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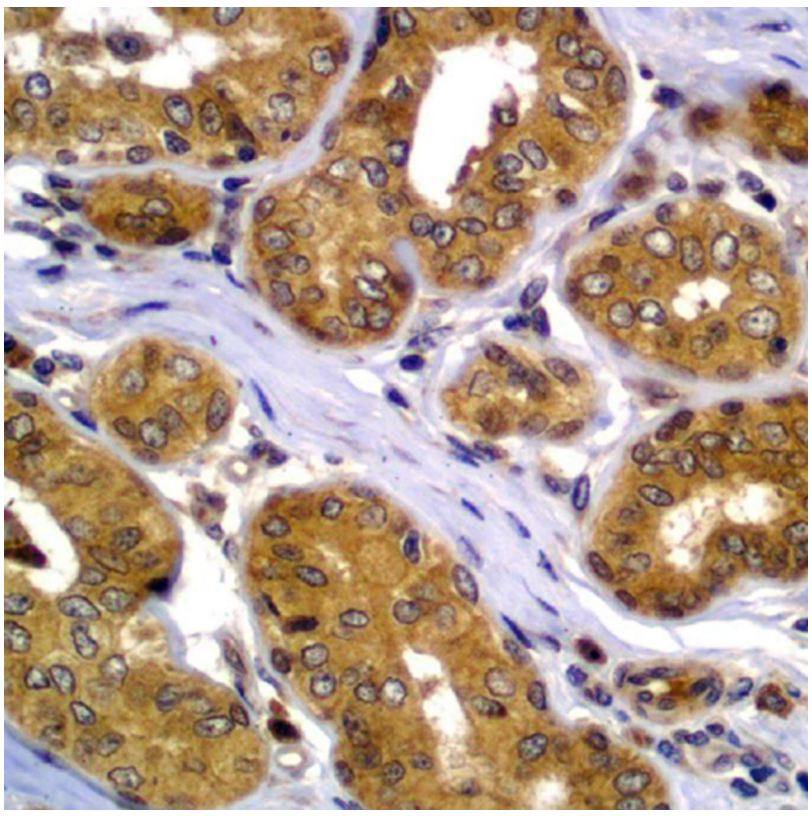

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Cover illustration: High osteopontin immunoexpression in canine mammary neoplasm. Immunohistochemistry, with DAB chromogen (Monteiro et al., p.213)

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Original Article
Livestock Diseases

Approaches for a field diagnosis of abamectin poisoning in calves[1]

Carolina C. Guizelini[2], Rayane C. Pupin[2], Tessie B.M. Möck[2], Dayane R. Morais[2], Juan A.C. Arredondo[2], Letícia L. Robalinho[2], Amanda Gimelli[2] and Ricardo A.A. de Lemos[2]*

ABSTRACT.- Guizelini C.C., Pupin R.C., Möck T.B.M., Morais D.R., Arredondo J.A.C., Robalinho L.L, Gimelli A. & Lemos R.A.A. 2020. **Approaches for a field diagnosis of abamectin poisoning in calves**. *Pesquisa Veterinária Brasileira 40(3): 155-157.* Laboratório de Anatomia Patológica, Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul, Avenida Senador Filinto Muller 2443, Vila Ipiranga, Campo Grande, MS 79074-460, Brazil. E-mail: ricardo.lemos@ufms.br

An approach for the diagnosis of an abamectin outbreak in calves in the field is described and discussed. In a Midwestern Brazilian property, nine out of a 52 newborn calves were affected and died, making up for morbidity, mortality, and lethality ratios of 17.3%, 17.3%, and 100%, respectively. Major clinical signs included tremors in various muscle groups, inability to stand, and difficult, wheezing breathing. Each affected calf had been treated subcutaneously with abamectin (0.4mg/kg/body weight). No lesions were found at necropsy or at histological examination. Major diseases of newborn calves were included in the differential diagnosis.

INDEX TERMS: Cattle diseases, toxic disorders, abamectin, poisoning, diagnosis approach, calf.

RESUMO.- [**Abordagem para um diagnóstico a campo da intoxicação por abamectina em bezerros**]. Uma abordagem para o diagnóstico de um surto de abamectina em bezerros a campo é descrita e discutida. Numa propriedade do Centro-Oeste brasileiro, nove de um lote de 52 bezerros de 3 dias de idade foram afetados e morreram, perfazendo quocientes de morbidade, mortalidade e letalidade, respectivamente, de 17,3%, 17,3% e 100%. Os principais sinais clínicos incluíam tremores em vários grupos musculares, incapacidade em se manter em pé, e respiração difícil e estertorosa. Cada bezerro afetado havia sido tratado por via subcutânea com abamectina, na dose de 0,4mg/kg/peso corporal. Não foram encontradas lesões na necropsia, nem no exame histológico. As principais doenças de bezerros recém-nascidos foram incluídas no diagnóstico diferencial.

TERMOS DE INDEXAÇÃO: Doenças de bovinos, distúrbios tóxicos, abamectina, intoxicação, abordagem diagnóstica, bezerros, bovinos.

INTRODUCTION

Abamectin-based products are widely used, most of the times safely, as antiparasitic in cattle, but cases of poisoning occur in young calves (Seixas et al. 2006, Ferreira et al. 2011, Guerra et al. 2011). Clinical signs associated with abamectin poisoning include somnolence, ataxia, progressive paresis, mydriasis, decreased tone of tongue and lips, drooling, decubitus, and coma. The outcome is usually fatal (Seaman et al. 1987, Button et al. 1998, Seixas et al. 2006, Swor et al. 2009). As this intoxication does not cause macroscopic and microscopic changes (Button et al. 1998), the diagnosis should be supported by the history of the use of the active ingredient in calves, observation of the clinical signs and ruling out other possible causes (Seixas et al. 2006), as organophosphate poisoning (Oliveira-Filho et al. 2010, Rivero et al. 2011).

In most cases, the intoxication occurs when the drug is used to control myiasis in newborn calves, using the same dosages recommended for ivermectin or doramectin-based deworming (Riet-Correa 2007). In outbreaks of abamectin toxicity, when abamectin-based products are reportedly being used, diagnosis can be made more quickly (Button et al. 1998, Guerra et al. 2011). However, in cases of iatrogenic intoxication, when the product is misused, and yet its use is not recorded in the clinical history, the diagnosis becomes challenging and requires intense epidemiological investigation including the

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collection of relevant data and reviewing comprehensively the sanitary procedures adopted for calves.

The aim of the present study is to describe an outbreak of iatrogenic intoxication by abamectin in 15-day-old calves, emphasizing the importance of proper diagnostic approach in cases where administration of the drug is not included in the initial history.

MATERIALS AND METHODS

An outbreak of neurological disease in cattle in a farm (latitude 20°08'30'' South and longitude 54°23'58'' West) located in Midwestern Brazil was studied. Clinical signs and epidemiological data were obtained from the animal caregiver and from the veterinary practitioner who looked over for the cattle in that farm. Also, clinical examination of affected cattle was performed by staff of the Laboratory of Anatomic Pathology of the School of Veterinary Medicine and Animal Husbandry of the "Universidade Federal de Mato Grosso do Sul" (LAP-FAMEZ). Four calves were necropsied. Tissue fragments from several organs were collected in 10% buffered formalin, processed routinely for histopathology, and stained by hematoxylin and eosin (HE). Brain and spleen impressions were performed looking for blood parasites (Tyler et al. 2002). Fresh fragments from the brain were sent to bacterial culture.

RESULTS

At the time of the outbreak, there were 52 calves with weights of 25kg at birth. The routine care with the calves was as follows: up to two days after birth the navel was treated with an antiseptic (Umbicura® - Pecuarista d'Oeste) and each calf received 1ml of a subcutaneous antiparasitic drug (Dorax® 1% - Agener).

By the morning of first day of the outbreak, the calves' caregiver found four 3-day-old calves dead. In the next day, three additional calves of the same age were found dead, and two were sick and died within 24 hours.

Clinical signs included tremors in several muscle groups, failure to stand and challenging, noisy breathing.

Due to a tentative diagnosis of tick fever and pneumonia, affected calves were treated with 44-diazoaminodibenzamidina diaceturate (Diaseg® - Coopers) and oxytetracycline (Terramicina LA® - Zoetis). Calves that did not have their navel disinfected two days after birth and were still considered vulnerable of navel inflammation received prophylactic treatment with a mixture of trichlorphon (Bertac® - Eurofarm) and fention (Tiguvon® - Bayer). However, none of the calves who became sick and died was treated with neither of these two drugs.

At necropsy of three of the four necropsied calves, there were no detectable lesions. In the other one, there was subacute suppurative omphalophlebitis; the liver was swollen, with rounded edges and imprinted with rib marks on its capsular surface. No significant microscopic lesions were observed in any organ of the four necropsied calves, except for the omphalophlebitis already detected clinically. Impression smears from the brain and spleen and culture from the brain resulted negative.

Well after the efforts for investigating the cause of the disease of the calves had begun, the calves caregiver admitted that for a certain period of time, which coincided with the period that calves became sick and died, he had used abamectin at 1% (Lancer®-Valée) (1ml/calf) because the supplies of doramectin has run out.

DISCUSSION

For various reasons, finding the causes of the current outbreak was challenging and constituted an excellent field diagnostic exercise. The collected data indicated that morbidity, mortality and lethality were, respectively, 17.3%, 17.3%, and 100%. The diagnosis was initially further complicated by the lack of a history of administration of any product with abamectin to the calves, which is a fundamental criterion for establishing the diagnosis (Riet-Correa 2007).

The initial suspicion of cerebral babesiosis was predominantly based on the neurological features which were somewhat similar to cerebral babesiosis, which is commonly observed in neonatal cattle in this region (Pupin et al. 2019). However, cerebral babesiosis was ruled out due to the absence of compatible macroscopic lesions (Rodrigues et al. 2005, Antoniassi et al. 2009) and in the absence of red blood cell parasites.

Neurological disease in newborn calves can be associated with failure of passive immunity transfer due to colostrum deprivation. This can result in septicemia and suppurative leptomeningitis from an infected navel (Fecteau et al. 2009), a condition known as neonatal bacterial suppurative meningitis or NBSM (Cantile & Youssef 2016), usually produced by *Escherichia coli* or *Streptococcus* spp. (Miller & Zachary 2017). This diagnosis was also at one time considered, but then ruled out on gross, histopathological, and bacteriological grounds. Although one of the calves had omphalophlebitis, all four calves lacked suppurative leptomeningitis, polyarthritis, choroiditis and endophthalmitis, which are hallmarks of NBSM. Furthermore, no bacterium was cultured from the brain.

The fact that all cases occurred within a short period (three days), and that all calves born on those three days were affected, led to the suspicion of a toxic condition. The clinical picture and the absence of macroscopic and microscopic lesions (Oliveira-Filho et al. 2010, Lopes et al. 2014.) led to the suspicion of organophosphate poisoning since this type of drug was used, at some time, to disinfect the newborn calves' navels.

The toxic mechanism of organophosphate and carbamates is the inhibition of acetylcholinesterase resulting in the accumulation of acetylcholine, which maintains the stimuli on its receptors, causing hyperstimulation of the parasympathetic nervous system (Kwong 2002). Therefore, one way to make a diagnosis of organophosphate and carbamates poisoning is to measure acetylcholinesterase in red cells and its activity in the plasma (Coye et al. 1987). However, these tests are not available in most laboratories. The diagnosis is then based on the history that indicates exposure to toxic substances, neurological clinical picture, and the absence of significant macroscopic and microscopic findings in necropsied cattle (Barros et al. 2006). All of these criteria were present in this outbreak. However, a closer look at the property records revealed that none of the affected calves in the outbreak had their navel treated with trichlorfon or fention.

The ruling out of all the diseases described above led us to investigate another possible cause for the neurological disease that lacked gross and histological lesions. We decided then to investigate further the possibility of the use, not reported previously by the calves' caregiver, of abamectin, due to similarity of the clinical signs with this toxicity. It was then found that a product (Lancer® - Valée) containing 1% of

abamectin had been administered (1ml/calf). If one considers the weight of calves at birth, the dose they received (0.4mg/kg/body weight - bw) was twice that recommended (0.2mg/kg/bw) by the manufacturer. Under experimental conditions, administration of abamectin, even at the manufacturer's recommended doses, in calves less than four months of age, was responsible for the development of clinical signs and death (Seixas et al. 2006).

Abamectin belongs to the group of avermectins, which includes ivermectin and doramectin. Abamectin usually does not cause toxic effects in adult cattle due to its high molecular weight, which prevents it from crossing the blood-brain barrier (Gerenutti & Spinosa 1997). In young calves (up to four-months-old), however, this barrier is immature and facilitate the entrance of the active principle into the central nervous system, resulting in severe intoxication characterized by neurological signs (Button et al. 1998).

The diagnostic approach adopted in this outbreak made possible to elaborate a clinical suspicion that led to the epidemiological investigation and the identification of the source of the intoxication. To reach the diagnosis we considered the set of signalment, history, clinical signs, necropsy and histopathological findings, and complementary microbiological and parasitic exams. This approach is considered to be ideal in the decision-making process to reach a definitive diagnosis (Maxie & Miller 2016). In this context, we evaluated the leading causes of death in calves with clinical signs compatible with those observed in this outbreak and, if necessary, ruled them out for not meeting the diagnostic criteria.

CONCLUSIONS

Abamectin (0.4mg/kg/bw) prove toxic for 3-day-old calves. The toxicosis induces an acute neurological disease with no associated gross or histological changes.

Clinical and epidemiological aspects are paramount for a field diagnosis with limited laboratory aid.

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

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Original Article
Livestock Diseases

Peritoneal fluid collection in healthy cattle and evaluation of changes in cell morphology during storage in refrigerated and non-refrigerated samples[1]

Nathali A.A. Sales[2], Karina K.M.C. Flaiban[3], Joandes H. Fonteque[4] and Júlio A.N. Lisbôa[5]*

ABSTRACT.- Sales N.A.A., Flaiban K.K.M.C., Fonteque J.H. & Lisbôa J.A.N. 2020. **Peritoneal fluid collection in healthy cattle and evaluation of changes in cell morphology during storage in refrigerated and non-refrigerated samples.** *Pesquisa Veterinária Brasileira 40(3):158-164*. Departamento de Clínicas Veterinárias, Centro de Ciências Agrárias, Universidade Estadual de Londrina, Campus Universitário, Cx. Postal 10011, Londrina, PR 86057-970, Brazil. E-mail: janlisboa@uel.br

This study aimed to evaluate the appropriate sites of abdominocentesis for peritoneal fluid collection in cattle and to investigate the time of cell viability in vitro, comparing three methods of sample conservation. Twenty-one healthy cattle (19 females and 2 males) were subjected to a laparocentesis procedure to obtain peritoneal fluid, with punctures in three defined sites: left cranial, right cranial, and right caudal. The total peritoneal fluid collected was divided into three aliquots and maintained under three preservation conditions: room temperature (26°C), refrigeration (4°C), and room temperature (26°C) with the addition of 1µL of 10% formaldehyde per 1mL of peritoneal fluid. The peritoneal fluid analysis performed immediately after collection consisted of: physical examination (color, appearance, volume, and specific gravity), biochemical measures (pH, total protein, fibrinogen, creatinine, and glucose), and cellularity (total and differential counts). The determination of proteins and the examination of cells were repeated in each separate aliquot at two, four, six, and eight hours after harvest. Data were analyzed through repeated measures ANOVA or Friedman test. The harvest was productive in 67% of cattle. The left cranial and the right cranial puncture sites were the most appropriate. Peritoneal fluid analyzed after collection, the total protein concentration ranged from 1.4 to 3.6g/dL, and number of leukocytes ranged from 54 to 1,322 cells/µL; 60 to 95% of leukocytes were lymphocytes. The protein concentration decreased, but the absolute values of leukocytes, lymphocytes, and segmented neutrophils did not change up to eight hours after collection, independent of the maintenance method. Cell lysis was delayed by cooling, and the addition of formaldehyde did not help preserve the integrity of cellular morphology. Laparocentesis is a safe and secure procedure in cattle and maybe more productive when performed in specific sites on the left or right sides of the cranial abdominal wall. Peritoneal fluid samples may be analyzed with reliable results for up to eight hours after collection when kept refrigerated and for up to six hours when kept at room temperature.

INDEX TERMS: Peritoneal fluid, healthy cattle, cell morphology, abdominocentesis, cell viability, conservation, cattle.

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RESUMO.- [**Colheita do fluido peritoneal de bovinos sadios e verificação das alterações na morfologia celular quando mantido refrigerado ou à temperatura ambiente.**] O estudo teve como objetivo avaliar os locais adequados de laparocentese para a colheita de fluido peritoneal de bovinos e estabelecer o tempo de viabilidade celular *in vitro,* comparando três métodos de conservação. Vinte e um bovinos hígidos (19 fêmeas e 2 machos) foram submetidos ao procedimento de laparocentese para obtenção de fluido peritoneal, com punção em três pontos definidos: cranial esquerdo, cranial direito e caudal direito. O volume total do líquido peritoneal foi dividido em três alíquotas mantidas sob três métodos de conservação: temperatura ambiente (26°C); refrigeração (4°C); e temperatura ambiente (26°C) com adição de 1µL de formol 10% para cada 1mL de líquido peritonial. A análise do líquido peritoneal realizada imediatamente após sua obtenção consistiu em: exames físico (cor, aspecto, volume e densidade); bioquímicos (pH, proteína total, fibrinogênio, creatinina e glicose); e da celularidade (contagens total e diferencial). A determinação de proteínas e o exame da celularidade foram repetidos, em cada alíquota separada, as duas, quatro, seis e oito horas após a colheita. Análise de variâncias de medidas repetidas ou teste de Friedman foram empregados para avaliação ao longo do tempo. A colheita foi produtiva em 67% dos bovinos e os locais de punção craniais esquerdo e direito foram os mais adequados. A concentração de proteína total variou de 1,4 a 3,6g/dL e o número de leucócitos de 54 a 1.322 células/µL, com predomínio de linfócitos (60 a 95% das células) no fluido peritoneal analisado logo após a colheita. A concentração de proteínas diminuiu, mas os valores absolutos de leucócitos, de linfócitos e de neutrófilos segmentados não se modificaram até oito horas após a colheita, independente do método de manutenção das amostras. A lise celular foi retardada pela refrigeração e a adição de formol não contribuiu para preservar a integridade da morfologia celular. A laparocentese é um procedimento seguro e de execução fácil em bovinos sendo mais produtiva quando realizada em locais específicos à esquerda ou à direita craniais da parede abdominal. Amostras de fluido peritoneal podem ser analisadas com resultados confiáveis quando mantidas refrigeradas por até oito horas após a colheita e quando mantidas à temperatura ambiente por até seis horas.

TERMOS DE INDEXAÇÃO: Fluido peritoneal, bovinos sadios, morfologia celular, laparocentese, líquido peritoneal, viabilidade celular, conservação.

INTRODUCTION

Peritoneal fluid analysis is useful for confirming the diagnosis of some conditions affecting the abdominal cavity (Hirsch & Townsend 1982, Divers & Peek 2008). Conditions such as peritonitis, uroperitoneum, hemoperitoneum, abdominal neoplasms, gastric, abomasal or intestinal ischemia, or rupture can determine physical, biochemical, and cellular changes in the peritoneal fluid that, when characteristic, allow the diagnosis to be completed (Oehme & Noordsy 1970, Wilson et al. 1985, Bohn & Callan 2007). Due to its unquestionable importance, the practice of collecting and examining peritoneal fluid is routinely performed in equine medicine. However, it is not commonly adopted by practitioners in bovine medicine.

Unlike the ease with which peritoneal fluid is harvested from horses, whose laparocentesis site is the midline at the lowest point of the abdomen (Larkin 1994), collecting samples from cattle is more complicated and this discourages the procedure. In cattle, the anatomical arrangement of forestomachs and abomasum allows fluid to accumulate in specific cavity sites (Dirksen 1993). Besides, pathological processes, notably peritonitis, are usually localized in this species (Fecteau 2005, 2009). As a result, the specimen collected from a single puncture site may not reflect disease elsewhere in the abdomen, leading to the need to perform laparocentesis at more than one predefined site (Kopcha & Schultze 1991b).

Analyses such as cell count and biochemical determination and evaluation of cell morphology need to be performed in the laboratory. This is another difficulty faced by the veterinarian who works in the field; the collected sample must be stored correctly, from its collection to its arrival in the laboratory, in order to preserve its characteristics. Refrigeration is a usual method used for sample conservation, but it does not prevent cells from degenerating (Larkin 1994). The length of cell viability in peritoneal fluid collected from cattle is unknown but assumed to be short (Oehme & Noordsy 1970, Hirsch & Townsend 1982, Wilson et al. 1985). Identifying effective methods that can prolong cell preservation is of practical importance.

In order to encourage the adoption of the procedure in cattle, this study aimed to evaluate possible laparocentesis sites suitable for obtaining peritoneal fluid and to establish the duration of cell viability in vitro, comparing conservation methods.

MATERIALS AND METHODS

Twenty-one healthy crossbred cattle (*Bos taurus* x *Bos indicus*) aged from 1.5 to 6 years old, 2 males and 19 females, were used in this study. All animals belonged to the herd of the Veterinary Teaching Hospital of the "*Universidade Estadual de Londrina*", which were kept in a semi-confinement system, received sorghum silage as their primary food, and had free access to water and mineral salt.

Laparocentesis was performed in each of the cattle at three defined sites: a) left cranial: in the reticular-ruminal recess, 5 to 6cm cranial and medial to the left mammary vein foramen (House 1992); b) right cranial: in the region of the abomasum, at the midpoint between the navel and the xiphoid appendix and to the right of the ventral midline, at the midpoint between this and the right mammary vein (Radostits et al. 2007); c) right caudal: medial to the fold of the right flank in a cranio-dorsal position to the udder (House 1992).

For the procedure, the animals were contained in individual trunks and kept in a quadrupedal position. The puncture sites were previously prepared with trichotomy and antisepsis with iodized alcohol. For the anesthetic button, 2% lidocaine without vasoconstrictor (Lidovet; Laboratório Bravet Ltda.) was used in a volume of 1 to 2mL, with deposition in the subcutaneous space and adjacent muscles. Peritoneal fluid was collected 10 minutes after anesthetic application, using a previously sterilized 80 x 15mm metal needle (BD; Becton, Dickinson Indústrias Cirúrgicas Ltda.). After perforation of the skin, abdominal muscles, and peritoneum, the needle mandrel was removed to allow peritoneal fluid to be harvested. For greater safety in performing this procedure, the needle body was kept fixed between the index and thumb fingertips and the mandrel was kept resting on the palm of the hand during

the puncture; this reduced the risk of sudden needle insertion and perforation of internal organs, such as the reticulum, abomasum, and intestinal loops.

Vacuum collection 5mL tubes containing ethylenediaminetetraacetic acid (EDTA) were used to receive the sample obtained from each puncture site. At the time of collection, the volume of fluid obtained from each site was measured. The samples were then combined into a single test tube to obtain a composite sample with total of all collection points.

Immediately after peritoneal fluid collection, blood was collected by jugular vein puncture, using a vacuum 5mL tube containing EDTA for subsequent determination of total protein, fibrinogen, and creatinine in plasma.

The first peritoneal fluid analysis was performed immediately after collection. Then, the original total volume was divided into three aliquots, which were kept under different storage conditions: a) kept on the bench at room temperature (26°C); (b) kept refrigerated (4°C); c) maintained at room temperature (26°C) with the addition of 1µL of 10% formaldehyde to each 1mL of peritoneal fluid.

Peritoneal fluid was analyzed at five moments after collection: immediately after and two, four, six, and eight hours later. Immediately after collection and before dividing the fluid into three aliquots, full analysis was performed; this included physical examination (color, appearance, and specific gravity), cellular examination (total cell count and differential leukocyte counts), and biochemical tests (pH, total protein, fibrinogen, glucose, and creatinine). At other times, the analysis consisted of total protein determinations and cellular examination.

The pH was determined by using indicator strips (pH-Indicator Strips; Merck, Germany). Specific gravity and total protein were measured by refractometry (Model 301 Manual Refractometer; Biobrix) and fibrinogen concentration was measured by using the heat precipitation method (Kaneko & Smith 1967). Creatinine and glucose concentrations were measured by colorimetric analysis using specific commercial reagents (Bioclin; Quibasa Química Básica Ltda), with spectrophotometric reading (Bio 2000/Plus; BioTécnica Indústria e Comércio Ltda). Total cell count was performed in a Fuchs-Rosenthal chamber. The total value of nucleated and red blood cells counted in the chamber was divided by three so that the obtained value was expressed in number of cells/µL. For differential counting, one drop of non-centrifuged liquid was deposited on a slide, and the smear was then prepared.

The smear was air dried and stained with Romanowsky stain (Instant Prov; Newprov Produtos para Laboratório Ltda). Differential cell counting was performed by light microscopy. The cellular and structural evaluated characteristics, as criteria of maintenance or not of cellular integrity, were: a) cytoplasmic membrane integrity; b) cell lysis, characterized by the extravasation of its content and fragmentation of organelles; c) presence of cytoplasmic vacuoles; d) nuclear membrane integrity; e) chromatin fragmentation (Curtin et al. 2002, Meinkoth et al. 2009). At least 50 fields were examined in each smear. The observed changes were classified into the following categories according to the percentage of cells that exhibited each change: 0) up to 5% of the affected cells; 1) from 6 to 25% of the affected cells; 2) from 26 to 50% of the affected cells; 3) from 50 to 75% of the affected cells.

Repeated measures ANOVA was used to evaluate the variation of total protein concentration in peritoneal fluid over time, using the Tukey test for multiple comparisons. For absolute leukocyte, neutrophil, and lymphocyte values, the Friedman test was used. For these evaluations, each conservation method adopted for sample maintenance was considered separately. The probability of a 5% error was assumed. SigmaStat for Windows 3.1 was used for the analysis.

RESULTS AND DISCUSSION

Laparocentesis was productive in 14 (67%) and unproductive in 7 (33%) cattle, with successful harvesting at the left (43% successful) and right (62% successful) cranial puncture sites (Table 1). Because of the difficulty in collecting peritoneal fluid from the right caudal puncture site (82% failure), attempts were performed only on the first 11 cattle.

The volume of peritoneal fluid obtained from the left and right cranial puncture sites was mostly equal to or greater than 5mL. This was more than enough for the routine laboratory test. Of the 13 cattle whose samples were collected from these cranial puncture sites, 8 (62%) had high peritoneal fluid volumes at both collection sites.

The results contradict claims that obtaining peritoneal fluid is difficult in cattle and that harvesting can be unproductive in many animals (House et al. 1992, Larkin 1994, Bohn & Callan 2007, Radostits et al. 2007). These statements serve to discourage clinicians from performing the procedure, depriving themselves of valuable information for completing the diagnosis of peritonitis. Contrary to the concept that the fluid volume is reduced in the abdomen of healthy cattle and this makes it difficult to collect (Hirsch & Townsend 1982, Wilson et al. 1985, Fecteau 2005, Divers & Peek 2008), the harvest was successful in most of the cattle used in this

Table 1. Results of the punctures performed in three different sites of the abdominal wall of healthy cattle (n=21), indicating the volume of peritoneal fluid obtained

Cattle	Left cranial	Right cranial	Right caudal
1	3.5mL	>5mL	1mL
2	>5mL	>5mL	Drops
3	0	0	Drops
4	Drops of blood	0	Drops
5	Drops	1.3mL	Drops
6	Drops of blood	0	>5mL
7	0	Drops	Drops
8	0	0	Drops
9	>5mL	3mL	Drops of blood
10	0	0	Drops of blood
11	Drops	1mL	0
12	0.5mL	5mL	Not collected
13	Drops	0	Not collected
14	>5mL	>5mL	Not collected
15	5mL	>5mL	Not collected
16	Drops	5mL	Not collected
17	>5mL	>5mL	Not collected
18	0	>5mL	Not collected
19	Drops	0	Not collected
20	5mL	>5mL	Not collected
21	>5mL	>5mL	Not collected

study despite them being healthy. In the clinical routine, the collection will be performed in suspected cases of peritonitis and in the initial phase of this condition the volume of exudate in the cavity is expected to increase, facilitating the collection (Oehme 1969, Wilson et al. 1985).

The presence of peritoneal fluid in the two cranial puncture sites can be explained by the gravitational effect generating its accumulation on the cavity floor, even under physiological conditions. The difficulty of harvesting at the right caudal puncture site, on the other hand, can be justified precisely because the cattle were healthy. This collection site is particularly recommended in cases of diffuse peritonitis, which are accompanied by omental bursitis, in which case the large exudate is sequestered within the space formed by the larger inflamed omentum, permeating the intestinal loops (Fecteau 2005). Therefore, in the studied healthy cattle, there was no accumulation of peritoneal fluid in this cavity site.

Regarding the adopted technique, it should be noted that obtaining peritoneal fluid samples was facilitated when the needle was left free after puncturing the cavity and removing the mandrel. Most of the time, the liquid did not flow immediately. However, the natural movement of the needle, resulting from reticular or abomasum contractions, precipitated spontaneous fluid outflow, dripping, or continuous flow, in variable volumes. Manual movements of the needle for repositioning and the effect of negative pressure produced by the plunger pull of a needle coupled to a syringe did not facilitate harvesting in the studied cattle.

Puncture accidents occurred in four cattle at the first harvest attempt; these accidents were two perforations of the reticulum and two of the abomasum. In these cases, the needle was immediately removed and replaced with a sterile one, and a new cavity puncture was performed at another location near the first one. In three of these cattle, the second harvest attempt was successful. None of them presented apparent later complications, remaining healthy and with a good appetite. The absence of problems related to accidental perforation of the viscera is cited as common in cattle (Wilson et al. 1985, Bohn & Callan 2007).

The physical, biochemical, and cytological characteristics of the peritoneal fluid obtained from the studied cattle (Table 2) were broadly compatible with those reported for healthy cattle (Hirsch & Townsend 1982, Dirksen 1993). Light yellow color and slightly cloudy appearance were the predominant findings. Specific gravity ranged from 1010 to 1020 and pH from 7.0 to 8.0, consistent with the health status of cattle (Fecteau 2005, Bohn & Callan 2007). Total protein concentration ranged from 1.4 to 3.6g/dL and can be considered physiological. Although there is some divergence as to the reference values for this variable, the vast majority of authors admit that protein concentration should be less than or equal to 3g/dL (Kopcha & Schultze 1991b, Belknap & Navarre 2000, Fecteau 2005, Bohn & Callan 2007). Fibrinogen concentration in the fluid did not exceed 200mg/dL and was within the accepted reference range for the species (between 100 and 400mg/dL) (Wilson et al. 1985), almost half of its plasma concentration (Table 2). Finally, creatinine concentration in the peritoneal fluid was close to that in the plasma, something that is expected in healthy cattle (Kopcha & Schultze 1991b, Bohn & Callan 2007).

Table 2. Physical, biochemical, and cytological characteristics of peritoneal fluid from healthy cattle (n=14), examined shortly after collection and some plasma variables in samples collected shortly after laparocentesis

	x	s	Md	Minimum	Maximum
Peritoneal fluid					
Color	Light yellow predominant (n = 11) and occasional orange (n = 3)				
Aspect	Slightly cloudy predominant (n = 9) and occasional cloudy (n = 5)				
Specific gravity	1,014.5	3.2	1,015	1,010	1,020
pH	7.22	0.40	7.0	7.0	8.0
Total protein (g/dL)	2.1	0.6	2.0	1.4	3.6
Fibrinogen (mg/dL)	112.5	34.1	100	100	200
Glucose (mg/dL)	55.9	5.4	55.3	48.7	66.6
Creatinine (mg/dL)	1.3	0.4	1.2	0.7	2.2
Leukocytes (/µL)	1,079	977	896	54	1,322
Segmented neutrophils (%)	15.2	9.3	14.0	5	40
Lymphocytes (%)	84.7	9.4	86.0	60	95
Segmented neutrophils (/µL)	182	230	127	4	303
Lymphocytes (/µL)	921	862	767	50	1,065
Plasma					
Total protein (g/dL)	7.8	0.6	7.5	7.2	9.4
Fibrinogen (mg/dL)	350	89,4	400	200	500
Creatinine (mg/dL)	1.5	0.4	1.6	0.9	2.5

The number of leukocytes varied widely (from 54 to 1,322 cells/μL), but can be considered physiological. As indicated in the literature, healthy cattle may have leukocyte counts from ≤5,000 cells/μL (Anderson et al. 1994) up to ≤10,000 cells/μL (House et al. 1992, Belknap & Navarre 2000). Lymphocytes were the predominant cells and represented 60 to 95% of them, followed by segmented neutrophils that represented 5 to 40% of the present cells. Eosinophils, basophils, and macrophages were not observed in this study and mesothelial cells were infrequently present and in small numbers. The results are consistent with those reported in healthy cattle (Oehme 1969, Oehme & Noordsy 1970), but contradict the claim that lymphocytes may be present in as few as 20% of leukocytes (Wilson et al. 1985, Anderson et al. 1994). For segmented neutrophils, percentages greater than 40% may be considered as indicative of bovine peritonitis (Wilson et al. 1985, House et al. 1992). The median ratio between mononuclear cells and polymorphonuclear cells observed in the studied cattle was 6:1, unlike the 1:1 ratio previously reported for healthy cattle (Oehme & Noordsy 1970, Bohn & Callan 2007). Finally, evidence that eosinophils may be present in larger numbers in the peritoneal fluid of healthy cattle, representing up to almost half of all cells (Wilson et al. 1985, Anderson et al. 1994), was not reinforced in the present study.

Peritoneal fluid from healthy animals should contain little or no red blood cells (Wilson et al. 1985, Stockham & Scott 2011). When present, red blood cells indicate abdominal hemorrhage (hemoperitoneum) or peripheral blood loss resulting from an accident during puncture (Kopcha & Schultze 1994a). In the studied cattle, the number of red blood cells in the peritoneal fluid ranged from a minimum of 85/μL to a maximum of 18,346/μL, with a median of 1,461/μL. This high number of red blood cells could be attributed to the accidental puncture of blood vessels during the laparocentesis procedure.

With respect to the changes that were observed in the peritoneal fluid over eight hours after collection, protein concentration decreased ($p<0.05$), but absolute leukocyte values, lymphocytes, and segmented neutrophils did not change ($p>0.05$) (Table 3). The method used for preserving the samples did not interfere with the results and cooling was not better than room temperature maintenance. Even the reduction in protein concentration detected four or six hours after harvest was very slight and may be considered insufficient to compromise the interpretation of the results. Therefore, it may be assumed that, regarding these variables, bovine peritoneal fluid presented reliable results when processed up to eight hours after its collection, even if it was not kept refrigerated. This contradicts the recommendations that the sample should be examined as soon as possible after collection (Oehme 1969) or that it should be kept refrigerated for storage (Oehme & Noordsy 1970, Hirsch & Townsend 1982). Some reports indicate that the refrigerated peritoneal fluid sample can be examined, with reliable results, up to 18 hours after collection (Wilson et al. 1985).

Regarding the evaluated criteria indicating cell integrity maintenance, it should be noted that the irregularity or rupture of the cytoplasmic membrane and cell lysis were the important changes observed; the irregularity or rupture of the nuclear membrane and the chromatin fragmentation were uncommon alterations, while cytoplasmic vacuoles were rarely present. The irregularity or rupture of the cytoplasmic membrane and cell lysis were already present since the first peritoneal fluid analysis, performed immediately after its

Table 3. Mean values (x ± s) of protein concentrations and median values of leukocytes in healthy bovine peritoneal fluid samples (n=12), maintained for up to eight hours after collection under different conditions: room temperature at 26°C (RT); refrigeration at 4°C (R); room temperature at 26°C with the addition of formaldehyde (RTF)

	0 h	2 h	4 h	6 h	8 h
			Proteins (g/dL)		
RT	1.95± 0.45[a]	1.91 ± 0.48[ab]	1.88±0.49[ab]	1.86±0.49[b]	1.84±0.50[b]
R	1.95 ± 0.45[a]	1.91 ± 0.48[ab]	1.88±0.49[b]	1.88±0.49[b]	1.85±0.51[b]
RTF*	1.95 ± 0.45[a]	1.91 ± 0.48[ab]	1.88±0.49[b]	1.88±0.49[b]	1.85±0.51[b]
			Leukocytes (/μL)		
RT	901[a]	880[a]	816[a]	826[a]	858[a]
R	901[a]	880[a]	805[a]	1,066[a]	944[a]
RTF*	901[a]	869[a]	896[a]	1,002[a]	1,002[a]
			Segmented neutrophils (/μL)		
RT	127[a]	109[a]	163[a]	66[a]	118[a]
R	127[a]	148[a]	89[a]	134[a]	124[a]
RTF*	127[a]	151[a]	128[a]	118[a]	118[a]
			Lymphocytes (/μL)		
RT	856[a]	674[a]	591[a]	715[a]	670[a]
R	856[a]	752[a]	653[a]	783[a]	771[a]
RTF*	856[a]	741[a]	774[a]	832[a]	863[a]

* 10% formaldehyde: 1μL for each 1mL of peritoneal fluid; [a,b] different letters indicate statistical difference ($p<0.05$).

collection. At this time, however, the number of affected cells was small. Overall, time elapsed after harvesting increased the percentage of cells exhibiting these two changes (Table 4).

The percentage of cells with irregular or ruptured cytoplasmic membranes increased four hours after collection in the samples in which formaldehyde was added and, apparently, eight hours after collection in those that did not receive formaldehyde. Refrigeration did not delay the appearance of this change in the cells. Cell lysis, in turn, increased six hours after collection and intensified eight hours after collection in samples that were kept uncooled. It can be stated that refrigeration substantially minimized the occurrence of this type of change (Table 4). The observed changes occurred earlier in segmented neutrophils than in lymphocytes, indicating that neutrophils are more susceptible to in vitro adverse conditions, and maintain their integrity for a shorter time outside the body (Stockham & Scott 2011).

It is reasonable to assume that the evaluation of cells for differential counting will not be compromised if the morphological changes that cells experience in vitro affect only up to a quarter of them. This motivated the definition of this limit in the present study (Table 4). It should also be noted that, among the observed alterations, cell lysis is the one that, in fact, impairs the cytological evaluation; while the irregularity or rupture of the cytoplasmic membrane creates difficulty for the examination, it does not always prevent the cell type to be defined by the examiner. Thus, the observed results reinforce the notion that refrigeration is the recommended conservation method and bovine peritoneal fluid can be analyzed with reliable results up to eight hours after harvest. Even in samples that have not been kept refrigerated, the test result can be reliable up to four or six hours after collection. Finally, it is clear that the addition of formaldehyde for the purpose of cell preservation is not justified because it does not fulfill its purpose.

One should not ignore the fact that the evaluated peritoneal fluid samples were from healthy cattle, therefore, they were transudates. It is likely that, in the case of exudates, the time to maintain cell viability is shorter than that observed in the present study. This statement is justified due to the presence of proteases, cytokines, and inflammation mediators (Kopcha & Schultze 1991a), in addition to the larger number of segmented neutrophils, which could be more reactive or already in a frank degeneration process (Rizzi et al. 2009). In cases of peritonitis, the main condition that justifies the collection and analysis of peritoneal fluid in cattle, in vitro conditions would be much more adverse than those present in this study. For this reason, it is logical to indicate that the sample taken must be kept under refrigeration.

Table 4. Changes observed in leukocytes in healthy bovine peritoneal fluid samples (n=12), maintained for up to eight hours after collection under different conditions: room temperature at 26°C (RT); refrigeration at 4°C (R); room temperature at 26°C with the addition of formaldehyde (RTF). Results expressed as the percentage of samples examined with changes present in up to a maximum of 25% of cells

	0 h	2 h	4 h	6 h	8 h
Cytoplasmic membrane irregularity or rupture					
RT	90	78	90	80	70
R	90	80	81	72	70
RTF*	90	77	60	50	66
Cell lysis					
RT	81	88	90	70	60
R	81	80	100	90	90
RTF*	81	88	90	70	55
Cytoplasmic vacuoles					
RT	100	100	100	100	100
R	100	100	100	100	100
RTF*	100	100	100	100	100
Nuclear membrane irregularity or rupture					
RT	100	100	100	100	100
R	100	100	100	100	100
RTF*	100	100	100	100	100
Fragmented chromatin					
RT	100	100	100	100	100
R	100	100	100	100	100
RTF*	100	100	100	100	100

* 10% formaldehyde: 1µL for each 1mL of peritoneal fluid.

CONCLUSIONS

Laparocentesis is a safe and easily performed procedure in cattle and peritoneal fluid collection is successful when puncture is performed at two specific points of the ventral abdominal wall: in the left cranial situation, corresponding to the space of the reticular recess and in the right cranial situation, corresponding to the ventral space of the abomasum at the lowest point of the abdomen.

After collection, peritoneal fluid from healthy cattle can be examined with reliable results for up to eight hours if kept refrigerated and for up to six hours if kept at room temperature. Refrigeration delays the degenerative process that cells experience in vitro, thus increasing cell viability.

This method should be used to preserve specimens during transport when peritonitis is suspected. Because of the ease, safety, and practicality involved, clinicians could adopt this diagnostic procedure whenever they suspect diseases located in the abdominal cavity. The distance to the laboratory cannot be considered a hindrance and pointed as justification for not performing the method.

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Original Article
Livestock Diseases

Detection of the *mcr-1* gene in Enteropathogenic *Escherichia coli* (EPEC) and Shigatoxigenic *E. coli* (STEC) strains isolated from broilers[1]

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ABSTRACT.- Lopes H.P, Costa G.A., Pinto A.C.L.Q., Machado L.S., Cunha N.C., Nascimento E.R., Pereira V.L.A. & Abreu D.L.C. 2020. **Detection of the *mcr-1* gene in Enteropathogenic *Escherichia coli* (EPEC) and Shigatoxigenic *E. coli* (STEC) strains isolated from broilers**. *Pesquisa Veterinária Brasileira 40(3):165-169*. Departamento de Saúde Coletiva Veterinária e Saúde Pública, Universidade Federal Fluminense, Rua Vital Brazil Filho 64, Santa Rosa, Niterói, RJ 24230-340, Brazil. E-mail: hugop_lopes@hotmail.com

Enteropathogenic *Escherichia coli* (EPEC) and Shigatoxigenic *E. coli* (STEC) strains are among the major pathotypes found in poultry and their products, which are capable of causing human enteric infections. Colistin has been claimed the drug of choice against diseases caused by multidrug-resistant Gram-negative bacteria (MDRGN) in humans. The *mcr-1* gene was the first plasmidial gene that has been described to be responsible for colistin resistance and has also been detected in birds and poultry products. Our study aimed to detect the *mcr-1* gene in enteropathogenic strains of *E. coli* in order to evaluate the resistance to colistin in broilers. The material was obtained from 240 cloacal samples and 60 broiler carcasses. The strains were isolated by the conventional bacteriological method and by the virulence genes, which characterize the enteropathogenic strains and resistance, and the samples were detected by polymerase chain reaction (PCR). Of the 213 isolated strains of *E. coli*, 57 (26.76%) were characterized as atypical EPEC and 35 (16.43%) as STEC. The *mcr-1* gene was found in 3.5% (2/57) of the EPEC strains and 5.7% (2/35) of the STEC strains. In this study, it was possible to confirm that the *mcr-1* resistance gene is already circulating in the broiler flocks studied and may be associated with the pathogenic strains.

INDEX TERMS: Detection, *mcr-1*, genes, enteropathogenic, Shigatoxigenic, *Escherichia coli*, strains, broilers, chicken, EPEC, STEC.

RESUMO.- [Detecção do gene *mcr-1* em estirpes de *Escherichia coli* Enteropatogênicas (EPEC) e Shigatoxigênicas (STEC) isoladas de frangos de corte.] *Escherichia coli* Enteropatogênica (EPEC) e Shigatoxigênica (STEC) estão entres os principais patotipos encontrados em aves e produtos avícolas que são capazes de causar doença entérica no homem. A colistina tem sido preconizada como droga de escolha para o tratamento de doenças causadas por bactérias Gram-negativas multirresistentes em humanos. O gene *mcr-1* foi o primeiro gene plasmidial a ser descrito como responsável pela resistência a colistina e tem sido descrito em aves e produtos avícolas. Este estudo tem como objetivo a detecção do gene *mcr-1* em estirpes de *E. coli* enteropatogênicas a fim de avaliar a resistência a colistina em frangos de corte. O material foi obtido a partir de 240 amostras cloacais e 60 carcaças de frango de corte. As estirpes foram isoladas pelo método bacteriológico convencional e os genes de virulência, que caracterizam as estirpes enteropatogênicas, e resistência foram detectados pela reação em cadeia pela polimerase (PCR). Das 213 estirpes de *E. coli* isoladas, 57 (26,76%) foram caracterizadas como EPEC atípica e 35 (16,43%) como STEC. O gene *mcr-1* foi encontrado em 3,5% (2/57) das estirpes EPEC e 5,7% (2/35) das estirpes STEC. Neste estudo foi possível confirmar que o gene de resistência *mcr-1* já está em circulação nos lotes de frango de corte estudados e pode estar associado às estirpes patogênicas.

TERMOS DE INDEZAÇÃO: Detecção, genes, *mcr*-1, estirpes, *Escherichia coli*, enteropatogênicas, Shigatoxigênicas, frangos de corte, EPEC, STEC.

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INTRODUCTION

The Enteropathogenic *Escherichia coli* (EPEC) and Shigatoxigenic *E. coli* (STEC) strains are pathogens of public health importance due to their ability to cause enteric diseases in humans (Ifeanyi et al. 2016, Fierz et al. 2017, Torres 2017) these pathogens are isolated from poultry and poultry products (Dutta et al. 2011, Alonso et al. 2012, Samanta et al. 2015, Doregiraee et al. 2016, Badi et al. 2018).

The pathotype of EPEC strains are divided into atypical (EPEC-a) and typical (EPEC-t). Both strains have genes that can cause a characteristic lesion to the intestinal epithelium (*eae* gene), called "attaching and effacing" (AE lesion), but only EPEC-t contains the plasmid of *E. coli* adherence factor (EAF) (EPEC adherence factor). The EAF plasmids carry the *bfp* gene that encodes the production of type IV fimbria called the bundle-forming pilus (BFP), which is responsible for the bacterial adherence to enterocytes (Nataro & Kaper 1998). In fact, the EPEC-a pathotypes host humans and a variety of animals, while EPEC-t occurs primarily in humans, but has also been described in some rare cases in captive-bred monkeys, coyotes, and dogs (Souza et al. 2016). These animals are possible reservoirs and sources of infection for humans and the environment. In humans, in most cases, EPEC-induced diarrhea is self-limiting and is treated with rehydration therapy, but requires the use of antimicrobials in persistent infections (Ifeanyi et al. 2016).

Shiga Toxin (STX) is the main virulence factor for the characterization of the STEC pathotype, and the toxins are divided into groups STX 1 and STX 2 and encoded by the genes stx_1 and stx_2, respectively. The STEC strains may present both genes alone or associated with. Patients infected with STX2-producing strains develop more frequently anemia, acute renal failure and thrombocytopenia, characterizing uremic hemolytic syndrome (UHS), than those infected with STX2-producing strains (Nataro & Kaper 1998). Some STEC strains are related to EPEC because they contain the *eae* gene, which is responsible for producing the AE lesion (Alonso et al. 2012). The presence of the *eae* gene increases the virulence of STEC strains carrying both the stx_1 and stx_2 genes, associated or not (Beutin & Martin 2012). As with EPEC, antibiotic therapy is indicated only in severe cases (Menne et al. 2012). These drugs should be avoided in the diarrheal phase of infection of this pathotype, as they may be responsible for the rupture of the bacterial membrane causing the release of STX, which may aggravate the cases of the disease (Wong et al. 2012).

Colistin antimicrobial, from the group of polymyxins, acts on these pathotypes on the bacterial cell wall (Giske 2015). Due to the emergence of resistance of these pathogenic bacteria to multiple antimicrobials, colistin has reappeared as the last treatment choice (Rolain et al. 2014). Evidence of colistin resistance involving chromosomal genes has been described in other bacteria, mainly *Klebsiella pneumoniae* (Cannatelli et al. 2013). However, Liu et al. (2016) were the first research team to highlight the role of the *mcr-1* plasmid gene that encodes an enzyme that alters bacterial cell wall structure by inhibiting the action of the antibiotic on the cell.

The recommended test for phenotypic characterization of colistin resistance is the broth microdilution test. However, due to this antimicrobial characteristic of adhering to various materials, including plastics, the phenotypic characterization using this technique, as well as the disc diffusion technique, does not guarantee reliable results (Karvanen et al. 2017). Given the consequent difficulty in phenotypic detection in clinical laboratories, the Agência Nacional de Vigilância Sanitária (ANVISA) (Brasil 2016a) recommends the detection of the *mcr-1* gene by molecular techniques.

Human contamination by multidrug-resistant (MDR) *E. coli* strains from poultry products has been confirmed by Johnson et al. (2007). In this context, the presence of the *mcr-1* gene becomes relevant due to the possibility of transferring colistin resistance to other bacteria of the human microbiota (Liu et al. 2016). In Brazil, the warning about the presence of these genes in bacteria isolated from animals and their products came from the detection of the *mcr-1* gene in *E. coli* and *Salmonella* spp., isolated from chickens and swine (Fernandes et al. 2016) and also in humans (Rossi et al. 2017).

The objective of this study was to evaluate the presence of the *mcr-1* gene in *E. coli* strains of EPEC and STEC pathotypes isolated from commercial broilers.

MATERIALS AND METHODS

The experimental protocol of this study followed the Ethical Principles in Animal Experimentation of the "*Sociedade Brasileira de Ciência em Animais de Laboratório"* (SBCAL) and obtained the approval of the Animal Use Ethics Committee (AUEC) of the Universidade Federal Fluminense (UFF), under no. 697.

Material collection. The samples analyzed were obtained from six slaughterhouses supervised by the State Inspection Service (SIE), in the eastern and southern regions of the state of Rio de Janeiro.

In the reception area of each slaughterhouse, 40 broiler chickens were selected to collect cloacal material. The material was collected as the aid of swabs, which were placed in groups of four swabs in tubes containing Cary Blair (OXOID®) medium, in a total of 10 tubes per batch. From the same batch, ten carcasses were randomly selected and removed from noria after dripping and individually wrapped in sterile bags. All samples were transported in recycled ice isotherms.

Conventional bacteriological isolation. In the laboratory, each pool of swab was washed and grown in tubes containing 10mL peptone saline (SSP) and 400mL of this solution added to the bags. Ten milliliters (10mL) from each carcass was removed and transferred to sterile tubes. All samples were incubated for 24h at 37°C according to the method recommended by the United States Department of Agriculture (USDA 1998).

After this period, all samples were seeded on MacConkey agar (HIMEDIA®) with subsequent incubation for 24h at 37°C. From each culture, three colonies with *Escherichia coli* compatible characteristics were selected for biochemical characterization using Triple Sugar Iron (TSI) (PRODIMOL®), Sulphide Indole Motility (SIM) (HIMEDIA®), Methyl Red Broth media (MR) and Voges Proskauer (VP) (MICRO MED®) and Citrate Agar (HIMEDIA®) (MacFaddin 2000).

PCR. All *E. coli* positive samples were subjected to DNA extraction by the thermal method (Andreatti Filho et al. 2011) and subsequently sent to the polymerase chain reaction (PCR) analysis for the detection of the virulence genes *eae*, stx_1, stx_2 and *bfp*, using gene-specific primer pairs (Table 1).

For the amplification reaction of the *eae* gene, 1X Buffer 10X was added to each 100ng of DNA extracted in the previous step; 1.5mM MgCl2, 0.2mM dNTP, 0.4μM of each primer (Table 1), 1U Taq Polymerase; totaling the final volume of 25μL.

In the reaction for detection of *stx-1* and *stx-2* genes, 1X of 10X Buffer was added to each 100ng of extracted DNA, 2mM MgCl2, 0.4mM

Table 1. Primer oligonucleotide sequence and size of PCR products obtained for detection of virulence genes of Enteropathogenic *Escherichia coli* (EPEC) and Shigatoxigenic *E. coli* (STEC) pathotypes and colistin resistance gene in broiler chickens

Primer oligonucleotides	Sequences	Size of PCR products	References
eae-F	5' GACCCGGCACAAGCA TAAGC 3'	384pb	Dutta et al. (2011)
eae-R	5' CCACCTGCAGCAACAAGAGG 3'		
stx_1-F	5' ATA AATCGCCATTCGTTGACTAC 3'	180pb	Dutta et al. (2011)
stx_1-R	5' AGAACGCCCACTGAGATCATC 3'		
stx_2-F	5' GGCACTGTCTGAAACTGCTCC 3'	255pb	Dutta et al. (2011)
stx_2-R	5' TCGCCAGTTATCT GACATTCTG 3'		
Bfp-F	5' GGAAGTCAAATTCATGGGGGTAT 3'	300pb	Vidal. (2005)
Bfp-R	5' GGAATCAGACGCAGACTGGTAGT 3'		
mcr1	5' CGGTCAGTCCGTTTGTTC 3'	309pb	Liu et al. (2016)
mcr1	5'CTTGGTCGGTCTGTA GGG-3'		

dNTP, 0.4μM of each specific primer (Table 1), 1U Taq Polymerase; totaling the final volume of 25μL.

For the *bpf* gene amplification reaction, every 100ng of DNA extracted in the previous step was added 1X 10X Buffer, 1.5mM MgCl2, 0.2mM dNTP, 0.4 of each primer (Table 1), 1U Taq Polymerase; totaling the final volume of 25μL.

Amplification was performed in a thermal cycler (Programmable Thermal, Controller-PTC-100). After prior denaturation at 94°C for 5 minutes, 30 cycles of 94°C for 45 seconds, 59°C for 45 seconds, 72°C for one minute and a final extension at 72°C for 6 minutes were used (Dutta et al. 2011).

For the amplification reaction of the *mcr-1* gene were added to each 2.0μL of DNA, extracted in the previous step, 1X of 10X Buffer, 1.5mM MgCl2, 0.2mM dNTP, 0.2mM of each primer (Table 1), 1U Taq Polymerase; totaling the final volume of 25μL.

Amplification was performed in a thermal cycler under the following conditions: 94°C for 15 minutes; followed by 25 cycles with denaturation at 94°C for 30 seconds; annealing of the primers at 58°C for one minute and 30 seconds and extension at 72°C for one minute. After these cycles, a final step of 72°C was followed for 10 minutes and the samples were kept for 30 minutes at a temperature of 4°C.

The PCR products were submitted to 1.5% agarose gel electrophoresis and observed in a transilluminator under ultraviolet light.

Statistical analysis. Fisher's exact test, with a significance level of 0.05, was used to evaluate the frequency association between EPEC and STEC strains and the presence of the *mcr-1* gene in cloaca and carcass.

RESULTS AND DISCUSSION

A total of 213 strains of *Escherichia coli* were isolated from the analyzed samples, being 107 isolated from carcasses and 106 isolated from cloacas.

EPEC and STEC characterization

Of the total strains isolated, 26.8% (57/213) were characterized as EPEC harboring only the *eae* gene. Of these, 53% (30/57) were isolated from the cloacas and 47% (27/57) from carcasses. None of the EPEC strains harbored the *bfp* gene and were therefore characterized as EPEC-a (Girón et al. 1993, Gomes & Trabulsi 2008), which was expected since only humans have been identified as EPEC-t hosts (Kaper et al. 2004) (Table 2). Probably the strains found in the study have reduced dissemination capacity in the poultry environment due to the absence of the *bfp* gene, also responsible for the adhesion of the bacteria at the binding site (Nataro & Kaper 1998).

The EPEC-a strains have been detected in birds reared in the conventional system and its products (Dutta et al. 2011, Alonso et al. 2012, Samanta et al. 2015, Doregiraee et al. 2016, Badi et al. 2018), as occurred in the present study.

Of the isolated strains, 16.4% (35/213) were characterized as STEC, being 54% (19/35) isolated from carcasses, and 46% (16/35) of the cloacas. The presence of both stx_1 and stx_2 genes characterizes the strains of the STEC pathotype, and genes may be present alone or associated, including the *eae* gene (Gomes & Trabulsi 2008).

Of the isolated STEC strains, 66% (23/35) presented the *stx1* gene associated with the *eae* gene, 20% (7/35) presented the stx_1 gene associated with the stx_2, 5.6% (2/35) presented only the stx_1 gene, 5.6% (2/35) presented the stx_2 gene and only 2.8% (1/35) the *stx2* gene associated with *eae* gene. No single strain had the stx_1 and stx_2 genes associated with the *eae* gene (Table 2). Among the STEC strains, the stx_1 gene in

Table 2. Frequency of virulence genes (*eae*, *bfp*, stx_1, stx_2) and colistin resistance gene (*mcr-1*) in strains of Enteropathogenic *Escherichia coli* (EPEC) and Shigatoxigenic *E. coli* (STEC) pathotypes isolated from broiler chickens

		EPEC	STEC
Virulence genes	*eae*	57 (100%)	-
	bfp	0	0
	stx_1	0	2 (5.6%)
	stx_2	0	2 (5.6%)
	stx_1 + stx_2	0	7 (20%)
	stx_1 + *eae*	0	23 (66%)
	stx_2 + *eae*	0	1 (2.8%)
	stx_1 + stx_2 + *eae*	0	0
TOTAL		57 (100%)	35 (100%)
Resistance gene	*mcr-1*	2/57 (3.5%)	2/35 (5.7%)

association with the *eae* gene is more frequent than reported by Dutta et al. (2011) and Samanta et al. (2015), demonstrating that these genes are in greater circulation in the STEC of birds and products studied. Although stx_2 gene is associated with higher virulence of STEC strains, the association of stx_1 gene with *eae* gene has been responsible for increased virulence of STEC strains (Beutin & Martin 2012).

There was a significant difference (p=0.0099), by Fisher's test, between the presence of EPEC and STEC strains presented in this study, being isolated a higher percentage of EPEC-a pathotype strains (26.8%) in relation to STEC (16.4%), corroborating with Alonso et al. (2012) who observed 3.9% and 3.3% percentages of EPEC-a and STEC in carcasses and 11.9% and 0.1% of these same pathotypes in cloacas, respectively. Regardless of the detected pathotype, both are of great public health importance because they are responsible for human enteric diseases (Blanco et al. 2006, Rasko et al. 2011, Canizalez-Roman et al. 2016).

Detection of the *mcr-1* gene. Of the strains isolated, 9/213 presented the *mcr-1* gene by PCR, 6/9 from carcasses, and 3/9 from the cloacas. There was no statistical difference between the percentage of strains carrying the *mcr-1* carcass or the cloaca gene (p=0.4984). Fernandes et al. (2016) and Irrgang et al. (2016) reaffirmed the circulation of the *mcr-1* gene in percentages from 0 to 5.3% in live and broiler chicken meat.

Although the first description of the *mcr-1* gene was in 2015 in China by Liu et al. (2016), this gene was detected in isolated *E. coli* strains in Germany in 2010 (Irrgang et al. 2016). Current work corroborated this study by describing the circulation of this gene in several countries to date (Fernandes et al. 2016, Trung et al. 2017, Clemente et al. 2019, Dominguez et al. 2019). Therefore, it is likely that the selective pressure of gene-bound that confers colistin resistance occurred even before its prohibition on animal production in some countries such as Brazil (Brasil 2016b).

Detection of the *mcr-1* gene in poultry and carcasses in the present study is of concern because sensitive strains present in humans may become resistant if plasmid transfers from strains containing the *mcr-1* gene from birds and products by manipulation or ingestion. Liu et al. (2016), suggested that the origin of plasmid from the *mcr-1* gene favors gene transfer to sensitive bacteria from the environmental microbiota, farm animals and humans; this fact may lead to increased resistance of these strains to colistin.

EPEC and STEC strains with the *mcr-1* gene. In this study, it was found that the *mcr-1* gene was present in 3.5% (2/57) of EPEC strains and 5.7% (2/35) of STEC strains (Table 2). The presence of the *mcr-1* gene in these strains is of concern because it is pathogenic to humans, posing a public health risk. The use of antimicrobials to treat infections caused by the EPEC and STEC pathotypes is recommended only in severe cases (Wong et al. 2012, Ifeanyi et al. 2016). As colistin has been cited as a last resort for the treatment of multidrug-resistant bacteria, resistance to this antimicrobial is very important, as it may be a limiting factor for the successful treatment of infections.

CONCLUSION

It was possible to detect the presence of EPEC and STEC strains and to confirm that the *mcr-1* gene is circulating in these strains in live broilers and carcasses, representing a potential public health risk. This study seems to be the first report of the circulation of the *mcr-1* resistance gene in the state of Rio de Janeiro.

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Original Article
Livestock Diseases

Adiposity and weight gain in Mangalarga Marchador horses subjected to hypercaloric diet[1]

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ABSTRACT.- Ribeiro R.M., Ribeiro D.S.F., Paz C.F.R., Gobesso A.A.O. & Faleiros R.R. 2020. **Adiposity and weight gain in Mangalarga Marchador horses subjected to hypercaloric diet**. *Pesquisa Veterinária Brasileira 40(3):170-175.* Graduate Program in Animal Science, Escola de Veterinária, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Cx. Postal 567, Campus Pampulha, Belo Horizonte, MG 31270-901, Brazil. E-mail: faleirosufmg@gmail.com

In recent years, several researchers have been studying obesity in national horse breeds; however, no studies demonstrating the dynamic of body and regional fat accumulation (adiposity) Mangalarga Marchador horses subjected to hypercaloric diets have been found. This study aimed to characterize the deposition of body and regional fat in horses with diet-induced weight gain. A total of nine Mangalarga Marchador adult horses with initial body condition score (BCS) of 2.9 ±1/9 (mean ±SD) were subjected to a hypercaloric, grain-rich diet for five months. Body weight and the following morphometric regional adiposity variables were analyzed: BCS, cresty neck scores (CNS), neck circumferences (NC) at 25, 50 and 75% of its length, and accumulation of subcutaneous adipose tissue at the base of the tail using ultrasonography (BTU). These data were collected at baseline and fortnightly after beginning the diet-induced weight gain until the end of the experiment. The effect of time on the variables was verified by analysis of variance (ANOVA) in randomized blocks or the Friedman's test, and the means were compared by the Tukey's test ($p \leq 0.05$). Exposure to hypercaloric diet promoted a mean weight gain of 27.45% ($p<0.001$). Significant values were observed for NC at 25 and 75% during the first 45 days of the experiment, and for NC at 50% during the first 30 days. BTU presented significant changes after 60 days, with an increase of 268% compared with the baseline value. These findings demonstrate the weight gain and the dynamic and magnitude of regional adiposity in Mangalarga Marchador horses subjected to hypercaloric diet.

INDEX TERMS: Adiposity, weight gain, Mangalarga Marchador, equine, hypercaloric diet, metabolic syndrome, regional fat, horses.

RESUMO.- [**Adiposidade e ganho de peso em equinos Mangalarga Marchador submetidos a dieta hipercalórica.**] Nos últimos anos vários pesquisadores têm estudado obesidade em raças nacionais, contudo não se encontram estudos que demonstrem a dinâmica do acúmulo de gordura corporal e regional em equinos marchadores submetidos a dietas hipercalóricas. O objetivo deste trabalho foi caracterizar a deposição de gordura corporal e regional em equinos com obesidade induzida. Foram utilizados nove equinos adultos, Mangalarga Marchador com escore de condição corporal (ECC) inicial de 2,9±1/9 (média±DP) submetidos a dieta hipercalórica por 5 meses. Foram avaliados o peso corporal, e as variáveis de adiposidade como o ECC, escore de acúmulo de gordura na crista de pescoço (ECP), circunferência do pescoço a 25%, 50% e 75% de seu comprimento e o acúmulo de gordura subcutânea na base da cauda por ultrassonografia (UBC). Coletados antes do início do experimento e quinzenalmente após o início da indução do ganho de peso. O efeito do tempo

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sobre as variáveis foi verificado por análise de variância em blocos ao acaso ou teste de Friedman e as médias foram comparadas pelo teste de Tukey (P≤0,05). O tratamento promoveu aumento médio de peso de 27,45% (P<0,001). Já as circunferências de pescoço 25% e 75% apresentaram valores significativos nos 45 dias de experimento e a circunferência a 50% se destacou nos primeiros 30 dias. A UBC apresentou alterações significativas aos 60 dias de experimento, com um acréscimo de 268% em relação ao valor basal. Tais achados demonstram o ganho de peso, a dinâmica e a magnitude do acúmulo de gordura localizada em equinos Mangalarga Marchador submetidos à dieta hipercalórica.

TERMOS DE INDEXAÇÃO: Adiposidade, ganho de peso, equinos, Mangalarga Marchador, dieta hipercalórica, síndrome metabólica, gordura regional.

INTRODUCTION

According to the World Health Organization (WHO), overweight and obesity are defined as abnormal or excessive fat accumulation that presents a risk to health (Geor 2008). In developed countries, prevalence of obesity is estimated at approximately 19-40% in horse populations (Wyse et al. 2008, Thatcher et al. 2012). To demonstrate how commonly obesity occurs in this species, a study evaluating 300 adult animals found that 19% of them were obese (body condition score (BCS) between 8 and 9) and 32% were overweight (BCS from 6.5 to 7.5) (Allan et al. 2000). Another study observed that 45% of the horses in a population of 319 randomly selected animals were obese or very obese (Robertson 2003).

Obesity is induced with the provision of high grain and forage diets (grass and hay) with high non-structural carbohydrate (NSC) contents, becoming a consequence of overfeeding, leading to excessive fat reserves in relation to body demand and the level of physical activity (Schott et al. 2001, Johnson 2002).

Fat accumulation (adiposity) can trigger exercise intolerance, thermoregulatory inefficiency, abnormal reproductive performance, and increased likelihood of developing mesenteric lipomas (Henneke et al. 1984, Cymbaluk & Christison 1990, Garlinghouse & Burrill 1999, Garcia-Seco et al. 2005). In addition to these changes, obesity is closely related to equine metabolic syndrome (EMS) and insulin dysregulation (ID) (Frank et al. 2010).

Several studies have shown that regional adiposity is closely correlated with ID, hyperinsulinemia and metabolic disorders such as dyslipidemia, high levels of leptin and non-esterified fatty acids, increased proinflammatory cytokine gene expression, high peripheral blood protein concentrations, and increased risk of developing laminitis (Johnson 2002, Sutherland et al. 2004, Vick et al. 2007, 2008, Carter et al. 2009). Thus, adipocytes located in different parts of the organism present different characteristics, and some adipocytes secrete endocrine signaling that can lead to metabolic dysfunction (Chaldakov et al. 2003, Lyon et al. 2003).

A relationship between obesity and evidence of metabolic disease in Brazilian horses has been confirmed by several studies (Paz et al. 2013, Magalhães et al. 2014, 2017, Xavier et al. 2014). However, the magnitude and dynamic of adiposity in national fattening horses are not yet known. The objective of this experiment was to characterize body and regional adiposity in Mangalarga Marchador horses with diet-induced weight gain.

MATERIALS AND METHODS

Nine healthy Mangalarga Marchador horses (five non-pregnant mares and four geldings) aged 48±5 months with initial weight of 316±62.68kg were used in this study. For standardization of diet administration, the horses were housed in duly identified individual stalls with sawdust-covered floor and access to water *ad libitum.* The experiment was carried out at the facilities of "Fazenda Modelo Pedro Leopoldo" of the "Universidade Federal deMinas Gerais" (UFMG).

Obesity was induced through provision of digestible energy (DE) in amounts 100% higher than the maintenance requirement established in the reference literature (NRC 2007). Digestible energy maintenance (DEm) was calculated based on the mathematical formula DE = 1.4 + (0.03 x Kg live weight [LW]), according to the NRC (2007), over a period of 150 days.

In order to avoid gastrointestinal disorders, it was previously established that animals would receive DEm once in concentrate form and once the forage form. To this end, the DE values for the feed available were used: commercial concentrate (Guabi Equitage Laminados) (DE: 3.650 Mcal/kg) and grass hay *Cynodon dactylon* (*L.*) *Pers.Var.* Coast cross (DE: 2.0Mcal/kg). Thus, the amounts of forage and concentrate were calculated considering the DEm of each animal divided by the DE concentration of each type of feed: Amount of Concentrate (kg): DE = (1.4 + (0.03 x kg LW))/3.650; Bulk Amount (kg): DE = (1.4 + (0.03 x kg LW))/2.

This calculation was repeated fortnightly after the animal weight was measured, thus maintaining the proportion of 100% on the DE requirement established by the NRC (2007) constant over the 150 days of the trial period. The diet was provided in individual feeders, in three (3) daily meals and equal parts offered at 6am, 12pm, and 18pm.

At baseline and fortnightly after beginning the diet-induced weight gain until the end of the experiment, the animals were evaluated walking and trotting to detect absence/presence of lameness. A sensitivity test was also performed using the hoof testers at baseline and at 30-day intervals until the end of the experiment.

Body weight and the following morphometric regional adiposity variables were assessed at baseline and fortnightly until the end of the experiment: body condition score (BCS), cresty neck score (CNS), and neck circumference (NC). To measure the BCS of the horses, a scale ranging from 1 to 9 (1 = emaciated and 9 = extremely obese) was used according to the grading proposed by Henneke et al. (1984). The CNS, which indicates metabolic disease, was verified using a 5-point scale (0 = crest not visible and not palpable and 5 = increased crest permanently drooping to one side), according to Carter et al. (2009).

Accumulation of subcutaneous adipose tissue at the base of the tail using ultrasonography (BTU) was measured at baseline and every 30 days until the end of the experiment.

A weighing scale was used to measure the body weight of the animals. For objective assessment of local fat deposition, neck circumference (NC) measurements were performed at three different points at the total neck length, according methodology described by Frank et al. (2006). These measurements were performed at dorsal points corresponding to 25, 50, and 75% of the neck length. The BTU measurements were obtained using an ultrasound device (Echovet, model KX 5100) equipped with a 5 MHz transducer, which was positioned on the right side of the base of the tail, 5 cm away

from the midline (Fig.1), according to the methodology described by Gentry et al. (2004).

For statistical analysis, the data were processed using the Sigma (SigmaStat, Systat) computer software. The effect of time on the variables with normal distribution was verified by analysis of variance (ANOVA) in randomized blocks , followed by the Tukey's test for comparison between the means. For the nonparametric data of the BCS and NC variables, repeated measures ANOVA over

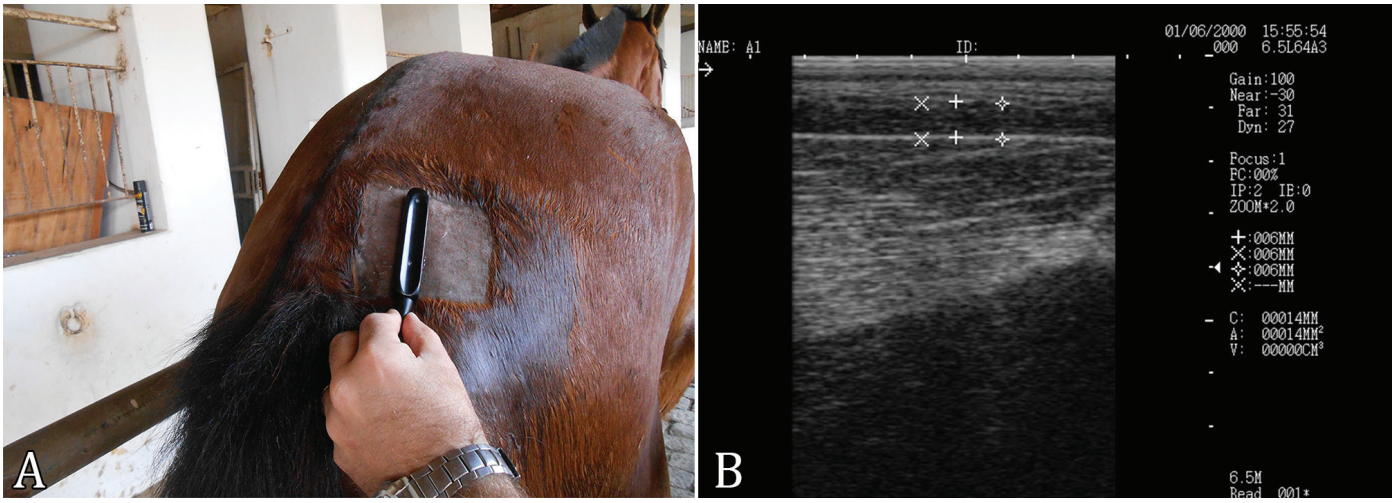

Fig.1. Procedure used to measure accumulation of subcutaneous adipose tissue at the base of the tail by ultrasonography (BTU). (**A**) Demonstration of transducer location of the ultrasound device. (**B**) Demonstration of the image obtained by an ultrasound device measuring three areas of the subcutaneous fat layer.

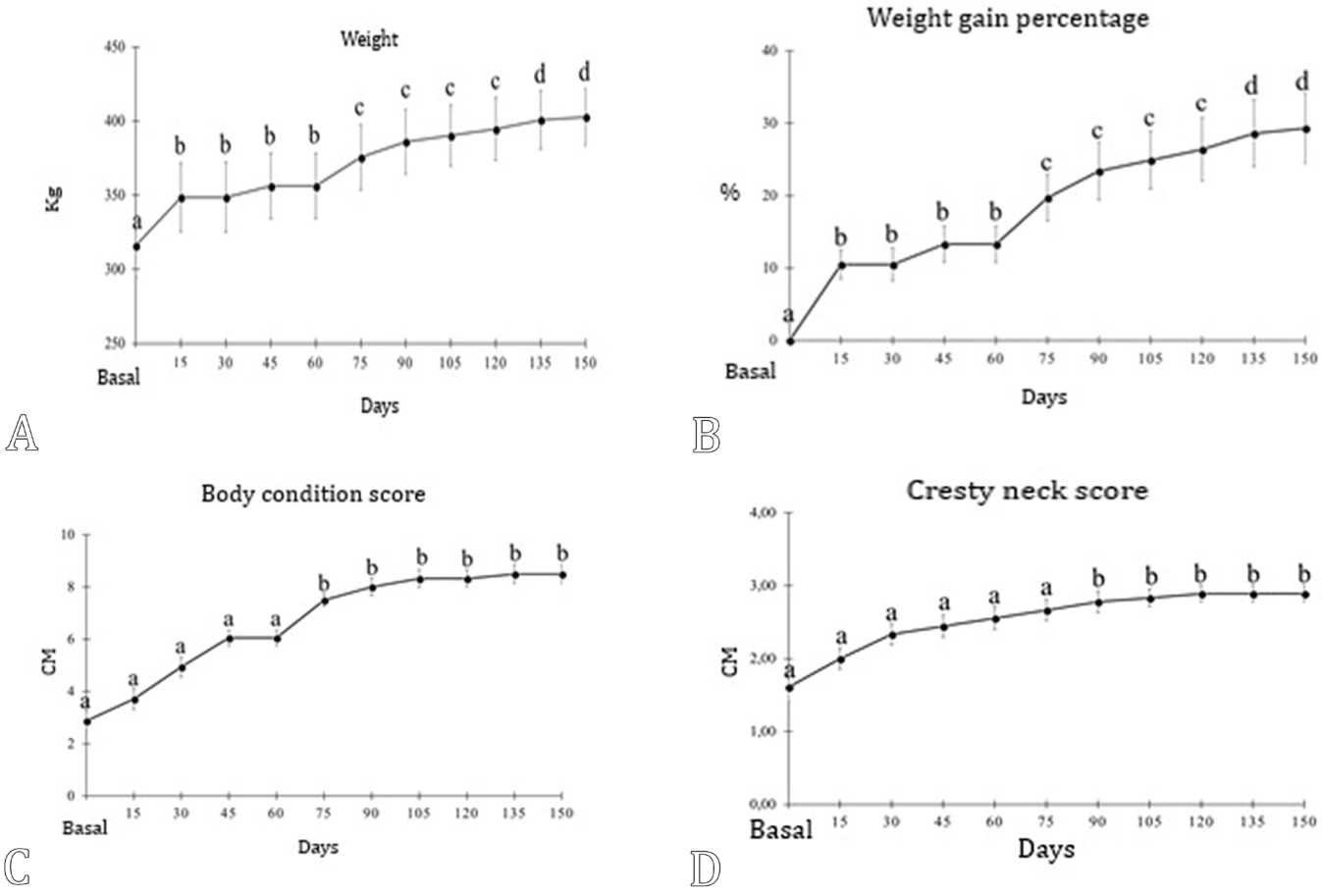

Fig.2. (**A**) Means and standard errors for weight gain, (**B**) weight gain percentage, (**C**) body condition score (BCS), and (**D**) cresty neck score (CNS) of Mangalarga Marchador horses subjected to hypercaloric diet. Means followed by the same letters do not differ by the Tukey's test for parametric variables and by the Friedman's test for nonparametric variables, at 5% probability.

time and the Friedman's test were used. A significance level of 5% ($p \leq 0.05$) was adopted for all statistical analyses.

RESULTS

During the experimental period, the horses consumed a daily average of 0.94±0.02% of concentrate and 1.72±0.04% of forage of body weight. There were no colic event, evidence of lameness, or signs of sensitivity by hoof tester exam.At the end of the experiment, the animals presented an average weight gain of 27.45%, with significant values ($p<0.001$) as of the second measurement (Fig.2). BCS and NC showed significant increases at 75 and 90 days, respectively (Fig.2), NC at 25 and 75% showed significant increases at 45 days, and NC at 50% present significant increase at 30 days (Fig.3). The BTU values showed significant alterations after 60 days, with an increase of 268% compared with the baseline value (Fig.3).

DISCUSSION

In the present study, it was possible to verify the effects of hypercaloric diet on the body weight and regional fat accumulation in Mangalarga Marchador horses. During the experimental period, the animals presented a weight gain of 27.5% compared with the initial values. As they were animals in the end of the developmental phase, this weight gain was reflected in the accumulation of subcutaneous adipose tissue promoting a 2.95-fold increase in BCS and a 1.7-fold increase in CNS.

It is known that the use of scores for the determination of adiposity in horses presents great individual variation, even when conducted by trained professionals (Young et al. 2004, Mottet et al. 2009). As noted by other authors, BCS is not accurate to assess regional fat accumulation because these adipose deposits often occur asymmetrically, hindering determination of the actual body situation of the animal (Geor 2008). However, these methods continue to be used in practice because they present significant correlations with metabolic disorders such as ID (Carter et al. 2007).

Objective methods, such as NC and BTU, were also used. Increases of 15, 23 and 21%, respectively, in circumference measures taken at 25, 50 and 75% of the neck length were observed. A study conducted by Frank et al. (2006) showed that neck circumference correlated closely with the area under the insulin curve, which is a well known risk factor for the development of insulin resistance (IR) in horses. All NC measures showed an increase throughout the experiment, proving that the use of NC is an objectively valid for the evaluation of regional fat accumulation. However, of these three measurements, NC at 50% was the one that presented the largest increase, being evidenced as the variable that stands out to measure animal weight gain, corroborating studies addressing Mangalarga Marchador (Lima et al. 2010) and police (Xavier et al. 2014) horses.

The BTU method should be highlighted in this study, because it directly determined subcutaneous fat deposition and presented a 7-fold increase from baseline. BTU has also proved to be a sensitive alternative to the early signaling of regional fat accumulation, presenting significant values after 60 days. The use of ultrasonography is effective in identifying regional accumulation of subcutaneous adipose tissue in horses and other farm animals (Westervelt et al. 1976, Gee et al. 2003, Dugdale et al. 2010, Silva & Cadavez 2012). Françoso et al. (2012) concluded that measuring subcutaneous fat at the base of the tail is the most reliable method to estimate adiposity, in agreement with the findings by Gentry et al. (2004), who reported that the tail region is where the most substantial fat deposition occurs in the body of a horse.

Changes in some biomarkers have been found to correlate with regional adiposity markers independently. This feature can be explained by a theory that defends that adipose tissue displays notably different effects depending on its location in the body. It has been theorized that adipocytes have different characteristics depending on their location (Lyon et al. 2003).

In this context, several authors have proposed that fat accumulation in some areas of equine subcutaneous tissue is responsible for the development of EMS. For example, accumulation of fat in the neck is associated with higher

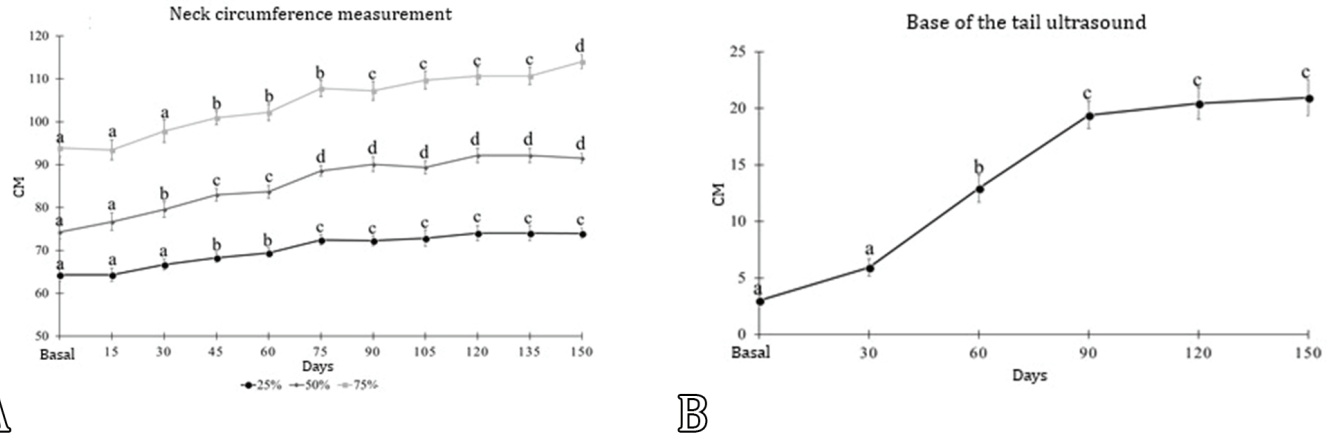

Fig.3. (**A**) Means and standard error for neck circumference (NC) measured at 25, 50 and 75% of the distance between the nape and the withers and (**B**) accumulation of subcutaneous adipose tissue at the base of the tail using of ultrasonography (BTU) of Mangalarga Marchador horses subjected to hypercaloric diet. Means followed by the same letters do not differ from each other by the Tukey's test, at 5% probability.

expression of the IL-1β and IL-6 genes, whereas visceral fat is linked to a larger number of active macrophages and a larger amount of leptin (De Luca & Olefsky 2008, Vick et al. 2008, Burns et al. 2010, Maury & Brichard 2010, Weber et al. 2013).

Increased adiposity on the neck and NC are closely correlated with IR. Packer et al. (2011) observed correlation between retro abdominal fat accumulation and leptin gene expression in horses, and Xavier et al. (2014) found correlation between BTU and BCS with radiographic alterations consistent with the spatial changes of the third phalanx.

Other studies have observed that adiposity parameters such as CNS, NC, BTU, weight gain, and BCS were positively correlated with metabolic changes and changes observed in the laminar tissue of horses, evidencing the value of the present work, considering that the horses used presented distribution of fat accumulation in the aforementioned places (Ribeiro et al. 2015a, 2015b, 2016). These measurements can assist with the monitoring of weight gain, and these obesity parameters can be used as reference to demonstrate whether the animals are presenting regional adiposity, thus being predisposed to possible metabolic changes.

CONCLUSION

Exposure to hypercaloric diet promoted weight gain and body fat deposition, with emphasis on body condition score (BCS), neck circumference (NC), and subcutaneous thickness at the base of the tail using ultrasonography (BTU). These findings demonstrated that these variables can be useful to monitor total and regional fat deposition in Mangalarga Marchador horses.

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Original Article
Small Animal Diseases

Clinical management of dogs with presumptive diagnosis of cervical intervertebral disc disease: 78 cases (2006-2017)[1]

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ABSTRACT.- Baumhardt R., Ripplinger A., Aiello G., Schwab M.L., Ferrarin D.A., Wrzesinski M.R., Moro S.S. & Mazzanti A. 2020. **Clinical management of dogs with presumptive diagnosis of cervical intervertebral disc disease: 78 cases (2006-2017).** *Pesquisa Veterinária Brasileira 40(3):176-180.* Departamento de Clínica de Pequenos Animais, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Av. Roraima 1000, Camobi, Santa Maria, RS 97105-900, Brazil. E-mail: alexamazza@yahoo.com.br

This study aimed to identify dogs with a presumptive diagnosis of cervical intervertebral disc disease (IVDD; C1-C5 or C6-T2) submitted to clinical management and evaluate the outcome. This study also aimed to demonstrate the age, sex, and treatment response according to the neurological degree, and verify whether those factors could potentially influence the outcome. The data were obtained from patients with a neurological dysfunction, admitted at the Veterinary Hospital from January 2006 to March 2017. In addition to patient records, the tutors answered a questionnaire related to the success of therapy. A hundred and seventy-seven neurological records were evaluated, and 78 were included in the study according to the inclusion criteria. The most frequent breeds were Dachshunds, followed by mixed-breed dogs. Regarding the neurological dysfunction degree, 58.97% presented grade I (only neck pain), 25.64% were grade II (ambulatory tetraparesis), and 15.38% grade III (nonambulatory tetraparesis). Absolute and partial space rest were performed in 75.64% and 24.36% of the cases, respectively. The minimum rest time was one week and could come up to four weeks. Most dogs were small-sized (≤15kg). The recovery was satisfactory in 87.17% of dogs and unsatisfactory in 12.83%. Regarding recurrence, we observed that 10.3% of dogs presented satisfactory recovery. The clinical treatment for dogs with cervical IVDD can be indicated with adequate clinical response to dysfunction degrees ranging from I to III, either at rest or in restricted space and with a low rate of relapse.

INDEX TERMS: Clinical management, dogs, diagnosis, cervical, intervertebral disc disease, IVDD, cage rest, extrusion, protrusion, clinics.

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RESUMO.- [**Tratamento clínico de cães com diagnóstico presuntivo de doença do disco intervertebral cervical: 78 casos (2006-2017).**] O objetivo desse estudo foi identificar cães com diagnóstico presuntivo de doença do disco intervertebral cervical (DDIV; C1-C5 ou C6-T2) submetidos ao tratamento clínico e avaliar a resposta a terapia instituída e o índice de recidiva. Esse estudo também visou demonstrar a idade, o gênero e a resposta ao tratamento de acordo com o grau neurológico, a fim de verificar se esses parâmetros podem ser utilizados como fatores prognósticos para a evolução clínica desses pacientes. Foram revisados os registros neurológicos

do Hospital Veterinário Universitário de janeiro de 2006 a março de 2017. Realizaram coleta de dados a partir dos registros e por meio de um questionário respondido pelos tutores. Avaliaram 177 fichas neurológicas de cães e obtidas informações para inclusão no estudo em 78 delas. As raças mais frequentes foram Dachshunds, seguido dos cães sem raça definida. Quanto ao grau de disfunção neurológica, 58,97% apresentavam grau I (somente dor), 25,64% estavam em grau II (tetraparesia ambulatória) e 15,38% em grau III (tetraparesia não ambulatória). O repouso absoluto e em espaço restrito foram realizados em 75,64% e 24,36% dos casos, respectivamente e com duração de no mínimo uma semana, podendo chegar a mais de quatro semanas. A maioria dos animais era de pequeno porte (≤15kg). A recuperação foi satisfatória em 87,17% dos cães e insatisfatória em 12,83%. Quanto à recidiva, esta foi observada em 10,3% dos pacientes com recuperação satisfatória. O tratamento clínico para cães com DDIV cervical pode ser indicado com adequada resposta clínica para graus de disfunção que variam de I a III, seja em repouso absoluto ou em espaço restrito e com baixo índice de recidiva.

TERMOS DE INDEXAÇÃO: Tratamento clínico, cães, diagnóstico, doença do disco, intervertebral cervical**,** DDIV, repouso absoluto, extrusão, protrusão, caninos, clínica.

INTRODUCTION

Intervertebral disc disease (IVDD) is a common neurological condition in dogs (Fluehmann et al. 2006, Ingram et al. 2013), occurring in the cervical region between 12.9% and 25.4% of cases (Goggin et al. 1970, Gage 1975, Chaves et al. 2014). It affects dogs of any breed and size, with the Dachshunds and Beagles being the most prevalent. Its average age of occurrence in the cervical region varies from six to eight years (Russell & Griffths 1968, Denny 1978, Cherrone et al. 2004, Levine et al. 2007, Ryan et al. 2008, Hillman et al. 2009, Santini et al. 2010, Schmied et al. 2011), for both extrusion and protrusion (Schmied et al. 2011). The minimum and maximum ages reported by Hakozaki et al. 2015 was one and 16 years, respectively, for chondrodystrophic an non-chondrodystrophic breeds.

Intervertebral disc extrusion (Hansen type I) or intervertebral disc protrusion (Hansen type II) may result in spinal cord compression with subsequent spinal cord compression, the protrusion being more frequent than the extrusion in the cervical region (Cherrone et al. 2004). Spinal hyperesthesia is the most reported clinical sign (Dallman et al. 1992, Cherrone et al. 2004, Ryan et al. 2008, Santini 2010). However, more severe signs such as proprioceptive 4-limb ataxia, ambulatory or non-ambulatory tetraparesis, never root signature in the affected thoracic limb, and tetraplegia are also described (Russell & Griffths 1968, Denny 1978, Seim & Prata 1982, Morgan et al. 1992, Tomlison 1996, Beal et al. 2001, Cherrone et al. 2004, Hillman et al. 2009). Nociception loss and breathing difficulty may occur but are rarely reported (Beal et al. 2001, Hillman et al. 2009).

Treatment for cervical IVDD can be clinical or surgical (Olby 2014). Clinical treatment consists of absolute cage rest between two and six weeks; this time would be the minimum necessary for annulus fibrous repair (Tipold et al. 2010, Dewey & Da Costa 2016) and is mainly indicated for dogs in grades I and II of neurological dysfunction (Tipold et al. 2010). Associated with rest, opioid analgesics, muscle relaxants, steroidal or non-steroidal anti-inflammatory drugs, and physical therapy may be used (Sharp & Wheeler 2005, Brisson 2010). Surgery is the treatment of choice for dogs with severe cervical pain, severe neurological disabilities, recurrences or clinical treatment failure, and chronic signs of the disease (Seim & Prata 1982, Cherrone et al. 2004, Hillman et al. 2009).

Surgical treatment of cervical IVDD is associated with a high rate of anesthetic-surgical complications, (Rossmeisl et al. 2013) with a mortality rate reaching 8% (Clark 1986, Posner et al. 2014). Some clinicians avoid cervical decompression surgery as much as possible (Hawthorne et al. 1999), and many tutors are discouraged to authorize the surgical procedure, opting for clinical treatment. Despite these facts, studies addressing the clinical treatment evolution of cervical IVDD in dogs are scarce (Russell & Griffths 1968, Janssens 1985, Levine et al. 2007).

This study aimed to evaluate the response to the clinical treatment instituted, the recurrence rate, and possible prognostic factors of dogs with a presumptive diagnosis of cervical IVDD. This study also aimed to demonstrate age, gender, and response to treatment according to neurological grade, in order to use these parameters as prognostic factors for the clinical evolution of these patients.

MATERIALS AND METHODS

Neurological records of a veterinary hospital were reviewed from January 2006 to March 2017. Only dogs with a presumptive diagnosis of IVDD in the C1-C5 and C6-T2 spinal segments were included. The presumptive diagnosis was defined by history, breed, age, clinical signs, neurological examination, plain radiography, myelography or computed tomography (Dewey & Da Costa 2016). In all dogs, serology tests were performed to exclude infectious diseases (toxoplasmosis, neosporosis and distemper). Each record was reviewed and certified that there were no other (non-neurological) conditions that could interfere with the results of the neurological exam.

Data on breed, age, sex, and neurological dysfunction degree at the time of examination were collected from neurological records. The other information was obtained by telephone contact with the tutors. The neurological dysfunction degree was rated from I to V, where: I) only pain on palpation of the cervical spine; II) ambulatory tetraparesis; III) non-ambulatory tetraparesis; IV) tetraplegia with intact deep pain perception caudal to the lesion site; V) tetraplegia without deep pain perception caudal to the lesion site (Kranenburg et al. 2013). The dogs were distributed amoung age groups up to three years old; four to six years old; seven to nine years old; and greater than or equal to ten years, like the distribution made by Chaves et al. (2014).

The prescribed clinical treatment by the SNNV consisted of absolute rest in a cage or transport box for a minimum of 30 days. The patient was removed three times a day for urination and defecation using a harness. Steroidal or non-steroidal anti-inflammatory drugs and opioid analgesics were also used.

Tutors of the selected patients were contacted by telephone and answered a two-part questionnaire, including: 1) information before hospital appointment; and 2) information after the appointment.

Data from the first part of the questionnaire were related to the recurrence or absence of signs; duration of clinical signs until the moment of appointment; performing absolute rest (in a cage

or a transport box) or partial rest in a restricted space, and the evolution of clinical signs until the appointment. Signal recurrence was considered in the presence of previous history of back pain and difficulty walking or climbing obstacles. The duration of clinical signs was classified as: less than or equal to one day; >1 and up to 7 days and >7 days. Regarding the evolution of clinical signs, the dogs were distributed in: satisfactory - when the dog showed improvement compared to the onset of signs; unsatisfactory - when there was no difference or worsening until appointment at the veterinary hospital. Data from the second part of the questionnaire refer to the treatment indicated by the physcian, and the clinical evolution of the treatment performed. Regarding clinical evolution, they were classified as: satisfactory - dogs that have recovered the ability to walk without hyperesthesia; unsatisfactory - when they did not recover motor function or remained with cervical hyperesthesia. Dogs that had satisfactory recovery were assessed for disease recurrence.

Those cases in which dogs died within a period shorter than two weeks or the tutors opted for euthanasia were excluded. All dogs had an interval of at least three months between the first appointment and the start of the survey; this was the minimum period for evaluation of clinical evolution. Any patient with more than seven days of evolution, who has not had rest before the appointment, was included in the control group (i.e., without rest). The treated group was composed of patients who received the treatment indicated by the physician, i.e., absolute cage rest or partial rest in a restricted space at the tutor's option.

Statistical evaluation was performed using the Chi-square test, and the following variables were evaluated: age, gender, recovery (satisfactory or unsatisfactory), type of treatment (absolute rest, rest in restricted space, or no rest), degree of neurological dysfunction, time of evolution.

RESULTS AND DISCUSSION

A total of 177 neurological records were evaluated, and 78 dogs with a presumptive diagnosis of cervical IVDD were included. Of this total, 52.56% (n=41) dogs were male and 47.43% (n=37) were female. Regarding breed, 39.74% were Dachshund (n=31), 23.07% mixed-breed (MB) (n=18), 8.97% Poodle (n=7), 6.41% Lhasa Apso (n=5), 5.13% Yorkshire (n=4), Maltese and Beagle breeds accounted for 2.56% of the total each (n=2 each), the other breeds with one representative each were Australian Cattle Dog, French Bulldog, Chow Chow, Cocker, Brazilian Terrier, Pomeranian Lulu, Alaskan Malamute and Shih Tzu (1.28% of the total of each breed). The animals' size ranged from small to medium, with body weights from 1.3kg to 25kg (average 8.51kg and median 8.10kg), 71 with weight less than or equal to 15kg and seven with a weight between 16 and 25kg. The animals' age ranged from one year and 11 months to 18 years (average 8.10 and median 8 years). As shown in Table 1, most of the animals in the study were old than four years old. The minimum and maximum age of the animals in this study is in agreement with studies of Schmied et al. (2011) and Hakozaki et al. (2015), where the minimum ages (three and one year old respectively) and maximum (twelve and sixteen year old respectively) are of dogs with cervical disc protrusion and extrusion in both chondrodystrophic and non-chondrodystrophic breeds. Thus, the population studied, regarding the prevalence of sex, age and breed of the patients, was like those found by previously published studies (Levine et al. 2007, Brisson 2010, Santini et al. 2010, Posner et al. 2014, Hakozaki et al. 2015, Schmied et al. 2011).

The recovery of dogs in different age groups and sex had similar proportions (without statistical difference), suggesting that these variables do not interfere with clinical recovery, which is in agreement with Levine et al. (2007). However, Itoh et al. (2008) found a higher sexual predisposition for male dogs in a janpanese study, which may be due to population variation in the region studied.

Tutors reported first time clinical sings in 84.6% (n=66) of the cases; signs were recurent in 15.38% (n=12). Tutors have administered anti-inflammatory drugs in 37,17% (n=29) before the first appointment, while 34.61% (n=27) have not used it. In 22 (28.2%) cases, the tutor did not know the answer. The neurological dysfunction degree was defined as grade I for 58.97% of dogs, grade II for 25.64% and grade III for 15.38% (Table 1). No dog with tetraplegia (grade IV or V) was observed. The higher prevalence of severe pain in the neck region in this study has been verified by Cherrone et al. (2004) as the main clinical sign of cervical DDIV.

The low rate of neurological deficits found for IVDD in the cervical region compared to the thoracolumbar region may be related to the greater width of the vertebral canal in the

Table 1. Representation of satisfactory recovery and relapse according to epidemiological variables evaluated in 78 dogs with presumptive diagnosis of cervical IVDD undergoing clinical treatment (2006-2017)

Variable	Number of dogs n	Satisfactory recovery n (%)	Relapse n (%)
Sex			
Female	37	33 (89.1)	4 (12.1)
Male	41	35 (85.3)	3 (8.5)
Age			
Up to 3 years	5	5 (100)	-
From 4 to 6 years	22	20 (90.9)	1 (5.00)
From 7 to 9 years	25	21 (84.0)	3 (14.2)
≥10 years	26	22 (84.6)	3 (13.6)
Duration of clinical signs			
≤1 day	5	5 (100)	-
>1-7 days	34	30 (88.2)	5 (16.6)
>7 days	39	33 (84.6)	2 (6.06)
Neurological dysfunction			
Grade I	46	43 (93.4)	4 (9.3)
Grade II	20	15 (75.0)	2 (13.3)
Grade III	12	10 (83.3)	-
Treatment			
Absolute rest	59	52 (88.1)	4 (7.6)
Restricted space rest	19	16 (84.2)	3 (18.7)
Rest duration			
1 week	7	5 (71.4)	-
>1-3.5 weeks	54	48 (88.8)	4 (8.3)
≥4 weeks	17	15 (88.2)	3 (20)

cervical region, so extruded disc contents need to be larger to severely compromise the spinal cord (Brisson 2010).

When the patients were distributed according to the rest performed before the appointment, 91% (n=71) of the dogs were not in absolute rest, and of these, 91.55% (n=65) were in an unsatisfactory state according to tutors. The signal duration was less than or equal to one day for 6.41% of dogs, >1 and up to 7 days for 43.59%, and >7 days 50% (Table 1).

After the appointment, 75.64% of cases complied with the absolute rest indicated, and 24.36% did not rest according to prescription but kept the dog in a restricted space of one room during the treatment period.

Clinical evolution was satisfactory in 87.17% (n=68) of dogs and unsatisfactory in 12.82% (n=10). The 10 dogs that did not recover from clinical treatment (three in grade I, five in grade II and two in grade III) were referred for surgery and confirmed disc extrusion (Hansen type I). Of the 68 dogs with satisfactory recovery, 52 were treated as absolute rest and 16 in restricted space (Table 1). There was no significant difference between the two types of rest (absolute or restricted) in the present group of animals studied. Bersan et al. (2017) also described success with clinical treatment involving controlled movements for dogs diagnosed with intervertebral disc foraminal extrusion in the cervical region; this suggests that the simple reduction of movement already allows partial or complete resolution of cervical IVDD. Even so, the authors suggest that the clinical treatment should be made in absolute rest since movement is more limited than rest in a restricted space and less likely to worsen the clinical outcome.

Of the dogs with satisfactory recovery, 10.3% (n=7) presented recurrence, a percentage lower than that observed by Levine et al. (2007) in which 29 of 43 patients who were submitted to clinical treatment and achieved recovery had recurrence. This may reflect the reduced follow-up time performed in part of the patients in the present study (minimum 3 months).

Table 1 shows the percentage of satisfactory recovery regarding gender, age group, degree of dysfunction, and duration of rest. There was no relationship between satisfactory recovery and the items described above.

The satisfactory response was significantly higher for patients treated with absolute rest (n=59) and partial rest (n = 19) when compared to the control group (n=39), i.e., without absolute rest ($p<0.05$). Reduction motion induced by rest is believed to minimize further extrusion of disc material into the spinal canal annulus fibrosus heal, and there is a reduction in the inflammatory reaction caused by extrusion during this rest period (Dewey & da Costa 2016). Spontaneous regression of disc extrusion has been described in humans (Weber 1983, Saal et al. 1990, Mochida et al. 1998) and is believed to occur in dogs as well (Steffen et al. 2014). Extruded disc material is likely reabsorbed as consequence of the inflammatory reaction and the action of activated macrophages (Haro et al. 1996). Komori et al. (1996) demonstrated in humans that the tendency for spontaneous regression of the herniated disc varied with its anatomical location; this may also be related to the degree of a satisfactory response to clinical treatment in dogs. Such information obtained through careful magnetic resonance imaging evaluations suggests larger studies in dogs with cervical IVDD undergoing clinical treatment so that it can be classified when this treatment is chosen for this species.

One of the limitations of this study was the presumptive diagnosis of IVDD. Levine et al. (2007) used simple radiography and myelography as diagnostic aid methods to exclude other causes such as discospondylitis, meningomyelitis fractures, and neoplasms. Moreover, we requested exams to investigate the main canine infectious diseases, in addition to computed tomography, considered an imaging exam with sensitivity higher than myelography in detecting spinal cord compression by IVDD (Brisson 2010). Even so, magnetic resonance imaging and, more precisely, surgical exploration that will reveal extruded content within the vertebral canal are recommended for the definitive diagnosis of IVDD (Levine et al. 2007). Therefore, one cannot rule out the possibility that some dogs with presumptive diagnosis of IVDD had another neurological disorder.

The clinical relevance of this study was to demonstrate the results of the clinical treatment in dogs with a presumptive diagnosis of cervical IVDD in Brazil and to verify that the recovery rate was satisfactory and with low recurrence when compared to the international literature (Levine et al. 2007). Even though the clinical therapy employed in this study was in line with the literature (Dewey & Da Costa 2016), tutors who did not follow SNNV guidance and optionally restricted movement (restricted space), the results were also satisfactory even for severe degrees of neurological dysfunction (Table 1).

CONCLUSION

Clinical treatment can be used in dogs with cervical IVDD with an adequate clinical response for dysfunction degrees ranging from I to III, either in absolute rest or restricted space and with a low recurrence rate. There is no difference in clinical response among dogs of different ages or genders, suggesting no prognostic influence of these factors on recovery.

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

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Original Article
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Concentrations of matrix metalloproteinase-2 and interleukin-1 β in the aqueous humor of dogs with normal and cataractous eyes[1]

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ABSTRACT.- Ruiz T., Almeida A.B.P.F. & Ribeiro A.P. 2020. **Concetrations of matrix metalloproteinase-2 and interleukin-1 β in the aqueous humor of dogs with normal and cataractous eyes.** *Pesquisa Veterinária Brasileira 40(3):181-187*. Faculdade de Medicina Veterinária, Universidade Federal de Mato Grosso, Av. Fernando Corrêa da Costa 2367, Boa Esperança, Cuiabá, MT 78060-900, Brazil. E-mail: alexandre.aleribs@gmail.com

We aimed to determine the concentration of MMP-2 and IL-1β in the aqueous humor of dogs with healthy eyes (n=8) and in those with mature (n=8) and hyper mature cataracts (n=8). Correlations between cytokines, cytokines, and intraocular pressure (IOP), as well as cytokines with ages of patients of each group, were also assessed. In patients with cataract, aqueous humor was collected at the end of the electroretinographic procedure. In healthy dogs, aqueous humor was collected before elective surgeries. Cytokine levels were determined using ELISA. IOP was assessed by applanation tonometry. IOP of patients with mature and hyper mature cataracts were lower than the ones measured in normal eyes (P=0.158). MMP-2 aqueous humor concentration was higher in patients with hyper mature cataracts, in comparisons with healthy patients (P=0.04). Average IL-1 β aqueous concentration was higher in patients with cataracts (P<0.0001). Significant higher values of IL-1 β were observed in patients with hyper mature, than in the ones with mature cataracts (P=0.0085). Correlations between MMP-2 and IL-1 β (r=-0.38, P=0.06), MMP-2 and IOP (r=-0.149, P=0.484), and IL-1 β and the ages of patients were not observed (P>0.05). IL-1 β and IOP correlated negatively (r=-0.42, P=0.04). MMP-2 and the ages of patients correlated only in dogs with mature cataracts (r=0.772, P=0.02). It can be concluded that the increment in the aqueous humor concentration of IL-1 β found in dogs with mature and hyper mature cataracts, in addition to the negative relationship of this cytokine with IOP, suggests that IL-1 β is involved in the pathogenesis of LIU. Despite dogs with hypermature cataracts presented significant higher concentrations of MMP-2 in the aqueous humor, such cytokine did not correlate with IL-1 β and IOP. In our study, a relationship between aqueous humor cytokines and the ages of patients was only confirmed between MMP-2 and the ages of dogs with mature cataracts.

INDEX TERMS: Metalloproteinase-2, interleukin-1 β, dogs, cataractous, eyes, lens-induced uveitis, inflammatory cytokines, capsular fibrosis, lens.

RESUMO.- [Concentração de metaloprotease-2 e de interleucina-1 β no humor aquoso de cães com olhos normais e cataratosos.] Objetivou-se determinar as concentrações da metalloprotease-2 (MMP-2) e de interleucina-1 β (IL-1 β) em cães com olhos saudáveis (n=8) e naqueles com catarata madura (n=8) e hipermatura (n=8). Correlações entre ambas as citocinas, entre as citocinas e a pressão intraocular (PIO), assim como entre as citocinas e a idade dos pacientes dentro de cada grupo foram averiguadas. Nos pacientes com catarata, o humor aquoso foi colhido ao final da eletrorretinografia. Nos cães saudáveis, o humor aquoso foi colhido antes do início de cirurgias eletivas. Os níveis das citocinas foram determinados por ELISA e a PIO por tonometria de aplanação. A PIO dos pacientes com catarata madura e hipermadura foram mais baixas que aquelas dos pacientes controle (P=0.158). A concentração de MMP-2 no humor aquoso foi maior nos pacientes com catarata hipermtura, comparativamente aos pacientes saudáveis (P=0.04). A concentração de IL-1 β no humor aquoso foi mais elevada

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nos cães com catarata (P<0.0001). Nos pacientes com catarata hipermatura, os valores de IL-1 β foram significativamente mais altos que aqueles dosados nos pacientes com catarata madura (P=0.0085). Correlações entre MMP-2 e IL-1 β (r=-0.38, P=0.06), MMP-2 e PIO (r=-0.149, P=0.484) e IL-1 β e as idades dos pacientes não foram observadas (P>0.05). A IL-1 β se correlacionou negativamente com a PIO (r=-0.42, P=0.04). Correlação entre MMP-2 e a idades dos pacientes foi observada apenas nos cães com catarata madura (r=0.772, P=0.02). Conclui-se que o aumento na concentração de IL-1 β no humor aquoso de cães com catarata madura e hipermatura, associado à correlação negativa entre essa citocina e a PIO, sugerem que a mesma está envolvida na patogênese da uveíte induzida pela lente. Apesar dos cães com catarata hipermadura apresentarem concentrações significativamente maiores de MMP-2 no humor aquoso, essa citocina não se correlacionou com a IL-1 β e a PIO. Em nosso estudo, correlação entre as citocinas dosadas no humor aquoso e a idade dos pacientes foi confirmada apenas entre MMP-2 e a idade dos cães com catarata madura.

TERMOS DE INDEXAÇÃO: Metaloprotease-2, interleucina-1 β, cães, olhos cataratoso, uveíte induzida pela lente, citocinas inflamatórias, fibrose capsular, lente.

INTRODUCTION

Cataract is defined the opacity of the eye lens, and is considered to be the leading cause of blindness in dogs. The affection can be classified based on the age of onset (congenital, juvenile, or senile), position in the lens (capsular, cortical, nuclear, axial, equatorial, or total), degree and stage of opacification (incipient, immature, mature, or hypermature), and the possible cause (inherited, traumatic, metabolic, or toxic) (Davidson & Nelms 2013, Renzo et al. 2014).

As the pathologic alterations associated with cataract progresses, hydrolytic and proteolytic enzyme activity increases within the lens. This process results in rupture of cell membranes, loss of low molecular weight proteins, and an increase in water content. Further degradation of proteins into amino acids and polypeptides allows small products of proteolysis to diffuse from the lens (Davidson & Nelms 2013). The loss of water and nitrogenous materials may cause the lens to shrink as the cataract progresses to the hypermature stage (Davidson & Nelms 2013). Such diffusion of lens material into the aqueous humor is responsible for the inflammation of the iris and the ciliary body. An immune-mediated inflammatory response against lens antigens occurs after deviation of the normal low level of T-cell-mediated tolerance to lens proteins (Van der Woerdt 2000, Davidson & Nelms 2013). This process is called lens-induced uveitis (LIU), which is considered to be the most frequent type of anterior uveitis in dogs. LIU has been associated with all stages of cataract maturity in this species (Van der Woerdt 2000, Renzo et al. 2014). Dogs with LIU, as those with uveitis due to other causes, present with lower intraocular pressure (IOP) because of the increased amounts of prostaglandins secreted by the inflammatory cells (Leasure et al. 2001, Renzo et al. 2014).

Pro-inflammatory cytokines, such as interleukin-1 alpha (IL-1α) and interleukin-1 beta (IL-1 β), have been shown to actively recruit leukocytes to sites of inflammation. Both IL-1 α and β are predominantly secreted by activated macrophages, B lymphocytes, and endothelial cells (Chiou et al. 1994, Medeiros et al. 2008, Ortencio et al. 2015). In animals and humans, IL-1 β seems to play a role in the pathogenesis of uveitis (Chiou et al. 1994, Nishi et al. 1994, El-Shabrawiet al. 2000, Medeiros et al. 2008, Ortencio et al. 2015, Wang et al. 2016, Aketa et al. 2017). One study demonstrated that the concentration of IL-1 β in the aqueous humor is correlated with the degree of iris damage in human patients who have undergone laser iridotomies and intraocular surgical procedures (Aketa et al. 2017). Another study reported that the concentration of IL-1 β in the aqueous humor was significantly increased in humans who have undergone femtosecond laser assisted cataract surgery, in comparison with the ones operated without laser assistance (Wang et al. 2016). With regard to veterinary ophthalmology, no studies have focused on quantifying the level of this cytokine in the aqueous humor of dogs with uveitis.

Matrix metalloproteinases (MMPs) are proteolytic enzymes capable of degrading the extracellular matrix (ECM) that is present in almost every tissue in the body. Such enzymes are involved in many ocular physiological processes, including embryogenesis, angiogenesis, and wound healing (Di Girolamo et al. 1996, Hodgkinson et al. 2007, Weinstein et al. 2007, Ribeiro et al. 2012, Jia et al. 2014). These enzymes are also up-regulated in several pathologic ocular disorders, including cataracts (Tamiya et al. 2000, West-Mays 2007, Alapure et al. 2012, Eldred et al. 2012). MMPs are capable of denaturing most components of the lens capsule ECM; hence, they can facilitate lens fiber migration and differentiation into myofibroblasts, which are responsible for the contraction and opacification of the anterior and posterior capsule of the lens (West-Mays 2007, Eldred et al. 2012). Higher activity of MMP-2 and MMP-9 was detected in both the lens epithelial cells and the serum of human patients with steroid-induced posterior capsular cataract (Alapure et al. 2012). In normal human lenses, gene expression (Hodgkinson et al. 2007) and concentration (Tamiya et al. 2000, Sachdev et al. 2004) of MMP-2 and -9 are very low, whereas the expression of tissue inhibitor of MMPs (TIMPs) is high. Even so, MMPs can be detected in the aqueous humor of humans with and without cataracts (Di Girolamo et al. 1996).

In humans with uveitis of different causes and in rabbits with experimentally induced anterior uveitis, concentrations of MMPs in the aqueous humor are elevated in comparison to controls (Di Girolamo et al. 1996, Määtää et al. 2006, Bauer et al. 2018). Weinstein et al. (2007) showed that only MMP-2 in its latent form was present in the aqueous humor of healthy dogs. One study showed there were no expressions of MMP-3 and -9 in the aqueous humor of dogs, before and after experimentally-induced uveitis (Pinard et al. 2011).

Identifying possible chemical mediators involved in the pathogenesis of anterior uveitis and LIU is important because could have direct therapeutic implications, and the knowledge can be used to minimize the potential vision-threatening sequelae that result from intraocular inflammation (Chiou et al. 1994, Pinard et al. 2011). Aqueous humor concentrations of prostaglandin E_2 (PGE_2), tumor necrosis factor-α, cicloxigenase-2, nitric oxide synthase, nitrites, nitrates, MMP-3 and -9 have been studied in dogs subjected to experimental breakdown of the blood-aqueous barrier (Ribeiro et al. 2010, Pinard et al. 2011, Gilmour et al. 2012). Nonetheless, to date, the only cytokines dosed in the aqueous humor of dogs with cataracts were the

vascular endothelial growth factor and PGE_2 (Abrams et al. 2011, Sandberg et al. 2012, Renzo et al. 2014). Therefore, we aimed to evaluate the concentrations of MMP-2 and IL-1 β in the aqueous humor of healthy dogs and those with cataracts at different stages. In addition, correlations between MMP-2 and IL-1 β, and between both cytokines and IOP, as well as both cytokines and the ages of patients were assessed.

MATERIALS AND METHODS

Approval from the "Universidade Federal do Mato Grosso", Ethics Committee for the Use of Animals in Research, as well as the consent from the patient owners were obtained prior to the experiment (23108.084384/2015-05). Male and female dogs of different breeds, with ages ranging from 6 to 13 years, which were referred to the Veterinary Ophthalmology Service of the University of Mato Grosso for cataract surgery, were enrolled in the study.

Patients underwent a full ophthalmological evaluation, which include Schirmer's test (Schirmer's Tear Test Strips, Ophthalmos, São Paulo, Brazil), biomicroscopy (XL-1 Slitlamp, Shin-Nippon, Japan), digital applanation tonometry (Tono Pen XL; Medtronic, Jacksonville/FL, USA), indirect binocular ophthalmoscopy (Oftalmoscopio binocular indireto FOH-5 - Eyetec S.A, São Carlos/SP, Brazil), fluorescein dye test (Fluorescein strips; Ophthalmos Ltda, São Paulo/SP, Brazil), ultrasound imaging (Ultra Scan, Alcon, Irvine/CA, USA), and flash electroretinography (Handheld Multi-species Electro- RetinoGraph; RetVetCorp, Columbia/MO, USA).

The criteria used to classify the stage of cataract maturity were described in the veterinary literature (Davidson & Nelms 2013). Only individuals with no ocular abnormalities other than mild LIU (no flare, mild to moderate conjunctival hyperemia and IOP ≤12mmHg), with mature (n=8) or hypermature (n=8) senile cataract, which were not under local or systemic steroidal or nonsteroidal anti-inflammatory therapy, were selected. Dogs with diabetic cataracts were not included in this study. A control group (n=8) composed of healthy dogs subjected to elective ovariohysterectomy (n=5) or orchiectomy (n=3), with ages ranging from 8 to 12 years, were also included. Dogs were included in the control group if no cataracts or other abnormalities were detected on ophthalmological evaluation. In addition, to be selected for the study, both healthy and cataractous dogs should have heart and respiratory rates, capillary refill time, temperature, skin turgor, bloodwork, and basic chemistry profile within the reference range for the species.

Anterior chamber paracentesis was performed under general anesthesia as previously described (Ribeiro et al. 2010, Gilmour et al. 2012) and 0.2mL of aqueous humor was slowly aspirated. In patients with cataract, the aqueous humor was collect at the end of the eletroretinographic examination. The aqueous humor of dogs was not sampled in dogs that had abnormal ERG readings. In control healthy patients, aqueous humor was collected before the beginning of the aforementioned procedures. In all groups, propofol (10mg/kg) was injected intravenously to effect for anesthetic induction (Propovan®, Cristália, São Carlos/SP, Brazil). In control healthy patients, isoflurane (1.5V%) was used for maintenance of anesthesia (Isofluorane; BioChimico, Itatiaia, Rio de Janeiro/RJ, Brazil), and analgesic drugs were administered as deemed necessary by the anesthesiologist after the aqueous humor had been aspirated. After paracentesis, the eyes of patients of each group were treated with one drop of the following medications: 1% atropine (q 12h), 0.3% tobramycine (q 6h), and 1% prednisolone (q 6h), for 48 hours. The aqueous samples from all dogs were transferred to microtubes that were labeled (Tubos criogênicos, 2mL, Adamo, Brazil), and frozen at -80°C until cytokine quantitation was performed.

In all groups, the IOPs that were considered for statistical analyses were measured in the morning of the day the aqueous humor was collected. We believe that this strategy minimizes the influences of diurnal fluctuation of IOP and obtained measurements that closely reflect this parameter at the time of aqueous humor sampling.

Concentrations of MMP-2 and IL-1 β in the aqueous humor were measured using a sandwich ELISA by means of paired cytokine-specific monoclonal antibodies to canine total MMP-2 (Quantikine® ELISA form total MMP-2 Immunoassay, RnDSystems, USA) and IL-1β (Canine Interleukin-1 β ELISA Kit®, Sigma Aldrich/Merck, Germany) according to the manufacturer's instructions. Known concentrations of recombinant MMP-2 and IL-1 β were added to each reaction, and the absorbance at 450 nanometers was read. The lower detection limits of the assays for MMP-2 and IL-1 β were 0.033ng/mL and 1pg/mL, respectively. Absorbance values read for MMP-2 and IL-1 β were converted to ng/mL and pg/mL, respectively; the estimated results were obtained from four-parameter logistic curves (http://www.myassays.com). None of the tested samples was thawed more than once.

Statistical analysis was performed using Prism version 7.04 (GraphPad Software, Inc., La Jolla, California, USA). Data were tested for normal distribution using the Shapiro-Wilk test. The concentrations of MMP-2 in the aqueous humor of healthy and cataractous eyes were compared using the Kruskal-Wallis test, followed by the Dunn test for multiple comparisons. Comparisons between healthy and cataractous eyes with respect to the concentration of IL-1 β in the aqueous humor were performed using the one way analysis of variance (ANOVA), followed by the Bonferroni test for multiple comparisons. ANOVA was used to assess differences in IOP between healthy and cataractous dogs; the same test was employed to assess differences between the ages. The Spearman´s test was employed to assess possible correlations between the concentrations of MMP-2 and IL-1 β, and between MMP-2 and IOP. The Person´s test was used to verify possible correlations between IL-1 β and IOP, and between cytokines and the ages of patients. On all occasions, a P value < 0.05 was considered statistically significant. Results are shown as mean ± standard deviation (SD) for parametric values, and as median and interquartile range for non-parametric values.

RESULTS

The ages of healthy controls, and those with mature and hypermature cataracts, was 9.75±2.43, 7.65±1.76, and 9.37±2.50 years, respectively (P=0.158). Breeds of selected dogs with cataracts were Poodle (n=8), Labrador (n=1), mixed breed (n=2), Pincher (n=3), Lhasa Apso (n=1), and Maltese (n=1). The breeds of healthy controls were York Shire (n=2), mixed breed (n=3), Shih Tzu (n=2), and Pincher (n=1). The IOP of control healthy dogs was 13.50±3.25mmHg. The IOP of patients with mature (-1.00mmHg) and hypermature (-2.87mmHg) cataracts were lower than the ones measured in healthy controls (Table 1). However, significant differences were not observed among the groups (P=0.158).

Median concentration of MMP-2 in the aqueous humor of healthy dogs was 1.67 (0.49-3.92) ng/mL. Median concentration of MMP-2 in the aqueous humor was 0.15 and 1.39ng/mL higher in patients with mature and hypermature cataracts, respectively, compared to healthy controls (Table 1, Fig.1). However, significant differences occurred only between healthy patients and those with hypermature cataracts (P=0.04) (Table 1, Fig.1).

The mean concentration of IL-1 β in the aqueous humor of healthy dogs was 5.95±0.02pg/mL. The concentration of IL-1β was significantly higher in patients with mature and hypermature cataracts than in healthy controls (P<0.0001) (Table 1, Fig.2). Significantly higher IL-1β concentrations were observed in patients with hypermature cataract, than in those with mature cataracts (P=0.0085) (Table 1, Fig.2).

The concentration of IL-1β did not correlate with the ages of dogs in any studied group (P>0.05). Regarding MMP-2, such cytokine also did not correlate with age in controls, and in dogs with hypermature cataracts (P>0.05). However, positive correlation was observed between MMP-2 and the ages of dogs with mature cataracts (r=0.772, P=0.02). Although there was a tendency toward a negative correlation between the concentrations of MMP-2 and IL-1 β (r=-0.38), statistical significance was not reached (P=0.06) (Fig.3). MMP-2 and IOP also did not correlate to each other (r=-0.149, P=0.484). However, IL-1 β and IOP correlated negatively (r=-0.42, P=0.04). (Fig.4)

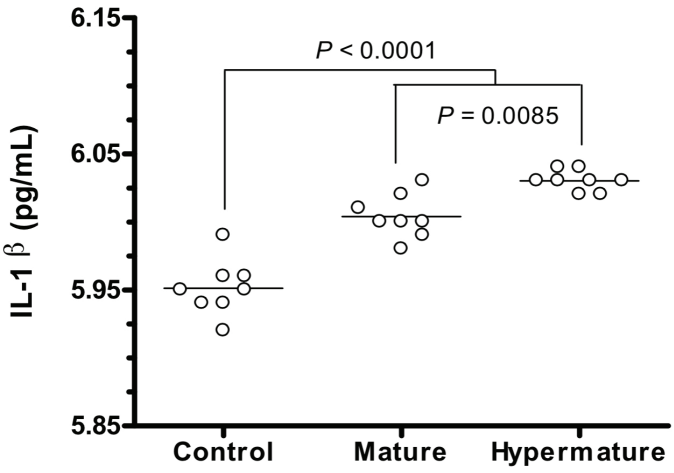

Fig.2. Mean (line) and individual dispersion (circles) of interleukin 1 β (IL-1 β) concentrations in the aqueous humor in studied dogs.

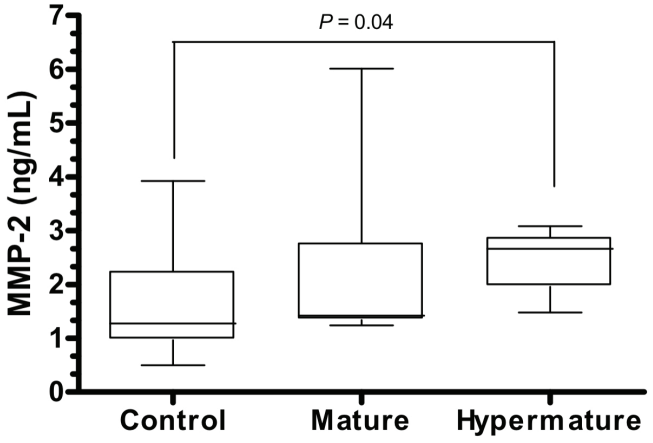

Fig.1. Box-and-whisker plots of matrix metalloproteinase-2 (MMP-2) concentrations in the aqueous humor in studied dogs. Each box represents the first and third quartiles, the horizontal line in each box represents the median value, and the whiskers represent the smallest and largest sample value within 1.5 times the interquartile range.

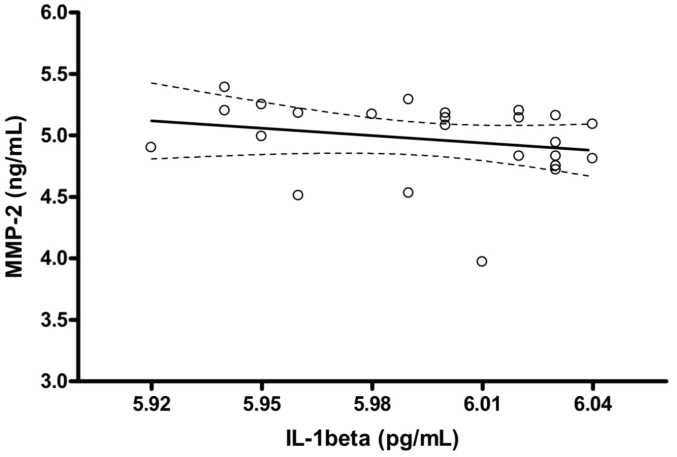

Fig.3. Linear regression curve between matrix metalloproteinase-2 (MMP-2) and interleukin 1 β (IL-1 β) concentrations in the aqueous humor in the studied dogs. Sperman´s correlation test (r=-0.38, P=0.06)

Table 1. Descriptive statistics of intraocular pressure (IOP), aqueous humor matrix metalloproteinase-2 (MMP2), and interleukin-1 β (IL-1 β) concentrations in the aqueous humor of dogs with normal eyes, and in those with mature and hypermature cataracts

	Healthy	Mature	Hypermature
IOP (mmHg)			
Mean ± SD	13.50±3.25	12.50±1.92	10.63±3.33
Coefficient of variation	24.08%	15.42%	31.39%
MMP-2 (ng/mL)			
Median (range)	1.27 (0.49-3.92)	1.42 (1.23-6.01)	2.66 (1.47-3.08)*
Coefficient of variation	65.77%	75.81%	24.31%
IL-1 β (pg/mL)			
Mean ± SD	5.95±0.02	6.00±0.01a	6.03±0.00aA
Coefficient of variation	0.34%	0.27%	0.13%

* Indicate significant difference between dogs with hypermature cataracts and healthy controls (P=0.04); a = lowercase letters indicate significant differences among cataractous and healthy dogs (P<0.0001), A = uppercase letters indicate significant difference between dogs with hypermature and mature cataracts (P=0.0085).

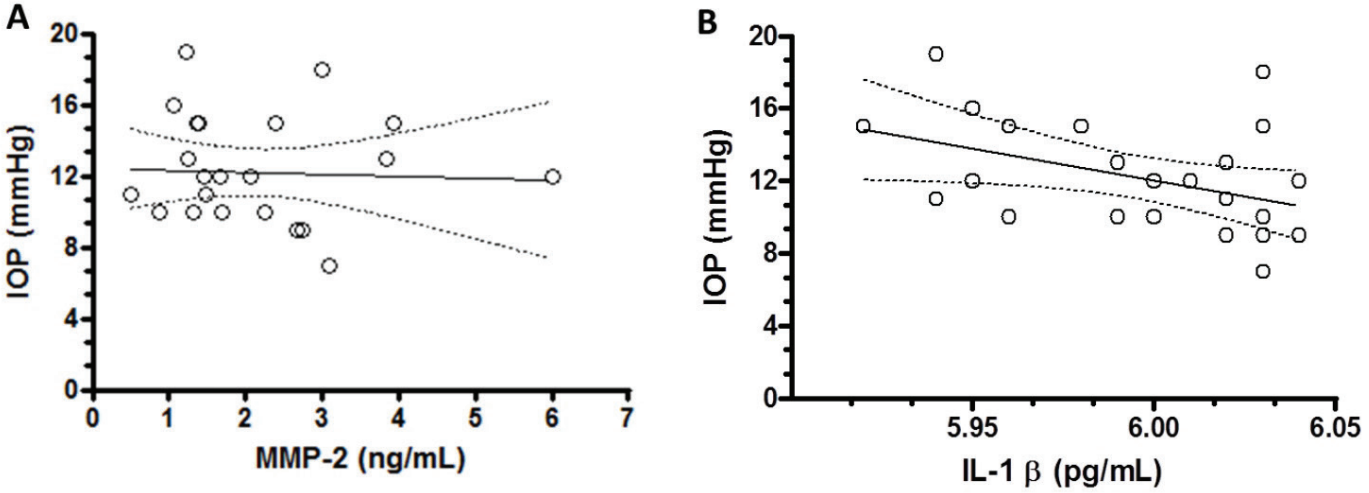

Fig.4. Linear regression curves between both cytokines dosed in the aqueous humor and intraocular pressure in the studied dogs. (**A**) Sperman´s correlation test (r=-0.38, P=0.06). (**B**) Person´s correlation test (r=-0.42, P=0.04).

DISCUSSION

Only two previous studies have focused on studying the relationship between cataract maturity and IOP (Leasure et al. 2001, Renzo et al. 2014). Leasure et al. (2001) reported that IOP tends to decrease as the cataracts progresses; however, significant results were only observed between dogs with immature and hypermature stages (Leasure et al. 2001). Renzo et al. (2014) also encountered lower IOP values in dogs with mature and hypermature cataracts in comparison to controls, with results that also did not reach statistical significance. Similarly, the dogs in our study showed the same tendency of lower IOPs in advanced stages of cataract maturity, but this tendency was not statistically significant.

This was the first study to evaluate the concentration of MMP-2 in the aqueous humor of dogs with cataracts. The decision to investigate MMP-2 was based on previous studies that reported that the latent form of such gelatinase was found to be the most relevant MMP in normal eyes of humans, rabbits, dogs, and chicken (Di Girolamo et al. 1996, Weinstein et al. 2007, Shimada et al. 2014). Although slightly increased concentrations of MMP-2 were observed in dogs with mature cataracts than in controls, significantly higher values were only detected in patients with the hypermature stage of this affection. In the current study we quantified the concentration of MMP-2 in the aqueous humor using ELISA sensitive for latent and active MMP-2, as well as MMP-2 complexed with TIMP-2. Therefore, comparisons of our results with those reported in a previous study performed on the eyes of normal and glaucomatous dogs cannot be made because MMPs were quantified using zymography (Weinstein et al. 2007). In the only study that compared healthy humans and those with uveitis, the researchers used zymography to quantify the concentration of MMPs in the aqueous humor (Di Girolamo et al. 1996).

Määtää et al. (2006) and Kara et al. (2014) used a similar ELISA assay that we used in our study (total MMP-2); in patients with cataracts, these authors reported that the average concentration of MMP-2 in the aqueous humor was 25ng/mL (Määtää et al. 2006, Kara et al. 2014). This was much higher than the concentrations observed in dogs with mature (1.42ng/mL) and hypermature (2.44ng/mL) cataracts in our study. Bauer et al. (2018) used multiplex bead immunoassay to quantify the concentration of MMP-2 in the aqueous humor of humans with cataracts and reported values that ranged from 0.25 to 0.75ng/mL; whereas in patients with juvenile idiopathic arthritis-associated anterior uveitis, median levels of such cytokine in the aqueous humor increased to 0.90ng/mL (0.30-1.60ng/mL). Reasonable explanations for the discrepancy between the results observed in humans studies with the ones found in our research can be related to interspecies variability, interassay variability, and for how long aqueous humor was stored. In addition, most of the studies conducted in humans focusing on the concentration of intraocular MMPs (El-Shabrawi et al. 2000, Määtää et al. 2006, Alapure et al. 2012, Bauer et al. 2018) and IL-1 β (Takay et al. 2012, Duvesh et al. 2017, Zheng et al. 2018) used individuals with senile cataracts as controls. In these studies, however, researches did not report the stages of the patients´ cataracts. Therefore, direct comparisons of our results with the ones obtained in human studies with regard to MMP-2 concentration and the stage of cataract maturity cannot be made.

MMPs present in the iridocorneal angle and in the aqueous humor play a role in controlling IOP by increasing the ECM turnover rate, which reduces the resistance to aqueous outflow through the meshwork (Bradley et al. 2001, Weinstein et al. 2007). Several studies have shown that the concentration of MMP-2 in the aqueous humor was significantly increased in human patients with uveitis of different causes (Di Girolamo et al. 1996, El-Shabrawi et al. 2000, Määtää et al. 2006, Alapure et al. 2012, Bauer et al. 2018). However, researchers in all aforementioned studies did not assess the relationship between the concentration of MMP-2 and IOP (El-Shabrawi et al. 2000, Määtää et al. 2006, Alapure et al. 2012). Only Määtää et al. (2006) reported that the concentration of MMP-2 in the aqueous humor did not correlate with the IOP in patients with cataracts, primary open-angle glaucoma and secondary glaucomas associated with uveitis. Although there was a tendency toward a negative correlation between the concentration of MMP-2 and IOP in our study, such parameters did not correlate significantly.

It has been reported that the immunoreactivity for MMP-2 in normal human lenses is weak and localized within the lens epithelium and intracellularly, within a few early differentiating fibers at the lens cortex (Sachdev et al. 2004). In the same study, no staining for MMP-2 was observed in cataractous lenses, while zymograms and ELISA assays revealed increased levels of this cytokine (Sachdev et al. 2004). MMPs are capable of denaturing most components of the lens capsule ECM facilitating lens fiber migration and differentiation into miofibroblasts, which are responsible for the contraction and opacification of the anterior and posterior capsule of the lens (West-Mays 2007, Eldred et al. 2012). These are the reasons because hypermature cataracts will have evidence of resorption, subcapsular plaques, lens-induced uveitis, and capsular wrinkling (Davidson & Nelms 2013). This can explain our findings with regard to higher levels of MMP-2 in the aqueous humor of dogs with hypermature cataracts. Although the degrees of anterior and posterior capsular opacification were not quantified in the dogs of our experiment, one can assume that the higher aqueous humor levels of MMP-2 detected in patients with hypermature cataracts can be associated with degradation of lens epithelium cells, as well anterior and posterior capsular fibrosis. Thus, further studies can be developed to evaluate the role of MMP-2 inhibitors in early stages of cataract maturity in order to prevent capsular fibrosis, which makes capsulorhexis harder to perform.

In our study, the concentration of IL-1 β in the aqueous humor varied very little among healthy controls and dogs with mature and hypermature cataracts. However, the concentration IL-1 β increased significantly in accordance with the degree of lens opacification. Contrary to our findings, it has been reported that the concentration of IL-1 β in the aqueous humor of humans with cataracts at stage II and III did not differ significantly; however, patients of that study received anti-inflammatory eye drops before phacoemulsification (Wang et al. 2016). Chiou et al. (1994) observed that the intraocular inflammation induced by lens proteins can be prevented with the use of interleukin-1 blockers; our results are consistent with these findings. There are no previous reports describing the concentration of IL-1 β in in the aqueous humor of dogs, cats, and horses. In rats, the concentration of IL-1 β in the aqueous humor quantified using ELISA was approximately 100pg/mL (Medeiros et al. 2008). In humans with cataracts, Wang et al. (2016) used ELISA to quantify the concentration IL-1β in the aqueous humor and reported concentrations that ranged from 14.9 to 19.4pg/mL; which were higher than the ones reported here for mature (6.00) and hypermature (6.03pg/mL) cataracts. In another studies that were also conducted in humans with cataracts in which multiplex bead immunoassay was used to quantify IL-1 β, much lower values of this cytokine were reported (0.002 to 1.0pg/mL) (Takay et al. 2012, Duvesh et al. 2017). Quantification of IL-1 β in the aqueous humor of cataractous human eyes assessed by cytometric bead array revealed concentrations that ranged from 2.7±0.0 to 5.29±0.9pg/mL (Aketa et al. 2017, Duvesh et al. 2017); which were closer to our results.

In dogs, LIU is most commonly associated with the presence of a hypermature cataract (Leasure et al. 2001, David & Nelms 2013, Renzo et al. 2014). Like in other causes of uveitis, dogs with LIU present lower IOPs because of the increased amounts of prostaglandins secreted by the inflammatory cells (David & Nelms 2013, Renzo et al. 2014). In one study, despite increased aqueous humor concentration of PGE_2 has been found in dogs with mature and hypermature cataracts, the levels of such cytokine did not correlate with IOP values (Renzo et al. 2014). In humans, the concentration of IL-1 β also did not correlate with IOP in subjects affected by cataracts, primary open-angle glaucoma and exfoliative syndrome (Takay et al. 2012). In dogs of our study, the concentration of IL-1 β correlated negatively with IOP, suggesting that this cytokine is involved in the pathogenesis of LIU. Therefore, further studies aiming to investigate the effects of anti-inflammatories to control of LIU should include IL-1 β as an inflammatory marker.

Aging is associated with the development of parainflammation, a term which describes a beneficial and controlled low level of inflammation in response to accumulating tissue stress such as oxidative and chemical stress (Zheng et al. 2018). Aqueous humor is an important immunological milieu of the anterior segment (Zheng et al. 2018). Therefore, we consider to asses possible correlations between the ages of our dogs with the cytokines dosed in the aqueous humor. It has been reported that the concentration of IL-1 β in the aqueous humor of children and adults affected by cataracts are similar, and that such cytokine did not correlate with the age of patients (Zheng et al. 2018). Herein, we also did not observe any correlations between IL-1 β and the ages of dogs in any studied group. In one report, MMP-2 dosed in the aqueous humor did not correlate with the ages of humans patients with cataracts and uveitis (Bauer et al. 2018). Similarly, significant correlations were not found between the concentration of this cytokine and the ages of dogs with normal eyes, and those with hypermature cataracts. In the ones with mature cataracts, however, MMP-2 correlated positively with age.

Although our findings showed that the concentrations of MMP-2 and IL-1 β were increased in parallel with the cataract stage, a relationship between these two cytokines was not encountered. It has been reported that the levels of MMP-2, MMP-9, IL-1 β, and IL-12 are positively correlated with the severity of uveitis in humans (Di Girolamo et al. 1996, El-Shabrawi et al. 2000, Hodgkinson et al. 2007, Aketa et al. 2017). Notwithstanding, we did not investigate possible correlations between the uveitis score and the other parameters evaluated because all enrolled patients had mild uveitis.

CONCLUSIONS

The increment in the aqueous humor concentration of IL-1 β found in patients with mature and hypermature cataracts, in addition to the negative relationship of this cytokine with IOP, suggests that IL-1 β is involved in the pathogenesis of LIU in dogs.

Although dogs with hypermature cataracts presented higher concentrations of MMP-2 in the aqueous humor, such cytokine did not correlate with IL-1 β and IOP.

In our study, a relationship between aqueous humor cytokines and the ages of patients was only confirmed between MMP-2 and the ages of dogs with mature cataracts.

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

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Original Article
Small Animal Diseases

Evaluation of left atrial function in asymptomatic dogs with chronic myxomatous mitral valve disease: two-dimensional feature-tracking echocardiography and Simpson's monoplanar methods[1]

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ABSTRACT.- Coelho M.R., Muzzi R.A.L., Dorneles E.M.S., Pereira G.G., Cabral R.M., Muzzi L.A.L., Abreu C.B. & Oliveira L.E.D. 2020. **Evaluation of left atrial function in asymptomatic dogs with chronic myxomatous mitral valve disease: two-dimensional feature-tracking echocardiography and Simpson's monoplanar methods.** *Pesquisa Veterinária Brasileira 40(3):188-196.* Departamento de Medicina Veterinária, Universidade Federal de Lavras, Av. Doutor Sylvio Menicucci 1001, Cx. Postal 3037, Lavras, MG 37200-000, Brazil. E-mail: marianacoelhorc@gmail.com

The present study evaluated the volume and function of the left atrium by two-dimensional echocardiographic feature-tracking imaging (2D-FTI) and Simpson's monoplanar modeling in dogs with asymptomatic degenerative mitral valve disease (DMVD). The study consisted of 80 dogs that were divided into the following three groups: Group 1, 21 dogs (A); Group 2, 30 dogs (B1) and Group 3, 29 dogs (B2). The variable strain (contraction phase) was significantly lower in Group 3 than in Group 1 (12.92±4.54 x 16.69±5.74, p=0.014), and significant differences in the contraction strain index (CSI) were observed between all of the groups that were evaluated (1 = 46.82±8.10, 2 = 39.88±8.03, 3 = 35.25±5.64, p<0.0001). The atrial diastolic volume index (AdVi) that was measured by 2D-FTI was significantly higher in Group 3 than in Group 1 (1.31±0.95 x 0.96±0.31, p=0.038), and the atrial cardiac index (ACI) was also higher in Group 3 than in Group 1 (102.38±80.18 x 78.19±33.38, p=0.030). Atrial function was assessed by Simpson's monoplanar method, which demonstrated an increase in the left atrial systolic volume, while the contractile function decreased with an increasing disease severity (Group 1 0.21±0.06; Group 2 0.25±0.06; Group 3 0.32±0.08, p<0.0001). The intraobserver and interobserver assessments showed low to moderate variability; most of the values for the coefficient of variation for the variables that were analysed with each method were below 25%. Thus, DMVD was determined to cause an alteration in atrial function, especially in the contraction phase, and even in asymptomatic animals, and the methods of 2D-FTI echocardiography and Simpson's monoplanar evaluation are sensitive and early methods for the detection of left atrial dysfunction.

INDEX TERMS: Evaluation, left atrial, asymptomatic dogs, myxomatous degeneration, mitral valve, echocardiographic, bidimensional tracking, left atrial volume, atrial strain, strain rate.

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RESUMO.- [**Avaliação da função atrial esquerda em cães assintomáticos com degeneração mixomatosa crônica de valva mitral: métodos ecocardiográficos bidimensional feature tracking e monoplanar de Simpson**]. O presente estudo avaliou o volume e a função atrial esquerda obtidos por meio da ecocardiografia bidimensional *feature tracking*

(2D-FTI) e pelo método monoplanar de Simpson em cães saudáveis e cães com DMVD assintomáticos. Foram avaliados 80 cães distribuídos em três grupos: Grupo 1, 21 cães (classe A); Grupo 2, 30 cães (classe B1) e Grupo 3, 29 cães (classe B2). A variável strain (fase de contração) foi significativamente menor no Grupo 3 que no Grupo 1 (12,92±4,54 x 16,69±5,74, p=0,014) e para a variável índice de *strain* de contração (CSI), houve diferença estatística entre todos os grupos avaliados (1 = 46,82±8,10; 2 = 39,88±8,03; 3 = 35,25±5,64, p<0,0001). O índice de volume diastólico atrial (iVdA) mensurado por meio do 2D-FTI foi significativamente maior no Grupo 3 que no Grupo 1 (1,31±0,95 x 0,96±0,31, p=0,038), assim como para o índice cardíaco atrial (iCA) também foi maior no Grupo 3 (102,38±80,18 x 78,19±33,38, p=0,030). A função atrial avaliada pelo método monoplanar de Simpson demonstrou um aumento do volume atrial esquerdo e do volume sistólico do átrio esquerdo, enquanto que a função contrátil diminuiu com o aumento da gravidade da doença (Grupo 1 0,21±0,06; Grupo 2 0,25±0,06; Grupo 3 0,32±0,08; p<0,0001). A avaliação intraobservador e interobservador, demonstrou variabilidade baixa a moderada, uma vez que a maioria dos valores de coeficiente de variação se concentraram abaixo de 25% para as variáveis analisadas em ambos os métodos. Dessa forma, conclui-se que a DMVD causa alteração na função atrial, principalmente na fase de contração, mesmo em animais assintomáticos e que a ecocardiografia 2D-FTI e o método monoplanar de Simpson são métodos sensíveis e precoces na detecção da disfunção atrial esquerda.

TERMOS DE INDEXAÇÃO: Avaliação, átrio esquerdo, cães assintomáticos, mixomatosa crônica, valva mitral, ecocardiográficos, bidimensional *feature tracking,* volume atrial esquerdo, Strain e Strain Rate atriais.

INTRODUCTION

Degenerative mitral valve disease (DMVD) is the most common heart disease in small dogs and causes mitral regurgitation (MR) and myocardial dysfunction. The progression of the disease occurs slowly, and its morbidity is directly related to the magnitude of valve insufficiency, volume overload and severity of atrial remodelling (Borgarelli & Buchanan 2012).

The left atrium (LA) controls ventricular filling via its three functions: as a reservoir, which occurs during ventricular contraction; as a conduit, which allows the blood to flow passively from the atrium to the left ventricle; and as a booster pump, which is involved in the contraction of the LA (Matsumoto et al. 2014).

The measurement of LA function is essential for cases of DMVD because LA remodelling is a physiological response to volume overload from MR (Fox 2012). However, quantifying LA deformation is challenging because of its complex anatomy, which includes the insertion of pulmonary veins, the presence of the atrial appendage, variable geometry, and small wall thickness (Hoit 2014).

These limitations can be overcome with new echocardiographic tools, including two-dimensional feature-tracking imaging (2D-FTI) echocardiography and the single-plane Simpson's method, which have all been used to assess atrial function. Nonetheless, few veterinary medicine studies to date have evaluated DMVD, demonstrating the need for additional investigation of this subject (Nakamura et al. 2017, Toaldo et al. 2017).

Therefore, the objectives of this study were (1) to determine the feasibility and reproducibility of 2D-FTI echocardiography in assessing LA function in dogs; (2) to compare the measurements of LA function and volume that were obtained from both 2D-FTI echocardiography and the single-plane Simpson's method between healthy dogs and asymptomatic dogs with DMVD; and (3) to correlate the variables that were obtained via 2D-FTI echocardiography to the left atrial to aortic root ratio (LA/Ao) measurements that were obtained by conventional echocardiography.

MATERIALS AND METHODS

Ethics statement. The data were obtained from the Cardiology Department of the Veterinary Hospital of the "Universidade Federal de Lavras", Lavras/MG, Brazil. The study was approved by the Animal Research Ethics Committee of our institution under Protocol No. 030/17.

Animals. A total of 1,160 echocardiographic examinations that were performed from February 2011 to July 2017 were evaluated, and images that were obtained from 80 dogs (*Canis lupus familiaris*) were selected for inclusion in the study. The animals were separated into three groups according to the classification proposed by Atkins et al. (2009) as follows: Group 1, class A animals (Control Group, breeds at risk of developing DMVD); Group 2, class B1 animals (presented with DMVD and MR without LA remodelling); Group 3, class B2 animals (presented with DMVD, MR, and cardiac remodelling - LA/Ao >1.5 and/or left ventricular diameter in normalised diastole >1.7 - without congestive heart failure). The study animals received a detailed physical examination, electrocardiography, echocardiography, and chest X-rays (when necessary, to exclude pulmonary edema). The inclusion criteria were a presence of systolic murmur in the mitral area, echocardiographic evidence of DMVD, and sufficient image quality to perform analyses using 2D-FTI echocardiography and the single-plane Simpson's method.

Conventional echocardiography. Examinations were performed using echocardiographic equipment (MyLab 40 Esaote®, Italy) with 4-10MHz multifrequency ultrasound transducers, an electronic sector scanning system, and simultaneous electrocardiographic recording on the monitor. All examinations were performed without sedation and with the animals gently restrained. The conventional echocardiographic variables that were measured included the LA diameter, aortic (Ao) diameter, and the left atrial/aortic root ratio (LA/Ao) in the parasternal short-axis view at the level of the great vessels. The conventional echocardiographic examinations were performed as recommended by Thomas et al. (1993). The measurements for the shortening fraction, LV diameter in normalised diastole (LVDd N), and LV diameter in normalised systole (LVDs N) were obtained in the M mode as previously described (Cornell et al. 2004). In pulsed Doppler mode, the E and A waves of the mitral flow, the E/A ratio, the isovolumic relaxation time (IVRT), the E/IVRT ratio, and the Em, Am and Em/Am variables by tissue Doppler were evaluated.

The atrial volume was measured in the three phases of the LA using a single-plane Simpson's method in the apical four-chamber view. LAEDV, LAESV, LA ejection fraction (LAEF), and LA cardiac output (LACO) were evaluated. The formulas that were used to measure volume in the three phases were as follows: i) reservoir (LAV_{max} - LAV_{min}/LAV_{max}); ii) conduit (LAV_{max} - LAV_{pre-a}/LAV_{max}); and iii) contraction (LAV_{pa} - LAV_{min}/LAV_{pre-a}) as described previously (Blume et al. 2011), where LAV is the left atrial volume and LAV_{pre-a} is preatrial contraction volume.

2D-FTI echocardiography. The indices representing atrial deformity and atrial myocardial velocity (strain and strain rate, respectively) were obtained by means of the 2D-FTI method (Fig.1) similar to the one described by Caivano et al. (2016). For this purpose, the two-dimensional images that were acquired in the left parasternal short-axis view and the apical four-chamber view were stored for off-line analysis using Xstrain software version 10.1 and the optical flow algorithm (ESAOTE®). Three consecutive cardiac cycles were analysed using continuous ECG monitoring, with a sampling rate between 50 and 100 frames/s.

The software automatically represented graphically and quantitatively atrial myocardial strain (St) and strain rates (StR) (Fig.2). It was obtained the mean value of the analyzed segments of strain (global St) in the reservoir, conduction and contraction phases, and to obtain the conduction phase the following calculation was used: conduction St = (reservoir St - contraction St). The mean StR of the three phases was automatically measured by the software. The contraction strain index (CSI), which represents the contribution of the active contraction of the LA to the LV filling phase, was calculated using two strain variables (Cameli et al. 2012) as follows: CS = (LAS contraction/LAS reservoir) × 100.

The diastolic volume, systolic volume, and cardiac output values were automatically determined by the 2D-FTI software. These parameters were correlated with body weight (HÖllmer et al. 2013) and were designated as the left atrial end-diastolic volume (LAEDV), left atrial end-systolic volume (LAESV), and cardiac index (CI). The LAEF was measured automatically by the 2D-FTI software.

Statistical analysis. The statistical analysis was performed using GraphPad Prism software version 5.0. The normality of the data distribution was assessed using the Shapiro-Wilk test. The data from different animal classes were analysed using one-way ANOVA and Tukey's post-hoc test. A two-tailed p-value smaller than 0.05 was used to define statistical significance. The intraclass correlation coefficient (ICC) and its 95% confidence interval were calculated using MedCalc® software version 16.8 to compare the values that were obtained from the two echocardiographic methods. The degree of correlation was defined as follows: ICC ≥0.75 excellent; 0.4≥ ICC <0.75 satisfactory to good; and ICC <0.4 poor (Fleiss 1981).

The intraobserver variability was obtained from 30% of the animals, and the interobserver variability was obtained from 60% of the animals; the animals were randomly assigned to each of these groups. The degree of variability was defined according to the

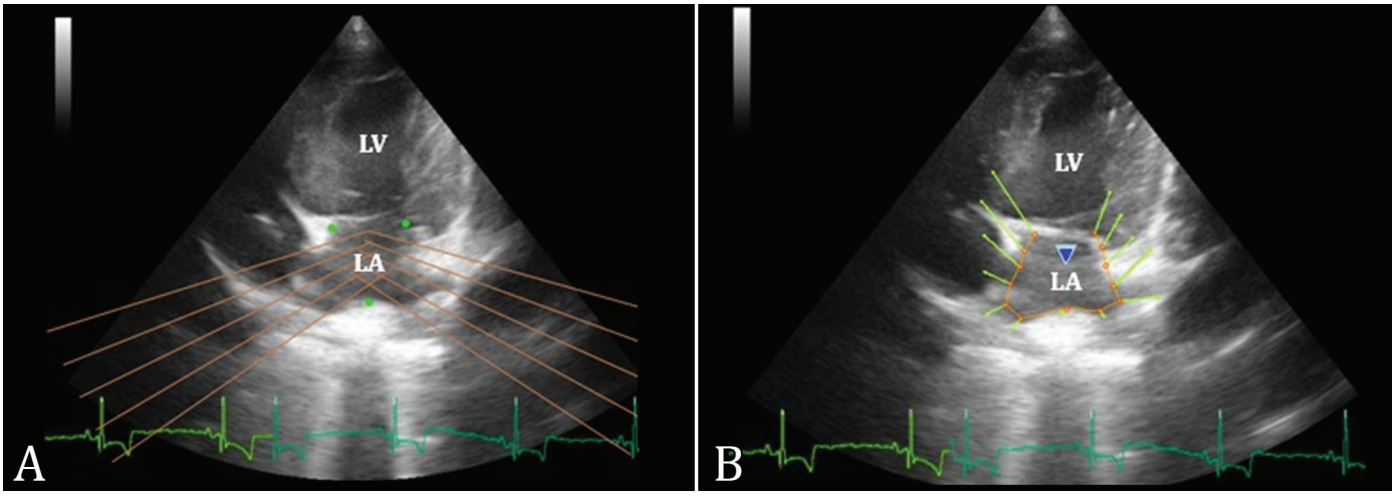

Fig.1. Two-dimensional echocardiographic image showing a semiautomatic tracing of the left atrium (LA) of a healthy dog (**A**) and velocity vectors (**B**) using 2D-FTI echocardiography.

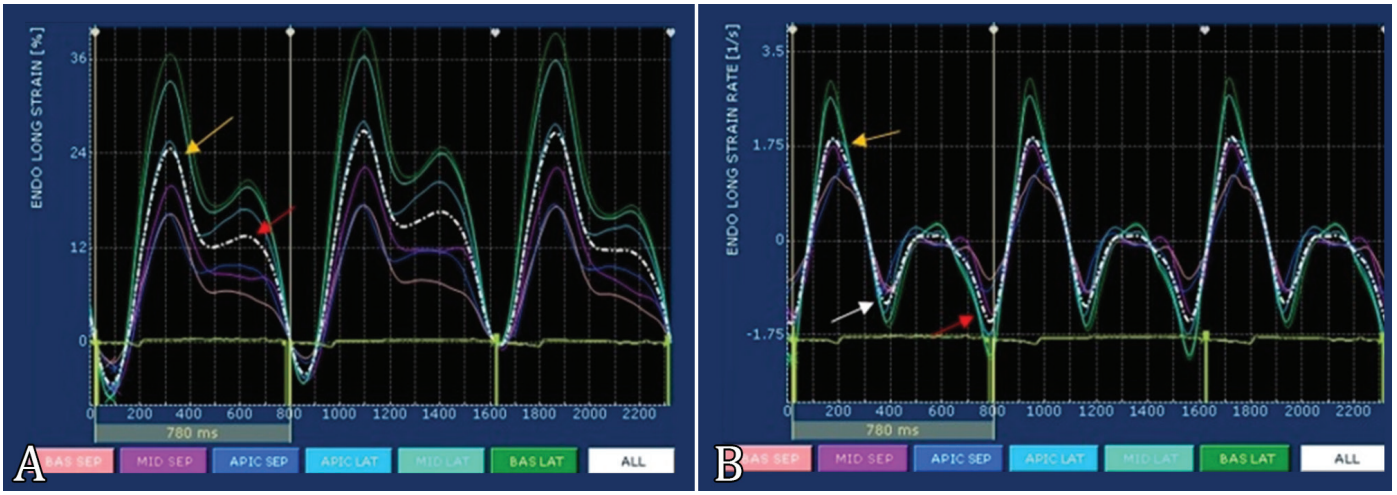

Fig.2. Graphical representation of atrial strain and strain rate in an 8-year-old 10-kg dog of Group 1 (class A). (**A**) Left atrial strain, (**B**) left atrial strain rate. Reservoir phase (yellow arrows), contraction phase (red arrows), conduction phase (white arrows).

coefficient of variation (CV) as follows: <15%, low; 15-25%, moderate; and >25%, high (Decloedt et al. 2011). The association between the variables that were derived from 2D-FTI echocardiography (LAEDV, LAESV, CI, CS, LAS, and LASR) and the LA/Ao was tested using a Pearson correlation analysis and was considered significant at p<0.05.

RESULTS

All of the dogs that were evaluated were of small to medium size and consisted of the following breeds: mixed breed (16), Poodle (14), Pinscher (10), Yorkshire (9), Beagle (8), Teckel (7), Maltese (4), Schnauzer (4), Shih Tzu (2), Cocker (2), Fox Terrier (1), Pug (1), Lhasa Apso (1) and Pekingese (1).

The data that were obtained from the conventional echocardiography methods and the clinical characteristics of the study sample are presented in Table 1. The variables for LA function and volume that were obtained by 2D-FTI echocardiography and the single-plane Simpson's method are shown in Table 2 and 3, respectively. For each animal, it was possible to obtain an adequate speckle tracking of the LA, with significant values for LA volume and LAS during contraction. With both the conventional and 2D-FTI echocardiography

Table 1. Clinical characteristics, mean and standard deviation of conventional echocardiography variables of dogs with DMVD at different stages

Variable	Group 1 - class A (n = 21)	Group 2 - class B1 (n = 30)	Group 3 - class B2 (n = 29)	Value p
Age (years)	8.5 ± 2.40	10.1 ± 2.63	11.3 ± 2.47	-
Sex (Male/Female)	6/14	13/17	11/18	-
Weight (kg)	6.24 ± 3.07	6.77 ± 3.30	7.93 ± 3.30	-
HR (bpm)	120 ± 26.80	113 ± 20.40	116 ± 20.11	-
LA/Ao	1.29^{a} ± 0.09	1.27^{a} ± 0.11	1.75^{b} ± 0.17	< 0.0001*
LVDd N (mm)	1.36^{a} ± 0.09	1.48^{a,b} ± 0.20	1.67^{b} ± 0.21	<0.0001*
LVDs N (mm)	0.82^{a} ± 0.09	0.94^{a,b} ± 0.14	0.97^{b} ± 0.19	0.002*
FS %	37.25 ± 4.72	35.25 ± 3.98	38.07 ± 5.53	0.074
E Wave (m/s)	0.66^{a,b} ± 0.17	0.58^{a} ± 0.13	0.72^{b} ± 0.16	0.003*
A Wave (m/s)	0.58 ± 0.13	0.55 ± 0.14	0.63 ± 0.19	0.153
E/A	1.18 ± 0.32	1.13 ± 0.39	1.20 ± 0.36	0.705
IVRT (ms)	74.86 ± 15.40	79.63 ± 12.02	79.38 ± 14.54	0.417
E/IVRT	0.93^{a,b} ± 0.33	0.75^{a} ± 0.22	0.96^{b} ± 0.36	0.025*
Em (m/s)	0.07 ± 0.02	0.06 ± 0.02	0.07 ± 0.02	0.123
Am (m/s)	0.08 ± 0.02	0.07 ± 0.02	0.08 ± 0.02	0.541
Em/Am	0.88 ± 0.33	0.92 ± 0.37	0.94 ± 0.29	0.848

DMVD - degenerative mitral valve disease., HR = heart rate, LA/Ao = relation between left atrium and aortic root, LVDd N = left ventricular diameter in diastole normalized by weight, LVDs N = left ventricular diameter in systole normalized by weight, FS = fractional shortening, IVRT = isovolumic relaxation time, E/IVRT = relation between E wave and isovolumic relaxation time, Em = early diastolic velocity of the septal mitral annulus, Am = late diastolic velocity of the septal mitral annulus; ^{a,b} different letters on the same line demonstrate statistical significance; * indicates significant difference between groups (P<0.05).

Table 2. Mean and standard deviation of 2D-FTI echocardiographic variables for left atrial function and volume of dogs with DMVD at different stages

Variable	Group 1 - class A (n = 21)	Group 2 - class B1 (n = 30)	Group 3 - class B2 (n = 29)	Value p
Strain (St)				
St reservoir	36.32 ± 12.58	36.14 ± 10.36	37.87 ± 16.18	0.866
St contraction	16.69^{a} ± 5.74	13.98^{a,b} ± 3.10	12.92^{b} ± 4.54	0.014*
St conduction	19.64 ± 7.79	22.16 ± 9.18	24.95 ± 12.00	0.081
Strain Rate (StR)				
StR reservoir	2.66 ± 0.81	2.62 ± 0.71	2.77 ± 0.97	0.763
StR contraction	1.75 ± 0.62	1.49 ± 0.74	1.36 ± 0.94	0.065
StR conduction	1.84 ± 0.68	2.01 ± 0.45	2.16 ± 0.60	0.366
CSI %	46.82^{a} ± 8.10	39.88^{b} ± 8.03	35.25^{c} ± 5.64	<0.0001*
LAdI (ml/kg)	0.96^{a,b} ± 0.31	0.89^{a} ± 0.42	1.31^{b} ± 0.95	0.038*
LAsI (ml/kg)	0.31 ± 0.14	0.28 ± 0.14	0.42 ± 0.38	0.108
ACI (mL/min/kg)	78.19^{a,b} ± 33.38	64.75^{a} ± 27.97	102.38^{b} ± 80.18	0.030*
LAEF%	67.87 ± 7.88	68.97 ± 7.82	68.86 ± 9.35	0.890

DMVD - degenerative mitral valve disease, CSI = contraction strain index, LAdI = left atrial diastolic volume index, LAsI = left atrial systolic volume index, ACI = atrial cardiac index, LAEF = left atrium ejection fraction; ^{a,b} different letters on the same line demonstrate statistical significance; * indicates significant difference between groups (P<0.05).

methods, the LA volume increased as the disease progressed, especially in Group 3, whereas the LAS during contraction and the CS decreased progressively in the groups of dogs with DMVD. The results using the single-plane Simpson's method indicated that LA volumes in the case of DMVD, regardless of the phase (LAV_{max}, LAV_{min}, or LAV_{pre-a}), were significantly higher in the animals of the B2 class.

The LA/Ao that was obtained from the conventional echocardiography method was significantly correlated with the LAESV (p = 0.0082), LAEDV (p = 0.0025), CI (p = 0.0010), and CS (p = 0025) (Fig.3).

The intraobserver and interobserver variabilities were low to moderate because most of the coefficients of variation for the variables that were analysed using both methods were less than 25%. The interobserver variability was high only for LAESV and LAVpre-a (measured using the single-plane Simpson's method) and the LASR conduit (measured by 2D-FTI) (Table 4).

Table 3. Mean and standard deviation of function and volume variables in the atrial phases measured by Simpson's monoplanar method in dogs with DMVD at different stages

Variable	Group 1 - class A (n = 21)	Group 2 - class B1 (n = 30)	Group 3 - class B2 (n = 29)	Value p
Left atrial ejection fraction (%)	65.46 ± 4.60	66.54 ± 5.72	68.72 ± 4.95	0.0767
Cardiac output (ml/kg/min)	64.25 ±16.57[a]	75.52 ± 35.47[a]	130.29 ± 74.73[b]	<0.0001*
Maximum volume (ml/kg)	0.78 ± 0.22[a]	0.92 ± 0.27[a]	1.58 ± 0.77[b]	<0.0001*
Atrial pre-contraction volume (ml/kg)	0.36 ± 0.11[a]	0.42 ± 0.13[a]	0.74 ± 0.44[b]	<0.0001*
Mnimum volume (ml/kg)	0.28 ± 0.09[a]	0.31 ± 0.09[a]	0.50 ± 0.29[b]	<0.0001*
Reservoir (ml/kg)	0.64 ± 0.04[a]	0.66 ± 0.06[a,b]	0.69 ± 0.04[b]	0.0028*
Conduction (ml/kg)	0.54 ± 0.05	0.54 ± 0.07	0.53 ± 0.06	0.9566
Contraction (ml/kg)	0.21 ± 0.06[a]	0.25 ± 0.06[a]	0.32 ± 0.08[b]	<0.0001*

DMVD - degenerative mitral valve disease; [a,b] different letters on the same line demonstrate statistical significance; * indicates significant difference between groups (P<0.05).

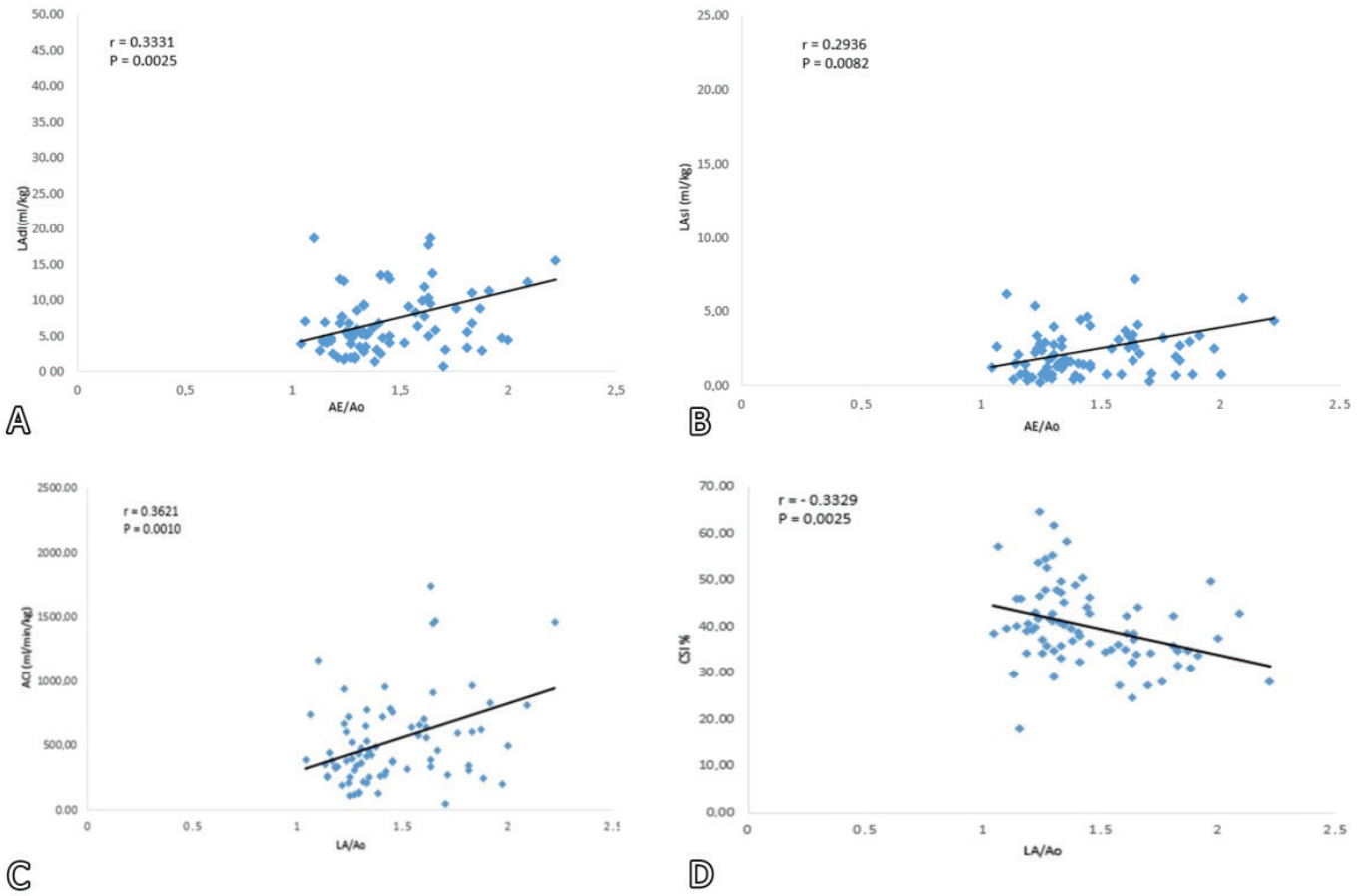

Fig.3. Scatter plots of four variables selected for atrial function correlated with LA/Ao ratio, analyzed by Pearson's correlation (p<0.05). LAsI = atrial systolic volume index, LAdI = atrial diastolic volume index, ACI = atrial cardiac index, CSI = contraction strain index.

Table 4. Intra and interobserver coefficient of variation (CV) of the echocardiographic variables analyzed by Simpson's monoplanar and 2D-FTI methods in dogs with DMVD at different stages

Variable	Simpson monoplanar method	
	CV (%) I Intraobserver	CV (%) Interobserver
Vd (ml)	1.62	20.36
Vs (ml)	3.09	30.31
Vpc (ml)	1.29	26.18
LAEF %	3.86	7.20
ACO (ml/kg/min)	2.58	12.57
Maxium volume (ml/kg)	4.25	10.46
Minimum volume (ml/kg)	3.17	22.16
Atrial pre-contraction volume (ml/kg)	1.57	17.58
Reservoir (ml/kg)	1.42	5.81
Conduction (ml/kg)	1.14	6.80
Contraction (ml/kg)	0.30	11.38
	2D-FTI method	
	CV (%) Intraobserver	CV (%) Interobserver
Vd (ml/kg)	4.35	11.09
Vs (ml/kg)	17.26	3.48
LAEF %	4.73	6.98
ACO (ml/kg/min)	0.34	18.10
St reservoir %	3.18	9.32
St contraction %	4.05	9.01
St conduction %	2.62	24.47
CSI	0.44	18.27
StR reservoir %	6.94	0.90
StR contraction %	7.37	17.39
StR conduction %	9.75	25.74

DMVD - degenerative mitral valve disease, Vd = diastolic volume, Vs = systolic volume, Vpc = preatrial contraction volume, LAEF = left atrium ejection fraction, ACO = atrial cardiac output, St = strain, CSI = contraction strain index, StR = strain rate, 2D-FTI = two-dimensional feature-tracking imaging.

Table 5. The intraclass correlation coefficient (ICC) variability of left atrial echocardiographic volumes and function measured by 2D-FTI and Simpson monoplanar methods

Variable	ICC	CI (95%)
LAEF (%)	0.2851	-0.1027 a 0.5383
ACI (ml/kg/min)	0.8680	0.7947 a 0.9153
LAdI (ml/kg)	0.8759	0.8065 a 0.9204
LAsI (ml/kg)	0.7967	0.6835 a 0.8694

2D-FTI = Two-dimensional feature-tracking imaging, ICC = intraclass correlation coefficient, CI = confidence interval, LAEF = left atrium ejection fraction, ACI = atrial cardiac index, LAdI = left atrial diastolic volume index, LAsI = left atrial systolic volume index.

The ICC was excellent for most of the LA volume variables that were measured using the two echocardiographic methods and was low for the LAEF (Table 5).

DISCUSSION

In the present study, 2D-FTI echocardiography and the single-plane Simpson's method proved useful for assessing the function of the LA by calculating the LAESV, LAEDV, CI, LAV_{max}, LAV_{min}, LAV_{pre-a}, LAS reservoir, LAS contraction, and cardiac conduction abnormalities in dogs with DMVD. The 2D-FTI analysis detected differences in the LAS of the healthy and class B2 dogs, which was indicative of early changes in the myocardial deformation of these animals. The images that were obtained were of sufficient quality to evaluate the dogs with structurally normal LA and dogs with LA remodelling. The animals were randomly selected according to the cardiology routine of our institute. For this reason, the number of females was higher in all of the groups that were studied, although previous studies showed that males are more commonly affected (Atkins et al. 2009, Fox 2012). However, the animal weight and age data indicated a mean value lower than 15kg and an age higher than 6 years because DMVD occurs more frequently in smaller breeds of dogs and those older than 6 years (Atkins et al. 2009, Boswood et al. 2016).

There were significant differences in some of the variables that were obtained from conventional echocardiography as reported by Nakamura et al. (2017). The E wave and the E/IVRT ratio, which are used to determine diastolic function and venous congestion, respectively, were significantly higher in Group B2 than in Group B1. An increased E wave indicates diastolic dysfunction with a pseudonormal filling pattern that is caused by an increased LA filling pressure and decreased ventricular compliance, which occurs with the progression of DMVD (Boon 2011).

The E/IVRT ratio in the B2 Group, although higher, is still below the predictive value indicating the development of

CHF (E/IVRT>2.5), but this increase is expected in the more advanced stages of the disease, since this index can be used to detect high ventricular filling pressure and CHF in dogs with DMVD (Schober et al. 2010). The LVDd N, LVDs N, and LA/Ao were used to classify the sample according to disease progression. These variables were higher in the B2 Group because the cardiac output is decreased and the hydrostatic pressure inside the cardiac chambers is increased in dogs with the more severe stages of DMVD, which activates neurohormonal systems (Oyama 2009). The long-term activation of these systems induces cardiac remodelling, which is characterised by eccentric myocardial hypertrophy (Bonagura & Schober 2009).

The variables that were used to assess the LA function by feature tracking (SLA reservoir, SLA contraction, SLA conduit, CI, SLAR reservoir, SLAR contraction, and SLAR conduit) were also used to characterise the physiological changes during the cardiac cycle (Caivano et al. 2016). In the clinical routine, the LA size is determined by LA/Ao. LA function can also be evaluated by measuring the diastolic and systolic volume by conventional two-dimensional echocardiography. However, the LA structure is complex (including the conical left atrium, pulmonary veins, variable geometry, and small wall thickness), thus limiting the measurement of linear dimensions (Toaldo et al. 2018). Volume-based methods can provide more accurate estimates of the LA than can linear one-dimensional or area measurements, but they have limitations regarding their ability to estimate the size, function, and pathology of heart chambers (Höllmer et al. 2013, 2016, Hoit 2014). This fact is demonstrated by the observation that dogs with and without CHF may have similar degrees of atrial remodelling, as confirmed by their LA/Ao values (Caivano et al. 2016). Therefore, an analysis of atrial deformation by 2D-FTI may provide additional and useful information about the phasic function of the LA and the clinical cardiac status in asymptomatic dogs with DMVD, as demonstrated in this study.

To date, no veterinary studies have evaluated the use of the feature-tracking tool to assess atrial volume. In the present study, the LAEDV and CI that were obtained by 2D-FTI were significantly increased in the B2 Group as a function of the severity of MR, which worsens as the disease progresses, thus corroborating previous findings (Osuga et al. 2016, Höllmer et al. 2017). This result was confirmed by the association between these variables and LA/Ao (Fig.3).

Although there was no significant correlation between LA/Ao and SLA during contraction, there was a significant difference in these variables in the B2 Group when compared to the Control Group, demonstrating a reduction in contractile function in these patients. This finding reinforces the idea that linear measurements do not accurately evaluate the entire extent of the LA because its remodelling is asymmetrical (Tidholm et al. 2011). Nakamura et al. (2017) found a significant relationship between the LA/Ao and SLA during contraction because the mean LA/Ao value (1.92) in the B2 Group was higher than the mean value found in the present study (1.75). In cases of major LA remodelling and severe MR, fractional shortening decreases as the atrial diameter and pressure increase, which compromises LA contractile function - Frank-Starling mechanism (Payne et al. 1971), and these changes are reflected in this correlation. However, LA contractile function is even reduced in the early stages of remodelling, as demonstrated in the present study.

It is important to emphasise that the atrial systolic function depends on multiple factors, including ventricular compliance and LV end-diastolic pressure. Ventricular diastolic dysfunction can be evaluated by conventional echocardiography using the E wave and E/IVRT, which were higher in Group B2 in this study. In DMVD, as ventricular filling pressure progressively increases with a worsening of diastolic dysfunction, the LA acts predominantly as a conduit, enhancing the reduction in contractile function (Prioli et al. 1998, Rosca et al. 2011).

In addition, atrial remodelling involves singular adaptive events, which include changes in the composition of the extracellular matrix that include excessive proliferation of fibroblasts, myocyte hypertrophy, necrosis, and apoptosis (Lee et al. 2015, Janus et al. 2016). These structural changes significantly affect the properties of the LA myocardial wall, including relaxation and contractility and lead to a reduction in LA phasic functions, as demonstrated by Cameli et al. (2012) using two-dimensional speckle tracking echocardiography (2D-STE) in humans and Toaldo et al. (2018) using 2D-STE in dogs with DMVD. Similar to the above studies, the present study showed that the reduction in atrial function could be observed early using 2D-FTI in asymptomatic dogs with DMVD.

The CSI represents the percent contribution of the active contraction of the LA to the filling phase of the LV (Cameli et al. 2012). There was a negative correlation between this variable and LA/Ao, as previously reported by Baron et al. (2017). In contrast to the previous studies, this study found a significant difference in the CSI between all study groups. An explanation for this result could be that the early effect of MR on LA morphology and function can be detected only by 2D-FTI. In humans with MR, the variables that are obtained by 2D-STE echocardiography have been strongly correlated with atrial fibrosis (Cameli et al. 2011). However, there is no information in the veterinary literature on the role of fibrosis in the LA of dogs at the initial stage of DMVD. Another contributing factor is the age of the animals in the Control Group, and an age-related effect cannot be excluded because this group was younger than the other groups.

With respect to the single-plane Simpson's method, the LA volumes increased as the disease severity increased, as observed by Höllmer et al. (2017), and they were significantly higher in the asymptomatic dogs of class B2. Furthermore, Toaldo et al. (2018) demonstrated that the use of a volumetric method has a prognostic value in assessing LA size in dogs with DMVD compared with standard linear measurement. Previous studies have evaluated several methods of measuring atrial volume in the two-dimensional mode in healthy dogs (Dickson et al. 2017) and in dogs with DMVD (Höllmer et al. 2016, 2017, Toaldo et al. 2018). The large LA volume in dogs with DMVD is mainly a reflection of volume overload due to mitral regurgitant flow. LA enlargement is an important compensatory mechanism to prevent pulmonary congestion and maintain adequate ventricular filling volume. Another important factor was LA contraction, which decreased with increasing disease severity (Group 1 0.21±0.06; Group 2 0.25±0.06; Group 3 0.32±0.08; $p<0.0001$) whereas LA volume and LA systolic volume were increased. The impaired LA contraction that occurs as DMVD progresses may be due to an increased afterload (end-diastolic LV filling pressure) and

the exhaustion of the Frank-Starling mechanism by severe volume overload (Höllmer et al. 2017).

Caivano et al. (2016) measured the variability and repeatability of 2D-FTI measurements and found that the inter-observer variability was high for some variables (CS, SLA during contraction, and SLAR during contraction). Since the software automatically generates the curves after feature tracking during the cardiac cycle, the high variability of some variables may depend on the software that was used. In addition, the need for high-quality images for analysing the 2D-FTI variables may increase the variability, whereas the measurements that require less computational power and lower quality images allow for higher repeatability, which was also observed by Baron et al. (2017).

The present study has some limitations; first this was a non-invasive study and was performed in companion animals. Therefore, invasive evaluations of the mechanical properties of the LA or afterload were not performed to confirm the reduced atrial function of the study animals. An alternative approach is magnetic resonance imaging, which is the gold standard in medicine to assess myocardial function and to validate the similarity between the methods. However, this analysis was not performed because of its high cost. Second, dogs that were affected with DMVD were older than those in the Control Group. However, it is difficult to obtain a group of older dogs without any degree of MR, given the pathophysiology of the disease.

Third, the results that we obtained may not be applicable to all clinical situations because of potential differences in echocardiography devices, software, equipment, examiner experience, and image quality, and these factors may affect measurements and data variability, as demonstrated in other studies (Caivano et al. 2016, Baron et al. 2017, Nakamura et al. 2017).

Notwithstanding, the study demonstrated that 2D-FTI echocardiography and the single-plane Simpson's method could be used to determine LA function in dogs with DMVD. The results we obtained provide new perspectives on LA deformation and other variables, allowing for a more comprehensive quantification and a better understanding of the role of the LA in the pathophysiology of DMVD.

CONCLUSIONS

The present study demonstrated that DMVD causes changes in atrial function, especially in the contraction phase, and that 2D-FTI echocardiography was a sensitive method that could be used for the early detection of LA dysfunction. The use of this tool allows for the measurement of atrial deformation even in asymptomatic animals, and the progressive decline in atrial function can be measured as the disease progresses.

Therefore, future studies can use 2D-FTI as a prognostic indicator in dogs with DMVD. The single-plane Simpson's method could differentiate between asymptomatic animals either with or without atrial remodelling, and its effectiveness was similar to that of 2D-FTI echocardiography.

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Original Article
Small Animal Diseases

Temperature gradients in domestic cats over seven-years-old: descriptive analysis[1]

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ABSTRACT.- Rabelo R.C., Pereira Neto G.B.., Carvalho V.J. & Carvalho G.J. 2020. **Temperature gradients in domestic cats over seven-years-old: descriptive analysis**. *Pesquisa Veterinária Brasileira 40(3):197-201*. Faculdade de Agronomia e Medicina Veterinária, Universidade de Brasília, Av. L4 Norte, Setor de Clubes Norte, Campus Universitário Darcy Ribeiro, Brasília, DF 70910-900, Brazil. E-mail: intensivet@gmail.com

The temperature gradients are dynamic and noninvasive monitoring techniques that provide information on peripheral blood flow and have been related to the prognosis of patients with circulatory shock. This study evaluated 47 elderly domestic cats' temperature gradients, and we measured central (rectal) and peripheral (palmar, plantar and medial region of the radio) temperatures. Values found in this study are compatible with studies in young felines and differ from dogs and humans. The mean gradients found were 7.5°C for the central-peripheral; 5.6°C for the peripheral-environmental; 2.7°C for the skin-diff; and 0°C for the member-diff and the variables age and gender do not seem to influence these measurements. To the authors' knowledge, there is no description of temperature gradients in elderly domestic cats, so this study pretends to clarify the vasoconstriction response in this group of animals.

INDEX TERMS: Temperature gradients, domestic cats, peripheral perfusion, vasoconstriction.

RESUMO.- [**Avaliação descritiva dos gradientes de temperatura em felinos domésticos com mais de sete anos.**] Os gradientes de temperatura são técnicas de monitoração dinâmicas e não-invasivas que fornecem informações sobre o fluxo sanguíneo periférico, e têm sido relacionados ao prognóstico de paciente com choque circulatório. O presente estudo avaliou os gradientes de temperatura em 47 felinos domésticos idosos aferindo as temperaturas central (retal) e periférica (palmar, plantar e região medial do rádio). Os gradientes encontrados foram 7,5°C para o centro-periférico; 5,6°C para o periférico-ambiental; 2,7°C para o *skin-diff*; e 0°C para o *member-diff*. As variáveis idade e sexo não pareceram influenciar as mensurações. Não há, em conhecimento dos autores, descrição prévia dos gradientes de temperatura em felinos domésticos idosos, e por isso esse estudo pretende contribuir com o entendimento sobre a capacidade de resposta de vasoconstrição nesse grupo de animais.

TERMOS DE INDEXAÇÃO: Gradientes de temperatura, felinos domésticos, gatos, deltas de temperatura, perfusão periférica, vasoconstrição.

INTRODUCTION

Proper perfusion and oxygen delivery to cells and tissues are the main functions of the cardiovascular system. When this does not occur, there is the onset ofshock syndrome. This syndrome is defined by a severe hemodynamic and metabolic imbalance caused by low tissue perfusion and consequent imbalance between oxygen supply (DO_2) and oxygen uptake (VO_2) (Le Dran 1743, Weil & Shubin 1971).

Hemodynamic monitoring aims at the early detection of perfusion and oxygenation failures, which allows rapid intervention to prevent organ dysfunction. Critically ill patients are highly predisposed to tissue hypoperfusion and multiple organ dysfunctions. When the goal is to anticipate microcirculatory decompensation, an evaluation through macro-hemodynamic parameters, such as blood pressure and central venous pressure is considered ineffective. According

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to Réa-Neto et al. (2006), about 30% of the circulating volume may be lost before there is central repercussion in humans, that is, blood pressure and central venous pressure may be supported by the sympathetic compensatory response. In this case, the patient will have a hemodynamic state called occult shock, initially represented by hyperlactatemia in a stable macrocirculatory environment (Réa-Neto et al. 2006, Rabelo & Ribeiro 2012, Cecconi et al. 2014).

During this shock, the blood is diverted to the vital organs through sympathetic activity and by reducing efferent vagal activity, which results in peripheral vasoconstriction. Given that peripheral perfusion is the first compensatory response line to change, it is important to note that after hemodynamic resuscitation, the critically ill patient completes his recovery with perfusion normalization (Genderen et al. 2012, Vincent et al. 2012, Morais 2016).

There are no 100% sensitive or specific methods for hemodynamic monitoring, so any clinical or laboratory evaluation that is related to the clinical context should be used. (Réa-Neto et al. 2006, Morais 2016).

Due to the self-regulation of vital central organs (heart, lung, and brain), the blood flow is diverted from less vital peripheral tissues (skin, intestines, and kidneys) by increasing resistance in the peripheral vascular bed. Due to the reduced blood flow, there will be a reduction in perfusion with a consequent decrease in skin temperature. Peripheral cells intensify anaerobic pathways; consequently, there is an increase in lactate production as a substrate of the alternative pathway (Rabelo & Ribeiro 2012, Morais 2016, Lima & Bakker 2005).

Less vital tissues may be early markers of changes in blood flow. Obtaining the temperature gradients, capillary refill time, mucosal staining, urinary output, and intestinal borborygmi usually occur through simple and non-invasive methods, allowing a quick and widespread use. Vincent et al. (2012) make an analogy of peripheral markers as "windows" through which we can see a "fire" (Lima & Bakker 2005, Vincent et al. 2012).

Since the 1960s, the skin temperature measurement has been suggested as a noninvasive method for blood flow monitoring. The prognostic value of the measurements was further described by Joly & Weil (1969), who reported the correlation between first toe temperature, cardiac output, and survival in humans. A physical examination that includes skin temperature should be the first step for the perfusional assessment of critically ill patients. Cold extremities, when associated with increased lactate, assist in identifying hypoperfusion (Joly & Weil 1969, Vincent et al. 1988, Kaplan et al. 2001, Lima & Bakker 2005).

Temperature gradients can be obtained by the difference between two body temperature measurements at different points: center-peripheral ($DeltaT_{CP}$), peripheral-environmental ($DeltaT_{PE}$) and the forearm to the tip of the first digit of the left pelvic limb ($DeltaT_{skin-diff}$) (Joly & Weil 1969, Réa-Neto et al. 2006, Genderen et al. 2012, Rabelo & Ribeiro 2012, Morais 2016).

The center-peripheral temperature gradient ($DeltaT_{CP}$) reported in humans between 3°C and 7°C should be less than 6.5°C in dogs and below 8°C in cats. Higher values indicate that the patient maintains a central (rectal) temperature through the vasoconstriction peripheral (Genderen et al. 2012, Beccon 2013, Morais 2016, Rabelo 2018).

In 2012, Vinkers et al. (2012) demonstrated the effect of stress on the peripheral temperature in humans as an important factor in reducing digit temperature. Morais (2016) observed such an effect on felines and justified the higher stress potential as a cause for the higher center-peripheral gradients in the species (Vinkers et al. 2012, Morais 2016).

Delta T_{PA} is the difference between the patient's ambient and the peripheral temperatures. Usually, these values should not be below 4°C-6°C when the animal is kept in thermoneutral temperatures (24°C), i.e., that do not influence the vasoconstriction of the skin. There is a reduction in survival rate when temperatures are kept below these values for more than 12 hours in the hospital setting. Morais (2016) and Rabelo (2018) describe a temperature of 7°C in cats up to seven years old and 7.43°C in dogs.

The Skin-Diff temperature gradient seems to be adequate in environments with extreme thermal variations and physiologically should be close to 0°C. In anesthetized patients with severe vasoconstriction, values were close to 4°C. In cats under seven years old, Morais (2016) reported temperatures around 2.6°C, and in dogs, the values were close to 1.3°C (Lima & Bakker 2005, Rabelo & Ferrari 2010, Morais 2016, Rabelo 2018).

Delta member-diff is the difference between the temperatures of the palmar and plantar regions of the homolateral limbs. The literature describes values close to 0°, but this parameter may be altered in cases of occlusive diseases, considering that the affected limb will have a drastic reduction in blood flow (Genderen et al. 2012, Morais 2016).

Finally, studies showed that a detailed physical examination assists in identifying changes in peripheral perfusion parameters that faithfully reflect the imminent risk of acute circulatory failure. Soares et al. (2018) demonstrated that patients with chronic stage C mitral valve disease had increased lactate values even if stable macro-hemodynamically, but with normalized temperature gradients, while animals in stage B2 had altered center-peripheral deltas. The two possible explanations for reducing the ability to perform peripheral vasoconstriction were the age of these animals or the use of the pimobendan vasodilator drug. Feger & Braune (2005) corroborated these data by reporting that the advancing age directly influences sympathetic responsiveness in humans, with a reduction in the physiological temperature gradient in elderly patients (Feger & Braune 2005, Lima et al. 2009, Soares et al. 2018).

So far, there has been no description of the normality of temperature gradients in domestic cats older than seven years old, so this study intends to collaborate with understanding the vasoconstriction response in this group of animals.

MATERIALS AND METHODS

Forty-seven male and female cats of different breeds, aged over seven years old, from the routine care of the "Serviço de Clínica de Felinos" of the "Hospital Veterinário" of "Universidade de Brasília" (UnB) were evaluated. Initially, we performed screening covering physical examination (heart rate - HR, respiratory rate - RR, mucosal membrane staining - mm, capillary refill time - CRT, lymph nodes, abdominal palpation, and cardiopulmonary auscultation). Cats with skin lesions that could interfere with the measurement of peripheral temperature were not part of the experiment. Sequentially, after acclimatization of the animals at room temperature for 15 minutes, all were gently placed in sternal decubitus and the following temperatures were recorded:

a) Environmental through the infrared thermometer with two laser tips (ST-700), the emissivity of 0.95 in °C, using the average between 4 points on the walls.

b) Peripheral of the left thoracic limb in two distinct points: palmar cushion and proximal radial region of the radius, always keeping the hairs apart; and peripheral pelvic limb in the plantar cushion through infrared thermometer (ST-700, Incotherm®), the emissivity of 0.98 in °C (Villaseñor-Mora et al. 2009). The distance from the thermometer to the measurement point was approximately 13 centimeters and the laser was left in contact with the skin for 10 seconds to perform each measurement. The cushions were chosen for the study because they are a glabrous distal region and easily accessible.

c) Central (rectal) in °C using a nine-second digital thermometer (Geratherm® Rapid).

The measured temperatures were the basis of the calculations of the following gradients, expressed in °C:

i. Center-peripheral temperature gradient ($DeltaT_{CP}$): the difference between the central (rectal) temperature and the left thoracic limb cushion temperature ($DeltaT_{CP1}$) and the difference between the central (rectal) temperature and the left pelvic cushion temperature ($DeltaT_{CP2}$);

ii. Same-limb temperature gradient ($DeltaT_{skin-diff}$ or $DeltaT_{SD}$): the temperature difference between the proximal region of the radius in the forearm of the left thoracic limb and the cushion;

iii. Temperature gradient between different limbs ($DeltaT_{member-diff}$ or $DeltaT_{MD}$): based on the temperature difference between the left thoracic limb cushion and the left pelvic limb cushion;

iv. Peripheral-environmental temperature gradient ($DeltaT_{PE}$): the difference between ambient and left thoracic cushion temperature ($DeltaT_{PE1}$); and environmental and left pelvic limb cushion ($DeltaT_{PE2}$) temperatures.

RESULTS

We performed descriptive analysis on 47 cats aged seven years old and over, of which 26 were female and 21 were male (Table 1).

Data normality was tested using graphical analysis, Shapiro-Wilk, Kolmogorov-Smirnov, and Anderson-Darling as a statistical basis. *P*-values were above 0.05 (95%) for all variables, ensuring normal distribution (Miot 2017).

The averages of each variable reported by Morais (2016) and Rabelo (2018) were the base for the hypothesis testing to conclude some statistical differences when evaluating felines over seven years old. There was a statistical difference for the $DeltaT_{skin-diff}$ variable and the $DeltaT_{PE1}$ and $DeltaT_{PE2}$ (Table 2).

We grouped the cats into males and females to study the effect of sex, and there was no statistically significant difference between the groups (Table 3).

The cats were allocated into three groups to study the effect of age. The first group was composed of animals aged 7 to 10 years old considered adults, the second group was composed of animals aged 11 to 14 years old called elderly and the third group was formed by geriatric animals older than 15 years old, according to Hoyumpa Vogt et al. (2010). There was no statistically significant difference (Table 4-6).

Table 1. Descriptive analysis of the obtained variables

Variable	Mean	Standard deviation	Minimum	Maximum	N	Median
Age	12.06	3.26	7	18	47	11
$DeltaT_{CP1}$	7.58	2.22	3.7	11.9	47	7.6
$DeltaT_{CP2}$	7.59	2.36	2.4	13.1	47	7.5
$DeltaT_{SD}$	2.74	2.11	-2.7	6.5	47	3.3
$DeltaT_{MD}$	0.01	1.67	-5.4	4.6	47	0.1
$DeltaT_{PA1}$	5.66	2.16	-0.5	9.1	47	6.1
$DeltaT_{PA2}$	5.65	2.02	0	9.9	47	5.8

N = Sample size.

Table 2. Comparison between values of this sample with Morais (2016) and Rabelo (2018)

Variable	Sample		Population	*P*-value
	Mean	S	Mean	
$DeltaT_{CP1}$	7.58	2.22	8⁺	0.201
$DeltaT_{CP2}$	7.59	2.36	8⁺	0.24
$Delta_{SD}$	2.74	2.11	0*	0.001
$DeltaT_{MD}$	0.01	1.67	0*	0.967
$DeltaT_{PA1}$	5.66	2.16	7⁺	0.001
$DeltaT_{PA2}$	5.65	2.02	7⁺	0.001

S = Standard deviation; *P*-value determined with T-test for a sample; *values reported by Rabelo (2018), ⁺ values reported by Morais (2016).

Table 3. Study of the sex effect on the studied variables

Variable	Sex				*P*-value
	Male		Female		
	Mean	S	Mean	S	
$DeltaT_{CP1}$	8.09	2.3	6.94	1.97	0.71
$DeltaT_{CP2}$	8.11	2.32	6.93	2.29	0.088
$Delta_{SD}$	3.06	1.94	2.32	2.28	0.244
$DeltaT_{MD}$	0.01	1.8	-0.001	1.52	0.982
$DeltaT_{PA1}$	5.19	2.29	6.23	1.88	0.094
$DeltaT_{PA2}$	5.17	2.03	6.24	1.87	0.067
FC	188.38	39.54	191.91	36.19	0.751

S = Standard deviation; *P*-value determined with T-test for two samples.

Table 4. Study of the age effect on the studied variables (adults x elderly)

Variable	Age				*P*-value
	Adults		Elderly		
	Mean	S	Mean	S	
$DeltaT_{CP1}$	7.75	1.92	7.46	1.92	0.659
$DeltaT_{CP2}$	7.26	2.44	7.87	2.09	0.797
$Delta_{SD}$	2.13	2.17	3.12	1.97	0.167
$DeltaT_{MD}$	-0.48	1.82	0.4	1.35	0.11
$DeltaT_{PA1}$	5.89	2.15	5.41	2.06	0.673
$DeltaT_{PA2}$	6.38	1.71	5	1.86	0.03
FC	187.19	47.39	192.1	30.54	0.714

S = Standard deviation; *P*-value determined with T-test for two samples.

Table 5. Study of the age effect on the studied variables (adults x geriatricians)

Variable	Age				*P*-value
	Adults		Geriatricians		
	Mean	S	Mean	S	
$DeltaT_{CP1}$	7.75	1.92	7.53	2.83	0.808
$DeltaT_{CP2}$	7.26	2.44	7.56	2.47	0.751
$Delta_{SD}$	2.13	2.17	2.92	1.96	0.992
$DeltaT_{MD}$	-0.48	1.82	0.03	1.65	0.452
$DeltaT_{PA1}$	5.89	2.15	5.74	2.19	0.858
$DeltaT_{PA2}$	6.38	1.71	5.7	2.19	0.364
FC	187.19	47.39	191.63	34.56	0.786

S = Standard deviation; *P*-value determined with T-test for two samples.

Table 6. Study of the age effect on the studied variables (elderly x geriatricians)

Variable	Age				*P*-value
	Elderly		Geriatricians		
	Mean	S	Mean	S	
$DeltaT_{CP1}$	7.46	1.92	7.53	2.83	0.935
$DeltaT_{CP2}$	7.87	2.09	7.56	2.47	0.711
$Delta_{SD}$	3.12	1.97	2.92	1.96	0.785
$DeltaT_{MD}$	0.4	1.35	0.03	1.65	0.5
$DeltaT_{PA1}$	5.41	2.06	5.74	2.19	0.675
$DeltaT_{PA2}$	5	1.86	5.7	2.19	0.348
FC	192.1	30.54	191.63	34.56	0.969

S = Standard deviation; *P*-value determined with T-test for two samples.

DISCUSSION

In this study with domestic cats over seven years old, the gradients $DeltaT_{CP1}$ and $DeltaT_{CP2}$ were 7.58°C and 7.59°C respectively. Currently, according to Rabelo & Ribeiro (2012), for dogs, values above 6°C are considered a warning sign, and values above 10°C are harmful and correlated with higher mortality. In another study, with healthy and young cats, it was demonstrated that these animals would have $DeltaT_{CP}$ alarm values close to 8°C (Rabelo & Ribeiro 2012, Morais 2016).

The results in this study differ from the expected since, with advancing age, there would be a reduction in sympathetic response and, thus, a reduction in gradients (Feger & Braune 2005).

Given that this sample is non-probabilistic for convenience, it is possible that the cats that were used, even if stable, were already showing some degree of vasoconstriction, keeping the value close to the reference of young animals. We suggest further studies only with elderly and healthy animals. Still, using the 8°C value for patient assessment is efficient and reduces the possibility of false negatives.

Regarding the temperature gradient from the forearm to digit tip or $DeltaT_{SD}$, the present study found an average value of 2.74°C. Human studies have reported $DeltaT_{SD}$ values close to 0°C when there is no vasoconstriction, and 4°C during severe vasoconstriction. For dogs, the expected value is 1.3°C and in young cats, a study found values of 2.6°C. The effect of stress on skin temperature may be the explanatory factor for the slightly higher results found in cats (Rabelo & Ribeiro 2012, Vinkers et al. 2012, Morais 2016).

$DeltaT_{member-diff}$ is the difference between the temperatures of the first digit of the left thoracic limb relative to that of the first digit of the left pelvic limb. The value found in this study was 0.01°C, with no statistical and biological difference with the literature reference. This parameter may be altered in cases of occlusive diseases, given that the affected limb will have a drastic reduction in blood flow (Genderen et al. 2012, Morais 2016).

The values for $DeltaT_{PE1}$ and $DeltaT_{PE2}$ were 5.66°C and 5.65°C and were statistically different from the 7°C value found by Morais (2016). Given that the patients were kept in a thermoneutral environment, these results may be the result of peripheral vasoconstriction related to the hemodynamic unhealthiness of the sampled patients (Morais 2016).

The sex effect study did not generate a statistical difference between the groups. However, males presented $DeltaT_{CP1}$ and $DeltaT_{CP2}$ at 8.09°C and 8.11°C, and for females, $DeltaT_{CP1}$ and $DeltaT_{CP2}$ were 6.94°C and 6.93°C respectively. Although in the absence of statistical significance, there is biological importance in the difference of 1°C, and further studies are needed to evaluate if females are more predisposed to stress vasoconstriction.

To study the effect of age, we divided the sample into three groups, according to Hoyumpa Vogt et al. (2010), that is adults (7 to 10 years), elderly (11 to 14 years), and geriatricians (>15 years). There was no statistical difference between the groups. Still, within this sample, we expected to find lower values in the older group (Hoyumpa Vogt et al. 2010).

CONCLUSIONS

The center-peripheral, skin-diff and member-diff temperature gradients in elderly domestic cats do not appear to differ from values found in young cats, at least in this non-probabilistic convenience sample.

Statistically, sex does not appear to affect temperature deltas; however, a 1°C difference was reported between males and females concerning the center-peripheral gradient.

The present study was unable to determine statistical differences for gradients in older animals compared to young animals. However, it reinforces the importance of the reference values already reported, which probably have good specificity.

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

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Original Article
Small Animal Diseases

Determination of the reference interval of the C-reactive protein/albumin ratio and its efficiency, CRP and albumin as prognostic markers in dogs[1]

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ABSTRACT.- Fabretti A.K., Siqueira R.C.S., Amaral H.F., Kemper D.A.G., Chaves R.O. & Pereira P.M. 2020. **Determination of the reference interval of the C-reactive protein/albumin ratio and its efficiency, CRP and albumin as prognostic markers in dogs.** *Pesquisa Veterinária Brasileira 40(3)202-209*. Universidade Estadual de Londrina, Rod. Celso Garcia Cid PR-445 Km 380, Londrina, PR 86057-970, Brazil. E-mail: akfabretti@gmail.com

The objective of this research was to creates a reference interval for C-reactive protein (CRP)/albumin ratio (CAR) in the canine species and to analyze the potential of CRP, albumin and the relationship between both, to serve as indicators of disease severity, length of hospital stay (LoS) and mortality in this species. For this, an outcome study was conducted in a Veterinary Teaching Hospital in southern Brazil. One hundred ninety dogs were included randomly, without distinction of gender, age, or breed, from June 2013 to November 2016. Plasma was collected from them and analyzed for assessment of CRP and albumin. The reference range stipulated for CAR in dogs was 0.36-0.60, as determined by the confidence interval of mean resamplings (in percentiles). The frequencies mean, and standard deviations of the variables, correlation analysis, and comparative analysis (Kruskal-Wallis in $\alpha = 5\%$) were calculated. Elevation (above reference) of CAR was determined to be proportional to the severity of the underlying disease, and CRP means were reasonable. Besides, hypoalbuminemia was indicative of systemic disease, but not of severity. Thus, CAR was a better marker of disease severity than were CRP and albumin, analyzed separately. Concerning LoS, there was a positive correlation with CAR ($p<0.01$) in patients, and the same was not observed with CRP and albumin. Concerning mortality, hypoalbuminemia was the only marker valid in animals with a critical illness ($p=0.04$). In conclusion, CAR is a better marker of disease severity and LoS in dogs than are CRP and albumin analyzed separately.

INDEX TERMS: C-reactive protein, albumin, prognosis, mortality, length of stay, disease severity, dogs.

RESUMO.- [Determinação do intervalo de referência e da eficiência da relação proteína C-reativa/albumina, PCR e albumina como marcadores prognósticos em cães.] O objetivo desta pesquisa foi determinar um intervalo de referência para a relação proteína C reativa (PCR)/albumina (R:PCR/ALB) na espécie canina e analisar o potencial de PCR, albumina e a relação entre ambas como indicadores de gravidade de doença, tempo de internação (TI) e mortalidade nesta espécie. Para isso, um estudo foi realizado em um Hospital Veterinário Escola no sul do Brasil. Cento e noventa cães foram incluídos aleatoriamente, sem distinção de sexo, idade ou raça, de junho de 2013 a novembro de 2016. O plasma foi coletado e analisado para avaliação da PCR e albumina. O intervalo de referência estipulado para o R:PCR/ALB em cães foi de 0,36-0,60, conforme determinado pelo intervalo de confiança da média das reamostragens (em percentis). Foram calculadas as frequências, médias e desvios-padrões das variáveis, análises de correlação e análises comparativas (Kruskal-Wallis em $\alpha = 5\%$). Notou-se elevação (acima da referência) da R:PCR/ALB proporcional à gravidade da doença

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de base, sendo normais as médias da PCR. Adicionalmente, a hipoalbuminemia foi indicadora de doença sistêmica, mas, não de gravidade. Dessa forma, a R:PCR/ALB foi melhor indicadora de gravidade de doença do que a PCR e albumina, analisadas separadamente. Em relação ao TI, houve correlação positiva com a R:PCR/ALB (p<0,01) em doentes, não sendo observado o mesmo com a PCR e albumina. Em relação à mortalidade, a hipoalbuminemia foi a única marcadora válida em animais com doenças críticas (p=0,04). Conclui-se, portanto, que a R:PCR/ALB é melhor marcadora de gravidade de doença e TI em cães do que a PCR e albumina analisadas separadamente.

TERMOS DE INDEXAÇÃO: Proteína C-reativa, albumina, prognóstico, mortalidade, tempo de internamento, gravidade de doença, cães.

INTRODUCTION

The strong bond of affection between humans and companion animals, coupled with easy access to information (especially online) has generated a high level of questioning of animal owners to veterinarians, with the clinical course of their sick animals (Rabelo et al. 2009, Fabretti et al. 2014). A similar scenario occurs in human medicine (Niewinski et al. 2014, Silva et al. 2014).

In this way, correctly determining a patient's prognosis is an increasingly valued medical action. Prognosis is defined as an early forecast of the patient's progression and chances of recovery based on clinical and diagnostic information from the patient. (Niewinski et al. 2014, Silva et al. 2014). This prediction is useful in several ways in human and veterinary medicine: it supports the type of treatment indicated (at home or in hospital, including intensive care); goal decisions regarding monitoring protocols; estimation of length of hospital stay (LoS); and when applicable, medical costs and chances of survival, as well as providing helpful information for making euthanasia decisions in animal patients (Cockcroft 2007, Rabelo et al. 2009, Davis et al. 2012, Fabretti et al. 2014).

As a tool that directs the patient's clinical conduct and serves as an argument for the veterinarian to receive authorization for expensive procedures, an erroneous estimate can have disastrous consequences, as a wrong choice of medical protocols can result in worse clinical outcomes (possibly death) in addition to the possibility of unnecessary expenses. As a result, conflicts may occur between veterinarians and their clientele (Cockcroft 2007, Rabelo et al. 2009, Fabretti et al. 2014).

For the prognosis to be as precise as possible, it is necessary to use effective markers to predict the severity of the underlying disease, clinical recovery time and chance of death in each case. Among the markers most commonly used in medicine, both human and veterinary, are serum levels of C-reactive protein (CRP) and albumin (Yeun et al. 2000, Cerón et al. 2008, Eckersall & Schmidt 2014, Fabretti et al. 2014, Wong et al. 2016).

C-reactive protein is the main positive acute phase protein in dogs and an excellent modulator and marker of initial inflammation, being measured as the magnitude of its elevation proportional to the inflammatory level. (Dabrowski et al. 2013, Kjelgaard-Hansen et al. 2013, Christensen et al. 2014, Eckersall & Schmidt 2014). In dogs and humans, their serum concentration increases four to six hours after injury, and their peak is reached in 24-48 hours. (Davis et al. 2012, Rubio & Schmidt 2014, Reimann et al. 2016). At this time, in dogs, C-reactive protein can reach serum concentrations above 100 to 1,000 times the reference range, and normalization occurs approximately one to two days after the end of the inflammatory stimulus (Jitpean et al. 2014, Tizard 2014, Venco et al. 2014). Healthy dogs have less than 5mg/L of this protein in plasma (Kuribayashi et al. 2003, Jitpean et al. 2014, Rubio & Schmidt 2014, Reimann et al. 2016).

As a prognostic marker in companion animals, C-reactive protein is especially useful because its increase precedes any leukocyte alteration, enabling the recognition of subclinical disorders (Anziliero et al. 2013, Karlsson et al. 2013, Viitanen et al. 2014). High serum concentrations have been associated with a higher death rate in dogs, with normalizing being associated with clinical recovery (Michelsen et al. 2012, Anziliero et al. 2013, McClure et al. 2013).

In addition, albumin is the most abundant plasma protein, with its main function being to transport nutrients, hormones, metabolites and pigments in blood and tissues (Cerón et al. 2008, Eckersall 2008, Thrall et al. 2015). It is a negative acute phase protein; therefore, its serum level decays in inflammatory processes (Cerón et al. 2008, Eckersall 2008). Albumin is considered, in human and veterinary medicine, a sensitive marker of morbidity, mortality and hospitalization time; in this way, the more serious the disease becomes, the lower the albumin concentration will be, and the worse the prognosis will be (Corkins et al. 2010, Fabretti et al. 2014, Ong et al. 2014, Qin et al. 2016).

One way to increase the accuracy of prognostic stipulation is to use both variables together in the CRP/albumin ratio (CAR). This relationship has been widely used in human medicine recently, with a large number of studies being published since 2013. It is considered an early, independent and reliable marker of prognosis (largely related to mortality), especially in cases of neoplasias or sepsis (Ranzani et al. 2013, Xu et al. 2015, Tao et al. 2016, Guo et al. 2017, Li et al. 2017).

There are authors, in human medicine who report that CAR is a better prognostic marker than several other indicators of inflammation, such as lymphocyte/neutrophil ratio, lymphocyte/platelet ratio, or the use of CRP and albumin separately (Ranzani et al. 2013, Liu et al. 2015, Wu et al. 2016). One advantage is that use is simple and has a high accuracy with a reduced cost compared with other prognostic indicators (Kinoshita et al. 2015, Park et al. 2016).

Although this marker is used in human medicine, there are no known studies, to the best of the authors' knowledge, of this relationship in veterinary medicine of companion animals. Thus, the present work creates a reference interval for CAR in dogs and analyzes the potential of CRP, albumin and the relationship between both as prognostic markers in dogs, studying their correlations with disease severity, LoS and mortality in this species. This study is, therefore, intended to innovate in the prognostic evaluation of dogs.

MATERIALS AND METHODS

One hundred ninety dogs admitted in a veterinary teaching school hospital, from June 2013 to November 2016, were randomly included in this study, regardless of gender, age or race and with client approval by the informed consent document. The project was approved in the institutional ethical committee, under registration 1679/2013. Exclusion criteria were: animals whose guardians did not agree to the study; animals with clinical dehydration above 8%; blood transfusion

within 21 days prior to project evaluation; aggressiveness, dyspnea or less than 2kg of body weight; nephropathies and proteinuria or liver disease with cirrhosis.

The following patient data were recorded: race, age, gender, hospitalization period (in days), reason for hospitalization and the outcome (discharge or death). The weight was measured in the electronic balance. The severity of the underlying disease was classified according to Disease Score (DS) as described by Muir (2007) and was a parameter for the distribution of dogs in three groups. The first group was the Control Group (CG), which was composed of 40 healthy and asymptomatic dogs. The second group was composed of 80 dogs classified in DS 2 or 3 and was called the moderate diseases group (MDG). These were patients with nondisabling systemic diseases who were able to move, at least at short distances. The third group was called the severe diseases group (SDG), consisting of 70 animals classified in DS 4 and 5, that is, with disabling and critical systemic diseases. These dogs remained in decubitus position.

Blood was collected from the jugular vein and stored in serum tubes (2mL). Samples were centrifuged within 15 minutes at 1500 x g for 10 minutes, and serum was immediately separated and stored at -80°C until analysis. All analyses were performed simultaneously on each sample.

C-reactive protein and serum albumin analyses were performed in veterinary laboratories. The albumin was measured by the bromocresol green method, analyzed by the semiautomatic apparatus BIO-2000 (Bioplus Products for Laboratories Ltda, Baurueri/SP, Brazil), using colorimetric tests with kits of Analisa brand (Gold Analisa Diagnostica Ltda, Belo Horizonte/MG, Brazil). Additionally, the CRP was analyzed by the ultrasensitive turbidimetric immunoassay technique in the Siemens Dimensions (automated clinical chemistry analyzer), model RXL, with test kits from the same company. The measurements were made according to the manufacturer's instructions and the kits used were validated for use on dogs (Christensen et al. 2014, Viitanen et al. 2014). The laboratory teams did not have access to other data of the studied patients and were blinded to the prognosis of these patients.

For the determination of the reference range of clinical laboratory variables in veterinary medicine, a sample between 40 and 120 is indicated (Friedrichs et al. 2012) This research had 40 samples (CG) for determination of the reference range of CAR; therefore, the data are significant. For that, the confidence interval (α = 95%) of the mean of the resamplings, based on percentiles, was calculated using the software BioEstat v. 5.0 (BioEstat - Institute of Sustainable Development Mamirauá/AM, Brazil).

In all groups, the correlation between CRP, albumin and CAR was tested with the prognostic measures of LoS and mortality. Comparative analysis of these variables between groups was allowed to investigate whether they were correlated to the severity of underlying disease.

The means and standard deviations of the variables for CG, MDG and SDG were also investigated. The Pearson correlation between the variables within each group was then analyzed. Finally, the Kruskal-Wallis nonparametric test at α = 5% (significant) and α = 1% (highly significant) was used to compare the groups (which did not have the same number of animals). Statistical software Action Stat Pro (Estatcamp - Statistical Consulting and Quality - São Carlos/SP, Brazil) was used for these analyses.

RESULTS

One hundred ninety dogs were evaluated in this study. The characteristics of each group are described in Table 1.

In relation to GC, 25/40 (62.5%) were admitted for checkup, 14/40 (35.0%) for elective castration and 1/40 (2.5%) for blood donation. The animal assessment and blood collection were performed before the surgeries. Regarding the reasons for attendance in MDG, 27/80 (33.75%) were due to gastroenteritis, 6/80 (7.5%) for pyometra, 3/80 (3.75%) due to pancreatitis and the remainder, 44/80 (55.00%), due to the presence of several other nondisabling systemic diseases. In SDG, these animals were taken to hospital care due to neoplasms 12/70 (17.14%); heart diseases 9/70 (12.85%), ehrlichiosis 8/70 (11.43%) and 41/70 (58.58%) due to other critical systemic diseases.

The description of CG relative to the values of CRP, albumin and CAR is shown in Table 2. The calculated reference range

Table 1. Description of the groups studied, in relation to sex, race, age and weight of the dogs

Group	Sex	Races	Age	Weight
CG[a] (n=40)	Males: 22/40 (55.00%) Females: 18/40 (45.00%)	Non-breed: 13/40 (32.50%) Different races: 27/40 (67.50%)	5 months - 10 years (44 ± 32[d])	2.3 to 60kg (15.84 ± 12.49[d])
MDG[b] (n=80)	Males: 35/80 (43.75%) Females: 45/80 (56.25%)	Non-breed: 36/80 (45.00%) Different races: 44/80 (55.00%)	2 months - 16 years (57.46 ± 55.50[d])	2.6 to 54.6kg (13.37 ± 11.59[d])
SDG[c] (n=80)	Males: 42/70 (60,00%) Females: 28/70 (40,00%)	Non-breed: 42/70 (60,00%) Different races: 28/70 (40,00%)	2 months - 20 years (92 ± 64,63[d])	2.0 to 35.0kg (12.0 ± 9.00[d])

[a] CG = Control group, [b] MDG = moderate diseases group, [c] SDG = severe diseases group, [d] mean ± standard deviation.

Table 2. Descriptive analysis of the control group of 40 healthy dogs in relation to the C-reactive protein, albumin and C-reactive protein/albumin ratio

Variable data	CRP[a] (µg/mL)	Albumin (g/dL)	CAR[b]
Minimum	0.10	2.40	0.02
1st quartile	0.70	2.90	0.19
Mean	1.64	3.30	0.48
Median	1.40	3.34	0.41
3rd quartile	2.30	3.60	0.71
Maximum	5.20	5.10	1.57
Standard deviation	1.26	1.27	0.08
Coefficient of variation	0.73	0.33	0.97

[a] CRP = C-reactive protein, [b] CAR = C-reactive protein/albumin ratio.

for CAR (using the GC of 40 dogs) was 0.36 to 0.60, with a mean of 0.48 (α = 5%) (Fig.1A).

Descriptive analyses of the CRP, albumin and CAR variables for sick animals (MDG and SDG) are shown in Tables 3 and 4, respectively. Likewise, the analysis of CAR values in these groups is shown in Figure 1B and 1C.

The comparative analysis of the means (and standard deviation) of the variables between groups are listed in Table 5 and allowed to evaluate their correlations with the severity of the underlying diseases. The correlations between the variables studied with LoS and mortality, for sick animals (MDG and SDG), are described in Table 6.

DISCUSSION

Severity of underlying disease

In this research, the dogs had a progressive increase in the CAR values as the severity of the underlying disease increased (Table 5), with the mean values in MDG (1.81) and SDG (2.62) being much higher than the reference interval suggested in this study (0.36-0.60). In fact, compared to the upper reference limit, the MDG mean was increased more than three times, and in SDG, this increase was greater than 4.3 times. As expected, the difference in means between groups was statistically significant, proving that CAR is a good marker of disease severity. There are several studies in human

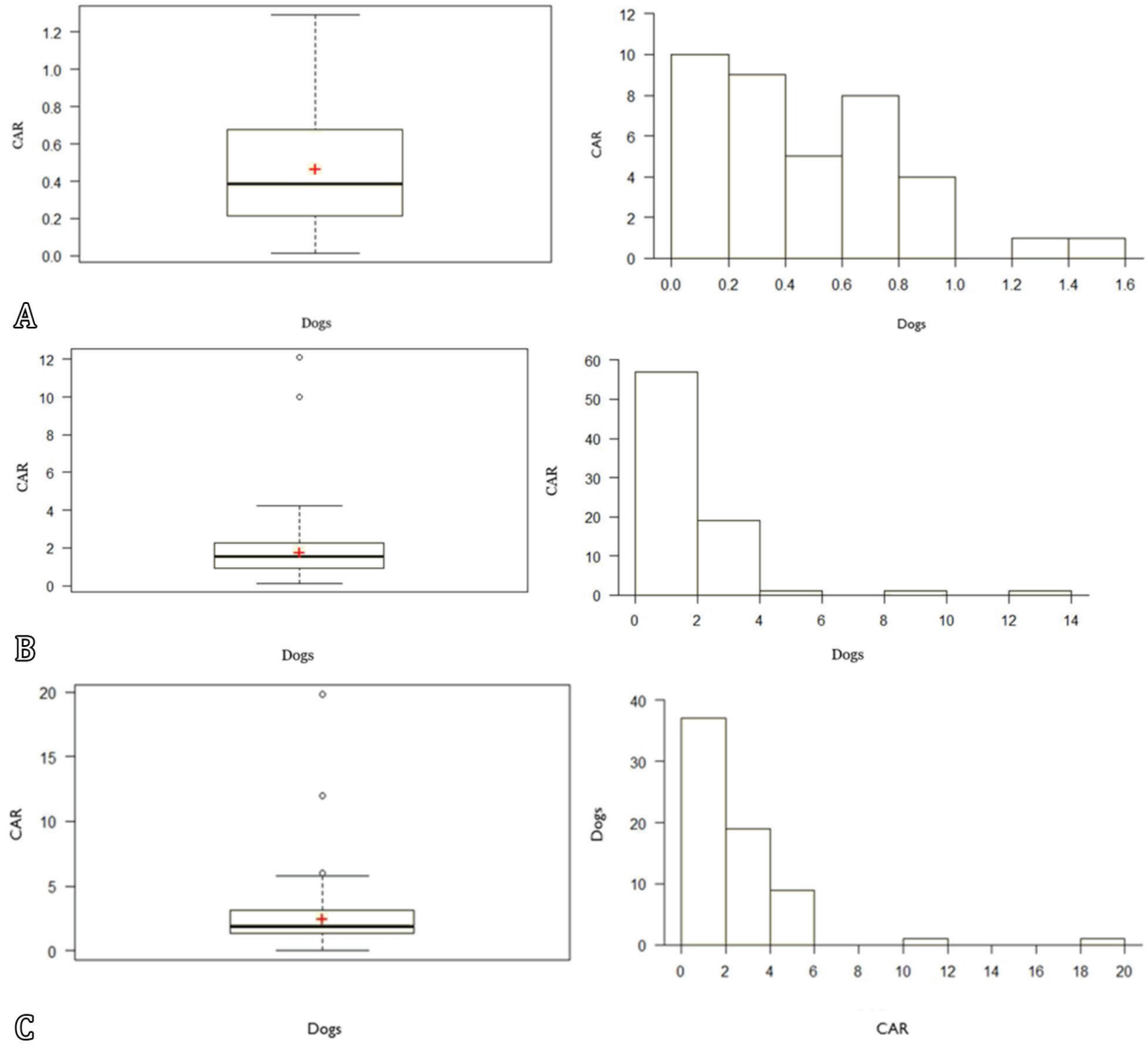

Fig.1. (**A**) Box chart and histogram of the C-reactive protein/albumin ratio (CAR) in the Control Group of 40 healthy dogs. (**B**) Box chart and histogram of the C-reactive protein/albumin ratio (CAR) in the group of 80 dogs with moderate diseases. (**C**) Box chart and histogram of the C-reactive protein/albumin ratio (CAR) in the group of 70 dogs with severe diseases. The central line of the box plot indicates the median value, the upper, and lower line of the box plot illustrate the range of the 25% and 75% of the values, the outer lines at the end of the vertical lines indicate the 95% and 5% range of the recorded values. The "+" sign represents the mean.

Table 3. Descriptive analysis of the group of 80 dogs with moderate systemic diseases in relation to the C-reactive protein, albumin and C-reactive protein/albumin ratio

Variable data	CRP[a] (µg/mL)	Albumin (g/dL)	CAR[b]
Minimum	0.40	0.30	0.12
1st quartile	3.00	1.90	0.93
Mean	3.61	2.54	1.81
Median	3.60	2.60	1.54
3rd quartile	4.10	3.20	2.33
Maximum	13.30	5.10	12.0
Standard deviation	1.73	0.90	1.72
Coefficient of variation	0.48	0.35	0.95

[a] CRP = C-reactive protein, [b] CAR = C-reactive protein/albumin ratio.

Table 4. Descriptive analysis of the group of 70 dogs with severe systemic diseases in relation to the C-reactive protein, albumin and C-reactive protein/albumin ratio

Variable data	CRP[a] (µg/mL)	Albumin (g/dL)	CAR[b]
Minimum	0.10	0.57	0.03
1st quartile	3.10	1.58	1.31
Mean	4.97	2.29	2.62
Median	3.90	2.20	1.88
3rd quartile	4.80	3.00	3.14
Maximum	59.40	6.30	19.80
Standard deviation	7.01	1.03	2.80
Coefficient of variation	1.41	0.45	1.07

[a] CRP = C-reactive protein, [b] CAR = C-reactive protein/albumin ratio.

Table 5. Comparative analysis of death rates and means (with standard deviation) of the variables: length of hospital stay, albumin, C-reactive protein and C-reactive protein/albumin ratio between the Control Group and the diseased dog groups

Variable	Frequency or means ± Standard deviation (s)		
	Control Group (n=40)	Moderate diseases group (n=80)	Severe diseases group (n=70)
Death	0 (0%)a	6 (7.5%)b	34 (48.57%)c
LoS (days)	0.34 ± 1.04a	3.18 ± 2.91b	6.50 ± 6.92c
CRP (µg/dL)	1.64 ± 1.26a	3.61 ± 1.73b	4.97 ± 7.01c
Albumin (g/dL)	3.81 ± 1.27a	2.54 ± 0.90b	2.20 ± 1.03b
CAR	0.48 ± 0.08a	1.81 ± 1.72b	2.62 ± 2.80c

LoS = Length of hospital stay, CRP = C-reactive protein, CAR = C-reactive protein/albumin ratio; a,b,c = equal letters indicate that the variables compared do not differ statistically, while distinct letters indicate a significant difference by the Kruskal-Wallis test at 5%. Reference values: albumin = 2.6 to 3.3g/dL (Thrall et al. 2015), CRP = <5,0-8,70µg/mL (Kuribayashi et al. 2003, Kaya et al. 2012, Anziliero et al. 2013).

Tabela 6. Analysis of correlations between C-reactive protein, albumin, and C-reactive protein/albumin ratio with length of hospital stay and death rate in sick dog groups

Correlation	Moderate diseases group		Severe diseases group	
	p-value	*r*	p-value	*r*
CRP[a] x LoS[b]	0.08	0.19	0.73	-0.04
CRP x Death	0.89	0.01	0.21	0.15
Albumin x LoS	0.12	-0.17	0.26	-0.13
Albumin x Death	0.42	-0.09	0.04	-0.24
CAR[c] x LoS	<0.01	0.45	0.86	0.02
CAR x Death	0.73	0.03	0.71	-0.01

r = Pearson correlation coefficient; [a] CRP = C-reactive protein, [b] LoS = length of hospital stay, [c] CAR = C-reactive protein/albumin ratio.

medicine that corroborate this assertion, demonstrating a proportional elevation of CAR in severe diseases (Wei et al. 2015, He et al. 2016, Toiyama et al. 2016, Wong et al. 2016, Zhang et al. 2017).

Although the CRP values increased proportionally associated with the severity of the underlying disease, in all groups the means were within the normal range (Table 5). That is, in this study, CRP was not a sensitive indicator of disease severity. There are some studies with similar results in human and veterinary medicine (Tvarijonaviciute et al. 2011, Squassoni et al 2011, Kaya et al. 2012, Qin et al. 2016).

It is known that CRP rises more intensely in cases of inflammation, neoplasms and autoimmune diseases. (Anziliero et al. 2013, Venco et al. 2014, Liu et al. 2015). In dogs, the half-life of only six hours causes the serum concentration to normalize rapidly, between one to two days after the lesion ends (Michelsen et al. 2012, Rubio & Schmidt 2014, Reimann et al. 2016). So as it is an acute phase protein with a short half-life, studied in this research in groups with heterogeneous disease, also containing chronic disorders or noninflammatory diseases, it is understandable their normal means.

In relation to albumin, the groups of sick animals presented subnormal values, with significant differences in relation to the CG, whose mean was within the reference values (Table 5). There was no significant difference between the MDG and SDG values. That is, hypoalbuminemia was indicative of systemic disease, but not of disease intensity. Despite this, works in human medicine report the association between low levels of albumin and disease severity. Thus, CAR was better as an indicator of disease severity than CRP and albumin analyzed separately. Researches in humans showed the same (Ranzani et al. 2013, Kim et al. 2015, Park et al. 2016, Wu et al. 2016).

Length of hospital stay

A positive correlation was also observed between CAR and LoS in MDG (Table 6), that is, animals with systemic diseases and values of CAR above the reference tended to recover more slowly (in this work, >3 days). The CAR was, therefore, the marker of the LoS in this group. In human medicine, this correlation has also been described. (Ranzani et al. 2013, Kim et al. 2015, Zhang et al. 2016, Li et al. 2017). It is interesting to note that neither CRP nor albumin was correlated with LoS in any of the groups studied, i.e., the CAR evaluation is superior as a predictor of LoS than these variables considered in isolation. This conclusion has been previously described (Ranzani et al. 2013, Zhang et al. 2016, Li et al. 2017).

However, the correlation between CAR and LoS was not noticed in SDG ($p = 0.86$, Table 6). This finding may be a consequence of high mortality in this group (approximately 50%). As the deaths occurred, mostly shortly after hospitalization, the mean LoS was reduced, impairing the correlation analysis between the aforementioned relationship and the time for clinical recovery in dogs with high lethality diseases. Despite this, the average LoS in the SDG (6.5 days) was significantly higher than that in the other groups, which shows that animals that did not die early (and had high CAR values) tended to have a prolonged hospitalization and delayed discharge, which reinforces the validity of this relationship as a prognostic marker. Several human studies support the efficacy and sensitivity of CAR as a prognostic marker (Wei et al. 2015, Xu et al. 2015, He et al. 2016, Guo et al. 2017, Yu et al. 2017).

Mortality

There was no correlation between the CAR and the death rate in this study (Table 6). However, articles in human medicine, especially in the area of oncology and in patients with sepsis show the opposite. A survey, aiming to analyze the residual effects of inflammation on survival after discharge from an intensive care unit, analyzed the mortality rate six months after a severe sepsis or septic shock event (Kim et al. 2015). Another group of researchers elaborated a similar study, analyzing mortality 90 days after discharge from the ICU with the same profile of patients (Ranzani et al. 2013, Kim et al. 2015). Both groups concluded that CAR was an excellent marker of mortality during follow-up periods (Ranzani et al. 2013, Kim et al. 2015). Numerous studies in oncology emphasize that CAR predicts the mortality of long-term human patients after cancer treatment (Kinoshita et al. 2015, Zhou et al. 2015, Tao et al. 2016, Toiyama et al. 2016, Zhang et al. 2017). Therefore, it is possible to infer that CAR is a better indicator of long-term mortality compared to observations only at the time of hospitalization.

Regarding CRP, there was also no significant correlation with the mortality rate. There are researches in dogs with immune-mediated hemolytic anemia and ehrlichiosis stating the same (Mylonakis et al. 2011, Griebsch et al. 2019). In humans, most published works demonstrate the inverse (Kunitoshi et al. 1999, Yeun et al. 2000, Kristine et al. 2009, Boulware et al. 2011).

However, there was a negative correlation between death and albumin levels in SDG (Table 6); therefore, in dogs with critical diseases, hypoalbuminemia indicates a higher chance of death. In the literature, reports of correlations between low concentrations of albumin and high mortality are extensive (Nakajima et al. 2014, Wang et al. 2014, Garwe et al. 2016, Slee 2016).

CONCLUSIONS

The reference range of CAR for normality in dogs in this study was 0.36 to 0.60. This relationship is a better marker of disease severity and LoS than the CRP or albumin analyzed separately.

However, CAR is not a sensitive marker of mortality during the hospitalization period, and serum albumin is better for dogs with critical diseases, for this purpose. Surveys are needed to assess the accuracy of CAR as a predictor of mortality in dogs over the long term (including postadmission).

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Original Article
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Osteopontin expression and its relationship with prognostic biomarkers in canine mammary carcinomas[1]

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ABSTRACT.- Monteiro L.N., Salgado B.S., Oliveira D.E., Rivera-Calderon L.G, Montoya-Flórez L., Sanctis P. & Rocha N.S. 2020. **Osteopontin expression and its relationship with prognostic biomarkers in canine mammary carcinomas.** *Pesquisa Veterinária Brasileira 40(3)210-219* Departamento de Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rua Prof. Dr. Valter Maurício Corrêa s/n, Botucatu, SP 18618-681, Brazil. E-mail: maomontoya53@yahoo.es, lmontoyaf@unal.edu.co

Osteopontin is a glycophosphoprotein implicated in different physiologic and pathologic processes and is known to be involved in progression and metastasis of various cancers in humans, but this relation is still little explored in the veterinary. The aim was to evaluate the expression of osteopontin in canine mammary carcinomas and its relation with well-established canine mammary tumor biomarkers. For that, expression of OPN, EGFR, HER2, and c-Kit were evaluated along with Ki67 rate in 43 mammary carcinomas. Osteopontin was demonstrated to be expressed by neoplastic epithelial cells in all carcinomas as well as in stromal cells from the tumor microenvironment. Relation between high osteopontin expression and EGFR positivity (P<0.001) and HER2 overexpression (P=0.012) was demonstrated. In conclusion, high OPN expression seems to be related to poor prognosis and MAPK pathway activation, given the association with EGFR and HER2, members of the MAPK signaling pathway.

INDEX TERMS: Dogs, cancer, osteopontin expression, prognostic biomarkers, canine mammary carcinomas, immunohistochemistry, epidermal growth factor receptor, human epidermal growth factor receptor 2.

RESUMO.- [**Expessão de osteopontina e sua relação com biomarcadores prognóstico nos carcinomas mamários caninos.**] A osteopontina é uma glicofosfoproteina implicada em diferentes processos fisiológicos e patológicos, sendo conhecida por estar envolvida na progressão e metástase de vários cânceres nos humanos, no entanto, essa relação é ainda pouco explorada na veterinária. O objetivo deste trabalho foi avaliar a expressão da osteopontina nos carcinomas mamários caninos e sua relação com biomarcadores bem estabelecidos para esta neoplasia. Para isto, foi avaliada a expressão de OPN, EGRH, HER2 e c-Kit juntamente com a taxa de Ki67 em 43 carcinomas mamários. A osteopontina foi expressa pelas células epiteliais neoplásicas em todos os carcinomas, assim como, nas células estromais do microambiente tumoral. Foi demonstrada uma relação entre uma alta expressão de osteopontina e positividade para EGFR (P<0.001) e superexpressão de HER2 (P=0.012). Em conclusão, alta expressão de OPN parece estar relacionada com mau prognóstico e ativação da via MAPK, devido a sua associação com EGRF e HER2, os quais são membros desta via de sinalização.

TERMOS DE INDEXAÇÃO: Cães, câncer, osteopontina, biomarcadores, carcinomas mamários caninos, imunohistoquímica, receptor de fator de crescimento epidérmico, receptor de fator de crescimento epidérmico humano 2.

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INTRODUCTION

Several studies have pointed cancer as the main cause of death for dogs in developed countries and 45% of dogs have over 10 years of age (Bronson 1982, Michell 1999, Proschowsky et al. 2003, Battisti et al. 2013, Dobson 2013, Daleck et al. 2016). In Brazil, the disease figures as the second most common cause of death for dogs (Fighera et al. 2008, Trapp et al. 2010, Andrade et al. 2012, Battisti et al. 2013, Daleck et al. 2016) and it is estimated that one in five dogs will develop cancer. The skin and subcutaneous tissue being the most prevalent followed by mammary, hematopoietic and bone tumors. Mammary tumors are the most common cancer diagnosed in women, likewise in female dogs (Dobson 2013, Pawlowski et al. 2013, Salas et al. 2015, Dias et al. 2016, Salas et al. 2016). Statistical surveys estimate that mammary neoplasms represent about 50% of all tumors afflicting female dogs, of which at least 40% are malignant (Brodey et al. 1983, Andrade et al. 2012, Feliciano et al. 2012, Battisti et al. 2013, De Nardi et al. 2013, Li et al. 2013, Pawlowski et al. 2013, Santos et al. 2013, Peña et al. 2014, Arias et al. 2015, Salas et al. 2015, Dias et al. 2016, Salas et al. 2016, Soler et al. 2016).

Advances in the diagnosis and therapy of the animals, the application of efficient measures in the prevention of infectious diseases through vaccination and deworming, in addition to improvements in the nutritional quality of dog food, have contributed towards a higher quality of life and longevity for dog (Dobson 2013, Salas et al. 2015), resulting in an increase in the diagnoses of neoplasms for the species (Pawlowski et al. 2013, Santos et al. 2013, 2014, Soler et al. 2016). Canine mammary tumours are highly heterogeneous in morphology and behavior and successful clinical management requires robust prognostic factors. The biological behavior of canine mammary neoplasms is widely variable in morphology and behavior, making validation and the use of tumor biomarkers to support the diagnosis and prognosis extremely important to successful clinical management (Graham & Myers 1999, Kandioler-Eckersberger et al. 2000, Arias et al. 2015, Damasceno et al. 2016b, Psyrri et al. 2017). Immunohistochemistry techniques may be useful to anticipate a diagnosis of cancer and to present prognostic information regarding the disease (Graham & Myers 1999, Kandioler-Eckersberger et al. 2000, Zacchetti et al. 2003, Peña et al. 2014, Santos et al. 2014, Arias et al. 2015, Damasceno et al. 2016a, Soler et al. 2016). Several biomarkers have been identified and associated with the survival rates of dogs afflicted with mammary neoplasms, such as the estrogen and progesterone hormone receptors (Sartin et al. 1992, Nieto et al. 2000), p53 (Lee et al. 2004) e-cadherin (Marmor et al. 2004), caspase-3 (West et al. 2008), cathepsin D (Lemmon & Schlessinger 2010) survivin (Bongiovanni et al. 2015), cell proliferation markers such as Ki-67 and PCNA (Zacchetti et al. 2003) epidermal growth factor receptor (EGFR) (Nieto et al. 2000, Rangaswami et al. 2006, Vollmann-Zwerenz et al. 2010, Arias et al. 2015, Elebro et al. 2016) and human epidermal growth factor receptor 2 (HER-2) (Sartin et al. 1992, Zacchetti et al. 2003, Carvalho et al. 2013, Ferreira et al. 2014, Peña et al. 2014, Silva et al. 2014, Burrai et al. 2015, Theocharis et al. 2015, Damasenco et al. 2016a, 2016b).

The receptors tyrosine kinase (RTK) of the ErbB family, known as EGFR/HER-1, erbB-2/HER-2, erbB-3/HER-3 and erbB-4/HER-4, play an important molecular control role as a signal for the development and maintenance of several organs and systems (Graham & Myers 1999, Nieto et al. 2000, Lee et al. 2004, Marmor et al. 2004, West et al. 2008, Damasceno et al. 2016b) since they are a group of primary mediators for the fundamental cell responses (Lemmon & Schlessinger 2010). Furthermore, it has been shown to have an important role to contribute to a better understanding of the progression mechanisms in malignant mammary tumors (Graham & Myers 1999, Bongiovanni et al. 2015, Damasceno et al. 2016b). Its role appears to be associated with increased angiogenesis and metastasis (Bongiovanni et al. 2015). In addition, another receptor tyrosine kinase (c-Kit) also plays an important role in cell proliferation and differentiation (Liang et al. 2013). These receptors have been widely studied due to recent discoveries regarding their involvement in the pathogenesis of hyperproliferative diseases such as cancer (Vollmann-Zwerenz et al. 2010, Liang et al. 2013, Elebro et al. 2016).

Recently, osteopontin (OPN), an adhesive glycophosphoprotein found in tissues and body fluids that is involved in both physiological and pathological processes (Rangaswami et al. 2006, Vollmann-Zwerenz et al. 2010, Liang et al. 2013, Shevde & Samant 2014, Elebro et al. 2016) has been widely pointed as a biomarker with potential prognostic implications for cancer due to its functional role over the tumor progression and metastasis control pathways (Rangaswami et al. 2006, Weber et al. 2010, Anborgh et al. 2011, Shevde & Samant 2014, Burrai et al. 2015, Damasceno et al. 2016a, Li et al. 2016, Psyrri et al. 2017, Wei et al. 2017). OPN belongs to the family of small integrin-binding glycoproteins related to N playing a key role in cell-matrix and cell-cell communication and interaction, modulating cellular behavior through autocrine and paracrine mechanisms (Fisher et al. 2001, Bellahcène et al. 2008). Studies have described its role in several development and differentiation processes in tissues, including bone (Yamate et al. 1997), skin (Chang et al. 2008) and mammary glands (Rittling & Novick 1997, Carvalho et al. 2013, Silva et al. 2014, Psyrri et al. 2017). In addition, it plays a key role in immune and inflammatory responses (Tuck & Chambers 2001, Rangaswami et al. 2006, Rittling & Singh 2015), including the healing process of wounds (Liaw et al. 1998).

The expression of OPN is up-regulated by many factors such as epidermal growth factor (EGF), transforming growth factor-Beta (TGF-β), tumor necrosis factor α (TNFα), interferon gamma (IFN-γ) and interleukin-1 β (IL-1β) (Rangaswami et al. 2006). Regarding pathological events, studies have shown that OPN is overexpressed in sepsis (Hirano et al. 2015), autoimmune diseases, cardiovascular diseases (Waller et al. 2010), neurodegenerative diseases (Carecchio & Comi 2011) and several tumors, especially carcinomas (Coppola et al. 2004, Anborgh et al. 2011, Shevde & Samant 2014). In the case of tumors, OPN's potential for predicting the prognosis was initially reported by Chambers et al. (1996), later, Tuck et al. (1997) have described the relationship between OPN overexpression and tumor progression in human mammary neoplasms, suggesting that OPN could be employed as a tumor prognostic marker both in tumor cells and in plasma.

There are few studies in the field of canine immunohistochemistry assessing the role of biomarkers in general, and OPN specifically, in tumor initiation and progression, as well as in the identification of patients with high disease recurrence risks. This study has analyzed the immunoexpression of OPN, EGFR, HER2, c-Kit

and Ki67 in *ex vivo* canine mammary carcinomas to obtain data supporting a better understanding of the role OPN plays and its relationship with other established biomarkers for this particular tumor.

MATERIALS AND METHODS

Tissue samples. The Brazilian Ethics Commission for the Use of Animals (protocol no. 88/2011 - CEUA) approved the study and the owners of all animals involved in the study have signed a Free and Clarified Consent Term authorizing the collection of material and the use of the data in research papers. Canine mammary carcinomas specimens (*n*=43) were collected at the time of surgical excision by research of the Investigative and Comparative Pathology Laboratory, "Universidade Estadual de São Paulo", Botucatu, Brazil, and it were used for the study. Samples were fixed in 10% neutral formalin and embedded in paraffin wax. Sections (4µm thick) were obtained and stained with hematoxylin and eosin for histological examination in order to confirm the diagnosis of mammary carcinoma. Tumor classification was defined according to the WHO classification of canine mammary tumors (Misdorp et al. 1999). Tumor sections were examined in an optical microscope (Zeiss® Axio Lab. A1) by three independent pathologists. For each slide, 10 fields were read with 400x magnification. Inter-observer variation was resolved by simultaneous re-evaluation.

Immunohistochemistry. For the immunohistochemistry assays, 3µm thick sections were obtained from paraffinized tissue blocks and subsequently deparaffinizated and rehydrated. The primary antibodies used in this study are summarized in Table 1. A polymer-based labeling system kit (NovoLink Polymer System, Novocastra Laboratories, Newcastle, UK) was used for detecting the antigen-antibody reaction and peroxidase and protein blockages. Antigen retrieval was carried out by heat treatment in 10mM citrate buffer, pH 6.0. After cooling (20 minutes at room temperature), sections were sequentially immersed in solutions provided in the kit according to manufacturer's instructions in order to block the endogenous activity of peroxidase and unspecific proteins. The slides were incubated overnight at 4°C with the specific antibodies. Subsequently, 3,3' diaminobenzidine (DAB) tetrahydrochloride was used as a chromogen in order to allow the visualization of antigen-antibody reaction. The slides were then counterstained using Harris's hematoxylin, dehydrated, and mounted for microscopic assessment.

Immunohistochemical results evaluation. The evaluation of the immunohistochemistry results was performed by three pathologists. OPN was considered positive whenever cytoplasmic staining was observed in the neoplastic and stromal cells. The assessment of OPN expression in neoplastic cells was performed semi-quantitatively using the Allred 8-unit system (Allred et al. 1993). In this scoring system, the tumor epithelial cells proportion score and intensity score were determined for each tumor, represented by one slide. The proportion score included the fraction of positively stained tumor cells and was as follows: 0 = none; 1 = <1/ 100th; 2 = 1/100th to 1/10th; 3 = 1/10th to 1/3; 4 = 1/3 to 2/3; 5 = >2/3. The estimated average staining intensity of positive tumor cells was expressed as follows: 0 = none; 1 = weak; 2 = intermediate; 3 = strong. For statistical purposes, an OPN score of 1–3 was considered low (+1), an OPN score of 4-6 was considered intermediate (+2), and an OPN score of 7-8 was considered high (+3). The expression of OPN was also evaluated in peritumoral inflammatory cells and tumor stromal cells.

The expression of HER2 was evaluated according to the Dako Cytomation Hercep Test scoring system: 0 = no staining or membrane staining in less than 10% of tumor cells; 1+ = faint, barely perceptible membrane staining in more than 10% of tumor cells, the cells are stained only in part of the membrane; 2+ = weak to moderate, complete membrane staining observed in more than 10% of tumor cells; and 3+ = strong, complete membrane staining in more than 10% of tumor cells. Cases were considered positive (overexpressed) for HER2 when immunostaining was characterized as 2+ or 3+. EGFR staining was classified in positive or negative, according to Dako's EGFR pharm Dx interpretation manual.

For Ki67, four categories were defined as follows: <10%, 10-25%, 26-50% and >50% of stained nuclei. For c-kit expression, the reaction product was evaluated along the cell membrane and in the cytoplasm. The quantity of immunoreactive cells was estimated according to the classification adopted by Biermann et al. (2007) none; <10%; 10–75%; and >75% of the cells. The level of immunoreactivity was assessed based on its predominant intensity: weak (+); moderate (++); and strong (+++).

The final immunoreactivity score was calculated as strong (4), when at least 75% of cells exhibited at least moderate immunoreactivity; in cases of weak immunoreactivity in <10% of all tumor cells, the final score was considered as (1). The score (0) was considered negative. The score (3) was assigned if weak immunoreactivity was present in >75% of tumor cells or if strong or moderate staining was observed in 10-75% tumor cells. All other cases were given a score of (2).

Positive and negative controls were included in each run in order to guarantee the reliability of the assays. For OPN, canine kidney was used. Additionally, a canine mammary carcinoma already recognized as HER-2 positive, a canine cutaneous mast cell tumor positive for c-Kit, and a canine cutaneous squamous cell carcinoma recognized as positive for EGFR and Ki67 were used. Rabbit and mouse IgGs (Dako, Carpinteria/CA) were used in tumor samples for negative control purposes.

Statistical analysis. Differences in the expression of proteins were compared using Fisher's exact test or Pearson's X2 test for qualitative variables. All statistical tests were two sided, and statistical significance was accepted at $P<0.05$. All analyzes were performed using the Prism GraphPad software version 5.0 (San Diego/CA).

Table 1. Antibodies used in the immunohistochemical study

Antibody	Clone	Dilution	Origin	Source
Osteopontin	LFMb-14	1:50	Novocastra Laboratories, UK	Mouse
Ki67	MIB1	1:50	Dako	Mouse
CD117	104D2	1:400	Dako	Mouse
HER-2	policlonal	1:2000	Dako	Rabbit
EGFR	NCL-EGFR	1:100	Novocastra Laboratories, UK	Mouse

RESULTS

Immunohistochemistry

Cytoplasmic OPN staining was observed consistently in neoplastic cells of all mammary tumors evaluated (43/43), always presenting an intermediate or high classification score (Fig.1); 12 of 43 (28%) samples presented intermediate score and 31 of 43 (72%) presented high score. We observed that all samples presented more than 50% of positive neoplastic cells and presented a high score variation due to the staining intensity. OPN positivity was observed mainly within the

cytoplasm, sometimes perinuclearly or, less commonly, in a cell surface distribution in the neoplastic cells. No sample was immunohistochemically negative for osteopontin.

Strong OPN expression was observed consistently in the epithelial component and in the myoepithelial cell layer, with cells presenting a cytoplasmic expression pattern. The surrounding stroma was usually OPN positive, since the OPN immunoexpression was also observed in the tumor stromal matrix component, stromal fibroblasts, vascular endothelial cells, muscular cells, macrophages and other inflammatory cells in all samples (Fig.2). Consistent OPN immunoexpression was also observed in areas with necrosis (Fig.3), chronic inflammation (Fig.4) and on invasive tumor borders (Fig.5). In addition, the weak OPN immunoexpression was observed in luminal mammary cells from the normal mammary tissue adjacent to neoplastic *foci* (Fig.5).

Relationship between osteopontin and other markers

From the 43 samples of mammary carcinoma evaluated, 34 (79.07%) were positive for HER2 and 36 (83.72%) were positive for EGFR. Additionally, 28 tumors with OPN high expression (90.3%) also presented HER2 overexpression, revealing a statistically significant association (P=0.012). For EGFR, 29 (93.5%) of the positive cases also presented high OPN expression, revealing a statistically significant relationship (P<0.001). All cases (43/43) were positive for c-Kit, presenting score variation between 1 and 4. There was no significant association with c-Kit (P>0.05). Likewise, 27 from the 31 cases with OPN overexpression (87%) revealed less than 25% neoplastic cells positive for Ki67. However, no statistically significant relationship could be noted between OPN and Ki67.

DISCUSSION

This study aimed at determining the expression level of the glycoprotein OPN in the stroma and neoplastic cells of canine mammary tumors, as well as its association with others proteins by means of immunohistochemistry assays. In humans, there are well documented reports showing the overexpression of OPN in malignant tissues and in the plasma, as well as the correlation with the tumor stage in the brain (Zakaria et al. 2016), mouth, esophagus, stomach, large intestine, liver,

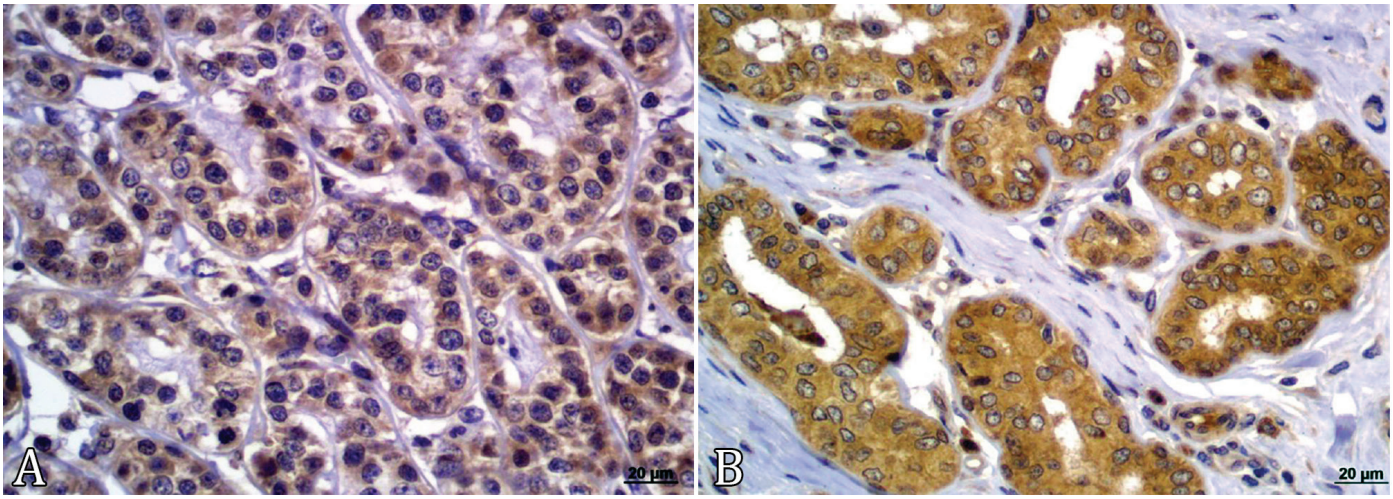

Fig.1. Osteopontin immunoexpression in canine mammary neoplasms. (**A**) Intermediate osteopontin immunoexpression, (**B**) high osteopontin immunoexpression. DAB immunohistochemistry, Harris hematoxylin counterstain, bar = 20µm.

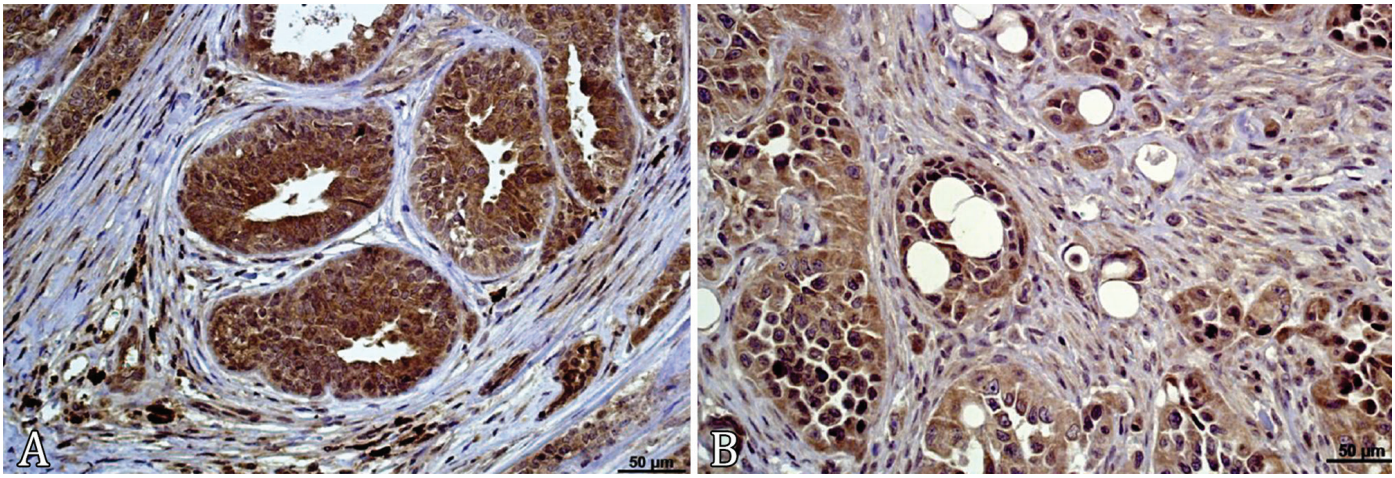

Fig.2. (**A**-**B**) Osteopontin-positive stromal cells, including inflammatory cells, can be observed within the neoplastic microenvironment. DAB immunohistochemistry. Harris hematoxylin counterstain, bar = 50µm.

pancreas, kidney (Coppola et al. 2004), ovaries, prostate (Tilli et al. 2014), lungs (Yan et al. 2015) and breasts (Bramwell et al. 2014). These studies clearly point towards the importance of this glycoprotein and its multidimensional ability to influence biological events associated with tumorigenesis and tumor progression, including the possibility of employing it as a molecular parameter in the prognostic evaluation of cancer patients. Since there are no similar reported studies in dogs, a clarification regarding its significance in canine mammary tumors was required. OPN-producing cells and OPN deposition in extracellular matrix were previously identified through IHC in canine bone and cartilage (Schnapper & Meyer 2004). Additionally, a quantitative real-time reverse transcription polymerase chain reaction array was established to quantify the expression levels of 49 genes relevant to carcinogenesis in laser-microdissected tumor cells of 10 benign and 13 metastatic canine mammary tumors (Klopfleisch et al. 2010). The study in question detected OPN in normal tissues, but observed no significant differences in the expression levels of osteopontin among any of the groups tested.

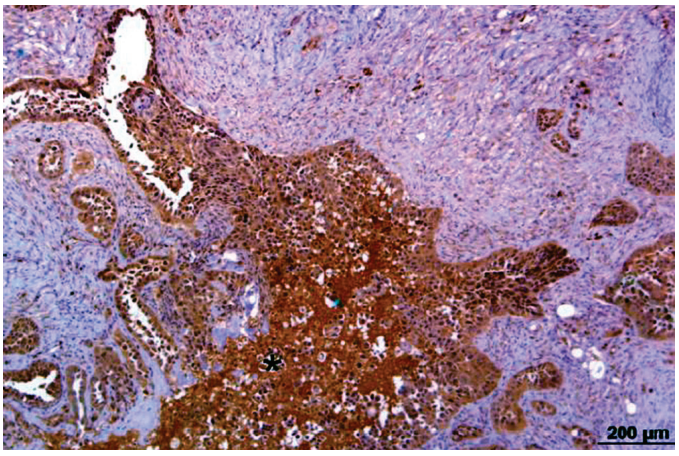

Fig.3. More intense OPN expression can be observed in necrotic areas (asterisk). DAB immunohistochemistry. Harris hematoxylin counterstain, bar = 200µm.

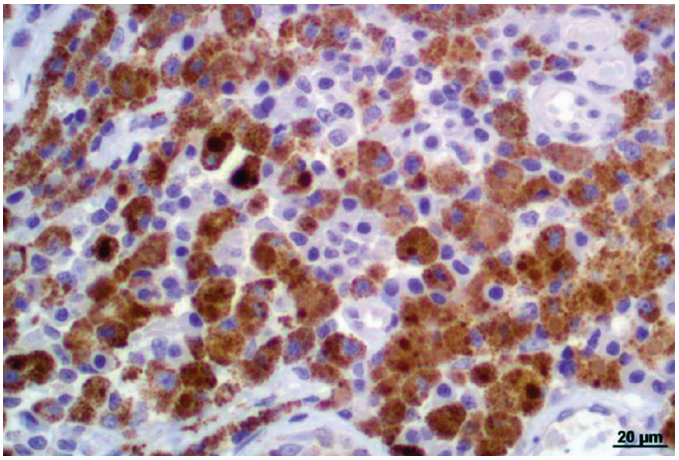

Fig.4. Osteopontin-positive macrophages. DAB immunohistochemistry. Harris hematoxylin counterstain, bar = 20µm.

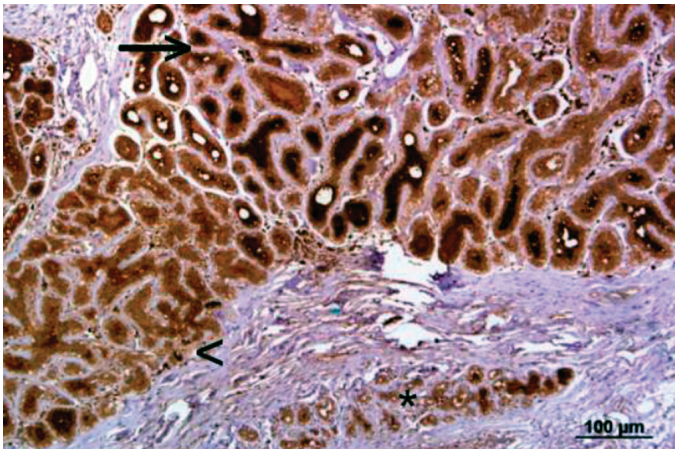

Fig.5. Osteopontin expression in normal (asterisk) tissue, neoplastic *foci* (arrows) and invasive tumors borders (arrowhead). DAB immunohistochemistry. Harris hematoxylin counterstain, bar = 100µm..

Presently, the scientific community fully accepts that OPN is expressed by tumor cells both in humans and animals and that it affects the malignant properties of neoplastic cells, specifically by affecting their ability to grow, invade, and metastasize. In addition, it is also known that OPN is expressed in both normal and malignant tissues (Shevde & Samant 2014, Ng et al. 2015) and effectively mediates many physiological and pathological events (Kahles et al. 2014).

In this study, we have found that all carcinomas were stained positively for OPN and, according to the immunohistochemical grading system employed; tumors presented consistently highly expression levels for the protein. This finding is in accordance to studies that focused on OPN levels in women breast tumor tissue (Tuck & Chambers 2001, Coppola et al. 2004, Rodrigues et al. 2009, Bramwell et al. 2014), since authors report a high frequency of OPN-positive samples. For example, some authors reported a positivity of 88.4% for OPN in metastatic human brain tumors (Zakaria et al. 2016) and a positivity of 100% in human hepatocellular carcinomas (Tsai et al. 2012) similarly to what was observed in this study. Although canine mammary tumors may occasionally be histologically and biologically different than human breast cancer, the results regarding OPN immunoexpression seems to be in concordance. This study and that of others (Tuck & Chambers 2001, Rodrigues et al. 2009, Luo et al. 2011) reported that OPN is expressed both in the epithelial and stromal components of neoplastic wounds. In humans, OPN immunoexpression is also observed outside neoplastic cells and reveals a variable staining pattern in which luminal cells from normal mammary tissue, luminal tumor cells, stromal fibroblasts, macrophages, lymphocytes, and blood vessels are weakly to highly stained (Kim et al. 1998, Tuck et al. 1998, Tuck & Chambers 2001, Rodrigues et al. 2009, Luo et al. 2011).

In this study, it was observed that cells from the tumor microenvironment and cells adjacent to the tumor presented OPN positivity in all samples. Studies have shown that, aside from being expressed in different cell types, including immune system cells, the expression of OPN is highly increased during the inflammatory process (Rittling & Singh 2015) and regulated positively through several growth factors and cytokines, including LPS, Ang II, NO, IL-1b, IL-2, IL-3, IFN-g, TNF-α and TGF-β (Denhardt et al. 2003). In our study, we observed a more intense OPN immunoexpression in cells near necrotic or inflammatory *foci*, as well as in invasive tumor areas, in

agreement with others reports (Brown et al. 1994, Hirota et al. 1995, Tuck et al. 1998). These findings suggest that those cell types may contribute to OPN production levels. However, our understanding regarding the molecular mechanisms involved in the regulation of OPN expression remains incomplete (Kahles et al. 2014). The presence of OPN in the tumor stroma and on the surface of tumor cells interfacing with the stroma suggests that this glycoprotein may participate in adhesive interactions at the tumor/normal tissue interface. Studies have shown that OPN overexpression, especially the OPNb and OPNc variants, at esophageal adenocarcinoma enhances tumor cell invasion and metastasis (Lin et al. 2015) and also contribute towards macrophage adhesion and migration (Brown et al. 1994).

In a previously reported study (Rodrigues et al. 2009) using invasive human breast cancer cases, no statistically significant association was reported between stromal OPN expression, major clinical and pathological parameters, and some of the most commonly used molecular markers for those tumors. Whether epithelial and stromal OPN has distinct roles during neoplastic development and progression is an important question to be further addressed, but it seems to be related to metastasis in various neoplasms (Brown et al. 1994).

In this study, different tumor markers were tested for their association with OPN in canine mammary carcinomas, but only EGFR and HER2 showed a statistically significant relationship with OPN-positive immunostaining scores. These components are part of the MAPK signaling pathway, which is recognized as an important pathway for carcinogenesis, particularly for the epithelia (Sebolt-Leopold & Herrera 2004). The findings suggest an osteopontin-associated activation of the MAPK pathway in canine mammary neoplasms, which is in agreement with the findings presented by other authors (Brown et al. 1994, Frey et al. 2007), revealing a possible relationship between OPN and the MAPK pathway in breast cancer (Tuck et al. 2003, Rodrigues et al. 2009), lung adenocarcinomas (Frey et al. 2007), hepatocellular carcinoma (Tsai et al. 2012) and in actinic keratosis/cutaneous squamous cell carcinomas (Luo et al. 2011) in humans. However, this finding was reported to be absent in other tumor histotypes such as mesothelioma (Frey et al. 2007), suggesting that this alteration may not be a distinctive feature for all tumor types.

In dogs, the expression of the erbB family components was previously evaluated in canine mammary tumors. A relatively high expression of the *ERBB1* and *ERBB2* genes suggests an important contribution to carcinogenesis in canine mammary tumors (Matsuyama et al. 2001, Singer et al. 2012). The overexpression of tyrosine kinase receptors EGFR and HER2 - proteins derived from *ERBB1* and *ERBB2* genes, respectively - is observed in many human cancers including bladder, breast, colon, and lung cancers (Eccles et al. 1995). HER2 overexpression is usually associated with poor prognosis indicators in canine mammary tumors such as tumor size, high histological grade, invasion, and high proliferation rates (De las Mulas et al. 2003).

Regarding the relationship between HER2 and OPN, Rodrigues et al. (2009) also did not find any association in women. However, this association was statistically significant in the tumors studied by our group. This finding may indicate the existence of a co-regulator expressed differently in human and canine mammary neoplasms, leading to the activation of this specific RTK in the canine counterpart.

In this study, 83.72% of the samples were positive for EGFR, a protein previously described to be related to reduced cure and overall survival rates in canine mammary tumors (Gama et al. 2009). We have also identified a statistically significant relationship between high OPN expression and EGFR positivity, which we believe to be a synergistic and complementary relationship between the molecules, in accordance to what was reported by other authors in human breast cancer (Tuck et al. 2003, Rodrigues et al. 2009) and hepatocellular carcinomas (Tsai et al. 2012).

For the latter, higher OPN and EGFR expression were significantly associated with advanced histological grades, advanced pathological stages, and poor survival rates (Hirota et al. 1995). Cell migration regulated by OPN is said to be dependent on the epidermal growth factor (EGF) and hepatocyte growth factor (HGF). OPN induces EGF receptor (EGFR) mRNA expression, EGFR tyrosine kinase activity, HGF receptor (Met) mRNA and protein expression, as well as increasing Met kinase activity during tumor cell migration in human mammary cancer cell lines (Tuck et al. 2003).

Previous reports indicated that ligation of OPN with integrin leads to c-Src-dependent transactivation of EGFR, resulting in the activation of downstream signaling pathways, including PI3-k, Ras–MAPK, phospholipase C, and protein kinase C (PKC) in cancer cells (Tuck et al. 2003). The transformation of epithelial cells induced by tissue-specific overexpression of EGFR *in vivo* provides direct evidence of the role EGFR plays in carcinogenesis (Yarden & Sliwkowski 2001).

These features which indicate a relationship between EGFR/HER2 and OPN overexpression are interesting since an arsenal of antibodies and tyrosine kinase inhibitors for growth factor receptors targeted to the MAPK pathway are currently either in development or already in clinical use, consequently being expected to be effective against tumors overexpressing OPN. Various therapeutic agents directed against EGFR and HER2 have provided promising alternatives to traditional chemotherapy in the search for better treatments for cancers overexpressing these tyrosine kinase receptors (Kamath & Boulamwini 2006). This could represent an evolution in the conventional treatment of canine mammary tumors focused in highly OPN expressing neoplasms.

CONCLUSION

Osteopontin overexpression is related to EGFR and HER2 expression in canine mammary tumors, probably by the activation of the MAPK signaling pathway. Although the mechanisms involving OPN and the progression of canine mammary carcinomas remain unknown, this study suggests that OPN, EGFR, and HER2 play important roles in canine mammary tumors carcinogenesis. These findings raise the question of whether is possible to use specific drugs to block the signaling pathway in OPN overexpressing tumors.

Conflict of interest. - The authors do not have any conflicts of interest to declare.

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Original Article
Wildlife Medicine

Investigation of *Mycoplasma* spp. in birds of the Rio de Janeiro Zoo by isolation and PCR[1]

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ABSTRACT.- Magalhães B.S.N., Pereira V.L.A., Dias T.S., Machado L.S., Silva M.M., Nascimento E.R., Mendes-de-Almeida F. & Almosny N.R.P. 2020. **Investigation of *Mycoplasma* spp. in birds of the Rio de Janeiro Zoo by isolation and PCR.** *Pesquisa Veterinária Brasileira 40(3):210-215*. Faculdade de Veterinária, Universidade Federal Fluminense, Rua Vital Brazil Filho 64, Niterói, RJ 24230-340, Brazil. E-mail: barbaraneil@hotmail.com

Brazil is one of the countries with the most abundant avifauna in the world. The confinement of birds associated with close contact with other animals and humans favor the spread of agents of respiratory diseases. Among them, mycoplasmas can cause asymptomatic or apparent disease that manifests in birds by coughing, sneezing, rales, conjunctivitis, ocular and nasal discharge. Several described mycoplasmas cause disease in birds, especially *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS). The diagnosis of *Mycoplasma* spp. can be done by clinical observation and laboratory analysis. Molecular diagnosis by PCR was boosted by its speed, sensitivity, and low cost of agent isolation techniques that take up to 21 days to complete. This study aimed to verify the occurrence of *Mycoplasma* spp. in birds of the Rio de Janeiro Zoo (Rio Zoo), by isolation and PCR. Of the total 635 birds from the Rio Zoo, 81 were studied for detection of *Mycoplasma* spp., when taken for routine health assessment exams. These birds belonged to the following orders: Psittaciformes (45), Accipitriformes (18), Galliformes (7), Piciformes (5), Strigiformes (4), Falconiformes (1) and Cariamiformes (1), all individuals already identified by microchip or leg-ring. There was no isolation of mycoplasmas in any of the samples tested, whereas, in the PCR, 62.96% (51/81) were positive, with 1.96% (1/51) identified as MG and 19.61% (10/51) as MS, representing 1.23% (1/81) and 12.34% (10/81) of the total population studied. PCR was shown to be a more effective technique than isolation in the detection of *Mycoplasma* spp. in birds. It was possible to detect mycoplasmas in birds from Riozoo with no clinical respiratory signs, with higher MS prevalence than MG. The positivities for *Mycoplasma* spp., MS, and MG were different among the orders studied, being the highest occurrence in birds of prey, followed by Galliformes and Piciformes. The presence of MG and MS in birds of Rio de Janeiro Zoo confirms the circulation of these agents and the need for further studies on the dissemination of mycoplasmas in zoos for the epidemiological analysis of these bacteria in these places.

INDEX TERMS: Investigation, *Mycoplasma* spp., birds, Rio de Janeiro, Brazil, zoo, captive.

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RESUMO.- [**Investigação de *Mycoplasma* spp. em aves do Zoológico do Rio de Janeiro por isolamento e PCR.**] O Brasil é um dos países com maior avifauna do mundo. O confinamento de aves associado ao contato próximo a outros animais e seres humanos favorece a disseminação de agentes etiológicos causadores de doenças respiratórias. Dentre eles, os micoplasmas podem causar doença assintomática ou aparente que se manifesta em aves por espirros, estertores,

conjuntivite, corrimentos oculares e nasais. São diversos os micoplasmas descritos causadores de doença em aves, com destaque para *Mycoplasma gallisepticum* (MG) e *Mycoplasma synoviae* (MS). O diagnóstico de *Mycoplasma* spp. pode ser feito pela observação clínica e análises laboratoriais. O diagnóstico molecular pela Reação em Cadeia da Polimerase (PCR) ganhou impulso por sua rapidez, sensibilidade e baixo custo em relação às técnicas de isolamento do agente que levam até 21 dias para conclusão do gênero *Mycoplasma*. Objetivou-se verificar a ocorrência da infecção por *Mycoplasma* spp. em aves no Zoológico do Rio de Janeiro (Rio Zoo), por isolamento e PCR. Do plantel de 635 aves do Rio Zoo, foram estudadas 81 para detecção de *Mycoplasma* spp., quando contidas para exames rotineiros de avaliação da condição de saúde. Essas aves eram pertencentes às ordens Psittaciformes (45), Accipitriformes (18), Galliformes (7), Piciformes (5), Strigiformes (4), Falconiformes (1) e Cariamiformes (1), todas já identificadas por microchip ou por anilha. Não houve isolamento de micoplasmas em nenhuma das amostras testadas, enquanto na PCR, 62,96% (51/81) foram positivas, sendo 1,96% (1/51) identificadas como MG e 19,61% (10/51) como MS, representando 1,23% (1/81) e 12,34% (10/81) da população total estudada. A PCR demonstrou ser uma técnica mais efetiva que o isolamento na detecção de *Mycoplasma* spp. em aves. Foi possível detectar micoplasmas nas aves do Riozoo sem sinal clínico respiratório, tendo MS maior prevalência do que MG. As positividades para *Mycoplasma* spp., MG e MS foram diferentes entre as ordens de aves estudadas, sendo a maior ocorrência nas aves de rapina, seguida dos Galliformes e dos Piciformes. A presença de MG e MS nas aves do Rio de Janeiro Zoo confirma a circulação destes agentes e a necessidade de mais estudos sobre a disseminação de micoplasmas em zoológicos para análise epidemiológica dessas bactérias nesse local.

TERMOS DE INDEXAÇÃO: Investigação, *Mycoplasma* spp., aves, zoológico, Rio de Janeiro, Brasil, PCR, cativeiro.

INTRODUCTION

The birds are the most studied and valued group of animals in the world with more than 11,000 different species and an extraordinary variety. Some occur in abundance, and others represented by a few remaining individuals. It is estimated that in Brazil, there are from 1600 to 1900 bird species, more than 10% of them endemic, which makes it to be considered one of the most avifauna countries in the world (Lewinsohn & Prado 2005). To present these different bird species to the public, zoos around the world have facilities for the maintenance of these captive animals. However, the confinement of animals, associated with their contact with visitors and breeders, make the transmission and dissemination of pathogens a specific risk in these places (Cubas 2008, Loria et al. 2008). Among these agents are those that cause respiratory diseases, with emphasis on mycoplasmas that can cause apparent or subclinical disease in birds (Nascimento & Pereira 2009). Its spread occurs directly or indirectly horizontally through people, other animals, feed, water, and fomites. In addition to this pathway, vertical transmission via egg or venereal may occur through mating or artificial insemination (Stipkovits & Kempft 1996, Nascimento & Pereira 2009).

Clinical signs commonly seen in wild and captive birds are sneezing, rales, eye and nasal discharge, unilateral conjunctivitis, or bilateral or not accompanied by enlargement of the infraorbital sinus and may be associated with chronic infections (Phalen et al. 2006). Among the main species of the genus *Mycoplasma*, stand out *M. gallisepticum* (MG) and *M. synoviae* (MS) which, cause an apparent or asymptomatic respiratory condition. But other species such as *M. gypis*, *M. vulturii*, *M. gallinarum*, *M. gallinaceum*, *M. iners* and *M. corogypsi* have been described as causing disease in birds (Poveda et al. 1990, Panangala et al. 1993, Fischer et al. 1997, Oaks et al. 2004).

To reduce the risk of these infections in bird populations and to assess the sanitary control of poultry, it is crucial to know and monitor these mycoplasmas in the environment by epidemiological surveys and laboratory tests associated with the development and implementation of strict standards and procedures in livestock (Vilani 2006). The mycoplasmic diagnosis can be presumptive with clinical and epidemiological, anatomopathological, serological, and etiological evaluations by agent isolation or PCR (Nascimento et al. 2005, Umar et al. 2017). Because these microorganisms are tedious, requiring enriched culture media, and they are slow to grow exponentially, bacteriological isolation has been used in conjunction with molecular techniques. PCR is fast, sensitive, requires a low laboratory cost, and does not allow the need for isolation from clinical specimens (Islam et al. 2011). Therefore, the objective was to verify the occurrence of *Mycoplasma spp.* in birds of different orders at the "Zoológico do Rio de Janeiro" (Rio Zoo) by isolation and PCR.

MATERIALS AND METHODS

The project was submitted and approved under no. 1017 by "Comissão de Ética no Uso de Animais" (CEUA) of "Universidade Federal Fluminense" and by "Sistema de Autorização e Informação em Biodiversidade" (SISBIO-ICMBio) under number 59538-1. In addition, the project was registered in "Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado" (SISGEN), no. A4E34FC.

Material collection. From the 635 birds of Rio Zoo, 81 were studied, belonging to the order Psittaciformes (45), Accipitriformes (18), Galliformes (7), Piciformes (5), Strigiformes (4), Falconiformes (1) and Cariamiformes (1) (Table 1), all already identified by microchip or washer. The animals were manually restrained for routine health assessment and samples collected of swab tracheal, which were conditioned in microtubes containing modified Frey's liquid medium (Nascimento 2000). The microtubes containing the swabs were kept refrigerated until the time of laboratory processing.

Isolation of *Mycoplasma* spp. An aliquot of 0.2mL of the collected sample was inoculated into 1.8mL of the modified Frey liquid medium. Serial dilutions were made until 10^{-5}, and the dilutions 10^{-3} and 10^{-5} were seeded on plates containing modified Frey solid medium (Nascimento 2000). All samples were incubated at 37°C under microaerophilic and observed for 21 days under a 100x magnification stereoscopic microscope (Razin et al. 1998).

DNA extraction. A 500µl aliquot of the collected sample was submitted to DNA extraction by the phenol-chloroform adapted method (Sambrook & Russell 2006). Each sample was then homogenized and centrifuged at 13,500rpm at 10°C for 20 minutes. After centrifugation, the supernatant was discarded, and 400µL of Tris Ethylenediaminetetraacetic acid (TE) dextrose, 30µl 10% sodium dodecyl sulfate (SDS) and 30µl proteinase K 240µg/µl were added to the pellet. The sample was taken to the thermal block at 50°C for

30 minutes with a subsequent ice bath for 5 minutes. Subsequently, 500µL of phenol was added to the samples, homogenized by inversion for 15 minutes, and then centrifuged at 13,500rpm at 10°C for 30 minutes. The supernatant was removed and added to a new microtube with the same volume of chloroform, followed by gentle homogenization for 3 minutes and centrifugation under the conditions already described. The supernatant was removed, added to a 1ml microtube of ethyl alcohol, and precipitated "overnight." The precipitated DNA was centrifuged at 13500rpm at 10°C for 20 minutes and the pellet after drying; it was resuspended in 100µL

Table 1. Order, species, common name and number of birds of the Rio de Janeiro Zoo evaluated for the detection of *Mycoplasma* spp.

Order	Species	Common name	Number	Total
Psittaciformes	*Amazona aestiva*	Real parrot	9	
	Amazona ocrocephala	Puffin parrot	1	
	Ara ararauna	Canary macaw	11	
	Ara macao	Scarlet macaw	3	
	Ara chloropterus	Red macaw	3	45
	Anodorhynchusleari	Lear's macaw	4	
	Guaruba guarouba	Ararajuba	10	
	Pyrrhura frontalis	Red-fronted tiriba	1	
	Pionus maximilliani	Green parrot	3	
Accipitriformes	Amadonastur lacernulatus	Pigeon hawk	3	
	Geranoaetus albicaudatus	White-tailed hawk	6	
	Leptodon cayanensis	Gray-headed hawk	1	18
	Heterospizias meridionalis	Leather-tailed hawk	4	
	Rupornis magnirostris	Carijó hawk	4	
Falconiformes	*Milvago chimachima*	Yellow-tailed hawk	1	1
Galliformes	*Pavo muticus*	Peacock	2	
	Crax alector	Black porcupine	2	
	Crax fasciolata	Pinum curassow	1	7
	Chrylophus pictus	Canary pheasant	1	
	Pavo cristatus	Blue peacock	1	
Piciformes	*Ramphastos toco*	Toucan toco	5	5
Strigiformes	*Strix huhula*	Black owl	1	4
	Pseudoscops clamator	Eared owl	3	
Cariamiformes	*Cariama cristata*	Seriema	1	1
TOTAL				81

Table 2. Primer oligonucleotides for PCR for detection of avian *Mycoplasma* with their sequences, amplified product size, and reference

"Primers"	Sequence	Product	Reference
Mspp GPO3	5´GGGAGCAAACAGGATTAGATACCCT3´	270 pb	Van Kuppeveld et al. (1992, 1993)
Mspp MGSO	5´TGCACCATCTGTCACTCTGTTAACCTC3´		
MG-f	5´CGTGGATATCTTTAGTTCCAGCTGC3´	481 pb	Nascimento et al. (2005)
MG-r	5´GTAGCAAGTTATAATTTCCAGGCAT3´		
MS-f	5´GAGAAGCAAAATAGTGATATCA3´	207 pb	Lauerman etal. (1993)
MS-r	5´CAGTCGTCTCCGAAGTTAACAA3´		

Mspp f and Mspp r = *Mycoplasma* spp., MG-f and MG-r = *Mycoplasma gallisepticum,* MS-f and MS-r = *Mycoplasma synoviae.*

of TE buffer, quantified in Biodrop Touch® (Biochrom) and stored at -20°C until PCR.

PCR. The extracted DNA was submitted to PCR for the detection of *Mycoplasma* spp., *Mycoplasma gallisepticum* (MG), and *Mycoplasma synoviae* (MS) respectively, according to Van Kuppeveld et al. (1992, 1993), Nascimento et al. (2005) and Lauerman et al. (1993). The PCR for detection of *Mycoplasma* spp. was performed in 25µl final volume containing 2µl isolated DNA, 1 × PCR buffer (10mM Tris-HCl, pH 8.0 and 50mM of KCl), 2mM MgCl2, 0.2mM deoxynucleotide triphosphate (dNTP), 0.2mM forward and reverse primers and 1U Taq polymerase. For MG detection the reaction contained: 1X PCR buffer; 2mM MgCl2; 0.2mM dNTP; 0.2nmol of each specific primer (Table 2); 1U Taq Polymerase (Ludwig, Brazil) and extracted DNA totaling 25µl. The MG PCR was performed under the following conditions: 95°C for 5 minutes, followed by 40 cycles of 95°C for 1 minute, 55°C for 2 minutes, and 72°C for 1 minute, with a final phase of 72°C for 5 minutes. The MS ATCC 25204 and MG ATCC 129 S6 strains were used as positive controls and as ultrapure water negative control. For MS the reaction contained 1X PCR buffer (10mM Tris-HCl, pH 8.0 and 50mM KCl); 1.5mM MgCl2; 0.2mM deoxyribonucleotide triphosphate (dNTP); 0.2nmol of each specific primer (Table 2); 1U Taq Polymerase, and extracted DNA, totaling 25µl. MS PCR was performed under the following conditions: 94°C for 1 minute, followed by 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, with a final phase of 72°C for 5 minutes.

Electrophoresis. The amplicons obtained in PCR were applied in 1.5% agarose gel, submerged in Tris-Borato-EDTA Buffer (TBE), and then submitted to electrophoretic run at 94V for 40 minutes. After the electrophoretic run, the gel was stained with ethidium bromide, and visualization of the amplicons was performed under ultraviolet light in a transilluminator.

Statistical analysis. Descriptive statistics were performed to obtain the percentages for *Mycoplasma* spp., MG, and MS for order taxonomic of birds. The Mann-Whitney non-parametric test was used to differentiate between the percentages obtained, which uses the median for comparative effects, with a significance level of 5% using the Bioestat 5.3® software (Ayres et al. 2007).

RESULTS AND DISCUSSION

Of the 81 birds studied, none showed clinical respiratory signs at the time of collection. All birds were negative for isolation for *Mycoplasma* spp., while PCR for *Mycoplasma* spp. detected 62.96% (51/81) of birds 1.96% (1/51) for MG and 19.61% (10/51) for MS, respectively representing 1.23% (1/81) and 12.34% (10/81) of the poultry total population studied (Table 3). In the statistical analysis by the Mann-Whitney test, the differences in percentages obtained between the orders about *Mycoplasma* spp. and MS, were significant ($p<0.05$), excluding the occurrence of MG because there was a single record during the study. Asymptomatic infections are the most common in mycoplasma-infected animals because these agents can escape of host immune system and remain dormant (Razin et al. 1998). Besides, the presence of low pathogenicity strains may cause a mild or inapparent clinical picture (Lecis et al. 2010).

The Falconiform and Cariamiform orders had only one individual tested in each of them, both being PCR positive for *Mycoplasma* spp., and the falconiform identified as MS positive. Without considering these specimens, the highest percentage of positive results for *Mycoplasma* spp. was observed in the Accipitriformes order, with 88.89% (16/18), 22.22% (4/18) identified as positive for MS. Then, in Galliformes, 71.43% (5/7) of positive birds were found, with 14.29% (1/7) characterized with MS; Piciform, 60.00% (3/5) for *Mycoplasma* spp. and 20.00% (1/5) for MS; Psittaciformes, 53.33% (24/45) for *Mycoplasma* spp., 2.22% (1/45) for *M. gallisepticum* (MG) and 6.67% (3/45) for MS. The order of lowest occurrence was Strigiformes with 25.00% (1/4) for Mycoplasma spp., but the species was not identified.

The results found for Psittaciformes contrast the studies of Silva et al. (2016) and Carvalho et al. (2017) when assessing the presence of *Mycoplasma* spp. in asymptomatic parrots under human care. Silva et al. (2016), when analyzing 85 parrots of different species from a zoo in Pernambuco, reported the presence of *Mycoplasma* spp. in 16.47% of the birds, however these mycoplasmas were not identified as MG or MS. Carvalho et al. (2017), when evaluating 300 samples of CETAS parrots, commercial and conservation breeding, observed positivity for *M. gallisepticum* (MG) in 21.6% (16/74) in CETAS, 15.7% (19/121) in commercial breeding, and 6.7% (7/105) in conservationist, while for MS the occurrences were 2.7 % (2/74) in CETAS, 0.0% (0/121) in commercial breeding and 1.9% (2/105) in conservationist. MG was still described with a high occurrence by Gomes et al. (2010) in parrots from the seizure of trafficking with the positivity of 85.4%.

Table 3. Isolation and PCR detection of *Mycoplasma* spp., *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by the order of the birds of Rio de Janeiro Zoo

Order	Isolation	PCR		
	Mspp.*	Mspp.*	MG**	MS***
Psittaciformes	0/45 (0%)	24/45 (53.33%)	1/45 (2.22%)	3/45 (6.67%)
Accipitriformes	0/18 (0%)	16/18 (88.89%)	0/18 (0.00%)	4/18 (22.22%)
Falconiformes	0/1 (0%)	1/1 (100.00%)	0/1 (0.00%)	1/1 (100.00%)
Galliformes	0/7 (0%)	5/7 (71.43%)	0/7 (0.00%)	1/7 (14.29%)
Piciformes	0/5 (0%)	3/5 (60.00%)	0/5 (0.00%)	1/5 (20.00%)
Strigiformes	0/4 (0%)	1/4 (25.00%)	0/4 (0.00%)	0/4 (0.00%)
Cariamiformes	0/1 (0%)	1/1 (100.00%)	0/1 (0.00%)	0/1 (0.00%)
TOTAL	0/81 (0%)	51/81(62.96%)	1/81 (1.23%)	10/81(12.3%)

* Mspp.= *Mycoplasma* spp., ** MG = *Mycoplasma gallisepticum*, *** MS = *Mycoplasma synoviae*.

Lecis et al. (2016) analyzed samples of 62 birds of prey from two Wildlife Centers and a Veterinary Hospital in Italy, obtaining positivity for *Mycoplasma* spp. in 41.9% (26/62) of them. In our study, we analyzed samples of three orders of birds considered of prey, Falconiformes, Accipitriformes, and Strigiformes in which we found positivities ranging from 25.00% to 88.89%, that is, with a mean positivity of 56.95%, with the prevalence obtained being higher than that presented by other authors. This difference may be due to the proximity between the nurseries, which may have favored or predisposed to mycoplasma infections (Kleven & Fletcher 1983). Lecis et al. (2016) observed clinical symptoms compatible with mycoplasmosis in a positive individual; in the present study, such occurrence was not observed, despite the high frequency of mycoplasma positive birds. The high frequency found in asymptomatic prey corroborates other studies where a high frequency of mycoplasma was observed in birds of prey with mild or inapparent clinical presentation (Lierz et al. 2008, Lecis et al. 2010, Ziegler et al. 2019).

For Kleven (1998), there may be a preference for MG and MS for Galliformes. However, in our study, the positivity for *Mycoplasma* spp. in this order was 71.43% (5/7), none positive for MG, and 14.29% (1/7) identified as MS in the wild Galliformes tested. Michiels et al. (2016) tested 15 wildlife Galliformes in Belgium, but none were positive; however, Haesendonck et al. (2014) demonstrated a high prevalence of 76.3% for MG and 36.0% for MS in Galliformes reared for ornamental purposes in the same country, suggesting that these birds may serve as a reservoir for these agents.

The high prevalence of *Mycoplasma* spp. in birds of the zoo, observed in this study, is important because birds with or in the subclinical state contribute to the maintenance of mycoplasmas in environments. Besides, its transmission occurs by diffusion in the form of aerosol or droplets, being the upper respiratory tract and conjunctiva the main entry doors of the pathogen (Nascimento & Pereira 2009, Stipkovits & Szathmary 2012). These agents are capable of causing respiratory signs, as well as severe reproductive disease in birds (Carnaccini et al. 2016), constituting a significant challenge for the conservation and rearing of wild and captive birds, because in addition to impairing rehabilitation, causes high mortality of embryos as well as young and adult birds (Gomes et al. 2010).

CONCLUSIONS

PCR was more effective than the isolation technique in detecting *Mycoplasma* spp. It was possible to detect mycoplasmas in birds of different orders without a clinical respiratory sign, obtaining *Mycoplasma synoviae* (MS) more prevalent than *Mycoplasma gallisepticum* (MG). The positivities for *Mycoplasma* spp., MG, and MS were different among the studied orders, being the highest occurrence in birds of prey, followed by Galliformes and Piciformes.

The presence of MG and MS in the birds of Rio Zoo confirms the circulation of these agents and the need for further studies on the dissemination of mycoplasmas in zoos for epidemiological analysis of these bacteria in this location.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3. **Apresentação das Figuras:**

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

b) Enviar cada figura separadamente.

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

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

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

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

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

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

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

Exemplos de Referências

➢ Artigos publicados em periódicos:

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

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

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

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

- Livros:

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

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

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

- Capítulos de livros:

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

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

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

- Dissertações e Teses:

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

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

- Resumos publicados em eventos:

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

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

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

Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

GUIDE FOR AUTHORS

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

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

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

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

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

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

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

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

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

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

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

The author rights of the accepted papers are preserved.

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

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

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

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

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

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

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

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

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

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

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

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

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

m) Conflict of interest or none should be mentioned.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3. **Figure presentation:**

a) Save images at 300 dpi, TIF files.

b) Send each figure separately.

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

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

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

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

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

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

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

Exemples for References:

➢ Articles published in scientific journals:

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

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

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

➢ Books:

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

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

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

➢ Chapters of books:

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➢ Dissertations and Theses:

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

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

➢ Abstracts published in Events:

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

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(Note: Consult entire papers instead of only Abstracts)

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