequilíbrio nutricional nas proporções de cálcio e fósforo dietético. A principal causa da morte dos suínos foi relacionada à desidratação e ao uroperitônio.

TERMOS DE INDEXAÇÃO: suíno, sistema urinário, urólitos, uroperitônio, desbalanço mineral, cálcio, fósforo.

INTRODUCTION

Obstructive urolithiasis is a disease characterized by the presence of uroliths in the urinary tract, with consequent obstruction of excretion pathways (Radostits et al. 2000, Cianciolo & Mohr 2016). Uroliths or calculi are macroscopic mineral concretions, which are composed by precipitated urinary solutes, associated with small amounts of organic matter (Drolet 2012, Cianciolo & Mohr 2016). Urolithiasis affects similarly males and females; however, urinary obstruction is a condition exclusively reported in males, mainly in castrated hogs (Radostits et al. 2000).

Case descriptions of urolithiasis affecting pigs are scarce when compared with other domestic species (Drolet 2012). Nevertheless, similarly to other species, males are more frequently affected due to specific features regarding their urinary tract morphologic anatomy (Maes et al. 2004). The condition is sporadically detected in pigs of all age groups and is occasionally observed as an incidental finding in pigs at the slaughter. Urinary calculi found in pigs may present various compositions, including calcium carbonate, calcium apatite, magnesium ammonium phosphate, uric acid, and urate (Drolet 2012).

Urinary calculi formation frequently results from the interaction of physiologic and nutritional factors with husbandry practices (Loretti et al. 2003), and is mainly related to the excessive or imbalanced ingestion of minerals present in the drinking water and feed (McIntosh 1978, Larson 1996, Radostits et al. 2000, Drolet 2012). Imbalance in the calcium and phosphorus ratio leads to high excretion of urinary phosphate, which is an important factor in the formation of phosphate calculi. Diets presenting high mineral concentration, associated with high levels of mucoproteins in the urine of fast growing animals, are likely the most important factor for calculogenesis (Radostits et al. 2000). Urinary pH, reduced water intake, urinary stasis, treatment with certain drugs, and preexisting urinary tract disease are also factors associated with the occurrence of urolithiasis. Such predisposing factors may act synergistically for calculi formation or play a role individually (Drolet 2012, Sobestiansky 2012). In the present study, the epidemiological and clinical-pathological findings of outbreaks of urolithiasis in growing-finishing pigs in Southern Brazil are reported.

MATERIALS AND METHODS

Clinical and epidemiological data were obtained directly with the farm owner and the referring field veterinarian. Out of forty finishing male pigs showing clinical signs of lethargy and dysuria submitted for necropsy during the outbreaks, four animals underwent histopathological examination. During the necropsy procedures, samples of various organs were collected for histological examination, fixed in 10% formalin, routinely processed and stained by hematoxylin and eosin (HE). In one pig submitted for necropsy, serum samples were collected in order to obtain total serum calcium and phosphorus values. Ration samples (ration for growing pigs, phases 1 and 2) were collected to determine the levels of calcium, phosphorus, magnesium, fluoride, sodium and moisture. In addition, a group of pigs with history of urolithiasis was monitored at the slaughterhouse. In this occasion, 20 urinary bladders were collected and urine samples were obtained for urinalysis. Uroliths collected during the necropsy procedures were tested using the qualitative determination technique for regular components of renal calculus (kit Cálculo Renal Bioclin®, Quibasa Química Básica Ltda, Belo Horizonte/MG, Brasil).

RESULTS

From April 2016 to September 2017, 22 growing-finishing pig farms integrated to the same company in the municipality of Harmonia, Rio Grande do Sul State, Brazil, reported the occurrence of obstructive urolithiasis in pigs ranging from 73 to 163 days of age. During the outbreaks, 44 pigs from 22 different growing-finishing sites died. Groups of pigs ranged from 400 to 600 hogs, with an average of 500 pigs allocated in the same farm. During the outbreaks, these pigs, originally from 3 farrowing farms, were fed the same ration. Feed and water access ad libitum were provided. The types of drinking systems varied from farm to farm, and were represented by drinking bowl (16/22), drinking bowl with nipple (4/22) and nipple drinkers (2/22).

All affected pigs were male, and the clinical manifestation included lethargy and dysuria, which was characterized by vigorous abdominal movements showing an attempt to urinate, causing marked rectal prolapse (Fig.1A), and progressing to distended abdomen (Fig.1B), peripheral cyanosis (distal extremities), and reluctance to move. Clinical signs were observed in pigs ranging from 15 days after allocation in the growing-finishing facilities until the animals reached age for slaughter. Clinical progression lasted from 12 hours to one week. Disease morbidity varied from 0.2 to 1% among different pig herds (average 0.6%), and lethality rate was 100%.

During the outbreaks, 40 growing-finishing crossbred castrated male pigs were submitted for necropsy in order to confirm the diagnosis of urolithiasis. Gross changes were

Fig.1. Obstructive urolithiasis in growing and finishing pigs. (**A**) Pig showing rectal prolapse. (**B**) Pig showing markedly distended abdomen and presenting persistent sternal decubitus position.

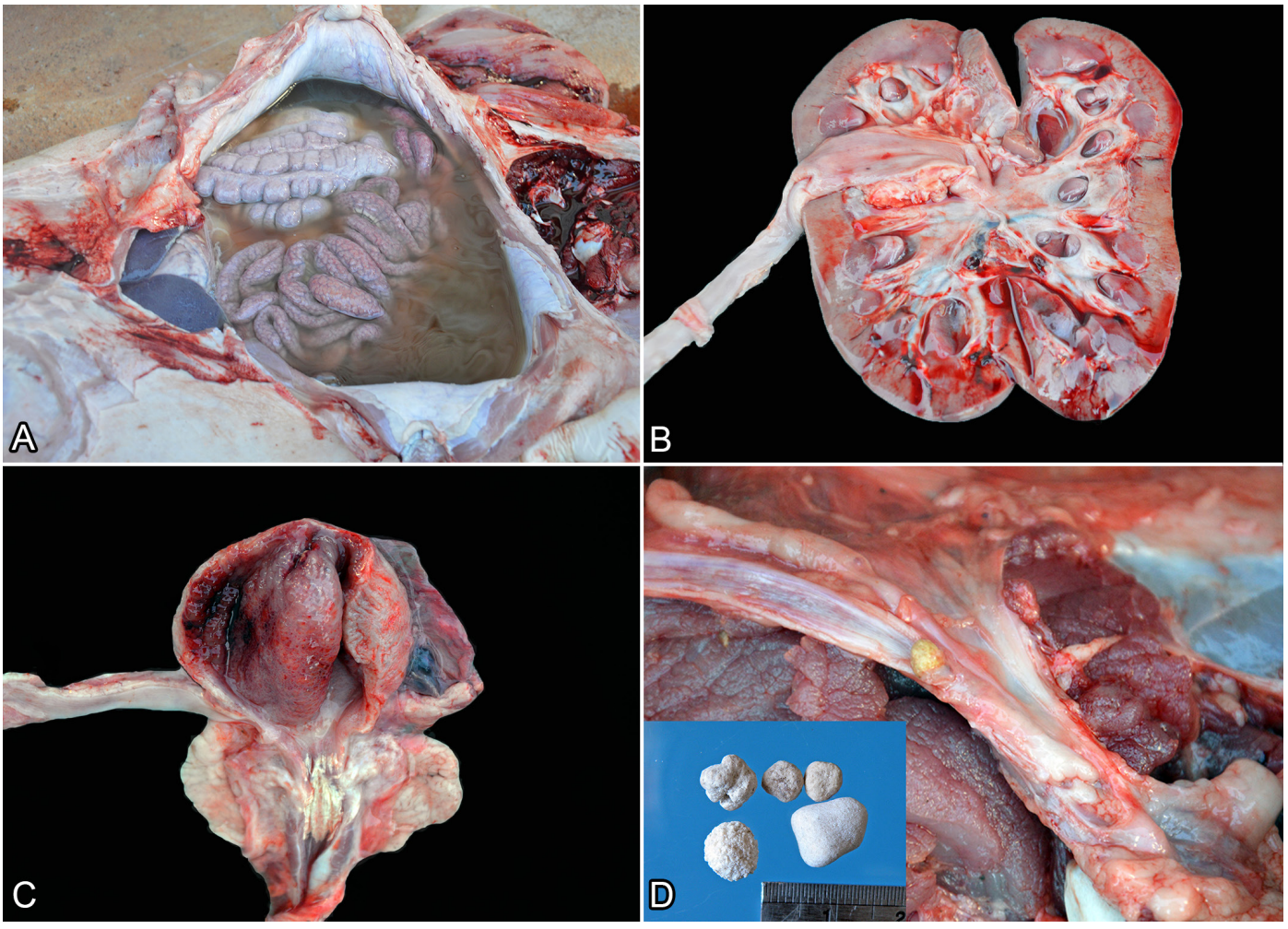

Fig.2. Obstructive urolithiasis in growing and finishing pigs. (**A**) Abundant amount of free yellowish fluid, presenting ammoniacal odor (urine) in the abdominal cavity due to urinary bladder rupture. (**B**) Marked dilatation of ureter (hydroureter), and moderate renal pelvis dilation (hydronephrosis). (**C**) Diffusely reddened, thickened, and irregular urinary bladder mucosa. In the urethra, a focally extensive area of fibrin deposition is noted. (**D**) Urethral luminal obstruction due to a 0.5cm in diameter calculus. Inset (left): size of uroliths.

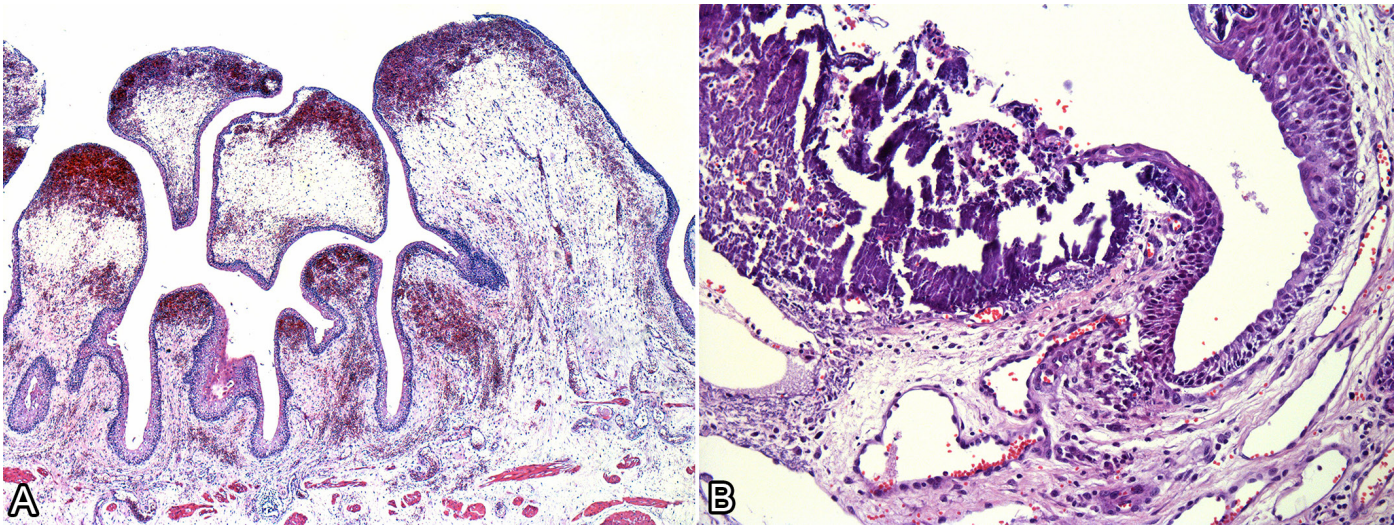

Fig.3. Obstructive urolithiasis in growing and finishing pigs. (**A**) Urinary bladder. Marked diffuse edema of the submucosa, associated with mucosal projections, as well as extensive areas of hemorrhage and multifocal inflammatory infiltrate. HE, obj.4x. (**B**) Urethra. Multifocal necrosis of the mucosal lining epithelium, associated with multifocal mild inflammatory infiltrate of neutrophils, lymphocytes and macrophages, as well as deposition of necrotic cell debris, foci of mineralization and bacillary basophilic bacterial aggregates. HE, obj.20x.'

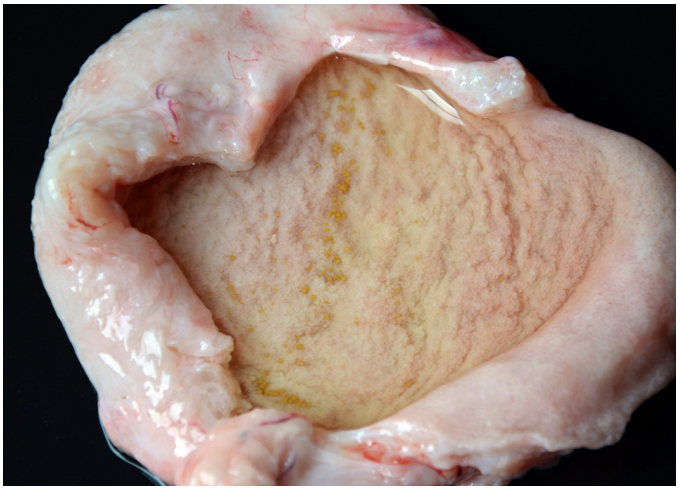

Fig.4. Obstructive urolithiasis in growing and finishing pigs. Urinary bladder presenting multiple uroliths ranging from 0.1 to 0.3cm in diameter. Sample collected during slaughterhouse monitoring conducted in a group of swine with the previous history of urolithiasis.

characterized by bladder rupture associated with abundant amount of free yellowish fluid presenting ammoniacal odor (urine) in the abdominal cavity, as well as mild fibrin deposition on the surface of abdominal organs (Fig.2A), marked bilateral dilatation of ureters (hydroureter), and moderate renal pelvis dilatation (hydronephrosis) (Fig.2B). The urinary bladder mucosa was diffusely reddened, thickened, and irregular and presented mild yellowish fibrillar material (fibrin) deposition. In the distal urethral segment, a focally extensive area of fibrin deposition was noted (Fig.2C). Uroliths ranging from 0.3 to 1cm in diameter were frequently observed obstructing the penile urethral lumen, and sometimes those were free in the abdominal cavity (Fig.2D). The urethral mucosa adjacent to the obstruction site was reddened and ulcerated.

Samples from four pigs were microscopically evaluated and showed diffuse marked edema in the urinary bladder submucosa, as well as extensive areas of hemorrhage and polypoid proliferation in the mucosa (Fig.3A). These lesions were associated with moderate inflammatory infiltrate of lymphocytes, plasma cells, and macrophages. In the penile urethra, marked multifocal necrosis of the mucosal lining epithelium, associated with deposition of necrotic cell debris, foci of mineralization, and bacillary basophilic bacterial aggregates were observed (Fig.3B). Mild to moderate fibrin deposition and infiltrate of neutrophils were noted in the peritoneum and in the serosa of the organs in the abdominal cavity. Marked mesothelial cell hypertrophy and mild fibrous connective tissue proliferation were evidenced in the serosa of the abdominal cavity organs, for instance, in the small and large intestine.

Ration samples of growing pigs (phases 1 and 2) showed magnesium, fluoride, sodium, and moisture levels within reference values recommended for the swine species. Nonetheless, the ration for growing pigs (phase 1) showed calcium and phosphorus values of 1589mg/Kg and 4513/Kg respectively, which corresponds to a Ca:P ratio of approximately 0.35:1 (1.2:1). Ration for growing pigs (phase 2) presented calcium and phosphorus values of 4404mg/Kg and 4169mg/Kg respectively, representing a Ca:P ratio of 1:0.95.

During the slaughterhouse monitoring, 20 urinary bladders were evaluated, of which only one presented uroliths (Fig.4). Six urine samples were submitted for analysis, and no significant alterations regarding density, pH, cellularity, and presence of crystals were noted except for one sample, which presented a small amount of triple phosphate crystals (magnesium ammonium phosphate).

Uroliths collected during the necropsy procedures were submitted to a mineral component qualitative test through the Cálculo Renal Bioclin® kit, which indicated that uroliths were composed of calcium carbonate and magnesium ammonium phosphate. Serum samples were collected from one of the pigs submitted for necropsy. Seric calcium and phosphorus levels were measured, revealing values of 7.05mg/dL (7.1mg/dL) and 13.6mg/dL (9.6mg/dL) respectively. After adjusting the dietary levels of calcium and phosphorus in the ration for growing-finishing pigs in the affected farms, clinical cases of obstructive urolithiasis were no longer observed.

DISCUSSION

The diagnosis of urolithiasis was based on the epidemiological, clinical, and pathological findings, along with the observation of uroliths in the urinary tract of affected animals. In the present study, obstructive urolithiasis was attributed to an imbalance in the ratio of calcium and phosphorus in the diet of growing pigs, since animals were fed the same ration, and the availability of drinking water in the facilities of affected farms was considered adequate in all outbreaks. Furthermore, after dietary levels of calcium and phosphorus in the ration for growing pigs were corrected in the affected farms, cases of obstructive urolithiasis were no longer reported.

Predisposing factors for the occurrence of urolithiasis include diet composition, mainly related to excess or imbalance of minerals, urinary pH, reduced water intake, urinary stasis, metabolic disturbances, and preexistent urinary tract disease. Such predisposing factors may act synergistically or play a role individually in calculi formation (Drolet 2012, Sobestiansky 2012). Excessive consumption of minerals may be associated with artesian wells, and with unbalanced diets, which contain particularly high phosphate concentrations. Sheep fed on diets with high phosphorus concentration present hyperphosphatemia, and consequently show increased phosphorus urinary excretion, which may favor the precipitation of the excess of such mineral in the urinary tract (Radostits et al. 2000).

The ration fed to the growing pigs presented low calcium levels in its composition, which resulted in a calcium to phosphorus ratio of approximately 0.35:1, while the adequate proportion for the swine species is approximately 1.2:1; however, increased calcium proportions, ranging from 1.5 to 2.0, have also been recommended (Radostits et al. 2000, Moreno et al. 2012). Serum levels of calcium (7.05mg/dL) were slightly below the recommended levels for the species, which is 7.1mg/dL, and seric levels of phosphorus (13.6/dL) were above the maximum reference values described for pigs, which is 9.6mg/dL (Radostits et al. 2000, Jackson & Cockcroft 2007).

Calcium carbonate uroliths are originated from calcium salts, which is the mineral type commonly found in pigs (Osborne et al. 1989, Maes et al. 2004), and horses (Neumann et al. 1994). However, differently from horses, which present large amounts of calcium carbonate crystals in the urine and frequently develop uroliths (Neumann et al. 1994), pigs rarely present spontaneous mineral precipitation (Maes et al. 2004). The formation of phosphate uroliths is directly related to the consumption of grain-based rations and mineral supplements, which lead to an increased excretion of phosphorus and magnesium through urine when compared with calcium. Increased levels of the referred minerals along with the availability of ammonia ions in the urine may act synergistically to promote calculogenesis (Manning & Blaney 1986). Uric acid and urate uroliths are frequently observed in dehydrated neonatal piglets, and piglets presenting negative energetic balance, which results from an increase in the catabolism of tissue proteins and purines. Such calculi are observed as orange precipitated mineral deposits, which are noted in the kidneys, ureters, and in the urinary bladder (Kakino et al. 1998, Cianciolo & Mohr 2016). Calculi originated from urinary tract infection are occasionally observed in sows (Drolet 2012). In these situations, crystalluria, characterized by abnormal microscopic precipitation, is considered an important risk factor for the development of urinary tract diseases, such as cystitis and pyelonephritis of bacterial origin (Carr et al. 1995, Drolet 2012, Chigerwe et al. 2013).

Although the occurrence of obstructive urolithiasis is considered to be sporadic, outbreaks affecting large numbers of animals have been reported (Manning & Blaney 1986, Radostits et al. 2000). Pigs rarely develop urolithiasis comparatively with other animal species. Sporadic cases of urolithiasis have been described in pigs of all age groups and occasionally as an incidental finding in swine at the slaughterhouse (Drolet 2012). Even though urinary sediment is frequently observed in both male and female adult pigs, obstructive urolithiasis leading to death of affected pigs is a disease exclusively reported in castrated males. This condition is probably related to the great length and small diameter presented by the urethra in the referred swine category (Carr et al. 1995, Maes et al. 2004).

Typical clinical signs of urinary obstruction by uroliths include reduced feed intake, oliguria or anuria, and abdominal distention and pain (Drolet 2012) similar to the findings described in the reported outbreaks. Lethality rate was high in all studied farms, since all pigs presenting clinical signs of obstructive urolithiasis died. In these cases, death usually results primarily from urinary bladder rupture and secondarily from complete urethral obstruction by calculi, which leads to progressive urine leakage to the abdominal cavity, culminating in a markedly distended abdomen (Radostits et al. 2000, Loretti et al. 2003). Hypertonic urine associated with uroperitoneum promotes the leakage of large amounts of extracellular fluid to the abdominal cavity, leading to severe dehydration, abdominal distention, as well as cardiovascular alterations (Gasthuys et al. 1993, Loretti et al. 2003). Urethral or urinary bladder rupture usually takes place within 2 to 3 days, and death results from uremia or secondary bacterial infection (Radostits et al. 2000, Maes et al. 2004), however, none of the pigs from this study had uremic lesions.

The treatment of pigs with obstructive urolithiasis is mainly surgical (Larson 1996, Van Meter et al. 1996); however, it is not considered economically feasible (Drolet 2012). The main preventive measure for urolithiasis is the supplementation of calcium and phosphorus in the ratio of 2:1 respectively,

in the complete diet (Radostits et al. 2000). Other measures include the addition of 4% sodium chloride in the diet, to stimulate water consumption and increase urine production (Radostits et al. 2000, Loretti et al. 2003), and to ensure that pigs raised in confinement facilities have access to adequate water supply and balanced diets, as well as to avoid urinary tract infections. After adjusting the dietary levels of calcium and phosphorus in the ration for growing-finishing pigs in the affected farms, clinical cases of obstructive urolithiasis were no longer observed.

CONCLUSIONS

In this study, the cause of obstructive urolithiasis was attributed to a nutritional imbalance in the ratio of calcium and phosphorus in the diet. The ration for growing animals fed to the affected pigs presented low calcium levels, which may have led to elevated phosphate excretion through the urinary tract. All affected animals were male and presented clinical signs of reduced feed intake, oliguria or anuria, abdominal distention and pain, with consequent death due to bladder rupture. Lethality rate reached 100%.

Mineral components which predominated in the uroliths found in the present study were calcium carbonate and magnesium ammonium phosphate. The chemical composition of uroliths and environmental risk factors must be considered to determine adequate preventive measures for urolithiasis. It is also crucial to point out the importance of appropriate nutritional management practices to prevent the occurrence of such condition in swine production systems.

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Original Article
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Concentrations of acute-phase proteins and immunoglobulins in serum and synovial fluid in clinically healthy heifers and steers[1]

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ABSTRACT.- Di Filippo P.A., Lannes S.T., Meireles M.A.D., Nogueira A.F.S. & Quirino C.R. 2019. **Concentrations of acute-phase proteins and immunoglobulins in serum and synovial fluid in clinically healthy heifers and steers.** *Pesquisa Veterinária Brasileira 39(6):388-392.* Laboratório de Clínicas e Cirurgia Veterinária, Centro de Ciências e Tecnologias Agropecuárias, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Av. Alberto Lamego 2000, Campos dos Goytacazes, RJ 28013-602, Brazil. E-mail: pdf@uenf.br

The aim of the study was to determine the concentration pattern of intra-articular acute phase proteins (APPs) and immunoglobulins in healthy crossbred cattle. Synovial fluid (SF) samples were collected from the radiocarpal joint of 25 heifers and 25 steers. Concentrations of APPs were measured by SDS-PAGE. The results were submitted to analysis of variance using the SAS statistical program, and means were compared by the Student-Newman-Keuls test ($P<0.05$). Thirty-seven proteins with molecular weights ranging from 7 to 37kDa were identified in SF of all animals. Eight were nominally identified with immunoglobulin A (IgA) and G (IgG), ceruloplasmin (Cp), transferrin (Tf), albumin (Ab), α_1-antitripsin (AAT), α1-acid glycoprotein (AGP), and haptoglobin (Hp). The α_1-antitripsin was only identified in the Sf of the heifers. The SF values of Cp, Hp, AGP and IgA were significantly higher in heifers than in steers. In sera, 34 proteins with molecular weights between 7 and 244kDa were identified in heifers and steers. Similar proteins were nominally identified in the sera, however the α_1-antitrypsin was identified only in SF. The serum values Tf, AGP and IgG were significantly higher in heifers compared with steers. In conclusion, the physiological acute-phase proteins concentrations in synovial fluid of healthy ruminants can be useful in the interpretation of samples from animals with joint diseases. The SF electrophoretic profile of healthy ruminants differs depending on gender. Similar proteins were nominally identified in the sera, but only the SF of α1-antitrypsin.

INDEX TERMS: Acute-phase protein, immunoglobulin, serum, synovial fluid, healthy animals, heifers, steers, arthritis, biomarkers, disease diagnosis, electrophoresis, lameness, cattle.

RESUMO.- [**Concentração de proteínas de fase aguda e de imunoglobulinas no soro e no líquido sinovial de bovinos clinicamente saudáveis.**] O objetivo do estudo foi determinar o padrão de concentração de proteínas de fase aguda e de imunoglobulinas intra-articulares (APPs) em bovinos mestiços saudáveis. As amostras de fluido sinovial (SF) foram coletadas da articulação radiocárpica de 25 novilhas e 25 novilhos. As concentrações de APPs foram mensuradas por SDS-PAGE. Os resultados foram submetidos à análise de variância usando o programa estatístico SAS, e os meios foram comparados pelo teste Student-Newman-Keuls ($P<0,05$). Trinta e sete proteínas com pesos moleculares variando de 7 a 37kDa foram identificadas no SF de todos os animais. Oito foram nominalmente identificadas como imunoglobulina A (IgA) e G (IgG), ceruloplasmina (Cp), transferrina (Tf), albumina (Ab), a1-antitripsina (AAT), glicoproteína a1-ácido (AGP) e haptoglobina (Hp). A α1-antitripsina foi identificada apenas no SF das novilhas. Os valores de Cp, Hp, AGP e IgA

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no SF foram significativamente maiores em novilhas do que em novilhos. No soro, 34 proteínas com pesos moleculares entre 7 e 244kDa foram identificadas nas novilhas e novilhos. Proteínas similares foram identificadas nos soros, mas apenas o SF das novilhas apresentou a α1-antitripsina. Os valores séricos de Tf, AGP e IgG foram significativamente maiores em novilhas em relação aos novilhos. Conclui-se que a mensuração das concentrações das proteínas da fase aguda no líquido sinovial de animais saudáveis pode ser útil na avaliação de amostras oriundas de bovinos com afecções articulares. O perfil eletroforético do SF de ruminantes saudáveis difere em função do gênero e as diferenças devem ser levadas em consideração na interpretação dos achados.

TERMOS DE INDEXAÇÃO: Proteínas de fase aguda, imunoglobulina, soro, líquido sinovial, animais saudáveis, artrite, biomarcadores, diagnóstico de doenças, eletroforese, claudicação, bovinos.

INTRODUCTION

Diagnosis of joint damage is based upon clinical orthopedic examination and radiographic assessment, both of which can be non-specific and insensitive in early joint pathologies (Hurter et al. 2005). Recent studies have investigated other methods to overcome these obstacles. Magnetic resonance imaging, computed tomography and evaluation of biochemical markers in synovial fluid have been used (Hegemann et al. 2002, Jacobsen et al. 2006a). Synovial fluid (SF) is a dialysate of plasma that contains proteins, electrolytes and hyaluronic acid, capable of reflecting infections, immunological, or inflammatory joint conditions by altering its composition and appearance (Basile et al. 2013).

Joint damage induces the production of nitric oxide, interleukin-1, interleukin-6, tumor necrosis factor, metalloproteinases, inhibitors of metalloproteinases, antibodies and others, which trigger the acute-phase response and production of acute-phase proteins (Gruys et al. 2005). The acute phase proteins (APPs) can be produced by both hepatocytes and peripheral tissues, and can be classified according to their concentration in positive APP, if they increase, or negative APP, if they decrease. The APPs are believed to play major roles in several aspects of the systemic reaction to inflammation, including the opsonization of several pathogens, the scavenging of potentially toxic substances and the overall regulation of different stages of inflammation (Petersen et al. 2004). The APPs are sensitive factors that allow the early and precise detection of inflammation in ruminants (Kent 1992).

Intra-articular injection of LPS induced systemic and local acute-phase response in horses (MacDonald & Benton 1996, Jacobsen et al. 2006b). Increases in interleukin-1, interleukin-6, and tumor necrosis factor-α have also been measured in cases of naturally occurring acute and severe chronic synovitis in horses (Bertone et al. 1993). By demonstrating SF-specific intraarticular serum amyloid (SAA) isoforms, the results obtained by Jacobsen et al. (2006b) suggested that acute-phase protein is synthesized locally in the inflamed equine joint, similar to what has been demonstrated in humans previously. Measuring local APP levels improves precision of diagnosis, because it provides information on inflammatory/infectious status of the particular organ of interest. However, this potential has been explored only to a very limited degree, and much more research is needed in this field (Jacobsen 2007). To our knowledge, SF-APPs have not been investigated specifically in cattle.

Limb pathology is also a serious problem in cattle, since lameness negatively affects welfare and economic production. However, the diagnosis of inflammatory processes in these animals is difficult because the clinical symptomatology is quite poor. Also, alterations in the classic parameters of the inflammatory response are relatively slight and not very specific. Serum amyloid A was useful for monitoring and early detection of arthritis in dairy cows (Jawor et al. 2008). In addition, severe sole ulcers and white line abscesses increased serum APPs in cows (Kujala et al. 2010). Based on the dynamic of haptoglobin, Smith et al. (2010) found which treatment was more effective in dairy cattle diagnosed with claw disorders.

Although these studies have demonstrated the importance of APPs in the diagnosis, monitoring and treatment of limb pathology in cattle, so far the local levels of APPs have not been detected. Therefore, the aim of this study was to determine the physiological concentrations of acute-phase proteins and immunoglobulins in synovial fluid (SF) and compare them to isoforms detected in serum of healthy heifers and steers.

MATERIALS AND METHODS

All procedures applied in this study were approved by the animal research ethics committee of "Universidade Estadual do Norte Fluminense Darcy Ribeiro" (protocol number 291). Fifty crossbred cattle with average age of 18±1.2 months and weighing 350±70kg, free from evidence of joint disease (as determined by thorough clinical examination, flexion tests, complete blood count and synovial fluid analysis) were used in the study (25 heifers and 25 steers). The animals were kept in pastures (*Brachiaria decumbens*) on the same farm. They had ad libitum access to water and mineral salt and received commercial feed containing 16% crude protein (min). The animals were immunized against clostridiosis (Poli-Star®, Vallé), rabies (Raivacel Multi®, Vallé) and foot and mouth disease (Bovicel®, Vallé).

Synovial fluid was collected at the radiocarpal joint site using a 25 x 0.7mm needle with the animals standing positioned in a head gate. Before arthrocentesis, the joint was shaved, washed using neutral detergent (three times), and rinsed with 70% alcohol. From each animal, 5 mL of jugular venous blood was drawn for serum samples. These were immediately centrifuged (8°C, 2,500g, 10 min at room temperature), and the supernatant was aliquoted and frozen within two hours of collection. The SF and serum samples were kept frozen in sterile Epperdorf tubes at -20°C until analysis.

Total proteins were determined by the Biuret method, and the serum and synovial proteinogram were obtained by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Laemmli 1970). Molecular weights and concentrations of protein fractions were determined by computed videodensitometry (CS 9000, Shimadzu Corp., Kyoto, Japan). Reference markers (Sigma Chemical Co., St Louis) were used to characterize proteins, with molecular weights of 29, 45, 66, 97.4, 116, and 205kDa. Also, electrophoretic migration of proteins was compared with that of pure proteins, including albumin, transferrin, haptoglobin, ceruloplasmin, IgA, IgG, α1-antitripsin and acidic glycoprotein.

Analysis of variance (Proc GLM) was performed with a model including the fixed effect of time, sex and simple interactions. Data were expressed as the mean ± standard deviation (SD). The means were compared by the Student-Newman-Keuls test (SNK) of SAS (SAS Institute Inc., Cary/NC, 2012). Differences were considered significant with $P<0.05$.

RESULTS

The concentrations of the serum and SF proteins are summarized in Table 1 and 2. Thirty-seven proteins with molecular weights ranging from 7 to 37kDa were identified in SF of all animals. Eight were nominally identified with immunoglobulin A (IgA) and G (IgG), ceruloplasmin (Cp), transferrin (Tf), albumin (Al), α_1-antytripsin (AAT), α1-acid glycoprotein (AGP), and haptoglobin (Hp). Cp, Hp, AGP and IgA levels in the synovial fluid were significantly higher in the heifers than in the steers. Only heifers SF presented α_1-antytripsin.

In sera, 34 proteins with molecular weights from 7 to 244kDa were identified in heifers and steers. Similar proteins were nominally identified in the sera, but only the SF contained α_1-antitripsin. The serum values of Tf, AGP and IgG were significantly higher in heifers than steers. APP levels in the synovial fluid were significantly lower than the levels in the serum of all animals.

DISCUSSION

The diseases that affect the locomotor system of cattle are one of the main health problems of the dairy industry and generate important questions about economic and animal welfare aspects (Warnick et al. 2001, Green et al. 2002). The economic losses result from lower milk production, ill temper of animals, fertility problems, increased need to cull animals and costs for diagnosis and treatment. Many studies have shown to the presence of an acute-phase response associated with locomotor infections or inflammation (Jawor et al. 2008, Smith et al. 2010, Tóthová et al. 2011). In horses, has been shown that APPs levels in synovial fluid can help distinguish infectious from non-infectious joint disease, and when synovial fluid APPs levels were measured sequentially in the same patient, levels reflected effect of treatment (Jacobsen et al. 2006a). This is the first study to determine the APPs levels in synovial fluid of healthy ruminants.

Ruminants are significantly different to other species in their acute phase response in that Hp is a major APP. In healthy cattle the serum Hp concentration is <20mg/L but can increase to >2g/L within 2 days of infection (Petersen et al. 2004). Elevations in this protein have also been reported in dairy cows with pododermatitis septica, pododermatitis circumscripta, interdigital necrobacillosis and papillomatous digital dermatitis. In the animals suffering from pododermatitis septica and interdigital necrobacillosis, the Hp concentrations decreased after treatment lasting one to five days, indicating the effectiveness of treatment for these diseases. In contrast, the treatment did not affect the concentrations of Hp in animals with pododermatitis circumscripta (Smith et al. 2010). Hp binds with free hemoglobin (Hb), which is toxic and pro-inflammatory, thus reducing the oxidative damages associated with hemolysis (Yang et al. 2003). Hp can inhibit the proliferation of mastocytes, prevent the spontaneous maturation of Langerhans cells and suppress the proliferation of T cells (Xie et al. 2000, Arredouani et al. 2003). It also has an inhibitory effect on granulocyte chemotaxis, phagocytosis and bactericidal activity (Rossbacher et al. 1999). In this study, regardless of sex, Hp concentrations were detected in serum and synovial fluid (Table 1 and 2). Hp was also detected in the synovial fluid of healthy horses (Basile et al. 2013).

Jawor et al. (2008) evaluated the concentrations of APPs at distinct times during the treatment of cows with limb diseases. The highest concentrations of Hp, serum amyloid A (SAA) and fibrinogen (Fbg) were observed at the start of treatment. The authors noted a gradual reduction in the concentration of APPs in the cows whose treatment occurred without complications. On the other hand, in cows showing additional complications (e.g., viral infections, bronchitis, occurrence of other inflammatory states of the limbs), they observed increases in one or two of the APPs measured. They concluded that monitoring APP concentrations can be a valuable complement for the clinical assessment of treatment and early detection of possible disease complication. Fibrinogen (Fbg) is involved in homeostasis, supplying a substrate for the formation of fibrin and tissue repair, in turn providing a matrix for migration of inflammatory cells (Thomas 2000). Serum amyloid A is involved in the recruitment of inflammatory cells to the infection site (Xu et al. 1995), promotes the neutralization of endotoxins, inhibits the proliferation of lymphocytes and endothelial cells, and deters the aggregation of platelets and the adhesion of T lymphocytes to the extracellular protein matrix (Urieli-Shoval et al. 2000). In horses, SAA is synthesized locally in the mammary gland and in joints, and the protein has been shown in normal colostrum and synovial fluid from horses with experimentally induced arthritis (MacDonald et al. 1996, Jacobsen et al. 2006b).

Table 1. Serum protein concentrations determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, in crossbred heifers and steers (mean ± SD)

Protein (mg/dl)	Bovines		P-value
	Heifers	Steers	
Total serum protein	8982.5±419.1A	8488.7±166.3A	0.28
Albumin	5142.1±157.4A	5021.7±98.8A	0.52
Ceruloplasmin	11.00±0,81A	13.96±1.46A	0.08
Transferrin	335.60±22.01A	267.54±22.01B	0.03
Haptoglobin	9.44±1.44A	7.65±0.92A	0.30
α1-Acid glycoprotein	13.42±0.73A	7.98±0.73B	0.00
Immunoglobulin A	174.90±17.81A	138.60±17.81A	0.15
Immunoglobulin G	2799.36±95.162A	2070.74±95.162B	0.00

P-value = significance of the differences in means; different letters in the same row indicate differences between groups (P<0.05).

Table 2. Synovial fluid protein concentrations determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, in crossbred heifers and steers (mean ± SD)

Protein (mg/dl)	Bovines		P-value
	Heifers	Steers	
Total serum protein	1195.00±394.33A	1116.50±510.38A	0.60
Albumin	514.92±389.47A	571.98±323.06A	0.62
Ceruloplasmin	1.56±0.92A	0.43±0.45B	0.0001
Transferrin	44.38±27.94A	44.51±24.26A	0.98
Haptoglobin	3.01±1.59A	1.64±1.26B	0.005
α1-Acid glycoprotein	3.25±1.20A	2.20±1.23B	0.01
Immunoglobulin A	17.35±12.48A	10.09±7.81B	0.03
Immunoglobulin G	224.88±100.23A	204.71±130.35A	0.59
α1-antitripsina	26.802±17.8	-	-

P-value = significance of the differences in means; different letters in the same row indicate differences between groups (P<0.05).

Significant increases in the serum concentrations of Hp, SAA and Fbg were found by Tóthová et al. (2011) in heifers suffering from hoof diseases (pododermatitis, laminitis, sole ulcer, and digital dermatitis). In turn, Laven et al. (2004) did not find a correlation between the presence of an acute-phase response and the development of sole hemorrhages in postpartum first-lactation heifers. According to them, only more severe pathologies, such as sole ulcers and interdigital necrobacillosis would be able to induce a systemic APR. The APPs measured by Laven et al. (2004) (albumin, Fbg, Hp, seromucoid and Cp) are considered to have high sensitivity to the species, despite the SAS being considered the most sensitive APP in bovines, with rapid increase in the blood after inflammatory stimulus (Werling et al. 1996, Kujala et al. 2010). The SAA is not measured by polyacrylamide gel electrophoresis and its determination has high costs, which often makes it difficult to determine it routinely. Therefore, Kujala et al. (2010) reported that cattle diagnosed with sole ulcers and/or e/or white line abscesses showed higher serum concentrations of SAA, although the concentrations of Hp remained unchanged. According to Young et al. (1996) and Kujala et al. (2010), Hp requires a stronger stimulus to induce an increase in the blood. Furthermore, the acute-phase response can vary between animals faced with the same challenge (Lomborg et al. 2008). Smith et al. (2010) and Jawor et al. (2008) reported that animals with the same hoof injuries presented high or undetectable serum concentrations of Hp. According to Jacobsen et al. (2004), the ability to produce determined APPs is an innate trait of the individual.

The analysis of the acute-phase proteins present at the site of interest (e.g., synovial fluid) increases the precision of diagnosis and helps to distinguish between inflammatory and infectious processes affecting the joints. It also allows evaluating the effectiveness of treatment (Jacobsen et al. 2006b). APPs have already been measured in the synovial fluid of equines (Jacobsen et al. 2006a, Basile et al. 2013, Di Filippo et al. 2014) and humans (Catterall et al. 2010). Nevertheless, little is known about the APPs present in the synovial fluid of cattle. In this study, we identified eight proteins in the SF - ceruloplasmin, transferrin, albumin, α_1-antitripsin, α1-glycoprotein acid, haptoglobin and immunoglobulin A and G. The identification and quantification of the APPs in the SF of healthy cattle can allow comparison with samples suffering from inflammatory and/or infections processes of the joints, as already performed in other species.

In this study, the male and female cattle studied were the same age and were raised on a single farm with the same management practices. Therefore, the differences between the levels of APPs present in the serum and SF between males and females can be attributed to gender. Differences between genders have already been described for competitive horses (Escribano et al. 2008, Cywinska et al. 2011, Di Filippo et al. 2016, Martins et al., 2017). Nothing has been demonstrated previously in ruminants, so further investigation is necessary. Thus, age, gender, and physiological condition should be considered when interpreting a measured APP concentration.

CONCLUSIONS

The establishment of the physiological acute-phase proteins concentrations in synovial fluid of healthy ruminants can be useful in the interpretation of samples from animals with joint diseases.

Further investigations are needed to establish the utility of synovial protein electrophoresis in bovine clinical practice in relation to inflammatory or infectious diseases.

The synovial fluid electrophoretic profile of young ruminants changes based on sex.

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Original Article
Small Animal Diseases

Feline lymphoma in the nervous system: pathological, immunohistochemical, and etiological aspects in 16 cats[1]

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ABSTRACT.- Mello L.S., Leite-Filho R.V., Panziera W., Bandinelli M.B., Sonne L., Driemeier D. & Pavarini S.P. 2019. **Feline lymphoma in the nervous system: pathological, immunohistochemical, and etiological aspects in 16 cats.** *Pesquisa Veterinária Brasileira 39(6):393-401*. Setor de Patologia 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: saulo.pavarini@ufrgs.br

The pathological, immunohistochemical (IHC), and etiological features of lymphoma involving the nervous system (NS) in cats were analyzed through a retrospective study (2004-2017) in Rio Grande do Sul State, Brazil. The NS involvement was observed in 16 (12.2%) of 125 felines with lymphoma. Young cats were mainly affected, with a median of 24 months old. Most cases were secondary central NS lymphoma, whereas in three cats, the NS involvement was primary. IHC revealed 14 (87.5%) FeLV-positive, six FIV-positive, and one FeLV/FIV-negative cats. Distribution of feline lymphoma in the NS was 8/16 in the spinal cord, 7/16 in the brain, and 1/16 in the paravertebral nerves and ganglia (neurolymphomatosis). The lymphoma pattern in the spinal cord was exclusively extradural, often focal (6/8), and located in the lumbar (3/6), sacral (1/6), thoracic (1/6), and cervical segments (1/6). Brain neuroanatomical patterns were: leptomeningeal lymphomatosis (4/7), lymphomatous choroiditis (2/7), and intradural lymphoma (1/7). The feline with primary neurolymphomatosis presented a marked thickening of paravertebral nerves and ganglia from the sacral region. B-cell lymphoma (75%) was often diagnosed, and diffuse large B-cell lymphoma (DLBCL) (11/16) was the main subtype. T-cell lymphoma (25%) was less commonly observed and was classified as peripheral T-cell lymphoma (PTCL) (3/16) and T-cell lymphoblastic lymphoma (T-LBL) (1/16).

INDEX TERMS: Feline, lymphoma, nervous system, immunohistochemistry, etiology, cats, neuropathology, FeLV, extradural lymphoma, leptomeningeal lymphomatosis, lymphomatous choroiditis, neurolymphomatosis, pathology.

RESUMO.- [**Linfoma no sistema nervoso de felinos: aspectos patológicos, imuno-histoquímicos e etiológicos em 16 gatos.**] Os aspectos patológicos, imuno-histoquímicos (IHQ) e etiológicos do linfoma envolvendo o sistema nervoso de felinos foram analisados através de um estudo retrospectivo (período de 2004-2017) no Estado do Rio Grande do Sul, Brasil. O envolvimento do sistema nervoso foi observado em 16 (12,2%) dos 125 felinos com linfoma desse estudo e afetou principalmente, jovens com idade mediana de 24 meses. A grande maioria dos casos o linfoma era secundário no sistema nervoso central e somente em três gatos o linfoma foi primário do sistema nervoso. Na IHQ, 14 (87,5%) casos foram positivos para FeLV, seis (37,5%) para FIV, e um foi negativo para ambos. A distribuição do linfoma no sistema nervoso foi em 8/16 felinos na medula espinhal, 7/16 no encéfalo e em 1/16 em nervos e gânglios paravertebrais (neurolinfomatose). Na medula espinhal, o padrão do linfoma foi exclusivamente extradural e frequentemente focal (6/8), localizadas nos segmentos lombares (3/6), sacrais (1/6), torácicos (1/6) e cervicais (1/6). No encéfalo, os padrões neuroanatômicos observados foram: linfomatose leptomeningeal (4/7), coroidite linfomatosa (2/7), linfoma intradural (1/7).

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No felino diagnosticado com neurolinfomatose primária, foi observado acentuado espessamento dos nervos e gânglios paravertebrais da região sacral. Os linfomas de células de células B (75%) foram os mais frequentes e o principal tipo foi o linfoma difuso de grandes células B (11/16). Os linfomas de células T (25%), menos observados, foram classificados como linfomas de células T periférico inespecífico (3/16) e linfoma linfoblástico T (1/16).

TERMOS DE INDEXAÇÃO: Linfoma, sistema nervoso, felinos, imuno-histoquímica, etiologia, gatos, neuropatologia, FeLV, linfoma extradural, linfomatose leptomeningeal, coroidite linfomatosa, neurolinfomatose, patologia.

INTRODUCTION

Lymphoma is one of the most common neoplasms in cats and typically young cats are affected, accounting for up to 90% of the hematopoietic tumors (Hardy 1981, Schmidt et al. 2010).

Lymphoma mainly arises from lymph nodes and less commonly from other sites, such as the spleen, liver, tonsils, gastrointestinal tract, and nasal cavity (Valli et al. 2017). Anatomically, multicentric, and alimentary lymphomas are the most common form of lymphoma (Reinacher 1989, Sato et al. 2014, Valli et al. 2016).

The main current classification used for lymphomas was formulated by the World Health Organization (WHO), adapted for animals and applied for the classification of feline lymphomas (Vezzali et al. 2010, Valli et al. 2016). The main purpose of this classification system is to correlate histotypes (phenotypic and immunophenotypic) and biological behavior (Vezzali et al. 2010, Valli et al. 2017). Lymphoma is a common CNS neoplasm in cats affecting the spinal cord more than the brain (Troxel et al. 2003, Tomek et al. 2006, Marioni-Henry et al. 2008).

Nervous system (NS) involvement occurs in approximately 12% of cats with lymphoma and usually as part of a generalized disease (Lane et al. 1994, Marioni-Henry et al. 2008). Lymphoma has a wide range of distribution patterns in the feline NS. However, there are limited papers that classify feline lymphoma in the NS, especially those that correlate different patterns and types of lymphoma (Mandara et al. 2016). Thus, the objectives of this study were to determine the epidemiological and anatomopathological aspects of feline lymphomas in the nervous system, in addition to classifying this neoplasm according to WHO, aiming to associate it with lymphoma distribution patterns in the NS.

MATERIALS AND METHODS

All post-mortem records of cats diagnosed with lymphoma were reviewed from January 2004 to January 2017, and cases with nervous system (NS) involvement were selected. All cats studied were from the metropolitan area of Porto Alegre, State of Rio Grande do Sul, Brazil. All data containing information were grouped, registered, and categorized according to age, breed, and sex. Additionally, gross distribution of lymphoma in the NS and extraneural sites were evaluated. The anatomic form was classified according to Gabor et al. (1998).

The anatomic distribution patterns of lymphoma in the central and peripheral NS were evaluated using histological slides stained with hematoxylin and eosin (HE) and characterized according to Mandara et al. (2016).

The classification of lymphoid neoplasms was carried out according to the system adopted by WHO as applied for use in animals (Valli et al. 2016). The NS tissue with neoplastic infiltrate were subjected to immunohistochemistry (IHC) for immunophenotypic analysis of neoplastic lymphocytes by applying primary antibodies CD79α (B-cell marker) and CD3 (T-cell marker). Additionally, FeLV and FIV IHC tests were carried out for the same tissues. The IHC technique was performed on tissue sections mounted on positively charged glass slides previously deparaffinized and dehydrated. The positive controls for IHC consisted of cat normal lymph node and spleen for lymphocyte markers (CD3 and CD79α) and previously tested lymph nodes from cats infected with the FeLV and FIV virus. Negative control sections were incubated with tris-buffered saline (TBS) in place of specific antibodies. Immunohistochemistry sections were counterstained with Harris hematoxylin. The data regarding techniques performed and antibodies employed is shown in Table 1.

RESULTS

Out of 125 cases of lymphoma, 16 (12.8%) exhibited NS involvement. Of the 16 cats, there were 9 females (56.3%) and 7 males (43.7%), 15 mixed breed (93.75%) and one Siamese. The ages ranged from 6 to 156 months, with a median of 24 months. According to anatomical forms, the feline lymphomas were classified into mixed (6/16), atypical (6/16), mediastinal (3/16), and abdominal (1/16). The immunostaining was positive in 14 (87.5%) cases for the FeLV antigen, 6 (37.5%) cases for the FIV antigen, and 1 was negative to both.

From the 16 cases of NS lymphoma, three were considered primary NS lymphoma and 13 secondary NS lymphoma (multicentric-form derived). The distribution of feline lymphoma in the NS was: spinal cord (8/16), brain (7/16), spinal nerves and paravertebral ganglia (1/16). Immunohistochemical

Table 1. Antibodies and immunohistochemistry procedures

Antibody	Antigen retrieval	Dilution	Detection method	Chromongen
Mouse antifeline leukemia virus gp 70[a]	40 min/100°C Tris EDTA buffer pH 9.0	1:500	MACH 4	AEC
Mouse antifeline immunodeficiency virus p24gag[a]	40 min/100°C Citrate buffer pH 6.0	1:200	MACH 4	AEC
Mouse antihumam CD79αcy[b]	20 min/100°C Tris EDTA buffer pH 9.0	1:100	MACH 4	DAB
Polyclonal rabbit antihuman CD3[b]	15 min/RT Protease XIV	Ready-to-use	MACH 4	AEC

[a] Bio-rad®, [b] Dako®; MACH 4 = Universal HRP-Polymer (Biocare®), AEC = 3-amino-9-ethylcarbazole (Dako), DAB = 3,3'Diaminobenzidine (Dako®), RT = room temperature.

analysis revealed that 75% of the cases (12/16) had immunostaining for CD79α (B cell lineage), whereas 25% (4/16) had immunostaining for CD3 (T cell lineage). All CD79α$^+$ cases were mature (peripheral) B-cell neoplasms, which were classified as diffuse large B-cell lymphoma (DLBCL, 11/16) and Burkitt's-like lymphoma (BLL-1/16). The 11 DLBCLs were further subtyped as immunoblastic (DLBCL-I, 7/11), centroblastic (DLBCL-C, 2/11), and T-cell rich (2/11). The four CD3$^+$ cases were classified as peripheral T-cell lymphoma unspecified (3/4, PTCLI, mature peripheral T-cell neoplasms) and T-lymphoblastic lymphoma (1/4, T-LBL, precursor T-cell neoplasm).

Spinal cord lymphoma (SCL)

All eight cases of SCL were positive for the FeLV antigen by IHC, of which, one was also positive for FIV. The ages ranged from 6 to 96 months with a mean with median of 38.5 and 24 months, respectively. The primary CNS lymphomas were identified in two cats (12 and 48 months) owing to the exclusive presentation on the spinal cord. Both the cats were FeLV positive and one was also FIV positive by IHC. The remaining six cases were secondary lymphomas with NS presentation only in the spinal cord, except in one case, which was also observed in the encephalic leptomeninges. The abdominal lymph nodes (5/6) were the most frequently affected in secondary lymphomas.

Feline SCL was predominantly located extradurally in the epidural space and associated with the epidural fat. Grossly, the neoplastic masses varied in size, were irregular, soft, and with white or yellow coloration. Focal distribution was identified in six of the eight cats (Fig.1A) and often involved the lumbar segments (3/6), followed by sacral (1/6), thoracic (1/6), and cervical (1/6) segments. The two remaining cats presented a multifocal spinal cord involvement observed in all spinal regions (Fig.1B).

Three cases (two cases of primary NS lymphoma) presented progressive hemorrhagic myelomalacia and affected the lumbosacral segments (Fig.1C). The grey matter was mainly affected, involving the dorsal and ventral horns. Gross lesions in these cases were characterized by bleeding and soft areas, in addition to occasional cavitation.

Histology showed a diffuse neoplastic infiltrate distributed extradurally and without infiltration in the meninges and spinal cord (Fig.1D). The eight SCLs were divided into B-cell lymphoma (6/8) and T-cell lymphoma (2/8), classified as: BLL (1/8), PTCLI (2/8), and DLBCL-I (5/8). Spinal cord injuries secondary to epidural lymphoma compression occurred in 50% of the SCLs. The main injury was progressive hemorrhagic myelomalacia (3/8) affecting the lumbosacral portion and extended until the thoracic segments. This lesion was found predominantly in grey matter and characterized by extensive hemorrhagic areas, neutrophilic vasculitis,

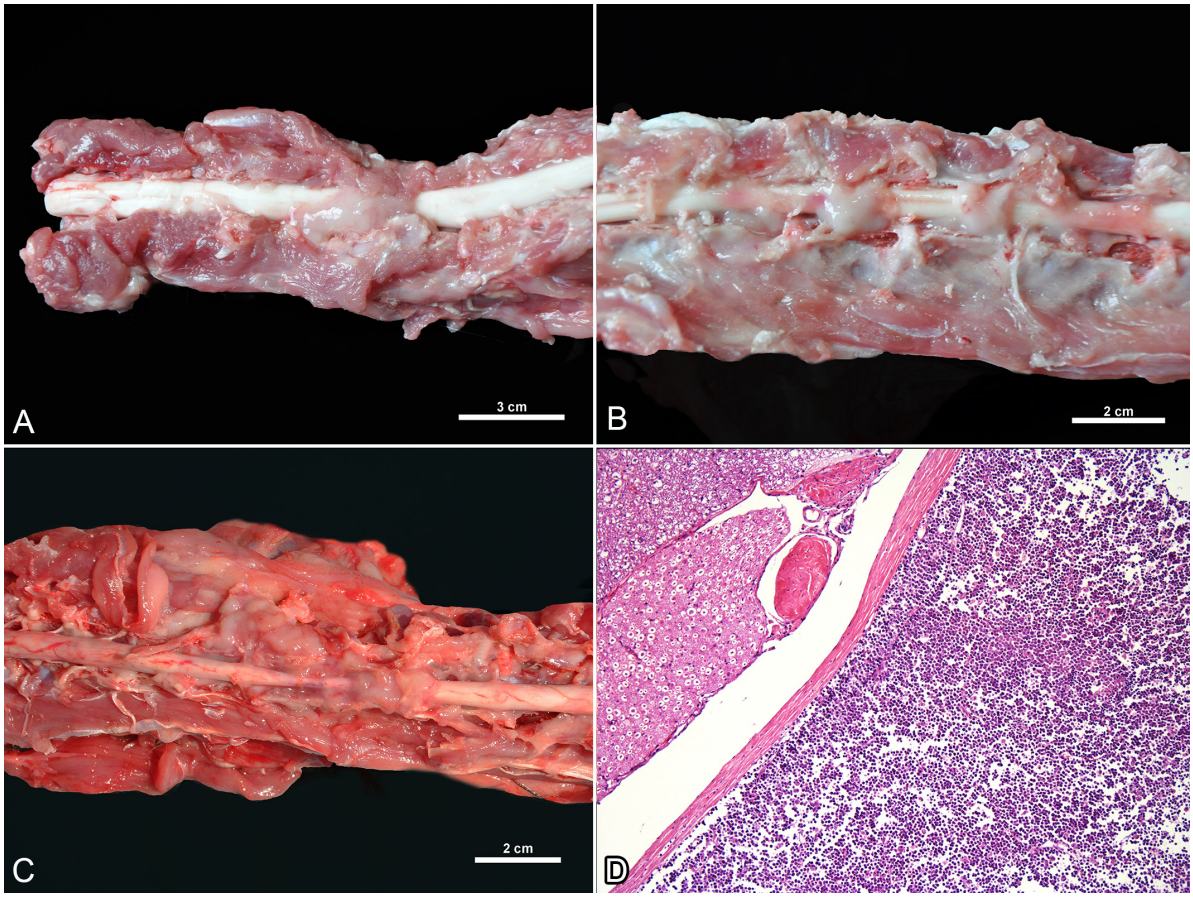

Fig.1. Feline lymphoma in the spinal cord. (**A**) Lymphoma in the cervical epidural space with focal distribution. (**B**) Lymphoma in the lumbar epidural space with multifocal distribution. (**C**) Extradural lymphoma extending to vertebral bodies and skeletal muscle. Hemorrhagic myelomalacia cranial to the tumor mass. (**D**) Extradural pattern of feline lymphoma in the spinal cord. HE, obj.10x.

gitter cell infiltrate, and malacia. Necrotic neurons showed retraction, a hypereosinophilic cytoplasm, and pyknotic nuclei. In the remaining white matter, axonal spheroids, Wallerian degeneration, and neutrophil infiltration were observed.

Brain lymphoma

Brain lymphoma affected six cats with ages ranging from 18 to 156 months with a mean and median of 41 and 24 months, respectively. Four cats were FeLV positive (Fig.2F), two were FIV/FeLV positive, one was FIV positive, and one was negative to both viral agents by IHC.

Grossly, brain lymphoma lesions were absent in most cases, except for a feline that presented a rough and thickened dura mater (Fig.2A). The brain lymphoma presented as a secondary form (multicentric presentation of the disease) in all seven cases, also affecting the liver (6/7), intestines (4/7), kidney (4/7), and lymph nodes (4/7).

The three neuroanatomic patterns found in the brain were characterized by the distribution of neoplastic infiltrates in the leptomeninges (leptomeningeal lymphomatosis, 4/7), choroid plexus (lymphomatous choroiditis, 2/7), and dura mater (intradural, 1/7).

The lymphomas in the leptomeningeal lymphomatosis pattern were classified as: DLBCL-I (2/4), PTCLI (1/4), and T-LBL (1/4). The neoplastic lymphocytes were widespread through the leptomeningeal space (Fig.2B) and expanded

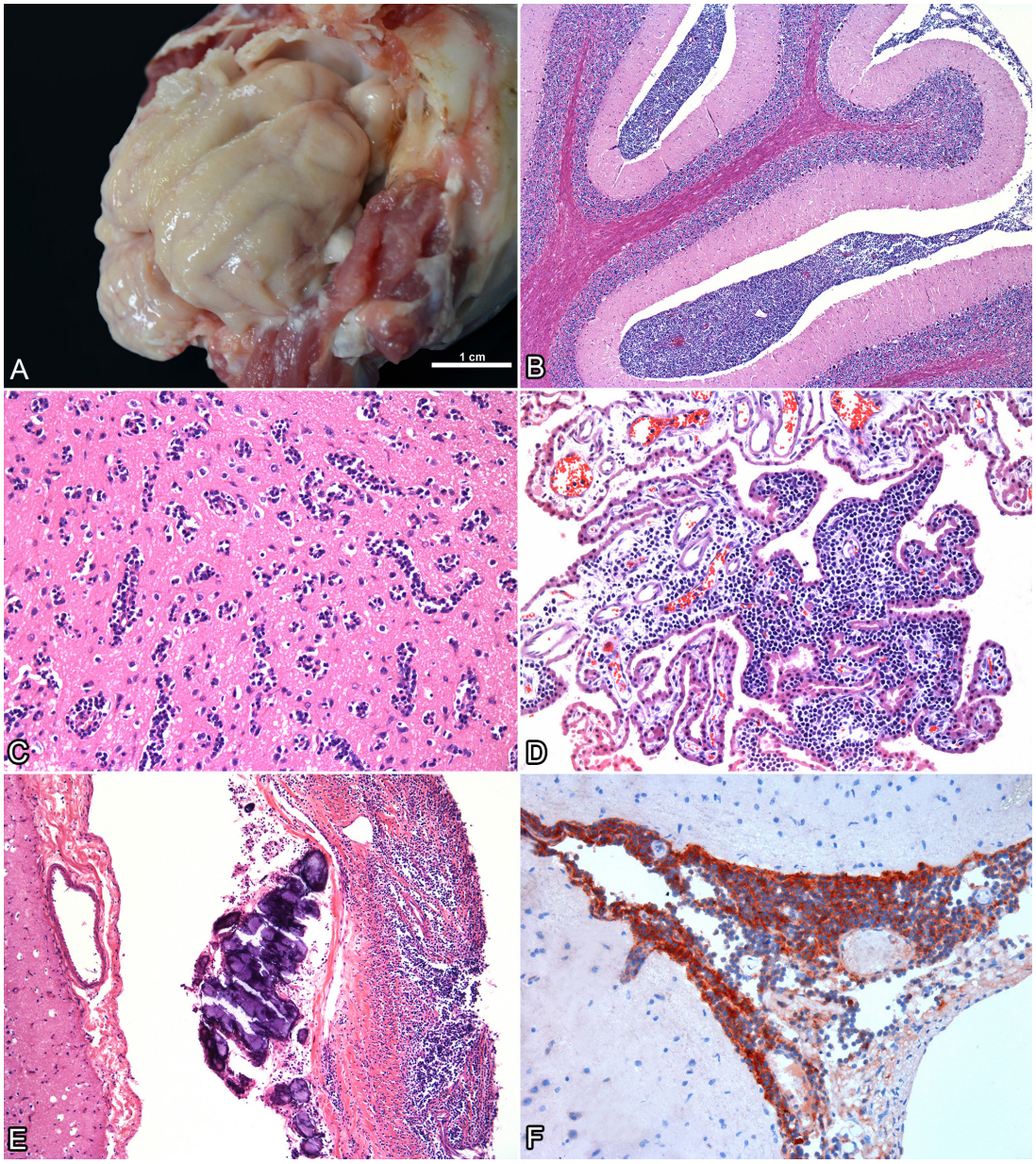

Fig.2. Brain feline lymphoma. (**A**) Brain with irregular and thickened dura mater. (**B**) Leptomeningeal lymphomatosis. Widespread leptomeningeal infiltration by neoplastic cells. HE, obj.4x. (**C**) Neoplastic lymphocyte infiltrating perivascular space of brain parenchyma in association with leptomeningeal lymphomatosis. HE, obj.20x. (**D**) Lymphomatous choroiditis. Diffuse infiltration of the choroid plexuses by neoplastic cells. HE, obj.20x. (**E**) Intradural lymphoma. Dura mater infiltrated by neoplastic lymphocytes and associated with multifocal mineralization. HE, obj.10x. (**F**) Brain leptomeninges, cytoplasmic immunostaining in lymphocytes. IHC for FeLV, obj.20x.

to perivascular spaces of the adjacent parenchyma (Fig.2C). Neoplastic cells were also found in the choroid plexus in one case of leptomeningeal lymphomatosis classified as DLBCL-I. In lymphomatous choroiditis cases, the choroid plexus of the fourth ventricle and lateral ventricles were diffusely infiltrated by neoplastic lymphoid cells classified as DLBCL-C (2/2) (Fig.2D). Additionally, congestion of choroid plexus vessels associated with proteinaceous effusion and exudation of cells into the ventricular lumen was observed. Intradural lymphoma was characterized by diffuse infiltration of DLBCL T-cells exclusively into the dura mater associated with multifocal calcification of the pachymeninges (Fig.2E). This presentation was responsible for the only case with alteration detected grossly in the brain.

Neurolymphomatosis

Neurolymphomatosis occurred in a 72-month-old, mixed breed, male cat that was FeLV positive by IHC and confined to the peripheral NS (primary NS lymphoma). Grossly, the sacral spinal nerves and paravertebral ganglia were bilaterally thickened with a soft consistency and yellowish coloration (Fig.3A). The malignant lymphoid cells classified as DLBCL T-cells were widespread along the sacral spinal roots, nerves, and ganglia and were associated to Wallerian degeneration with formation of axonal spheroids and digestion chambers (Fig.3B-D). The neoplastic cells were diffusely distributed in the leptomeningeal and perivascular spaces of the spinal cord segment adjacent to affected nerves.

DISCUSSION

Lymphoma is the most common neoplasm in cats, presenting a higher incidence in cats than other species, such as humans and dogs (Jarrett et al. 1966, Hardy 1981, Vail & Macewen 2000). Feline lymphoma occurs spontaneously and by viral oncogenic action. FeLV is the main viral agent implicated in lymphoma development. In studies conducted up to the 1990s, more than 80% of leukemias and lymphomas in cats were related to FeLV (Cotter et al. 1975, Francis et al. 1977, Francis et al. 1979, Hardy 1981, Reinacher 1989, Shelton et al. 1990). The prevalence of lymphoma associated with FeLV has been declining over the years in several parts of the world, possibly owing to the implementation of elimination and vaccination programs (Louwerens et al. 2005, Hartmann 2012, Meichner et al. 2012). Nevertheless, the high rate of cats infected with FeLV in the present study remains similar to proportions presented in studies published prior to the 1990s, when the prevalence of FeLV infection in cats with lymphoma was higher (Hardy 1981, Lane et al. 1994,

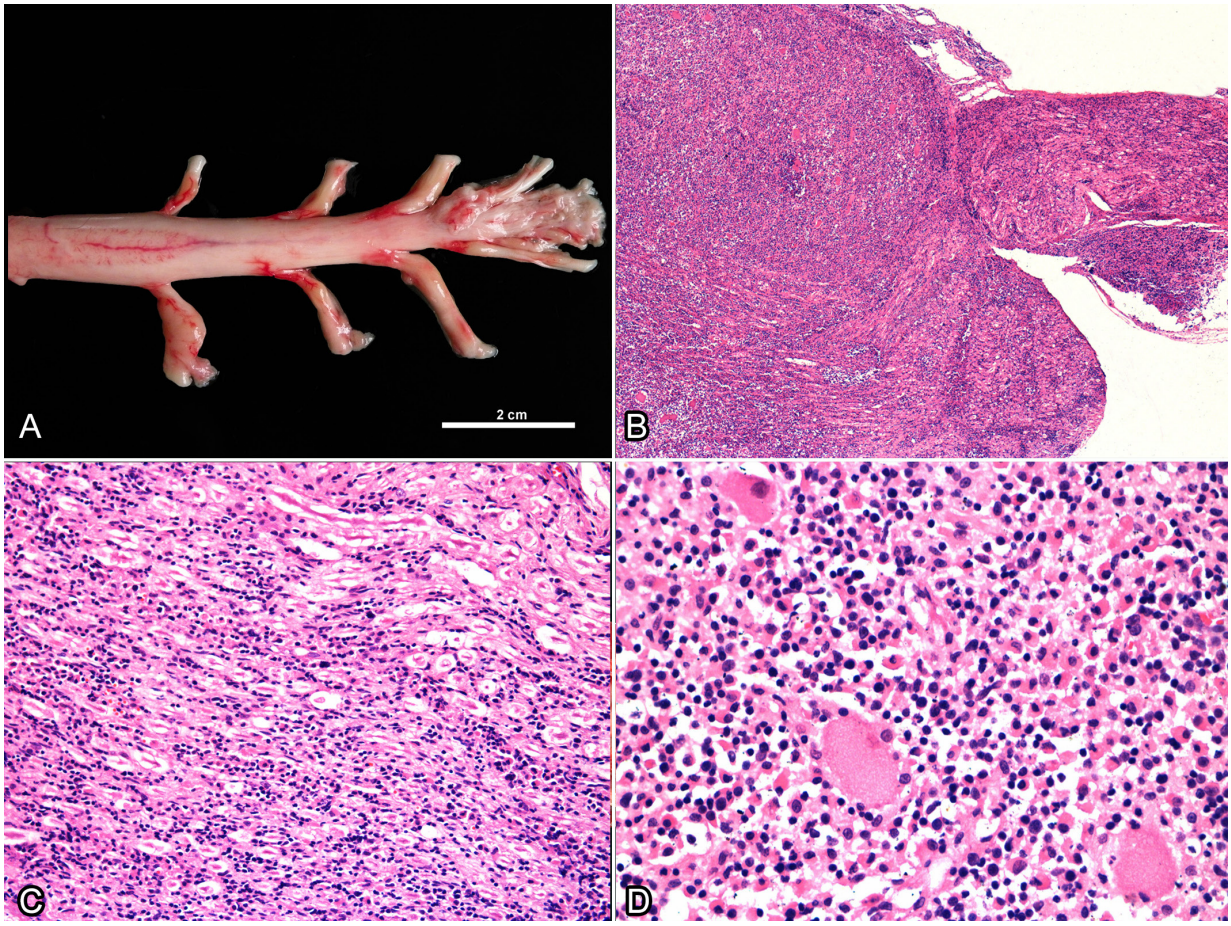

Fig.3. Neurolymphomatosis. (**A**) Bilateral thickened of spinal nerves (sacral region) presenting soft consistency and yellowish coloration. (**B**) Marked thickening of the nerve and paraspinal ganglia by neoplastic lymphocytic infiltrate. HE, obj.4x. (**C**) Neoplastic cells infiltration in the paravertebral ganglia. HE, obj.40x. (**D**) Lymphocytic neoplastic infiltrate along the spinal nerve associated with axonal spheroids, Wallerian degeneration. HE, obj.20x.

Meichner et al. 2012). These data suggest that the regional prevalence of infected cats is possibly still high.

The relationship between FeLV and lymphoma was particularly important in the present study since approximately 87.5% of the cats were infected by this virus. FeLV infected cats were approximately 62 times more likely to develop lymphoma, whereas concurrent FeLV/FIV infection confers approximately 80 times the risk for lymphoma development in relation to negative cats (Shelton et al. 1990, Hartmann 2012). The infection by FIV in the present study occurred in co-infection with FeLV, except for one cat only positive to FIV. Despite the indirect role of FIV in feline lymphoma development, this virus can exert a direct oncogenic role although less frequently (Diehl & Hoover 1992, Beatty et al. 1998, Beatty et al. 2002) with the peak in young cats allegedly attributable to FeLV infection.

Feline lymphoma generally has a bimodal age distribution. The first group more often seen in the present study affected young cats usually under 3 years and widely associated with FeLV infection (Hardy 1981, Lane et al. 1994, Gabor et al. 1998, Schmidt et al. 2010). The second group comprised older cats generally with FeLV infection absent, as seen in the two only FeLV-negative cats that were older than 8 years of age (Hardy 1981, Gabor et al. 1998, Louwerens et al. 2005). Although the incidence of lymphoma may be particularly higher or lower in certain breeds, indicating a probable genetic predisposition, these factors could not be evaluated in the present study owing to the high prevalence of mixed breeds (Gabor et al. 1998, Lyons 2010).

Lymphoma is a common CNS neoplasm in cats and occurs more frequently in the spinal cord than in the brain (Bradshaw et al. 2004). Predominantly, CNS lymphomas are a secondary manifestation of disease that are also presented by other organs (Lane et al. 1994, Tomek et al. 2006, Marioni-Henry et al. 2008). Primary CNS lymphoma (PCNSL) is originated and confined to the CNS and unlike the secondary form is rare in cats (Valli et al. 2017). Despite all the PCNSL cases in the present study being FeLV-positive, this viral relationship is not considered relevant to the frequency of this type of lymphoma (Valli et al. 2016). Unlike cats, the majority of PCNSLs in humans occur in immunocompromised patients by HIV infection (Rubenstein et al. 2008). These lymphomas are often identified as B-cell in both humans and cats (Ferracini et al. 1993, Vernau et al. 2001, Haldorsen et al. 2008, Nakamoto et al. 2009), and rarely as T-cells (Fondevila et al. 1998, Morita et al. 2009).

Feline lymphoma is the most frequent spinal cord neoplasm and the second most common disease of the spinal cord (Lane et al. 1994, Bradshaw et al. 2004, Marioni-Henry et al. 2004, Bradshaw et al. 2004, Marioni-Henry et al. 2008). Lymphoma is predominantly situated extradurally to the spinal cord and this presentation is frequently associated with young FeLV-positive cats (Zaki & Hurvitz 1976, Northington & Juliana 1978, Spodnick et al. 1992, Lane et al. 1994, Bradshaw et al. 2004, Marioni-Henry et al. 2008). Lymphoma involved in all regions of the spinal cord in the studied cats and, as described in the literature, exhibited predilection for lumbar segments (Marioni-Henry et al. 2004). Similarly to those described by Spodnick et al. (1992) and Lane et al. (1994), the epidural masses were often focal. The extension to vertebral bodies or underlying skeletal muscle has been reported in extradural lymphomas (Spodnick et al. 1992, Lane et al. 1994). Lymphoma establishments in the spinal cord possibly develops through direct expansion from the paravertebral region to the epidural space through the vertebral foramen, despite also being possible through hematogenous spread via the epidural venous system (Harrington 1986, Maccauro et al. 2011). Hemorrhagic myelomalacia, described in three cases, is a neurovascular disorder from a secondary compressive medullar injury related to extradural masses (De Lahunta & Glass 2009). In cats this lesion associated with extradural lymphoma has been poorly reported (Laisse et al. 2017).

Lymphoma is the second most common intracranial neoplasm in cats (Troxel et al. 2003, Bradshaw et al. 2004). Leptomeningeal lymphomatosis or lymphomatous meningitis was the most frequent anatomical distribution of lymphoma in the brain. The neoplastic infiltration in leptomeninges could occur through the direct spread of primary or metastatic tumors from the parenchyma, or by hematogenous spread through arachnoid vessels (Grossman & Krabak 1999). This pattern has also been described in humans and is strongly associated with HIV (Levitt et al. 1980, Mamidi et al. 2002). Leptomeningeal lymphomatosis, as seen in cats studied with this pattern, usually cannot be perceived grossly, appearing sometimes as a mild leptomeningeal thickening (Mandara et al. 2016). The lymphomatous choroiditis was the less frequent pattern found. Grossly, in these cats, mass formation was not observed, unlike in the case reported by Zaki & Hurvitz (1976). DLBCL was the main lymphoma type found in leptomeningeal lymphomatosis and lymphomatous choroiditis. DLBCL also was described in dogs as the main type in leptomeninges and choroid plexus (Sisó et al. 2016). However, intradural lymphoma is poorly described in veterinary medicine. The presentation restricted to pachymeninges in CNS is also rarely described in humans (Matmati et al. 2010). The intradural lymphoma was the only case in the brain grossly detected owing to mineralized areas in the dura mater. The dural calcification related to lymphoma in young cats has rarely been reported (Mandara 2003). Calcifications of brain pachymeninges have been reported in humans related both to primary CNS lymphoma and chemotherapeutic treatment (Jenkins & Colquhoun 1998, Apter et al. 2002). However, the cat from the present case had no previous history of chemotherapy treatment. Meninge calcification has also been reported in FIV-infected cats and this association could also possibly be a cause of this lesion in the present study (Hurtrel et al. 1992). Similar aspects have been observed in the human SNC with metastatic lung carcinoma (Inomata et al. 2012). Although not fully known, the calcification mechanism involved in these cases possibly occurs owing to: calcified scar tissue; dystrophic calcification in areas of tumor necrosis; metastatic calcification owing to the high production of calcium phosphate; and deposition of calcium as a result of the tumor secretory function (Mahoney et al. 1990).

Primary neurolymphomatosis, presented by one cat, is the term referred to diffuse infiltration of neoplastic lymphocytes along the peripheral nervous system (PNS) (Grisariu et al. 2010). The gross findings often diverge from the common lymphoma presentation in other organs (Linzmann et al. 2009). Lymphoma is the main secondary tumor in the feline CNS and neurolymphomatosis is a rare condition in cats, other animals, and humans (Allen & Amis 1975, Hankenson et al. 1998,

Kuntzer et al. 2000, Mellanby et al. 2003, Higgins et al. 2008, Choi et al. 2013, Shree et al. 2016). The neoplastic infiltration in leptomeninges (leptomeningeal lymphomatosis) and perivascular space from medullar parenchyma, as observed in the present study, is a common consequence relative to spinal nerve neurolymphomatosis (Lane et al. 1994, Schaffer et al. 2012, Mandrioli et al. 2012, Rupp et al. 2014, Sakurai et al. 2016). The unusual feature of this condition can lead to confusion with other neurological conditions, such as vasculitis and mononeuritis (Mandrioli et al. 2012). PNS lymphoma in humans and animals is often identified as B-cell (Higgins et al. 2008).

DLBCL is the main type classified in dogs and humans (Friedberg & Fisher 2008, Vezzali et al. 2010). FeLV-related lymphomas are mainly T-cell lymphomas, and more precisely, lymphoblastic, and occur as a mediastinal form (Valli et al. 2017). In a study performed on FeLV-positive cats, approximately 30% of the lymphomas originated from B lymphocytes (Jackson et al. 1996). Lymphomas in cats infected with FIV and FIV/FeLV coinfection are often from a mature B-cell lineage, as in that observed in the felines analyzed in the present study (English et al. 1994, Callanan et al. 1992, Terry et al. 1995, Callanan et al. 1996, Hartmann 2012, Beatty et al. 2002). In addition, B-cell lymphoma has been linked to FIV pro-viral DNA even in the absence of antigen detection, which may justify the high frequency of this type of neoplasm in the present study despite the frequency of 35% FIV-positive cats (Beatty et al. 1998). As observed in dogs, most of the lymphomas observed in the CNS of cats analyzed in the present study were B cells, classified predominantly as DLBCLs (Sisó et al. 2016).

CONCLUSIONS

The nervous system (NS) involvement was observed in 12.8% of cats with lymphoma and mainly young cats with a median age of 24 months were affected.

Lymphomas occurred more frequently in the spinal cord than in the brain.

Secondary lymphomas were the predominant form that affected the NS.

The NS primary lymphomas were restricted to the spinal cord.

All spinal cord lymphomas were FeLV positive and B-cell lymphomas were the most common.

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Original Article
Small Animal Diseases

Investigation of Norovirus genogroups (GI, GII and GIV) in stool of pet dogs with diarrhea[1]

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ABSTRACT.- Sokel S. & Kale M. 2019. **Investigation of Norovirus genogroups (GI, GII and GIV) in stool of pet dogs with diarrhea.** *Pesquisa Veterinária Brasileira 39(6):402-408*. Departamento de Virologia, Faculdade de Medicina Veterinária, Mehmet Akif Ersoy University, Antalya Burdur Yolu, 15030 Yakaköy Köyü/Burdur, Merkez/Burdur, 'Burdur, Turkey. E-mail: drmkalex@yahoo.com

In this study, we searched the existence of human norovirus (NoV) GI, GII and GIV in the stool of 128 pet dogs with diarrhea, of different sex, age and breed, in Burdur, Turkey, using Real-Time PCR method. Human NoV GII was found in only 5 of the 128 dog stool samples (3.91%). It was discovered that human NoV existed most in crossbreed, female and aged 24 months or over dogs. These dogs found with human NoV GII were either bought from pet shops, stray dogs or taken as puppy of another pet dog. The sheltering conditions of these dogs were moderate and they were fed with home food residue and dry food. It was also found that most of them were vaccinated and had certain walking sites. The owners of the animals detected with infection generally did not have the habit of washing their hands or changing their clothes before or after caring their pets. We strongly advice that dog owners' personal hygiene, the necessity of changing their clothes during their contact with animals, the environment provided for the dog, the sensitivity in caring, use of strong and effective disinfectant, keeping the dogs away from toilets and sewerage systems, as well as not feeding them with food residues are crucial issues in dogs' care. Owners of the dogs with NoV GII were middle aged or elderly people, male, and there were no children in their houses. As these dogs are treated like the owner's child, it is assumed that they could be transmitted with NoV GII as a result of close interaction with their owner.

INDEX TERMS: Norovirus, genogroups GI, GII, GIV, pet dogs, diarrhea, human, real-time PCR, stool.

RESUMO.- [Investigação de genogrupos de norovírus (GI, GII e GIV) nas fezes de cães de estimação com diarréia.] Neste estudo pesquisamos a existência de norovírus humano (NoV) GI, GII e GIV nas fezes de 128 cães com diarréia, de diferentes sexos, idades e raças, em Burdur, Turquia, utilizando o método de PCR em tempo real. NoV GII humano foi encontrado em apenas 5 das 128 amostras de fezes de cães (3,91%). Foi descoberta NoV humana, principalmente em cruzamentos, fêmeas e cães com idade igual ou superior a 24 meses. Os cães encontrados com NoV GII humano foram comprados de lojas de animais, eram vira-latas ou foram tomados como filhotes de outro cão de estimação. As condições de abrigo desses cães eram moderadas. Os cães foram alimentados com restos de comida caseira e comida seca. Verificou-se também que a maioria dos animais foi vacinada e tinham locais adequados para caminhada. Os donos dos animais detectados com infecção geralmente não tinham o hábito de lavar as mãos ou trocar de roupa antes ou depois de cuidar de seus animais de estimação. Aconselhamos que a higiene pessoal dos donos, a necessidade de trocar de roupa durante o contato com animais, o ambiente fornecido para o cão, a sensibilidade no cuidado, o uso de desinfetantes eficazes, manter os cães longe de banheiros e esgotos, assim como evitar alimentá-los com resíduos alimentares, são questões cruciais no cuidado dos cães. Os proprietários dos cães com NoV GII são de meia-idade ou idosos, a maioria do sexo masculino, e não havia crianças em suas casas. Como esses cães são tratados como um filho, presume-se que eles foram infectados com o NoV GII como resultado de uma interação próxima com o proprietário.

TERMOS DE INDEXAÇÃO: Norovírus, genogrupo GI, GII e GIV, cães de estimação, diarreia, humanos, tempo real PCR, fezes.

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INTRODUCTION

Acute gastroenteritis (AGE) is seen all over the world in all age groups. A non-membraneous virus family, Caliciviridae, transmitted by fecal and oral ways, causes epidemic diseases. Norovirus (NoV) and Sapovirus from Calicivirus family are the most important viruses causing foodborne epidemic and AGE in all age groups (Rodriguez-Lazaro et al. 2012). NoV, which is very contagious, transmits from person to person by contaminated food and/or water and contact with contaminated surfaces. Particularly, symptoms of stomach ache, nausea-vomiting and diarrhea affect children and elderly people (Centre for Disease Control and Prevention 2017). NoVs have been reported to have been responsible for nearly 50% of AGE epidemics seen around the world. Zheng et al. (2006) standardized NoV strains genetically. With this study, they classified NoVs as strain, genotype and genogroup and detected 29 genotypes in 5 genogroups. GII was determined as the most common genogroup amongst studies on human NoV epidemics seen around the world. As a result of the studies conducted in late 1990s and 2001, NoV GII.4 was seen (White et al. 2002, Atmar & Estes 2006, Bull et al. 2006). Like influenza virus, NoV also causes epidemics with new strains every 2-3 years (Turkey 2017). In studies performed from 2014 to 2015 epidemic seasons, human NoV GII.17 was reported to have caused natural infections with mutations in rhesus monkeys and probably been a new host (He et al. 2017). According to NoV phylogenetic research results, the new dominant NoV epidemic strain detected in AGE cases during 2016 winter season in European countries was reported as GII.P16-GII.2 (Niendorf et al. 2017).

NoV infection is effective in many animals such as calves, pigs, dogs, cats and monkeys other than humans. Gastroenteritis case is seen together with diarrhea in almost all cases (Karst et al. 2015). Canine NoV, genetically resembling NoV GIV in puppies with diarrhea, was reported in Italy (Pistoia) in 2006. Bari/170/07/ITA, a canine NoV prototype, was found 96.7% similar to Pistoia/387/06/ITA lion NoV nucleic acid and 90.1% similar to amino acids of capsid proteins (Martella et al. 2008, 2011). Zoonotic disease factor of canine NoV reveals its potential depending on the facts that dogs have been living together with humans like a family member in recent years (the rate of dogs living in homes in England is 31%) and there have been studies detecting that canine NoV uses the same receptors as human NoV while entering into the cell (Caddy et al. 2015, Karst et al. 2015).

There is not enough evidence about the zoonotic transmission of NoV between humans and animals. However, the fact that contamination of foods and environment by animal/human waste happens in indirect way leads to consideration of the agent as a zoonotic character. Studies detecting that human NoV and canine NoV use the same cell receptors make zoonotic risk possible (Karst et al. 2015, Rodriguez-Lazaro et al. 2012). The fact that animals are a crucial reservoir for human NoVs is a strong hypothesis. The study about the antibodies detected against human NoVs amongst pigs in Venezuela supports this case. However, although animal reservoir and zoonotic transmission is a known fact, difference between receptors and genetic distance does not support this case (Farkas et al. 2005).

In our study, we aimed to search the presence of NoV infection, which is a major public health problem, in possessed dogs showing diarrhea symptoms, to detect its genotype and to study nutrition and life conditions.

MATERIALS AND METHODS

Dog stool samples

In central Burdur (37°43'13" N, 30°17'27" E), stool samples were collected from 128 owned domestic dogs of various race and gender showing diarrhea symptoms kept in homes and gardens. Stool samples were stored in sample containers by the owners and brought to the lab under cold chain conditions by us. Stools taken from stool samples by sterile plastic spoon (nearly 1g) were removed into a glass tube were added by 5mL RNAfter™ (GeneMark, GMbiolab Co., Ltd., Taichung, Taiwan) solution and vortexed for 5min. Later, this mixture was kept in a -20°C deep freezer until testing.

The owners were given a poll during collecting dog stool samples. The topics were as information about dogs (age, race, way of feeding, treatment for diarrhea and vaccination case), life conditions of the dog and presence of walking areas.

Preparing dog stool samples for RNA extraction

Stool samples collected under suitable conditions and kept in RNAfter™ solution under -20°C were solubilized at ambient temperature. 1g of stool from the solubilized mixture was taken by the help of sterile plastic spoon and was removed into previously prepared sterile eppendorf tubes. 1mL Phosphate Buffer Solution (PBS) was added into these tubes. This new mixture in the eppendorf tube was vortexed for ten minutes. Later, the eppendorf tubes were centrifuged at 4°C at 2500rpm for 25min. After centrifugation, 0.25mL of supernatants in the eppendorf tubes was taken into another sterile eppendorf tube.

RNA extraction protocol in dog stool samples

Homogenization. RNA extraction protocol was applied in order to detect virus genome in stool samples. 750µl Trizol (GeneMark, GMbiolab Co., Ltd., Taichung, Taiwan) was transferred onto 250µl pretreated sample supernatant that was taken into eppendorf and was mixed by pipetation process. This mixture was centrifuged at 4°C at 978 x *g* for 25 minutes.

RNA phase separation. 200µl chloroform (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added into sample and trizol mixture. After centrifugation at 4°C at 12000 x *g* for 15min, organic molecules (cell proteins, DNA, lipid etc.) were taken into supernatant clean eppendorf tubes as RNA was located in the aqueous part in the sediment.

RNA precipitation. 500µl isopropanol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added in order to provide positive loading of RNA and precipitation. It was incubated at room temperature for 10min and centrifuged at 4°C at 12000 x *g* for 10min and supernatant was poured. For washing, 1000µl 75% ethanol was added and mixed thoroughly and centrifuged at 4°C at 7500 x *g* for 15min and this process was repeated once. Supernatant was poured and left for drying. After drying, 40µl RNAase free water was added and incubated in the heater (Benchmark Scientific, Edison, New Jersey, USA) at 58.5°C for 15min. RNA extracted samples were stored at -20°C.

Detecting NoV genome by Real-Time PCR

In stool samples of domestic dogs, foodproof® Norovirus Detection Kit-5'Nuclease-(BIOTECON Diagnostic®, Potsdam, Germany) kits were used for detecting NoV GI, GII and GIV by Real-Time PCR method. Amplification stage was initiated by LightCycler® 480 II Systems (Roche Diagnostics Ltd., Mannheim, Germany) device whose temperature-time program was set according to the determinants of the producing company of the

kit. The data obtained by fluorescent reading in LightCycler® 480 II Systems device was evaluated according to the result evaluation table (Table 1) of the producing company of the kit.

Statistical analysis

Poll questions asked to dog owners were inserted into SPSS 15.0 (2007) statistical analysis program. Within the data, identifier analysis was carried out as frequency, average and standard deviation and was accepted as $p<0.05$.

RESULTS

Samplings from different dog races were carried out for the study. The most frequent three races were Golden Retreiver (21.6%), Shepherd's Dog (17.6%) and Akbash-Kangal (11.8%). 47% of the dogs were male and 53% was female. The dogs were 36.5 months old on average (minimum 1.0, maximum 156.0). The way of owning the dogs was classified as from pet shops, animal shelters, streets and private sources and those not included in these groups were stated as others. The most common way of dog owning was found as private sources (41.7%, 53/128).

Dog owners evaluated the housing conditions as 6.7% bad, 42.3% moderate and 51% good. In the poll applied in the study, the questions were on nutrition habits of the dogs, convenience dry food, home-made food, waste home food and as others and multiple options could be ticked. Correspondingly, participants claimed that 67.3% of the dogs were fed with convenience dry food, 66.3% with waste home food and 7.7% with home-made food. 51% of the dogs were stated as full-vaccinated, 30% as under-vaccinated and 18% as not-vaccinated 21.2% of the dogs had no walking area.

The average age of the dog owners was stated as 39.1 (minimum 12, maximum 69). 75.5% (97 persons) of the dog owners was male and 24.5% (31 persons) was female. When child presence was observed in dog-keeping houses, 49% (62 persons) was positive and 51% (66 persons) was negative. The number of children living in dog-keeping houses was mostly two children at a rate of 58.8% (37 persons).

The household was responsible for caring the dog and multiple options could be ticked. Accordingly, the sequence was as 69.2% fathers, 41.3% mothers and 23.1% children. The average of the children living in the houses was found as 15.2+7.5, 12.6+5.4 and 10.8+5.2 for the first, second and third child respectively.

In order to detect the behaviors and attitudes of people during contact for caring the dogs, four point likert scale prepared with the options "always, sometimes, rarely and never". 66.3% of the participants did not wash their hands before contact with the dogs and 60.6% of them had no habit of changing clothes. 62.5% of the participants had the habit of washing hands after caring the dog and 26.9% of them had no habit of changing clothes.

Human NoV GII was found in 5 of 128 dog stool samples examined by Real-time PCR (Fig.1). However, no positivity for NoV GI and GIV was found in samples with NoV GII. NoV GI, GII and GIV presence could not be detected in the

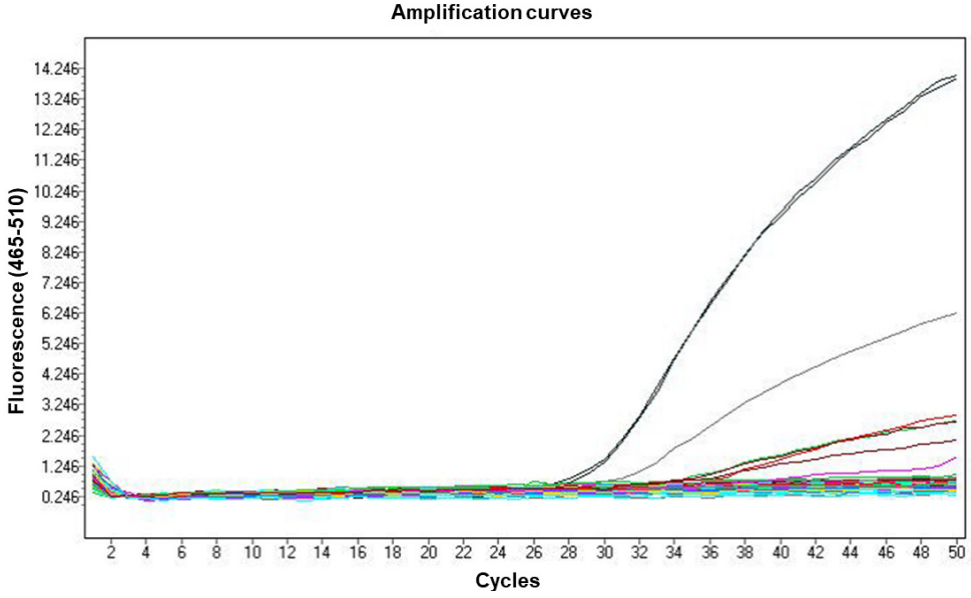

Fig.1. Real-time PCR results. PC1 = Positive Control 1 (CP:30.25), PC2 = Positive Control 2 (CP:30.07), S1 = Sample 1 (CP:33.20), S2 = Sample 2 (CP:39.92), S3 = Sample 3 (CP:40.52), S4 = Sample 4 (CP:39.48), S5 = Sample 5 (CP:42.76).

Table 1. Result evaluation table depending on the fluorescent wave length of HEX, FAM and ROX channels

NoV GI HEX channel	NoV GII FAM channel	Proses Control ROX channel	Interpretations
Positive	Positive	Positive /Negative	NoV GI and II/IV Positive
Negative	Positive	Positive /Negative	NoV GII Positive
Positive	Negative	Positive /Negative	NoV GI Positive
Negative	Negative	Positive	NoV GI nd II/IV Negative
Negative	Negative	Negative	Invalid

other 123 dog stool samples. The evaluation was performed using commercial kit result evaluation table according to the condition of fluorescent wave length of HEX, FAM and ROX channels.

The distribution of dogs detected as NoV GII positive was shown in Table 2. No diarrhea in the last 72 hours among the household (Table 2).

DISCUSSION

NoV is related with intestinal diseases of humans, sheep, pigs, mice and dogs. Canine NoV was first discovered in Italy in 2007. Later, during the studies carried out in Portugal, Greece and USA, it was found in dog stools (Caddy et al. 2014). Human NoVs are classified as GI, GII and GIV whereas canine NoVs are classified as GIV and GVI. Amino acid similarity is seen at a rate less than 85% between human and canine NoVs. Therefore they are divided into two different genotypes, IV.1 and IV.2 (Martella et al. 2009). Due to the facts that humans and dogs live together in close relationship, they share the same environment and this condition is highly common, the presence of human NoV in cat and dog populations has been searched by various studies. On the other hand, canine NoV antibody presence was detected for 22.3% of 373 vets dealing with small animal medicine and for 5.8% of 120 individuals (Mesquita et al. 2010).

Summa et al. (2012) used Real-time PCR method to detect NoV GI, GII and GIV presence in stool samples of owned dogs living in the same house with their owners. At the end of the study, human NoV presence was found in stool samples of four dogs in contact with humans showing diarrhea symptoms. Soma et al. (2015) searched for NoV presence in the stool of 97 dogs with diarrhea in Japan using RT-PCR. In the study, NoV presence was detected in two samples. Caddy et al. (2015) searched for human NoV presence in the stool samples of owned dogs in England using RT-PCR. They found human NoV antibody presence in 43 of 325 dog blood samples. Di Martino et al. (2017) performed research by ELISA developed on the basis of human NoV GII.4, GIV.1 and dog NoV GIV.2 and GVI.2 like virus particles in 516 dog blood serum. Mesquita et al. (2010) searched for NoV presence in 105 dog stools. In the study, diarrheal stools of 63 dogs and normal stools of 42 dogs were examined using RT-PCR. At the end of the examination, a new kind of NoV presence was detected at a level of 40% in stools of diarrheal dogs and 9% in stools of non-diarrheal dogs.

In our literature survey, we couldn't find a study on searching human NoVs in diarrheal dog stools. That's why our results are claimed to be the first from this aspect.

In this study, NoV GII was found in 5 (3.91%) of 128 dog stool samples. In these samples, no positivity was detected for NoV GI and GIV. NoV GI, GII and GIV presence could not be detected in 123 dog stool samples. Summa et al. (2012) stated that they found GII.4 in three and GII.12 in one of the positive samples particularly. Mesquita et al. (2010) tested blood samples of 308 dogs living in European countries against human NoV GI.1 and GII.4. In the study, 20 serum samples (19 GII.4 and 1 GI.1) showed positivity and 3.91% rate of human NoV GII obtained from dog stools was parallel to 4.3% rate of human NoV GII obtained by Summa et al. (2012) from dog stools and to 2.2% rate obtained by Martella et al. (2009) for all NoVs in dog stools. However, during the studies were obtained higher prevalence results both in dog serum samples and in dog stools: 40% (Mesquita et al. 2010), 11% (Azevedo et al. 2012) and 10.1% (Di Martino et al. 2017).

In Real-time PCR analysis in the study, we found Cp (Crossing point) values of the positive samples between the ranges of 33.20-42.76 (average 39.18). Summa et al. (2012) found Cq (Quantification cycles) values of the positive samples between the ranges of 23-37 (average 30.98) in Real-time PCR analysis. These results were close to each other. By the way, Ct (Treshold cycle), Cq and Cp expressions used in Real-time PCR define the same conditions with each other.

Summa et al. (2012) detected human NoV GII presence in 1 Irish setter, 1 Dachsund and two poodle races while Soma et al. (2015) detected that in 1 poodle and 1 Borzoi race. In this study, human NoV GII presence was detected in two crossbreed, 1 poodle, 1 golden retriever and 1 Rottweiler races. At the end of these three studies, Poodle race came into prominence. Human NoV presence was most seen in crossbreed race.

When infection was examined according to gender, Summa et al. (2012) detected distribution in three males and one female while Soma et al. (2015) found it in 1 male and one female. In the study, it was detected in one male and four females.

Mesquita et al. (2010) stated that the percentage of Canine NoV positive dogs rose until three years of age, decreased later and this condition depended on the decrease of immunity with aging. Summa et al. (2012) determined the distribution of age of dogs with human NoV GII as 1 for three year-olds, 2 for five year-olds and 1 for 6 year-olds. Soma et al. (2015)

Table 2. The results of NoV GII positive dogs

No.	Breed	Gender	Age (months)	Way of dog owning	Vaccination condition	Walking area of the dog	Hand washing before caring	Clothes changing before caring	Hand washing after caring	Clothes changing after caring
1	Golden Retriever	Female	40	Stray dog	Full	Got	Rarely	Sometimes	Always	Sometimes
2	Rottweiler	Female	24	Pet shop	Not	Got	Always	Sometimes	Sometimes	Sometimes
3	Miniature Poodle	Female	27	Pet shop	Under	Got	Never	Never	Never	Never
4	Crossbreed	Female	30	Owned puppy	Full	None	Rarely	Never	Sometimes	Never
5	Crossbreed	Male	36	Stray dog	Full	None	Never	Never	Never	Never

detected NoV presence in two 2 month-old puppies. In our study, NoV presence was detected in 5 dogs whose average was 31.40 between 24.40 months. Di Martino et al. (2017) detected positivity from 7 to 9% in dogs younger than 1 year of age for NoV GII.4 and GVI.2 and 15% in adult dogs older than 12 years of age. Different from these conditions, Caddy et al. (2015) stated that they could not detect human NoV presence in 131 dogs from 56 different races with 5.1 years of age range on average that were brought into vet clinics and in 117 healthy looking dogs with 5.6 years of age range on average.

In this study, it was realized that dogs detected with human NoV GII were obtained from pet shops as stray dogs and owned puppies. Their housing conditions were at medium level and they were fed with waste home food (60%) and convenience dry food (100%). 60% was full vaccinated, 20% undervaccinated and 20% not vaccinated. 60% of the dogs had walking areas while 40% did not. 100% of the dogs with human NoV GII was diarrheal and had diarrhea treatment.

The average age of dog owners was 53.4 (minimum 36, maximum 62) and was classified in classes of middle-aged and elderly. The gender of dog owners was 80% male and 20% female. There were no children in houses with dogs. In contradiction to this condition, Summa et al. (2012) stated that little children lived in houses of positive detected dogs and that human NoV contamination from children to animals might be caused by uncontrolled vomiting of children onto surfaces and beds. In our study, 80% of fathers and 20% of mothers were responsible for feeding the dog. In dogs with human NoV GII, the fact that dog owners were classified in classes of middle-aged and elderly and were mostly male revealed that feeding and hygiene conditions were not satisfying.

Personal habit of hand washing of owners of dogs with human NoV GII before caring was stated with the rates as 40% rarely, 40% never and 20% always. The habit of clothes changing of owners before caring was found as 60% never and 40% sometimes. The habit of hand washing of owners after caring was stated with the rates as 40% never, 40% sometimes and 20% always. The habit of clothes changing of owners after caring was found as 60% never and 40% sometimes. During the period when sampling was carried out, no diarrhea case was seen among the household in the last 72 hours. According to the data obtained here, owners of dogs with human NoV GII did not have the habits of hand washing and clothes changing before and after caring the dogs.

Human NoVs first need to bind complex carbonhydrates known as histo-blood group antigens (HBGAs) in order to enter cells (Marionneau et al. 2002). As in the case of erythrocytes, they are replicated at epithelial cell surfaces of gastrointestinal, genitourinary and respiratory system organs and HBGAs and are released by cells within body fluids including saliva (Marionneau et al. 2001). Human NoV agents were experimentally proved to enter cells by binding HBGAs. It was revealed that there is susceptibility between HBGAs replication and human NoV in gastrointestinal system (Lindesmith et al. 2003, Hutson et al. 2005).

Since human NoVs are sensitive to dogs, HBGAs replicate in gastrointestinal organs of dogs. Even though dog blood types do not resemble human systems, dog erythrocytes are not hemagglutinated by human NoVs (Hutson et al. 2003). For the time being, HBGAs production is visible in saliva and intestinal epithelial cell surfaces of dogs (Caddy 2017).

Contact is a crucial way of disease transmission from animals to humans or from humans to animals. During dog care, behaviors of individuals are important especially in terms of infections of zoonotic diseases. There is no sufficient data on zoonotic transmission of NoVs between humans and animals. However, some studies show that potential transmission is possible. Recent observations on canine NoV reveal that it uses the same cell receptors as human NoV and that transmission of animal NoVs to humans might be possible. Thus, the detection of anti-canine NoV antibodies by veterinarians is one of the examples showing this transmission (Karst et al. 2015). Besides, as the exact opposite of this condition, the detection of human NoV presence in stools of animals (cattle, pigs and dogs) is considered similarly (Peasey et al. 2004, Mattison et al. 2007, Caddy et al. 2015). It was stated that 45% of dog owners in America and Europe share their beds with their dogs and they might have zoonotic factors (Chomel & Sun 2011). NoVs and other factors are said to transmit as a result of kissing their animals on the mouth or licking their owners by dogs (Chomel & Sun 2011). Intense virus dispersion from stools of humans infected by NoV and vomiting that appears in acute phase of the infection is effective in transmission (Patel et al. 2009). Transmissions on surfaces inside or outside the house, unwashed dirty dishes, not using strong disinfectants in cleaning are crucial for dispersion of the factor (Weber et al. 2010). While dogs are walking outside, their furs, mouths, noses and paws are highly important in transmission of the factor to humans, animals and the environment (Summa et al. 2012).

In this study, dogs infected by human NoV are thought to have transmission by living spaces, environment, waste food and hand-mouth-body contact. Among the most important reasons, we reckon that especially toilets, sewerage systems, waste food by dog owners, home-environment walking areas and close contact between the dogs and their owners are in the foreground.

CONCLUSIONS

At the end of the study, human NoV GII was found in 5 (3.91%) of 128 dog stool samples. No positivity for NoV GI and GIV was detected in samples with NoV GII. NoV GI, GII and GIV presence could not be found in the other 123 dog stool samples. The obtained data was parallel to this kind of studies in recent years. This research seems to be the first study in our country in this manner.

Human NoV presence was mostly detected in crossbreed dog races, female dogs and dogs over 24 months old. Dogs with human NoV GII were obtained from pet shops as stray and owned puppies. Housing conditions of the dogs were at a medium level. They were fed with home food waste and convenience dry food. The majority had full vaccination and walking areas. 100% of the dogs with human NoV GII had diarrhea and diarrhea treatment.

Owners of dogs with human NoV GII often did not have the habit of hand washing and clothes changing before and after caring for the dog. Therefore, it is highly crucial to give importance to housing conditions, nutritional elements, living spaces, cleaning and hygiene conditions of dogs in their relation with the environment. Some basic precautions must be taken: personal hygiene of dog owners, the obligation to change clothes in contact with dogs, the environment prepared

for the dog, the sensitivity in caring, usage of strong and effective disinfectants, keeping the dogs away from toilets and sewerage systems and not feeding them with waste food.

At the end of the study, the owners of dogs with human NoV GII were classified in middle-aged and elderly groups as mostly males and there were no children in these houses. Since it was estimated that dogs took the place of children as individuals in houses, it is natural that these dogs and their owners are in close contact (eating together, hugging, kissing etc.). In this regard, NoV GII, not seen in dogs by contact as a result of close relations of dogs and their owners, might possibly transmit from humans to dogs.

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Original Article
Small Animal Diseases

Non-invasive ECG recording and QT interval correction assessment in anesthetized rats and mice[1]

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ABSTRACT.- Botelho A.F.M., Joviano-Santos J.V., Santos-Miranda A., Menezes-Filho J.E.R., Soto-Blanco B., Cruz J.S., Guatimosim G. & Melo M.M. 2019. **Non-invasive ECG recording and QT interval correction assessment in anesthetized rats and mice.** *Pesquisa Veterinária Brasileira 39(6):409-415*. Departamento de Clínica e Cirurgia Veterinária, Escola de Veterinária, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Belo Horizonte, MG 30123-970, Brazil. E-mail: mariliamm@ufmg.br

Rats and mice are the most common species used in experimental cardiac electrophysiology studies. Electrocardiogram (ECG) recording shows paramount importance for monitoring arrhythmias and cardiac function in several disease models, including QT syndrome. However, the lack of standardized reference values and QT correction formula for different animal species and lineages represent a challenge for ECG interpretation. The aim of this study is to provide an improved method for ECG recording, establishing reference range values and determine the QT formulas with higher correlation to heart rate (HR). A total of 10 Wistar rats, 10 Swiss mice, 10 C57BL/6 mice and 10 FVB/NJ mice were used in the study. Animals were submitted to anesthesia with isoflurane and ECG recording was performed using a six-channel non-invasive electrocardiograph. QT was corrected using the following formulas: Bazzett, Fridericia, Mitchell, Hodges, Van der Water and Framingham. Normal range values for ECG parameters were established in all animals studied. Pearsons' correlation defined Hodges formula as the most suitable for QT correction. This study demonstrated an improved method of ECG recording with reference values for Swiss, FVB/NJ, C57BL/6 mice, and Wistar rats. Hodges' formula was the most effective formula for QT correction in rodents, whereas Bazett's and Friderica formulas were ineffective for such animals. The present work contributes to arrhythmias investigation in experimental cardiology and may reduce misinterpretations in rodents' ECG.

INDEX TERMS: Non-invasive ECG, QT, interval correction, rats, mice, anesthesia, Hodges, Bazzet, Mitchell, QT correction formula.

RESUMO.- [**Eletrocardiograma não invasivo e avaliação da correção do intervalo QT em roedores anestesiados.**] Ratos e camundongos são as espécies mais comumente utilizadas em estudos experimentais de eletrofisiologia cardíaca. O registro do eletrocardiograma (ECG) é de suma importância para o monitoramento de arritmias e função cardíaca em vários modelos de patologias. No entanto, a falta de valores de referência padronizados e a fórmula de correção do QT para diferentes espécies e linhagens animais representam um desafio para a interpretação do ECG. O objetivo deste estudo é fornecer um método melhorado para o registro de ECG, estabelecendo valores de referência e determinar as fórmulas QT com maior correlação com a freqüência cardíaca (FC). Um total de 10 ratos Wistar, 10 camundongos Swiss, 10 camundongos C57BL/6 e 10 camundongos FVB/NJ foram utilizados no estudo. Os animais foram submetidos à anestesia com isoflurano e o registro de ECG foi realizado com eletrocardiógrafo

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não invasivo de seis canais. O QT foi corrigido usando as seguintes fórmulas: Bazzett, Fridericia, Mitchell, Hodges, Van der Water e Framingham. Os valores da normalidade para os parâmetros do ECG foram estabelecidos em todos os animais estudados. A correlação de Pearson definiu a fórmula de Hodges como a mais adequada para a correção do QT. Este estudo demonstra um método melhorado de registro de ECG com valores de referência para camundongos Swiss, FVB/NJ, C57BL/6 e Wistar. A fórmula de Hodges foi a mais eficaz para correção de QT em roedores, enquanto as fórmulas de Bazett e Friderica apresentaram valores mais baixos de correlação. O presente trabalho contribui para a investigação de arritmias em cardiologia experimental e pode reduzir interpretações erradas no ECG de roedores.

TERMOS DE INDEXAÇÃO: Eletrocardiograma não invasivo, correção do intervalo QT, roedores, anestesia, Hodges, Bazzet, Mitchell, fórmula de correção do QT.

INTRODUCTION

Rats and mice are the most common species used in experimental cardiac physiology and electrophysiology studies (Zaragoza et al. 2011, Nerbonne 2014, Surikow et al. 2017). Several disease models including myocardial infarction (Heywood et al. 2017), diabetic cardiomyopathy (Bugger & Abel 2009), hypertrophic and dilated cardiomyopathy (Xu et al. 2014, Yu et al. 2014), Chagas' disease (Roman-Campos et al. 2013) and QT syndrome (Drum et al. 2014) are well established in such animals. Some number techniques can be used to monitor and characterize cardiovascular pathologies, specially the electrocardiogram (ECG) exam.

The ECG is a valuable tool for cardiac electrical function evaluation and paramount importance for the diagnostic and monitoring of arrhythmias and conduction disturbances. Changes in the ECG waves and intervals, as well as waveform alterations and arrhythmias are particularly important in pharmacological and toxicological investigations of drugs, especially aiming to determine the safety of its therapeutic use (Ruppert et al. 2016).

ECG is routinely used in human medicine and experimental research. Since its description by Einthoven (1895) several adjustments and improvements have been made to increase its' precision and to reduce interferences (Wilson et al. 1934, Holter & Generelli 1949). Several methodologies have been described in the search for the most adequate technique, including telemetry, invasive ECG, non-invasive ECG and Holter monitoring. Both conscious and anesthetized models were also studied over the years with its' positive and negative points to be considered in each model (Ho et al. 2011, Kumar et al. 2017). However, in the last few years computerized non-invasive ECG introduced new forms of analyzing its' parameters, with greater accuracy and storage capacity than conventional technique (Camacho et al. 2010, Pinto et al. 2010).

Enhanced data collection allows researchers to further investigate waves and intervals alterations and provide insights into discrete changes. One of the most important intervals evaluated in mice and rat models is the QT. It is known that QT interval represents depolarization and repolarization of ventricular action potential, and several diseases and drugs are implicated in its' prolongation. However, QT interval is dependent of the heart rate and different formulas throughout the years have tried to correct such variations, but no consensus was achieved (Bazett 1920, Fridericia 1920, Hodges et al. 1983, Van der Water et al. 1989, Sagie et al. 1992, Mitchell et al. 1998, Vandenberk et al. 2016).

Understanding the biological basis of QT correction is important to improve interpretation of ventricular abnormalities associated with progressive and fatal arrhythmias (Harada et al. 2010, Niemeijer et al. 2014). In fact, QT prolongation and its shortening are present in several pathologic disturbances, including intoxication with cardioactive drugs, myocardial infarction and QT syndrome (Yap & Camm 2003, Nachimuthu et al. 2012). It is important to highlight that the correction is widely recommended in clinical practice and experimental studies, but the best correction formula for computerized electrocardiographic studies with anesthetized animals is undetermined.

Currently, the best animal model for QT interval studies uses guinea pigs (*Cavia porcellus*) due to the functional expression of channels responsible for potassium current known as Ikr (Rees & Curtis 1993). However, the majority of studies still uses rats and mice, because of the easy access to such species and previously established diseases models (Chu et al. 2001, Andrag & Curtis 2013). ECG interpretation in rodents is a challenge, due to the difficulty in determining the isoeletric point of the tracings. Some authors suggest evaluation of QT interval at 90% of repolarization (Rees & Curtis 1993). On the other hand, this value is only accurately measured in isolated hearts in Langendorff system, which makes the experimental conduction and ECG interpretation even more challenging (Stables & Curtis 2009, Andrag & Curtis 2013).

Considering the animal models research importance, the increasing use of computerized electrocardiography and the QT formula discrepancies, the aim of this study is to provide an improved method for ECG recording, to establish normal range values and to determine the QT formulas with higher correlation to heart rate (HR).

MATERIALS AND METHODS

Ethics statement. The experiment was conducted at Veterinary School, UFMG, Brazil, in compliance with the Ethics Principles in Animal Experimentation, approved by the Ethics Committee on Animal Experimentation (CEUA-UFMG) (Protocols #74/2017 and 25/2015). Health and welfare were evaluated through clinical examination by a trained veterinary.

Experimental design. All animal used in this study were healthy adults (4-8 months), provided by the Central Bioterium of "Universidade Federal de Minas Gerais" (UFMG), Brazil. Ten Wistar rats (*Rattus novergicus albinus*), 10 Swiss mice (*Mus musculus*), 10 C57BL/6 mice (*M. musculus*) and 10 FVB/NJ mice (*M. musculus*) were selected. They were kept in the Laboratory of Animal Experimentation of Veterinary School (UFMG) in Belo Horizonte, Minas Gerais, Brazil. Groups of 4-6 animals were kept in plastic cages (36x26x15cm), under controlled environment, with 12 hours light/dark cycle and room temperature at 23°C. They had free access to water and food.

ECG recording. A six-channel non-invasive electrocardiograph (ECG-PC version 2.07®, Tecnologia Eletrônica Brasileira (TEB), Belo Horizonte/MG, Brazil) was used. The animals were induced with 2.5% isoflurane and maintained with 1.5% (VetCase - Incotec, Serra/ES, Brazil). Rodents were placed in a dorsal recumbence position on a wooden table covered with plastic material; electrocardiographic gel was applied, and four alligator clip electrodes were attached

to the skin in the forelimbs and hindlimbs (Botelho et al. 2016). All procedures were performed in a quiet room to minimize stress.

ECG trace analysis. All ECGs were performed and analyzed by the same veterinary according to standard methods. Tracings were recorded in six leads of the frontal plan, with 50mm/s of velocity and sensitivity of 1cm=2mV (2N). In each tracing three segments, containing five beats (lead II) were selected for quality (clean baseline with no artifacts) and mean values for heart rate (HR), and amplitude and length of P-QRS-T deflections were determined. The parameters evaluated were heart rate (HR) and cardiac rhythm, duration of the P wave (Pms), QRS complex (QRS), PR and QT intervals, and amplitude of P waves (PmV), R waves and T waves. Morphology patterns and rhythm were evaluated in every lead, and P-QRS-T measurements were conducted in lead II. QT corrected values were obtained from the following equations (Table 1).

Statistical analysis. All measurements are expressed as mean ± standard deviation (SD). The influence of the different QT formulas was compared by the Pearson's test (variables normally distributed). In all the analyses differences were considered to significant when $P<0.05$.

RESULTS

ECG tracings of the distinct species/lineages are shown in (Fig.1) and similar wave morphology and deflections amongst the derivations is observed. Predominantly in all animals DI, DII, DIII and aVF are positive whilst avR and aVL are negative. Rats presented the most challenging evaluation of the ST segment and all lineages/species presented discrete Q wave. Mean, standard deviation, minimum and maximum values of the electrocardiographic parameters (HR, Pms, PmV, PR, WRS, R, T, QT) of Swiss mice, C57BL/6 mice, FVB/NJ mice and Wistar rats are described in Table 2 to 5.

Pearson's test evaluated the correlation amongst QT correction formulas, QT, HR and RR presented in Table 6. In all evaluated animals, Van der Water's formula had the perfect correlation when compared with uncorrected QT. However, the strongest correlation with RR and HR was obtained by Hodge's formula, presenting all r values higher than 0.9 (Fig.2).

Table 1. QT correction formulas used in this study and their respective references

QT formula	Reference
$QTcB = QT*(RR)^{1/2}$	Bazett 1920
$QTcF = QT*(RR)^{1/3}$	Fridericia 1920
QTcV = QT + 0.087(1- RR)	Van der Water et al. 1989
QtcFr = QT + 0.154*(1-RR)]	Sagie et al. 1992
QTcM = QT/square root of (RR/100)	Mitchell et al. 1998
QTcH= QT + 0.00175 x (HR-60)	Hodges et al. 1983

Table 2. Mean, standard deviation, minimum and maximum values of the electrocardiographic parameters of Swiss mice. Tracings were recorded in six derivations of the frontal plan, with 50mm/s of velocity and sensitivity of 1cm=2mV (2N) in healthy adult anesthetized mice (*Mus musculus*) using a computerized ECG

Parameters	Mean ± SD	Min	Max
HR (bpm)	434.90 ± 68.43	321	502
P (ms)	29.29 ± 2.62	25	33
P (mV)	0.028 ± 0.005	0.02	0.03
PR (ms)	36.20 ± 3.22	32	40
QRS (ms)	41.66 ±5.02	33	53
R (mV)	0.22 ± 0.05	0.19	0.27
T (mV)	0.028 ± 0.011	0.020	0.05
QT (ms)	83.16 ± 8.37	63	92

HR = Heart rate, Pms = duration of the P wave, PmV = amplitude of P wave, PRms = duration of the PR segment, QRSms = duration of the QRS complex, RmV = amplitude of R wave, TmV = amplitude of T wave, QTms = duration of QT interval.

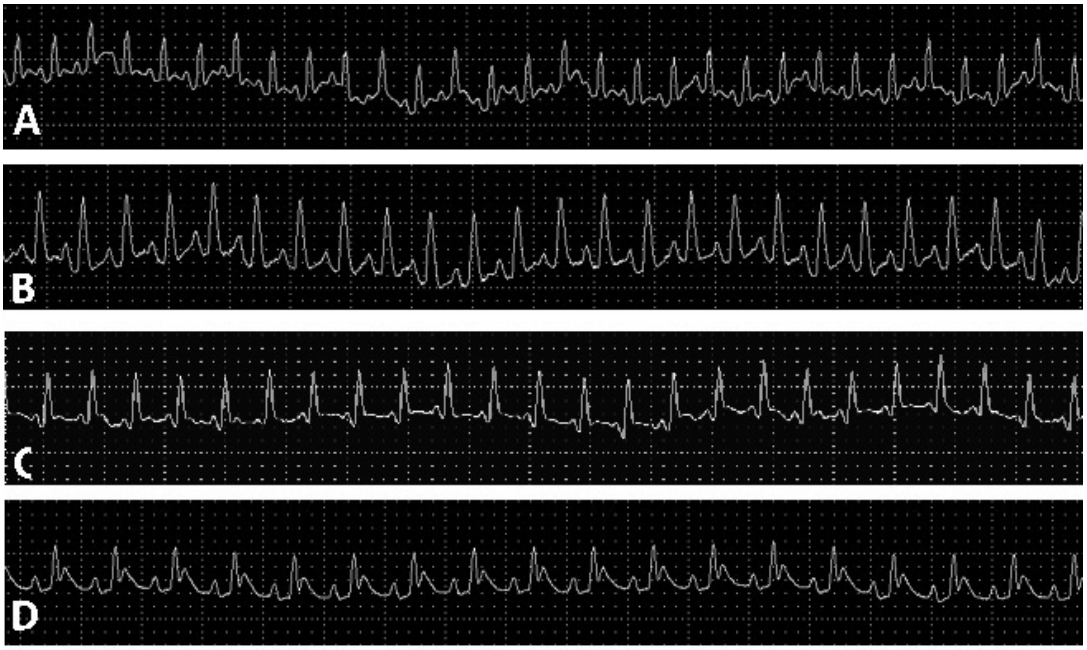

Fig.1. Computerized electrocardiographic tracings of healthy anesthetized (**A**) Swiss, (**B**) C57BL/6, (**C**) FVB/NJ mice and (**D**) Wistar rat. Recordings of the second deviation of the frontal plane (DII), with velocity of 50mm/s and amplitude 2N. Red tracings determine the QT interval.

Table 3. Mean, standard deviation, minimum and maximum values of the electrocardiographic parameters of C57BL/6 mice. Tracings were recorded in six derivations of the frontal plan, with 50mm/s of velocity and sensitivity of 1cm=2mV (2N) in healthy adult anesthetized mice (*Mus musculus*) using a computerized ECG

Parameters	Mean ± SD	Min	Max
HR (bpm)	432.75 ± 81.79	281	510.4
P (ms)	31.23 ± 3.67	25.2	37
P (mV)	0.034 ± 0.008	0.02	0.055
PR (ms)	46.13 ± 5.77	38.2	38.00
QRS (ms)	43.39 ± 4.58	38.2	51.80
R (mV)	0.15 ± 0.04	0.09	0.227
T (mV)	0.033 ± 0.014	0.02	0.594
QT (ms)	88.53 ± 8.37	75	123

HR = Heart rate, Pms = duration of the P wave, PmV = amplitude of P wave, PRms = duration of the PR segment, QRSms = duration of the QRS complex, RmV = amplitude of R wave, TmV = amplitude of T wave, QTms = duration of QT interval.

Table 4. Mean, standard deviation, minimum and maximum values of the electrocardiographic parameters of FVB/NJ mice. Tracings were recorded in six derivations of the frontal plan, with 50mm/s of velocity and sensitivity of 1cm=2mV (2N) in healthy adult anesthetized mice (*Mus musculus*) using a computerized ECG

Parameters	Mean ± SD	Min	Max
HR (bpm)	478.58 ± 69.06	374	516.3
P (ms)	31.12 ± 3.89	27	38
P (mV)	0.038 ± 0.008	0.02	0.054
PR (ms)	32.09 ± 3.51	29	37
QRS (ms)	40.55 ± 3.30	34.5	46
R (mV)	0.20 ± 0.08	0.15	0.363
T (mV)	0.03 ± 0.006	0.02	0.041
QT (ms)	82.26 ± 6.87	71	95

HR = Heart rate, Pms = duration of the P wave, PmV = amplitude of P wave, PRms = duration of the PR segment, QRSms = duration of the QRS complex, RmV = amplitude of R wave, TmV = amplitude of T wave, QTms = duration of QT interval.

Table 5. Mean, standard deviation, minimum and maximum values of the electrocardiographic parameters of Wistar rats. Tracings were recorded in six derivations of the frontal plan, with 50mm/s of velocity and sensitivity of 1cm=2mV (2N) in healthy adult anesthetized rats (*Rattus novergicus*) using a computerized ECG

Parameters	Mean ± SD	Min	Max
HR (bpm)	326 ± 57.45	189	378
P (ms)	34.40 ± 5.01	25	43
P (mV)	0.050 ± 0.015	0.03	0.082
PR (ms)	54.50 ± 10.14	48	75
QRS (ms)	34.10 ± 4.58	27	42
R (mV)	0.182 ± 0.064	0.13	0.293
T (mV)	0.046 ± 0.02	0.03	0.086
QT (ms)	75.60 ± 11.89	62	98

HR = Heart rate, Pms = duration of the P wave, PmV = amplitude of P wave, PRms = duration of the PR segment, QRSms = duration of the QRS complex, RmV = amplitude of R wave, TmV = amplitude of T wave, QTms = duration of QT interval.

Table 6. Pearson's correlation values between QT correction formulas of adult healthy Swiss mice, C57BL/6 mice, FVB/NJ mice and Wistar rats. The following formulas were used: Bazett, Fridericia, Van der Water, Framingham, Hodges and Mitchell. Recordings were made in six derivations of the frontal plan, with 50mm/s of velocity and sensitivity of 1cm=2mV (2N) using a computerized ECG

Swiss	RR	HR	QT
QTcB	-0.536	0.559	0.717
QtcFri	-0.341	0.365	0.852
QTcV	0.2	-0.175	1
QTcH	-0.988	0.998	-0.107
QtcFra	-0.256	0.277	0.896
QTcM	-0.461	0.458	0.682
C57BL/6	RR	HR	QT
QTcB	0.05	-0.035	0.689
QtcFri	0.397	-0.380	0.9
QTcV	0.756	-0.735	1
QTcH	-0.977	-0.998	-0.687
QtcFra	0.502	-0.482	0.946
QTcM	0.033	-0.017	0.676
FVB/NJ	RR	HR	QT
QTcB	-0.329	0.267	0.613
QtcFri	-0.012	-0.053	0.833
QTcV	0.541	-0.594	1
QTcH	-0.966	0.999	-0.553
QtcFra	0.191	-0.251	0.902
QTcM	-0.344	0.286	0.573
Wistar	RR	HR	QT
QTcB	-0.222	0.334	0.754
QtcFri	-0.002	0.120	0.880
QTcV	0.473	-0.359	1
QTcH	-0.954	0.993	-0.243
QtcFra	-0.019	0.125	0.713
QTcM	0.226	0.339	0.750

QTcB = QT*(RR)1/2 (Bazett 1920), QTcFRI = QT*(RR)1/3 (Fridericia 1920), QTcV = QT + 0.087(1- RR) (Van der Water et al. 1989), QTcFRA = QT + 0.154*(1-RR)] (Sagie et al. 1992) and QTcM = QT/ square root of (RR/100) (Mitchell et al. 1998).

DISCUSSION

ECG is routinely used in both medicine and experimental research and although it defined as a simple technique, its interpretation may often be challenging. The lack of standardization for ECG parameters is an important gap for research comparisons. Different experimental designs, such as type of electrodes and anesthesia, age and size of the animals partake in its misinterpretation (Konopelski & Ufnal 2016).

The present work aims to contribute to such studies using the most common species and lineages evaluated in cardiovascular research. The use of a non-invasive six-channel ECG (Botelho et al. 2016) and isoflurane anesthesia is a well-known technique that provides harmless, safe and low-cost recording with high repeatability (Murakami et al. 2014). Ten animals were evaluated considering the similarity amongst rodents as previous works evaluating ECG variations showed such number is sufficient to establish correlations of its parameters (Roussel et al. 2016).

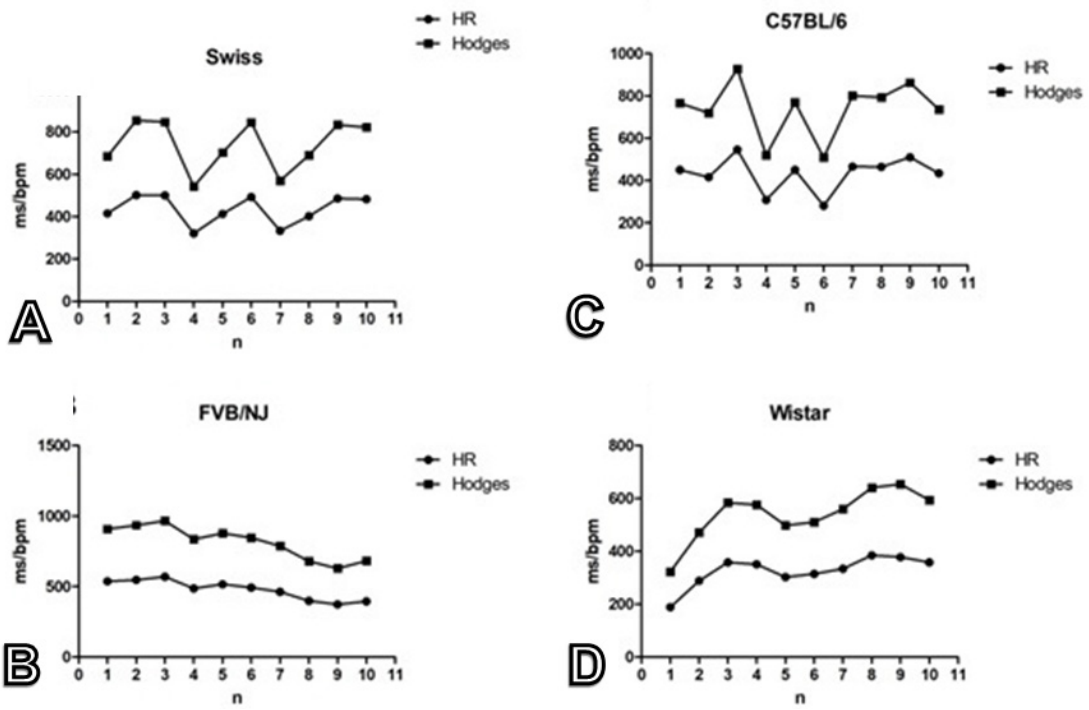

Fig.2. Pearson's correlation between Hodge's QT correction formula and hear rate (HR) studied in healthy adult anesthetized (**A**) Swiss, (**B**) C57BL/6, (**C**) FVB/NJ mice and (**D**) Wistar rats.

Mice mean HR of the lineages study was about 450bpm, similar to previous studies (Shintaku et al. 2014). It is noteworthy that HR is affected by the type of anesthesia used in all rodent species. For example, ether anesthesia of Wistar rats defines HR between 290-378 bpm (Fraser et al. 1967), under urethane anesthesia from 357-452bpm (Buschmann et al. 1980), while ketamine and xylazine from 242-336bpm (Miranda et al. 2007). The present work describes 326±57.45bpm in accordance to values previously found (320±6 bpm) (Murakami et al. 2014).

PR interval is also affected by anesthesia and was reported in Wistar rats to be 39 to 78ms and of 35.7±0.9ms in mice (Shintaku et al. 2014). The present study revealed similar mean values of 34.4 for rats and 35.7 for mice. Previous work has described important electrophysiological differences amongst animals (Boukens et al. 2014), especially regarding the expression of ionic channels that may influence the atrioventricular conduction and therefore PR interval. Although anatomy and weight of the mice are similar, the singularities in electrophysiological parameters amongst lineages may be responsible for the differences in PR value.

Concerning QT correction, the different correlations amongst formulae reveals the importance in establishing the most adequate for animal models and methodology. Previous studies used formulas more suitable for human parameters, may have generated misguidedly values (Costa et al. 2008) as each formula is more suitable for a specific HR range. Our findings reveal that Hodge's formula has the best correlation to HR/RR and therefore should be used for this model. The lowest correlations were found for Bazett and Fridericia as they are more appropriate to HR lower than those found in rats and mice. This finding is particularly important for hypokalemia evaluation, ischemia, myocardial infarction, channelopathies, drug toxicity, and overall pathologies that disturb cardiomyocyte repolarization. As QT prolongation is one of the predictors of ventricular arrhythmias, its thorough evaluation may provide insights into rodent ECG alterations and better correlate human disease to animal models. A recent study using anesthetized mice showed that QT has little correlation to HR (Roussel et al. 2016); however, there is no consensus on whether anesthetized models require adjustment of QT to HR (Hayes et al. 1994, Kmecova & Klimas 2010), as the majority of rat studies uses one of several formulas to calculate QTc (Hamdy & Brocks 2009). Nonetheless, the present work using different anesthetized rodents showed there is a moderate to high correlation between corrected QT and RR/HR.

CONCLUSIONS

Considering the proposed model for ECG evaluation, our study describes the references intervals for Swiss, FVB/NJ, C57BL/6 mice and Wistar rats. It also suggests that Hodge's formula is the most suitable QT correction formula and that Bazett's and Fridericia formulas used for such animals should be questioned.

The present work contributes to electrocardiographic investigation in experimental cardiology and may reduce misinterpretations in rodents' ECG.

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Original Article
Wildlife Medicine

Fatal hemothorax caused by pleural mesothelioma in a lion[1]

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ABSTRACT.- Rocha P.R.D., Duarte R.P., Laranjeira M.G., Melo T.A.L., Azevedo E.Z., Garcia S.D. & Machado G.F. 2019. **Fatal hemothorax caused by pleural mesothelioma in a lion**. *Pesquisa Veterinária Brasileira 39(6):416-418.* Faculdade de Medicina Veterinária, Universidade Estadual Paulista "Julio de Mesquita Filho", Rua Clóvis Pestana 793, Dona Amélia, Araçatuba, SP 16050-680, Brazil. E-mail: giselem@fmva.unesp.br

Mesothelioma is considered a malignant neoplasm caused by the proliferation of mesothelial cells mostly from the pleura, peritoneum and pericardium. Here we described a case of fatal hemothorax caused by pleural mesothelioma in a lion by means of necropsy, histopathology and immunohistochemistry. Gross inspection of the thoracic cavity showed hemothorax with about 4 liters of blood. Microscopically, numerous, randomly distributed, soft, red-pink, irregular masses with up to 1cm in diameter were observed in both visceral and parietal pleurae. Microscopically, a papillary structure pattern was observed in the thoracic masses, composed mainly by one layer of cubic mesothelial cells, which presented eosinophilic cytoplasm, central nucleus and evident nucleolus, supported by a low cellular fibrovascularstroma. Neoplastic cells were positive for both cytokeratin and vimentin by immunohistochemistry. This seems to be the first report of fatal hemothorax caused by pleural mesothelioma in a lion.

INDEX TERMS: Hemothorax, pleural mesothelioma, immunohistochemistry, *Panthera leo*, lion, mesothelioma, zoo animal, wildlife animals, pathology.

RESUMO.- [**Hemotórax fatal causado por mesotelioma pleural em um leão.**] O mesotelioma é considerado um neoplasma maligna causada pela proliferação de células mesoteliais, principalmente da pleura, peritôneo e pericárdio. O presente caso descreve os achados macroscópicos, microscópicos e imuno-histoquímicos do hemotórax fatal causado por um mesotelioma pleural em um leão. Macroscopicamente, na cavidade torácica, foi observado cerca de 4 litros de sangue. Além disso, foram observadas numerosas massas macias, vermelho-rosa, irregulares, com até 1cm de diâmetro e distribuídas aleatoriamente pelas pleuras parietal e visceral. Microscopicamente, as massas torácicas apresentavam estruturas papilares, compostas por uma camada de células mesoteliais, que apresentavam citoplasma eosinofílico, núcleo central e nucléolo evidente, suportada por um estroma fibrovascular pouco celular. A imuno-histoquímica foi positiva para ambas citoqueratina e vimentina nas células neoplásicas. Este trabalho descreve o que parece ser o primeiro relato de um hemotórax fatal causado por um mesotelioma pleural em um leão.

TERMOS DE INDEXAÇÃO: Hemotórax, imuno-histoquímica, *Panthera leo*, leão, mesotelioma pleural, animais de zoológico, animais silvestres, patologia.

INTRODUCTION

Mesothelioma is a cancer that arises from mesothelial cells of the pleura, peritoneum and pericardium, and occasionally from the tunica vaginalis testis (Gibbs & Berry 2008, Bollo et al. 2011). This tumor can have a localized to more diffuse distribution and may present as multiple to coalescing nodular, and sessile to more pedunculated structures (Head et al. 2002). Mesothelioma is rare in all species but has been recorded most frequently in humans, especially associated with exposure to

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asbestos and erionite (Gibbs & Berry 2008, Bollo et al. 2011) and it has also been experimentally induced in laboratory rodents, primates and dogs by exposure to asbestos fibers (Bollo et al. 2011). Mesothelioma is more frequent in cattle and dogs, but has been occasionally reported in horses, cats, pigs, a mouse and a goat (Krametter et al. 2004, Stoica et al. 2004, Brown et al. 2007). Although there is one description of pleural mesothelioma in a lion (Bollo et al. 2011), so far there is no association of this neoplasm with hemothorax in wild or domestic cats. The present case described a fatal hemothorax caused by pleural mesothelioma in a lion by means of necropsy, histopathology and immunohistochemistry.

CASE REPORT AND DISCUSSION

A 25 year old, male lion (*Panthera leo*), kept in a Zoo was referred for necropsy because of sudden death. The day before, the animal presented apathy, anorexia, dyspnea and an episode of emesis. On necropsy, the animal was in good body condition but markedly dehydrated. Oral and ocular mucosae were markedly pale. Numerous randomly distributed, soft, red-pink, irregular masses with up to 1cm in diameter were observed in both visceral and parietal pleurae. The thoracic cavity contained approximately 4 liters of blood hemothorax (Fig.1A). No masses were observed outside the thoracic cavity. Marked, diffuse emphysema was observed in the left lungs (Fig.1), whereas the right side was diffusely firm, dark-redand hemorrhagic (post-mortem hypostasis, figure not shown). No significant lesions were observed in other organs. Fresh samples of the thoracic masses, lungs, heart, brain, spinal cord, duodenum, liver and kidneys were fixed in 10% buffered formalin. Subsequently, tissues were routinely processed, embedded in paraffin and 4µm tissue sections were cut for histopathologic and immunohistochemical (IHC) evaluations. The sections for histopathology were stained with hematoxylin and eosin (HE).

Microscopically, the masses in the thoracic cavity had a papillary structure, composed mainly by one layer of cubic mesothelial cells, which presented eosinophilic cytoplasm, central nucleus and evident nucleolus, supported by a low cellular fibrovascular stroma, with a marked amount of collagen (Fig.1B). Mitosis or vascular invasion was absent. Marked emphysema, hyperemia, atelectasis and congestion were observed in the left lung. Erythrocytes in the bronchiolar lumen were observed, whereas in the right lungs, diffuse post-mortem hydrostatic congestion was present.

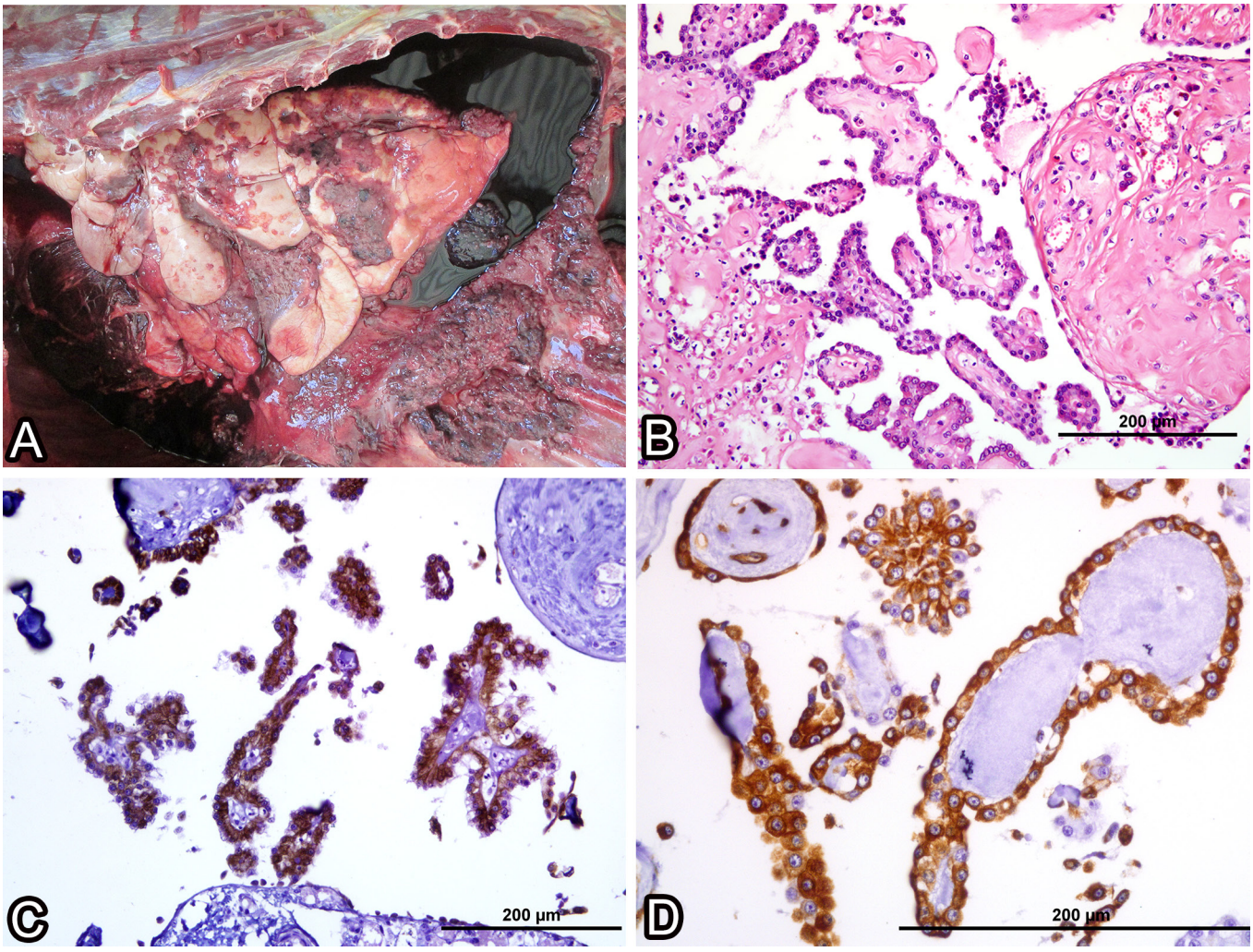

Fig.1. Fig.1. (**A**) Thoracic cavity and lungs. Mesothelioma in a lion (*Panthera leo*). Multifocal, soft, red-pink, irregular masses with up to 1cm in diameter in both visceral and parietal pleurae, associated with hemothorax. (**B**) Lungs, mesothelioma. Papillary mass, composed mainly by one layer of cubic mesothelial cells, supported by a low cellular fibrovascular stroma. HE, bar = 200µm. (**C**) Mesothelioma, cytoplasmic staining for cytokeratin in mesothelial neoplastic cells. IHC, bar = 200µm. (**D**) Mesothelioma, cytoplasmic immunolabeling for vimentin in mesothelial neoplastic cells. IHC, bar = 200µm.

Sections of thoracic masses were submitted for immunohistochemical analysis with anti-cytokeratin and anti-vimentin antibodies using commercial immunohistochemical kits. Briefly, indirect immunohistochemistry was performed using monoclonal primary antibodies against cytokeratin (DAKO, Carpinteria, USA, Code M3515, 1:200) and vimentin (DAKO, Code M7020, 1:100). For antigen retrieval, all sections were incubated in citrate buffer (pH 6.0) at 125°C for 10 min. The secondary detection system was a polymer LSAB2-HRP kit (Code K0679, DAKO, USA) was applied. Subsequently, all tissue sections were incubated with3,3'-diaminobenzidine chromogen (DAB, DAKO, USA) for 1 min at room temperature. Finally, the sections were counterstained using Mayer's hematoxylin. Positive controls included a mammary carcinoma and normal lung from two cats, respectively. Sections of the neoplasm herein described and the positive controls without the incubation of each primary antibody were used as negative controls. The cytoplasm of mesothelial cells was positive for both cytokeratin and vimentin (Fig.1B-C); the supportive connective tissue cells were also positive for vimentin (Fig.1D).

The gross and histologic findings in the lion of the present report were consistent with pleural mesothelioma. The diagnosis was confirmed by the immunolabeling of neoplastic cells for cytokeratin and vimentin. Immunohistochemical detection of both vimentin and cytokeratin are useful in distinguishing mesotheliomas from other epithelial or non-epithelial neoplasms (Brown et al. 2007). In domestic animals, mesothelioma is notable because it occurs most frequently as a congenital neoplasm in fetal or young cattle. In pigs, mesotheliomas are particularly more frequent as pleural, whereas in cattle they are more peritoneal (Krametter et al. 2004, Stoica et al. 2004, Brown et al. 2007). In wild cats, pleural mesotheliomas have been described in tigers (*Panthera tigris*) (Wiedner et al. 2008), leopards (*Neofelis nebulosa*) (Cunningham & Dhillon 1998), and a lion (Bollo et al. 2011). Even though metastasis were not observed in the present case, pleural mesothelioma may spread and implants within the pleural cavity to cause persistent thoracic effusion, and often invades the underlying tissue or reaches the abdominal cavity via lymphatics vessels (Caswell & Williams 2007). Moreover, pleural and peritoneal mesotheliomas can have a multicentric origin, and transcoelomic implantation with invasion of the underlying tissue is possible, although distant metastasis can seldom be found (Stoica et al. 2004).

Respiratory insufficiency associated with hypovolemic shock due to the massive hemothorax was most likely the causes of the death of this lion. Hemothorax is rare in human medicine and typically involves rupture of intrathoracic masses or underlying vascular pathology such as an aneurysm (Martinez et al. 1992). Bleeding associated with thoracic neoplasm has been attributed to several mechanisms, including: 1) direct exsanguination of the tumor into the pleural space, 2) acute bleeding due to rupture of the primary tumor, and 3) tumor invasion into larger vessels including arteries, causing vascular lesions (Nakamura et al. 2008, Snaebjornsson et al. 2011). In humans, spontaneous neoplasm-associated hemothorax has most commonly been associated with neurofibromatosis type 1, pseudomesotheliomatous carcinomas, angiosarcoma, hepatocellular carcinoma and lung carcinoma, the latter an uncommon cause of hemothorax (Snaebjornsson et al. 2011). In dogs, traumatic injuries and coagulopathies have been described as the most common causes of hemothorax. *Dirofilaria immitis*, *Spirocerca lupi*, hemangiosarcoma, mesothelioma, lung lobe torsion and pancreatitis may be other causes of hemothorax in dogs, even though the two latter are less commonly reported (Nakamura et al. 2008). To the best of the authors' knowledge, there are no descriptions of pleural mesothelioma associated with hemothorax in domestic or wild felids.

The differential diagnosis for pleural mesothelioma should include metastatic carcinomas, adenocarcinomas with diffuse peritoneal involvement and sarcomas (Stoica et al. 2004, Caswell & Williams 2007). In the abdominal cavity, mesothelioma must be differentiated from peritoneal tuberculosis, peritoneal metastases from other tumors, and bacterial and parasitic granulomas (Krametter et al. 2004).

No microscopic evidence of bacterial, parasitic or a different type of neoplasm was observed in the lion of the present case. In conclusion, this seems to be the first report of fatal hemothorax caused by pleural mesothelioma in a lion.

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

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Original Article
Animal Morphophysiology

Musculoskeletal ultrasonography of the elbow joint in dogs: applicability and evaluation protocol[1]

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ABSTRACT.- Bellegard G.M.C., Lopes E.R., Bisetto S.P. & Hage M.C.F.N.S. 2019. **Musculoskeletal ultrasonography of the elbow joint in dogs: applicability and evaluation protocol.** *Pesquisa Veterinária Brasileira 39(6):419-428*. Setor de Diagnóstico por Imagem, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade São Paulo, Avenida Duque de Caxias Norte 225, Zona Rural, Pirassununga, SP 13635-900, Brazil. E-mail: gabi_bellegard@hotmail.com

The elbow is a complex joint and has great clinical relevance in small animal medicine. Previous research in this area has been performed using radiographic and tomographic methods; however, there are limited studies on ultrasonography. The aims of this study was suggesting an evaluation protocol for elbow scan and describe the ultrasonographic anatomy of the elbow joint in dogs. Ten cross-breed dogs weighing 5-15kg underwent radiography and were selected for this ultrasonographic study. The protocol was established for the ultrasonographic description dividing the articular areas in the proximal, middle, and distal, lateral, cranial, medial, and caudal faces. The approach was performed in the longitudinal, transverse and oblique planes and the musculoskeletal structures were described according to the architecture, echogenicity and echotexture. Computed tomography and magnetic resonance imaging scans were obtained for one animal for comparison. Ultrasonography was effective in visualizing and analyzing muscles, tendons and ligaments. Bone contours and regions that have clinical significance such as the medial coronoid process and anconeus process were identified, but with limited access. Prior knowledge of the normal sonographic anatomy of the elbow joint, as well as its technical advantages and limitations will allow further studies related to the identification of musculoskeletal disorders.

INDEX TERMS: Musculoskeletal ultrasonography, elbow joint, canine, articulation, diagnostic imaging, standardization, dogs, morphology.

RESUMO.- [Ultrassonografia musculoesquelética da articulação do cotovelo em cães: aplicabilidade e protocolo de avaliação.] O cotovelo é uma articulação complexa e tem grande relevância clínica na medicina veterinária de pequenos animais. Pesquisas prévias nesta área foram realizadas utilizando radiografias e tomografia computadorizada, entretanto há limitados estudos com ultrassonografia. O objetivo desse estudo é sugerir um protocolo de avaliação da articulação do cotovelo e descrever sua anatomia ultrassonográfica. Dez cães sem raça definida, pesando 5-15kg foram submetidos à radiografias e foram selecionados para o estudo ultrassonográfico. O protocolo foi estabelecido para a descrição anatômica ultrassonográfica dividindo as articulações em proximal, média e distal, faces lateral, cranial, medial e caudal. A abordagem foi realizada nos planos longitudinal, transverso e oblíquo e as estruturas foram descritas de acordo com a arquitetura, ecogenicidade e ecotextura. Tomografia computadorizada e ressonância magnética foram realizadas em um animal para comparação. A ultrassonografia foi efetiva na visualização e análise de músculos, tendões e ligamentos. Os contornos ósseos e regiões com significado clínico como o processo coronóide medial e o processo ancôneo foram identificados, mas com acesso limitado. Conhecimento prévio da anatomia ultrassonográfica normal da arquitetura do cotovelo, bem como suas vantagens e limitações, irão permitir estudos adicionais relacionados à identificação de desordens musculoesqueléticas.

TERMOS DE INDEXAÇÃO: Ultrassonografia musculoesquelética, articulação do cotovelo, caninos, articulação, diagnóstico por imagem, padronização, morfologia.

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INTRODUCTION

Ultrasonography is a modality of diagnostic imaging that can be used to complement radiographic imaging in evaluating the musculoskeletal system (Samii & Long 2002, Knox et al. 2003, Villamonte-Chevalier et al. 2015b).

There is prevalent interest in finding alternative techniques to evaluate the joints, and ultrasound can pass easily through soft tissue and provides details of the bone surfaces. High definition equipment can provide information similar to that obtained with magnetic resonance imaging (Nazarian 2008). This is a dynamic imaging tool that can be used both during flexion and extension, and it is cost-effective, non-invasive, and does not require sedation or anesthesia (Samii & Long 2002, Knox et al. 2003).

Thoracic limb injuries in dogs with clinical signs of claudication are often related to elbow diseases (Cook & Cook 2009a). This joint has a complex structure and bio-mechanical physiology, and is of great importance to clinicians (Van der Meulen 2013). The elbow joint is commonly evaluated by radiography initially, especially in elbow dysplasia (Gielen et al. 2012), but apparently normal elbows in radiographs could be false negative in some cases (Sendyk-Grunkraut et al. 2017). Computed tomography and magnetic resonance imaging are also techniques that can be used, but these modalities are expensive and most of the times requires anesthesia (De Rycke et al. 2002, Cook & Cook 2009b). Even though these techniques are considered the gold standard (Sendyk-Grunkraut et al. 2017), they should be considered in cases when alternative technique findings are inconclusive (Brienza & Lacreta Júnior 2013).

Previous research in this area has been conducted using radiography and computed tomography; however, there are few studies using ultrasonography. Our study intended to evaluate the normal elbow joint of 10 healthy dogs by using ultrasonography and thereby suggest a suitable protocol that can help students and professionals to identify the elbow structures and reproduce the images for learning and help in their diagnosis.

MATERIALS AND METHODS

The project was approved by the Animal Experimentation Ethics Committee of FZEA-USP with protocol number 14.1.1490.74.0. Ten cross-breed dogs, weighing 5-15kg with no signs or history of claudication were selected. Before ultrasonographic evaluation, the animals underwent a complete blood count and measurement of the levels of urea and creatinine, alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase. Dogs with normal blood test results were forwarded to radiographic examination by analogical method, anesthesia was made when it needed. The thoracic limbs were evaluated in the mediolateral, craniocaudal and craniolateral-caudomedial directions as well as in the mediolateral direction during flexion. Dogs that showed any radiographic alteration were excluded from the ultrasonographic screening. Clinical, radiographic and ultrasonographic analysis was made by the postgraduate student, ex-resident student, in imaging diagnosis (G.M.C.B) with master's supervisor (M.C.F.N.S.H) care. During the project was notice a learning curve of the techniques that were performed.

Ultrasonographic study

Ultrasonography was performed with an Esaote® My Lab Class C Vet, equipped with a high frequency linear transductor (LA 533 8-13 MHz), for this study 10 MHz frequency was the most used. For a better image quality, hair from the middle humerus to the middle forearm was shaved in all dogs, except one, for which we used only alcohol and acoustic gel. All dogs were held in the lateral (right or left) decumbency position for the examination, with the limbs in extension or flexion depending on the region that was evaluated. The use of *standoff* was not necessary in this study.

Evaluation protocol for ultrasonography examinations

The elbow joint was divided into the proximal (distal humerus), middle (elbow joint) and distal regions (proximal forearm). The screening was started at the middle third of the humerus until the middle third of the radius and ulna. The joint was also divided into cranial, lateral, medial, and caudal faces.

Lateral face. The evaluation was performed with the limbs extended. Initially, the probe was placed in the longitudinal plane of the proximal region and the screening was performed from the cranial to caudal direction, approaching the cranio-lateral and caudo-lateral aspect. Then, the probe was placed in the middle region, taking the lateral epicondyle of the humerus as a reference, and the screening was performed in the same way. Finally, the transductor was placed in the distal region and the procedure was performed in the same way (Fig.1A).

Cranial face. The thoracic limbs were evaluated in the extension position. Screening of the middle aspect was performed with the probe placed in the longitudinal plane from the lateral to medial direction, approaching the cranio-lateral and cranio-medial faces (Fig.2A).

Medial face. The evaluation was made with the limb extended in the longitudinal plane. Initially, the proximal region was screened from the cranial to caudal direction, approaching the craniomedial and caudo-medial faces. Then, the probe was placed in the middle region, taking the medial epicondyle of the humerus as a reference, and the screening was performed in the same way. Finally, the same procedure was performed in the distal region (Fig.3A).

Caudal face. The evaluation was started with the probe placed in the middle region, with the limb extended in the longitudinal plane. The screening was first performed from the lateral to medial direction (Fig.4A). Then, the screening was performed in the same way but with the limb in flexion (Fig.5A). Subsequently, the probe was placed on the edge of the proximal and middle region in a transverse plane with the limb extended. The screening was performed from the proximal to distal direction (Fig.6A).

Complementary studies

The Veterinary Anatomy Laboratory of the Faculty of Animal Science and Food Engineering of the University of São Paulo (FZEA-USP, Pirassununga/SP, Brazil) provided anatomical structures and sections in transverse and longitudinal planes for elbow joint anatomical study. The sections were photographed and images of the anatomical structures were compared with the images obtained by radiography, ultrasonography, MRI, and CT scans of the region. Radiographic studies of the selected dogs were conducted before the mean study to a previous evaluation. Computed tomography[1] and

[1] CT images were acquired using Shimadzu®, helical, model SCT-7800.

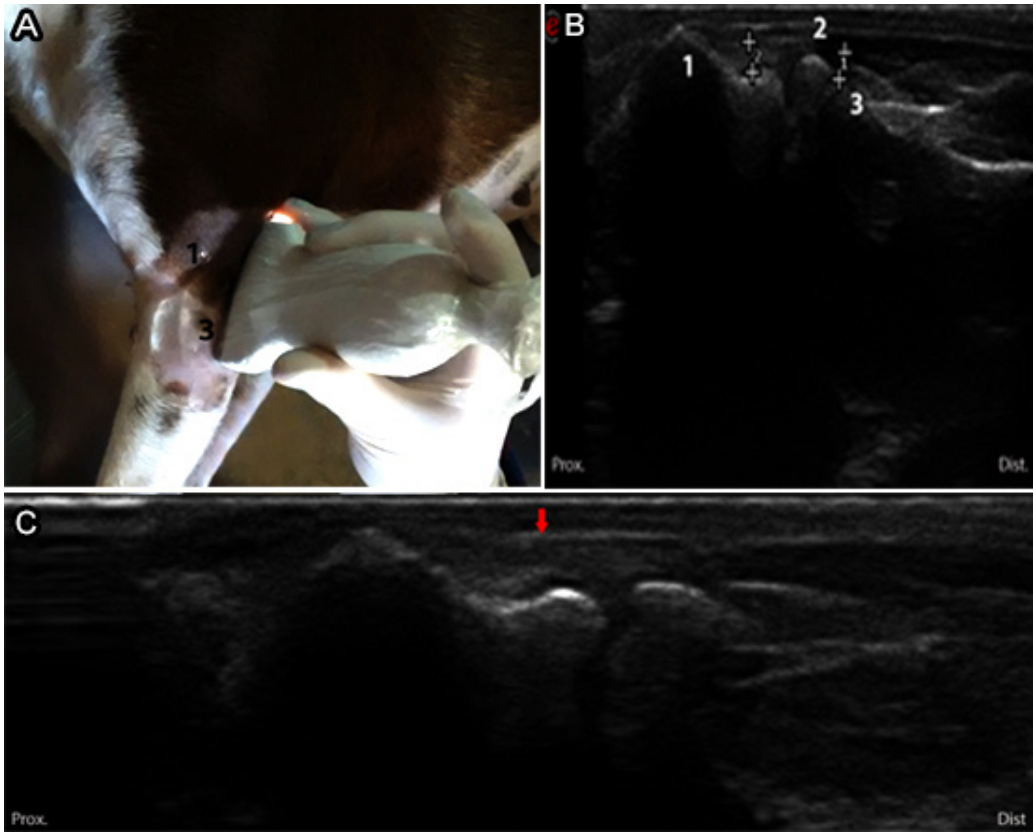

Fig.1. (**A**) Transducer positioning showing the topographic region of the structures of the lateral aspect of the elbow. Cranial (Cra.) and caudal (Cau.). (**B**) Ultrasonographic image corresponding to the assessed region. Lateral epicondyle of the humerus (1), lateral collateral ligament (2) and radius head (3). (**C**) Ultrasonographic image of the lateral collateral ligament (red arrow).

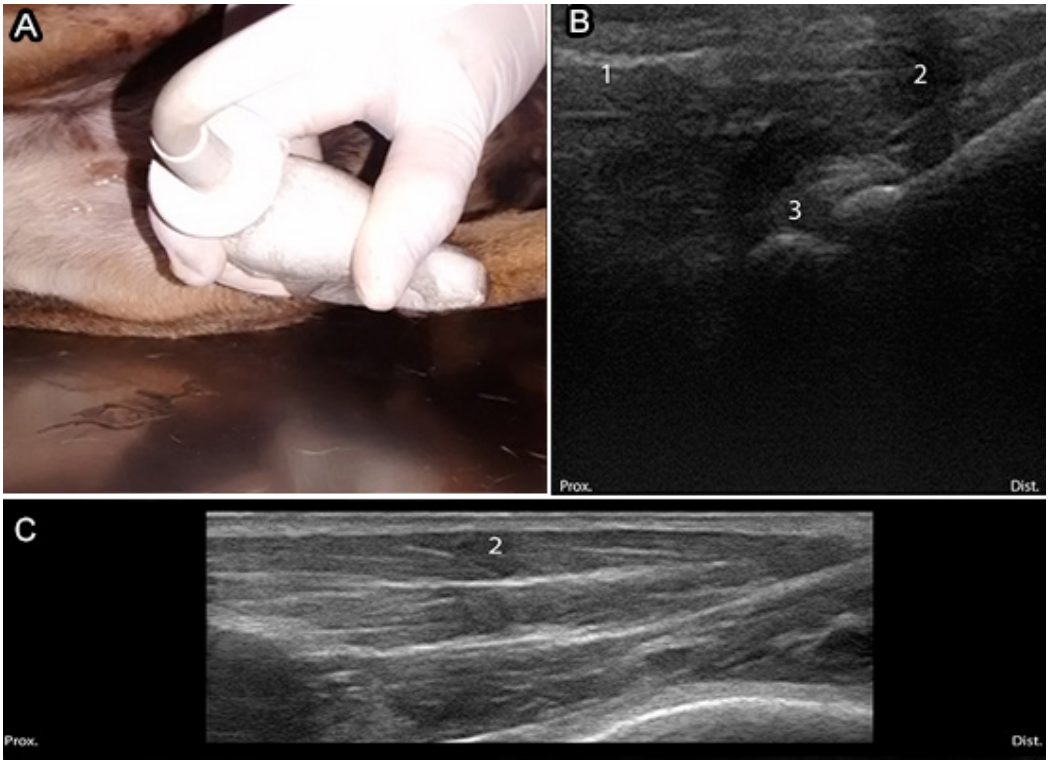

Fig.2. (**A**) Transducer positioning showing the topographic region of the structures of the cranial aspect of the elbow. (**B**,**C**) Ultrasonographic image corresponding to the assessed region. Brachial/brachial biceps muscle (1), extensor carpi radialis muscle (2) and joint space (3).

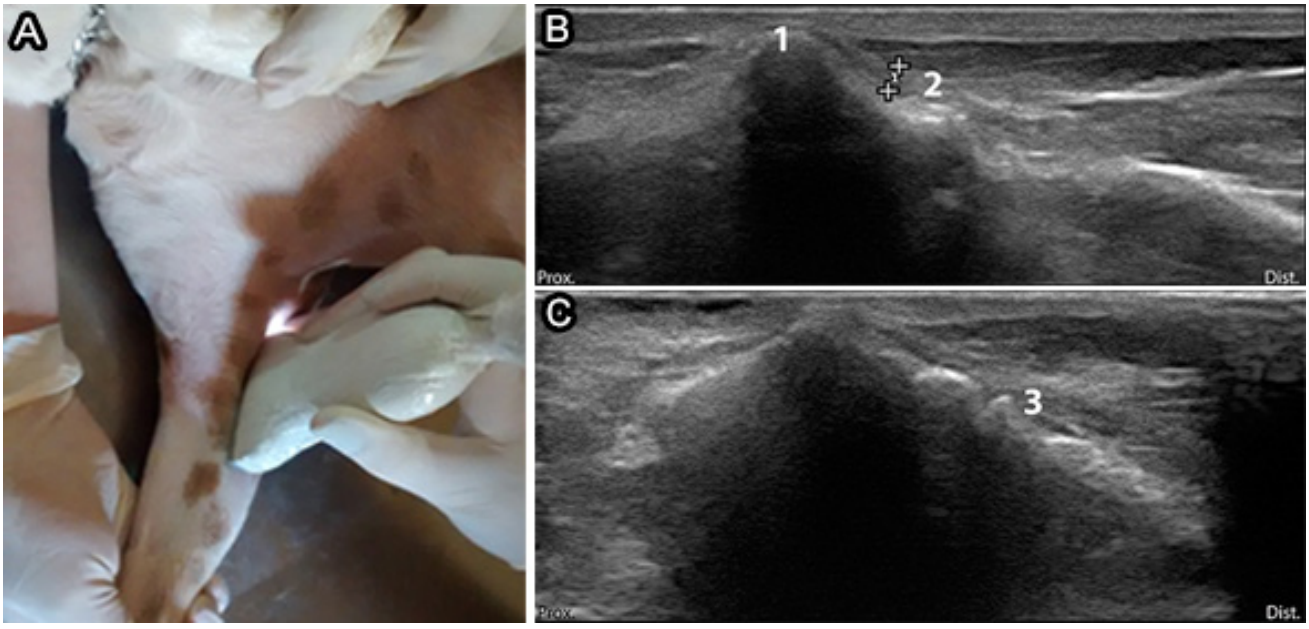

Fig.3. (**A**) Transducer positioning showing the topographic region of the structures of the medial aspect of the elbow. (**B**,**C**) Ultrasonographic images corresponding to the assessed region, with the limb in extension. Bone surface of the distal humerus (1), medial collateral ligament (2) and medial coronoid process of the ulna (3).

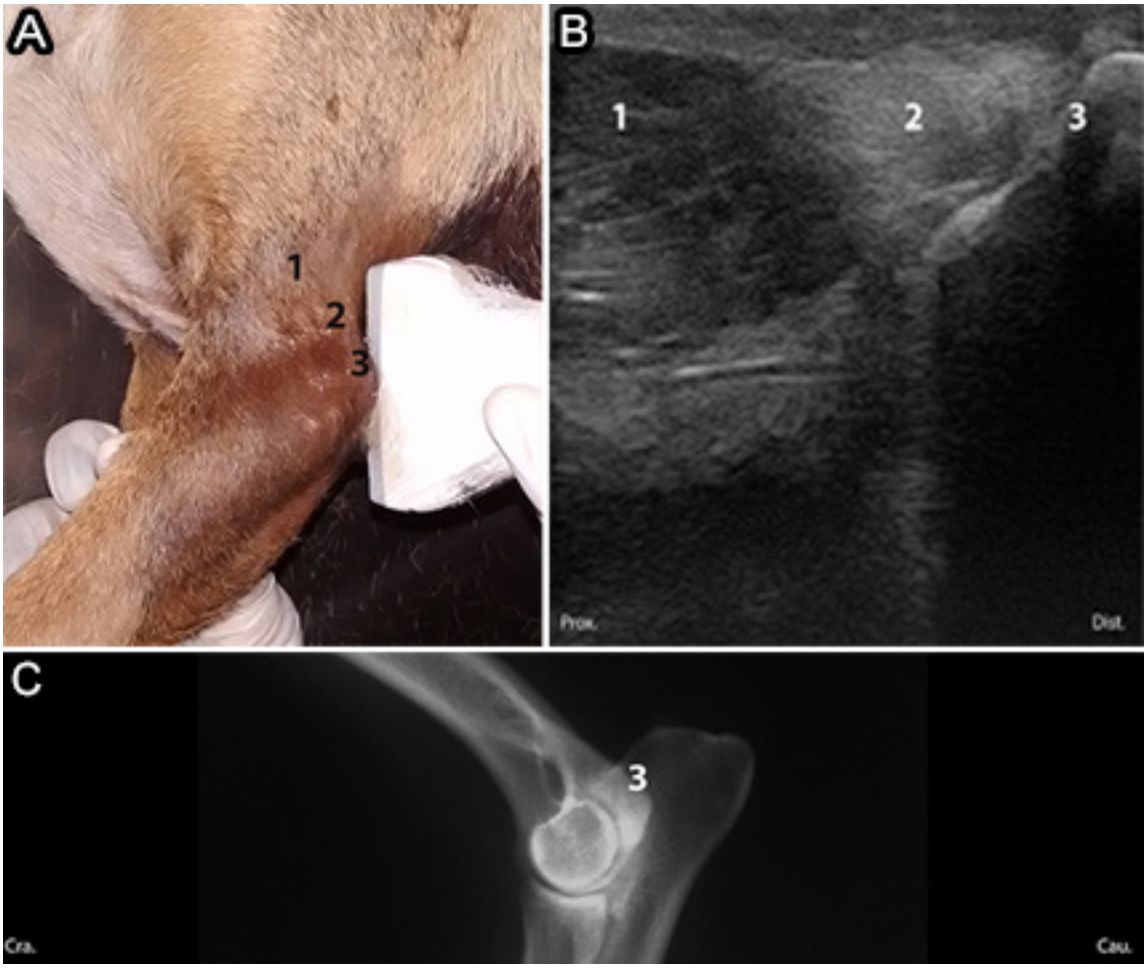

Fig.4. (**A**) Transducer positioning showing the topographic region of the structures of the caudal aspect of the elbow in extension. (**B**) Ultrasonographic image corresponding to the assessed region. Triceps brachii muscle (1), triceps brachii muscle tendon (2) and olecranon (3). (**C**) Radiographic image in the mediolateral projection of the elbow joint in a neutral position for comparison.

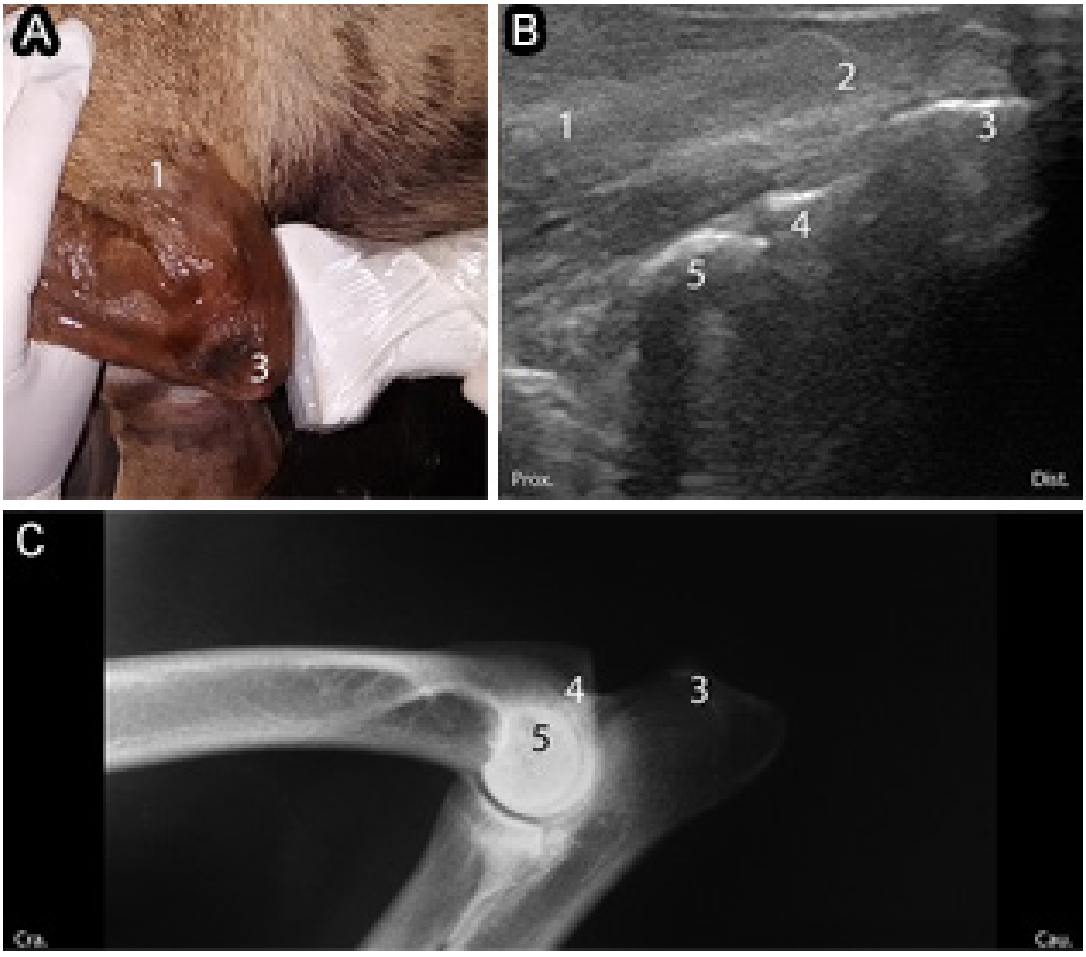

Fig.5. (**A**) Transducer positioning showing the topographic region of the structures of the caudal aspect of the elbow in flexion. (**B**) Ultrasonographic image corresponding to the region evaluated with the limb. Triceps brachii muscle (1), triceps brachii muscle tendon (2), olecranon (3), anconeal process (4) and humeral condyle (5). (**C**) Radiographic image in the mediolateral projection of the elbow joint in flexion for comparison.

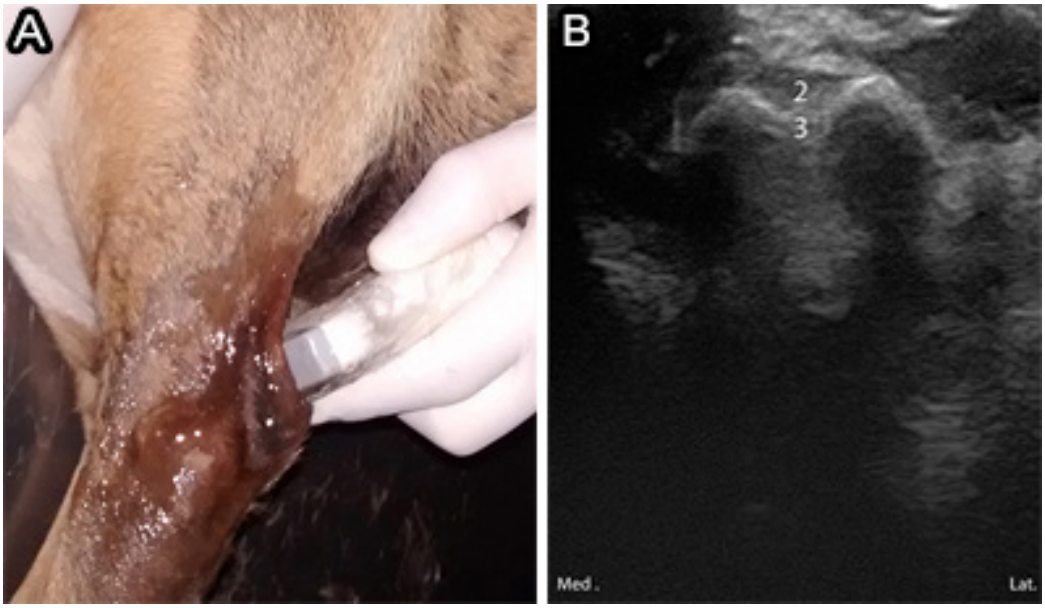

Fig.6. (**A**) Transducer positioning showing the topographic region of the structures of the caudal aspect of the elbow. (**B**) Ultrasonographic image corresponding to the region evaluated in the transverse plane. Triceps brachii muscle tendon (1), subtendinous bursa of the tendon of the triceps brachii muscle (2) and olecranon (3).

Table 1. Identification of the structures of the elbow joint by the ultrasonographic method in comparison to the other diagnostic imaging techniques

Structures	Imaging technique			
	XR	US	TC	MR
Humeral surface	+++	++	+++	++
Lateral condyle of the humerus	++	+	+++	++
Lateral collateral ligament	-	+++	-	+
Articular space	+	++	+++	+++
Radius surface	+++	++	+++	++
Ulnar surface	++	++	+++	++
Medial condyle of the humerus	++	+	+++	++
Medial collateral ligament	-	+++	-	+
Medial coronoid process of the ulna	+	+	+++	++
Flexors muscles	-	+++	+	++
Triceps brachii muscle	-	+++	+	++
Triceps brachii muscle tendon	-	+++	-	+++
Olecranon	++	+	+++	++
Anconeal process	++	+	+++	++
Subtendinous bursa of the triceps brachii muscle	-	++	-	-
Extensors muscles	-	+++	+	++
Brachii/biceps brachii muscle	-	+++	+	++

XR = x-ray, US = ultrasound, CT = computed tomography, MR = magnetic resonance; - unidentified structure, + poorly visible structure, ++ moderately visible structure, +++ clearly visible structure.

magnetic resonance imaging[2] scans were obtained for one animal as an additional part of this study to compare the additional findings of these techniques.

In this study, different diagnostic imaging tests were compared regarding the quality of visibility of the previously described structures identified on ultrasonography. The analysis was performed by checking the images; these were analyzed by the examiner and compared with the information in the literature. They were divided into unidentified structures, structures with poor visibility, structures with moderate visibility, and structures with excellent visibility (Table 1).

A subjective evaluation was made considering the superinposition or not of the studied structures, the complete or partial identification and the detailing, according to the limitation of each technique.

RESULTS

During the ultrasonography examinations, all the dogs were co-operative, except one, which required tranquilization for radiographic and ultrasonographic evaluation. Radiographic study was used as an exclusion criterion and it allowed the initial evaluation of the bone and articular structure, even though this technique did not exclude all the articular injuries. One limb each from four of the animals was excluded from the experimental group because of the presence of periarticular osteophytes and pre-existing fracture.

Ultrasonographic assessment was performed according to the proposed evaluation protocol. All the dogs that have been evaluated didn't show ultrasonographic changes.

The lateral face of the elbow joint was the first region of study. Muscular structures were visible as hypo-echogenic areas with parallel hyper-echogenic lines in between. The brachii muscle was located in the cranio-lateral section adjacent to the humeral surface. In the caudo-lateral section, the distal portion of the triceps brachii muscle was identified; the extensor muscles were distal to the elbow joint in the cranio-lateral section of the forearm. The bone surfaces of the humerus and radius head were characterized as intense hyper-echogenic lines with acoustic shadows. The collateral lateral ligament was observed as fine hyper-echogenic parallel lines originating in the lateral humerus epicondyle and adjacent lateral bone surfaces (Fig.1B).

In the longitudinal plane of the cranial face, the articular space was identified as a gap between the bone surfaces of the distal region of the humerus condyle and the proximal region of the radius. The biceps brachii muscle and brachii muscle were observed in the proximal portion in relation to the elbow joint. Distally, in the forearm region, the carpus radialis extensor muscle was identified (Fig.2B).

The triceps brachii muscle was seen in the longitudinal plan of the proximal region in the caudo-medial portion of the medial face of the elbow joint. In the craniomedial portion in the proximal region of the medial face, the biceps brachii muscle could be seen. In the forearm region in the caudo-medial portion, it could be identified the flexors muscle group.

In the longitudinal plane at the middle region of the medial face, a curved hyper-echogenic surface was characterized as the distal portion of the humerus, corresponding to the medial epicondyle. The medial collateral ligament had the same characteristics as the lateral collateral ligament and originated in the medial humerus epicondyle. Oblique sections were obtained for characterization of the medial coronoid process, because it could not be seen at the same plane as the collateral medial ligament (Fig.3B).

The caudal face of the elbow joint was analyzed with the thoracic member in extension and flexion. The triceps brachii muscle and its tendon were identified in the longitudinal plane. Hyper-echogenic parallel lines with insertion in the olecranon were observed in the middle region of the joint. The anconeal process was seen as a hyper-echogenic curved line, forming acoustic shadows, identified on flexed limbs (Fig.4B and 5B).

In the transverse cross-section of the middle region of the caudal face of the elbow, the triceps brachii muscle tendon, subtendinous bursa of the triceps brachii muscle, and the olecranon surface were identified. A hyper-echogenic structure was observed in the superficial region, corresponding to the tendon and subtendinous bursa, which was iso-echogenic to the muscle region between the tendon and the olecranon bone surface (Fig.6B).

Computed tomography (CT) could evaluate the elbow joint in the transverse section without superpositioning. This is an important advantage over radiographic and ultrasonography exams. Furthermore, could clearly characterized the bone structure, especially in the region of the medial coronoid process (Fig.7B,C) and provides a great perception of elbow congruence.

[2] MRI images were acquired using Esaote® model Vet MR Grande - 0.25 Tesla.

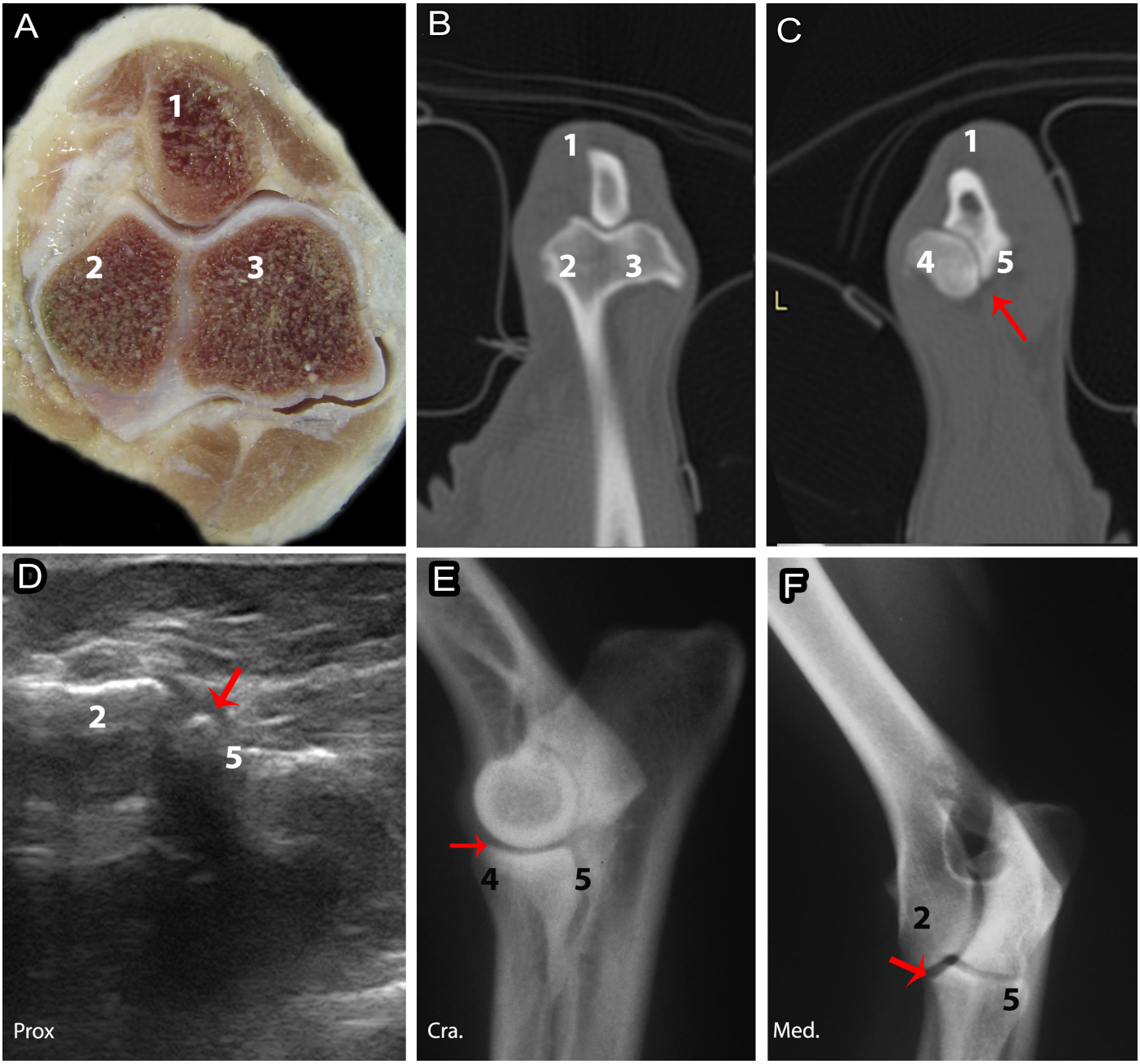

Fig.7. (**A**) Cross-section anatomy of the region of the humeral condyles. (**B**) Computed tomography image of the region of the humeral condyles in the transverse plane. (**C**) Computed tomography image in the transverse plane of the region of the medial coronoid process of the ulna (red arrow). (**D**) Ultrasonography image in the longitudinal oblique section (craniomedial) in the middle region of the joint with visualization of the medial coronoid process of the ulna (red arrow). (**E**,**F**) Radiographic images of the elbow joint in the mediolateral and craniocaudal oblique projections, showing the region referring to the medial coronary process of the ulna (red arrow). Ulnar neck (1), medial humeral condyle (2), lateral humeral condyle (3), radio (4), ulna (5).

Magnetic resonance imaging could be used to examine soft tissues such as muscles, tendons, and ligaments, but the size of the elbow region made it difficult to observe the details. Even as CT could evaluate in transverse sections, being advantage over others techniques. The bone marrow, articular space, and subchondral bone could be clearly visualized, which cannot be fully characterizes in ultrasound technique (Fig.8B-E).

Ligaments (lateral collateral and medial collateral), tendons (triceps brachii muscle tendon) and muscles (triceps brachii, brachii/biceps brachii, flexores and extensors) were the structures that have most clearly visibility in ultrasound in this study. Bones and articular space have limited access, ultrasound only allowed the surface vision with a superficial evaluation.

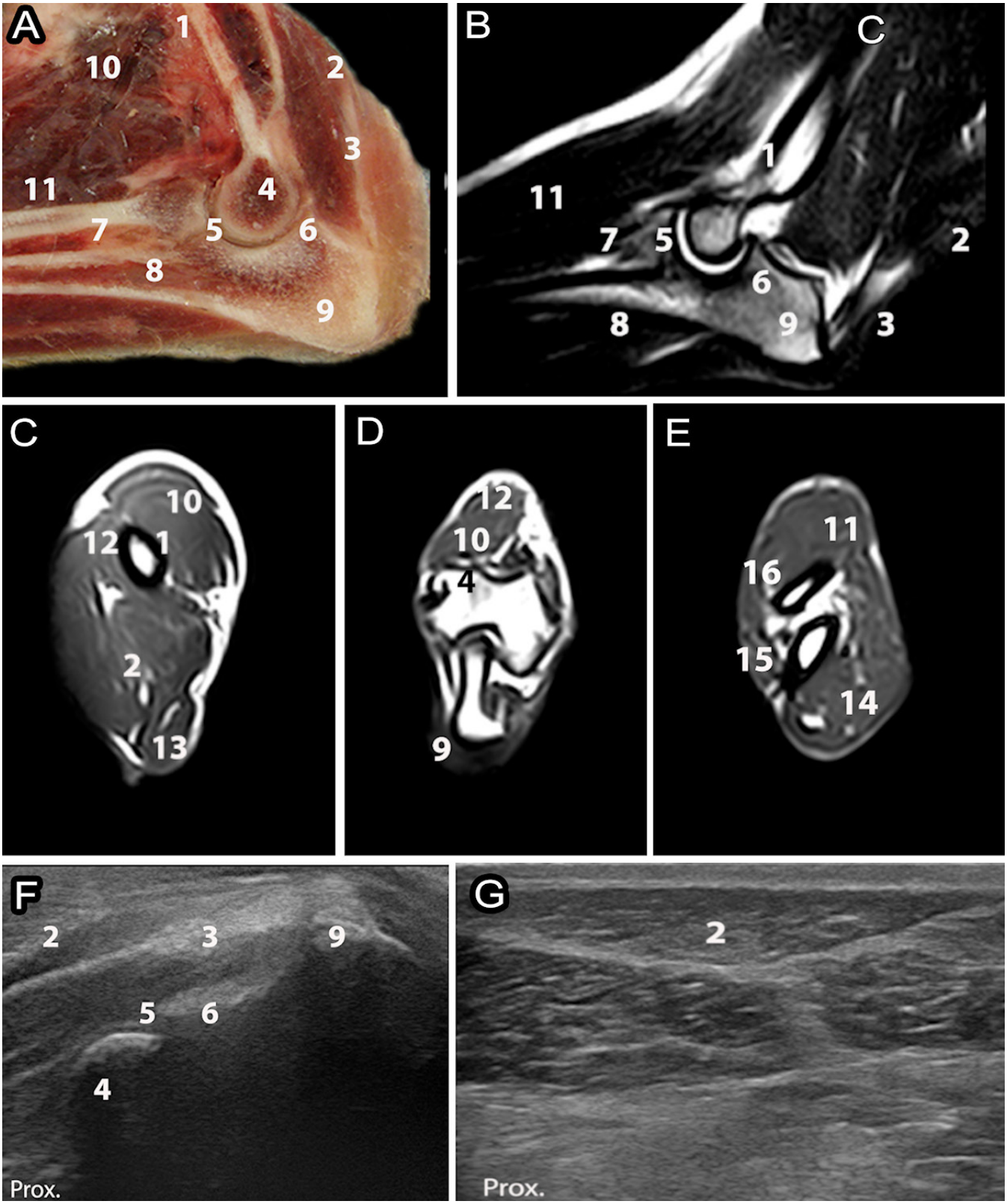

Fig.8. (**A**) Longitudinal anatomy of the elbow joint region. (**B**) Magnetic resonance imaging of the longitudinal plane elbow joint on T2-weighted fast spin echo (FSE) sequence. (**C**) Magnetic resonance imaging in the transverse plane of the proximal region, (**D**) region of the humeral condyles and (**E**) distal region in relation to the elbow joint in 3D sequence Hyce. (**F**) Ultrasonographic image of the caudal face of the elbow region and (**G**) lateral face in longitudinal plane. Humerus (1), triceps brachial muscle (2), triceps brachial muscle tendon (3), humeral condyle (4), joint space (5), anconeus process (6), radius (7), ulna (8), olecranon (9), biceps brachii muscle (10), carpal radial extensor muscle (11), brachial muscle (12), tensor muscle of the forearm fascia (13), flexor muscles (14), lateral ulnar muscle (15), common digital extensor muscle (16).

DISCUSSION

Most of the dogs evaluated in this study were co-operative during the examination, showing the possibility of routine use of ultrasonography. The animals used in this study were healthy, although sedation or anesthesia may be required depending on the level of pain. No previous studies have evaluated this issue.

The ultrasonographic evaluation protocol was created based on previous literature (Lamb & Wong 2005). The anatomical references used were the same, but this study suggests a refinement of regions division, a detailed description of the structures and transductor position, intended to do a didactic approach as a "step by step".

Results showed identification of most of the structures that was intended to demonstrate by the suggested protocol. Musculoskeletal ultrasonography examination is essential for the identification of structures. Standardization of the ultrasonographic procedure is one of the prerequisites for proper evaluation (Kramer et al. 2001). In this study, prior knowledge of anatomical structures was essential for the identification and description of the structures.

Ligaments showed significant characterization, highlighting collateral ligaments, which can be described as multiple linear hyper-echogenic structures, in agreement with the findings of Villamonte-Chevalier et al. (2015a).

Tendons and muscular structures were identified along with their points of origin and insertion. Ultrasonography allows evaluation of muscle fibers, their morphology, echogenicity, and echotexture. Muscles were described as hypo-echogenic structures with multiple fine hyper-echogenic lines, and the tendons as hyper-echogenic parallel lines, contiguous to the muscle inserted in the bone surface (Knox et al. 2003, Villamonte-Chevalier et al. 2015b).

The articular space was observed as a hypo-echogenic space between the surfaces of the humerus, radius, and ulna (Knox et al. 2003, Lamb & Wong 2005, Villamonte-Chevalier et al. 2015b). The identification of the articular space is useful when considering interventional procedures guided by ultrasonography.

The subtendinous bursa of the triceps brachii muscle was identified earlier in the study by Villamonte-Chevalier et al. (2015a). The subtendinous bursa was not observed in every animal in this study.

Bone structures were characterized by a hyper-echogenic interface, forming acoustic shadows, which have already been described in literature. It's believed that the technique can identify some discreet changes as bone fragments (Kramer et al. 1997), and could be used as additional technique to radiographic exam.

The medial coronoid process was identified in the oblique plane as a hyper-echogenic region, with acoustic shadows, adjacent to the articular space (Knox et al. 2003). Some morphological changes could be observed on ultrasonography as previously described by Seyrek-Intas et al. (2009). However, computed tomography can provide additional information regarding this region (De Rycke et al. 2002).

Ultrasound is effective to identify the joint space and evaluation of soft tissues specially ligament, tendons and muscles. For bone structures, CT is the chosen technique when radiographs are not sufficient for the diagnosis, CT can show similar information as arthroscopy and identify premature lesions in cases of fragmentation of coronois process. Besides that, is the gold standard technique for elbow incongruence evaluation (Sendyk-Grunkraut et al. 2017). Magnetic ressonance presents a great soft tissues diferenciation, identifying tendons, cartilage and other soft tissues injury (Gielen et al. 2012).

Ultrasound should be used for soft tissues evaluation in places that are not easily accessible using computed tomography and magnetic resonance imaging. One of the factors that lead to limited use of ultrasonography for musculoskeletal evaluation is the small number of professionals who perform this type of evaluation. Musculoskeletal ultrasonography is a difficult, operator-dependent modality with a long learning curve (Nazarian 2008); therefore, it depends on the training of the professionals in the area to disseminate the use of this technique.

CONCLUSIONS

Ultrasonography is useful for complementary assessment of the ligaments and tendons, and may also provide data about muscular structures. However, further studies are needed to evaluate its capacity for identifying musculoskeletal injuries.

The idea of suggesting a protocol with a description of the structures and anatomical references is interesting to stimulate veterinary students and professionals to deepen their knowledge in this area. A standard protocol is also useful in research.

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Original Article
Animal Morphophysiology

Seasonal influence on testicular morphophysiological parameters of bat *Carollia perspicillata* in fragments of the Atlantic Forest, northeastern Brazil [1]

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ABSTRACT.- Arandas M.J.G., Teixeira A.A.C., Texeira V.W., Silva F.R., Marinho K.S.N., Lima Junior N.B., Júnior F.C.A.A & Santos K.R.P. 2019. **Seasonal influence on testicular morphophysiological parameters of bat *Carollia perspicillata* in fragments of the Atlantic Forest, northeastern Brazil.** *Pesquisa Veterinária Brasileira 39(6):429-433.* Departamento de Morfologia e Fisiologia Animal, Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. E-mail: julianaarandas@hotmail.com

Bats belong to the order Chiroptera, family Phyllostomidae, and present a wide diversity of reproductive strategies. However, information on the reproductive biology of male bats is scarce, mainly in the Northeast Region of Brazil. Thus, this study evaluated the seasonal testicular histomorphometry of the bat *Carollia perspicillata* in fragments of the Atlantic Forest in Pernambuco state. To this end, adult males were collected, euthanized for removal of the testicles, and later submitted to a routine histological technique. Histomorphometric analysis included assessment of the areas of tubular and intertubular compartment occupation, as well as quantification of spermatocytes, rounded spermatids, elongated spermatids, and Sertoli and Leydig cells. Results indicated that this bat species presents reproductive seasonality, because significantly higher averages of the testicular parameters were observed in the rainy season, which is a period of greater availability of food resources. Such inferences indicate that there is a synchrony between peak spermatogenesis and hormonal inversion in the months of high precipitation; furthermore, a higher carrying capacity of the Sertoli cells is noted. *C. perspicillata* males possibly present greater sperm and androgenic activity in the rainy season, associated with increased tubular area and number of spermatogenic cells, as well as with the intertubular area and number of Leydig cells, respectively.

INDEX TERMS: Morphophysiology, *Carollia perspicillata,* Chiroptera, Phyllostomidae, Atlantic Forest, Brazil, bat, climatic factors, histomorphometry, testicles.

RESUMO.- [**Influência sazonal sobre os parâmetros morfofisiológicos testiculares de *Carollia perspicillata* (Chiroptera: Phyllostomidae) em fragmentos florestais de Mata Atlântica, Nordeste do Brasil.**] Os morcegos pertencem a ordem Chiroptera, família Phyllostomidae, e apresentam ampla diversidade de estratégias reprodutivas. Entretanto, as informações relacionadas à biologia reprodutiva dos machos são escassas, principalmente no Nordeste do Brasil. Dessa forma, o trabalho avaliou a histomorfometria sazonal testicular de *Carollia perspicillata* em fragmentos de Mata Atlântica de Pernambuco. Para tanto, os machos adultos foram coletados, eutanasiados para a remoção dos testículos, e posteriormente submetidos à técnica histológica de rotina. As análises histomorfométricas avaliaram as áreas de ocupação do compartimento tubular e intertubular, assim como a quantificação dos espermatócitos, espermátides arredondadas, espermátides alongadas, células de Sertoli e de Leydig. Os resultados indicaram que a espécie apresenta sazonalidade reprodutiva, visto que maiores médias significativas dos parâmetros testiculares foram encontradas na estação chuvosa, que é um período de maior disponibilidade de recursos alimentares. Tais

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inferências indicam que existe uma sincronia entre o pico de espermatogênese e investimento hormonal nos meses de alta precipitação, atrelado a isso, nota-se ainda, uma maior capacidade de suporte das células de Sertoli. Os machos de *C. perspicillata* possivelmente apresentam uma maior atividade espermática e androgênica na estação chuvosa, associadas ao aumento da área tubular e do número de células espermatogênicas, assim como da área intertubular e do número das células de Leydig, respectivamente.

TERMOS DE INDEXAÇÃO: Morfofisiologia, *Carollia perspicillata,* Chiroptera, Phyllostomidae, Mata Atlântica, Nordeste do Brasil, fatores climáticos, histomorfometria, morcego, testículo.

INTRODUCTION

Bats belong to the order Chiroptera, family Phyllostomidae, present a wide diversity of species, and evolved standards and reproductive strategies that correlate to the occurrence area, climate conditions, and food availability (Neuweiler 2000, Zórtea 2003). In tropical regions, bats reproductive events are associated with periods of increased precipitation (Fleming et al. 1972, Lima Junior et al. 2014).

Despite bat diversity, there is limited information on function, physiological control, and cyclicality of spermatogenesis (Krutzsch 2000). Testicular positioning is an aspect commonly reported in studies addressing male bat reproduction, as these organs present seasonal variation between the inguinal and abdominal region (Lima Junior et al. 2014); however, sexual activity cannot be established only with the external morphological features of the gonads, reason why evaluation of the testis morphological parameters can present inferences about the dynamics of gonadal development and species reproductive capacity (Beguelini et al. 2009, 2010, 2011, 2013a, 2013b, 2013c, 2013d, 2014, 2015, 2016, Morais et al. 2013a, 2013b, 2013c, 2014a, 2014b, Lima Junior et al. 2014, Farias et al. 2015, Notini et al. 2015).

Bats of the Phyllostomidae family are present in temperate and tropical areas, with records of variable reproductive strategies, including male reproductive cyclicality during the whole year (Handley Junior et al. 1991, Zortéa 2003, Oliveira et al. 2009, Duarte & Talamoni 2010, Notini et al. 2015). The species *Carollia perspicillata* (Linnaeus, 1758) belongs to a family widely distributed throughout Brazil, with preferably frugivorous food habit, and thus of great importance for seed dispersion (Charles-Dominique 1991, Mello et al. 2004). Regarding the reproductive biology, studies have indicated bimodal polyestry as the predominant reproductive cycle; their spermatogenesis show ultra-structural features similar to those of other species of the Phyllostomidade family (Mello et al. 1999, Mello & Fernandez 2000, Beguelini et al. 2014).

However, there are few or no studies addressing the seasonal testicular histomorphometry of these bats. Thus, this article evaluated the testicular histomorphometry of the bat *Carollia perspicillata* in the dry and rainy seasons in fragments of the Atlantic Forest in Pernambuco state, Brazil.

MATERIALS AND METHODS

Area of study. The study was conducted in fragments of the Atlantic Forest in the municipality of Sirinhaém (08º 35'27" S; 35º06'58" W), Pernambuco state - submontane and montane, dense ombrophilous (open and seasonal semi-deciduous) forest (Veloso et al. 1991).

Animal collection. Adult male bats (*Carollia perspicillata,* Chiroptera: Phyllostomidae) were captured monthly, during three consecutive nights between 5 PM and 5 AM, using mist nets, from September 2008 to October 2009.

Animals were captured using mist nets (12x3m) authorized by the Chico Mendes Institute of Biodiversity Conservation (ICMBio) and the Biodiversity Information and Authorization System (SISBIO) (no. 2800740). The study was approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Pernambuco (UFPE) under protocol no. 23076.037360/2014-92.

Meteorological data and weather stations. The dry and rainy seasons were defined by the National Institute of Meteorology (INMet 2008/2009) as per analysis of temperature, humidity and rainfall (Table 1). Two groups were considered: dry season (September to February) and rainy season (March to August).

Reproductive stage. Adult males (n=60) were classified on the basis of testicular position: descending testis (located in the inguinal region) and non-descending testis (located in the abdominal region) (Gannon & Willig 1992).

Euthanasia. The animals selected for histomorphometric analysis were anesthetized with sodium pentobarbital at a concentration of 40mg kg^{-1} intraperitoneally, followed by a potassium chloride saturated solution at 40mg/kg^{-1}.

Histomorphometric and statistical analyses. A total of 26 adult males, comprising at the most two bats per month, were randomly selected according to the two established groups as follows: dry season (n=13) and rainy season (n=13).

After surgical incision from the abdominal region to the inguinal region, the testes were removed and had their tissues fixed in 10% neutral buffered formalin (NBF), routinely processed for histology, and embedded in paraffin (Behmer et al. 1976, Rieder & Schmidt 1987). 5µm-thick sections were obtained, stained with hematoxylin and eosin (HE), and analyzed under optical microscopy.

Table 1. Monthly averages of precipitation, air temperature and humidity determined by the National Institute of Meteorology (INMET) in a fragment of the Atlantic Forest, Pernambuco state, Brazil

Month	Year	Monthly averages		
		Precipitation (mm)	Temperature (ºC)	Humidity (%)
September	2008	47.6	25.3	78
October	2008	53.6	26.2	75
November	2008	16.0	26.9	69
December	2008	18.3	27.2	69
January	2009	85.2	27.3	70
February*	2009	376.1	26.5	79
March	2009	142.8	27.2	76
April	2009	351.8	26.4	83
May	2009	410.1	25.8	88
June	2009	333.0	25.0	86
July	2009	386.8	24.6	86
August	2009	290.2	24.6	82

*Although rainfall was high, it was concentrated in few days, which were followed by dry days, but the temperature patterns were similar to those of the dry season (APAC 2009).

Histological slides were photographed with a total of 100X and 400X magnification using the ScopePhoto software coupled to a camera positioned between the optical microscope and the computer. Thus, 10 photomicrographs were used per animal at each magnification increase.

Testicular histomorphometric analysis was performed using the ImageJ 1.44 software. The following parameters were assessed: number spermatocytes, rounded spermatids, elongated spermatids, and Leydig and Sertoli cells with 400X magnification, as well as percentage of tubular compartment and intertubular compartment occupation areas with 100X magnification.

Variables were submitted to Student's *t*-test and processed using the Statistical Package for the Social Sciences 15.0 software (SPSS Inc., Chicago, IL, USA) for comparison between data for the dry and rainy seasons. Values were considered statistically significant when $p<0.05$.

RESULTS

During the studied period, 60 *Carollia perspicillata* males were captured, 25 in the dry season, of which 80% (n=20) showed descendent testes and 20% (n=5) had non-descending testes, and 35 in the rainy season, of which 65.71% (n=23) presented descendent testes and 34.29% (n=12) showed non-descending testes (Table 2).

Testicular histological results indicated that *Carollia perspicillata* males with descending and non-descending testes observed in the dry and rainy seasons showed Sertoli cells of the spermatogenic lineage at different stages of maturation (spermatogonia, spermatocytes, rounded and elongated spermatids), as well as Leydig cells, regardless of testicular position (Fig.1).

Testicular histomorphometric analysis showed statistically significant differences with respect to tubular compartment occupation area ($p<0.001$), intertubular compartment occupation area ($p<0.001$) and number of spermatocyte ($p<0.001$), rounded spermatids ($p<0.001$), elongated spermatids ($p<0.001$), and Sertoli ($p<0.001$) and Leydig ($p<0.001$) cells, with the highest averages observed in the rainy season (Table 3).

Table 2. Relative frequency (RF) of *Carollia perspicillata* males with descending and non-descending testes in the dry and rainy seasons in a fragment of the Atlantic Forest, Pernambuco state, Brazil

	Dry season		Rainy season	
	RF (%)	N	RF (%)	N
Descending testis	80.00	20	65.71	23
Non-descending testis	20.00	5	34.29	12
Total	100	25	100	35

N= number of individuals.

Table 3. Mean and standard deviation of the areas of tubular compartment (TCOA) intertubular compartment (ITCOA) occupation, quantification of spermatocytes (SPC), rounded spermatids (RS), elongated spermatids (ES), and Sertoli (SC) and Leydig (LC) cells of *Carollia perspicillata* testes in the dry and rainy seasons in a fragment of the Atlantic Forest, Pernambuco state, Brazil

Parameter	Rainy season	Dry season	*p*-value
TCOA %	64.61 ± 4.51^{b}	58.83 ± 7.52^{a}	<0.001
ITCOA %	18.75 ± 4.15^{b}	13.87 ± 2.77^{a}	<0.001
SPC	36.74 ± 6.29^{b}	21.89 ± 3.68^{a}	<0.001
RS	40.38 ± 6.39^{b}	25.86 ± 4.26^{a}	<0.001
ES	46.77 ± 15.48^{b}	28.49 ± 7.04^{a}	<0.001
SC	14.10 ± 3.09^{b}	11.29 ± 2.38^{a}	<0.001
LC	36.89 ± 8.74^{b}	24.31 ± 5.61^{a}	<0.001

[a, b] Different letters in the same line are statistically different by the Student's *t*-test ($p<0.05$).

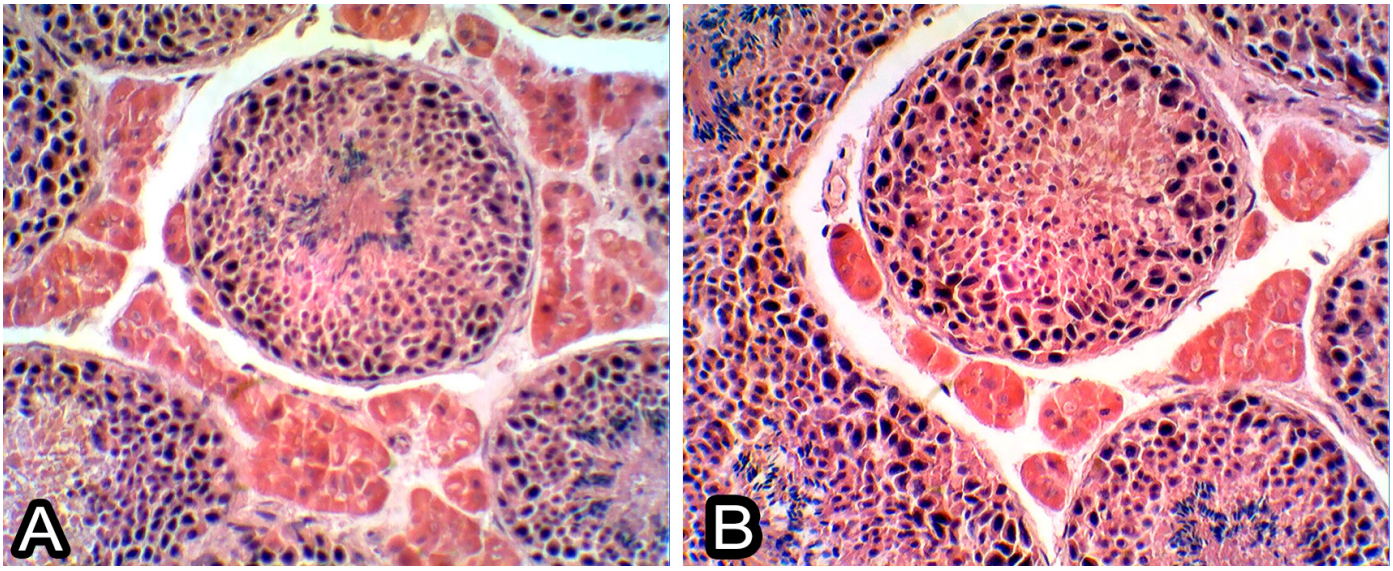

Fig.1. (**A**) Testis in the dry season, (**B**) testis in the wet season. Note the spermatogonia (yellow arrow), spermatocytes (black arrow), rounded spermatids (arrowhead), elongated spermatids (two-headed arrows), and Leydig (asterisks) and Sertoli (blue arrow) cells. HE, obj.40x, bar=20µm.

DISCUSSION

A higher proportion of male bats with descending testes was observed in both seasons; however, the external reproductive characteristics were limited, impairing verification of sexual activity. Recently, a study conducted at the same site of the present research reported that *Phyllostomus discolor* male bats present spermatogenic activity regardless of testis position (Lima Junior et al. 2014). Thus, studies performing testicular histomorphometric analysis become relevant to establish the reproductive dynamics of bats (Morais et al. 2013a, 2013b, 2013c, 2014a, 2014b, Farias et al. 2015, Lima Junior et al. 2014, Notini et al. 2015). Associated with this information, histology of *Carollia perspicillata* testis presents morphological characteristics similar to those described for other bat species (Beguelini et al. 2009, 2011, Bordignon & França 2012).

The higher testicular histomorphometric averages observed in the rainy season indicate an investment in both spermatogenesis and hormone production. These characteristics evidenced the reproductive strategy of this species in generating viable spermatozoa for mating during the months of greater precipitation; in addition, this species presents synchrony of spermatogenesis with the period of greater availability of food resources.

Thus, the larger sperm production may be synchronized with the greater sexual receptivity of females, because *Carollia perspicillata* bats are characterized by polygynous mating system with harem formation (Mello & Fernandez 2000), to which a greater androgenic and spermatogenic investment in the rainy season is essential. These data are associated with the reproductive characteristics of female bats in areas of the Atlantic Forest in the Southeastern Region of Brazil, because there is simultaneity of the reproductive peaks and the rainy season, which is the most favorable period of the year due to the greater availability of food resources (Mello et al. 1999, Mello & Fernandez 2000).

In addition, it is noteworthy the importance of testosterone and Sertoli cells in the maintenance of *C. perspicillata* spermatogenic lineage, especially evidenced by the process of spermatogenesis involving a series of complex biochemical, molecular and cellular events (Mruk & Cheng 2004), and depending on the bat species, Sertoli cells are essential for reproduction, mainly because of the support functions of germ cells, nutrition, and growth factors, as well as formation of the blood-testis barrier, thus providing greater protection to spermatozoa under development (Crichton 2000, Griswold & Skinner 2004, Fijak & Meinhardt 2006).

CONCLUSION

Seasonality influences the testicular morphological and physiological parameters of *Carollia perspicillata* male bats, as evidenced by the increased tubular and intertubular compartment occupation areas, and the increased number of spermatogenic, Leydig and Sertoli cells in the rainy season, suggesting that these bats present greater sexual activity in this period.

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Erratum

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