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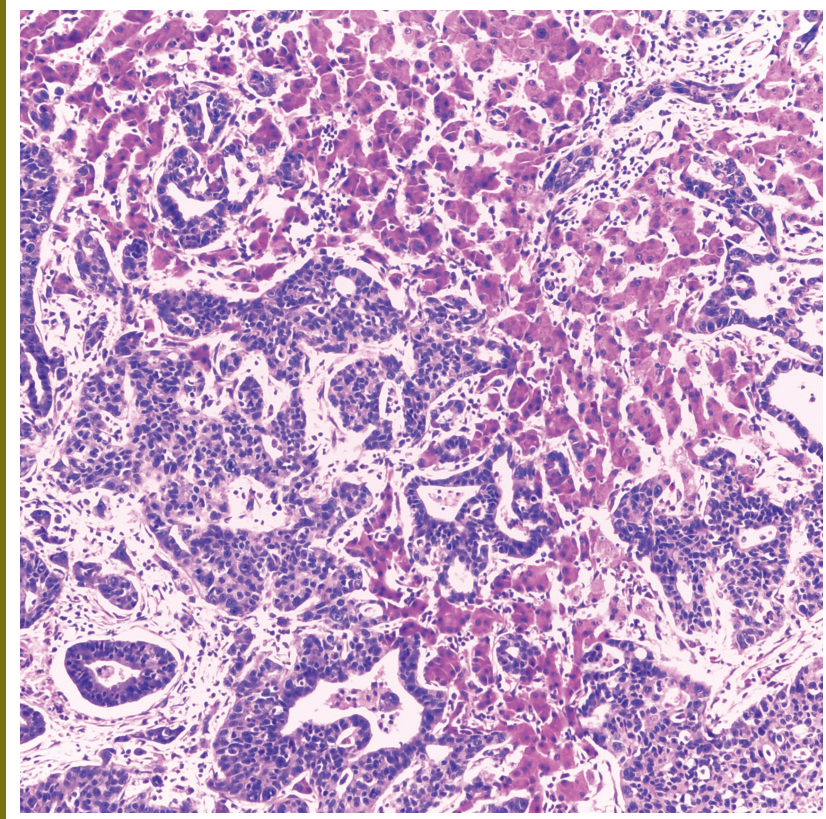

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Cover illustration: Cholangiocarcinoma in a cat. Replacement of the hepatic parenchyma by neoplastic epithelial cells arranged as acni and ducts (Argenta et al., p.49)

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Original Article
Livestock Diseases

Spontaneous and experimental poisoning by *Froelichia humboldtiana* in cattle[1]

Givaldo B. Silva Filho[2], Hisadora A.S. Chaves[2], Raquel F. Albuquerque[2], Paulo E. Souza[3], Maria E.Q. Vieira[4], Ana L.O. Nascimento[5], Stephanie C. Lima[5] and Fábio S. Mendonça[5]*

ABSTRACT.– Silva Filho, G.B.S., Chaves H.A.S., Albuquerque R.F., Souza P.E., Vieira M.E.Q., Nascimento A.L.O., Lima S.C. & Mendonça F.S. 2020. **Spontaneous and experimental poisoning by *Froelichia humboldtiana* in cattle.** *Pesquisa Veterinária Brasileira 40(1):1-6.* Laboratório de Diagnóstico Animal, Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. E-mail: fabio.mendonca@pq.cnpq.br

The aim of this work was to describe the epidemiological, clinical and pathological aspects of two outbreaks of spontaneous poisoning caused by *Froelichia humboldtiana* in cattle in Pernambuco, northeastern Brazil and reproduce experimentally this poisoning in cattle. Spontaneous poisonings of primary photosensitization occurred in two farms at the municipalities of Cachoeirinha and São Caetano and affected twenty-two adult bovines and two suckling calves after the rainy season. All bovines have recovered 21 days after they were removed from the pasture. To reproduce experimental poisoning, three cows and a calf were maintained in a pasture with 1ha composed by *F. humboldtiana* during 14 days. Clinical signs and skin lesions were similar in both spontaneous and experimental poisoning and consisted of cutaneous itching and hyperemia of non-pigmented areas of skin that evolved into edema, exudative dermatitis and extensive areas of skin necrosis. Serum levels of aspartate aminotransferase (AST), gamma glutamyltransferase (GGT), total, direct and indirect bilirubin were normal in all cattle examined. Histologically, lesions consisted of epidermal necrosis, hyperkeratosis with large amounts of degenerate neutrophils and acanthosis. In the dermis, edema and inflammatory infiltrate composed of eosinophils, lymphocytes and plasma cells mainly around the blood vessels were observed. In the experimental group, clinical signs of photosensitization were observed after the third day of *F. humboldtiana* consumption. The suckling calf displayed mild clinical signs of photodermatitis on the 8th day of the experiment. It was estimated that the average consumption of *F. humboldtiana* necessary to initiate clinical signs in each adult bovine was 78kg.

INDEX TERMS: Spontaneous poisoning, experimental poisoning, *Froelichia humboldtiana*, cattle, poisonous plants, skin, photodermatitis, poisoning, toxic plants, toxicoses.

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RESUMO. [Intoxicação espontânea e experimental por *Froelichia humboldtiana* em bovinos.] Os objetivos deste trabalho foram descrever os aspectos epidemiológicos, clínicos e patológicos de dois surtos de intoxicação por *Froelichia humboldtiana* em bovinos em Pernambuco e reproduzir experimentalmente essa intoxicação em bovinos. Intoxicações espontâneas foram observadas após o início do período chuvoso nos municípios de Cachoerinha e São Caetano. Vinte e dois bovinos apresentaram sinais clínicos e lesões cutâneas compatíveis com fotossensibilização primária,

dentre os quais, dois bezerros lactentes. Todos os bovinos se recuperaram totalmente cerca de 21 dias após serem retirados da pastagem. Para reproduzir experimentalmente a intoxicação, três vacas, uma delas com bezerro ao pé, foram mantidas em um piquete de 1ha composto por *F. humboldtiana* por 14 dias consecutivos. O quadro clínico e as lesões tegumentares, tanto nos bovinos intoxicados nos surtos espontâneos, quanto nos bovinos do experimento consistiram em prurido e hiperemia em áreas despigmentadas de pele, que evoluíam para edema, dermatite exsudativa e necrose de áreas extensas de pele. Em todos os bovinos examinados, os níveis séricos de aspartato aminotransferase (AST), gamaglutamiltransferase (GGT), bilirrubina total, direta e indireta estavam normais. Histologicamente, as lesões consistiram em necrose da epiderme, hiperqueratose com grande quantidade de neutrófilos degenerados e acantose. Na derme havia edema e infiltrado inflamatório composto por eosinófilos, linfócitos e plasmócitos principalmente ao redor dos vasos sanguíneos. Nos bovinos do experimento, sinais clínicos de fotossensibilização foram observados após o terceiro dia de consumo de *F. humboldtiana*. O bezerro lactente apresentou sinais clínicos leves de fotodermatite no 8º dia do experimento. Estimou-se que o consumo médio de matéria seca de *F. humboldtiana* necessário para iniciar os sinais clínicos em cada bovino adulto foi de 78kg.

TERMOS DE INDEXAÇÃO: Intoxicação espontânea, intoxicação experimental, *Froelichia humboldtiana*, bovinos, plantas tóxicas, pele, fotodermatite, toxicoses.

INTRODUCTION

Photosensitization is an important cause of dermatitis resulting from the interaction between a photosensitizing agent and ultraviolet (UV) radiation or visible light (Tokarnia et al. 2012). It can be classified in two types in farmed animals: primary, as a result of photodynamic substances ingestion and, secondary, or hepatogenic, as a aftereffect of liver damage mainly generated by saponins and sapogenins (Pimentel et al. 2007, Tokarnia et al. 2012, Knupp et al. 2014, 2018, Santos et al. 2017, Amado et al. 2018, Moreira et al. 2018).

Froelichia humboldtiana (Amaranthaceae), commonly known as "ervanço", is widely distributed of the Brazilian northeastern region (Pimentel et al. 2007, Souza et al. 2012, Santos et al. 2017, Amado et al. 2018, Knupp et al. 2018), being thus responsible for primary photosensitization outbreaks. Horses are mainly poisoned (Pimentel et al. 2007, Knupp et al. 2014, Amado et al. 2018), however cattle, sheep and goats could be also affected (Pimentel et al. 2007, Souza et al. 2012, Santos et al. 2017).

The most noticeable clinical signs are hyperemia and itching on depigmented skin patches. These areas are swollen, presented exudative dermatitis, ulcers, tissue necrosis with extensive areas skin loss (Pimentel et al. 2007, Souza et al. 2012, Santos et al. 2017, Amado et al. 2018, Knupp et al. 2018). Weight loss and decreased milk production in cows and does occur concurrently (Santos et al. 2017, Knupp et al. 2018).

Photosensitization outbreaks are common in semiarid region of Brazil. However, despite its importance for ruminants and horses (Amado et al. 2018), the disease is not diagnosed frequently in the State of Pernambuco. This study aimed to describe the epidemiological, clinical and pathological aspects of two outbreaks of spontaneous poisoning caused by *F. humboldtiana* in cattle in Pernambuco, northeastern Brazil and reproduce experimentally this poisoning in cattle.

MATERIALS AND METHODS

Spontaneous poisoning. Outbreaks of spontaneous poisoning by *Froelichia humboldtiana* (Fig.1) occurred in the municipalities of Cachoeirinha and São Caetano in the dry region of the State of Pernambuco. Epidemiological data was obtained through interviews with the owners during the visit in the farms. Additionally, in order to find poisonous plants, the pastures of these farms were inspected. During the visits, affected cattle were clinically evaluated and skin biopsies and blood samples were collected. Skin biopsies were performed on three most severely affected animals of each property and the fragments fixed in 10% formalin, routinely processed for histopathology, stained with hematoxylin and eosin (HE) and evaluated by light microscopy. Blood samples were collected by jugular venipuncture to determine serum levels of aspartate aminotransferase (AST), gamma glutamyltransferase (GGT), total, direct and indirect bilirubin.

Experimental poisoning. For the experiment the guides related to animal welfare and ethics were followed, as recommended by

Fig.1. *Froelichia humboldtiana*, an erect or decumbent sub-bushes with 0.5-1.5m of high. Inset: *F. humboldtiana* sprout. Cachoeirinha/PE, August 2017.

the National Council for the Control of Animal Experimentation (CONCEA) and the Ethics Committee on the Use of Animals of the "Universidade Federal Rural de Pernambuco" (CEUA-UFRPE) (Authorization 014/2013).

The experiment was conducted on a farm in the municipality of Salgado de São Félix, State of Paraíba. Three adult crossbred Holstein cows, mean weighing 300kg were used. One of them had a two months suckling calf. Before the beginning of the experiment, all animals were clinically evaluated by examination of behavior, heart and respiratory rates, ruminal motility and inspection of the body surface for the occurrence of skin lesions. This physical examination was repeated daily until the end of the experiment.

The animals were fasted for 24 hours before being introduced into a grazing enclosure of 1 hectare (10000 square meters) consisting of *F. humboldtiana*, where they remained for 14 days. In this area there were no other species of plants causing photosensitization. Throughout the experimental period the animals received commercial mineral salt for cattle and water *ad libitum*.

Samples of *F. humboldtiana* were collected and sent to the zootechnical department of UFRPE for bromatological analysis. In order to estimate the amount of *F. humboldtiana* required to induce clinical signs, the dry matter daily intake was considered at 3% of cow's body weight (Hargreaves & Kerr 1981) and the estimated dry matter daily intake weight was 9kg per adult cow. This data was applied to the following formula:

$$F = \frac{9Kg \ x \ 100}{DM}$$

Where: F = Forage daily intake and DM = % *F. humboldtiana* dry matter.

Skin biopsies were performed on the 16th day of the experiment in all adult cattle. These samples were fixed in 10% formalin, routinely processed for histology and evaluated by light microscopy. Blood samples were collected at the beginning and at the end of the experiment to determine serum levels of AST, GGT, total, direct and indirect bilirubin as previously described.

RESULTS

Spontaneous poisoning

Two outbreaks occurred in August 2017 during the rainy season and farmers reported these were the first outbreaks of this disease in the municipalities. Due to the prolonged drought period farmers had not seeded the pastures and after first rains, *Froelichia humboldtiana* strongly invaded cattle's grazing areas. In both farms a large amount of *F. humboldtiana* with cattle grazing signs were observed.

The farm located in the municipality of Cachoeirinha had 50 Girolando dairy cattle grazing in approximately 147ha. Clinical signs were only noted by the owner 8 days after introduction of the herd in a pasture composed of native vegetation with large amount of *F. humboldtiana.* Fourteen animals showed clinical signs: two males, ten females and two suckling calves, whose mothers also had photosensitization. These clinical signs consisted of photophobia, intense itching, hyperemia, alopecia, ulcers and crusts on skin, mostly in white skin areas. Additionally, dairy cows showed intense hyperemia, ulceration of teats/udder, decreased milk production and weight loss.

In São Caetano eight from a total of 60 crossbred Nelore cattle were affected in a farm with approximately 200ha. The first clinical signs began 11 days after introduction of the herd into a pasture previously composed of *Brachiaria* spp. and native vegetation; however, after prolonged drought and with the first rains, *F. humboldtiana* invaded the pasture areas. The clinical signs consisted of licking and alopecia due to intense skin´s itching that evolved to ulcerative, necrotizing and exudative dermatitis with loss of extensive areas of skin, especially in the flanks and inguinal region (Fig.2).

Microscopically the lesions were similar in the two outbreaks and consisted of epidermal necrosis (Fig.3), hyperkeratosis with large amounts of degenerated neutrophils and moderate to severe acanthosis. In the dermis edema, inflammatory infiltrate consisting of eosinophils, lymphocytes and plasma cells mainly around blood vessels and thrombosis were observed. Serum levels of AST, GGT and total, direct and indirect bilirubin concentrations were within the reference values for the bovine species in all blood samples analyzed.

After primary photosensitization diagnosis, all cattle have been removed from the pasture invaded by *F. humboldtiana* to a shaded facility with a supply of napier grass (*Pennisetum purpureum*). The cutaneous lesions of affected cattle in both outbreaks regressed in 10 days.

Fig.2. (**A**) Several cattle from a herd showing alopecia, ulcerative, necrotizing and exudative dermatitis, with loss of extensive areas of the epidermis, especially in the non-pigmented areas of skin in the (**B**) hindlimbs and (**C**) flanks.

Experimental poisoning

From the second day of experiment (DE) cattle showed discrete clinical signs consisting of restlessness and an attempt to remain as long as possible in shaded areas. Between 3rd and 5th DE, the cows repetitively compressed flank, lateral side of the face and posterior region against fences and tree trunks due to itching. In this period, it was possible to observe cutaneous lesions that consisted of alopecia and hyperemia of depigmented areas of skin, mostly in the head, dorsal portion of the neck, withers and proximal portion of the hind limbs. In 8th DE, the calf presented slight lesions of photodermatitis, which consisted of alopecia and hyperemia of the barb and other areas of white skin. In cows, lesions evolved to edema and skin necrosis between the 8th and 17th DE (Fig.4).

No changes were observed in the heart rate, respiratory or ruminal motility in experimental cattle. The microscopy lesions consisted of areas of skin ulceration. The epidermis was covered by fibrin with neutrophil infiltrate and several superficial bacterial aggregates. Inflammatory infiltrate composed of mast cells, lymphocytes and some plasma cells were observed in the superficial dermis, especially around vessels. The serum levels of AST, GGT and concentrations of total, direct and indirect bilirubin were normal in all experimental cattle.

It was estimated that the average daily DM consumption of each adult animal was 9kg. As *F. humboldtiana* DM was estimated at 35%, it is suggested that each experimental cattle had ingested approximately 26kg of *F. humbolditiana* per day. The amount of *F. humboldtiana* ingested until present the first clinical signs on the 3rd DE was approximately 78kg per adult cattle.

Once the experiment ended, all cattle were removed from the pasture containing *F. humboldtiana*, placed in shaded facility and received buffel grass (*Cenchrus ciliaris*), napier grass (*Pennisetum purpureum*), commercial -ration, mineral salt for cattle and water. From 7 to 12 days later, the skin lesions regressed and totally recovered after 23 days.

DISCUSSION

The diagnosis of primary photosensitization due to *Froelichia humboldtiana* consumption in cattle of this study was based on epidemiological, clinical and histopathological findings that was similar to those reported previously (Pimentel et al. 2007, Souza et al. 2012, Knupp et al. 2014, Santos et al. 2017, Amado et al. 2018). *Malachra fasciata,* a species of poisonous

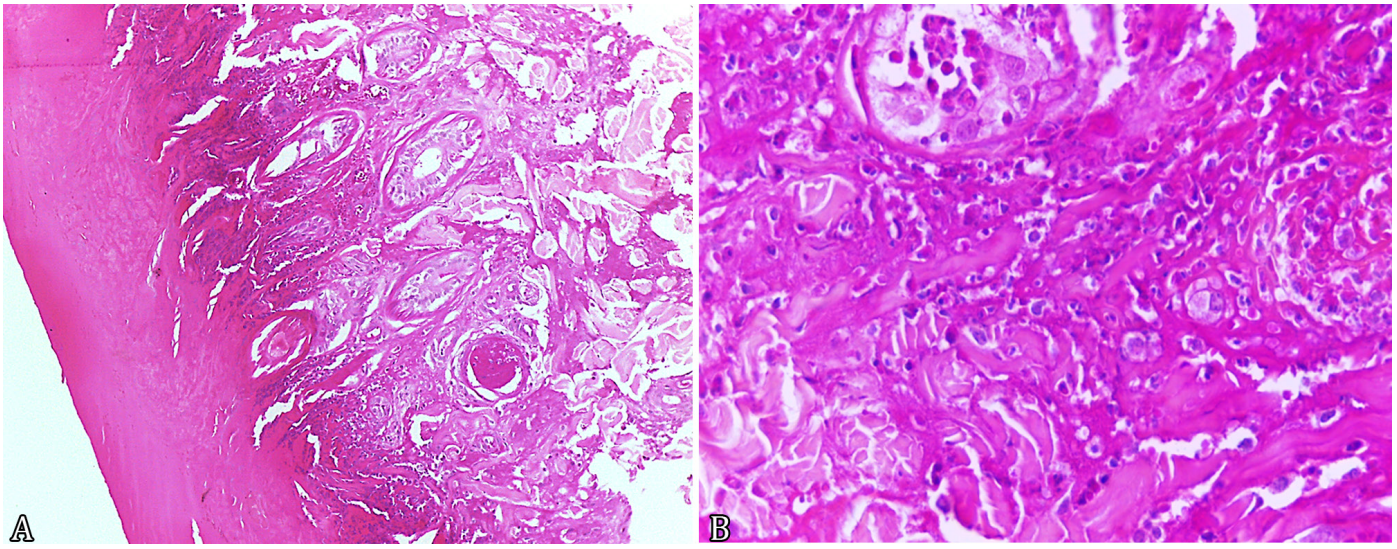

Fig.3. (**A**) Extensive coagulation necrosis of the epithelial tissue with thrombosis in the superficial dermis. HE, obj.10x. (**B**) Infiltrate composed by lymphocytes, plasma cells and eosinophils in superficial dermis. HE, obj.40x.

Fig.4. (**A**) Cutaneous lesions of *Froelichia humboldtiana* poisoning in the 8th day of experiment. Note the alopecia and hyperemia of depigmented areas of the skin near to the dorsal portion of the neck of the cow and (**B**) edema and alopecia of the calf barb. (**C**) Cow showing photophobia, remaining the most part of the day in a shaded area on the 3rd day of experimental poisoning.

plant that causes primary photosensitization also occurs in Pernambuco. However, this species was not found during pasture inspections and nowadays cases of photodermatitis were only described in sheep (Araújo et al. 2017). In northeastern Brazil, poisonings by *Brachiaria* spp., *Crotalaria* spp. and *Enterolobium contortisiquum* are also important for cattle (Amado et al. 2018). However, these plants cause secondary photosensitization, and hepatic injuries can be diagnosed by biopsies or by serum biochemistry analysis.

Serum levels profiles of AST, GGT and bilirubin's, are important tools to distinguish primary and secondary photosensitization because elevated concentrations are observed when significant damages are present in the liver (Knupp et al. 2014, 2018). These profiles remain within the normal range in cases of primary photodermatitis (Souza et al. 2012, Knupp et al. 2016, 2018).

The extended period of drought in the region studied, along with the occurrence of first rains, the lack of knowledge about the good palatability and toxicity of *F. humboldtiana* for cattle and the incorrect management of the pastures were important factors for the occurrence of primary photosensitization outbreaks described in this study. In these cases, correct pasture management could have prevented outbreaks, as it would not allow *F. humboldtiana* grows too much in the grazing lands.

In this study, affected cows as well as their suckling calves presented a clinical picture and characteristic lesions of primary photosensitization. This information, reported by farmers, was observed in two calves from spontaneous outbreaks and was reproduced in a calf from the experiment that presented mild dermatitis. The toxic principle of *F. humboldtiana* is unknown, but it is suggested that naftodiantrones may be related to the pathogenesis of tegumentary lesions (Pimentel et al. 2007). Other toxins from Brazilian plants can be excreted in milk, such as pyrrolizidine alkaloids, steroidal saponins, monocrotaline, ptaquiloside, and tremogenic toxin from *Ipomoea asarifolia* (Dickinson et al. 1976, James 1994, Medeiros & Górniak 1995, Lemos et al. 1998, Lopes et al. 2014, Lucena et al. 2014).Therefore, the possibility of *F. humboldtiana* toxin(s) being excreted by the milk of poisoned cows should be investigated better, since this characteristic was not observed in previous studies in cows (Knupp et al. 2018) and dairy goats (Santos et al. 2017).

In this study, it was estimated that each cow used in the experiment consumed 26kg of *F. humboldtiana* per day and showed clinical signs on the 3rd DE. This is an important result because although there are other experimental studies demonstrating the toxicity of *F. humboldtiana* to cattle, none of them have suggested what its toxic dose would be (Souza et al. 2012, Medeiros et al. 2014).

None of cattle in spontaneous outbreaks died or even in experimental poisonings. This characteristic is expected in cases of primary photosensitization, since the lesions are restricted only to the skin (Pimentel et al. 2007, Souza et al. 2012). Cases of death have been reported in asinines and were associated with self-mutilation or opportunistic agents, such as bacteria and myiasis (Knupp et al. 2014). Secondary photosensitization frequently occurs only in light-haired animals, but as a result of hepatic injury and high mortality has been reported in animals regardless of coat color (Macêdo et al. 2008, Knupp et al. 2016, Amado et al. 2018, Moreira et al. 2018).

CONCLUSION

It was concluded that *Froelichia humboldtiana* intake was indicated as the cause of primary photosensitization in cattle in the municipalities of Cachoeirinha and São Caetano in the State of Pernambuco. Suckling calves whose mothers graze on pasture areas invaded by the plant also be affected.

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

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Original Article
Livestock Diseases

Neonatal diarrhea and rotavirus A infection in beef and dairy calves, Brazil, 2006-2015[1]

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ABSTRACT.- Medeiros T.N.S., Lorenzetti E., Massi R.P., Alfieri A.F. & Alfieri A.A. 2020. **Neonatal diarrhea and rotavirus A infection in beef and dairy calves, Brazil, 2006-2015**. *Pesquisa Veterinária Brasileira 40(1):7-11*. Laboratório de Virologia Animal, Departamento de Medicina Veterinária Preventiva, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, Campus Universitário, Cx. Postal 10011, Londrina, PR 86057-970, Brazil. E-mail: alfieri@uel.br

Calf diarrhea causes substantial economic losses in the cattle industry worldwide. Bovine rotavirus A (RVA) is the main viral agent that leads to enteric infection and diarrhea outbreaks in calves throughout the world. The aim of this retrospective (2006-2015) study was to determine the frequency of RVA detection in diarrheic fecal samples from beef and dairy calves from the three main cattle-producing regions of Brazil. Diarrheic fecal samples (*n*=1,498) of 124 beef and 56 dairy cattle herds from the Midwest, South, and Southeast geographical regions of Brazil were evaluated using the silver-stained polyacrylamide gel electrophoresis (ss-PAGE) technique. RVA double stranded-RNA was identified by the ss-PAGE technique in 410 (27.4%) fecal samples. The frequency of positive samples found in beef calves (31.9%; 328/1,027) was higher than the frequency found in diarrheic fecal samples from dairy calves (17.4%; 82/471). RVA infection was identified in calves from the three Brazilian geographical regions analyzed. However, the frequency of positive diarrheic calves in the Midwest region (39.4%), predominantly beef calves, was higher than in the South (19.4%) and Southeast (17.6%) regions. The temporal distribution of RVA-infected calves evaluated by two five-year periods (2006-2010, 24.5%; 2011-2015, 28.8%) demonstrated a very similar frequency of RVA in both periods. Considering the wide regional and temporal scope of this study, it can be concluded that RVA remains an important etiology of neonatal diarrhea in calves of Brazilian cattle herds.

INDEX TERMS: Newborns, diarrhea, rotavirus A, infection, beef cattle, dairy cattle, calves, Brazil, cattle, enteric infection, RVA, ss-PAGE, epidemiology.

RESUMO.- [Diarreia neonatal e infecção por rotavírus A em bezerros de corte e leite, Brasil, 2006-2015.] A diarreia neonatal ocasiona perdas econômicas importantes na pecuária bovina em todo o mundo. Rotavírus A (RVA) é o principal agente etiológico viral de infecções entéricas e surtos de diarreia em bezerros de rebanhos de corte e leite. O objetivo deste estudo retrospectivo (2006-2015) foi determinar a frequência de detecção de RVA em amostras de fezes diarreicas de bezerros de corte e leite das três principais regiões produtoras de bovinos do Brasil. Amostras de fezes diarreicas (*n*=1.498) de 124 rebanhos bovinos de corte e 56 rebanhos bovinos de leite das regiões Centro-Oeste, Sul e Sudeste do Brasil foram avaliadas utilizando a técnica de eletroforese em gel de poliacrilamida (EGPA). O genoma segmentado de RVA foi identificado pela técnica de EGPA em 410 (27,4%) amostras de fezes. A frequência de amostras positivas encontrada em bezerros de rebanhos de corte (31,9%; 328/1.027) foi maior que a frequência identificada em amostras de fezes diarreicas de bezerros de rebanhos leiteiros (17,4%; 82/471). A infecção por RVA foi identificada em bezerros das três regiões geográficas brasileiras analisadas.

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No entanto, a frequência de bezerros com diarreia positivos para RVA na região Centro-Oeste (39,4%), predominantemente de bezerros de rebanhos de corte, foi maior que nas regiões Sul (19,4%) e Sudeste (17,6%). A distribuição temporal dos bezerros infectados com RVA avaliados por dois períodos de cinco anos (2006-2010, 24,5%; 2011-2015, 28,8%) demonstrou uma frequência muito semelhante em ambos os períodos. Considerando a amplitude regional e temporal deste estudo, pode-se concluir que RVA continua sendo uma importante etiologia de diarreia neonatal em bezerros de rebanhos bovinos brasileiros.

TERMOS DE INDEXAÇÃO: Diarreia neonatal, infecção, rotavírus A, bezerros de corte, bezerros de leite, Brasil, bovinos, infecção entérica, RVA, EGPA, epidemiologia.

INTRODUCTION

Neonatal diarrhea is a major health problem in livestock production worldwide (Smith 2012). Diarrhea outbreaks in calves have severe direct and indirect economic consequences due to morbidity, mortality, reduced growth rates, increased age at first calving, treatment costs, and time spent caring for the affected calves (Alfieri et al. 2006, Windeyer et al. 2014).

Diarrhea in calves has a multifactorial etiology (Blanchard 2012). Virus, bacteria, and protozoa infection, as well as immunological status and management factors (housing, feeding, and hygienic conditions) play an important role as determinants and predisposing factors, respectively (Alfieri et al. 2006, Blanchard 2012, Windeyer et al. 2014). With regard to the determinant factors, enteropathogens such as *Escherichia coli* K99, *Cryptosporidium parvum*, *Clostridium perfringens*, bovine coronavirus, and rotavirus A (RVA) have been widely recognized in cases of intestinal infections in young calves, including in Brazil (Alfieri et al. 2006, Oliveira Filho et al. 2007, Bartels et al. 2010, Blanchard 2012, Lorenzetti et al. 2013, Coura et al. 2015).

Rotavirus is one of the most important etiological viral agents of severe gastroenteritis in young humans and many mammalian and avian species (Estes & Greenberg 2013). In Brazil, several studies related to the etiology of neonatal diarrhea point to infection with RVA as one of the major causes of enteric infections in newborn calves (Langoni et al. 2004, Alfieri et al. 2006, Oliveira Filho et al. 2007, Coura et al. 2015).

Rotaviruses belong to the Reoviridae family, genus *Rotavirus*. The virus is 70-100nm in diameter and is characterized by a non-enveloped triple-layered protein capsid with a genome composed of 11 segmented double-stranded RNA (dsRNA) translated into six structural (VP1-VP4, VP6-VP7) and six non-structural (NSP1-NSP5/6) proteins (Estes & Greenberg 2013). Based on the antigenic properties and genetic characteristics of the VP6 gene that composes the middle layer of the viral capsid, rotaviruses are classified into nine species that are designated RVA to RVI (Mihalov-Kovács et al. 2015, ICTV 2017). A new species described in bats, named RVJ, have been suggested (Bányai et al. 2017).

Infections by RVA, B, and C have already been reported in cattle (Ghosh et al. 2010, Otto et al. 2015). However, RVA is the most common RV species that causes neonatal diarrhea outbreaks in calves in a number of different countries, including Brazil (Papp et al. 2013, Medeiros et al. 2014, Otto et al. 2015).

For the diagnosis of rotavirus, both the viral particle and proteins and genomes identification can be performed by several laboratory methods (Alfieri et al. 2004, 2006, Buzinaro et al. 2009, Medeiros et al. 2014, Otto et al. 2015, Rocha et al. 2017).

Silver-stained polyacrylamide gel electrophoresis (ss-PAGE) is a simple, fast, and low-cost method for RV dsRNA detection in diarrheic feces. This technique is commonly used for RV identification in acute infections due to specificity and sensitivity (Herring et al. 1982, Alfieri et al. 2006, Medeiros et al. 2014, Coura et al. 2015, Rocha et al. 2017).

This retrospective study (2006-2015) aimed to describe the frequency of RVA diagnosis in diarrheic fecal samples from beef and dairy calves in the three main cattle-producing regions of Brazil.

MATERIALS AND METHODS

Ethics statement. The study was submitted to Ethics Committee on Animal Experiments of the "Universidade Estadual de Londrina" (UEL) and approved under the identification number 6371.2013.43. All applicable institutional guidelines for the care and use of animals were followed.

Study population. The diarrheic fecal samples included in this study were obtained from a collection of fecal samples that were sent to the Laboratory of Animal Virology, UEL, Londrina, Paraná, Brazil. For the analysis, all fecal samples collected from January 2006 to December 2015 in beef and dairy herds from the three main cattle-producing regions of Brazil (Midwest, South, and Southeast) were selected. A total of 1,498 diarrheic fecal samples (beef calves *n*=1,027; dairy calves *n*=471) from 124 beef and 56 dairy cattle herds were included in the study. Only samples collected from diarrheic calves up to 60 days of age were included in the study. Based on the number of diarrheic fecal samples evaluated each year, the temporal distribution of bovine RVA infection in calves was not uniform. To reduce this sampling bias, we chose to analyze the results in two five-year periods, represented by the years 2006-2010 (first) and 2011-2015 (second). Additional information about the origin of the fecal samples is presented in Table 1. The fecal samples were stored at -80°C until analysis.

Table 1. Diarrheic fecal samples for rotavirus diagnosis according to the origin (geographical region) and type (beef/dairy) of cattle production, Brazil, 2006-2015

Region	State	County (*n*)	Herds			Samples		
			Beef	Dairy	Total	Beef	Dairy	Total
South	RS/SC/PR	43	38	32	70	250	327	577
Southeast	SP/MG	33	20	20	40	174	122	296
Midwest	MS/GO/MT	44	66	4	70	603	22	625
Total		120	124	56	180	1,027	471	1,498

RS = Rio Grande do Sul, SC = Santa Catarina, PR = Paraná, SP = São Paulo, MG = Minas Gerais, MS = Mato Grosso do Sul, GO = Goiás, MT = Mato Grosso.

Table 2. Rotavirus A identified by ss-PAGE in diarrheic fecal samples from calves according to the geographical origin of the cattle herds, Brazil, 2006-2015

Region	Fecal samples		
	Positive (%)	Negative	Total
South	112 (19.4)	465	577
Southeast	52 (17.6)	244	296
Midwest	246 (39.4)	379	625
Total	410 (27.4)	1,088	1,498

Table 3 Temporal distribution of rotavirus A identified by ss-PAGE in diarrheic calves, Brazil, 2006-2015

Period	Fecal samples		Total
	Positive (%)	Negative	
2006-2010	123 (24.5)	378	501
2011-2015	287 (28.8)	710	997
Total	410 (27.4)	1,088	1,498

Nucleic acid extraction and ss-PAGE. Fecal suspensions 20% (w/v) in buffer Tris-Ca^{2+} pH7.4 (50mM Tris-HCl; 10mM NaCl; 1.5mM 2-mercaptoetanol; 3mM $CaCl_2$) were homogenized and centrifuged at 2,000 x *g* for 5min at 4°C. Aliquots of 500µL of supernatant were collected and treated with SDS (sodium dodecyl sulfate) at a final concentration of 1%. The nucleic acid was extracted using a combination of the methods phenol/chloroform/isoamyl alcohol (25:24:1) and silica/guanidinium isothiocyanate (Alfieri et al. 2006). The nucleic acid was eluted in 50µL of ultrapure diethylpyrocarbonate (DEPC)-treated water (Invitrogen Life Technologies, Carlsbad/CA, USA) and briefly stored at -20°C. The cell culture adapted bovine RVA NCDV-Lincoln strain and aliquots of Tris-Ca^{2+} buffer were included as positive and negative controls, respectively, in all nucleic acid extraction procedures. The presence of RVA dsRNA in diarrheic fecal samples was evaluated by the ss-PAGE technique (Herring et al. 1982, Pereira et al. 1983).

RESULTS

The dsRNA of bovine RVA was identified in 27.4% (410/1,498) of the diarrheic fecal samples included in this study. The rate of positive samples was higher in calves from beef (31.9%, 328/1,027) than in calves from dairy (17.4%, 82/471) herds.

RVA infection was identified in calves from three geographical regions in Brazil. However, the frequency of positive diarrheic calves in the Midwest region (39.4%) was higher than that in the South (19.4%) and Southeast (17.6%) regions (Table 2).

The frequencies of RVA diagnosis by ss-PAGE in diarrheic calves according to the temporal distribution (2006-2010 and 2011-2015) is presented in Table 3.

DISCUSSION

The current retrospective study evaluated over a period of 10 years (2006-2015) the frequency of RVA diagnosis in diarrheic fecal samples of beef and dairy calves from three geographical regions that represent approximately 65% of the Brazilian cattle industry (Brasil 2015). The detection rate of ss-PAGE RVA-positive fecal samples (27.4%) was similar to that identified in other studies performed in Brazil (Alfieri et al. 2006, Buzinaro et al. 2009), Argentina (Badaracco et al. 2012), and Iran (Madadgar et al. 2015). Therefore, the present information highlights the similar frequency of RVA infection in calves from different countries, despite differences in the management system, temperature, humidity, breed, and other calf diarrhea risk factors.

The frequency of RVA-positive fecal samples in diarrheic calves from beef herds (31.9%) was higher than in dairy herds (17.4%). The use of fixed-time artificial insemination during the two- or three-month breeding season is widespread in extensive beef cattle herds and herds with the highest number of cows. Due to this reproduction management practice, there is a temporal concentration of calves born and the number of animals susceptible to infection is higher. Thus, the challenge, and consequently the risk of RVA infection, is even greater, and in the field, outbreaks of diarrhea in beef calves are common in the main Brazilian cattle producing regions (Buzinaro et al. 2003, Medeiros et al. 2014, 2015).

Cattle herds from three Brazilian geographical regions with different management systems were evaluated in this study. Although in the South region was evaluated a higher number of diarrheic fecal samples in relation to the Southeast region, the frequency of RVA-positive samples identified in these two regions was similar.

The frequency of diarrhea caused by RVA infection identified in the Midwest region was higher than in the other regions. Some herd and management characteristics that are present in most of the farms in this region may have contributed to the higher frequency of diarrhea. In this context, we highlight several potential risk factors: I) a short breeding season with a concentration of calving; II) herd size, as this region is characterized by the presence of large herds with fixed-time artificial insemination; III) greater frequency of crossbreeding producing calves (*Bos indicus* x *Bos taurus*) with lower resistance than Nelore (*B. indicus*) calves; IV) use of maternity pens; and V) population density (Aono et al. 2013, Pereira et al. 2013, Alfieri & Alfieri 2017).

Although more fecal samples (*n*=997) were evaluated in the second period (2011-2015) compared to the first period (2006-2010) (*n*=501), the frequency of RVA-positive results found in both time periods was very similar.

A study performed from 1998-2002 by our research group detected bovine RVA in 19.4% (369/1,898) of the samples collected in calves with diarrhea from beef and dairy cattle herds in four Brazilian geographical regions (South, Southeast, Midwest, and North). The proportion of positive samples collected was 22.8% (205/899) and 16.4% (164/999) from beef and dairy cattle herds, respectively (Alfieri et al. 2006). Comparing the results, it can be observed that in nearly 20 years, the frequency of RVA diagnosis in Brazilian dairy cattle herds has remained practically the same. However, in beef cattle herds, the frequency of RVA diagnosis has increased considerably, possibly due to the reproductive management system, including reproduction biotechniques, used in the beef cattle herds.

It is likely that the highest rate of diagnosis of RVA in beef herds in the Midwest region is due to changes in reproductive management practices, such as fixed-time artificial insemination, which leads to an increased risk of enteric infections in neonates.

Protocols for the control of neonatal diarrhea in calves have been implemented in some countries, and studies indicate that the commercial vaccines currently in use are

appropriate for providing protection against RVA infection in cattle (Collins et al. 2014).

Considering the quantitative and qualitative increase in the Brazilian cattle industry, measures to control and prevent bovine neonatal diarrhea outbreaks are necessary and urgent. The mitigation of risk factors and the implementation of a vaccination program against calf diarrhea are two ways to achieve this goal.

CONCLUSIONS

The frequencies of RVA detection when evaluating two five-year periods (2006-2010 and 2011-2015) were similar.

The RVA infection rate was higher in calves of beef herds than in those of dairy cattle herds.

In the Midwest region, where predominately diarrheic fecal samples from beef herds were evaluated, the rate of RVA-positive diagnosis was higher than in the other Brazilian geographical regions (South and Southeast) evaluated in the study.

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

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Original Article
Livestock Diseases

Bovine tuberculosis: diagnosis in dairy cattle through the association of analyzes[1]

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ABSTRACT.- Dametto L.L., Santos E.D., Santos L.R., & Dickel E.L 2020. **Bovine tuberculosis: Diagnosis in dairy cattle through the association of analyzes.** *Pesquisa Veterinária Brasileira 40(1):12-16.* Graduate Program in Bioexperimentation, Faculdade Agronomia e Medicina Veterinária, Universidade de Passo Fundo, Rodovia BR-285, Bairro São José, Passo Fundo, RS 99052-900, Brazil. E-mail: lldametto@yahoo.com.br

Tuberculosis is a chronic anthropozoonosis of worldwide occurrence, caused by the bacterium *Mycobacterium tuberculosis* and its variants. In Brazil, the National Program for the Control and Eradication of Brucellosis and Tuberculosis in cattle, is responsible for diagnosing and the correctly allocate positive animals, but there is still a lack of definitive diagnosis of the disease. This study described the use of five diagnostic tools that can be used, preferably together, for the confirmation of suspected cases. These tools included the clinical examination comparative cervical tuberculin test, macroscopic findings during the slaughtering and histopathology of the damaged tissues followed by histochemistry. We evaluated a total of 211 dairy cattle, where 15.1% (32/211) had classic clinical signs of bovine tuberculosis, 74 (35%) showed reactivity in the comparative cervical tuberculin test. Of the total number of animals, 141 (66.8%) were referred for sanitary slaughter due to legal and control issues in the outbreaks of the disease. In the follow-up of slaughtering and inspection of viscera and carcasses, 74 (52.5%) had macroscopic lesions compatible with bovine tuberculosis, while 67 (47.5%) showed no visible changes. During the inspection, fragments of lymph nodes and liver and lung parenchyma were collected from five cattle with macroscopic lesions and five with no lesions. The histopathological analysis showed numerous areas of caseous necrosis with or without central calcification and granulomatous inflammatory infiltrate. In the special staining of Ziehl-Neelsen, numerous acid-fast bacilli were evidenced in all cases.

INDEX TERMS: Bovine, tuberculosis, diagnostic, dairy cattle, zoonosis, *Mycobacterium* spp., cattle, bacterioses.

RESUMO.- [**Tuberculose bovina: diagnóstico em bovinos leiteiros através da associação de análises.**] A tuberculose é uma antropozoonose crônica de ocorrência mundial, causada pela bactéria *Mycobacterium tuberculosis* e suas variantes. No Brasil existe o Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose em bovinos que viabiliza o diagnóstico e a destinação correta dos animais positivos, porém ainda há carência quanto ao diagnóstico da doença. Assim, este trabalho descreve a utilização de cinco ferramentas diagnósticas para a confirmação de casos suspeitos de tuberculose. As ferramentas utilizadas compreenderam o exame clínico, teste tuberculínico cervical comparativo, os achados macroscópicos durante o abate sanitário e a histopatologia dos tecidos lesados seguido de histoquímica. O estudo avaliou um total de 211 bovinos leiteiros, dos quais 15,1% (32/211) apresentaram sinais clínicos clássicos de tuberculose bovina, 35,1% (74/211) apresentaram reatividade no teste tuberculínico cervical comparativo, e 143 animais (67,8%) foram encaminhados para abate sanitário devido a questões legais e de controle nos focos da doença. No acompanhamento do abate e inspeção sanitária de vísceras e carcaças verificou-se que 51,8% (74/143) dos bovinos abatidos apresentavam lesões macroscópicas compatíveis com tuberculose bovina, enquanto 48,2% (69/143) não

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apresentavam alterações visíveis. Durante a inspeção foram coletados fragmentos de linfonodos e parênquima de fígado e pulmão de cinco bovinos com lesões macroscópicas e de cinco sem lesões, que na análise histopatológica apresentaram numerosas áreas de necrose caseosa com ou sem calcificação central e infiltrado inflamatório granulomatoso. Na coloração de Ziehl-Neelsen foram evidenciados numerosos bacilos álcool-ácido resistentes em todos os casos. Assim, diante dos resultados obtidos verifica-se que as análises empregadas no presente estudo foram de extrema importância para o diagnóstico acurado de tuberculose em bovinos.

TERMOS DE INDEXAÇÃO: Tuberculose, bovinos, diagnóstico, bovinos leiteiros, zoonose, *Mycobacterium* spp., bacterioses.

INTRODUCTION

Tuberculosis is a chronic anthropozoonosis of worldwide evolution (Acha & Szyfres 2005). It is caused by the bacterium *Mycobacterium tuberculosis* and its variants, comprising *M. tuberculosis* var. *tuberculosis*, *M. tuberculosis* var. *africanum*, *M. tuberculosis* var. *canetti*, *M. tuberculosis* var. *bovis*, *M. tuberculosis* var. *caprae*, *M. tuberculosis* var. *microti*, *M. tuberculosis* var. *pinnipedii*, *M. tuberculosis* var. *mungi*, *M. tuberculosis* var. *orygis* and *M. tuberculosis* var. *suricattae* (Riojas et al. 2018). According to the World Health Organization (WHO), tuberculosis is the infectious disease that causes the most deaths, even surpassing HIV. In 2016, about 10.4 million people fell ill from tuberculosis worldwide, and about 1.3 million died (WHO 2017). In Brazil, in 2016, 4,426 deaths were registered, and in 2017 69,569 new cases of tuberculosis were reported (Brasil 2018).

In cattle, the disease is caused by *M. tuberculosis* var. *bovis* and is one of the leading infectious diseases of the species (Acha & Szyfres 2005, Radostits et al. 2007, Riojas et al. 2018). The disease causes a reduction in meat and milk production and a high rate of slaughter carcass condemnation, as well as economic losses due to embargoes on the marketing of animals and animal products (Demelash et al. 2009, Asil et al. 2012, Paes & Franco 2016). Its transmission occurs mainly through direct contact with contaminated animals, consumption of infected water, and ingestion of raw milk and/or dairy products made from raw milk, such as clandestine cheese (Corrêa & Corrêa 1992). The national average of tuberculosis occurrence in cattle is 2.64%, with heterogeneity and fluctuation within and between the evaluated states, and in the southern region, highlighting milk production, Rio Grande do Sul, Santa Catarina and Paraná presented 2, 8%, 0.06% and 2.15%, respectively (Ferreira Neto et al. 2016).

In Brazil, since 2001, the "Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose" (PNCEBT, National Program for the Control and Eradication of Brucellosis and Tuberculosis) for cattle and buffaloes, created to establish norms and procedures for the diagnosis of these diseases (Brasil 2006). According to PNCEBT, the basis for the diagnosis of *in vivo* tuberculosis is given by intradermal tuberculinization in cattle and buffaloes older than six months. Tuberculinization is immunologically characterized by late-type IV hypersensitivity reaction and can be performed using caudal, simple cervical or comparative cervical fold techniques (Brasil 2006, 2017a).

Brazilian legislation recommends sanitary slaughter of the positive tuberculin test animal, which must be performed in refrigerators under official inspection so that *post mortem* observations can be performed and, mainly, for the correct disposal of the carcass (Brasil 2017b). However, there are cases of nonspecific reaction where cattle show reaction to tuberculin without presenting the disease. There may also be cases in which the animal had contact with the agent, without tuberculosis lesions characteristic in the *post mortem* inspection, as well as negative animals in the test and presenting compatible lesions in the *post mortem*. These situations become questionablefor official agents who have to decide the fate of animals (Paes & Franco 2016). We used the comparative cervical tuberculin test, followed by thorough inspection of animals submitted to sanitary slaughter and tissue collection for histological and histochemical examination for diagnosis of bovine tuberculosis.

MATERIALS AND METHODS

The application of tests for diagnosis of brucellosis and tuberculosis was part of the routine of the PNCEBT-qualified veterinarian and collaborator of the study, whose services were hired by the owners of the herds. The study evaluated 211 dairy cattle of different ages, from November 2017 to April 2018, coming from small farms of three municipalities of the north of Rio Grande do Sul. The importance of the diagnosis was explained to the owners, as well as clarified the legal measures in case there are positive animals in the herd. Thus, upon clarification, all farm animals were evaluated by a general clinical examination and subsequently submitted to tuberculin test.

The comparative cervical tuberculin test (CCTT) is considered a confirmatory test. We performed trichotomy in two areas: 1) cervical region, in front of the scapular spine and at 20cm from the withers, where the avian tuberculin was inoculated; 2) in the cervical region, behind the scapula spine, and 20cm from the withers, where bovine tuberculin was inoculated. Initially, a minimum distance from 15 to 20cm was respected between these two points, and the skinfold thickness of each point was measured with a cutimeter. Subsequently, we inoculated 0.1ml of avian tuberculin PPD (purified protein derivatitis) and 0.1ml of bovine PPD tuberculin. After 72 hours of inoculation, the skinfold thickness was checked again, and the differences were interpreted according to the criteria defined by the PNCEBT Technical Regulation (Brasil 2017a), in order to conclude which animals were reactive, nonreactive or inconclusive.

As recommended by the PNCEBT, all cattle considered reactive or inconclusive in the tuberculin test were marked on the right side of the face with a "P" in a circle of eight centimeters of diameter, and intended for slaughter in a slaughterhouse under official inspection. For sanitary and control reasons, cattle considered negative, but coming from properties where more than 50% of the lot was positive or inconclusive, were also destined for slaughter in a slaughterhouse under official inspection. The carcass and viscera inspection of all animals destined for sanitary slaughter was followed in order to locate and identify macroscopic lesions suggestive of bovine tuberculosis, especially caseous and/or calcified lesions. The analysis was performed by visual inspection, palpation and serial sectioning of the lymph nodes (mediastinal, bronchial, retropharyngeal, parotid, mandibular, mesenteric and hepatic), lung parenchyma and hepatic parenchyma and carcass.

Among the reactive CCTT cattle sent for slaughter, five animals with characteristic tuberculosis lesions and five without any caseous or calcified lesions were selected. From these, we collected lymph node fragments (retropharyngeal, mediastinal, bronchial, mesenteric and hepatic) and pulmonary and hepatic parenchyma fragments. The samples were placed in vials with 10% buffered formalin and

sent for processing and laboratory analysis with histopathological examination (hematoxylin and eosin, HE) and histochemical analysis using the special Ziehl-Neelsen stain to identify acid-fast bacilli (AFB).

RESULTS AND DISCUSSION

In the clinical evaluation, 15.1% (32/211) of dairy cattle presented classic clinical signs of bovine tuberculosis, such as decreased milk production, difficulty in conception and difficulty breathing, prostration and cachexia (Fig.1A). Subsequently, they were submitted to CCTT (Fig.1B), which showed that 35.1% (74/211) had reactivity, being 63.5% (47/74) positive and 36.5% (27/74) inconclusive. The test also showed that 64.9% (137/211) of the cattle were negative for bovine and avian tuberculin, although kept with test-reactive animals. Table 1 summarizes the results of the clinical evaluation, the CCTT, the frequency of animals destined for sanitary slaughter by the municipality and the number of macroscopic lesions found during *post mortem* inspection.

Due to its chronic nature, the wide variability of manifestations makes the clinical diagnosis of tuberculosis a relative value, since the cattle may be locally infected and look healthy (Corrêa & Corrêa 1992). Animal studies inoculated with *Mycobacterium tuberculosis* var. *bovis* in contact with uninoculated animals showed no clinical signs compatible with the disease in either group (Cassidy et al. 1999). In cases of advanced tuberculosis, the clinical diagnosis assumes greater importance, since the animals have decreased milk production, conception difficulty, difficulty breathing, elimination of nasal discharge, prostration, and cachexia (Roxo 1997, Brasil 2006, Garbaccio et al. 2018).

The sensitivity of the tuberculin test in cattle ranges from 32 to 99% and the specificity ranges from 75.5 to 99.9% (Vitale et al. 1998). In the present study, the sensitivity was 35% (74/211) of reactive animals. The tuberculin test may yield false-positive results (Monaghan et al. 1994), as animals infected with *M. avium*, *M. tuberculosis*, *M. avium* subsp. *paratuberculosis*, *Nocardia farcinius* or other mycobacteria may be reactive to bovine PPD (Monaghan et al. 1994, Souza et al. 2016, Garbaccio et al. 2018). Thus, we recommend the CCTT since, compared to the simple tuberculin test, there is a reduction in the possibility of such cross-reactions (Collins et al. 1994). However, the occurrence of 12.8% (27/211) inconclusive animals demonstrates its limitation, as well as the need to use complementary methods for an accurate result (Monaghan et al. 1994, Romero et al. 1999).

Some animals, although infected, do not respond to tuberculin tests (Brasil 2006), which may have occurred in the present study. In municipalities 1 and 3, 100% of the tested animals were sent for sanitary slaughter control, of which 52% (27/52) and 47% (42/89) were negative in

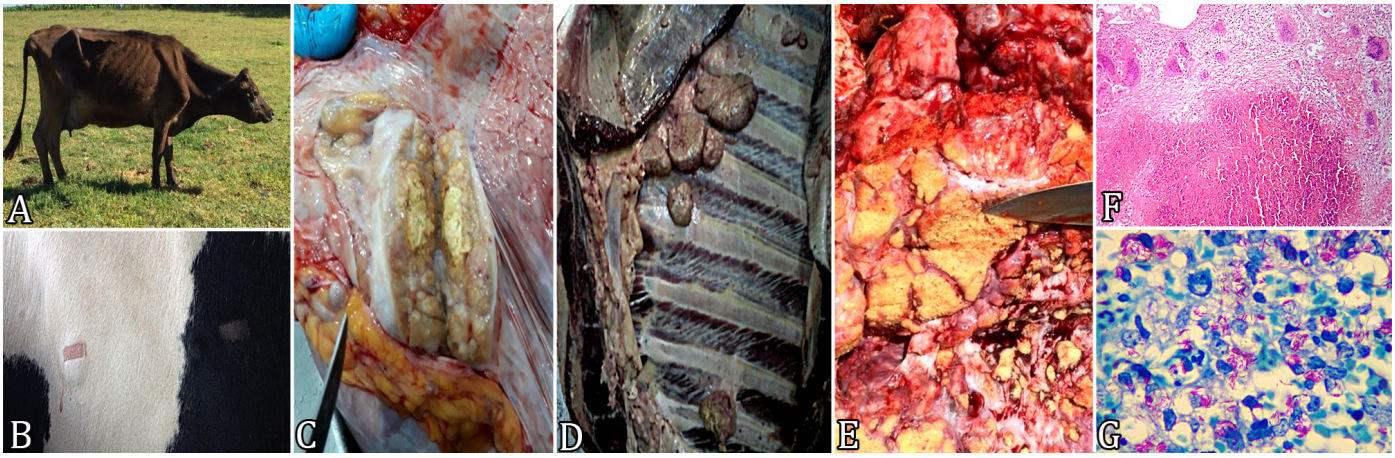

Fig.1. Diagnostic strategies for bovine tuberculosis. (**A**) Bovine female with difficulty breathing, prostration, and cachexia. (**B**) Cattle showing positive reaction on CCTT. (**C**) Caseous lesion in the mediastinal lymph node. (**D**) Caseous nodules adhering to the parietal pleura. (**E**) Extensive areas of caseous and mineralized lesions in the pulmonary parenchyma. (**F**) Lung with lesion with central area of caseous necrosis and granulomatous inflammatory infiltrate composed of epithelioid macrophages, multinucleated giant cells, and lymphocytes. HE, obj.10x. (**G**) Acid-resistant bacilli (AFB) in giant cell cytoplasm in bovine pre-scapular lymph node lesion with tuberculosis. Ziehl-Neelsen, obj.100x.

Table 1. Clinical evaluation, cervical comparative tuberculin test (CCTT), animals destined for sanitary slaughter by the municipality, and number of animals with macroscopic lesions found in *post mortem* inspection during sanitary slaughter

Municipality	Clinical evaluation		Tuberculin test			Sanitary slaughter	*Post mortem* inspection	
	With signs	Without signs	Positive	Inconclusive	Negative	No. of animals	With lesions	Without lesions
Municipality 1	13	39	15	10	27	52	44	8
Municipality 2	-	70	2	-	68	2	-	2
Municipality 3	19	70	30	17	42	89	30	59
Total	32	179	47	27	137	143	74	69
%	15.1	84.9	22.3	12.8	64.9	67.8	51.8	48.2

CCTT although they were together with animals positive, respectively. In animals from the same municipalities and sent for slaughter, 85% and 33.7% of macroscopic lesions suggestive of tuberculosis during *post mortem* inspection, respectively, were evidenced. Factors such as recent infection, late pregnancy, malnutrition, and advanced disease may cause false negatives in the tuberculin test. Also, such results may occur due to variations inherent in the test itself or due to variations in the reading and interpretation of the exam (Brasil 2006). It is important to note that animals in advanced stages of infection may manifest the phenomenon called anergy, defined as absence of skin reactivity to tuberculin (Roxo 1996).

Based on the CCTT results, the guidance of the PNCEBT (Brasil 2006) and the Veterinary Inspectorate was that, in addition to the reactive cattle, CCTT negative animals coming from properties with more than 50% of reactive cattle for the bovine and avian tuberculin should also be slaughtered, accounting for 67.8% (143/211) of the animals examined. Slaughter was carried out in a slaughterhouse under official inspection, where all processes were suppervised and recorded, including the *post mortem* inspection of carcasses and viscera. The *post mortem* inspection showed that 51.8% (74/143) of the cattle presented purulent or caseous lesions, with or without mineralization. This occurred at one or more sites, mainly in the pre-scapular, retropharyngeal, retromammary, hepatic, mesenteric, and apical and/or mediastinal pulmonary lymph nodes (Fig.1C), in addition to the parietal (Fig.1D) and visceral pleurae and pulmonary (Fig.1E) and hepatic parenchyma. However, 48.2% (69/143) of the cattle showed no visible lesions.

Carcasses were considered positive when at least one organ had lesions suggestive of tuberculosis, as in the study by Furlanetto et al. (2012). In the present study, of the 143 cattle slaughtered, 51.8% (74/143) presented suggestive lesions, a result inferior to that reported by Fráguas et al. (2008), who observed 72% of carcasses with lesions in a similar study. However, it was higher than that reported by Pinto et al. (2004), who found 44% of bovine tuberculosis-reactive cattle also presenting tuberculous lesions. Corner et al. (1990) reported that about 58% of tuberculosis animals have unique lesions, and detailed inspection of lymph nodes (head, thoracic, mesenteric and carcass) as well as liver, lung, spleen, kidneys, udder, and genitals increases the possibility visualization of these lesions (Corner 1994). Some aspects may be involved with the non-detection of suggestive lesions in CCTT-reactive animals, such as: early stage of the disease, contact with mycobacteria other than *M. tuberculosis* var. *bovis*, time and attention for insufficient post mortem inspection (Souza et al. 1999, Corner 1994, Brasil 2006, Medeiros et al. 2012).

Of the ten cattle selected for collection and requested for histopathological and histochemical examination, five had yellowish lesions with purulent or caseous content surrounded by a fibrous capsule and sometimes calcified appearance at cut, as found by Souza et al. (2016). Tissue fragments were also collected from five other reactive CCTT cattle, which did not present macroscopic lesions on inspection. As found by Furlanetto et al. (2012), in the microscopic analysis the ten cattle presented lesions compatible with bovine tuberculosis. In the present study we identified severe or moderate lesions, delimited, with central area of caseous necrosis, sometimes mineralized, and presence of granulomatous inflammatory infiltrate composed of epithelioid macrophages, multinucleated giant cells, and lymphocytes (Fig.1F).

Granulomatous inflammatory processes with macroscopic tuberculosis-like characteristics may occur in cattle, but with different etiologies such as lymphosarcoma, nonspecific lymphadenitis, actinobacillosis and nocardiosis (Kantor et al. 1981, Reis et al. 1995, Roxo 1997). Macroscopic inspection can be subjective for carcass judgment (Reis et al. 1995, Brasil 2017b), indicating that sanitary inspection of carcasses should be performed carefully by well-trained professionals, reducing the risk of suspicious food arriving consumers or carcasses to be unnecessarily condemned for non-tuberculous lesions (Fraguás et al. 2008, Brasil 2017b).

In the present study, Ziehl-Neelsen staining provided evidence of numerous acid-fast bacilli (AFB) in all tissue samples examined (Fig.1G), including those without macroscopic lesions. However, these findings differ from studies that consider low sensitivity Ziehl-Neelsen staining when applied to histological sections (Fráguas et al. 2008). Andrade et al. (1991) observed AFB in only 9.1% of the histological samples examined in their study, while Salazar (2005) and Furlanetto et al. (2012) reported absence of AFB in tissue samples with macroscopic lesions suggestive of tuberculosis and confirmed on histopathological examination. According to Rodriguez et al. (2004), these divergences may occur because the test reveals the presence of AFB in concentrations higher than 104 mycobacteria in a histological section.

As in the study Silva et al. (2018), the results obtained in the present study allowed us to infer that the clinical examination, CCTT, macroscopic *post mortem* inspection, histopathological examination and identification of AFB are useful tools for the diagnosis of bovine tuberculosis. The present study highlights the histopathological and histochemical analysis in tissues with lesions suggestive of bovine tuberculosis, which allows characterizing the microscopic alterations of the lesions, as well as identifying the AFB. It is also noteworthy that the study was conducted in a dairy herd, demonstrating the circulation of the disease, which poses a threat to people who come into direct contact and/or consume meat and milk from these animals. Besides, positive cattle are also a source of infection for those working in the industry, as they will be exposed to the etiological agent when handling animals or meat products.

CONCLUSIONS

The present study demonstrated that the combination of low-cost alternatives increases the diagnostic accuracy for bovine tuberculosis, contributing to the good functioning of PNCEBT and prevention of this zoonosis in public health.

The clinical examination identified symptomatic animals, the CCTT identified reactive animals with suggestive lesions in the slaughterhouse, while the histopathology of the suggestive lesions showed caseous lesions, and Ziehl-Neelsen staining showed AFBs, even in CCTT reactive cattle and without macroscopic lesions.

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

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Original Article
Livestock Diseases

Influence of early use of antimicrobial on the health and performance of Holstein calves in the first month of life[1]

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ABSTRACT.- Martin C.C., Basqueira N.S., Ramos J.S., Silva K.N. Baccili C.C., Brandão P.E. & Gomes V. 2020. **Influence of early use of antimicrobial on the health and performance of Holstein calves in the first month of life.** *Pesquisa Veterinária Brasileira 40(1):17-28.* Departamento de Clínica Médica, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Orlando Marques de Paiva 87, Cidade Universitária, Butantã, SP 05508-270, Brasil. E-mail: camilacmartin@gmail.com

The early use of antimicrobial therapy has been introduced in many farms to prevent diarrhea and respiratory disease in young calves; however, there is controversy about whether this practice has a beneficial effect on the health of these animals. This study evaluated the influence of the early use of antimicrobials on the health and performance of neonatal Holstein calves. Twenty-six Holstein calves were screened and divided into two groups, according to the administration (ATB+), or not (ATB-) of tulathromycin (2.5mg/kg, subcutaneously) within the first 12 hours of life. Calves were evaluated by general clinical examination, fecal score, respiratory score, and external palpation of the umbilical region, besides fecal output of dry matter. Anemia was determined by using an automatic system and, also, using a commercial kit for iron dosage. Diarrhea was diagnosed by a centrifuge-flotation technique using a sugar solution (*Cryptosporidium*) and multiplex semi-nested RT-PCR (rotavirus/coronavirus). The performance of the calves was estimated by Daily Weight Gain (DWG). The young dairy calves were evaluated within 12 hours of birth (≤12h) and at 3-5th (D3-5), 7-9th (D7-9), 13-15th (D13-15), 20-23rd (D20-23), and 27-30th (D27-30) days of life. No difference was noted between the ATB+ and ATB- groups concerning heart rate, respiratory frequency, and rectal temperature. Erythrogram showed a higher frequency of anemia in ATB- group (P=0.016) at the D3-5 check-up; lower values of serum iron were also observed simultaneously (P=0.051). Thirteen cases of respiratory disease were detected during this study; however, no significant difference was observed between the groups in this regard. The frequency of diarrhea (fecal score 2-3) was high in both groups, peaking at D13-D15. No differences were noted between the groups regarding the frequency of diarrhea when considering the dry fecal matter. The predominant etiological agent for diarrhea was *Cryptosporidium* spp.. The DWG was similar between groups, with maximum weight reduction on D13-15. The administration of tulathromycin in prophylactic dose (2.5mg/kg) at birth decreased the frequency of anemia but did not influence weight gain or the prevalence of diarrhea.

INDEX TERMS: Antimicrobial, health, performance, Holstein calves, tulathromycin, prophylaxis, sanity, cattle.

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RESUMO.- [**Influência do uso precoce de antimicrobiano na sanidade de bezerras holandesas no primeiro mês de vida.**] O uso precoce de antimicrobianos tem sido adotado em muitas fazendas para profilaxia das diarreias e doença respiratória em bezerras, no entanto existem controvérsias sobre os beneficios desta prática na saúde desses animais. Esta

pesquisa avaliou a influência do uso precoce de antimicrobiano na sanidade e desempenho de bezerras holandesas recém-nascidas. Para tanto foram selecionadas 26 bezerras Holandesas distribuídas de acordo com a aplicação (ATB+) ou não (ATB-) de tulatromicina (2,5mg/Kg) por via subcutânea até 12h de vida. As bezerras foram examinadas por meio de exame clínico geral, escore fecal, escore respiratório e palpação externa da região umbilical, além da matéria seca fecal. A presença de anemias foi determinada pelo eritrograma utilizando sistema automático e além da dosagem de ferro utilizando kit comercial. O diagnóstico etiológico das diarreias foi investigado por meio da técnica de flutuação em solução saturada de sacarose (*Cryptosporidium*) e multiplex semi-nested RT-PCR (rotavírus/coronavírus). O desempenho das bezerras foi estimado pelo ganho de peso. As bezerras foram avaliadas até doze horas após o nascimento (≤12h); 3-5º (D3-5); 7-9º (D7-9); 13-15º (D13-15); 20-23º (D20-23); e 27-30º dias de vida (D27-30). Não foram encontradas diferenças entre os grupos ATB+ e ATB- em relação à frequência cardíaca, frequência respiratória e temperatura retal. O eritrograma revelou maior frequência de anemias no grupo ATB- (P=0,016) no D3-5. Neste momento também foram observados menores valores de ferro sérico (P=0,051). Foram detectados treze casos de doença respiratória durante o estudo, no entanto não foi possível detectar diferença entre os grupos. A frequência de diarreias (escore fecal 2 e 3) foi alta em ambos os grupos, observando-se pico no D13-15 (ATB+=92,3%; ATB-=92,3%). Não observamos diferenças entre os grupos em relação a frequência de diarreia considerando-se a matéria seca fecal. O agente etiológico predominante nas diarreias foi o *Cryptosporidium*. O ganho de peso diário foi igual entre grupos, com intensa redução no GPD no D13-15. A administração de tulatromicina na dose profilática (2,5mg/Kg) ao nascimento diminuiu a frequência de anemias e não influenciou no ganho de peso e prevalência de diarreias.

TERMOS DE INDEXAÇÃO: Antimicrobiano, bezerras holandesas, desempenho, tulatromicina, profilaxia, sanidade, bovinos.

INTRODUCTION

The cost of the 22- to 24-month calf production cycle is around $ 1124.06, being the second only for the cost of feeding lactating cows. All this investment, in addition to expressing the maximum genetic potential of females, can be diluted by nutritional and health management errors (Gabler et al. 2000, Heinrichs & Heinrichs 2011). Increasing age at first calving is related to the treatment of antimicrobial diseased calves (Heinrichs et al. 2005); also the number of days of disease or treatment before four months of age significantly alters milk production in the first lactation (Heinrichs & Heinrichs 2011).

Calves are born hypogammaglobulinemia and have an adaptive *näive* immune system, and are immunosuppressed due to the interaction between hormones and cytokines present in the maternal-fetal environment. Due to these factors, the postnatal immune response is slow and low in intensity, making these animals dependent on maternal protection transferred by colostrum and susceptible to management errors that predispose to the establishment of agents that cause diarrhea, umbilical inflammation and bronchopneumonia (Barrington & Parish 2001, Chase et al. 2008). Morbidity from diarrhea in neonatal calves can be as high as 90 to 100% in animals up to three weeks of age and the mortality rate observed in the suckling phase is approximately 3.5%, with a peak death rate around 19 days of age life (Langoni et al. 2004, Windeyer et al. 2014).

In an attempt to decrease mortality, improve health, and increase performance, many producers use long-acting prophylactic antimicrobials because of the difficulty in early identification of sick animals (Ives & Richeson 2015). Prophylactic use of antimicrobials has been widely used in confinements for respiratory disease prophylaxis, and there are currently eight injectable antimicrobials (danofloxacin, enrofloxacin, tulathromycin, ceftiofur, tilmicosin, florfenicol, tildipirosin and gamithromycin), plus four oral antimicrobials (oxytetracycline, tilmicosin, chlortetracycline (CTC), and CTC in combination with sulfadiazine (SDZ), internationally approved for prophylactic use for respiratory disease in cattle (Ives & Richeson 2015).

Antimicrobials have also been ostensibly used to treat or prevent diarrhea in calves. Walker et al. (2012), in a study in the United States of America (USA) by evaluating antimicrobial practices on dairy farms, found that 83% of the animals were treated with diarrhea antimicrobials, and 50% of calves diarrhea received more than one antimicrobial type. The authors also found that 56% of the properties used antimicrobial milk, and 54% of the properties used antimicrobials to prevent diarrhea or respiratory disease. Only 65% of the farms had written guidelines on the use of antimicrobials to treat the main diseases affecting calves.

The complexity of etiology of diarrhea in neonatal calves has been the basis for questions about the cost and benefit of antimicrobial use for prophylaxis of this disease. Diarrhea is caused by different groups of microorganisms among which some pathotypes of *Escherichia coli* (from 15.6 to 28.5%), rotavirus (from 3.5 to 6.2%), coronavirus (from 3.1 to 21.6%), *Salmonella* spp. (from 16.4 to 43.7%) and *Cryptosporidium* spp. (100 and 75%) (Silverlås et al. 2010, Carvalho et al. 2014, Gomez et al. 2017). The benefit of the therapeutic use of antimicrobials for cases of enteritis due to *Salmonella* spp. or *E. coli* is distinct; however, there is no argument for its use in cases of rotavirus, coronavirus, and cryptosporidiosis (Berge et al. 2009, Smith 2015).

The use of antimicrobials affects not only harmful bacteria but also commensal and symbiotic bacteria that gradually colonize the gut in the postnatal period. Recent studies in humans have shown that the intestinal microbiota has a lot of benefits to the host, especially related to energy metabolism through digestion of undigested carbohydrates, production of vitamins B and K, antibacterial and immunomodulatory activities, through different mechanisms: competition for binding sites; physiologically restricted environment production (pH and O_2 pressure); production of antimicrobial substances; formation of the intestinal barrier by mucin production, reducing the permeability of the epithelium; development of immune tolerance to commensal/symbiotic microorganisms and immune response to harmful bacteria (Wang et al. 2012, Aziz et al. 2013, Fernández et al. 2013, Vaziri et al. 2015). Thus the balance of the intestinal microbiota, as well as its diversity, has been associated with intestinal health.

Despite the many studies already conducted on the use of antimicrobials for calves, primarily associated with milk, these studies presented conflicting data regarding their

efficacy. Berge et al. (2009) demonstrated that the use of antimicrobial supplemented milk increased the risk for diarrhea development by 28%, decreased weight gain, and concentrate intake. However, Donovan et al. (2002) found no differences in performance and incidence of diarrhea in calves fed antimicrobial supplemented milk.

Besides, the influence of antimicrobials on immunity and health and their misuse has been associated with the selection and spread of resistant bacteria. Resistance is not a problem in itself, but the transfer of resistance elements to zoonotic pathogens has severe implications for human and animal health (Kaneene et al. 2008, Smith 2015). Once resistance is established in non-pathogenic bacteria, they can transfer these genes via plasmids and integrons to pathogenic organisms. Gene transfer that gives the pathogen selective host advantage has the potential to be rapidly spread within the bacterial population (Kaneene et al. 2008).

The current concern with the use of antimicrobials for disease prophylaxis and metaphylaxis in newborn calves has encouraged the scientific community to seek explanations about their real effect on the development of these animals and their implications for animal health and welfare (Berge et al. 2009, Smith 2015). Much research has been conducted to evaluate the parenteral use of long-acting antimicrobials in the prevention of respiratory disease in confined cattle, but little is said about their effect on newborn calves, especially their impact on intestinal health. Thus, this research aimed to investigate the influence of early parenteral antimicrobial use on the health profile and performance of calves in the neonatal period.

MATERIAL AND METHODS

Farm and Animals. This research was approved by the Animal Use Ethics Committee of "Faculdade de Medicina Veterinária e Zootecnia" of Universidade de São Paulo (USP), protocol no. 2596070715.

The calves of this research were from a high production commercial farm located in the state of São Paulo. The field phase of the study was conducted from May to July 2016. The farm had assisted births in farrowing stalls with adequate hygiene and colostration conditions, in addition to breastfeeding with pasteurized milk and without antimicrobials associated with raising calves in individual suspended stalls with periodic hygiene and disinfection.

Cows that were near the expected date of calving were transferred from the pre-calving paddock to sand-bed maternity pens where they remained until calving. Staff veterinarians or farm collaborators monitored all deliveries. Soon after birth, the calves were separated from the cows and transferred to the transitional stalls, where navel healing and bottle-feeding were performed.

The cows were milked in the stalls by individual mechanical milking, after cleaning the ceilings with a chlorinated solution and drying with individual paper towels. All colostrum supplied to calves was assessed for immunological quality by indirect evaluation of immunoglobulin concentration by colostrum balls (Ms colostrum balls, Nutri Support©, 3705010) (density 1045-1075) and Brix refractometer (Model 300001; SperScientific, Scottsdale/AZ) (23-32°). Calf colostration was performed as soon as possible after birth, with the first breastfeeding given at the latest at six hours of life and the second generally performed within 6 hours of the first breastfeeding, according to the calf's need. Calves were given a minimum volume of colostrum equivalent to 10% of live weight.

Navel healing was performed shortly after birth by complete immersion of the umbilical cord in a container containing commercial antiseptic solution based on iodoform, phenol, picric acid, and dichlorvos (DDVP) (Umbicura - Pec®) for 1½ minutes, repeating the process after 12 hours. This process was repeated twice a day until complete umbilical cord mummification occurred around eight to 10 days of life of the calves.

Healthy heifers from eutocic calves were selected for this research. Twenty-six calves were randomly assigned to two groups of 13 animals: Group 1- received a dose of tulathromycin antimicrobial (2.5mg/Kg) subcutaneously, in the caudal thoracic region, the right scapula, up to twelve hours after birth (ATB+); Group 2- received a dose of physiological solution, subcutaneously up to twelve hours after birth (ATB-).

Stool specimens were evaluated and collected at the following times: up to twelve hours after birth (≤12h); 3-5th day of life (D3-5); 7-9th day of life (D7-9); 13th -15th day of life (D13-15); 20-23rd day of life (D20-23); and 27-30th days after birth (D27-30). The choice of these intervals was based on the dynamics of the main events and conditions affecting calves in the neonatal period. By 12 hours after birth, most calves were still eliminating meconium, at D3-5 is the period when animals are transferred from Maternity Pens (MP) to the calf after colostration and have a higher frequency of umbilical disorders, D7-9 to D13-15 is the peak of diarrhea, and from D20-23 to D27-30, post-diarrhea follow-up was performed.

The calves remained in the transitional pens for approximately three days, receiving only colostrum and transitional milk in the bottle, and were offered around three liters of transitional milk per breastfeeding. After this period, the calves were transferred to the hutches, where they remained until weaning. In the hutches, the calves were kept in individual stalls suspended from metal and wood with hay beds and were fed by bucket twice a day with a total of six liters of milk from postpartum cows. In the case of milk shortage, replacer (Natimilk, Auster®) was used to complete daily milk volume, and the same replacement volume was used for all calves. Also, the calves received commercial feed and water *ad libitum*. These products contained no antimicrobial residues and growth promoters. All calves from four to six days old were dehorned using caustic paste.

Periodic clinical evaluation. Calves were evaluated at all times by clinical examination, assessing vital functions, and mucosal coloration (Feitosa 2014). The diarrhea score was also used according to the Calf Heath Scoring Criteria of University of Wiscosin - Madison (McGuirk 2008).

The stool score was classified from 0 to 3 according to fecal consistency, being 0 - normal consistency, 1 - pasty and semi-formed consistency, 2 - aqueous consistency with larger amount of water, with fecal content adhered to the perineum and tail, 3 - liquid with fecal content adhered to the perineum and tail. Calves with a 0 or 1 score are considered without diarrhea, while animals with a score 2 or 3 have diarrhea.

The assessment of the presence of respiratory disease was performed according to the procedures described by Poulsen & McGuirk (2009). Scores from 0 to 3 were assigned according to intensity for rectal temperature, cough, nasal and ocular discharge, and ear position. Finally, the scores were added to obtain a final value. Calves whose sum of scores was ≥4 were screened for the diagnosis of respiratory disease.

During all evaluations, the external umbilical region was evaluated by inspection and palpation for the detection of inflammatory signs such as redness, presence of purulent discharge, tenderness, increased

umbilical volume, umbilical cord thickening, and abdominal tension (Figueiredo 1999).

Performance. To evaluate the impact of the use of antimicrobial on calf performance, the animals were weighed using calf weight tape. For this, the thoracic perimeter of an animal was measured, placing tape around the chest with the animal in season.

Collection and storage of samples. Stool samples were collected directly from the rectal ampoule with sterile latex gloves and packaged in sterile universal collectors. In the laboratory, stool samples were aliquoted into microtubes (1.5mL) in the laminar flow in the presence of a Bunsen burner, and then stored in a freezer at -80°C for Rotavirus and Coronavirus screening. The second aliquot of fresh feces, approximately 2 grams, was used in the diagnosis of Cryptosporidiosis by the sucrose saturated flotation technique. The remainder was frozen inside the freezer collection cups at -20°C for later determination of the amount of dry matter in the feces. Blood samples were collected from calves by external jugular vein puncture, using a vacuum system, in tubes without anticoagulant (10mL) and with anticoagulant Ethylenediaminetetraacetic Acid (EDTA). All samples were kept refrigerated between sample collection and transport to the laboratory.

Erythrogram. The hematological components belonging to the erythrogram were obtained by automatic system (BC 2800Vet®).

Iron. Serum aliquots were thawed in the refrigerator (overnight), homogenized in an automatic homogenizer for further testing on an automated biochemical analyzer (Daytona Model, Randox Laboratories Ltd., Co. Antrium, UK). To measure iron concentration, a commercial kit (UIBC Iron, Randox®, Cat. No. 357599) was used using the methodology described by the manufacturer.

Determination of dry stool matter. Correctly identified metal pots were used for weighing about 5 grams of feces on a high precision analytical balance. After weighing, the samples were transferred to a greenhouse at 103°C, where they remained for 24 hours. After this period, the samples were removed from the greenhouse and transferred to the desiccator for 30 minutes to cool. The already cooled samples were weighed, and calculations were performed to determine the sample dry matter percentage (% Dry matter = [dry sample mass/wet sample mass] x 100). Cases of diarrhea were considered as feces containing a percentage of ≤15% of dry matter (Dirksen et al. 1993).

Detection of *Cryptosporidium*. The detection of *Cryptosporidium* spp. was performed by the sucrose saturated flotation technique in stool samples with scores 2 and 3. To do so, initially, the feces were washed to remove the fat and facilitate the observation of protozoa. One to two grams of feces were added to a collecting beaker with 8 to 9mL of distilled water; with the aid of a glass stick, the feces were utterly diluted. The solution was sieved, and the liquid fraction added into a 15mL plastic tube, then 4 mL of ether was added to the tube and then homogenized. Samples were centrifuged for five minutes at 796 x g. After this process, the fat layer and the supernatant were removed with the aid of a Pasteur pipette, and the walls of the plastic tube were wiped with gauze. The stool "pellet" was added in 9mL saturated sucrose solution and homogenized and centrifuged for ten minutes at 264 x g (Ogassawara & Benassi 1980). The supernatant was removed with a metal handle and placed on a glass slide, covered with a coverslip, and observed under a 400X magnification optical microscope.

Multiplex semi-nested RT-PCR for detection of group coronavirus and rotavirus A. Fecal score 2 and 3 samples were screened for detection of Rotavirus and Coronavirus by the system multiplex semi-nested RT-PCR, according to the technique described by Asano et al. (2010). Fecal samples were first prepared as 50% (liquid stool) or 20% (pasty stool) suspensions in DEPC-water (*Diethylpyrocarbonate*) and clarified at 5,000 x g/15min at 4°C, taking the supernatant as a sample.

Total RNA extraction from the fecal sample suspensions was performed with TRIzolReagent™ (Invitrogen, Carlsbad/CA, USA), according to the manufacturer's instructions. Kakegawa (Akashi et al. 1980) and 8209 (Rodriguez et al. 2004) strains were used as positive controls for bovine coronavirus (BCoV) and rotavirus, respectively, and water-DEPC as negative control.

For the detection of BCoV, A set of three primers directed to the BCoV N-nucleocapsid protein-coding gene was used. The RT-PCR reactions for rotavirus detection were performed using three primers directed to the viral protein-coding gene VP1 (Table 1).

Each RNA-containing tube was brought to 95°C for 5 minutes for RNA denaturation, then immersed in ice and added from the 1x FirstStrand Buffer®, 1mM of each dNTP, 10mM reverse transcription combination reagent DTT, 1μM of each primer (BCOV1 + BCOV2 for BCoV, ROT1 + ROT2 for rotavirus) and 400U M-MLV Reverse Transcriptase® (Invitrogen®) for a final volume of 40μL, reverse transcribed at 42°C/60 minutes.

After obtaining the complementary DNA, the PCR reaction was performed in a thermal cycler. To this end, 2.5 μL of the respective cDNA was added to the PCR reagent combination containing 1x PCR Buffer® (Invitrogen®), 0.2 mM from each dNTP, 0.25μM from each pair of primers (BCOV1 + BCOV2 and BCOV1 + BCOV3 for BCoV, ROT1 + ROT2 and ROT1 + ROT3 for), 1.5mMMgCl2 and 0.5U Platinum Taq DNA Polymerase® (Invitrogen®) to a final volume of 25μL supplemented with DEPC-water.

The tubes were then brought to the thermal cycler for initial denaturation at 94°C/4min, followed by 35 cycles of 94°C/30sec (denaturation), 55°C/30sec with gradient 5°C (hybridization) and 72°C/45sec (polymerization), followed by the cycle, 72°C/5min

Table 1. Primers used for the detection of bovine coronavirus N-nucleocapsid protein-coding gene and its hybridizing nucleotide regions in the Mebus sample N gene (U00735), primers used for the detection of rotavirus VP1 viral protein-coding gene group A and nucleotide regions hybridizing to genomic segment 1 of sample KJ44 (DQ494405.1), fusion temperatures (Tm) and their amplicons (in base pairs); and nucleotide regions hybridizing to accession sequence GenBank NC_006853, melting temperatures (Tm) and their amplicon (in base pairs)

Target	Primer	Sequence (5'-3')	Region	Tm	Amplicon
Coronavirus	BCOV1 (sense)	AAGAGCTCAAYCCAAGCAAACTGY	123-146	60°C	463pb
	BCOV2 (antisense)	AGCAGACCTTCCTGAGCCTTCAAT	562-585	60°C	463pb
	BCOV3 (antisense)	TCAATRTCGGTGCCATACTGGTCT	405-428	59.9°C	306pb (with BCOV1)
Rotavirus	ROT1 (sense)	CTCTGGCAAARCTGGTGTCA	737-753	59.7°C	492pb
	ROT2 (antisense)	CATTCGACGCTGATGACATY	1206-1225	59.7°C	492pb
	ROT3 (antisense)	ARCAATCRACCAACCASTCCTGTA	938-961	59.8°C	228pb (with ROT1)

for the final extension. After electrophoresis on 1.5%, agarose gel stained with 0.5µg/mL ethidium bromide and observed under ultraviolet light.

The hemi-nested PCR for BCoV and rotavirus was performed by adding 2.5µL of the first amplification product to the PCR reagent combination containing 1x PCR Buffer® (Invitrogen®), 0.2mM from each dNTP, 0.25µM of each primer (BCOV1 + BCOV3 for BCoV, ROT1 + ROT3 for rotavirus), 1.5mMMgCl2 and 0.5U Platinum Taq DNA Polymerase® (Invitrogen®) and sterile ultrapure water (to a final volume of 25µL), leading to the thermal cycler initial denaturation of 94°C/4min followed by 25 cycles of 94°C/30sec (denaturation), 55°C/30sec with gradient of 5°C (hybridization) and 72°C/45sec (polymerization) followed by 72°C/5min for the final extension. After electrophoresis on 1.5%, agarose gel stained with 0.5µg/mL ethidium bromide and observed under ultraviolet light.

Statistical analysis. Statistical analysis was performed using the SPSS 19.0 statistical program (IBM Corp. Released 2011, IBM SPSS Statistics for Windows, Version 19.0, Armonk/NY).

Variables with quantitative values were evaluated for distribution concerning the Gaussian curve by the Shapiro-Wilk test. Data that did not present normal distribution were submitted to $\log_{10}$, square root, or inverse transformation to verify the possibility of performing a parametric analysis. Mean and standard deviation measurements represent parametric data. Student's t-test for independent samples was used to compare the values obtained in the group ATB- and group ATB+ in every moment. Time analysis was assessed by one-way ANOVA calculations for repeated time measurements within the same group with Tukey post hoc test. In the case where the transformations occurred, the *p* presented in the tables refers to the tests related to the transformed values, while the described values are real. The nonparametric variables were represented by the median, quartile range, minimum and maximum values; the comparison between groups was performed by the Mann-Whitney test. Qualitative data (scores and presence of diseases) were presented in absolute values and frequencies, and the Chi-square test performed the comparison between groups. The analyzes were considered significant when P≤0.05 (*).

RESULTS

This study evaluated the influence of prophylactic use of tulathromycin provided up to 12 hours after birth on the performance and health of calves in the neonatal period through clinical evaluation, disease score, weight gain, and diarrhea diagnosis.

Clinical evaluation

Mean values and standard deviation for vital functions, weight gain, and frequency of changes in mucosal color are shown in Figure 1.

Heart rate was higher in ATB+ group (156.9±23.9bpm) compared to ATB- (138.2±13.6bpm) in the first assessment

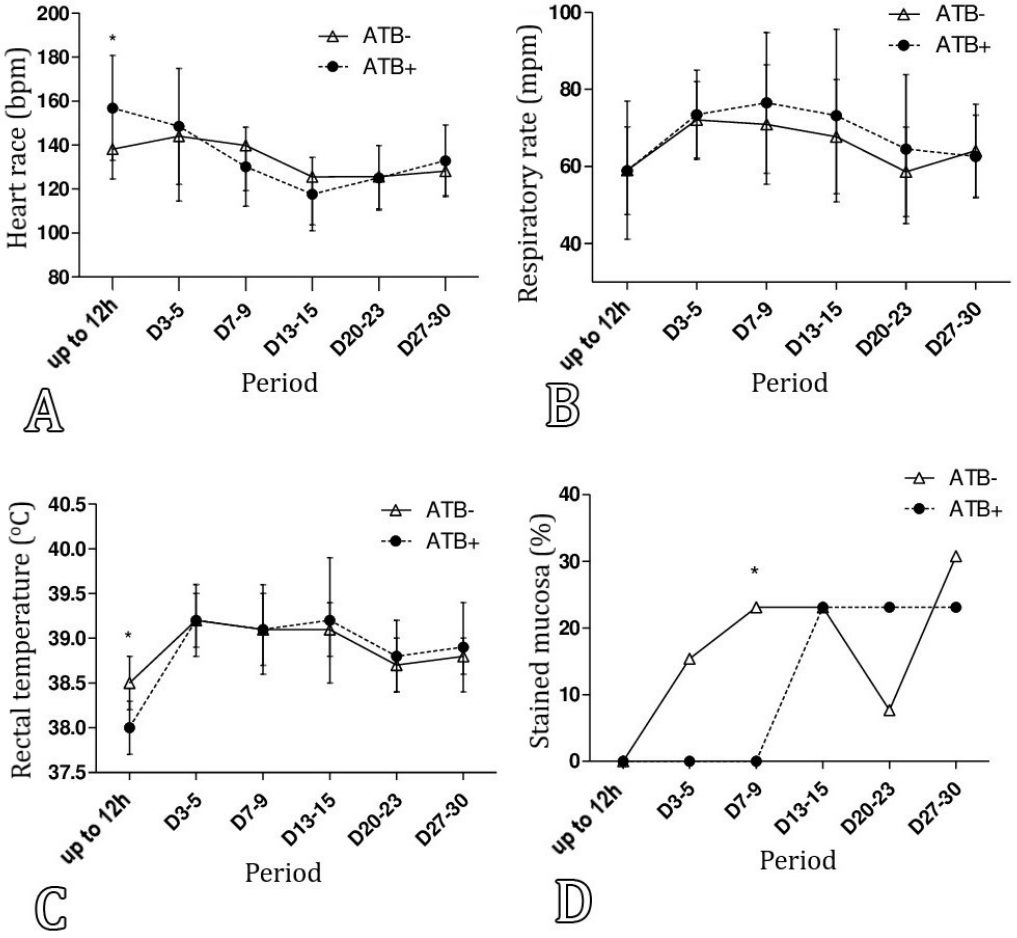

Fig.1. Mean values and standard deviation for (**A**) heart rate, (**B**) respiratory rate, (**C**) rectal temperature and (**D**) percentage of stained mucosa in ATB- and ATB+ Holstein calves in the first month of life.

performed in the first 12 hours of life (P=0.022) (Fig.1A). In general, time analysis detected a decrease in heart rate from ≤12h to D13-15 in ATB+ group (P=0.000). Respiratory rate was similar between groups at one month of life (P≥0.358) (Fig.1B). The respiratory rate in ATB- group increased from ≤12h to D3-5. After this period, there was a progressive decrease from D3-5 to D20-23 and an increase in D27-30 (P=0.047). In ATB+ group, there was an increase in respiratory movements from ≤12h to D7-9, with a progressive decrease until D27-30 (P=0.048).

The T°C of the calves was higher in ATB- group (38.5±0.3°C) about the ATB+ (38.0±0.3°C) in the first evaluation moment (≤12h of life) (P=0.002). (Fig.1C). In general, time analysis revealed an increase in T°C from ≤12h to D3-5 in both groups, with a decrease in values in ATB+ group at subsequent times (P=0.000). T°C was more oscillating from D3-5 in ATB- group, with an increase in values from D3-5 to D13-15 with a slight decrease in D20-23 and D27-D30 (P=0.000).

All calves in both groups were hydrated (dehydration ≤5%) and presented Capillary Refill Time (CRT) ≤2.0 at all times. The calves' mucous membranes of both experimental groups were rosy in the first evaluation performed between birth and 12 hours of life (≤12h), but only the ATB- group presented hypocolored mucous membranes in D3-5 and D7-9 (Fig.1D)., most often from animals with hypocolored mucosa in ATB- group at time D7-9 (ATB- = 23.1%; ATB+ = 0.0%). The ATB+ calves began to show stained mucous from D13-15, but the frequencies between groups were similar from that moment.

Erythrogram

Mean values and standard deviations for erythrogram components are shown in Figure 2. The mean values of red blood cells were similar between the ATB- and ATB+ groups (P≥0.236) (Fig.2A). Mean hemoglobin values were similar between ATB- and ATB+ groups (Fig.2B). At D3-5, it could

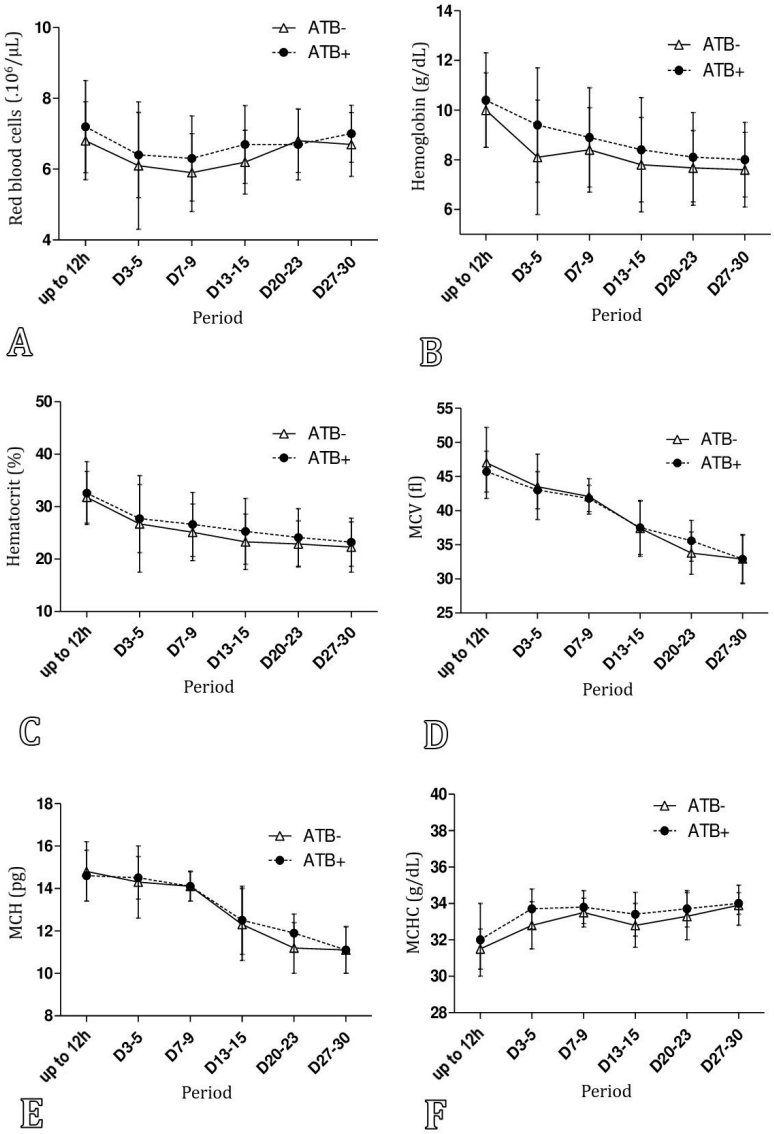

Fig.2. Mean values for (**A**) red blood cells (x106/µL), (**B**) hemoglobin (g/dL), (**C**) hematocrit (%), (**D**) Mean Corpuscular Volume (MCV) (fl), (**E**) Mean Corpuscular Hemoglobin (MCH) (pg) and (**F**) Mean Corpuscular Hemoglobin Concentration (MCHC (g/dL)) in ATB- and ATB+ Holstein calves in the first month of life.

be observed higher hemoglobin values in calves receiving a tulathromycin dose at birth but without statistical difference (P=0.071). The time profile revealed a gradual decrease in hemoglobin levels in ATB+ (P=0.005) and ATB- (P=0.011) groups. The mean hematocrit values were similar between the ATB- and ATB+ groups (P≥0.359) (Fig.2C), time analysis revealed a gradual decrease of the values in the first month of life in ATB+ (P=0.001) and ATB- groups (P=0.001).

It was not possible to observe differences in the comparison between groups for hematimetric indices (Fig.2F). Time analysis showed a progressive decrease in the mean values of Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCH) in both groups in the first month of life (Figs.2D,E). The mean values of Mean Corpuscular Hemoglobin Concentration (MCHC) also presented time variations in ATB+ (P=0.001) and ATB- (P=0.000) groups, with a gradual increase in the values from 12 hours after birth to D27-30 in both experimental groups.

The anemia frequencies in ATB+ and ATB- groups are shown in figure 3. Individual evaluation of erythrograms at different times was performed to detect anemia, according to the reference values established by Brun-Hansen et al. (2006). The frequency of anemia was higher in the ATB- group (61.5%) than the ATB+ (15.4%) in D3-5 (P=0.016), showing statistical difference.

Iron

The mean values and standard deviation of serum iron concentration for ATB- and ATB+ calves are shown in Figure 4. There was no difference between groups in mean iron concentrations, despite the higher values in ATB+ compared to ATB- in D3-5 (P=0.051). The maximum iron peak for both groups was up to 12 hours after birth (ATB- = 14.43±8.82μM/L, ATB+ = 15.06±8.88μM/L), values up to D27-30 (ATB- = 3.9±2.01μM/L, ATB+ = 3.95±2.52μM/L) (P=0.000).

Disease scores

The diarrhea frequencies assessed by fecal score and dry matter, umbilical inflammation, and percentage of dry matter in calf feces are described in Figure 5 and 6.

Diarrhea started soon after breastfeeding at ≤12h, with a maximum prevalence between D13-15 (Fig.5A). Higher frequency of diarrhea was observed in ATB+ group through the fecal dry matter classification (ATB+ = 84.6%, ATB- = 53.8%) (Fig.5B). The frequency of diarrhea decreased at D20-23 (Score: ATB+ = 15.4%, ATB- = 23.1%; Dry Matter: ATB+ = 23.1%, ATB- = 15.4%) and D27-30 (Score: ATB+ = 30.8%, ATB- = 7.7%; Dry Matter: ATB+ = 30.8%, ATB- = 15.4%). Quantitative comparison of the percentage (%) of dry matter in calf feces revealed differences between the groups, with a higher average in ATB- calves at birth (P=0.046). Moderate Spearman correlation (r=0.633) was observed between detection of diarrhea by fecal score and percentage of dry matter (P=0.000).

Umbilical changes were detected at D3-5 (ATB- = 38.5, ATB+ = 7.7%, P=0.063), D7-9 (ATB- = 23.1, ATB+ = 15.4%, P=0.619) and D13-15 (ATB- = 23.1, ATB+ = 0.0%, P=0.066) (Fig.5C). In general, the ATB- group had a higher frequency of umbilical inflammation.

Thirteen cases of respiratory disease were detected during the study period, eight in ATB- group and five in ATB+ group (Fig.5D). D27-30 presented the highest frequency of bronchopneumonia, with 23.1% of the animals from the affected ATB- group and 7.7% of the animals from the ATB+ group. No statistical difference was observed between the groups at any of the evaluated moments.

Performance

Daily weight gain was similar between experimental groups (P≥0.241) (Fig.7). Both groups showed weight gain above 1kg in D3-5 and D7-9, but there was a marked reduction in weight gain in D13-15 in ATB+ (0.4±0.2kg) and ATB- (0.3±0.5kg), when it was observed the highest frequency of diarrhea in calves. After diarrhea peak (D13-15) the animals restored weight gain D20-23 (ATB- = 1.0±0.6kg, ATB+ = 0.7±0.4kg), D27-30 (ATB- = 1.0±0.5kg, ATB+ = 1.0±0.5kg).

Diarrhea etiology

The results obtained for the detection of *Cryptosporidium* spp. and rotavirus are shown in Figure 8. The dynamics of *Cryptosporidium* spp. were similar between experimental

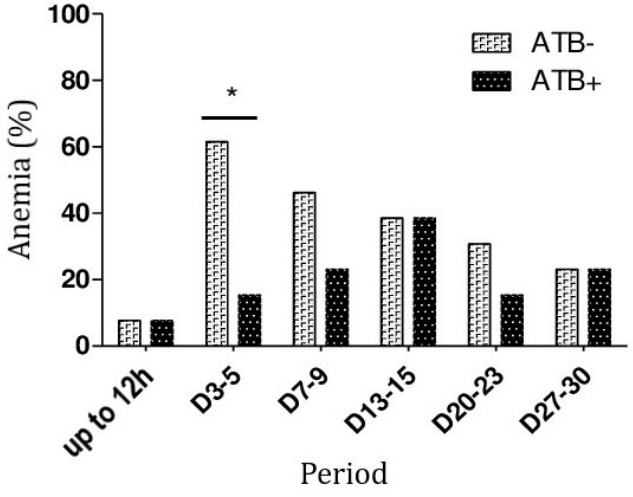

Fig.3. Frequency of Holstein calves presenting with anemia in ATB- and ATB+ groups in the first month of life.

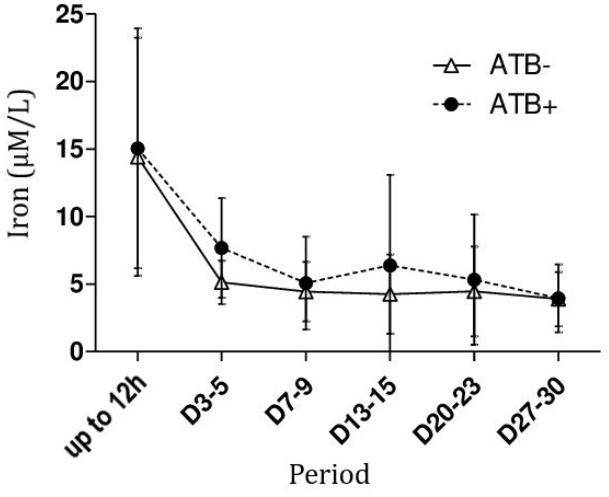

Fig.4. Mean iron values (μM/L) in the serum of ATB- and ATB+ Holstein calves in the first month of life.

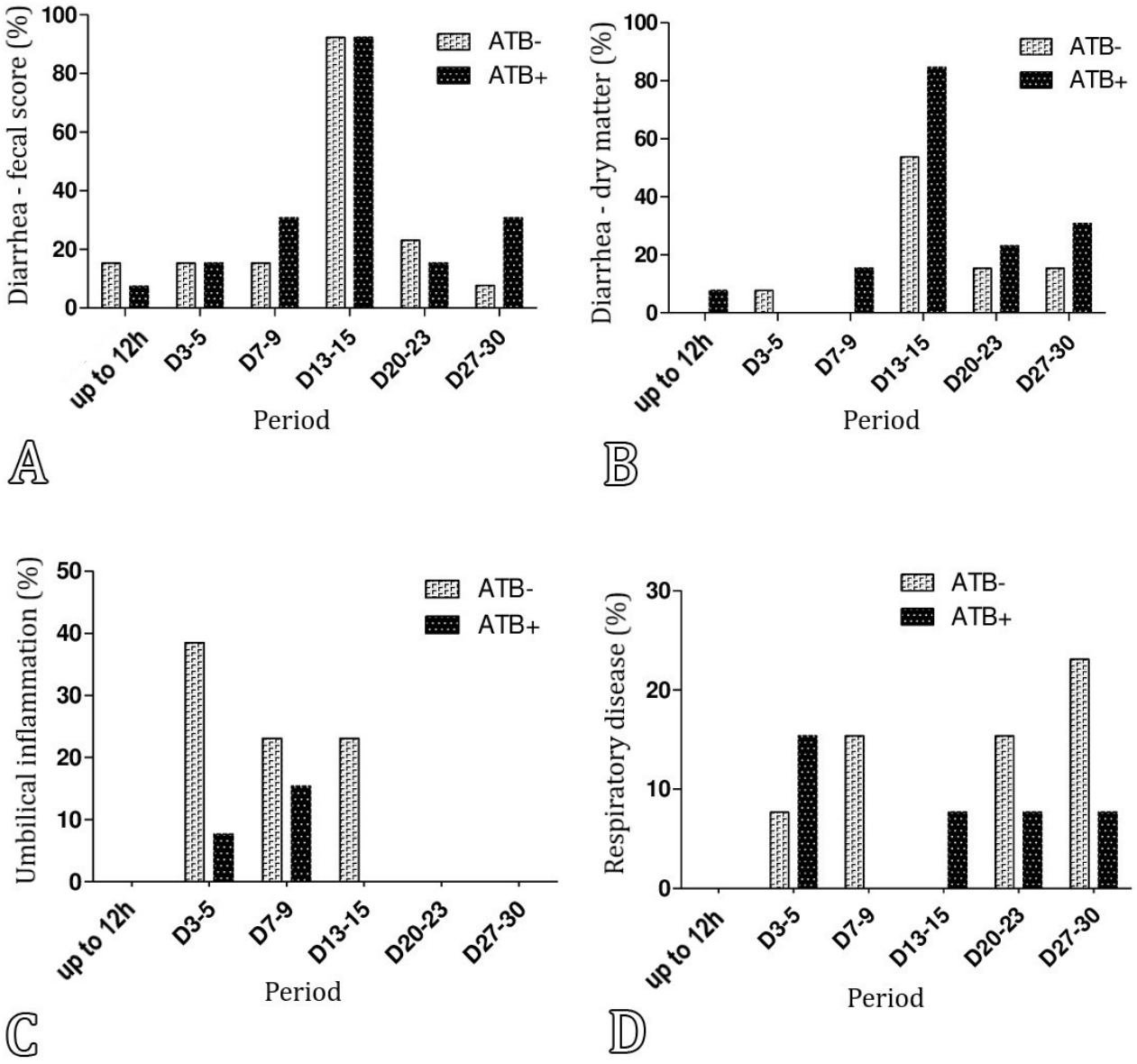

Fig.5. Frequency (%) of Holstein ATB- and ATB+ Holstein calves presenting (**A**) diarrhea by fecal score evaluation, (**B**) diarrhea by fecal dry matter evaluation, (**C**) umbilical inflammation and (**D**) respiratory disease in the first month of life.

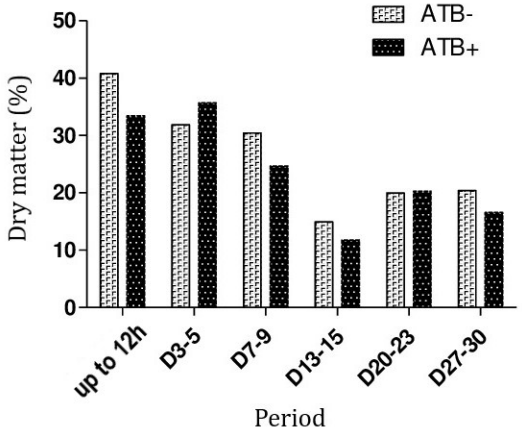

Fig.6. Mean values and standard deviations for the percentage of dry matter in feces of ATB- and ATB+ Holstein calves in the first month of life.

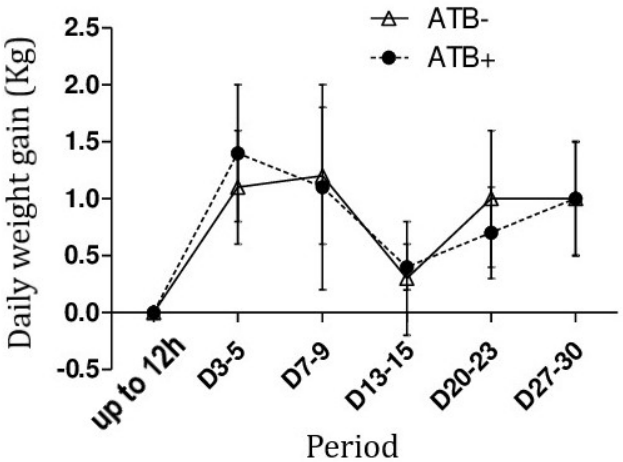

Fig.7. Daily Weight Gain (DWG) of ATB- and ATB+ Holstein calves groups in the first month of life.

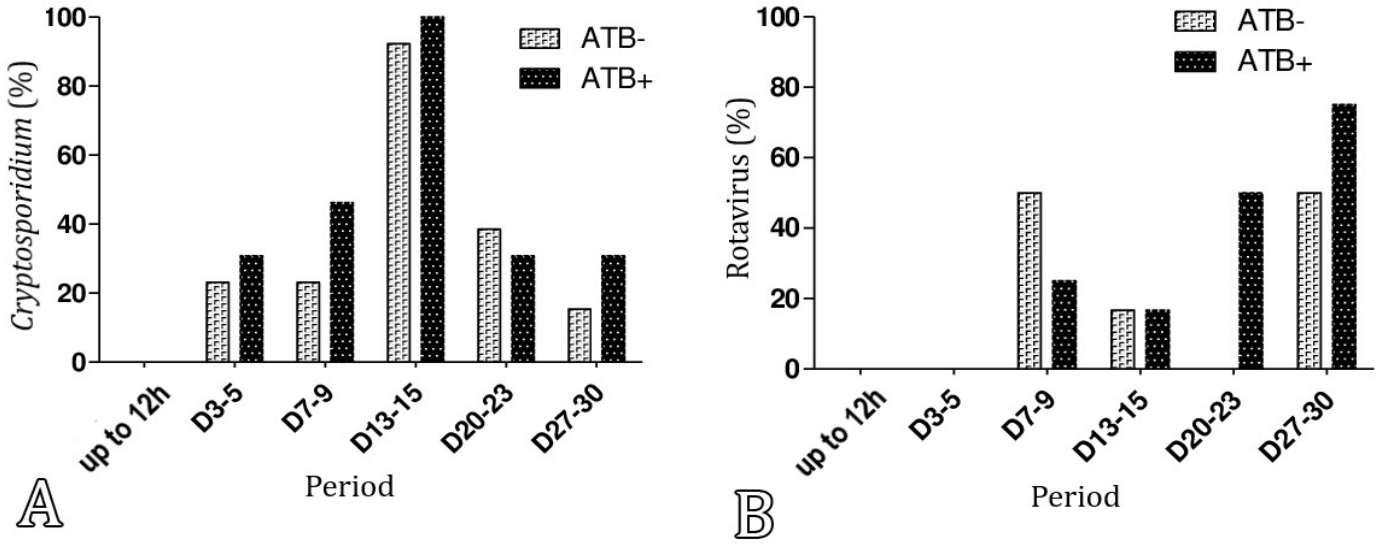

Fig.8. Frequency (%) of (**A**) *Cryptosporidium* spp. and (**B**) rotavirus in the feces of Holstein calves presenting with diarrhea in ATB- and ATB+ groups in the first month of life.

groups from D3-5 to D27-30 (Fig.8A). At ≤12h, two calves with ATB- diarrhea and one calf with ATB+ diarrhea were observed, however this clinical manifestation was not caused by *Cryptosporidium* spp. In the subsequent time (from D3-5 to D20-23), all calves ATB+ and ATB- with diarrhea were positive for fecal *Cryptosporidium* spp. (100%). In D27-30 frequencies of 100% (1/1) and 75% (3/4) for *Cryptosporidium* spp. were observed in ATB- and ATB+ groups, respectively.

The frequency of rotavirus in the fecal samples with a score of 2 or 3 was similar between groups and ATB- and ATB+ (P≥0.171) (Fig.8B). Rotavirus positive tests were detected at D7-9 (ATB- = 50%, ATB+ = 25%), D13-15 (ATB- = 16.7%, ATB+ = 16.7%), D20-23 (ATB- = 0%, ATB+ = 50%) and D27-30 (ATB- = 50%, ATB+ = 75%). Coronaviruses were not detected in the feces score 2 and 3 of the calves of both groups.

DISCUSSION

The proposal for this study came initially with questions from farmers about the effectiveness of prophylactic treatment with calf antimicrobials. Thus, the objective of this study was to evaluate the influence of early use of antimicrobial (tulathromycin) on the health and performance of calves during the neonatal period.

Thus, it could not be observed statistical differences between the groups regarding the clinical examination resulting from the use of antimicrobial. The variations observed in the first evaluation reflect the adaptation of the calves to the extrauterine environment as a result of the interval between birth and first evaluation (Mee 2008). The heart rate profile throughout the study was similar to that found by Novo et al. (2017), who also observed a decrease in values up to 14 and 21 days and an increase in subsequent moments. Respiratory rate showed an increasing behavior in the first week of life with progressive reduction until D27-30, corroborating the findings of Silva et al. (2016). These variations in time reflect the physiological homeostatic variability during the neonatal period, as well as the incomplete development of the anatomical and functional functions of neonates. The temperature variation profile over time is within the physiological values for the species and agrees with Silva et al. (2016). After birth, calves need to activate thermogenic mechanisms for temperature control. This regulation depends on the environment in which this animal is housed, and the colostrum intake, and the temperature tends to stabilize within 48 and 72 hours (Leone et al. 2009).

Red blood cell analysis revealed lower hemoglobin and MCHC concentrations in D3-5 in ATB- group calves. These data are consistent with the lower iron content and higher frequency of stained mucosa and anemia in ATB- group. Low iron concentration in calves in the neonatal period is due to low iron concentration in milk. However, all calves used in this experiment received the same diet, and it could be observed lower iron levels in ATB- group. These data suggest the effect of chronic inflammatory processes arising from umbilical inflammation in ATB- group. Ganz (2005) states that this type of anemia may be a side effect of the host defense response to control bacterial multiplication, considering that iron is fundamental to its metabolism. The erythrogram showed classic variations over time due to age. Hemoglobin, hematocrit, MCV and MCH values decreased over time as described by Novo et al. (2015). Red blood cells decreased to the D7-9, progressively increasing from that time until the end of the experiment.

Umbilical inflammation was detected early between D3-5 to D13-15. Throughout this period, the disease was more frequent in ATB- calves. Affections related to the structures that constitute the umbilical cord are among the main factors affecting the development of newborns in the 1st week of life. We can suggest that the differences observed between the groups regarding the frequency of umbilical changes are due to the protection provided by the antimicrobial provided within the first 12 hours of life. Tulathromycin acts by inhibiting protein synthesis essential for bacterial metabolism; its action is highest against Gram-positive bacteria, especially during its 10-day period of action (Stanton et al. 2010, Ives & Richeson 2015). Unfortunately we did not culture umbilical secretion to determine which microorganisms were responsible for

inflammation in this region. However, we know that among the main agents involved in the etiology of umbilical infections are *Staphylococcus* spp., *Streptococcus* spp., *Micrococcus* spp., *Bacillus* spp., and *Actinomyces pyogenes* (Rengifo et al. 2006), which are Gram-positive bacteria and exhibit antimicrobial sensitivity used in this research.

The main disease which affected calves this experiment was diarrhea. Through the evaluation of the scores it can be observed that the diarrhea started soon after the colostrum breastfeeding at ≤12h, with maximum peak in D13-15, and frequency decrease in later moments. The frequency of diarrhea through the percentage of dry matter in the stool (≤15%) was similar to that estimated by the scoring system. The average values of dry matter content presented lower percentage in the feces of ATB+ calves at moments D7-9 and D27-30, but without statistical difference.

Supplementation of oxytetracycline and neomycin antimicrobials in milk is the main route used to prevent diarrhea and increase calf weight gain. In general, research found null (Donovan et al. 2002) or unsatisfactory (Berge et al. 2009) effect with oral antimicrobial use, which agrees with the absence of positive results observed in this research for parenteral use of tulathromycin with the goal of decreasing the prevalence of diarrhea.

In our research, we observed a larger number of animals presenting diarrhea by evaluating the dry matter at time D13-15 for the ATB- group and we observed no effect on calves weight gain. Stanton et al. (2010) conducted research evaluating the effects of parenteral tulathromycin on disease occurrence and performance of calves and found different results from those found in our research. The authors found an 80% frequency of diarrhea in the treated group, while the control group had a rate of 87%, corresponding to 1.8 (P=0.005) times more likely to have diarrhea. The weight gain of the calf group that received tulathromycin was 0.02±0.01kg/day (P=0.010) higher than the control group animals.

The effect of prophylactic use of tulathromycin observed in this research did not show significant difference in relation to diarrhea, despite the higher frequency observed in ATB+ group, there were also no significant differences in animal performance. However, we must consider that the number of animals evaluated was small, which may have influenced this result, which is a limitation of this study. Thus, further research is needed to investigate the effect of antimicrobial on short and long term performance of calves, as well as its influence on intestinal microbiota.

Some mechanisms have been postulated in the association between diarrhea and antimicrobial use, such as: growth of opportunistic pathogens; changes in microbial metabolic functions; colonization by resistant bacteria and inability of microbiota to resist overgrowth of pathogenic microorganisms (McFarland 1999, Young & Schmidt 2004). Changes in bacterial composition and quantity, even in the absence of overgrowth of pathogenic microorganisms, can lead to changes in colonic metabolism leading to diarrhea. Young & Schmidt (2004) described that colonic bacteria, especially certain anaerobes, metabolize dietary carbohydrates and use them as an energy source to produce lactic acid and short chain fatty acids, the latter being absorbed by the colon. Loss of these bacteria due to antimicrobial treatment can lead to the accumulation of these carbohydrates that are not absorbed in the colon, leading to osmotic diarrhea, short chain fatty acids mainly butyrate is an important source of energy for the distal colon mucosa, reduced butyrate productions due to decreased anaerobic bacterial populations can cause direct mucosal functional disorders.

For the diagnosis of diarrhea, we performed the research of *Cryptosporidium* spp., rotavirus and coronavirus. A high frequency of ositive animals for *Cryptosporidium* spp. was observed, and the dynamics of infection were similar between experimental groups from D3-5 to D27-30. Regarding rotavirus, it was possible to identify 11 positive calves during the study period. Rotavirus positive tests were detected from D7-9 until the end of the study, in which higher frequency of rotavirus was observed in ATB+ group on D27-30, despite the absence of statistical differences. Coronaviruses were not detected in the feces score 2 and 3 of the calves of both groups. Several studies have shown that *Cryptosporidium* spp. associated or not with Rotavirus is the main agent involved in neonatal diarrhea, corroborating the findings of this research.

A study conducted in France with diarrhea dairy calves showed that only 6.1% of the animals studied were infected by *E. coli* K99, 14.3% by rotavirus, 6.8% by coronavirus, 0.3% by *Salmonella* spp. and 50% were excreting *Cryptosporidium parvum* oocysts. The peak of oocyst elimination reported in this study was on the seventh day of life (Naciri et al. 1999), different from what we observed in our study with higher prevalence in D13-15.

Despite *Salmonella* spp. being among the main causative agents of diarrhea we did not perform research on this agent. Previous work by this team in São Paulo found only two positive animals for *Salmonella enteritidis* on the second day of life. Due to the low frequency of this agent in São Paulo, associated with the need for specific means of collection and transportation of stools for a reliable diagnosis, is decided not to search this agent in this work. However, a study conducted in the state of Minas Gerais showed high frequencies (50 to 66%) of *Salmonella* spp. in calves during lactation (Carvalho et al. 2014), which differs from the results found in other studies (Costa et al. 1979, Langoni et al. 2004, Ferreira et al. 2009). This finding may be associated with the type of rearing animals were submitted to, with high stocking rate, high humidity, organic matter accumulation, high temperature and age difference between the animals used in the different researches. Other studies also report the importance of *Salmonella* spp. in public health, salmonellosis being one of the leading causes of intestinal infections in humans (Pelkonen et al. 1994). Thus, we know that the lack of diarrhea diagnosis by *Salmonella* spp. is a limitation of this study.

At ≤12h there were two calves with diarrhea in ATB- group and one calf with diarrhea in ATB+ group, however this clinical manifestation was not associated with *Cryptosporidium* spp. or to the rotavirus. Enterotoxigenic *E. coli* infections usually occur in animals less than five days old, so this bacterial group may have participated in the development of diarrhea observed in calves. Most strains of *E. coli* present in the gastrointestinal tract are non-pathogenic commensals and participate in the normal process of colonization of the intestine (Manzoor et al. 2015, El-Seedy et al. 2016). Unfortunately we did not perform *E. coli* research and identify virulence factors or search for genes that encode toxins (Andrade et al. 2012, Costa et al. 2014). Thus, we can suggest that the diarrhea observed in

these animals at the very first moment of collection may have occurred due to the laxative effect of colostrum responsible for clearing the intestine through the elimination of meconium (Embrapa 2005).

Cows from this farm were vaccinated in the pre-partum period for *E. coli*, rotavirus and coronavirus, the low rate of rotavirus positive animals and absence of coronavirus may indirectly indicate the prevention of infection by passive antibody transfer by colostrum ingestion. It is worth noting that there are some differences regarding the effectiveness of vaccines used against agents that cause diarrhea. Meganck et al. (2015) reported that the quality of colostrum may reflect the success of the vaccination protocol. Many risk factors are associated with diarrhea, and identification of the etiological agent is important. Bartels et al. (2010) reported that the main risk factors for the presence of *Cryptosporidium* spp. were mixed infections; the presence of rotavirus in the feces increased the chances of detection of *C. parvum* by 2.2 times. Farms where calves with diarrhea were routinely treated with antimicrobials (orally or injected) were 3.2 times more likely to detect *C. parvum*.

The farm used for this research has excellent sanitary management and has protocols directed to cleaning and disinfecting the calf. The calves remained in individual cages with slatted wooden floors with a replaced hay bed once a day. Twice a week, all cages were sprayed with the chlorhexidine-cetrimide solution. After weaning, the stalls were washed with water, and after drying, fire, brooms, and lime were used. The stalls remained empty for about 30 days depending on the number of births despite proper sanitary management, a high frequency of *Cryptosporidium* spp. infection was observed, possibly because the product used in disinfection (chlorhexidine-cetrimide) has no action against this agent, despite its broad spectrum for bacteria, fungi, algae, and viruses.

Based on the data obtained in this research, it was possible to verify that the early use of antimicrobials near birth did not bring benefits about the prevalence of neonatal diarrhea, because it has no direct action on *Cryptosporidium* spp. and rotavirus. Thus, the establishment of etiological diagnosis is fundamental for definition therapeutic and prophylactic protocols with or without the use of antimicrobials. The cost of prophylactic treatment using one dose of tulathromycin (price of medication and application costs), not counting the work of collaborators, is around R$ 7,50 (reais) per animal. The cost and benefit of using this prophylactic protocol are unlikely to compensate financially as no improvement in diarrhea performance or prevalence could be observed. The only benefit detected was the decreased frequency of anemia and umbilical inflammation; however, these infections were mild, transient, and did not compromise the development of the animals.

CONCLUSION

The administration of prophylactic tulathromycin (2.5mg/kg) at birth decreased the frequency of anemia in calves receiving the antimicrobial. This practice did not influence calf weight gain and diarrhea rates.

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

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Original Article
Livestock Diseases

Characteristics of virulence, resistance and genetic diversity of strains of *Salmonella* Infantis isolated from broiler chicken in Brazil[1]

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ABSTRACT - Mendonça E.P., Melo R.T., Oliveira M.R.M., Monteiro G.P., Peres P.A.B.M., Fonseca B.B., Giombelli A. & Rossi D.A. 2020. **Characteristics of virulence, resistance and genetic diversity of strains of *Salmonella* Infantis isolated from broiler chicken in Brazil.** *Pesquisa Veterinária Brasileira 40(1):29-38.* Laboratório de Biologia Molecular, Faculdade de Medicina Veterinária, Universidade de Uberaba, Av. Nenê Sabino 1801, Bloco 2D, sala 52, Universitário, Uberaba, MG 38055-500, Brazil. E-mail: eliane_vet@yahoo.com.br

Salmonella Infantis is frequently associated with human infections worldwide and is transmitted by consumption of contaminated foods, particularly those of animal origin, especially the chicken meat. We aimed to evaluate virulence characteristics, antimicrobial resistance and the genetic similarity of 51 strains of *S.* Infantis isolated from samples of poultry origin. The strains were isolated from 2009 to 2010 in a company with full cycle of broiler's production in the state of São Paulo, Brazil. The antimicrobial susceptibility test was performed and, by PCR, we evaluated the presence of the genes *lpf*A (hem-adhesion), *agf*A (hem-biofilm) and *sef*A (hem-adhesion) and resistance genes to beta-lactams (bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{AmpC}). The phylogenetic relationship was determined by RAPD-PCR method. Among the drugs tested, the highest percentages of resistance were to amoxicillin (35.3%) and to sulfonamide (15.7%). Eleven antimicrobial resistance patterns were identified (A1 to A11), none of them presented a multiresistance profile (> 3 antimicrobials classes). There was 100% of positivity for the *agf*A gene, 92.2% for the *lpf*A gene, and no strain presented the *sefA* gene. Most of the isolates showed similarities in virulence potential, since they were simultaneously positive for two studied genes, *agfA* and *lpfA* (92.2%, 47/51). Of the 18 (35.3%) strains resistant to antimicrobials of the β-lactam class, 10 (55.5%) were positive to bla_{AmpC} gene, five (27.8%) for bla_{CTX-M}, two (11.1%) to bla_{SHV} and no strain presented the bla_{TEM} gene. The phylogenetic evaluation has shown the presence of five clusters (A, B, C, D and E) with similarity greater than 80%, and three distinct strains which were not grouped in any cluster. Cluster B grouped 33 strains, all positive for *lpf*A and *agf*A genes, from both, the broiler farming facility and the slaughterhouse, persistent throughout all the study period. This cluster also grouped 18 strains clones with genetic similarity greater than 99%, all isolated in the slaughterhouse. The presence of virulence genes associated with persistent strains clones for a long period, warns to the possibility of *S.* Infantis to form biofilm, and should be constantly monitored in broilers' production chain, in order to know the profile of the strains that may contaminate the final product and evaluate the hazards that represents to public health.

INDEX TERMS: Virulence genes, resistance genes, genetic diversity, strains, *Salmonella* Infantis, broiler chicken, Brazil, RAPD-PCR, antimicrobial resistance, *Salmonella*, genes, chickens.

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RESUMO.- [**Características de virulência, resistência e diversidade genética de estirpes de *Salmonella* Infantis isoladas de frangos de corte no Brasil.**] *Salmonella* Infantis é frequentemente associada a infecções humanas no mundo todo sendo transmitida pelo consumo de alimentos contaminados, principalmente aqueles de origem animal, com destaque para a carne de frango. Objetivou-se avaliar características de virulência, resistência antimicrobiana e a similaridade genética de 51 estirpes de *S*. Infantis isoladas em amostras de origem avícola. As estirpes foram isoladas no período de 2009 a 2010 em uma empresa com ciclo completo de produção de frango de corte, localizada no estado de São Paulo, Brasil. Foi realizado o teste de susceptibilidade antimicrobiana e pela técnica de PCR, foi avaliada a presença dos genes *lpf*A (fímbria-adesão), *agf*A (fímbria-biofilme) e *sef*A (fímbria-adesão) e os genes de resistência aos beta-lactâmicos (bla_{TEM}, $bla_{SHV,}$ $bla_{CTX-M\ e}$ bla_{AmpC}). A relação filogenética foi determinada pelo método de RAPD-PCR. Dentre as drogas testadas, os maiores percentuais de resistência foram para amoxacilina com 35,3% e sulfonamida com 15,7%. Onze perfis de resistência aos antimicrobianos foram identificados (A1 a A11), sendo que nenhum deles apresentou perfil de multirresistência (>3 classes de antimicrobianos). Houve 100% de positividade para o gene *agf*A, 92,2% para o gene *lpf*A e nenhuma estirpe apresentou o gene *sef*A. A maioria dos isolados apresentaram semelhanças no potencial de virulência, pois foram positivos simultaneamente para dois genes estudados, *agf*A e *lpf*A (92,2% - 47/51). Das 18 (35,3%) estirpes resistentes aos antimicrobianos da classe dos β-lactâmicos, 10 (55,5%) foram positivas para o gene bla_{AmpC}, cinco (27,8%) para bla_{CTX-M}, duas (11,1%) para bla_{SHV} e nenhuma estirpe apresentou o gene bla_{TEM}. A avaliação filogenética demonstrou a presença de cinco *clusters* (A, B, C, D e E) com similaridade superior a 80%, e três estirpes distintas que não foram agrupadas em nenhum dos *clusters*. O *cluster* B agrupou 33 estirpes, todas positivas para os genes *lpf*A e *agf*A, provenientes tanto do aviário quanto do matadouro frigorífico, persistentes durante todo o período do estudo. Este *cluster* ainda agrupou 18 estirpes clones com similaridade genética superior a 99%, todas isoladas no matadouro frigorífico. A presença dos genes de virulência, associada à persistência das estirpes clones durante um longo período do estudo, alertam para a possibilidade de *S*. Infantis em formar biofilme, devendo ser constantemente monitorada na cadeia de produção avícola, especialmente no ambiente de abate, de forma a conhecer o perfil das estirpes que podem contaminar o produto final e assim avaliar os perigos que representam para a saúde pública.

TERMOS DE INDEXAÇÃO: Virulência, genes de resistência, diversidade genética, estirpes, *Salmonella* Infantis, frangos de corte, Brasil, *Salmonella*, RAPD-PCR, resistência antimicrobiana.

INTRODUCTION

Salmonellosis is one of the most common food-borne diseases, considered a complex zoonosis that affects global public health. In Brazil, it is the primary cause of outbreaks in which the etiological agent is identified (Brasil 2008a). The intestinal tract of a wide variety of animals is the reservoir of this bacterium, which can still survive in diverse environments, explaining its high potential for dissemination. The broiler chicken is one of the main reservoirs of this pathogen, with a high frequency of contamination of the final product in the slaughterhouse (EFSA 2014, 2015). Thus, the consumption of chicken meat is considered a risk factor for human infection by *Salmonella* (FAO-WHO 2009). In order to reduce the prevalence of this agent and establish an adequate level of consumer protection, the Ministry of Agriculture recently implemented a controlling and monitoring program for *Salmonella* sp. both in commercial establishments of broiler chickens and turkeys, as in slaughter and breeding environments (Brasil 2016).

More than 2,600 serotypes of *Salmonella* are known, but a limited number are associated with most human diseases, and the prevalence of different serovars may change over time (EFSA 2014). Since the late 1970s, serovar Infantis has been increasingly registered in countries such as Argentina, Australia, Brazil, the Netherlands, Finland, Canada, Hungary, Japan, New Zealand and Russia (Miller et al. 2010). Along with *S.* Enteritidis and *S.* Typhimurium, *S.* Infantis has been reported for involvement in human cases of the disease, and is the most frequently isolated in the live bird as well as in the chicken meat (EFSA 2015). In Brazil, this serovar is also among those most isolated from broiler samples of producing farms (Medeiros et al. 2011, Voss-Rech et al. 2015).

The long-term use of antimicrobials in animal husbandry exerts selection pressure on bacteria population, thus favoring the survival of resistant strains of *Salmonella*, which can be transferred to humans through the consumption of contaminated food and can lead to antibiotic therapy failure (Lai et al. 2014). The dynamics of resistance transmission and the evolution of populations of resistant bacteria are difficult to elucidate and are associated with the genetic transfer of so-called resistance genes (Alekshun & Levy 2007). Several international health authorities consider the occurrence of antimicrobial resistance in zoonotic micro-organisms as one of the major emerging problems of importance to public health (Moore et al. 2006).

The extended-spectrum β-lactamase (ESBL) is an enzyme that allows bacteria to become resistant to a wide variety of penicillins and cephalosporins, and the bacteria that contain this enzyme are resistant to the β-lactam penicillins, cephalosporins of 3rd and 4th generations and monobactams, remaining sensitive to carbapenems, cephamycins (2nd generation of cephalosporins) and β-lactamase inhibitors. In the United States an estimated 26,000 infections occur annually due to ESBL-producing bacteria, resulting in about 1,700 deaths, generating large hospital costs in the country (CDC 2014).

The molecular mechanisms involved in the pathogenicity of *Salmonella* spp. are also complex, and investigations on virulence factors have shown that pathogenic strains are differentiated from those that are not by the presence of pathogenicity specific genes, which are located in the Pathogenicity Islands (PI) (Kaur & Jain 2012). Furthermore, it is known that there is a genetic differentiation in isolates of the same serotype, determining variations for virulence in different strains (Borges et al. 2013). These genes provide the microorganism with adhesion, invasion, colonization, survival and multiplication in the host cells, determining a series of events that trigger the disease (Suzuki 1994, Vieira 2009). There are many types of fimbriae that help *Salmonella* in the intestinal adhesion including long polar fimbriae (Lpf), aggregative fimbriae (Agf), whose operon is highly conserved among the isolates of this pathogen, and the

S. Enteritidis (Sef) fimbria, identified in serotypes of group D (Bäumler et al. 1997).

The evaluation of the spread of pathogens along the food chain is of great importance for industries to act in the prevention and implementation of more effective control programs. Molecular typing methods for *Salmonella* have been widely used in order to establish the phylogenetic relationship between bacterial isolates and provide important data for the understanding of epidemiology. In this sense, Random Amplified Polymorphic DNA (RAPD-PCR) is a tool widely used for epidemiological studies in several countries because of its high discriminatory power (Turki et al. 2014, Vázquez-Garcidueñas et al. 2014).

The aim of this study was to evaluate the resistance characteristics of β-lactams by evaluating the presence of the bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{AmpC} genes and also to investigate the virulence genes *agfA*, *sefA* and *lpfA* that are involved in the adhesion and the process of biofilm formation, in strains of *S.* infants isolated from samples of poultry origin, from the broiler breeding environment to the slaughterhouse. It was also evaluated their dissemination within the production chain, in order to estimate the danger they pose to human health.

MATERIALS AND METHODS

Origin of the strains. *Salmonella* Infantis strains were derived from a previous study that monitored *Salmonella* spp. in two units of the same company, with a complete cycle of broiler chicken production and integration system, located in the state of São Paulo (SP) and Mato Grosso do Sul (MS), during the period from 2009 to 2010. The slaughterhouse was inspected by the federal inspection service and the chicken meat produced was marketed throughout the national territory and exported. Samples were collected at all stages of the production cycle, from the poultry rearing environment to the ready-to-trade industrialized final product, including samples from the slaughter environment.

Considering the total of 239 strains, 187 were isolated in SP and 52 in MS. *S.* Infantis was not identified in the MS unit, but in SP, it was the most prevalent serovar, representing 27.27% (51/187) of the isolates.

Among the 51 strains of *S.* Infantis used in this study, 14 were isolated in the environment of broiler poultry (samples of disposable foot socks and sampling of drag swabs of the shed were taken when the chickens were with approximately 30 days). Thirty-seven samples were obtained from the slaughterhouse and collected at the points required by the Pathogen Reduction Program - PRP (Brasil 2003) and, in addition, other points with a higher frequency of isolation of *Salmonella* in the routine of the industries studied, including samples of meat cuts (1), mechanically separated meat (MSM) (10), scalding water (1) and water from the pre-cooling tanks (1).

The antigenic typing was carried out by the "Fundação Instituto Oswaldo Cruz" (Fiocruz) in the state of Rio de Janeiro.

Antimicrobial sensitivity test. The susceptibility of the strains to the antimicrobial agents was evaluated by the disc diffusion technique, using a protocol recommended by the Clinical and Laboratory Standards Institute (CLSI 2013). The antimicrobials choices were based on the use of these drugs in veterinary and human medicine and the occurrence of resistance in both areas. The antimicrobials and microgram concentrations tested were: amoxicillin (10μg) (β-lactam/penicillin), norfloxacin (10μg) (fluorquinolone), neomycin (30μg) (aminoglycoside), gentamicin (10μg) (aminoglycoside), trimetropin (5μg) (pyrimidine), ceftazidime (30μg) (β-lactam/cephalosporin), chloramphenicol (30μg) (phenicol), imipenem (10μg) (β-lactam/carbapenem), tetracycline (30μg) (tetracycline), sulfonamide (300μg) (sulfonamide) (LABORCLIN®). Inhibition zones were measured and the results were classified as sensitive, intermediate or resistant according to CLSI (2013) recommendations. *Salmonella* isolates that were resistant to three or more classes of antimicrobials were defined as multiresistant (Brasil 2008b). The strain *Escherichia coli* ATCC 25922 was used as a quality control of the sensitivity tests.

Research on virulence genes and antimicrobial resistance. Three virulence genes were investigated in *S.* infantis, related to the phases of adhesion and consequent lesion in intestinal cells (Table 1).

Extraction of the bacterial DNA was performed using the commercial DNA Purification Kit (Promega) according to the manufacturer's instructions. DNA quantification was done in a spectrophotometer (Femto 750®) with a wavelength of 260nm.

For the polymerase chain reaction (PCR) analyzes of the virulence genes, the strain *S.* Enteritidis ATCC 13076 was used as the positive control. PCR reactions were performed from a final volume of 25μL containing 1μL of the DNA sample, 2.5μl of 10X buffer, 0.75μl of 50mM $MgCl_2$, 1.25μl of 10pmol/μl of the forward and reverse sequence of each primer (Invitrogen®), 0.25μl of 20mM of the mix of dNTPs (Invitrogen®), 0.25μL of Taq (5U/μL)

Table 1. Virulence and resistance genes, primer sequences and amplicon sizes (bp)

Gene	Primers	Molecular weight[a]	Reference
*sef*A	F:5'GATACTGCTGAACGTAGAAGG3' R:5'GCGTAAATCAGGATCTGCAGTAGC3'	488 bp	Oliveira et al. (2003)
*agf*A	F:5'TCCACAATGGGGCGGCGGCG3' R:5'CCTGACGCACCATTACGCTG3'	350 bp	Collinson et al. (1993)
*lpf*A	F:5'CTTTCGCTGCTGAATCTGGT3' R:5'CAGTGTTAACAGAAACCAGT3'	250 bp	Bäumler & Heffron (1995)
bla_{TEM}	F: 5'CAGCGGTAAGATCCTTGAGA3' R: 5'ACTCCCCGTCGTGTAGATAA3'	643 bp	Chen et al. (2004)
bla_{SHV}	F: 5'GGCCGCGTAGGCATGATAGA3' R: 5'CCCGGCGATTTGCTGATTTC3'	714 bp	Chen et al. (2004)
bla_{CTX-M}	F: 5'ATGTGCAGYACCAGTAARGTKATGGC3' R: 5'TGGGTRAARTARGTSACCAGAAYCAGCGG3'	593 bp	Monstein et al. (2007)
bla_{AmpC}	F: 5'CCCCGCTTATAGAGCAACAA3' R: 5'TCAATGGTCGACTTCACACC3'	634 bp	Shahid (2010)

[a] bp = base pairs.

(Invitrogen®) and 17.75µL of ultrapure H_2O. The samples were submitted to the following amplification cycles: initial denaturation at 94°C for 5 minutes, amplified in 35 denaturation cycles at 94°C for 45 seconds, annealing at 50°C for 30 seconds (*sefA* and *lpfA*), 66°C for 30 seconds (*agfA*), extension at 72°C for 90 seconds, final extension at 72°C for 10 minutes.

The strains that showed resistance to the antimicrobials of the β-lactam group were also evaluated for the presence of resistance genes described in Table 1. A *Klebsiella pneumoniae* field strain previously tested was used as the positive control for the presence of the four studied genes, provided by the "Laboratório de Microbiologia Molecular" of the "Universidade Federal de Uberlândia". The preparation of the mix for the PCR reactions was the same as for the virulence genes.

The amplification conditions followed the steps: initial denaturation at 94°C for 5 minutes, 30 denaturation cycles at 94°C for 45 seconds, annealing at 50°C for 45 seconds (*blaTEM*), 56°C for 45 seconds (*blaSHV*), 58°C for 1 minute (*blaCTX-M*) and 54°C for 1 minute (*blaAmpC*), extension at 72°C for 90 seconds, followed by a final extension of 72°C for 10 minutes.

Evaluation of genetic similarity between the strains. The isolates were submitted to gene analysis by RAPD-PCR using the protocol described by Oliveira et al. (2007). As a positive control, the *S.* Enteritidis strain ATCC 13076 was used.

RAPD-PCR reactions were performed with two primers, individually, which were described by Lin et al. (1996): 23L (5'-CCGAAGCTGC-3 ') and P1254 (5'-CCGCAGCCAA-3').

The RAPD-PCR technique was performed from a final volume of 25µL containing 1µL of DNA sample at 50ng/µL, 2.5µL of 10X buffer, 0.75µL of 50mM $MgCl_2$, 0.25µL of 20mM dNTP mix (Invitrogen®), 0.25µL of Taq (5U/µL) (Invitrogen®) and 17.75µL of ultrapure H_2O. The concentration of the primers was 50pmole for P1254 and 30pmole for 23L (Invitrogen®). The PCR reaction was conducted under the following conditions: one cycle of 94°C for 4 minutes, followed by 35 cycles of 94°C for 1 minute, 35°C for 1 minute, 72°C for 2 minutes, and final extension at 72°C for 5 minutes.

All PCR reactions were performed on the thermal cycler (Eppendorf®) and the amplified products separated in 1.5% agarose gel electrophoresis for 120 minutes. The gel was stained with Syber Safe (Invitrogen®) and visualized in UV translucent (Loccus Biotechnology®).

Data analysis. The results were tabulated and submitted to analysis through descriptive statistics, calculating the percentages of antimicrobial resistance and the presence of virulence genes. For the analysis of genetic similarity between the strains, computational analysis was used by the GelCompar II Program (Comparative Analysis of Electrophoresis Patterns), version 1.5, Applied Maths Korthrijk, Belgium. The profiles obtained in the gel captured by the program were considered in the analysis and the similarity matrix was obtained by comparing pairs of strains using the Dice similarity coefficient, adopting 1% of tolerance for each primer separately. The final analysis was based on the average of experiments. For the analysis of all the studied strains, the UPGMA (Unweighted Pair Group Method with Arighmetic Mean) method was used to construct the dendogram, in which the isolates were grouped in clusters when they presented homology equal or higher than 80% or in different genotypes when they had less than 80% of homology. The strains were compared considering the local of isolation, date of collection, profile of antimicrobial resistance and presence of virulence genes and resistance to β-lactams. Groups with homology greater than 99% were classified as clones.

RESULTS AND DISCUSSION

The occurrence of *Salmonella* Infantis only in the industrial unit of São Paulo, and not in the unit of Mato Grosso do Sul, is possibly due to the fact that the birds acquired by these units have different origins and come from different hatcheries.

Figure 1 presents the results of the resistance percentages for the antimicrobials tested in the 51 strains of *S.* Infantis, being these percentages the sum of the isolates classified as resistant and intermediate by the disc diffusion test. The highest resistance indexes were for amoxicillin (class of β-lactams/penicillin), with 35.3% (18/51), and for sulfonamide (sulfonamide class), with 15.7% (8/51). The lower percentages of resistance were found for tetracycline (9.8%, 5/51) (tetracycline class) and ceftazidime (5.9%, 3/51) (β-lactam/cephalosporin of 3rd generation). All these antimicrobials belong to the exclusive veterinary classes in therapeutics, being prohibited their use as zootechnical additives of performance enhancers or as preservatives in animal feed, according to Normative Instruction no. 26 (Brasil 2009). All isolates were sensitive to norfloxacin, neomycin, gentamicin, trimethoprim, chloramphenicol and imipenem, thus suggesting an adequate use of these drugs in poultry production.

High levels of resistance to penicillin were also observed by Medeiros et al. (2011), in a study with *Salmonella* isolated from frozen carcasses in Brazil, where they found 44.8% of ampicillin-resistant strains. The high number of penicillin resistant strains, amoxicillin and ampicillin, warns of a major public health problem, since these drugs are considered the first choice for treatment of diseases in human medicine (WHO 2011). The serious public health problem is due to the possible transfer of resistant strains to man via contaminated food, especially those of poultry origin, which could lead to an ineffective therapy by the use of these drugs.

Lower percentage of resistance to sulfonamide, as found in this study, was identified by Rowlands et al. (2014) when evaluating strains of *Salmonella* spp. isolated from food in Brazil, where they found 2.1% of strains resistant to this drug. This result was not expected, since previous studies in Brazil reported a high frequency of resistance to this antimicrobial in *Salmonella* (Ghilardi et al. 2006, Bessa et al. 2007), suggesting the judicious use of this drug currently.

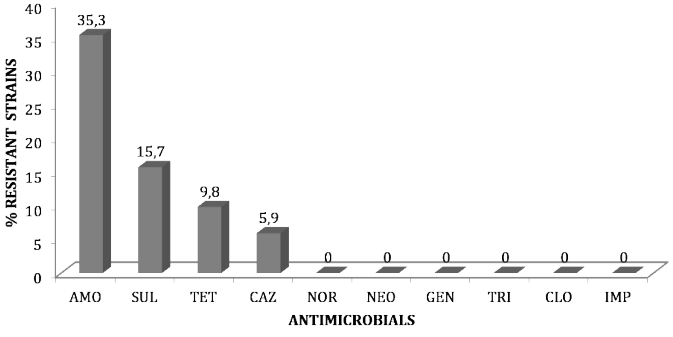

Fig.1. Frequency (%) of antimicrobial resistance in *Salmonella* Infantis isolated in a poultry production chain located in the state of São Paulo, Brazil. AMO = amoxicillin (10µg), SUL = sulfonamide (300µg), TET = tetracycline (30µg), CAZ = ceftazidime (30µg), NOR = norfloxacin (10µg), NEO = neomycin (30µg), GEN = gentamicin (10µg), TRI = trimethoprim (5µg), CLO = chloramphenicol (30µg), IMP = imipenem (10µg).

In Brazil, tetracycline have been banned as additives in animal feed since 1998, however, they are still used therapeutically and therefore may exert a selective pressure on microorganisms (Brasil 2009, Voss-Rech et al. 2015). Although levels of resistance to tetracycline, ceftazidime and sulfonamide are not considered alarming, it is critical to constantly evaluate the susceptibility of the isolates to these drugs, and thus to verify the resistance characteristics of *Salmonella*, to infer if the drugs administered in the birds are being properly used in animal production. Careful administration of antimicrobial agents and continuous surveillance are important initiatives that help define the best treatment and inhibit or hinder the selection and propagation of resistant strains between the flocks (Voss-Rech et al. 2015).

A study developed by Asgharpour et al. (2014), with strains of *S.* Infantis isolated from broiler chicken in Iran, found higher levels of resistance than those found in this study, with 70% for amoxicillin, 66% for trimethoprim-sulfamethoxazole, 100% for tetracycline, 28% for ceftazidime and 64% for chloramphenicol. According to Lai et al. (2014), the increase of resistance to sulfonamide and tetracycline is probably due to the use of these antimicrobials in animal feed, at subtherapeutic or therapeutic levels, to prevent diseases or to promote the animal growth.

The emergence of antimicrobial resistant bacteria to the classes of cephalosporins and fluoroquinolones is of great concern because both are used to treat serious human infections, and resistance to these drugs may cause serious complications in treatment (Hur et al. 2012, Kilonzo-Nthenge et al. 2013, Lai et al. 2014). Positive results were found in the present study, with only 5.9% of the strains showing resistance to ceftazidime, a third generation cephalosporin, whereas all *Salmonella* strains were sensitive to norfloxacin, a fluoroquinolone.

Eleven antimicrobial resistance profiles were identified (A1 to A11), being 27 (53%) strains with resistance or intermediate resistance to one or two drugs, and there were no isolates with a multiresistant profile (Table 2). The most frequent profiles were A11 (47%), with strains sensitive to all antimicrobials tested, and A2 (19.6%), showing strains with intermediate resistance for amoxicillin.

Voss-Rech et al. (2015), in a study with strains of *Salmonella* spp. isolated from broiler chicken in Brazil, also did not find multiresistance profiles for *S.* Infantis, which was the second most isolated serovar in the study, with 14.63%. In another study performed in different Brazilian cities, *S.* Infantis was also the second most isolated serovar in chicken carcasses with 7.6%. Of these, 57.8% were resistant to one or two antimicrobials, while 42.1% showed multiresistance profiles, with a higher resistance index for sulfonamide with 94.7% (Medeiros et al. 2011). On the other hand, a study developed by Thai et al. (2012) in North Vietnam, found that 78.2% of the strains of S. infants isolated from both, chicken and pork meat, presented resistance from four to 13 antimicrobials, demonstrating the prevalence of the multiresistance characteristic for this serovar in this country.

The occurrence of multidrug resistant strains may be associated, in addition to inappropriate use of antimicrobials in industrial poultry, to the spread of antimicrobial resistance genes. The PREBAF, a monitoring program of prevalence and antimicrobial susceptibility profile of *Salmonella* spp. isolated from frozen chicken carcasses marketed in Brazil, emphasizes the importance of characterizing multiresistant clones as to their ability to host and disseminate antimicrobial resistance genes (Brasil 2008b).

Among the resistance genes encoding ESBL are bla_{AmpC}, $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV}, which have been detected in *Salmonella* isolates from animal products in several countries (Rodriguez et al. 2009, Tamang et al. 2011). Of 18 (35.3%) antimicrobial resistant strains of the β-lactam class (amoxicillin and ceftazidime), 11 (61.1%) harbored one or more resistance genes, of which 10 (55.5%) were positive for the bla_{AmpC} gene, five (27.8%) for $bla_{CTX\text{-}M}$, two (11.1%) for bla_{SHV} and no strain showed the bla_{TEM} gene. Two strains showed the genes bla_{AmpC} and $bla_{CTX\text{-}M}$, and two other genes were positive for bla_{AmpC}, $bla_{CTX\text{-}M}$ and bla_{SHV}, concomitantly (Table 3).

All 10 strains harboring the bla_{AmpC} gene showed intermediate resistance or sensitivity to amoxicillin. The presence of the *AmpC* gene in *S.* infantis isolated from chicken meat is a concern for Japanese public health agencies because this serovar is a major cause of human salmonellosis, and chicken meat is

Table 2. Resistance profiles of 51 strains of *Salmonella* Infantis isolated in a poultry production chain located in the State of São Paulo, Brazil

Profiles	Antimicrobial resistance[a]	Number of classes[b]	Number of strains (%)
A1	AMO	1	4 (7.8)
A2	(AMO)	1	10 (19.6)
A3	SUL	1	2 (3.9)
A4	(SUL)	1	2 (3.9)
A5	TET	1	1 (2.0)
A6	(TET)	1	1 (2.0)
A7	AMO CAZ	2	2 (3.9)
A8	AMO (CAZ)	2	1 (2.0)
A9	(AMO) SUL	2	1 (2.0)
A10	TET SUL	2	3 (5.9)
A11	Multi sensitive	-	24 (47.0)
TOTAL			51 (100.0)

[a] Profiles in parentheses = strains with intermediate resistance to antimicrobials, [b] Number of classes of antimicrobials to which the isolates showed resistance; AMO = amoxicillin, CAZ = ceftazidime, TET = tetracycline, SUL = sulfonamide.

Table 3. Relationship of resistance profiles and occurrence of resistance genes in 18 strains of *Salmonella* Infantis resistant to β-lactam antibiotics, isolated in a poultry production chain located in the State of São Paulo, Brazil

Resistance profiles[a]	Resistance genes	Number of strains (%)
AMO	bla_{AmpC}	2 (11.1)
AMO	bla_{AmpC} $bla_{CTX\text{-}M}$ bla_{SHV}	2 (11.1)
(AMO)	bla_{AmpC}	4 (22.2)
(AMO)	bla_{AmpC} $bla_{CTX\text{-}M}$	2 (11.1)
(AMO)	-	5 (27.7)
AMO CAZ	$bla_{CTX\text{-}M}$	1 (5.6)
AMO CAZ	-	1 (5.6)
AMO (CAZ)	-	1 (5.6)
TOTAL		18 (100.0)

[a] Profiles in parentheses = strains with intermediate resistance to antimicrobials, AMO = amoxicillin, CAZ = ceftazidime.

the main source of human infection in the country (Aviv et al. 2014, Noda et al. 2015).

Although phenotypically resistant to the antimicrobial agents of the β-lactam group, seven strains did not present any of the investigated genes (Table 3). This result indicates that resistance may be associated with the presence of other β-lactamases whose genes have not been evaluated in this study and/or other mechanisms of resistance to these antimicrobials, such as: efflux pumps, loss of porine expression, changes in penicillin-binding proteins (PBPs), presence of multiple or even new β-lactamases (Babic et al. 2006, Jacoby 2009).

The strains presented 100% of positivity for the *agfA* gene, 92.2% (47/51) for the *lpfA* and no strain showed the *sefA* gene.

The presence of the *agfA* in all studied strains suggests its ability to bind during the infection process, in addition to being associated with biofilm formation (Yoo et al. 2013). The detection of this gene in isolates of chicken meat cuts, carcasses, chiller and scalding water warns the danger of their presence during processing due to their possible capacity to produce biofilm, which can lead to contamination of the final product in poultry slaughterhouses.

The absence of *sefA* gene is consistent with the literature. The SefA fimbria encoded by this gene is described as being restricted to group D of *Salmonella*, which includes Enteritidis, Dublin, Moscow and Blegdon serotypes (Amini et al., 2010). However, knowing the possibility of horizontal transfer of fimbrial genes in serovars of the *Salmonella* genus, which allows their adaptation to different colonization situations (Rotger & Casadesús 1999), it was proposed to evaluate the presence of this gene in *S.* infantilis. According to Bäumler et al. (1997), the acquisition of different fimbrial operons may have been one of the mechanisms by which *Salmonella* serovars succeeded in expanding their host range.

Most isolates of *S.* Infantis (92.2% - 47/51) showed similarities in virulence potential, since they were positive simultaneously for two genes studied *agfA* and *lpfA*. This indicates that these strains may be efficient in the adhesion process and in the formation of biofilms, which is associated with the presence of both genes. The presence of fimbriae, mediated by these genes, is extremely important in the infection process. It is possible that there are additive effects of adhesives Lpf and Agf on colonization of the intestine and expression of virulence in the host, indicating potential risk after infection. These findings were similar to other data obtained in previous studies that studied different serotypes of *Salmonella* (Borsoi et al. 2009, Cesco 2010, Borges et al. 2013).

The characteristics of resistance and virulence in *S.* Infantis shows that this serovar can be considered as potentially pathogenic and that genes related to these characteristics should be constantly monitored to understand and follow the process of adaptation of these strains throughout the productive process of the broiler and, consequently, in the human host. These strains can acquire and lose virulence genes over time, thus determining the spread of different genetic profiles (Moussa et al. 2013, Suez et al. 2013).

The genetic similarity analysis of *S.* Infantis showed a high proximity between the strains (Fig. 2), indicating that there are probably common sources of contamination.

Five clusters and three isolates with distinct profiles were identified, which could not be grouped with the other strains due to the genetic proximity being less than 80%.

Cluster A grouped seven strains with homology of 80.4%, all from the aviary, from environmental swab samples. This profile was present for a period of five months in the aviary environment. The common identification of the *lpfA* and *agfA* genes suggests the potential of these strains to fix themselves on surfaces and to produce biofilms in the environment of the aviary, allowing their maintenance in the place for long periods. The identification of the three resistance genes studied (SHV, CTX-M and AmpC) indicates the risk of the horizontal spread of resistance genes among the strains that can thus present multiresistant profiles. Therefore, it is necessary to establish more efficient and rigorous hygiene and biosafety measures to guarantee the control of this agent in the aviary's environment. According to Moura et al. (2014) the neglecting of biosecurity standards within the industry is a decisive factor for the maintenance of the microorganism in the environment.

The cluster with the highest number of isolates was B, composed of 33 strains with homology of 83.7% and therefore considered the main problem of this industry. This profile was isolated from the environmental swab of the aviary and the meat matrixes in the slaughterhouse. This genotype was identified over the two years of samples collection (2009 and 2010). The long period of permanence suggests that there was infection in successive animal flocks, associated to the maintenance of the microorganism in the aviary environment, and consequent contamination of the product in the slaughterhouse, which indicates that the cross contamination seems to be important in the dissemination of this genotype along the production chain. The presence of this profile in samples of swabs and disposable foot socks of the aviary, besides the scald and chiller water, suggests the negligence to the biosafety norms in the production unit that contributed to the contamination of the samples of carcasses and chicken meat cuts

Some authors state that there is influence of the environment on the contamination of the final product (Von Ruckert et al. 2009, Colla et al. 2012). Chiller and scald water are considered important factors in the dissemination of *Salmonella* in the slaughterhouse, since a large number of carcasses pass in the same water tank, increasing the chances of cross contamination (Mead et al. 2000).

The *lpfA* and *agfA* genes were also common in group B, suggesting the ability of the strains to produce biofilms, making it difficult to eliminate them within the industry.

Six clonal subgroups (>99% similarity) were identified in Cluster B (B1 to B6) composed of 18 strains. The B1 subgroup has isolates of chicken thigh, cartilage and disposable foot socks of the aviary. In B2 the strains are all of breast clipping. The B3 subgroup was detected in carcasses and neck. B4 contains isolated strains of chiller and scald water and cartilage. In B5, strains from thigh and chicken breast were grouped. Finally, B6 is composed of strains isolated from mechanically separated meat (MSM). According to Chu et al. (2009) the detection of clones in different samples demonstrates the propagation of *Salmonella* in the productive chain and the risk of transmission to humans.

The profile C showed similarity of 94.1% composed of four strains, all of these samples from chicken's breast and skin in the slaughterhouse. Three of these strains were isolated in the same period (August of 2009), indicating that their

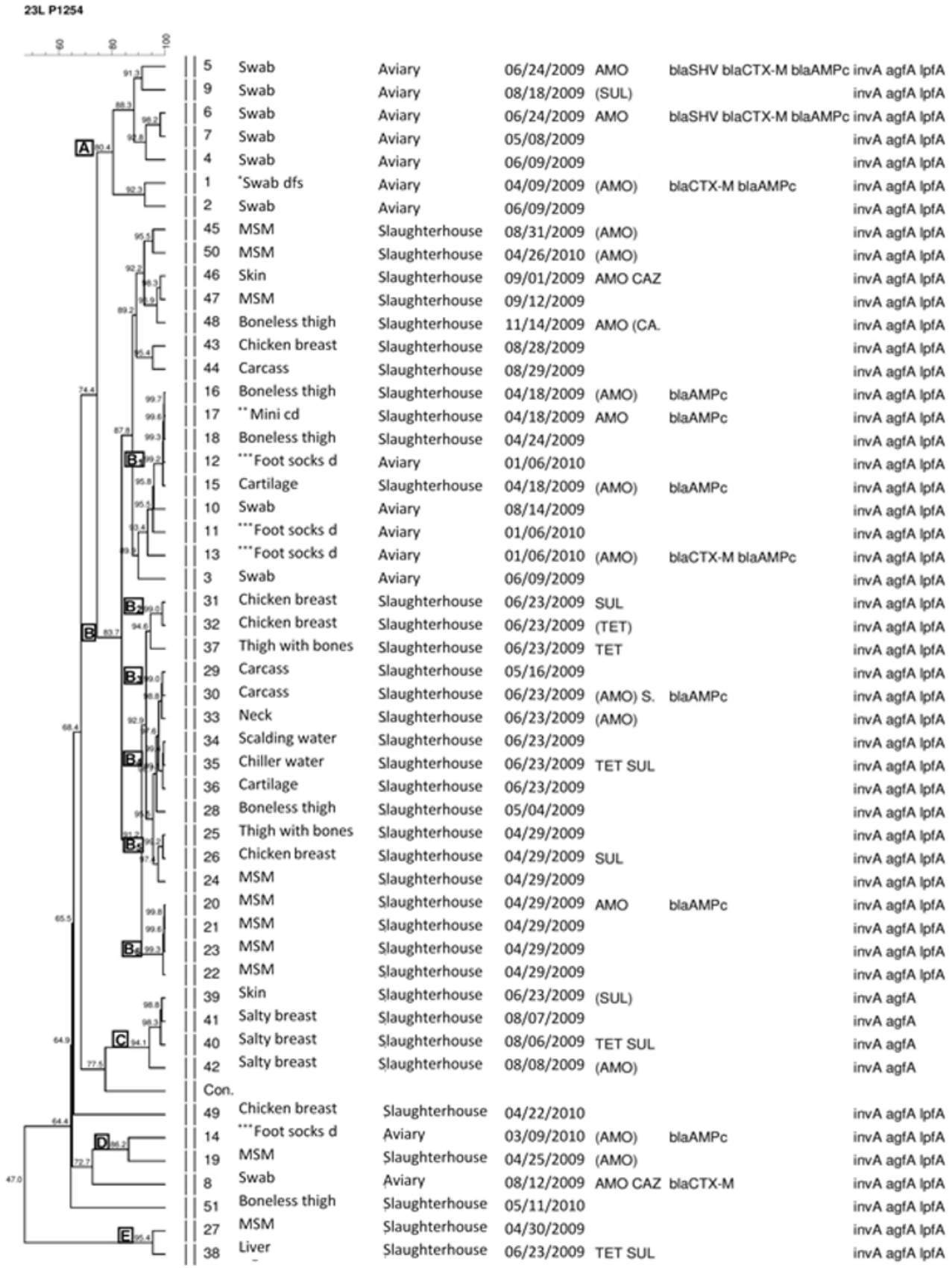

Fig.2. Comparative dendrogram of *Salmonella* Infantis using dice similarity coefficient with 1% of tolerance and UPGMA method with 0.80% of optimization. Profiles A to E = different clusters, with homology higher than 80%; profiles B1 to B6 = clonal groups, with homology greater than 99%. * Swab with disposable foot socks, ** mini chicken drumsticks, *** disposable foot socks.

permanence was temporary and that there is a possibility of greater ease in their control. In addition, the presence of the *agfA* gene alone in this profile may justify the shorter residence time, different from that found in other clusters. This fact does not eliminate the potential of biofilm formation of the strains; however, the absence of the *lpfA* gene may be the determining factor for its lesser ability to persist in the environment. The *lpfA* gene is involved in the process of adhesion to surfaces and epithelial cells that characterize an essential stage and prior to the biofilm formation process (Gibson et al. 2007).

Due to the fact that only two strains formed Clusters D and E, a more in-depth analysis of the data found was not possible.

CONCLUSIONS

The high percentage of resistance to amoxicillin warns of a risky condition, since this is a drug commonly used in human and veterinary medicine.

The high positivity for virulence genes, associated with the presence of β-lactam resistance genes in some isolates, suggests the pathogenic potential of *Salmonella* Infantis, the possibility of causing clinical disease in humans and the complications that can lead to the treatment of severe cases of salmonellosis.

Phylogenetic evaluation showed that strains from the aviary were fairly close to those isolated from the slaughterhouse, and persisted throughout the study period.

The presence of the *lpfA* and *agfA* genes associated with the persistence of strains in the environment warns of the potential of *S.* Infantis to form biofilms and should be constantly monitored in the poultry production chain and rigorous cleaning and decontamination measures applied in breeding environments, especially in the slaughterhouse, in order to minimize the contamination of the final product and the hazards to public health.

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Original Article
Livestock Diseases

Insulin dysregulation in horses with induced obesity[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. **Insulin dysregulation in horses with induced obesity**. *Pesquisa Veterinária Brasileira 40(1):39-45*. Graduate Program in Animal Science, Escola de Veterinária, Universidade Federal de Minas Gerais, Campus Pampulha, Av. Antônio Carlos 6627, Cx. Postal 567, Belo Horizonte, MG 31270-901, Brazil. E-mail: faleirosufmg@gmail.com

Insulin deregulation (ID) is a central player in the pathophysiology of equine metabolic syndrome (EMS), which is associated with generalized and/or regional obesity. The objective of this experiment was to characterize the alterations in the hormonal profile in horses exposed to a hypercaloric diet. A total of nine Mangalarga Marchador adult horses with initial body condition score (BCS) of 2.9±1/9 (mean±SD) were submitted to a high calorie grain-rich diet for 5 months. The data was collected before the start of the experiment and every 15 days until the end of the experiment and glucose and insulin concentrations were measured in the plasma. Proxies G:I, RISQI, HOMA-IR and MIRG were calculated. The low-dose oral glucose tolerance test (OGTT) was performed and the total area under the glucose (GTA) and insulin (ITA) curves at three different timepoints (before inducing obesity, after 90 days and after 150 days) was used. Analysis of variance of the results was performed considering the time effects and the means were compared with repeated measures by the Tukey's test (P≤0.05). The ID was observed during the first 90 days of the experiment and was characterized as a decompensated ID, showing an increase of basal glucose and insulin plasma levels, changes in all proxies and a significant increase in GTA (P<0.001) and ITA (P<0.05). However, a clear compensation of the ID was evident after 150 days of experiment, which was supported by data from the insulin secretory response of β cells of the pancreas that showed an increase in insulin plasma levels, after fasting or exposure to gastric glucose, with a concomitant decrease in fasting glucose and fructosamine levels, and a decrease of GTA and marked increase of ITA (P<0.0001) in the dynamic test. These findings confirm the occurrence of hyperinsulinemia associated with insulin deregulation in Mangalarga Marchador horses exposed to hypercaloric diets.

INDEX TERMS: Insulin dysregulation, horses, obesity induction induced metabolic syndrome, insulin resistance, overweight, Mangalarga Marchador horses.

RESUMO.- [**Desregulação da insulina em equinos com obesidade induzida.**] A desregulação insulínica (DI) é o ponto central dos mecanismos fisiopatológicos da síndrome metabólica equina (SME), que é associada à obesidade generalizada e/ou regional. O objetivo deste experimento foi caracterizar as alterações no perfil hormonal em equinos submetidos à dieta hipercalórica. Foram utilizados nove equinos Mangalarga Marchador adultos com escore corporal (EC) médio (±DP) inicial de 2,9±1 (escala de 1-9) submetidos à dieta hipercalórica atingindo um EC de 8,3±1 após cinco meses. Os dados foram coletados antes do início do experimento e com o intervalo de 15 dias até o final do experimento, os valores plasmáticos foram obtidos para mensuração das concentrações de glicose e insulina. Foram calculados os proxies G:I, RISQI, HOMA-IR e o MIRG. Foi realizado o teste de baixa dose de glicose oral (TBDGO) utilizando a área total sob a curva de glicose (ATG) e insulina (ATI) em três momentos, antes da

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indução a obesidade, após 90 e 150 dias. Os resultados foram submetidos à análise de variância considerando-se os efeitos de tempo e as médias comparadas com medidas repetidas pelo teste de Tukey, com o valor P≤0,05. A DI foi observada nos primeiros 90 dias de experimento, se caracterizando como um quadro de DI descompensada, apresentando um aumento dos níveis plasmáticos basais de glicose e insulina, pelas alterações em todos os proxies e com um aumento significativo da ATG (P<0,001) e ATI (P<0,05). Contudo, ficou evidente uma compensação do quadro de DI após 150 dias de experimento, sendo demonstrado pelos dados da resposta secretória insulínica das células β do pâncreas, que se manifestaram pelo aumento dos níveis plasmáticos de insulina pós-jejum ou exposição à glicose gástrica com concomitante redução nos níveis de glicose e frutosamina pós-jejum e pela redução da ATG e pela marcada elevação de ATI (P<0,0001) no teste dinâmico. Tais achados comprovam a ocorrência de hiperinsulinemia associada à desregulação insulínica em equinos Mangalarga Marchador expostos a dietas à dieta hipercalórica.

TERMOS DE INDEXAÇÃO: Desregulação insulínica, equinos, obesidade, síndrome metabólica induzida, indução da obesidade, cavalos, resistência à insulina, sobrepeso, insulina, Mangalarga Marchador.

INTRODUCTION

Equine obesity has become a challenge for equine veterinarians and can be observed in up to 40% of equine populations (Wyse et al. 2008, Thatcher et al. 2012). Obesity induction occurs due to the supply of diets rich in grains and forages (grass and hay) with high levels of non-structural carbohydrates and is a consequence of excess feed supply, which exceeds the metabolic requirements for the horses' physical activity (Schott et al. 2001, Johnson 2002).

Among the main alterations related to obesity in horses are exercise intolerance, thermoregulatory inefficiency, abnormal reproductive performance and increased probability of developing mesenteric lipomas (Henneke et al. 1983, Cymbaluk & Christison 1990, Garlinghouse & Burrill 1999, Garcia-Seco et al. 2005). In addition to these alterations, the obese equines remain in a persistent chronic inflammatory state, which can occur due to maximum storage of lipids by the adipocytes of overweight animals, leading to changes in energy efficiency, inflammatory processes and cellular stress (Goossens 2008).

Several studies show that regional fat accumulation closely correlates with insulin dysregulation (ID), hyperinsulinemia, dyslipidemia, elevated leptin levels, elevated non-esterified fatty acids, gene expression of proinflammatory cytokines, increased protein concentration in the peripheral blood and increased risk of developing laminitis (Johnson 2002, Sutherland et al. 2004, Vick et al. 2007, 2008, Carter et al. 2009).

ID is a central mechanism in the pathophysiology of equine metabolic syndrome (EMS) and most of the affected horses and ponies will show generalized or regional obesity. However, not all horses with EMS are obese and not all obese animals develop ID (Treiber et al. 2006).

There are two theories to explain the correlation between obesity and ID. The first theory is based on the decrease in insulin signaling caused by the action of adipokines and cytokines produced by the adipose tissue and the second is based on the intracellular accumulation of lipids in insulin sensitive tissues. This situation can occur after the supply of a diet rich in glucose, which is converted to fat through lipogenesis. The increased circulating fat concentration will be stored in the intracellular region of non-adipose tissues, such as the skeletal muscle, liver and pancreas, which occurs when the storage capacity of the adipose tissue is exceeded, leading to accumulation of lipids within these cells and causing changes in normal functions, such as impairment of insulin receptor signaling (Summers 2006).

With the hypothesis that the induction of obesity in horses can promote metabolic changes and lead to the development of insulin resistance (IR), the objective of this study was to examine the physiological alterations that can trigger the development of ID caused by obesity in horses to better understand the pathophysiology and provide an early diagnosis of IR.

MATERIALS AND METHODS

This study was approved by the Ethics Committee on Animal Experimentation (CETEA) of the "Universidade Federal de Minas Gerais" (UFMG), protocol number 49/2014. In total, nine Mangalarga Marchador healthy horses were used (5 non-pregnant females and 4 castrated males) with mean age (±SD) of 48±5 months and initial weight of 316±62.68kg. To standardize the supply of the diet, the horses were housed in properly identified individual stalls, with the floor covered with sawdust and access to water ad libitum. The experiment was carried out at Pedro Leopoldo Model Farm of the UFMG.

Obesity was induced by supplying digestible energy (DE) in quantities 100% higher than the maintenance requirement value established by the reference literature (NRC 2007). The maintenance DE (DEm) was calculated based on the mathematical formula DE=1.4 + (0.03 x kg live weight [l.w.]), as demonstrated by the NRC (2007) over a period of 150 days.

To avoid gastrointestinal disorders, it was previously established that the animals would receive the DEm once as concentrate and once as forage. For this, the following digestible energy values for the provided food were used: commercial concentrate (Guabi Equitage Laminados) (DE = 3.650Mcal/kg) and grass hay *Cynodon dactylon* (L.) Pers.Var. "Coast cross" (DE = 2.0Mcal/kg). Thus, the amounts of hay and feed were calculated by considering the DEm of each animal divided by the digestible energy concentration of each type of feed: Amount of concentrate (kg): DE = (1.4 + (0.03 x kg of l.w.))/3.650; Amount of coast cross (kg): DE = (1.4 + (0.03 x kg of l.w.))/2.

This calculation was repeated every fifteen days, after measuring the weight of the animals, to maintain the 100% increase regarding the DE requirement established by the NRC (2007) during the 150 days of the experimental period. The diet was provided in individual feeders, in 3 (three) daily meals, distributed in equal parts ad supplied at 6 a.m., 12 a.m. and 6 p.m. (Table 1).

Before inducing obesity and fortnightly until the end of the experiment, the weight of the animals (W) was determined, the animals were examined for claudication and the hoof sensitivity was performed before inducing obesity and every 30 days until the end of the experiment.

To analyze basal insulin and glucose, venous blood samples were collected through venipuncture of the left external jugular vein using a vacuum collection system (vacutainer), placed in vials without anticoagulants and with sodium heparin, respectively, and immediately centrifuged and stored at -18ºC. Samples were

collected between 7 and 9 a.m., with the first sample collected before inducing obesity (which was called basal) and samples collected throughout the experimental period (which were called based on the time - 15, 30, 45, 60, 75, 90, 105, 120, 135 and 150). Samples were collected after a fasting period of 12 hours to determine basal glucose values for all samples collected every 15 days and basal insulin levels were analyzed in samples collected every 30 days until completing the 150 days of the experiment.

The assays were performed using a spectrophotometric identification system in an automated biochemical analyzer (Cobas Mirage-Roche Diagnostic System®) using specific commercial kits (Labtest®). The GOD-Trinder methodology was used to determine plasma glucose levels (ref. 133) (Lindåse et al. 2016). Serum insulin was determined by a radioimmunoassay (RIA) technique (Milipore's Porcine Insulin RIA), performed in a commercial laboratory. Tinworth et al. (2011) observed that Porcine Insulin RIA is the most accurate assay for measuring equine insulin concentrations.

To determine if the animals used in this experiment presented insulin dysregulation, the values of the proxies were calculated and the low-dose oral glucose tolerance test (OGTT) was performed.

The proxies used in this experiment were based on the glucose and insulin basal values and included the ratio of glucose to insulin (G:I), insulin sensitivity (RISQI), insulin secretory response by pancreatic β cells (MIRG) and the homeostasis evaluation model (HOMA-IR), following the methodology by Treiber et al. (2005), with the respective formulas described below:

$$G:I = \text{glucose/insulin}$$
$$RISQI = 1/\sqrt{\text{insulin}} = \text{insulin} - 0.5$$
$$MIRG = (800 - 0.3\ [\text{insulin} - 50]\ 2)/(\text{glucose} - 30)]$$
$$HOMA\text{-}IR = \text{glucose (mg/dL)} \times 0.0555 \times \text{insulin (µU/mL)}/22.5$$

As normality parameters, a G:I>10, compensatory IR for values between 4.5 and 10 and severe IR for values <4.5 were considered (Treiber et al. 2005, 2006). Regarding RISQI, it was considered normal for values >0.32, IR for values between 0.22 and 0.32, and severe IR for values <0.22. For MIRG, it was considered normal if <5.6 and, for HOMA-IR, IR was considered for values >2.71 (Geloneze & Tambascia 2006).

The low-dose oral glucose tolerance test (OGTT) was performed before supplying the experimental diet (basal), and 90 and 150 days after such diet supple. After a 12-hour fasting period, glucose (0.25g/kg) was administered through a nasogastric tube. Blood samples were collected at the following timepoints: basal and 30, 60, 90, 120, 150 and 180min after administrating dextrose. Blood samples were used to measure glucose and insulin plasma levels.

To interpret the results, it was considered that healthy animals show a glucose peak of 90 to 120mg/dL between the samples collected at 60 and 90min, and insulin should be below 20µIU/mL after the samples collected at 60 and 90min (Ralston 2002). The total area (TA) under the curve was also calculated, defined as the areas under the glucose and insulin curves, until the x axis. These were obtained by calculating the integral of the curve and expressed in mg/dL/min, derived from the glucose (GTA) and insulin (ITA) values obtained in the OGTT (Correa et al. 2007).

The Sigma (Sigma Stat, Systat) software was used for the statistical analysis; the data with a normal distribution was analyzed by analysis of variance in randomized blocks with measures repeated by the time, followed by the Tukey's test to compare the means. For all tests, a significance level of P≤0.05 was considered.

Table 1. Bromatological composition of the concentrate (Guabi Equitage Laminados®) and hay (*Cynodon dactylon* (L.) Pers.Var. "Coast cross") and their respective ingredients used during the experimental period

Ingredients	Concentrate	Hay
Energy		
DE (Mcal/kg)	3.50	2.0
Nutrients (%)		
DM	87	89.4
MM	10	5.48
CP	12	17
NDF	10	56.8
Corn flour	27	1.5
EE	9	1.43
Ca	1,6	0.48
P	0.5	0.35
Energetic ingredients (%)		
Corn	19	-
Oats	12	-
Oil	5	-

DM = Dry matter, MM = mineral matter, CP = crude protein, NDF = neutral detergent fiber, EE = ethereal extract, Ca = calcium, P = phosphorus; data provided by suppliers.

RESULTS

During the experimental period, the horses consumed a daily mean of 0.94% (±0.02) of concentrate and 1.72% (±0.04) of coast cross in relation to their live weight. There were no cramps, evidence of claudication or signs of hoof sensitivity.

At the end of the experimental period, the animals showed a mean weight gain corresponding to 27.45% of the original, with significant values (P<0.001) (Table 2).

There were significant alterations in plasma glucose concentrations only in samples collected after 45 days of the experiment (P<0.0001). Regarding basal plasma insulin concentrations, there was an increase of up to 156.67% in the first 30 days of the experiment (P>0.05) and the concentration remained higher than the basal level until the end of the experiment (Table 3).

Among the proxies, G:I and RISQI showed a similar trend, with a significant reduction in relation to the basal values already in the first samples collected after supplying the hypercaloric

Table 2. Mean and standard deviation of weight of Mangalarga Marchador horses subjected to obesity induction

Time (days)	Weight (Kg)	
	Mean	SD
Basal	316	62.68
15	348.44**	69.6
30	348.55**	71.18
45	356.22**	66.2
60	356.22**	66.2
75	375.55**	66.4
90	386**	65.15
105	390.22**	61.88
120	394.55**	63.56
135	400.77**	58.77
150	402.77**	58.31

Tukey's test, significant P value ≤0.05: * P<0.05, ** P<0.001, *** P<0.0001

diet (P<0.0001), which was maintained until the end of the experiment. However, the MIRG showed significant increases after 75 and 105 days of experiment (P<0.05), it was not significant for samples collected after 135 days of experiment (P=0.18) and was again significant for samples collected after 150 days of experiment (P<0.0001). The HOMA-IR showed significant increases for sample collected at 45 (P<0.05), 75 (P<0.001), 105 (P<0.001), 135 (P<0.001) and 150 (P<0.05), with a decreased value at the last collection (Table 4).

Insulin results obtained for the dynamic OGTT test are shown in Figure 1. The curve shows the period prior to the supply of the diet and an insulin peak from 60 to 90 minutes after glucose supply, with the respective values of 16.67 and 19.36mg/dL, decreasing to 10.45mg/dL in samples collected after 180 minutes. The analysis performed at 90 days showed the two highest values for samples collected at 60 (31.12mg/dL) and 90 (33.52mg/dL) minutes and the subsequent values remained elevated until the end of the test. At the end of the experiment, there was an increase in insulin concentration levels already after 30 minutes (46.45mg/dL), with a peak at 60 minutes (50.17mg/dL), and the values remained elevated until the collection performed at 120min (42.05mg/dL) (Fig.1).

Glucose results obtained for the dynamic OGTT test are shown in Figure 2. Before the diet supply, the two highest values are observed after 60 (135.5mg/dL) and 90 (131.5mg/dL) minutes, with a decrease to 123.0mg/dL for samples collected after 120 minutes. At 90 days, a high basal value of 105.94mg/dL was observed in relation to the others, with a peak after 90 minutes (172.62mg/dL) and a decrease after 150 minutes (128.86mg/dL). At the end of the experiment, the basal value was 65.82mg/dL, with the peak after 60 minutes (149.12mg/dL) and starting to decrease after 120 minutes, reaching its lowest value after 180 minutes (69.05mg/dL).

The results of the areas under the insulin and glucose curve are shown in Table 5. For insulin, there was a significant increase already after 90 days (P<0.05), which was maintained until the 150 days of experiment (P<0.001). Regarding glucose, there was an increase after 90 days (P<0.001) and a value that was considered a trend after 150 days of experiment (0.06).

Table 3. Mean and standard deviation of glucose and insulin plasma values in Mangalarga Marchador horses subjected to obesity induction and their respective reference values

Time (days)	Glucose (mg/dL)		Insulin (µU/mL)	
	Mean	SD	Mean	SD
Basal	102.37	18.92	5.87	3.4
15	88.01	14.74	-	-
30	130.24	22.44	15.02*	2.84
45	147.54***	34.07	14.42	7.49
60	102.04	21.34	-	-
75	120.07	25.01	20.03**	6.28
90	100.2	18.85	-	-
105	109.23	20.4	18.69**	7.44
120	126.36	16.83	-	-
135	115.43	11.43	18.61**	5.52
150	73.65	13.91	16.74*	11.64
Reference values	70 - 135[a]	8.5	<20[b]	-

Tukey's test, significant P value ≤0.05: * P<0.05, ** P<0.001, *** P<0.0001; [a] Kaneko et al. (1997), [b] Hassel et al. (2009).

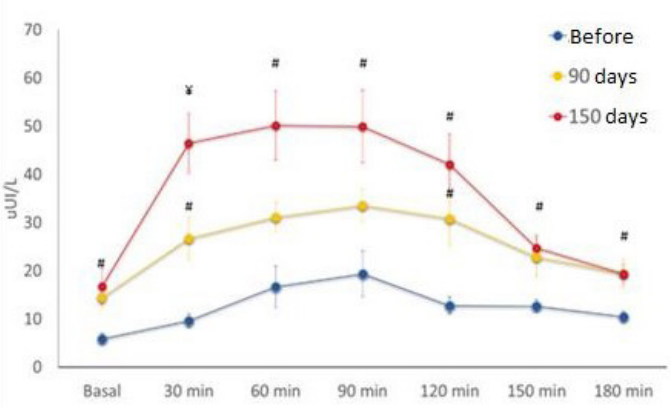

Fig.1. Means and standard errors of insulin plasma concentrations after gastric glucose administration (0.25g/kg) in Mangalarga Marchador horses before and 90 and 150 days after obesity induction. Significant values in relation to the results of the previous time point (#), significant values in relation to the results of the previous time point and 90 days (¥).

Table 4. Mean and standard deviations of the proxies plasma values of Mangalarga Marchador horses subjected to obesity induction

Time (days)	G:I (mg/dL/µU/mL)		RISQI ($\mu U/mL^{-0,5}$)		MIRG ($\mu U_{ins}^{2}/[10.L.mg_{gli}]$)		HOMA-IR	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Basal	22.87	11.5	0.46	0.13	3.07	1.4	1.5	0.92
30	8.93***	2.32	0.26***	0.03	4.44	0.92	4.85*	1.29
45	12.1***	4.69	0.29***	0.07	3.53	1.2	5.51*	3.66
75	6.29***	1.4	0.23***	0.03	6.04*	1.59	6.02**	2.36
105	6.37***	1.68	0.24***	0.05	6.28**	1.21	5.28*	2.95
135	7.27***	2.26	0.24***	0.03	5.28	1.27	5.79*	1.83
150	6***	2.69	0.28***	0.07	10.07***	3.85	3.24	2.76

Tukey's test, significant P value ≤0.05: * P<0.05, ** P<0.001, *** P<0.0001; G:I = ratio between glucose and insulin, RISQI = insulin sensitivity, MIRG = secretory insulin response, HOMA-IR = homeostatic evaluation model.

Table 5. Mean and standard deviation of the areas under the curve regarding insulin and glucose plasma concentrations after gastric glucose administration (0.25g/kg) in Mangalarga Marchador horses before and 90 and 150 days after obesity induction

	Before		90 (days)		150 (days)	
	Mean (µUI or mg/dL/min)	SD	Mean (µUI or mg/dL/min)	SD	Mean (µUI or mg/dL/min)	SD
Insulin	27545.9	10509.93	56327.64*	24164.73	78569.93**	29015.53
Glucose	25990.22	32361.21	32042.15**	38933.51	22787.52	30514.51

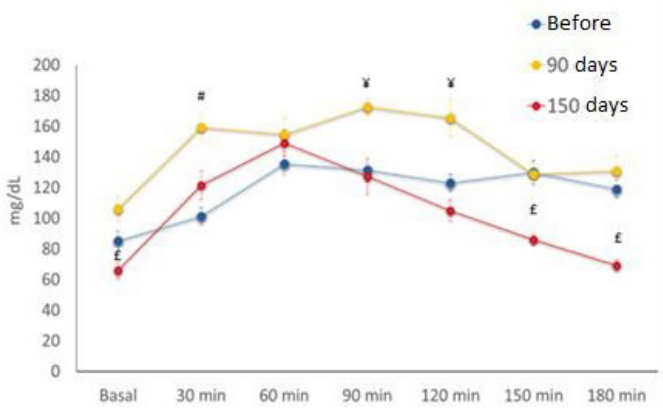

Fig.2. Means and standard errors of glucose plasma concentrations after gastric glucose administration (0.25g/kg) in Mangalarga Marchador horses before (control) and 90 and 150 days after obesity induction. Significant values in relation to the results of the previous time point (#), significant values in relation to the results of the previous time point and 90 days (¥), significant values in relation to the values of the time points 150 and 90 days (£).

DISCUSSION

The mean basal glycemia of the animals used in this experiment showed an increase of up to 27.22% in the first 30 days of the experimental period, with a significant peak after 45 days, demonstrating a metabolic impairment in these animals and a possible insulin resistance that was still decompensated up to this point. However, after this period, the values returned to basal values. Such subtle changes in basal glucose values has been observed in other studies with obese equines and can be due to the compensatory production of insulin to counteract insulin resistance in the tissues (Waller et al. 2011, Morgan et al. 2015).

On the other hand, basal insulin plasma values showed a significant increase in the first 30 days of the experiment, but in a more consistent and lasting manner after 75 days. This finding is in agreement with the glycemia results. In the first 75 days, increases in blood insulin concentrations are more discrete and insufficient to prevent glycemia from rising above the normal range, accounting for a decompensated insulin resistance condition. However, from this time point onwards, insulinemia reached its peak and kept its values above 16µU/mL, which were able to maintain glycemia within the reference range (Waller et al. 2011).

This increase in insulin plasma values can be interpreted dysregulated insulin responses to oral glucose developed by these animals during the weight gain period, a finding confirmed by the low-dose oral glucose tolerance tests (OGTT). This was evidenced by the total area under the insulin curve, which showed a significant increase when comparing the OGTT data on days 90 and 150 with the control and could be interpreted as a sign of a decompensated insulin resistance condition in the first 90 days of the experiment. Analysis of the total area under the glucose curve showed a significant increase in glucose only on day 90. This finding shows that the animals evolved to a compensated IR condition after a three-month period, since the increased production of insulin was able to maintain the glucose values close to the control.

The values obtained with the PROXIES confirm the idea of the compensated insulin resistance condition at the end of the experimental period. The ratio of glucose to insulin (G:I) and the RISQI showed significant results from day 30 of the experiment, with P values lower than 0.0001 in both cases, revealing that the animals developed a metabolic alteration that is in agreement with an insulin resistance condition, with G:I results showing a compensatory insulin resistance condition.

The MIRG proxy accounts for the insulin secretory response of the pancreatic β cells in response to glucose, demonstrating the ability of the pancreas to respond to tissue insulin resistance and its results were significant from day 75 ($P<0.05$) and 105 ($P<0.001$), with an improvement at day 135 and a considerable worsening at day 150 ($P<0.0001$) of the experiment, thus showing that the animals presented a secretory response after 75 days of experiment.

The MIRG trend was confirmed by the HOMA-IR values, which were significant between samples collected at day 30 and day 135 ($P<0.05$) of the experiment, with the most significant value at day 75 ($P<0.001$), indicating that the animals presented IR in the initial period and evolved to a compensatory IR at the end of the experiment.

The present study is in agreement with the data obtained in Standardbred horses, which were submitted to a hypercaloric diet for 6 weeks, presenting a decrease in insulin sensitivity that was compensated by an increase in plasma insulin production (Stewart-Hunt et al. 2010). This decrease in insulin sensitivity is consistent with other studies in equines where an adaptation to a diet rich in non-structural carbohydrates has been observed, which may alter insulin signaling and change the metabolism of glucose and fatty acids (Jenkins et al. 1987, Hoffman et al. 2003, Samaha et al. 2003, Treiber et al. 2005, Pratt et al. 2006).

This is the first study that shows the development of insulin resistance in Mangalarga Marchador horses and is in agreement with international studies, confirming that fat accumulation is associated with insulin resistance, hyperinsulinemia and dyslipidemia (Johnson 2002, Sutherland et al. 2004, Vick et al. 2007, 2008, Carter et al. 2009). This association of fat accumulation with insulin deregulation can be

explained by the decreased expression of insulin receptors in insulin-dependent cells. Decreases expression of the receptors can be explained by two hypotheses: the first is that the adipokines and cytokines produced by the adipose tissue act on insulin-dependent cells and the second is based on the accumulation of lipids in insulin-sensitive tissues that are sensitive to insulin as Summers (2006) observed. In addition to this, the insulin receptor has a negative feedback in response to circulating insulin and higher plasma insulin concentration lead to a lower availability of receptors in the cell membranes (Shanik et al. 2008).

Another pathway that could interfere with GLUT-4 translocation would be the one caused by the toxic effects of plasma hyperglycemia. Glucose is influenced by glutamine to be converted into glucosamine-6-phosphat and this excess glucosamine can impair GLUT-4 translocation (Sposito et al. 2007). The impaired insulin action ultimately leads to a glucose intolerance condition, with a relative increase in glucose concentrations. However, hyperglycemia values are mild in horses with IR and this can be explained in horses as an increase in insulin production seems to compensate for its reduced action, leading to a compensated insulin resistance condition (Cosentino & Luscher 1998).

CONCLUSIONS

Exposure to the hypercaloric diet promoted weight gain and had an important impact on the metabolism of the horses studied, with an increase in blood glucose and insulin concentrations.

Insulin deregulation was already evident in the first 75 days due to an increase in plasma glucose and insulin levels, with a considerable increase in glucose levels during this period, which can be considered a decompensated insulin resistance condition. In the first 75 days, it was possible to observe the effects of this resistance on glucose metabolism, through increases in basal glucose and fructosamine concentrations. However, from that point onwards, there was a compensation of the insulin secretory response by pancreatic β-cells, which was confirmed by the increase in fasting insulin plasma levels, with a concomitant decrease in glucose levels.

These findings confirm the occurrence of hyperinsulinemia associated with insulin deregulation in Mangalarga Marchador horses exposed to a hypercaloric diet and that insulin dysfunction does not occur uniformly, and thus further studies are necessary to better understand the dynamics of this phenomenon.

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Conflict of interest statement.- We have no conflict of interest to declare.

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Original Article
Small Animal Diseases

Pathological and immunohistochemical aspects of primary hepatobiliary neoplasms in cats[1]

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ABSTRACT.- Argenta F.F., Mello L.S., Caprioli R.A., Pavarini S.P., Driemeier D. & Sonne L. 2020. **Pathological and immunohistochemical aspects of primary hepatobiliary neoplasms in cats.** *Pesquisa Veterinária Brasileira 40(1)46-54*. Setor de Patologia Veterinária, Departamento de Patologia Clínica Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Prédio 42505, Porto Alegre, RS 91540-000, Brazil. E-mail: lusonne@yahoo.com.br

Primary hepatobiliary neoplasms (PHN) are uncommon in cats, and originate in hepatocytes, intra- and extrahepatic bile ducts, mesenchymal cells, and cells of neuroendocrine origin. The aim of this study was to determine the frequency of PHN in cats diagnosed in the metropolitan region of Porto Alegre (RS), Brazil, for a period of 17 years, determining their epidemiological, anatomopathological and immunohistochemical aspects. Necropsy reports of 2.090 cats were analyzed, 125 were diagnosed with primary hepatobiliary diseases, of which 15 were cases of PHN, representing 12% of the specific hepatobiliary conditions and 0.7% of the necropsies. All PHN were malignant, of which 93.3% had epithelial origin and 6.7% presented mesenchymal origin. Cholangiocarcinoma was the most commonly diagnosed neoplasm, followed by hepatocellular carcinoma and hemangiosarcoma. In general, cats with no defined breed were the most affected. Concerning sex, 60% were females and 40% males. Age ranged from five to 18 years, with a mean age of 10.5 years (median of ten years). Grossly, cholangiocarcinoma and hemangiosarcoma were multinodular and hepatocellular carcinoma was massive. Microscopically, cholangiocarcinomas were arranged in acini and ducts, whereas hepatocellular carcinomas were arranged in solid sheets or trabeculae. On immunohistochemistry, cholangiocarcinomas, hepatocellular carcinomas, and hemangiosarcomas were positive for the antibodies CK 7, Hep Par-1, and vimentin and von Willebrand factor, respectively.

INDEX TERMS: Felines, pathology, immunohistochemistry, hepatobiliary carcinoma, neoplasm, cats, cholangiocarcinoma, hepatic hemangiosarcoma.

RESUMO.- [**Aspectos patológicos e imuno-histoquímicos de neoplasias hepatobiliares primárias em gatos.**] Neoplasias hepatobiliares primárias (NHP) são incomuns em gatos e se originam de hepatócitos, células dos ductos biliares intra e extra-hepáticos, células mesenquimais e ainda células de origem neuroendócrina. O objetivo do trabalho foi determinar a frequência das NHP em gatos diagnosticados na Região Metropolitana de Porto Alegre, no período de 17 anos, abordando seus aspectos epidemiológicos, anatomopatológicos e imuno-histoquímicos (IHQ). Foram analisados os laudos de necropsia de 2.090 gatos sendo que 125 foram diagnosticados com doenças hepatobiliares primárias, destes 15 foram casos de NHP, representando 12% das condições hepatobiliares específicas e 0,7% do total de necropsias. Todos os diagnósticos de NHP eram malignos, destes 93,3% apresentaram origem epitelial e 6,7% mesenquimal. Colangiocarcinoma foi a neoplasia mais diagnosticada, seguido do carcinoma hepatocelular e hemangiossarcoma. De uma maneira geral, os gatos sem raça definida foram os mais acometidos. Em relação ao sexo 60% eram fêmeas e 40% machos. A idade variou de cinco a 18 anos, com a idade média de 10,5 anos (mediana de 10 anos). Macroscopicamente o colangiocarcinoma e hemangiossarcoma eram multinodulares, e o carcinoma hepatocelular, maciço. À histologia, houve predomínio do

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arranjo acinar e ductal nos colangiocarcinomas e sólido, no carcinoma hepatocelular. Na IHQ os colangiocarcinomas foram reativos para CK 7, carcinoma hepatocelular para Hep Par-1 e hemangiossarcoma para vimentina e fator de von Willebrand.

TERMOS DE INDEXAÇÃO: Felinos, patologia, imuno-histoquímica, carcinoma hepatobiliar, colangiocarcinoma, hemangiossarcoma hepático.

INTRODUCTION

Primary hepatobiliary neoplasms (PHN) are uncommon in cats (Cullen & Stalker 2016), and originate in hepatocytes, intra- and extrahepatic bile duct cells, mesenchymal cells, and cells of neuroendocrine origin (Head et al. 2003). They usually affect older cats, with no predisposition for breed or sex (Cullen 2017). Anorexia, lethargy, weight loss, hepatomegaly identified on abdominal palpation, and jaundice may be present, but clinical signs are often nonspecific, making clinical diagnosis difficult (Rutgers 1998, Barros 2016). The use of immunohistochemistry assists with the diagnosis of PHN (Patnaik 1992), and treatment and prognosis are determined by gross pattern and histological features, with indication for surgical resection in cases of neoplasms confined to only one hepatic lobe (Stonehewer 2006). The present study aimed to determine the frequency of PHN in cats diagnosed in the metropolitan region of Porto Alegre (RS), Brazil, for 17 years, addressing their epidemiological and pathological aspects.

MATERIALS AND METHODS

PHN cases in the necropsy reports of cats archived at the Department of Veterinary Pathology of the "Universidade Federal do Rio Grande do Sul", from January 1999 to December 2016, were reviewed and selected. Data described in necropsy requests, such as breed, sex, age and gross lesions, were reviewed and compiled. All cases analyzed were from the metropolitan region of Porto Alegre. Of the selected cases, the archived paraffin-embedded blocks were searched for the preparation of 3 μm-thick sections on histological slides for subsequent staining by the hematoxylin and eosin technique and visualization by optical microscopy.

The gross pattern and histological classification of the present study followed the criteria established by World Health Organization (WHO) (Head et al. 2003). For evaluation of the degree of fibrosis and quantification of mucin expression, histological sections were subjected to special Masson's trichrome (MT) and periodic acid-Schiff (PAS) stains. The special staining techniques followed the protocols described in the records of the Armed Forces Institute of Pathology (Gaffney 1992, McElroy 1992).

Neoplasm sections were submitted to immunohistochemistry (IHC) for biliary epithelium cells (cytokeratin 7 - CK 7), hepatocytes (Hepatocyte Paraffin 1 - Hep Par-1), mesenchymal cells (vimentin), and vascular endothelium (von Willebrand factor). The primary antibodies and protocols used are specified in Table 1.

RESULTS

Necropsy reports of 2.090 cats were analyzed, and 125 were diagnosed with primary hepatobiliary diseases, of which 15 were cases of PHN, representing 12% of the specific hepatobiliary conditions and 0.7% of the necropsies. All PHN (100%, 15/15) were malignant; of these, 93.3% (14/15) had epithelial origin and 6.7% (1/15) presented mesenchymal origin. Cholangiocarcinoma was identified in 66.6% (10/15), hepatocellular carcinoma in 26.7% (4/15), and hemangiosarcoma in 6.7% (1/15) of the cats.

In general, cats with no defined breed (NDB) were the most affected, representing 80% (12/15) of the cases, and the Siamese breed represented the remaining 20% (3/15) of the cases. Regarding sex, 60% (9/15) were females and 40% (6/15) males. Age ranged from five to 18 years, with a mean age of 10.5 years (median of ten years).

Cholangiocarcinoma

Cholangiocarcinoma was diagnosed in 66.6% (10/15) of the cases. It affected NDB cats in 80% (8/10) and Siamese cats in 20% (2/10). As for sex, 50% (5/10) were females and 50% (5/10) males. Age ranged from five to 13 years, with a mean age of 10.3 years (median of 11 years). In 80% (8/10) of the cases, cholangiocarcinoma originated in intrahepatic bile ducts and in the remaining 20% (2/10) from extrahepatic ducts (13.3%, 2/15). Grossly, the multinodular pattern was identified in 60% (6/10), and was characterized by multifocal to coalescent nodules, firm on palpation, and whitish with red areas (Fig.1A,B), and sometimes presented central depression (umbilicated aspect). The massive pattern was observed in 20% (2/10) of the cases, with the right lateral lobe affected in one case (50%, 1/2) and the left medial lobe affected one other case (50%, 1/2). These were firm on palpation and red interspersed with whitish areas (Fig.1C). The extrahepatic cases involved the cystic duct and were characterized by whitish to yellowish masses, firm on palpation, and with biliary flow obstruction (Fig.1D). Extrahepatic gross findings were mainly characterized by poor body condition and free slightly reddish

Table 1. Antibodies and immunohistochemical protocols used in hepatobiliary neoplasms in cats

Antibody	Clone/Code	Antigen retrieval	Dilution	Detection method	Chromogen
Monoclonal Anti-Vimentin	V9, Zymed	3 min/125°C, Citrate buffer pH 6.0	1:200	MACH 4, Biocare Medical	DAB, Dako
Monoclonal Anti-Cytokeratin 7 (CK 7)	M7018, Dako	Proteinase K, 10 min	1:40	MACH 4	DAB
Monoclonal Anti-Hep Par-1	M7158, Dako	40 min/96°C, Citrate buffer pH 6.0	1:100	MACH 4	DAB
Polyclonal Anti-von Willebrand factor	A0082, Dako	3 min/125°C, Citrate buffer pH 6.0	1:200	MACH 4	DAB

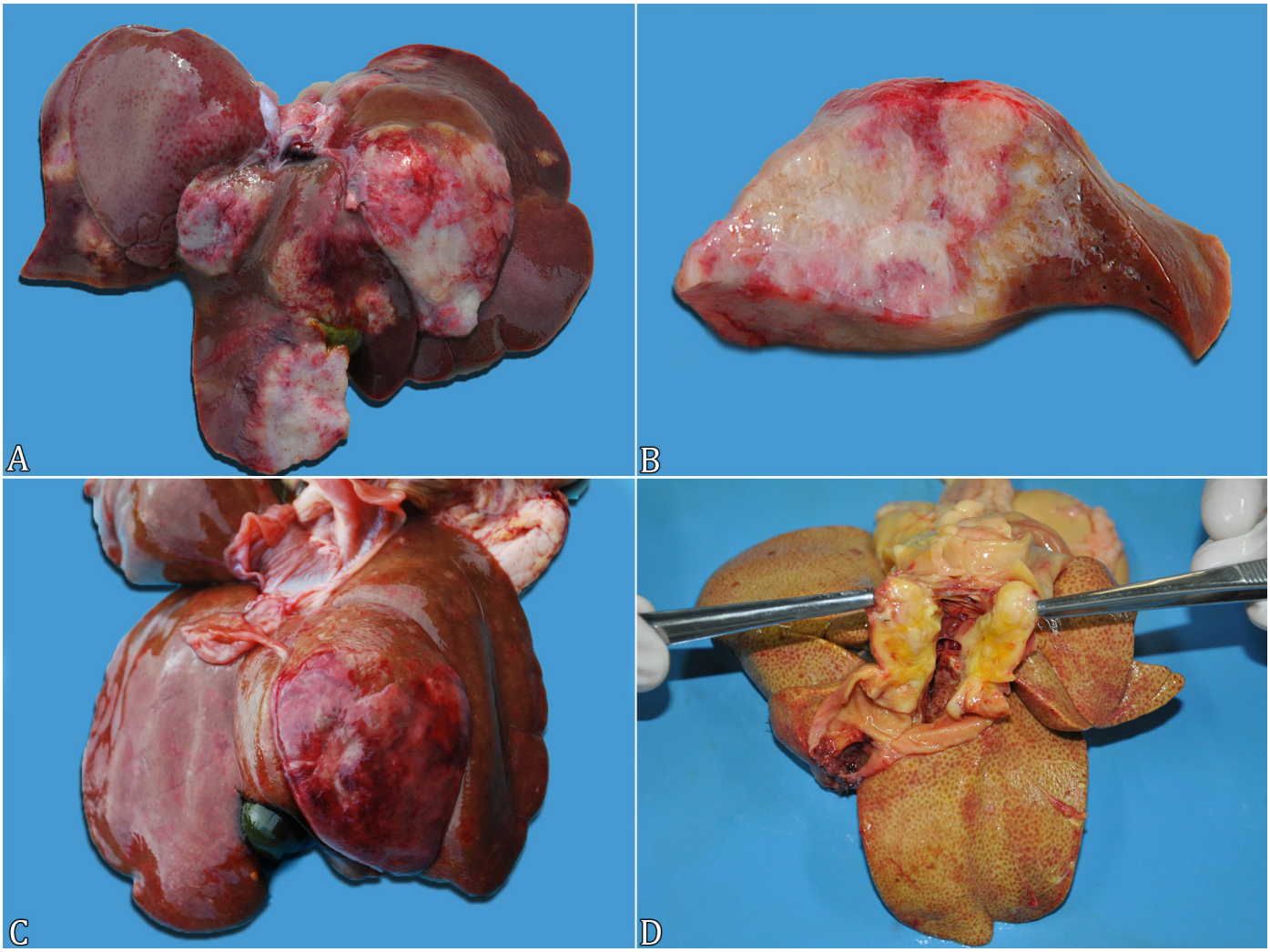

Fig.1. Gross aspects of cholangiocarcinoma in cats. (**A**) Multinodular pattern. Multifocal to coalescent nodules of different sizes, whitish interspersed with red areas. (**B**) Cholangiocarcinoma cutting surface of Figure 1A. (**C**) Massive pattern. Extensive focal mass located in the left medial lobe, red with lighter areas and central depression (umbilicated aspect). (**D**) Extrahepatic cholangiocarcinoma. Yellowish mass located in the cystic duct.

serous fluid in the abdominal cavity (ascites), identified in 50% (5/10) of cats with cholangiocarcinoma. In 40% (4/10) of the cases, nodules with gross appearance similar to those of the liver were identified in various organs, such as regional (hepatic) lymph nodes (75%, 3/4), peritoneum, diaphragm, intestinal and gastric serosa, lungs (50%, 2/4 each), and kidneys (25%, 1/4). Yellow oral and conjunctival mucosa, skin and subcutaneous tissue (jaundice) and yellow slightly diminished liver with lobular pattern accentuation were observed in cats with extrahepatic cholangiocarcinomas.

Histologically, 60% (6/10) of the cases were characterized by replacement of the hepatic parenchyma by proliferation of non-delimited and non-encapsulated epithelial cells, with formation of acini and/or irregular ducts (Fig.2A). In 30% (3/10) of the cases, the solid pattern was predominant. One case (10%, 1/10) was identified as biliary cystadenocarcinoma, characterized by formation of numerous cysts, with papillary projections to their lumen (Fig.2B). Cells were cuboid to rounded, with relatively undefined cytoplasmic borders, moderate and eosinophilic cytoplasm, round nucleus with chromatin, which ranged from dense to finely stippled, and with one to two conspicuous nucleoli (Fig.2C). Cellular pleomorphism was moderate in 50% (5/10), marked in 30% (3/10), and mild in 20% (2/10) of the cases. The mitotic index per high power field (HPF, 400x) was discrete (1/HPF) in 60% (6/10) of the cases, and moderate (2/HPF) in the remaining 40% (4/10) of the cases. Fibrous connective tissue proliferation, visualized mainly using MT staining, was marked in 50% (5/10) (scirrhous pattern) (Fig.2D), moderate in 30% (3/10), and mild in 20% (2/10) of the cases. Mucin expression was quantified as moderate in 40% (4/10), discrete in 20% (2/10), and marked in 10% (1/10) of the cases. This histological change was not identified in the remaining 30% (3/10) of the cases. Mucin was characterized by amorphous, slightly basophilic material within the acinar and ductal structures, which was intensely stained pink by PAS (Fig.2E). Intratumoral necrosis and hemorrhage were observed in 70% (7/10) and 60% (6/10) of the cases, respectively. Tumor invasion in lymphatic and/or blood vessels was identified in 40% (4/10) of the cases, and extrahepatic metastases were found mainly

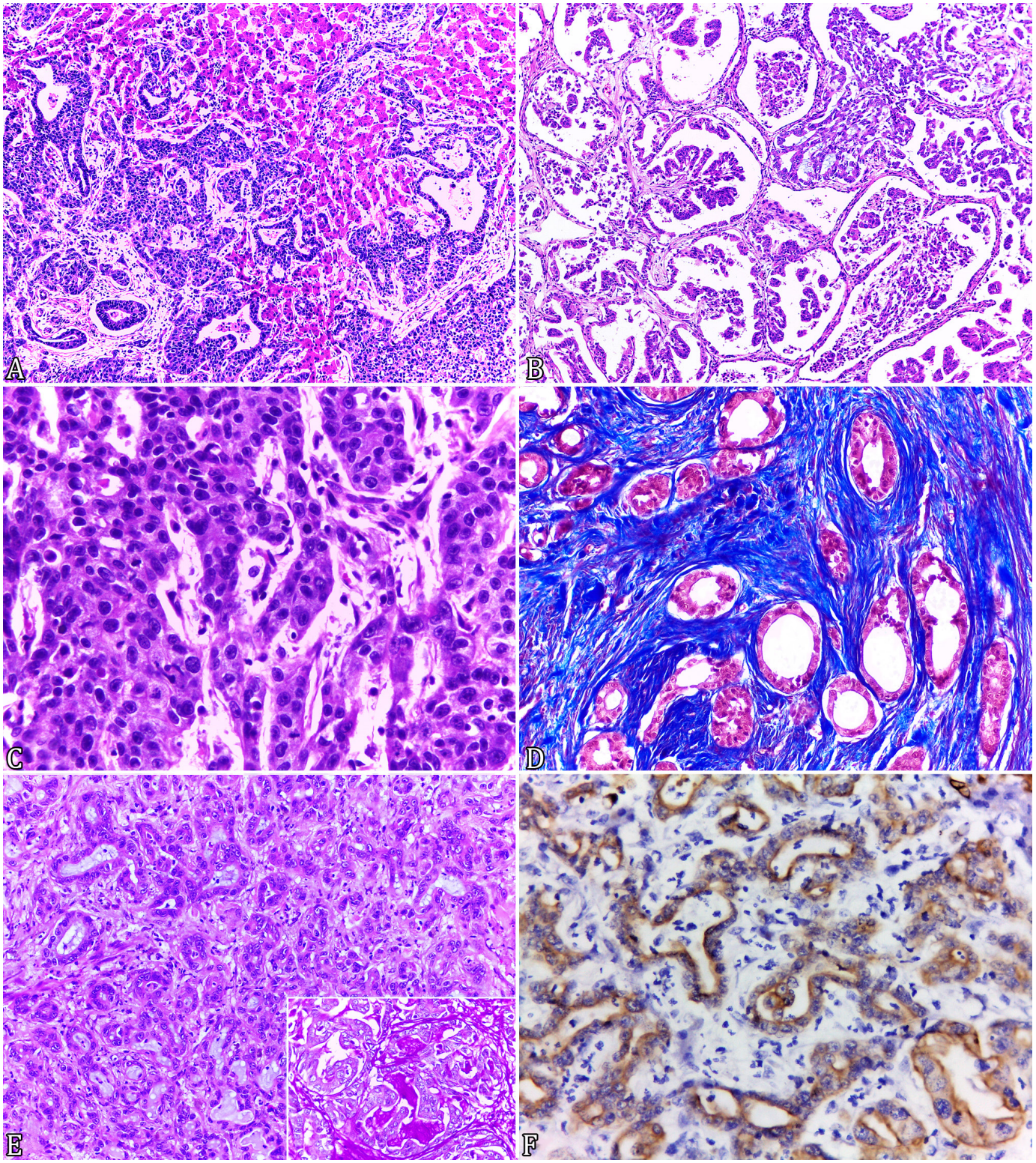

Fig.2. Histological and immunohistochemical aspects of cholangiocarcinoma in cats. (**A**) Replacement of the hepatic parenchyma by neoplastic proliferation of epithelial cells, with formation of acini and irregular ducts, supported by moderate connective stroma. HE, obj.10x. (**B**) Biliary cystadenocarcinoma. Neoplastic proliferation of epithelial cells with formation of cystic structures, sometimes with papillary projections to the lumen. HE, obj.10x. (**C**) Enlargement of Figure 2A showing neoplastic epithelial cells with moderate pleomorphism. HE, obj.40x. (**D**) Proliferation of duct-forming epithelial cells (red), interspersed with marked proliferation of fibrous connective tissue (blue). MT, obj.20x. (**E**) Proliferation of epithelial cells arranged in ductal and acinar pattern, with amorphous slightly basophilic material within these structures (mucin). HE, obj.20x. Inset: evidence of mucin intensely pink stained by PAS. PAS, obj.40x. (**F**) Marked anti-CK 7 staining in the cytoplasm of neoplastic epithelial cells, sometimes more intense near the plasma membranes. IHC, DAB, obj.40x.

in hepatic lymph nodes (4/10), spleen (3/10), lungs (3/10), stomach and intestinal serosa (2/10), peritoneum (1/10), diaphragm (1/10), and kidneys (1/10).

Immunohistochemistry (IHC) showed that intracytoplasmic and membrane staining for CK 7 was marked in 50% (5/10) (Fig.2F), moderate in 40% (4/10), and discrete in 10% (1/10) of the cases. No cases of cholangiocarcinoma showed IHC positivity for Hep Par-1.

Hepatocellular carcinoma

Hepatocellular carcinoma represented 26.7% (4/15) of the diagnoses. All affected cats were NDB (100%, 4/4). Females corresponded to 75% (3/4) and males to 25% (1/4) of the cases. Age ranged from 10 to 18 years, with a mean age of 12.5 years (median of 11 years).

The massive gross pattern was identified in all cats (100%, 4/4), characterized by large masses, predominantly located in the quadrate lobe with extension to the right medial and lateral lobes (75%, 3/4) (Fig.3A). The left medial lobe was also affected in one case (25%, 1/4). The masses were friable, whitish to yellowish, with irregular surface and red multifocal areas. Extrahepatic gross findings were characterized by poor body condition (75%, 3/4), ascites, jaundice in mucosa and subcutaneous tissue, and large amounts of free blood and clots in the abdominal cavity (hemoperitoneum) (25%, 1/4 each).

Histologically, there was disorganization of the organ architecture caused by proliferation of neoplastic hepatocytes with solid pattern (75%, 3/4), which were characterized by dense mantle cells (Fig.3B), sometimes with formation of trabeculae of variable thickness (25%, 1/4). Cells were polygonal, with relatively distinct cytoplasmic borders, abundant and eosinophilic cytoplasm, round nucleus, with finely stippled chromatin and one to two conspicuous nucleoli (Fig.3C). Marked intracytoplasmic vacuolization was identified in 50% (2/4) of the cases. Cell pleomorphism ranged from moderate to severe (50%, 2/4 each), and binucleated cells were identified in 50% (2/4) of the cases.

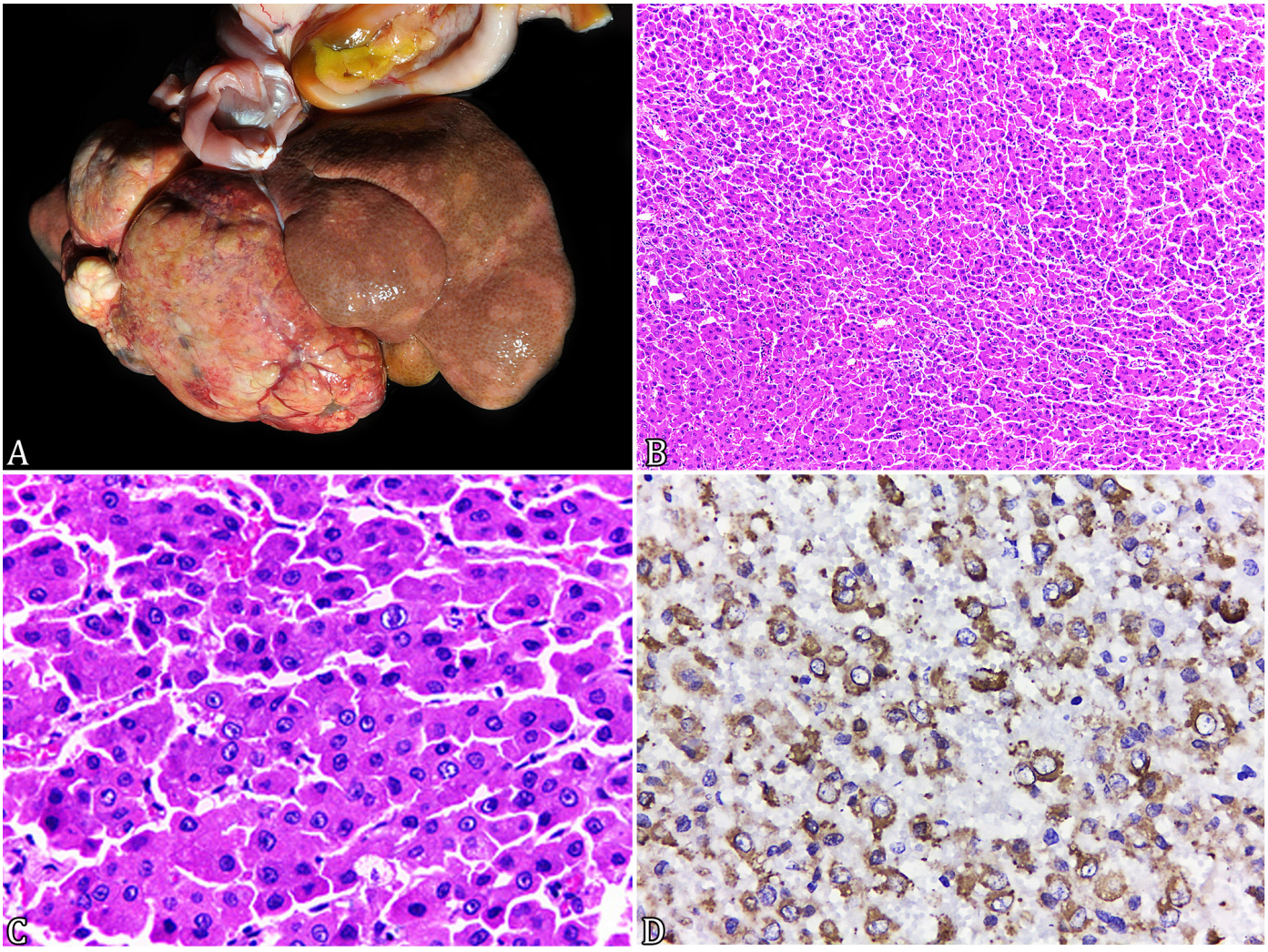

Fig.3. Anatomopathological and immunohistochemical aspects of hepatocellular carcinoma in cats. (**A**) Gross pattern. Large whitish mass with multifocal red and yellowish areas located in the quadrate lobe extending to the right medial and lateral lobes. (**B**) Histological aspect. Neoplastic proliferation of hepatocytes arranged in a solid pattern. HE, obj.10x. (**C**) Enlargement of Figure 3B showing neoplastic hepatocytes with moderate pleomorphism. HE, obj.40x. (**D**) Intense anti-Hep Par-1 staining in the cytoplasm of neoplastic hepatocytes. IHC, DAB, obj.40x.

The mitotic index HPF (400x) was discrete (1/HPF) in all cases (100%, 4/4). Fibrous connective tissue proliferation was classified as mild, and no mucin expression was identified in all cases (100%, 4/4). Intratumoral necrosis and hemorrhage were visualized in 100% (4/4) and 75% (3/4) of the cases, respectively. No tumor invasion in the blood and/or lymphatic vessels or extrahepatic metastases was identified.

All cases (100%, 4/4) showed marked intracytoplasmic, sometimes granular, staining for Hep Par-1 (Fig.3D). No cases of hepatocellular carcinoma presented IHC positivity for CK 7.

Hemangiosarcoma

Only one animal was diagnosed with primary liver hemangiosarcoma, accounting for 6.7% (1/15) of the cases. It was a five-year-old male NDB cat.

At necropsy, the liver showed multiple dark red nodules (Fig.4A) and soft on palpation. On the cutting surface, numerous cystic areas containing blood, interspersed with whitish firm areas were observed. The cat presented good body condition and pale mucous membranes. In abdominal cavity, a large amount of free blood and clots was observed.

Microscopic analysis was characterized by non-delimited and non-encapsulated endothelial cells proliferation, arranged in a solid pattern, often forming vascular structures, and sustained in a discrete conjunctival stroma (Fig.4B). The cells were spindle-shaped, with indistinct cytoplasmic borders, discrete and eosinophilic cytoplasm, oval to elongated nucleus, stippled chromatin, and evident single nucleoli (Fig.4C). Cellular pleomorphism was moderate, and the mitotic index HPF (400x) was discrete (1/HPF). An extensive area of necrosis and intratumoral hemorrhage was observed. No tumor invasion in vessels or extrahepatic metastases was identified.

IHC showed marked multifocal intracytoplasmic staining for vimentin and moderate for von Willebrand factor (Fig.4D).

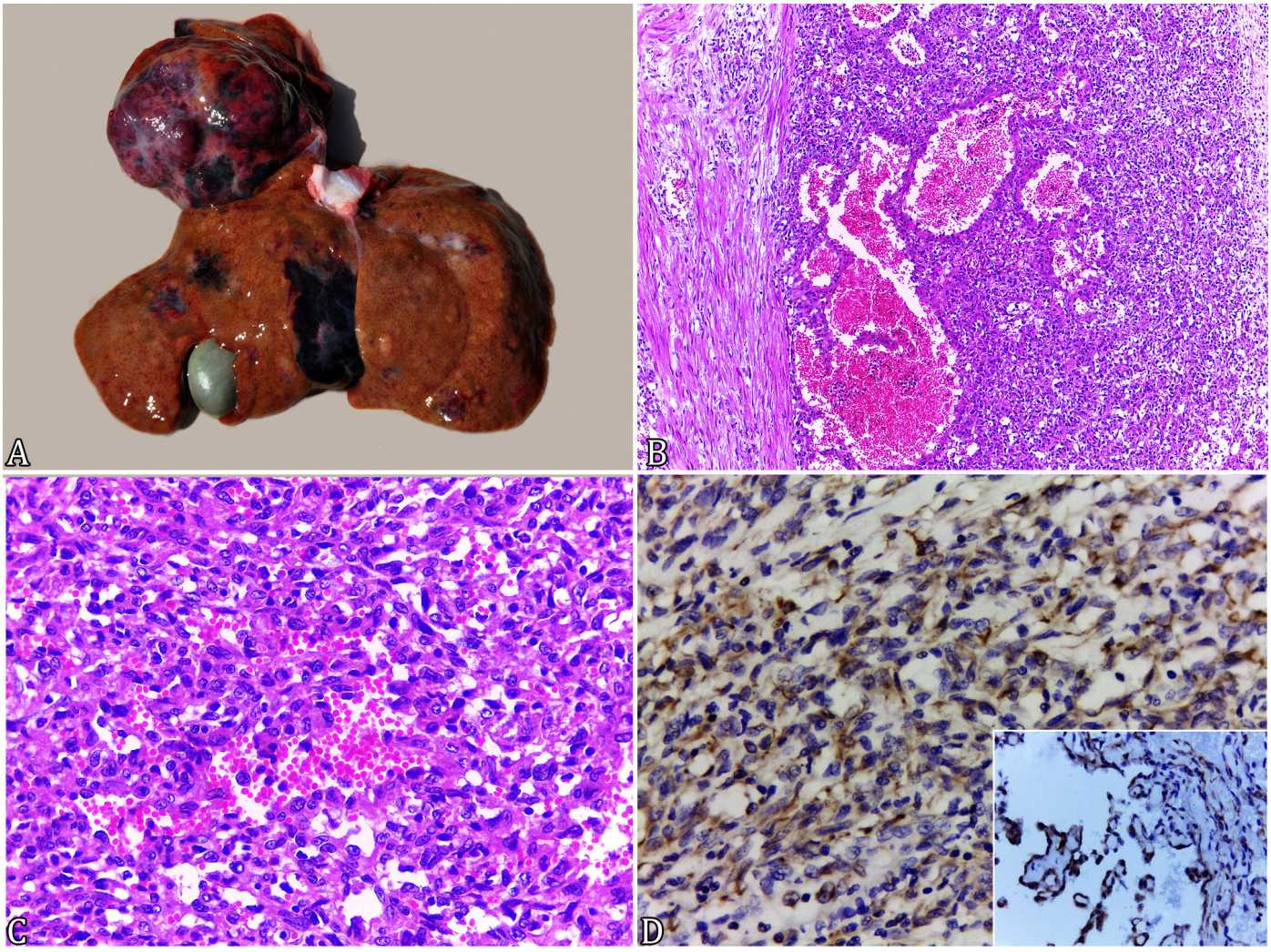

Fig.4. Anatomopathological and immunohistochemical aspects of hepatic hemangiosarcoma. (**A**) Gross pattern characterized by multifocal dark red nodules of varying sizes. (**B**) Histological aspects. Neoplastic proliferation of spindle cells arranged in a solid pattern, sometimes with formation of vascular structures of different sizes and filled with red blood cells. HE, obj.10x. (**C**) Enlargement of Figure 4B showing endothelial neoplastic cells. HE, obj.40x. (**D**) Intracytoplasmic accentuated multifocal staining for vimentin. Inset: intracytoplasmic staining for von Willebrand factor. IHC, DAB, obj.40x.

DISCUSSION

Frequency of PHN in the present study was 0.7% of the necropsies in cats, and when analyzed only the category of hepatobiliary diseases, the frequency was 12%. In similar studies, PHN frequency ranged from 0.7 to 2.3% of the necropsies (Schmidt & Langham 1967, Engle & Brodey 1969, Patnaik et al. 1975, Martins 2016). In the analyses of biopsy samples, these neoplasms corresponded to 9.7 to 11.3% (Gagne et al. 1996, Hirose et al. 2014). Researchers have reported that PHN corresponded to 1.0 to 5.7% of all cat neoplasms (Hammer & Sikkema 1995, Rutgers 1998, Andrade et al. 2012, Van Sprundel et al. 2014). NDB cats were the most affected in this study; however, no breed predisposition to PHN has been described (Balkman 2009). The larger number of NDB cats is probably due to the fact that they are the most assisted in the metropolitan region of Porto Alegre and, consequently, referred to the Department of Veterinary Pathology. There was no apparent sex predisposition, as previously described (Lawrence et al. 1994, Van Sprundel et al. 2014). Elderly cats were the most affected, corroborating the literature (Patnaik 1992, Post & Patnaik 1992, Lawrence et al. 1994, Andrade et al. 2012, Cullen 2017), and this may be associated with their longevity (O'Neill et al. 2015).

In the present study, all PHN were malignant, as described by Patnaik et al. (1975). However, numerous researchers have reported that the benign form is most commonly found (Post & Patnaik 1992, Lawrence et al. 1994). Cholangiocarcinoma was the most commonly diagnosed neoplasm, followed by hepatocellular carcinoma, corresponding to 66.6 and 26.7% of the cases, respectively. In cats, neoplasms originating in bile duct cells occur more frequently than those originating in hepatocytes (Van Sprundel et al. 2014, Otte et al. 2017). Several studies have reported cholangiocarcinoma as the main PHN in cats (Engle & Brodey 1969, Patnaik 1992, Post & Patnaik 1992, Andrade et al. 2012, Martins 2016, Cullen 2017). However, some researchers have described hepatocellular carcinoma (Patnaik et al. 1975) and biliary adenoma (Post & Patnaik 1992, Lawrence et al. 1994) as the most common. Cholangiocarcinomas may develop from intra- or extrahepatic bile ducts (Crawford & Liu 2010), with intrahepatic ducts as the most frequent (Patnaik 1992, Cullen 2009, 2017). In the present study, approximately 13% of the cases originated in extrahepatic ducts, corroborating the literature (Patnaik et al. 1975, Patnaik 1992). Extrahepatic bile duct and gallbladder neoplasms are rare in cats (Feldman et al. 1976, Cullen 2017), but researchers have found a similar frequency between intra- and extrahepatic tumors (Lawrence et al. 1994).

In this study, approximately 6.0% of the cases were of mesenchymal origin. Primary hepatobiliary sarcomas are rare in cats (Balkman 2009). Primary liver hemangiosarcoma varies widely in frequency (Scavelli et al. 1985, Patnaik 1992, Post & Patnaik 1992). This variability can result from two factors: the low incidence of this neoplasm and the difficulty in establishing the primary site when more than one organ is involved (Barros 2016, Cullen 2017). Researchers have reported that hemangiosarcoma is the second most common malignant neoplasm in cats (Post & Patnaik 1992).

Cholangiocarcinoma and hepatocellular carcinoma present different gross appearance (Head et al. 2003, Cullen & Stalker 2016). In the present study, the multinodular pattern was identified in 60% of cholangiocarcinomas, corroborating the literature, which describes this as the most common gross presentation in dogs and cats (Patnaik 1992, Cullen & Stalker 2016). In a survey with dogs, 83% of the cases were multinodular (Flores et al. 2013). In the present study, 20% of cholangiocarcinomas were classified as of massive pattern, and were characterized by large masses that obliterated the entire hepatic lobe (Cullen 2017). In dogs, this presentation has been identified in approximately 17% of bile duct neoplasms (Flores et al. 2013). It is not yet clear whether multiple nodules result from intrahepatic metastases or primary lesions in different *foci* (Barros 2016, Cullen & Stalker 2016). Firm consistency and whitish color are common gross features (Head et al. 2003, Barros 2016, Cullen 2017), and are attributed to the large amount of fibrous stroma present in cholangiocarcinomas (Head et al. 2003, Cullen & Stalker 2016). The umbilicated aspect is probably attributed to intratumor necrosis (Head et al. 2003, Cullen & Stalker 2016, Cullen 2017). Occasionally multiple cystic areas are observed, and when there is predominance of this presentation, the neoplasm is named biliary cystadenocarcinoma (Head et al. 2003, Cullen 2017), as observed in a cat of the present study. Hepatocellular carcinomas exhibited massive gross pattern. This presentation is the most commonly found, and is characterized by large masses involving a single hepatic lobe or extending to adjacent lobes (Barros 2016, Cullen 2017). This neoplasm varies in size and gross appearance, and may be present in massive, nodular or diffuse patterns (Cullen & Stalker 2016). In the present study, quadrate, right medial and lateral lobes were the most affected. According to Patnaik (1992), there is no predilection for hepatic lobe, but the left side is involved in more than two-thirds of hepatocellular carcinomas in dogs (Liptak et al. 2004). Color and consistency vary according to the degrees of hemorrhage and necrosis of the neoplasm and vacuolization of neoplastic cells (Barros 2016, Cullen 2017).

Extrahepatic gross findings were mainly characterized by poor body condition, ascites, and jaundice. These changes are frequent in cases of liver disease (Barros 2016). It has been suggested that ascites occurs as a result of portal hypertension owing to compression caused by neoplasms. Jaundice resulted from bile flow obstruction with consequent intra- and extrahepatic cholestasis (Charles et al. 2006). In the literature, approximately 20% of the cases presented jaundice (Lawrence et al. 1994). Hemoperitoneum as a result of rupture of neoplasms was observed in two cats: one with hepatocellular carcinoma and one with hemangiosarcoma. Generally, when the neoplasm is friable, there is rupture with consequent hemoperitoneum, anemia, and sudden death (Barros 2016, Cullen & Stalker 2016, Cullen 2017). Hemoperitoneum, ascites, and jaundice have been frequently reported in dogs with PHN (Flores et al. 2013).

The histological characteristics of PHN vary according to the degree of differentiation (Head et al. 2003, Cullen 2017). Well-differentiated cholangiocarcinomas are composed of cells that resemble the normal biliary epithelium and present a tubular or acinar arrangement (Barros 2016). As they become undifferentiated, the solid pattern is more prevalent (Head et al. 2003, Cullen 2017). Biliary cystadenocarcinoma is a variation histologically characterized by numerous cysts of varying sizes,

which often contain mucin and present papillary projections to the lumen (Cullen & Stalker 2016). Although the acinar/ductal arrangement was the most frequently found in this study, well-differentiated cholangiocarcinomas were not identified, since their cellular pleomorphism ranged from moderate to severe. Hepatocellular carcinoma usually has three microscopic patterns: trabecular, adenoid and solid (Head et al. 2003, Barros 2016). According to Cullen (2017), the solid pattern is poorly differentiated and characterized by pleomorphic cells (Crawford & Liu 2010, Flores et al. 2013). Vacuolization in the cytoplasm of neoplastic hepatocytes is a frequent finding, and is associated with glycogen or lipid deposition (Cullen 2017). Hemangiosarcomas are composed of neoplastic endothelial cells, often with formation of vascular spaces, but the solid arrangement may be found in some situations (Head et al. 2003, Barros 2016). Hemorrhage, necrosis and thrombus formation are frequent (Cullen 2017), and these findings are associated with the gross presentation, characterized by soft dark red nodules. The mitotic index was discrete in most PHN cases; however, researchers have reported that the mitotic index is more pronounced in cholangiocarcinoma than hepatocellular carcinoma (Head et al. 2003), and can be used to differentiate between them (Barros 2016).

Fibrous connective tissue and mucin are histological features frequent in cholangiocarcinomas and uncommon in hepatocellular carcinomas (Patnaik 1992, Head et al. 2003, Barros 2016, Cullen & Stalker 2016, Cullen 2017). Cholangiocarcinomas may vary in the amount of fibrous connective tissue proliferation, and researchers have reported a scirrhous pattern when there is marked fibrosis (Head et al. 2003). As observed in the present study, researchers have reported that mucin stains strongly with PAS (Barros 2016, Cullen 2017). Multiple *foci* of necrosis and hemorrhage are common in cholangiocarcinomas and hepatocellular carcinomas (Patnaik 1992, Cullen 2017). In the present study, these characteristics were visualized in most of the PHN.

Vascular invasion and extrahepatic metastases were identified in 40% of cholangiocarcinomas. In a similar study, most cholangiocarcinomas showed invasion in lymphatic and/or blood vessels. In dogs, vascular invasion was identified in 64% of these neoplasms (Flores et al. 2013). In hepatocellular carcinomas, invasion in vessels is not a common feature (Flores et al. 2013, Cullen 2017), and this finding corroborates those of the present study. Several authors have described that cholangiocarcinoma is the most commonly found metastatic PHN (Patnaik 1992, Lawrence et al. 1994, Head et al. 2003, Cullen 2017), and that hepatocellular carcinoma metastasis is uncommon in all animal species (Cullen 2009, Cullen & Stalker 2016). Regional lymph nodes are the major sites of metastatic PHN (Cullen & Stalker 2016). Especially in cats, cholangiocarcinomas can invade the Glisson capsule, with implantation of neoplastic cells in the peritoneum and serosa of various organs of the abdominal cavity (Cullen 2017). In a similar research, the frequency of metastatic cholangiocarcinomas ranged from 67 to 80% of the cases (Patnaik 1992, Lawrence et al. 1994). In a study with dogs, metastases were observed in approximately 78% of the cases, with lungs, lymph nodes, and abdominal cavity (omentum, mesentery and parietal peritoneum) as the most frequently affected organs (Flores et al. 2013), corroborating the findings of the present study.

All cholangiocarcinomas expressed CK 7, whereas hepatocellular carcinomas expressed Hep Par-1 in immunohistochemical analysis. In humans, the Hep Par-1 and CK 7 IHC assessments are used to differentiate PHN and in cases of metastatic carcinomas (Lau et al. 2002). Several studies in dogs have reported that CK 7 immunostaining in ductal epithelial cells has good sensitivity and specificity (Ramos-Vara et al. 2001, Flores et al. 2013). Cholangiocarcinoma does not show immunoreaction for Hep Par-1 (Shimonishi et al. 2000, Lau et al. 2002), as observed in the present study. Hep Par-1 is a highly specific and sensitive marker of normal, hyperplastic and/or neoplastic hepatocytes. Therefore, the Hep Par-1 associated with the CK 7 IHC techniques establish the diagnosis of PHN (Ramos-Vara et al. 2001). Hemangiosarcoma showed immunostaining for vimentin and von Willebrand factor, which are mesenchymal and endothelial cell-specific antibodies, respectively (Mello & Alves 1999, Bertazzolo et al. 2005).

In humans, hepatocellular carcinoma is the major PHN, because there are several associated etiological factors, such as viral infection, chronic alcoholism, nonalcoholic steatohepatitis and food contaminants as aflatoxins (Crawford & Liu 2010). In cats, the etiology of PHN is unknown (Lawrence et al. 1994), but *Platynosomum fastosum* infection has been described in the literature as a predisposing factor for the development of cholangiocarcinoma (Santos et al. 1981, Andrade et al. 2012). In the present study, no hepatobiliary trematodes were identified, because occurrence of *Platynosomum* sp. in the metropolitan region of Porto Alegre is infrequent (Michaelsen et al. 2012).

CONCLUSIONS

All primary hepatobiliary neoplasms (PHN) presented characteristics of malignancy and affected mainly elderly cats.

Cholangiocarcinoma was the most commonly diagnosed neoplasm, followed by hepatocellular carcinoma and hemangiosarcoma. Most cases of cholangiocarcinoma originated in intrahepatic bile ducts.

Grossly, cholangiocarcinoma and hemangiosarcoma presented predominance of the multinodular pattern, whereas hepatocellular carcinoma showed predominance of the massive pattern.

Extrahepatic gross findings were mainly characterized by poor body condition, ascites, and jaundice.

Histologically, there was predominance of acinar/ductal arrangement in cholangiocarcinomas and solid arrangement in hepatocellular carcinoma.

PHN showed moderate to severe cellular pleomorphism and mild mitotic index.

Proliferation of fibrous connective tissue and presence of mucin, identified by MT and PAS staining, respectively, were common histological findings in cholangiocarcinoma.

The use of hepatocyte and bile duct epithelial cells specific antibodies, such as Hep Par-1 and CK 7 assisted with the diagnosis of PHN.

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

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Original Article
Small Animal Diseases

Clinical management of dogs with presumptive diagnosis of thoracolumbar intervertebral disc disease: 164 cases (2006-2017)[1]

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ABSTRACT.- Baumhardt R., Ripplinger A., Aiello G., Schwab M.L., Ferrarin D.A., Wrzesinski M.R., Rauber J. & Mazzanti A. 2020. **Clinical management of dogs with presumptive diagnosis of thoracolumbar intervertebral disc disease: 164 cases (2006-2017).** *Pesquisa Veterinária Brasileira 40(1):55-60.* 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 presumptive diagnosis of cervical intervertebral disc disease (IVDD) submitted to clinical management and to evaluate the outcomes. Data were obtained from the medical records of patients with neurological dysfunction assisted at a University Veterinary Hospital from 2006 to 2017. In addition to the patients' records, dog owners responded to a questionnaire on the success of therapy. Four hundred and thirteen neurological records were evaluated, and 164 met the inclusion criteria of the study. The most common breed was Dachshund, followed by mongrels. Classification of neurological dysfunction in the study sample was as follows: 15.9% with grade I, 25.6% with grade II, 26.8% with grade III, 8.5% with grade IV, and 23.2% with grade V. Outcome was satisfactory in 71.6% of the dogs and unsatisfactory in 28.4% of them. Recurrence was observed in 27.7% of those with satisfactory outcomes. The clinical treatment of dogs with thoracolumbar IVDD is satisfactory, particularly for animals with milder disease grades (I, II, and III). There is possibility of recurrence with conservative therapy and clinical signs may be more severe.

INDEX TERMS: Clinical management, dogs, diagnosis, thoracolumbar intervertebral, disc disease, rest, corticosteroids, NSAID, extrusion, protrusion.

RESUMO.- [**Tratamento clínico de cães com diagnóstico presuntivo de doença do disco intervertebral toracolombar: 164 casos (2006-2017).**] O objetivo desse estudo foi identificar cães com diagnóstico presuntivo de DDIV toracolombar submetidos ao tratamento clínico, a fim de avaliar a resposta à terapia instituída. Foram revisados os registros neurológicos de cães atendidos pelo Serviço de Neurologia e Neurocirurgia Veterinária no período de 2006 a 2017 de um Hospital Veterinário Universitário. Foi realizada coleta de dados a partir dos registros e por meio de um questionário respondido pelos tutores. Foram avaliadas 413 fichas neurológicas de cães e obtidas informações para inclusão no estudo em 164 delas. As raças mais frequentes foram dachshunds, seguido de cães sem raça definida. Quanto ao grau de disfunção neurológica foi definido como grau I para 15,9% dos cães, grau II para 25,6%, grau III para 26,8%, grau IV para 8,5% e grau V para 23,2%. A recuperação foi satisfatória em 71,6% dos cães e insatisfatória em 28,4%. Dos que se recuperaram satisfatoriamente, 27,7% tiveram recidivas. Com base nos resultados obtidos pode-se concluir que o tratamento clínico em repouso absoluto e administração de anti-inflamatórios e analgésicos opióides para cães com DDIV toracolombar é efetivo, principalmente para cães em graus mais leves da doença (grau I, II e III). Há possibilidade de recidiva com esse tipo de terapia cujos sinais clínicos poderão ser mais graves.

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TERMOS DE INDEXAÇÃO: Tratamento clínico, cães, diagnóstico, doença do disco, intervertebral toracolombar, repouso, corticoide, AINE, extrusão, protrusão.

INTRODUCTION

Intervertebral disc disease (IVDD) is a common condition in the neurological clinic of dogs, and its most frequent site of occurrence is the thoracolumbar region (T3-L3) (Dewey & Da Costa 2016). Dogs of chondrodystrophic breeds are the most affected, especially Dachshunds. There is no gender predisposition, and the average age varies between three and six years (Brisson 2010, Santos et al. 2012, Kranenburg et al. 2013).

Clinical signs range from spinal hyperesthesia to severe neurological disabilities such as loss of caudal nociception (deep pain) to injury (Sharp & Wheeler 2005, Brisson 2010, Dewey & Da Costa 2016).

Clinical treatment for IVDD is generally indicated for dogs with hyperesthesia associated or not with minimal neurological disabilities (Brisson 2010, Kranenburg et al. 2013, Dewey & Da Costa 2016) and consists of absolute cage rest for four to six weeks (Sharp & Wheeler 2005), assuming that this time would be the minimum required for fibrous annulus repair (Jerram & Dewey 1999). Opioid analgesics, muscle relaxants, steroidal or nonsteroidal anti-inflammatory drugs (NSAID), and passive physical therapy are indicated, associated with rest (Sharp & Wheeler 2005, Levine et al. 2007, Mann et al. 2007). Surgery is the treatment of choice for paraplegic dogs with or without nociception (Ferreira et al. 2002, Sharp & Wheeler 2005, Dewey & Da Costa 2016).

In contrast to the large number of retrospective studies addressing the efficacy of surgical treatment of dogs with thoracolumbar IVDD (Ferreira et al. 2002, Kazakos et al. 2005, Santos et al. 2012, Kranenburg et al. 2013, Jeffery et al. 2016), research evaluating the response to clinical treatment is scarce in the literature, especially nationally.

Therefore, the objective of this study was to identify dogs with presumptive diagnosis of thoracolumbar IVDD submitted to clinical treatment in order to evaluate the therapy outcomes and recurrence rates. This study also aimed to describe the age, sex, and response to treatment according to neurological grade, so that these parameters can be used as prognostic factors for the clinical evolution of these patients.

MATERIALS AND METHODS

Neurological records of dogs assisted at the Veterinary Neurology and Neurosurgery Service (SNNV) of a University Veterinary Hospital (HVU) from 2006 to 2017 were reviewed. Presumptive diagnosis was defined by history, breed, age, clinical signs, and neurological examination. All patients underwent imaging exams, namely, plain radiography (X-ray), myelography, or computed tomography (CT), in order to discard other conditions such as discospondylitis, fractures, and neoplasms (Dewey & Da Costa 2016). Serology was performed in all dogs to exclude probable infectious diseases (toxoplasmosis, neosporosis and distemper). Only dogs that presented a presumptive diagnosis of IVDD in the T3-L3 and L4-S3 segments of the spinal cord, and without other (non-neurological) diseases that could interfere with neurological evaluation were included in the survey.

Data were collected from the clinical and neurological records of patients and by telephone contact with owners. Dogs that underwent clinical treatment were selected for the study. Neurological dysfunction was classified from grades I to V, where grade I, only hyperesthesia; grade II, outpatient paraparesis; III grade, non-ambulatory paraparesis; grade IV, paraplegia with deep caudal pain perception; grade V, paraplegia with absent deep caudal pain perception (Sharp & Wheeler 2005). The dogs were distributed in age the following age groups: ≤3 years; 4-6 years; 7-9 years, and ≥10 years, according to the distribution by Chaves et al. (2014).

The clinical treatment prescribed to the dogs by the SNNV at the time of consultation consisted of absolute cage rest for at least 30 days, taken three times a day for urination and defecation with the use of a movement restriction guide associated with opioid analgesics and prednisone steroidal anti-inflammatory drug at a dose of 0.5-1.0 mg/kg every 24 h or nonsteroidal meloxicam at a dose of 0.1-0.2 mg/kg every 24 h (Dewey & Da Costa 2016).

Owners responded to a two-part questionnaire: i) information before consultation and ii) information after consultation. Some information was obtained from the patients' medical records and supplemented with the owner's information at the time of the telephone contact.

Data from the first part of the questionnaire were related to the recurrence or not of clinical signs; duration of clinical signs until the time of consultation; use of anti-inflammatory drugs; absolute rest or maintenance in a confined space; evolution of clinical signs until the time of consultation. Recurrence was considered when there was previous history of dorsal pain and/or difficulty in walking or climbing obstacles. Duration of clinical signs was classified as follows: ≤1 day; 2-7 days; 8-30 days; >30 days. Regarding the evolution of clinical signs, dogs were distributed as follows: improved - when there was an improvement compared with the onset of signs; stable - when no difference between the times was observed; progressive worsening - gradual worsening until the time of consultation; rapid worsening - paraplegia observed in less than five days.

Data from the second part of the questionnaire referred to the treatment indicated by the SNNV, administration of anti-inflammatory drugs, absolute rest or maintenance in a restricted space, and clinical evolution of treatment performed. Regarding the anti-inflammatory drug, it was asked whether it was steroidal (corticosteroid) or nonsteroidal (NSAID). Clinical evolution was classified as satisfactory - for dogs that recovered the ability to walk without hyperesthesia, and unsatisfactory - for those that did not recover motor function or remained with hyperesthesia. Dogs that had a satisfactory recovery were evaluated for the occurrence of disease recurrence.

Dogs whose owners opted for euthanasia or died within less than two weeks were excluded from the clinical evolution assessment. Dogs that presented paraplegia without deep pain perception (grade V) at the time of consultation and whose owners rated their recovery as satisfactory returned to the SNNV for further neurological examination to discard acquisition of involuntary spinal locomotion (spinal walking) (Gallucci et al. 2017).

The study sample was mainly composed of Dachshunds - 59.1% (n=97). Other breeds included Poodle (n=10), Pinscher (n=3), Shih Tzu (n=7), Lhasa Apso (n=5), Pekingese (n=4), Cocker Spaniel (n=2), Beagle (n=2), French Bulldog (n=2), American Staffordshire Terrier (n=1), Basset Hound (n=1), Boxer (n=1), Brazilian Mastiff (n=1), Brazilian Terrier (n=1), German

Shepherd (n=1), Pug (n=1), Schnauzer (n=1), and Yorkshire Terrier (n=1). The sample was included 23 mongrels. Age ranged from two to 15 years, and distribution was as follows: 13.4% (n=22), >3 years; 44.5% (n=73), 4-6 years; 29.9% (n=49), 7-9 years; 12.2% (n=20), ≥10 years. Regarding gender, 43.9% (n=72) were male and 56.1% (n=92) female.

RESULTS

The survey identified 413 neurological records with a presumptive diagnosis of thoracolumbar and lumbar IVDD. Of these, 213 (51.5%) owners responded to the telephone contact and agreed to answer the questionnaire and 164 confirmed clinical treatment was the first option. Of the latter, first-time presentation of clinical signs was observed in 80.5% (n=132), recurrent (relapse) clinical signs were observed in 18.9% (n=31), and 0.6% of the owners could not answer the question. In 65.9% (n=108) of these dogs, anti-inflammatory drugs were administered before the consultation, and 55.5% (n=60) of them had received NSAID and corticosteroids, associated or not. In 26.2% (n=43) of the animals, anti-inflammatory drugs were not administered and 7.9% (n=13) of the owners could not answer the question. Of the dogs that received anti-inflammatory drugs, 70.4% presented worsening clinical signs (n=53 with progressive worsening and n=23 with rapid worsening).

Regarding gender, 70.6% of the females and 70.8% of the males showed satisfactory recovery. In the age groups, 75% of the dogs aged <3 years, 65.1% of those aged 4-6 years, 79.6% of those aged 7-9 years, and 80% of the animals aged ≥10 years presented satisfactory recovery.

As for the evolution of clinical signs and treatment prior to consultation, absolute cage rest was observed in 12.2% (n=20) of the dogs, 27.4% (n=45) of the animals rested in restricted spaces, and 60.4% (n=99) of them did not rest at all. Clinical signs improved in 9.7% (n=16), remained stable in 17.1% (n=28), progressively worsened in 50.6% (n=83), and rapidly worsened in 22.6% (n=37) of the dogs. Neurological dysfunction was classified as grade I in 15.9% (n=26), grade II in 25.6% (n=42), grade III in 26.8% (n=44), grade IV in 8.5% (n=14), and grade V in 23.2% (n=38) of the animals.

When neurological dysfunction was distributed according to the rest performed prior to the consultation, 85.7% of the dogs with milder disease grades (I and II) and that underwent absolute cage rest showed improvement of clinical signs or remained stable. When rest occurred in restricted spaces, 42.9% and 37.5% of the dogs with grades I and II of neurological dysfunction presented worsening of clinical signs, respectively; and when no rest occurred, 56.2% and 66.7% of the dogs with grades I and II of neurological dysfunction showed worsening of clinical signs. Of the dogs with grade III, 22.2% of those that had absolute cage rest improved, 22.2% remained stable, and the others had worsening signs. Of those that rested in restricted spaces, 100% showed worsened clinical signs, and those that did not rest at all, 18.5% remained stable and 81.5% presented worsened clinical signs. All dogs with grades IV and V of neurological dysfunction, regardless of the occurrence of rest, presented worsened clinical signs, except for one dog with grade V, which remained stable (Table 1).

Of the total number of dogs with clinical treatment prescribed by the SNNV, 85.6% (n=141) of the owners agreed to perform the therapy on the day of consultation. Clinical evolution (Table 2) was satisfactory in 71.6% (n=101) and unsatisfactory in 28.4% (n=40) of the animals. Of those with satisfactory recovery, 76 (75.3%) underwent absolute cage rest, 19 (18.8%) rested in restricted spaces restriction, and six (5.9%) did not rest at all.

As for unsatisfactory recovery, 25 dogs (62.5%) were under absolute cage rest, 11 (27.5%) rested in restricted spaces, and four (10%) did not rest at all (owners' option). Disease recurrence was observed in 28 (27.7%) dogs: 25% (n=7) of those had more severe clinical signs, 46.4% (n=13) showed similar signs, and 6% (n=8) presented milder signs.

Duration of clinical signs was ≤1 day in 9.2% (n=13), 2-7 days in 39.0% (n=55), 8-30 days in 41.2% (n=58), and >30 days in 10.6% (n=15) of the dogs. Most of the animals with clinical signs lasting up to 30 days recovered satisfactorily (<1 day = 76.9%; 2-7 days = 76.4%; 8-30 days = 70.7%). Only 53.3% of the dogs with clinical signs lasting >30 days presented satisfactory recovery.

Of the 141 dogs that should undergo clinical treatment as prescribed by the SNNV, absolute cage rest was observed in 62.3% (n=102). In 30 dogs (21.3%), rest occurred in restricted spaces, and nine (6.4%) animals did not rest at all. Recovery was satisfactory in 88.0, and 80.5% for dogs with grvII of neurological dysfunction, respectively. Of the dogs

Table 1. Evolution of clinical signs in relation to pre-consultation treatment and neurological grade of 164 dogs with presumptive diagnosis of thoracolumbar IVDD

Previous treatment	Total n	Improved n	Stable n	Progressive worsening n	Rapid worsening n
Grade I	25	3	10	12	-
Rest AB	2	1	1	-	-
Rest RS	7	-	4	3	-
Without rest	16	2	5	9	-
Grade II	42	11	10	21	-
Rest AB	5	3	1	1	-
Rest RS	16	7	3	6	-
Without rest	21	1	6	14	-
Grade III	45	2	7	35	1
Rest AB	9	2	2	5	-
Rest RS	9	-	-	9	-
Without rest	27	-	5	21	1
Grade IV	14	-	-	5	9
Rest AB	1	-	-	-	1
Rest RS	4	-	-	2	2
Without rest	9	-	-	3	6
Grade V	38	-	1	10	27
Rest AB	3	-	-	-	3
Rest RS	9	-	-	3	6
Without rest	26	-	1	7	18
AI	108	13	19	53	23
Without AI	42	3	6	23	10
CNA	14	-	3	7	4
TOTAL	164	16	28	83	37

n = number of dogs; AB = absolute; RS = restricted space; AI = anti-inflammatory drugs; CAN = could not answer.

Table 2. Clinical evolution regarding clinical treatment outcome and neurological grade of the 141 dogs with presumptive diagnosis of thoracolumbar IVDD

	Number of dogs n=141	Unsatisfactory recovery n	Satisfactory recovery n
Sex			
Female	80	22	58
Male	61	18	43
Age			
<3 years	19	5	14
4-6 years	69	24	45
7-9 years	37	8	29
>10 years	16	3	13
Duration of clinical signs			
<1 day	13	4	9
2-7 days	55	14	41
8-30 days	58	16	42
>30 days	15	7	8
Rest			
Absolute	102	26	76
Restricted space	30	11	19
Without rest	9	3	6
Neurological dysfunction			
Grade I	25	3	22
Grade II	40	8	32
Grade III	41	8	33
Grade IV	12	6	6
Grade V	23	15	8
TOTAL	141	40	101

n = number of dogs.

with grades IV and V, 50.0% and 34.8% showed satisfactory recovery, respectively.

Of the 141 dogs, 111 (78.7%) received anti-inflammatory drugs prescribed by the SNNV, 62.4% (n=88) were treated with corticosteroids, and 16.3% (n=23) received NSAID. Of the dogs receiving corticosteroids, 70.5% (n=62) had satisfactory recovery, and 78.3% (n=18) of those receiving NSAID recovered satisfactorily. Twenty-four (17.0%) dogs were not prescribed with anti-inflammatory drugs by the SNNV; however, they had already received medication for the time recommended before the consultation and, therefore, only opioid analgesic was prescribed.

DISCUSSION

Prevalence of gender, age, and breed of the patients studied is similar to that reported in previously published studies (Levine et al. 2007, Brisson 2010, Kranenburg et al. 2013).

Recovery of dogs in the different age groups had similar proportions, suggesting that there was no interference of age in this variable, corroborating the findings of previous studies (Olby et al. 2004, Penning et al. 2006); however, some authors have reported better (Macias et al. 2002, Levine et al. 2007) and faster (Olby et al. 2003) recovery in young dogs. Variation in methodology and difference in study samples sizes may have interfered with divergent results.

Male and female dogs had similar recovery times, in agreement with the results found by Macias et al. (2002), who found no gender influence on recovery. Experimental studies have described that the female hormone would have a neuroprotective effect (Marchetti et al. 2000, Bjorling et al. 2002), and retrospective studies have found higher occurrence of IVDD in male dogs (Ferreira et al. 2002, Penning et al. 2006). A population study of the region is essential to conclude about gender predisposition in IVDD.

According to Levine et al. (2007), duration of clinical signs was significant for therapeutic response in dogs treated clinically for thoracolumbar IVDD. The studies by Ferreira et al. (2002), Macias et al. (2002), Olby et al. (2003), Kazakos et al. (2005) have demonstrated that the duration of clinical signs until treatment did not influence the patients' recovery. In this study, the duration of clinical signs up to 30 days did not seem to influence clinical evolution; however, when compared with dogs with that presented signs for >30 days, worse recovery results were observed.

There was indication of surgery for all grade IV dogs (n=14) and for seven grade V animals. However, two dogs with grade IV and five with grade V neurological dysfunction were euthanized on the owners' choice. Santos et al. (2012) reported that some of these paraplegic dogs might show satisfactory recovery in the long term. In the present study, 34.7% of the dogs with grade V that underwent clinical treatment presented satisfactory recovery. Although 65.3% of the dogs with grade V did not recover with treatment, five developed spinal walking, considered by most owners as satisfactory recovery. Owners should be advised that there is a possibility of motor function recovery even without recovering from nociception. In a retrospective study, Gandini et al. (20017) found that of the 81 paraplegic dogs without deep pain perception, 59% (n=49) developed spinal walking, and that intense physical therapy enabled dogs to acquire this type of involuntary movement. They also noted that young and lightweight dogs were more likely to develop spinal walking.

In grades I and II of neurological dysfunction, absolute cage rest prior to the consultation allowed most dogs to recover a clinical condition better than that observed at the onset of clinical signs, or to remain with stable signs. For those that did not rest before the consultation, at all neurological dysfunction grades, 79.8% arrived at the SNNV with worsening clinical signs (Table 1). Clinical treatment outcomes were as follows: of the 102 dogs that underwent absolute cage rest, 76 (74.5%) had satisfactory recovery, showing that dogs kept in cages or transport boxes are 2.5 times more likely to respond better to treatment (Table 2) compared with those that rested in restricted spaces or did not rest at all.

Cage rest for approximately four to six weeks is indicated for the satisfactory recovery of dogs treated for IVDD due to healing of ligamentous structures, lower chance of extrusion of the nucleus pulposus, and reduced likelihood of accidental traumatic injury (Sharp & Wheeler 2005, Dewey & Da Costa 2016). Although Levine et al. (2007) did not observe a relationship between clinical improvement of patients and duration of cage rest when comparing resting with non-resting dogs, they found that those that rested presented a 1.6-time greater chance of successful recovery. Based on these results, they suggested that resting is associated with better recovery,

but that absolute restriction of exercise over extended periods in dogs with IVDD is not ensured by treatment with medications.

Approximately 65.9% of the dogs in this study received anti-inflammatory drugs before the consultation, and 70.4% of these presented worsening of clinical signs. In addition, 87.8% of the dogs did not undergo absolute cage rest. These results suggest that only anti-inflammatory drugs may not be sufficient for satisfactory evolution, as they do not act on the dynamic component of the disease. The effects of NSAID and corticosteroids may decrease the discomfort of patients with spinal cord disease and encourage them to excessive activity (Platt et al. 2005).

The results obtained with respect to recovery and recurrence of neurological signs were similar to those found by Levine et al. (2007), who justified their satisfactory results with the fact that most dogs presented milder grades of neurological dysfunction (I, II and III). In this study, dogs with grades I, II, and III showed better therapeutic response compared with that of dogs with grades IV and V, suggesting that clinical treatment is satisfactory is for milder disease grades.

Corticosteroids are useful in the treatment of vasogenic edema of the spinal cord (Kraus 1996). However, their use in IVDD has been questioned both for their effectiveness in treatment (Levine et al. 2007, Mann et al. 2007) and for the decreased resulting quality of life, which is better in dogs that receive NSAID (Levine et al. 2007). In the present study, recovery in dogs receiving NSAID or corticosteroids was similar, but this assessment was impaired because of the relatively larger number of animals that had received anti-inflammatory drugs before the first consultation.

Mann et al. (2007) associated the use of corticosteroids with higher recurrence rate when compared with the use of NSAID. Thus, these authors suggest that the use of corticosteroids may influence the healing of the fibrous annulus of the intervertebral disc, contributing to recurrence in the same site, or on different disks owing to the adverse effects of collagen synthesis and degradation, especially if the disc is already undergoing a degenerative process. No higher recurrence rates were observed in the group receiving corticosteroids.

One of the limitations to this study was the presumptive and indefinite diagnosis of IVDD. Levine et al. (2007) used plain radiography and myelography as diagnostic methods to exclude other causes such as discospondylitis, meningomyelitis, fractures, and neoplasms. In this study, in addition to myelography and plain radiography, CT was performed, which is considered an imaging examination with sensitivity superior to that of myelography in detecting spinal cord compression caused by IVDD (Brisson 2010). Even so, magnetic resonance imaging (MRI) is used for the definite diagnosis of IVDD and, more precisely, surgical explorations that will reveal the extruded content inside the vertebral canal (Levine et al. 2007). Therefore, the possibility that some dogs in this study with presumptive diagnosis of IVDD had another neurological disorder cannot be discarded.

The clinical relevance of this research was to demonstrate the results of clinical treatment of dogs with presumptive diagnosis of thoracolumbar IVDD in Brazil and to verify whether recovery rate was satisfactory mainly in dogs with milder (I, II and III) and more severe (IV and V) neurological dysfunction grades. This type of therapy was not effective, and surgical decompression was recommended immediately (Dewey & Da Costa 2016). Regarding recurrence, the results found were similar to those described in the international literature (Levine et al. 2007). The clinical therapy employed in this study, with absolute cage rest, was as recommended in the literature (Dewey & Da Costa 2016) and promoted satisfactory recovery in most patients. Owners who did not follow the instructions of the SNNV and opted for rest in restricted spaces, also obtained satisfactory results, but in a smaller number of cases (Table 1).

CONCLUSIONS

Clinical treatment with absolute cage rest and administration of anti-inflammatory drugs and opioid analgesics for dogs with thoracolumbar IVDD is effective, especially for dogs with milder disease levels (grades I, II, and III).

There is possibility of recurrence with conservative therapy using nonsteroidal anti-inflammatory drugs (NSAID), and clinical signs may be more severe. No change in response between dogs of different age or sex was observed, suggesting that there is no prognostic influence of these factors on recovery.

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Original Article
Small Animal Diseases

Primary nonlymphoid gastrointestinal neoplasms in dogs in Rio Grande do Sul[1]

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ABSTRACT.- Slaviero M., Argenta F.F., Ehlers L.P., De Lorenzo C., Pavarini S.P., Driemeier D. & Sonne L. 2020. **Primary nonlymphoid gastrointestinal neoplasms in dogs in Rio Grande do Sul.** *Pesquisa Veterinária Brasileira 40(1)61-71*. Setor de Patologia Veterinária, Departamento de Patologia Clínica Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Prédio 42505, Porto Alegre, RS 91540-000, Brazil. E-mail: lusonne@yahoo.com.br

Gastrointestinal neoplasms (GIN) are uncommon in dogs, but they mainly show malignant behavior and poor prognosis. The types of GIN in dogs and their frequency, as well as their epidemiological and histopathological characteristics were analyzed through a retrospective study of biopsies from 24.711 dogs from 2005 to 2017. Additionally, histological sections of neoplasms were subjected to immunohistochemistry (IHC) using antibodies against pancytokeratin, vimentin, smooth muscle actin, c-Kit, S-100, CD31, CD79αcy, and neuron-specific enolase. Of the total samples from dogs analyzed, 88 corresponded to GIN. Neoplasms occurred more frequently in purebred dogs (64.8%, 57/88), males (53.4%, 47/88), with a median age of 10 years. The intestine was affected by 84.1% (74/88) of the cases. Of these, the large intestine was the most affected (67.6%, 50/74). Most of the neoplasms had malignant behavior (88.6%, 78/88). Regarding the classification of neoplasms, 46.6% (41/88) of the diagnoses corresponded to epithelial, 46.6% (41/88) were mesenchymal, 5.7% (5/88) were hematopoietic, and 1.1% (1/88) was neuroendocrine. The most frequently diagnosed neoplasms were papillary adenocarcinoma (19.3%, 17/88), leiomyosarcoma (17.0%, 15/88), gastrointestinal stromal tumors (GISTs) (12.5%, 11/88), and leiomyoma (5.0%, 8/88). Adenocarcinomas were located mainly in the rectum, whereas leiomyosarcomas and GISTs developed mainly in the cecum. Epithelial neoplasms showed a greater potential for lymphatic invasion whereas mesenchymal neoplasms appeared to be more expansive with intratumoral necrosis and hemorrhage. Immunohistochemistry was found to be an important diagnostic technique for the identification of infiltrating cells in carcinomas and an indispensable technique for the definitive diagnosis of sarcomas.

INDEX TERMS: Primary nonlymphoid, gastrointestinal neoplasms, dogs, Rio Grande do Sul, Brazil, canine, gastric neoplasm, intestinal neoplasm, histopathology, immunohistochemistry.

RESUMO.- [**Neoplasmas gastrointestinais primários não linfoides em cães no Rio Grande do Sul.**] Neoplasmas gastrointestinais (NGI) são pouco comuns em cães, mas possuem principalmente comportamento maligno e prognóstico reservado. Os tipos de NGI em cães e sua frequência, bem como características epidemiológicas e histopatológicas foram analisados por meio de um estudo retrospectivo dos exames de biópsias de 24.711 cães entre os anos de 2005 a 2017. Adicionalmente, cortes histológicos de NGI foram submetidos à técnica de imuno-histoquímica (IHQ), utilizando os anticorpos anti-pancitoqueratina, vimentina, actina de músculo liso, c-Kit, S-100, CD31, CD79αcy e enolase neurônio específica. Do total de cães analisados, 88 corresponderam a NGI não linfoides. Os neoplasmas ocorreram com maior frequência em cães de raça pura (64,8%, 57/88), machos (53,4%, 47/88), com mediana de idade de 10 anos. O intestino foi acometido em 84,1% dos casos (74/88). Destes, o intestino grosso foi o segmento mais afetado (67,6%, 50/74). A maior parte dos

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neoplasmas tinha comportamento maligno (88,6%, 78/88). Quanto à classificação, 46,6% (41/88) dos diagnósticos corresponderam a neoplasmas epiteliais, 46,6% (41/88) mesenquimais, 5,7% (5/88) hematopoiéticos e 1,1% (1/88), neuroendócrino. Os neoplasmas mais frequentemente diagnosticados foram adenocarcinoma papilar (19,3%, 17/88), leiomiossarcoma (17,0%, 15/88), tumor estromal gastrointestinal (GIST) (12,5%, 11/88) e leiomioma (12,5%, 8/88). Adenocarcinomas localizavam-se principalmente no reto, enquanto leiomiossarcoma e GISTs desenvolveram-se principalmente no ceco. Os neoplasmas epiteliais demonstraram um potencial maior de invasão linfática enquanto que os mesenquimais aparentaram ser mais expansivos, com necrose e hemorragia intratumorais. A imuno-histoquímica mostrou ser uma técnica diagnóstica importante para a identificação de células neoplásicas infiltravas no caso dos carcinomas e uma técnica indispensável para o diagnóstico definitivo de sarcomas.

TERMOS DE INDEXAÇÃO: Neoplasmas gastrointestinais, cães, Rio Grande do Sul, Brasil, caninos, neoplasma gástrico, neoplasma intestinal, histopatologia, imuno-histoquímica.

INTRODUCTION

Gastrointestinal neoplasms (GIN) are uncommon in dogs, but show malignant behavior and a poor prognosis in most cases (Swann & Holt 2002, Uzal et al. 2016). The stomach and intestine have the same basic histological structures and therefore exhibit similar primary neoplasms, which are classified as epithelial, neuroendocrine, hematopoietic mesenchymal, and non-hematopoietic mesenchymal tumors (Head et al. 2003).

Although reports of GIN cases and individualized studies of each neoplastic type are not uncommon (Patnaik et al. 1978, 1980a, 1980b, Fonda et al. 1989, La Rock & Ginn 1997, Ozaki et al. 2002, Paoloni et al. 2002, Bettini et al. 2003, Cohen et al. 2003, Frost et al. 2003, Kupanoff et al. 2006, Hayes et al. 2013), retrospective studies combining all neoplastic types of the gastrointestinal tract of dogs and address histological characteristics and immunohistochemical techniques (IHC) are still scarce (Patnaik et al. 1977, Frgelecová et al. 2014). Little is known about the epidemiological data, anatomical locations of occurrence, and behavior of these neoplasms.

In addition, non-hematopoietic mesenchymal tumors have very similar histological characteristics and until recently, due to the absence of criteria, there have been controversies in defining these neoplasms in veterinary medicine (Munday et al. 2017). This results in inconsistent classification of these neoplasms as gastrointestinal stromal tumors (GISTs), smooth muscle tumors, neural tumors, and fibroblast tumors, which have a wide variety of biological behaviors (La Rock & Ginn 1997). Human GISTs are currently recognized as a distinct tumor entity with neoplastic cells originating from the interstitial cells of Cajal and expressing c-Kit protein (Kindblom et al. 1998, Miettinen & Lasota 2001). The correct definition and identification of GISTs has become important after the introduction of Kit-selective tyrosine kinase inhibitor imatinib mesylate for the treatment of these tumors (Van Oosterom et al. 2001, Demetri et al. 2002). Since then studies have reclassified canine gastrointestinal mesenchymal neoplasms based on the human classification criteria mainly through IHC (Frost et al. 2003, Maas et al. 2007, Hayes et al. 2013) with the objective of establishing an accurate diagnosis and determining a more accurate prognosis and treatment.

This study aimed to determine the frequency and types of nonlymphoid GIN in dogs from biopsy specimens diagnosed in the Metropolitan Region of Porto Alegre and to describe their epidemiological, pathological, and immunohistochemical features.

MATERIALS AND METHODS

From January 2005 to December 2017 the biopsy files of dogs were reviewed, and cases with GIN were selected. Epidemiological aspects such as breed, age, and sex, as well as anatomical location and histopathological characteristics were analyzed and compiled. Gastric and intestinal lymphomas were excluded in the present study. The cases were mainly from the metropolitan area of Porto Alegre, Rio Grande do Sul, Brazil.

The GIN diagnoses of the present study were standardized according to the histological criteria established by the World Health Organization (Head et al. 2003) and the classification proposed by Hayes et al. (2013) for nonlymphoid and nonangiogenic gastrointestinal sarcomas. The mesenchymal neoplasms that are part of the hematopoietic system were separated into a specific category of hematopoietic neoplasms.

Additionally, histological sections of neoplasms were subjected to histopathological routine tests, stained with hematoxylin and eosin (HE), and to immunohistochemistry (IHC); the primary antibodies and the protocols used are specified in Table 1. When necessary, sections of GIN with mesenchymal origin were subjected to special histochemical staining with Masson's Trichrome (MT) and Red-Congo, to identify collagen and amyloid fibers, respectively.

RESULTS

Epidemiological and general morphological aspects

From January 2005 to December 2017, 24.711 canine biopsies were processed and evaluated, of which 14.603 had diagnoses of neoplasms. In total, 88 samples corresponded to GIN, accounting for 0.4% of the total canine biopsies and 0.6% of the total neoplasm diagnoses. GIN affected pure-bred dogs at 64.8% (57/88) and mixed breed dogs at 32.9% (29/88). In two cases, the breed was not informed. The most affected breeds were Poodle (8/88), Boxer (5/88), Dachshund (5/88), and Labrador Retriever (4/88). The median age at the time of diagnosis was 10 years with a range of 1.0-17.0 years. The dogs included 47 (53.4%) males and 39 (44.3%) females. In two cases, the sex was not informed.

Neoplasms involved the intestine in 84.1% (74/88) of the cases and the stomach in 15.9% (14/88). The large intestine (LI) was the most affected segment (67.6%, 50/74), and the small intestine (SI) corresponded to 29.7% (22/74) of cases. In two cases, the affected intestinal segment was not reported and the sample was too small to be determined by histology. Regarding biological behavior, 88.6% (78/88) of the neoplasms were malignant and 11.4% were benign (10/88).

Epithelial neoplasms corresponded to 46.6% (41/88) of the diagnoses, mesenchymal to 46.6% (41/88), hematopoietic to 5.7% (5/88), and neuroendocrine to 1.1% (1/88). The main diagnoses of nonlymphoid GIN in dogs of the present study were papillary adenocarcinoma (19.3%, 17/88), leiomyosarcoma (17.0%, 15/88), and GIST (12.5%, 11/88). The complete morphological diagnoses of canines nonlymphoid GIN are described in Table 2, along with the anatomical location and the total number of cases.

Table 1. Antibodies and immunohistochemical protocols used in canine nonlymphoid gastrointestinal neoplasms

Antibody/code	Clone	Antigenic recovery	Diluition	Detection system	Chromogen
Alpha smooth muscle actin[a] (M0851)	Monoclonal (1A4)	20 min/120°C, plus Tris-EDTA pH 9.0	1:50	MACH4[b]	AEC[a]
CD31[a] (A0082)	Monoclonal (JC70A)	3 min/125°C, plus citrate, pH 6.0	1:800	MACH4[b]	AEC[a]
CD79αcy[a] (M7051)	Monoclonal (HM57)	20 min/96°C, plus Tris-EDTA pH 9.0	1:50	MACH4[b]	AEC[a]
CD117 (c-Kit)[a] (A04502)	Polyclonal	40 min/96°C, plus citrate pH 6.0	1:300	MACH4[b]	AEC[a]
Neuron-Specific Enolase[a] (M0873)	Monoclonal (BBS/NC/VI-H14))	40 min/96°C, plus citrate, pH 6.0	1:200	MACH4[b]	AEC[a]
Pancytokeratin[a] (M3515)	Monoclonal (AE1/AE3)	3 min/125°C, plus citrate, pH 6.0	1:80	MACH4[b]	AEC[a]
S-100[a] (Z0311)	Polyclonal	20 min/96°C, plus citrate, pH 6.0	1:50	MACH4[b]	AEC[a]
Vimentin[c] (18-002)[c]	Monoclonal (V9)	3 min/125°C, plus citrate, pH 6.0	1:200	MACH4[b]	AEC[a]

Acquisition sources: [a] Dako, [b] Biocare Medical, [c] Invitrogen; MACH 4 = Universal HRP-Polymer, AEC = 3-amino-9-ethylcarbazole.

Table 2. Morphological diagnoses, location and number of cases of nonlymphoid gastrointestinal neoplasms in canines from January 2005 to December 2017

Diagnoses	Location			Total	% Total
	Stomach	SI	LI		
Epithelial					
Papillary adenocarcinoma	0	2	15	17	19.3%
Acinar/tubular adenocarcinoma	2	4	4	10	11.4%
Mucinous adenocarcinoma	1	3	2	6	6.8%
Undifferentiated adenocarcinoma	2	1	2	5	5.7%
Signet-ring cell adenocarcinoma	2	0	0	2	2.3%
Tubular adenoma	1	0	0	1	1.1%
Mesenchymal					
Leiomyosarcoma	1	4	9	15*	17.0%
Gastrointestinal stromal tumors (GIST)	0	3	7	11*	12.5%
Leyomioma	4	1	3	8	9.2%
Non-GIST/non-leiomyosarcoma	0	3	2	5	5.7%
Hemangiosarcoma	0	0	1	1	1.1%
Fibroma	0	0	1	1	1.1%
Hematopoietic					
Mast cell tumor	0	1	2	3	3.4%
Plasma cell tumor	0	0	2	2	2.3%
Neuroendocrine					
Neuroendocrine carcinoma (carcinoid)	1	0	0	1	1.1%
TOTAL	14	22	50	88*	100%

* In two cases (a leiomyosarcoma and a GIST) the affected intestinal segment was not informed and the sample was too small to be determined histologically; SI = small intestine, LI = large intestine.

Histological and immunohistochemical aspects of epithelial neoplasms

Neoplasms of epithelial origin corresponded to 46.6% (41/88) of the diagnoses of nonlymphoid GIN, of which carcinomas were the most frequently diagnosed (97.6%, 40/41). They were mainly located in the LI (57.5%, 23/40). Papillary adenocarcinoma was the main diagnostic, representing 41.5% (17/41) of epithelial neoplasms, and they mainly involved the rectum. These neoplasms were mostly characterized by papillary projections lined by multiple layers of anaplastic columnar cells (Fig.1A). They were usually limited to the mucosa and neoplastic cells were observed throughout the submucosa in three cases and throughout the muscular region in one case.

Acinar (intestine) or tubular (stomach) adenocarcinoma was the second most common epithelial neoplasm, representing 24.4% (10/41) of the cases. Histologically, they were characterized by variably sized acinar structures replacing the mucosa, arising from hypercellular crypts, and often infiltrating the submucosal, muscular, and serous layers as groups or individual acinar structures (Fig.1B). A large amount of extracellular mucinous material was observed in 14.6% (6/41) of the cases, forming multiple agglomerates that sometimes replaced the mucosa and often invaded the submucosal, muscular, and serous layers, to be classified as mucinous adenocarcinoma (Fig.1C).

In 12.1% (5/41) of the cases, neoplastic epithelial cells were arranged in a solid pattern with moderate to severe pleomorphism and without glandular differentiation; these were classified as undifferentiated carcinomas. These neoplasms presented an infiltrative character with invasion of the submucosa and muscular layers (3/5), and occasionally the serous layer (2/5).

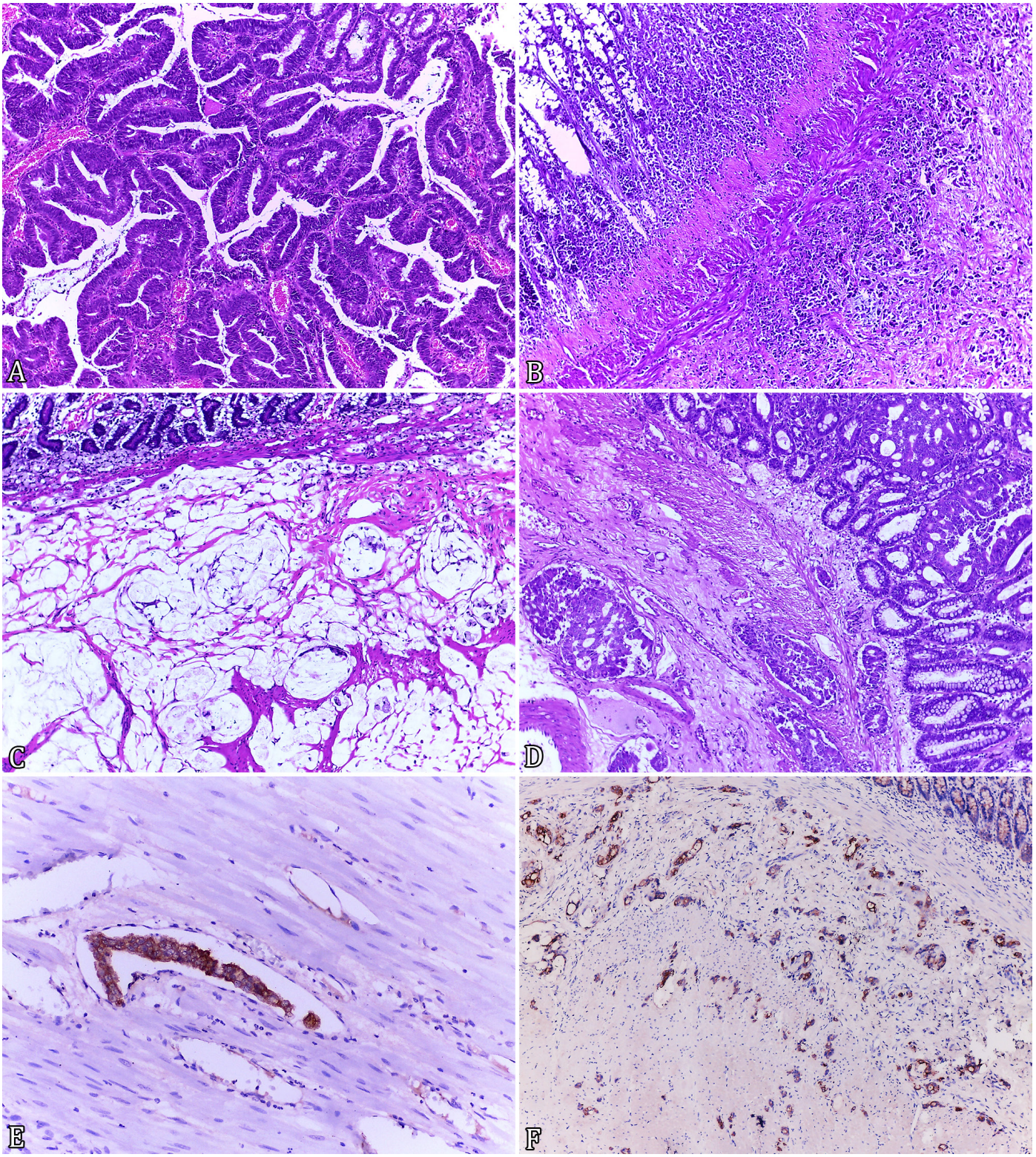

Fig.1. Epithelial gastrointestinal neoplasms. (**A**) Papillary adenocarcinoma. Neoplastic cells form multiple papillary projections that protrude into the intestinal lumen. HE, obj.20x. (**B**) Acinar adenocarcinoma. The mucosa is partially replaced with acinar structures, which invade the submucosa and muscular layers. HE, obj.10x. (**C**) Mucinous adenocarcinoma. The submucosal and muscular layers are replaced with large accumulations of extracellular mucin. HE, obj.10x. (**D**) Submucosal lymphatic vessels markedly distended and filled with neoplastic cells in an acinar adenocarcinoma. HE, obj.10x. (**E**) Acinar adenocarcinoma. Neoplastic cells evidenced inside the lymphatic vessels of the submucosa layer with staining for anti-pancytokeratin. IHC, 3-amino-9-ethylcarbazole (AEC), obj.20x. (**F**) Infiltrative neoplastic cells in an acinar adenocarcinoma highlighted in the submucosa and muscular layers by anti-pancytokeratin immunolabeling. IHC, AEC, obj.10x.

In 4.9% (2/41) of the cases, neoplasms were classified as signet-ring cell carcinoma, in which the mucosa was replaced by an abundance of epithelial cells with a large cytoplasm filled with mucinous material that displaced the nucleus peripherally. These cells infiltrated the submucosa and muscular layers.

Neoplastic cells were often observed in the lymphatic vessels in epithelial nonlymphoid GIN (68.3%, 28/41) (Fig.1D). These infiltrative cells were visualized in all neoplasms with acinar, mucinous, and signet-ring pattern, whereas only five of the 17 cases with papillary arrangement presented lymphatic invasion.

All carcinomas presented cytoplasmic or membrane immunolabeling against pancytokeratin, which varied from discrete to intense with no differences between the arrangements (Fig.1E,F).

Histological and immunohistochemical aspects of mesenchymal neoplasms

Mesenchymal neoplasms were diagnosed in 46.6% (41/88) of the nonlymphoid GIN cases. Of these, the most frequently diagnosed were leiomyosarcoma (36.6%, 15/41), GIST (26.8%, 11/41), and leiomyoma (19.5%, 8/41). Additionally, 78.0% were malignant (32/41) and 22.0% were benign (9/41).

Leiomyosarcomas (36.6%, 15/41) mainly occupied the LI (60.0%, 9/15). Of these, six were located in the cecum. Histologically, they were characterized by the proliferation of neoplastic cells, arranged in bundles in multiple directions, with high cell density and moderate to severe cellular and nuclear pleomorphism (Fig.2A,B).

Extensive areas of intratumoral necrosis, sometimes associated with hemorrhage and mineralization, were present in 46.7% (7/15) of the cases. They were primarily localized in the muscular layer and in 66.7% (10/15) they infiltrated the serosa, the submucosa in 53.4% (8/15), and extended to the mucosa in 40.0% (6/15). Invasion of neoplastic cells was occasionally observed in lymphatic vessels (20.0%, 3/15). These were characterized immunohistochemically by intense, diffuse, and intracytoplasmic labeling against smooth muscle actin (Fig.2C).

In leiomyomas (19.5%, 8/41), neoplastic cells formed a well-defined nodular structure restricted to the muscular layer. They were arranged in bundles mimicking normal smooth muscle tissues, with few pleomorphism (Fig.2D).

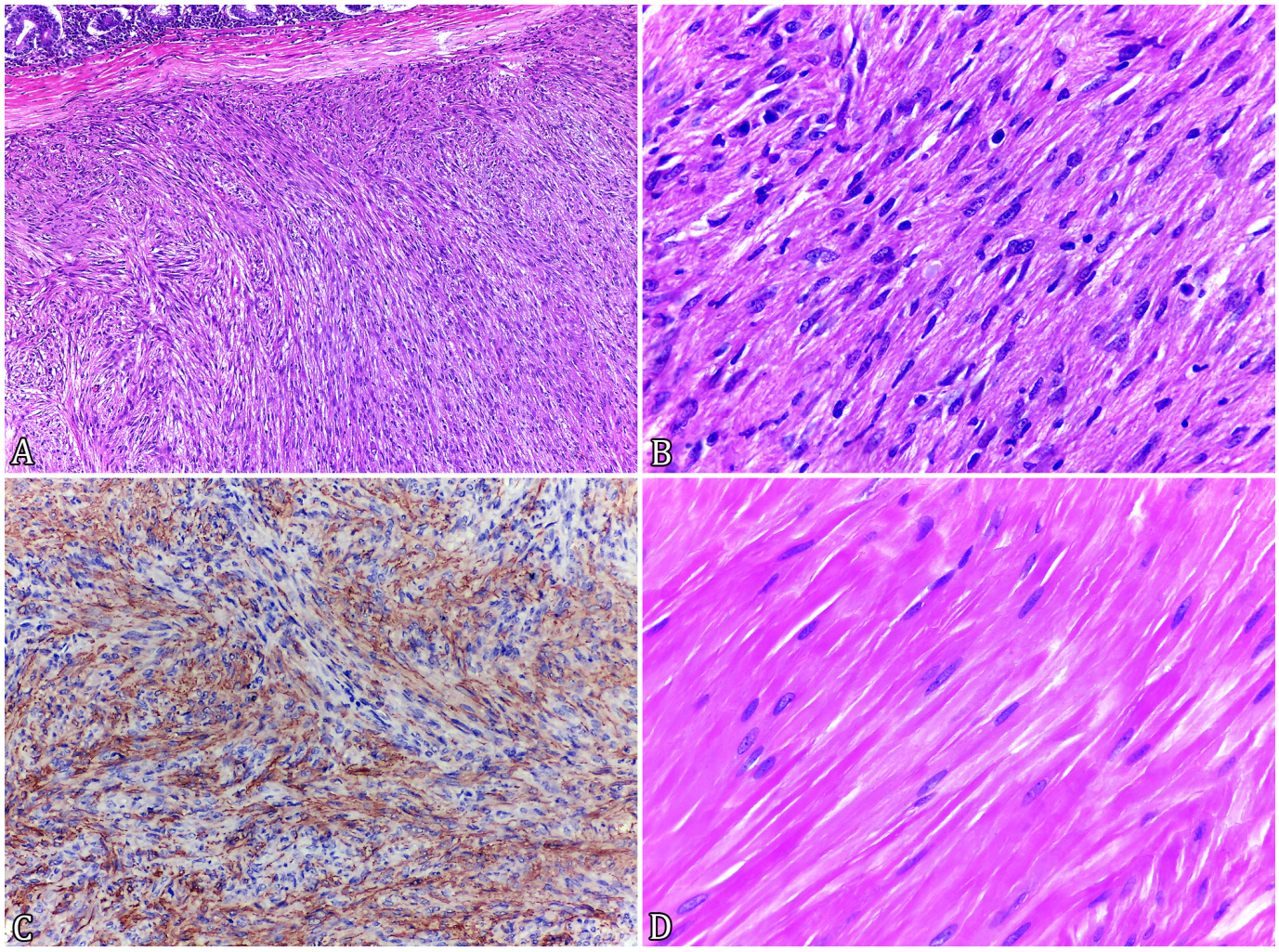

Fig.2. Mesenchymal gastrointestinal neoplasms of smooth muscle origin. (**A**) Leiomyosarcoma. Neoplastic cells are arranged in multidirectional bundles, replacing the muscular layer and infiltrating the submucosa. HE, obj.10x. (**B**) High cell density and pleomorphism in a leiomyosarcoma. HE, obj.40x. (**C**) Leiomyosarcoma. Neoplastic cells present intense immunolabeling for smooth muscle actin. IHC, 3-amino-9-ethylcarbazole (AEC), obj.20x. (**D**) Leiomyoma. Low cell density and low pleomorphism were observed. HE, obj.40x.

All cases showed intense staining on IHC for smooth muscle actin, which was predominantly diffuse and discrete with multifocal areas of moderate and intracytoplasmic marking.

GISTs (26.8%, 11/41) were mostly visualized in the LI (63.6%, 7/11), of which five were in the cecum. Histologically, 54.5% (6/11) presented a fusiform pattern (Fig.3A) and 36.4% (4/11) showed an epithelioid pattern (Fig.3B). One GIST was highly pleomorphic and had a marked number of vacuoles, and was classified as pleomorphic (Fig.3C). All GISTs were densely cellular and occasionally showed perinuclear vacuolization. Extensive areas of intratumoral necrosis, sometimes with hemorrhage, were visualized in 81.8% (9/11) of the cases. One case presented invasion of lymphatic vessels. Immunohistochemically, the neoplasms showed positive labeling for c-Kit (Fig.3D), which was intracytoplasmic (11/11) and diffuse (10/11) or multifocal (1/11), and ranged from discrete (5/11), moderate (2/11), and intense (4/11).

Five (12.2%) of the mesenchymal neoplasms were classified as non-GIST/non-leiomyosarcomas. Of these, all neoplasms showed immunoreactivity for vimentin and three were also positive for the S-100 protein; these were located in the SI and showed two different morphological patterns. Two of them were composed of mesenchymal cells arranged in bundles in different directions and were densely cellular with moderate anisocytosis and anisokaryosis. There were also extensive areas of intratumoral hemorrhage and necrosis. The other pattern was characterized by few mesenchymal cells set in a loose network of fibers with a myxoid stroma and marked anisocytosis and anisokaryosis. In all these cases, moderate and multifocal cytoplasmic immunostaining against S-100 protein was observed.

Hemangiosarcoma was diagnosed in 2.4% (1/41) of the mesenchymal neoplasms, in which neoplastic cells occasionally formed irregular vascular spaces filled with red blood cells, with solid areas. The neoplasm was located in the LI and presented multifocal and discrete immunolabeling for CD31.

Fibroma corresponded to 2.4% (1/41) of the mesenchymal neoplasms and showed well-delimited neoplastic proliferation of fusiform cells arranged in bundles in the submucosa. Immunohistochemically, the neoplasm showed discrete staining for vimentin and lack of smooth muscle actin immunolabeling. On Masson's Trichrome staining, the neoplasm showed an intense reaction, marked in blue.

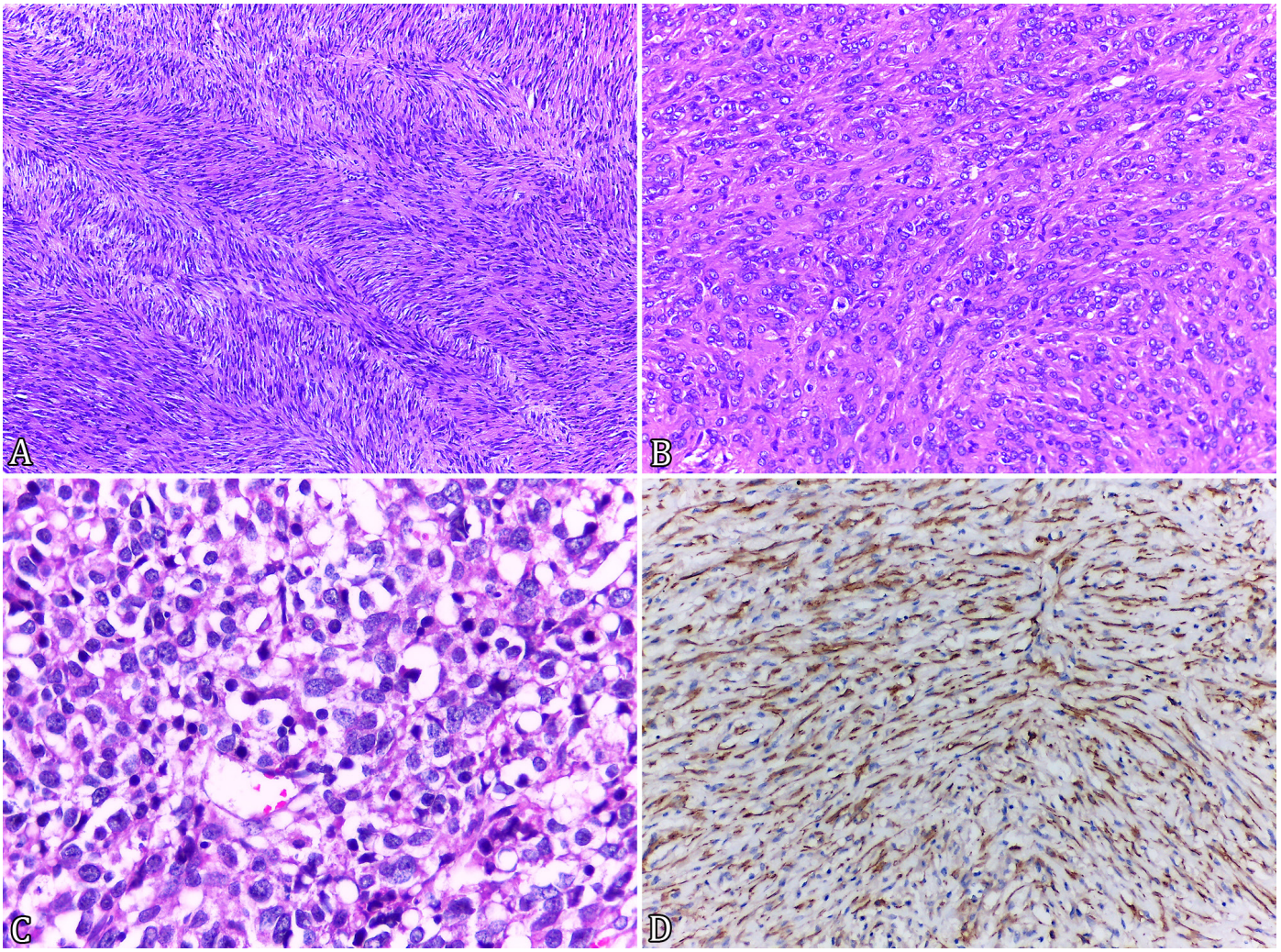

Fig.3. Mesenchymal gastrointestinal neoplasms, gastrointestinal stromal tumors (GISTs). (**A**) GIST with fusiform pattern. HE, obj.10x. (**B**) GIST, epithelioid pattern. HE, obj.20x. (**C**) GIST exhibiting high pleomorphism, with a marked quantity of vacuoles. HE, obj.40x. (**D**) Intense and diffuse IHQ anti-c-Kit staining on a fusiform GIST. IHC, 3-amino-9-ethylcarbazole (AEC), obj.20x.

Histological and immunohistochemical aspects of hematopoietic neoplasms

Hematopoietic neoplasms corresponded to 5.7% (5/88) of all nonlymphoid GIN. Of these, 80.0% (4/5) were located in the colorectal region. Sixty percent (3/5) of the cases were diagnoses of mast cell tumors and 40.0% (2/5) were plasma cell tumors.

The mast cell tumors (60.0%, 3/5) were located in the submucosa and had invaded through the mucosa, muscular, and serous layers. They were mostly characterized by round cells rarely containing intracytoplasmic granules, with moderate pleomorphism, and moderate and focal immunolabeling for intracytoplasmic c-kit (Fig.4A,B).

Plasma cell tumors (40.0%, 2/5) were characterized by round cells with broad and eosinophilic cytoplasm, eccentric nuclei, and were located in the submucosa, extending to the mucosa and muscular layers (Fig.4C). In one case, abundant deposition of amyloid was observed, which was evidenced by the histochemical stain Red-Congo (Fig.4D), and one case presented discrete and multifocal CD79αcy immunolabeling in the IHC. No invasion of lymphatic vessels was observed.

Histological and immunohistochemical aspects of neuroendocrine neoplasms

Carcinoma of neuroendocrine origin was found in 1.1% (1/88) of the cases. Neoplastic cells were characterized by poorly delimited proliferation in the submucosa and muscular layers of the stomach, formed by cells arranged in solid nests supported by thin fibrovascular stroma with moderate pleomorphism. Multifocal areas of intratumoral necrosis and mineralization were visualized. In IHC, discrete intracytoplasmic multifocal marking was observed for neuron-specific enolase.

DISCUSSION

The present study describes the epidemiological, pathological, and immunohistochemical findings of canine nonlymphoid GIN. Nonlymphoid GIN constituted 0.6% of neoplasms and 0.4% of total canine biopsies in the analyzed period, confirming the fact that these neoplasms involved an uncommon diagnosis in this species. Previous studies have shown frequencies of 0.2% to 3.0% (Patnaik et al. 1977, Penninck et al. 1998, Munday et al. 2017).

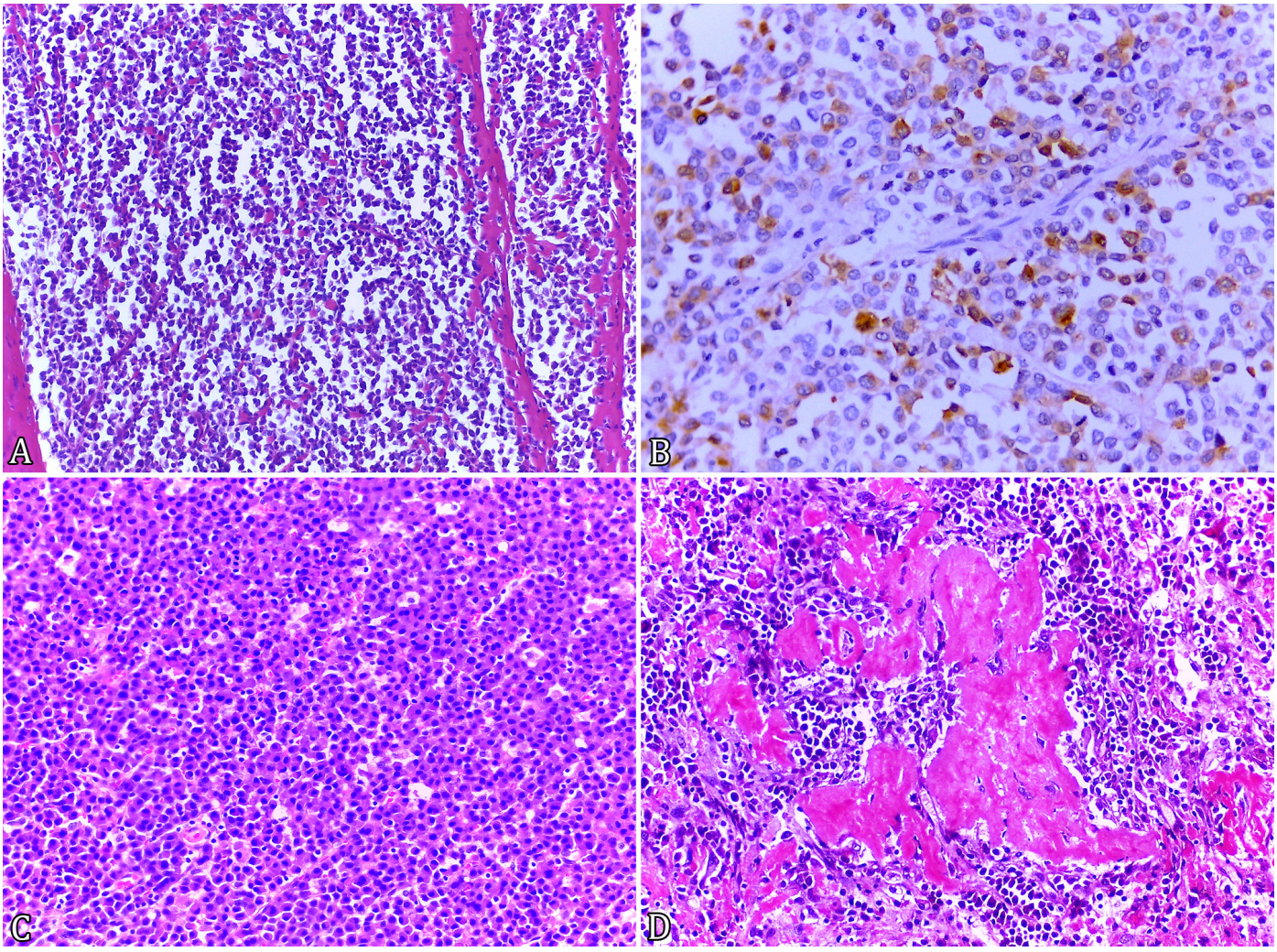

Fig.4. Hematopoietic mesenchymal neoplasms. (**A**) Mast cell tumor. Neoplastic proliferation of round cells arranged in cords infiltrating the muscular layer. HE, obj.20x (**B**) Mast cell tumor. Moderate and multifocal cytoplasmic staining for c-Kit. IHQ, 3-amino-9-ethylcarbazole (AEC), obj.20x. (**C**) Plasma cell tumor, characterized by round neoplastic cells containing broad and eosinophilic cytoplasm. HE, obj.20x. (**D**) Plasma cell tumor. Deposition of amyloid to neoplastic cells evidenced by Red-Congo histochemical staining, obj.20x.

Pure-bred dogs were more affected than non-breed dogs, as reported by other studies (Patnaik et al. 1977, Frost et al. 2003, Maas et al. 2007). The age varied from 1 to 17 years, with a median age of 10 years, which concurs with the fact that these neoplasms mostly occur in middle-aged to older dogs (Patnaik et al. 1977, LaRock & Ginn 1997, Swann & Holt 2002, Bettini et al. 2003, Russell et al. 2007, Hayes et al. 2013, Munday et al. 2017).

Although some studies have reported that the occurrence of GIN of epithelial origin is more common than that of mesenchymal origin (Bettini et al. 2003), the frequency of mesenchymal diagnoses in this study was similar to that of epithelial GIN. This shows that mesenchymal neoplasms are also important in dogs and should always be considered as a differential diagnosis in the species. However, when the diagnoses were individually analyzed, carcinomas were the most frequent, similar to those described in the literature (Patnaik et al. 1977, Head et al. 2003, Munday et al. 2017).

Both epithelial and mesenchymal neoplasms mainly involved the intestine. Although the LI comprises the smallest segment of the canine intestinal tract, it was the main site of development for both carcinomas and sarcomas. The reason for the low occurrence of neoplasms in the SI compared to the LI is still uncertain, but some hypotheses based on microenvironmental differences have been proposed in humans (Maguire & Sheahan 2018). The much quicker transit time of intestinal contents in the SI than in the LI reduces mucosal exposure to carcinogenic agents, and the concentration of these is diluted in the largest volume of secretions (Lowenfels 1973, Crawshaw et al. 1998). Furthermore, the bacterial population in the SI is smaller and metabolically inactive, and therefore may not be able to transform pre-cancerous substances into carcinogens (Lowenfels 1973, Pan & Morrison 2011).

Malignant neoplasms were the most frequent observed in this study. Researchers report that malignant presentation is considered more common in the gastrointestinal tract compared to benign presentation (Patnaik et al. 1977). Regardless of cellular origin, benign tumors are differentiated from carcinomas and sarcomas based on morphologic features such as increased cellularity with a high nucleus/cytoplasm ratio, anisocytosis and anisokaryosis, abundant or atypical mitosis figures, and the presence of invasion or metastasis (Gillespie et al. 2011).

The benign neoplasms corresponded almost entirely to neoplasms of smooth muscle origin. The leiomyomas equally involved the stomach and intestine, diverging from what is mentioned in literature, where authors report that the most common site of this neoplasm is the stomach (Patnaik et al. 1977, Bettini et al. 2003, Munday et al. 2017). However, considering that biopsy samples were used in this study and that leiomyomas usually do not lead to clinical signs and are not surgically removed, the number of gastric leiomyomas was probably underestimated in this study. Benign neoplasms such as fibroids and adenomas are rarely reported in dogs (Head et al. 2003, Munday et al. 2017). As in humans, adenomas are considered pre-malignant lesions, and it can be speculated that adenomas occur in dogs much more frequently than is recognized, but are detected only when their transformation to adenocarcinoma has occurred, resulting in clinical disease (Munday et al. 2017).

Adenocarcinomas are the most common intestinal neoplasms in dogs (Head et al. 2003, Uzal et al. 2016). In this study, these neoplasms corresponded to half of the diagnoses in the stomach, and almost half of the diagnoses in the intestine, occurring predominantly in the rectum. These results contrast with those of other authors who mention that adenocarcinomas occur predominantly in the SI, mainly in the duodenum and jejunum (Crawshaw et al. 1998, Paoloni et al. 2002, Munday et al. 2017).

Adenocarcinomas are often in an advanced stage of evolution due to late diagnosis, and already occupy deeper layers of the stomach and intestine (Munday et al. 2017), which explains the large number of cases with intestinal layer infiltration and lymphatic invasion in epithelial neoplasms in this study. In humans, the type of arrangement seems to be involved in the chance of metastases, since neoplasms that project to the lumen have much smaller capacity for lymphatic invasion and metastasis than those that grow first toward the gastric or intestinal wall (Goldblum 2018). In this study, of the 17 papillary adenocarcinomas only five infiltrated the deeper layers and lymphatic vessels, whereas those with an acinar, mucinous, signet ring, and undifferentiated arrangement often invaded and replaced the intestinal layers, and all invaded the lymphatic vessels. Therefore, neoplasms in these arrangements may present a greater invasive potential compared to that of the papillary arrangement.

All carcinomas showed immunostaining for pancytokeratin. IHC for pancytokeratin is an important diagnostic tool to confirm an epithelial origin, especially in cases where neoplasms are undifferentiated, as well as to facilitate the identification of infiltrative cells in deeper layers or to highlight small numbers of neoplastic cells in small biopsy samples (Munday et al. 2017).

Leiomyosarcomas corresponded to the second most common neoplasm and to the main sarcoma. They were mainly located in the LI, which differs from the observation in some studies that consider these neoplasms rare in the intestine (Bettini et al. 2003) and common in the stomach (Russell et al. 2007). Another study describes the cecum as the main localization of leiomyosarcomas (Cohen et al. 2003). Leiomyosarcomas have slow growth and often have areas of necrosis and hemorrhage (Munday et al. 2017), as observed in this study.

Leiomyosarcomas often resemble GISTs in histopathology, especially when GISTs have a fusiform pattern. Therefore, IHC is indicated for a more definite diagnosis of mesenchymal gastrointestinal neoplasms (Munday et al. 2017). Immunostaining for c-Kit defines the diagnosis of GIST, whereas lack of c-Kit expression and immunolabeling for smooth muscle actin defines a leiomyosarcoma (Maas et al. 2007, Hayes et al. 2013). However, IHC reactivity for smooth muscle actin alone should not be used for a definitive diagnosis for leiomyosarcoma, because a part of GISTs may also express smooth muscle markers and neural origin (Bettini et al. 2003, Frost et al. 2003, Maas et al. 2007, Hayes et al. 2013).

Prior studies on the identification of GISTs consider that the most common nonlymphoid mesenchymal neoplasms of the gastrointestinal tract in dogs are neoplasms of smooth muscle origin (Patnaik et al. 1977, Birchard 1986, LaRock & Ginn 1997, Crawshaw et al. 1998). However, after the discovery of c-Kit expression by GISTs, some studies have reclassified the tumors using IHC, and have demonstrated that the

majority of these tumors corresponded to GISTs (Maas et al. 2007, Russell et al. 2007, Hayes et al. 2013) and that these leiomyosarcoma diagnoses are overestimated. However, in the present study, despite the use of IHC, leiomyosarcomas were more frequent, which corroborates with the findings in literature (Patnaik et al. 1977, Birchard 1986, LaRock & Ginn 1997, Crawshaw et al. 1998).

GISTs comprised the second most frequent mesenchymal neoplasm, different from that described by some authors (Bettini et al. 2003, Maas et al. 2007, Hayes et al. 2013). In humans, GISTs are more common in the stomach (Miettinen & Lasota 2001) whereas in dogs, GISTs are found preferentially in the intestine with occasional reports in the stomach (Bettini et al. 2003, Frost et al. 2003, Maas et al. 2007, Gillespie et al. 2011, Hayes et al. 2013). These neoplasms can be classified histologically based on four morphological patterns, as described in humans: fusiform, myxoid, fascicular, and epithelioid (Head et al. 2003, Miettinen & Lasota 2003). Only the fusiform and epithelioid types are described in dogs, and the fusiform pattern is reported as the most common (Hayes et al. 2013), as observed in this study. Additionally, perinuclear vacuolization is a common feature of GISTs and is occasionally very prominent (Miettinen & Lasota 2003), and may be considered as a factor to histologically differentiate them from other sarcomas.

Five neoplasms were classified as non-GIST/non-leiomyosarcoma based on the lack of expression for c-Kit and smooth muscle actin. Immunolabeling for vimentin was observed, similar to that found by other authors (Russell et al. 2007, Hayes et al. 2013). In addition, three cases were also immunoreactive for the S-100 protein, and had a histological pattern of peripheral nerve sheath tumors, and were reminiscent of an Antoni A and/or Antoni B pattern. However, as a neurogenic origin of these neoplasms often cannot be confirmed, it is currently suggested that they be included in the non-GIST/non-leiomyosarcoma group, with S-100 immunostaining (Hayes et al. 2013). Although relatively common in other locations, nerve sheath tumors are rarely described in the gastrointestinal tract of dogs (Schöniger & Summers 2009).

Mast cell tumors are uncommon in dogs and are generally more aggressive than their cutaneous counterparts (Patnaik et al. 1980b, Munday et al. 2017). They may involve any intestinal segment, but are more common in the SI (Head et al. 2003). When cytoplasmic granules are not easily visualized, c-Kit immunohistochemistry may aid in their diagnosis (Reguera et al. 2000, Ozaki et al. 2002).

Extramedullary plasma cell tumors located in the gastrointestinal tract are rare in dogs and are found more frequently in the colorectal region (Ramos-Vara et al. 1998, Head et al. 2003). Histologically, they are easily recognized as tumors of round cells with eosinophilic cytoplasm and eccentric nuclei (Kupanoff et al. 2006, Munday et al. 2017); however, anti-CD79αcy IHC for B lymphocytes can be used as an auxiliary diagnostic method when there is little differentiation (Ramos-Vara et al. 1998). Although uncommon, plasma cell tumors may show amyloid deposition (Rowland et al. 1991, Kupanoff et al. 2006). A neoplasm in this study did not show reactivity for CD7αcy, probably due to the prolonged fixation time of the sample, but the cellular morphology along with amyloid deposition evidenced by red-Congo staining was considered sufficient for the diagnosis. Most neoplasms tend to be restricted to the submucosa, but some plasma cell tumors exhibit a more aggressive behavior including invasion of the muscular tunica (Uzal et al. 2016), as observed.

Neuroendocrine neoplasms originate from gastrointestinal enteroendocrine cells and are very uncommon in the gastrointestinal tract (Munday et al. 2017). Although there are few reports, these tumors show an aggressive behavior. In one study evaluating four intestinal carcinoids, all cases had distant metastases (Patnaik et al. 1980a). Use of cell morphology is not indicated to evaluate prognosis, because despite the malignant behavior that these neoplasms often demonstrate, they present cellular atypia and high mitotic indexes only in some cases (Munday et al. 2017). To confirm the neuroendocrine origin, IHC for chromogranin A, neuron-specific enolase, synaptophysin, or PGP 9.5 is indicated (Head et al. 2003, Sako et al. 2003). In the present study, labeling for neuron-specific enolase was sufficient to confirm the diagnosis.

CONCLUSIONS

Nonlymphoid gastrointestinal neoplasms occurred mainly in middle-aged to older dogs, mostly purebred, and male. They affected the intestine more than the stomach, especially the LI. The rectum was the main site for the development of carcinomas whereas the cecum was the main site for sarcomas. The stomach was an important site for the development of smooth muscle neoplasms, mainly leiomyomas.

Malignant neoplasms were more common, and the main neoplasm observed was adenocarcinoma, followed by leiomyosarcoma and GIST. Epithelial neoplasms showed a greater potential for lymphatic invasion whereas mesenchymal cells appeared to be more expansive with intratumoral necrosis and hemorrhage.

Immunohistochemistry proved to be an important tool for confirming the cellular type involved in gastrointestinal neoplasms, as well as for the identification of infiltrating neoplastic cells in the case of carcinomas, and an indispensable technique for the definitive diagnosis of sarcomas.

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Original Article
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Plasma cholinesterase activity as an environmental impact biomarker in juvenile green turtles (*Chelonia mydas*)[1]

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ABSTRACT.- Fonseca L.A., Orozco A.M.O., Souto P.C., Dornelas L.R.S., Filho W.P.C., Girardi F.M., Ermita P.A.N. & Fagundes V. 2020. **Plasma cholinesterase activity as an environmental impact biomarker in juvenile green turtles (*Chelonia mydas*).** *Pesquisa Veterinária Brasileira 40(1):72-76.* Departamento de Veterinária, Universidade Federal de Viçosa, Av. Peter Henry Rolfs s/n, Campus Universitário, Viçosa, MG 36570-900, Brazil. E-mail: leandroabreu@ufv.br

The objective of this study was to evaluate the enzymatic activity of plasma cholinesterase in *Chelonia mydas* marine turtles belonging to two populations, according to their capture sites, under the absence and probable influence of anthropic effects. A total of 74 animals were used and later divided into two groups, based on the capture site. Blood samples were collected from all captured animals, which were then released into the sea at the site of capture. A descriptive statistical analysis of the plasma cholinesterase activity values and an analysis comparing these values based on the capture site were performed. Samples of heparinized plasma from animals captured at the two different sites were analyzed. Plasma cholinesterase activity ranged from 121 to 248U/L, with a mean and standard deviation of 186.1±30.68U/L. When comparing plasma cholinesterase activity values in individuals based on the capture site, a significant difference was observed. Establishing reference values for different sea turtle populations is necessary to interpret future sampling results and to allow sea turtles to be used as sentinels of ecosystem health. Future studies are needed to evaluate other populations and the activity of plasma cholinesterase in juvenile marine turtles, in relation to environmental contamination.

INDEX TERMS: Plasma cholinesterase, environmental impact, biomarker, green turtles, *Chelonia mydas*, cholinesterase, marine turtle, biochemical markers, turtles, wildlife animals.

RESUMO.- [**Atividade da colinesterase plasmática como biomarcador de impacto ambiental em tartarugas verdes juvenis (*Chelonia mydas*).**] O objetivo desse estudo foi avaliar a atividade enzimática da colinesterase plasmática em tartarugas marinhas da espécie *Chelonia mydas* em duas populações de acordo com o local de captura, sob ausência e provável influência de efeito antrópico. Foi utilizado um total de 74 animais e posteriormente divididos em dois grupos de acordo com o local de captura. Foram coletadas amostras de sangue de todos os animais capturados e em seguida liberados ao mar no mesmo local. Foi realizada uma análise estatística descritiva dos valores da atividade plasmática de colinesterase do total de animais e análise comparando os valores de acordo com o local de captura. Foram analisadas amostras de plasma heparinizado de animais capturados em dois locais distintos. Os valores da atividade plasmática de colinesterase variaram de 121 a 248U/L, com média e desvio padrão de 186.1±30.7U/L. Quando comparados os valores de atividade plasmática da colinesterase nos indivíduos de acordo com o local de captura, foi observada diferença significativa. O estabelecimento de valores de referência para diferentes populações de tartarugas marinhas são necessários para

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interpretar os futuros resultados amostrais e permitir que as tartarugas marinhas sejam usadas como sentinelas da saúde do ecossistema. Estudos futuros são necessários para avaliar outras populações e a atividade da colinesterase plasmática de tartarugas marinhas juvenis em relação à contaminação ambiental.

TERMOS DE INDEXAÇÃO: Colinesterase plasmática, biomarcador, impacto ambiental, tartarugas verdes, *Chelonia mydas*, tartaruga marinha, marcadores bioquímicos, animais selvagens.

INTRODUCTION

With modernization, industrial and urban development has been expanding, which brings important environmental impacts. In light of this, it is necessary to control the emission of pollutants, since they can compromise water resources, which are of great importance to ecosystems (Freitas 2009). The production of potential synthetic compounds from industrial, agricultural and domestic activities is responsible for much of the contamination of the waters that reach rivers and oceans. Accordingly, a multitude of chemical products in various concentrations (Freire et al. 2008) accumulate in animals living in marine environments (Coito et al. 2007).

Monitoring the aquatic environment is necessary. For this, biomarkers have been used to identify the presence of contaminating agents and their interaction with the organisms (Sarkar et al. 2006) that use these environments, in a predictive manner, thus avoiding the occurrence of irreversible environmental damage (Cajaraville et al. 2000). Some environmental monitoring programs have used the inhibition of acetylcholinesterase enzymatic activity in fish and other animals as a biomarker indicative of environmental contamination by organophosphates and carbamates (Oliveira et al. 2007).

Cholinesterases are enzymes that are responsible for the hydrolysis of choline esters. There are two types of cholinesterases: I) acetylcholinesterase (AChE) or erythrocyte cholinesterase, which is most concentrated in the central nervous system, striated muscle and erythrocyte membrane; and II) butyrylcholinesterase (BChE) or plasma cholinesterase, which is most abundant in plasma (Kramer & Hoffmann 1997). According to other studies, acetylcholinesterase may also be inhibited by heavy metals and surfactants (Coito et al. 2007).

According to Fonseca et al. (2015),marine turtles of the *Chelonia mydas* species can be used as sentinels of exposure to pollutants that inhibit the enzymatic activity of plasma cholinesterase, since these animals have detectable activities of this enzyme. However, these authors suggest evaluating the enzymatic activity in this species under different conditions and exposures, both *in situ* via comparison between sites and *ex situ* through bioassays.

The objective of this study was to evaluate the enzymatic activity of plasma cholinesterase in marine turtles belonging to the *C. mydas* species; these were in two populations, according to their capture sites, under the absence and probable influence of anthropic effects.

MATERIALS AND METHODS

Experimental design. A total of 74 animals were used in our study and later divided into two groups based on their capture sites: G1 and G2. All captured animals were banded; biometric data and blood were collected immediately after capture. The animals were then released into the sea at the same site. The animals were manually captured at low tide by fishing, using a hand net, or diving, in strategic locations and monitored by GPS. Licenses for the collection, transport and handling of biological material were granted by the Biodiversity Information and Authorization System (SISBIO)/ICMBio - IBAMA, under registration number 26080.

A descriptive statistical analysis of the plasma cholinesterase activity values of the animals and an analysis comparing these values based on the capture sites were performed. The G1 comprised 35 individuals captured in the Bay of Sueste in the Archipelago of Fernando de Noronha, state of Pernambuco (3°51´26" S and 32°25´33" W), Brazil. The region is characterized as an important feeding and spawning area of *Chelonia mydas* (Bellini et al. 1996, Baptistotte 2007). Because the animals were in the open sea, the manual capture involved continuous, prolonged chases and required diving and pursuit durations of over 60 minutes until the individuals could be captured and transported to the beach. During this pursuit, the capture was realized when the individuals, worn out by the fugue, rose to the surface to breathe and could not escape the capture. Following the capture, the animals were transported to the beach for blood collection, banding, biometry measurements, and subsequent release.

The G2 comprised 39 individuals captured from the final effluent of a steelworks company in the city of Vitória, state of Espírito Santo (20°15'49" S and 40°13'43" W), Brazil. In this region, seawater is collected and used for cooling in industrial operations, thus causing an increase in the local average temperature of the water, in addition to receiving domestic and industrial sewage after treatment. The high water temperature and the richness of organic matter favor the growth of algae. According to Torezani et al. (2010), these conditions lead to noticeable growth, mainly of green algae *Enteromorpha flexuosa*, but also of *Pterocladiella* sp., *Jania* sp., *Arthrocardia* sp. and *Chaetomorpha* sp. Four capture points were previously defined on both sides of the effluent channel. This effluent channel is 500m long and approximately 33m wide, with an average depth of 2m.

Table 1. Characteristics of study groups (G1 and G2) of the present study

Criteria	G1	G2
Capture and manipulation time	Average over 60 minutes	Average less than 60 minutes
Average water temperature	28°C[a] (82.4°F)	22°C (71.6°F), increased by 8.75°C[b] (47.8°F)
Capture method	Diving with continuous pursuit	Fishing hand net
Number of animals	35	39
Site	Fernando de Noronha/PE	Vitória/ES

[a] Accoding to Mendes (2006), [b] According to Torezani (2010).

As the animals were aggregated in the effluent channel, and by the use of fishing hand nets, the process involved surprising the animals without persecution (Table 1).

The areas used in our study were chosen and characterized using the information described by Dos Santos et al. (2010), who evaluated the environmental quality of both localities using the ecological evaluation index (EEI) (Orfanidis et al. 2001, 2003) and concluded that the area used in our study to capture individuals from G1 was classified as of good quality, in contrast to the area used to capture individuals from G2 that was characterized as of bad quality.

Collection of blood and biometric data. From all the captured individuals, carapace size data (in centimeters); curvilinear carapace length (CCL) and weight (in grams) were obtained to characterize the age of the animal, and blood was collected. Recaptured animals were evaluated, measured, weighed, and released without further blood collection. The sex of the animals was not determined due to the absence of external sexual dimorphism at this stage of the life cycle. Peripheral blood collection was performed by venipuncture of the cervical sinus or jugular vein, preceded by adequate cleaning and antisepsis with iodinated alcohol. Blood was immediately transferred to a 2ml heparin-containing tube (Vacutainer®) and was kept cool in an ice container until arrival at the laboratory.

Sample processing. In the laboratory, heparin tube-conditioned blood was centrifuged for 10 minutes at 4000rpm, and the obtained plasma was transferred to 1.7ml microtubes for cholinesterase dosing. Heparinized plasma was frozen at -20°C (68°F) until the samples were processed. To measure the cholinesterase enzyme activity, we followed the method of Ellman et al. (1961), modified by Sturm et al. (1999), which allows for the quantification of enzyme activity in plasma samples (Fonseca et al. 2015). The determination of cholinesterase plasma activity was performed using the kinetic enzymatic method. The reaction occurs due to the catalytic action of cholinesterase on the hydrolysis of butyrylthiocholine to thiocoline and butyrate. Thus, thiocholine reduces hexacyanoferrate III (which is yellow in color) to hexacyanoferrate II (colorless). The decrease in absorbance was measured at 405nm and represents the intensity of the enzymatic activity. For this dosage, the test had a minimum detection limit of 50U/L and the reaction was linear up to 25000U/L.

Statistical analysis. The results were submitted to descriptive analysis to obtain means and standard deviations. The symmetry of the data distribution was evaluated using a Shapiro-Wilk test, and the homogeneity of the variances was evaluated using a Levene's test. For the determination of the reference range, the outliers were detected and discarded, according to the Tukey procedure, where a result is defined as extreme when its value is less than the first quartile minus 1.5 times the interquartile range, or greater than the third highest quartile plus 1.5 times the interquartile range, similar to that used by Friedberg et al. (2007). After the outliers were removed, the 95% confidence interval was obtained. The procedure was performed using SPSS 20 software (IBM SPSS Statistics). The influence of the capture site on plasma cholinesterase levels was assessed using a Student's t-test for independent samples. The statistical package Minitab 17 (Minitab Inc.) was used and significance was considered when $p<0.05$.

RESULTS

All captured animals were juveniles, according to the criteria proposed by Hirth (1971), i.e., curvilinear carapace length (CCL) was less than 73.5cm. The animals presented CCLs ranging from 28.4 to 56.3cm, with a mean and standard deviation of 51.8±11.3cm.

Heparinized plasma samples from the 74 animals captured from two different sites were analyzed. All analyzed samples showed some enzymatic activity of plasma cholinesterase. Plasma cholinesterase activity ranged from 121 to 258U/L, with a mean and standard deviation of 186.1±30.7U/L. The results of the descriptive statistical analysis of plasma cholinesterase activity are presented in Table 2 and a graphical representation is presented in Figure 1. When comparing plasma cholinesterase activity values between individuals according to the capture site, a significant difference was observed.

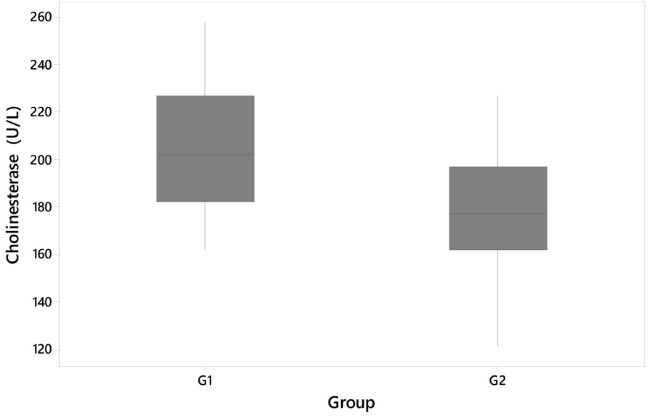

Fig.1. Graphical representation for cholinesterase activity (U/L) in green turtle (*Chelonia mydas*) from Brazil.

Table 2. Descriptive statistics for cholinesterase activity (U/L) in green turtle (*Chelonia mydas*) from Brazil

Statistic	Plasma cholinesteraseactivity (U/L)		
	Total	Group 1	Group 2
Number of samples	74	35	39
Mean + SD	186.1 + 30.7	178.2 ± 26.9[a]	194.1 ± 32.5[b]
95% Lower confidence interval	178.8	200.1	170.3
95% Upper confidence interval	193.4	232.7	186.7
Minimum	121	162	121
Maximum	258	258	227

SD = Standard deviation; [a,b] Different letters on the same line demonstrate significant statistical difference.

DISCUSSION

In veterinary medicine, the determination of reference biochemical values is indispensable for the monitoring of the health status of individuals of different species. Some of these parameters can be used as biomarkers, and according to Atkinson Junior et al. (2001), their measurement serves as an indicator of a normal biological process, pathogenic, or in response to a therapeutic intervention. In this study, the method used to measure cholinesterase activity was the same as that used by Fonseca et al. (2015) and all animals in the study had some type of activity for this enzyme.

As the animals in the G1 were captured in an environmental conservation unit and did not present any evidence that could directly or indirectly interfere with plasma cholinesterase activity, the data obtained in our study can be proposed as a reference for this site for future studies of *Chelonia mydas* juveniles, as there is no data in the current literature for the studied species and age group.

Animals are often exposed to toxins of various origins. The evaluation of biomarkers permits the evaluation of an individual's exposure to these substances and their health effects on the body (Myers et al. 2017). The difference in the values found between the groups in our study demonstrates that the activity of this enzyme can vary according to the environmental conditions in which the animals live.

Cholinesterase activity has been used as an indicator of environmental contamination in several species and situations. In dogs, the inhibition of cholinesterase activity is an indicator of exposure to organophosphorus toxins (Ferré et al. 2015) and carbamates (Saldeña et al. 2017). In birds, studies have evaluated the activity of this enzyme. In northeast Mexico, cholinesterase activity decreased by 29-49% because of possible exposure to pestivcides (Ruvalcaba-Ortega et al. 2017). In Spain, Oropesa et al. (2017) observed a decrease in plasma cholinesterase activity under *in vitro* conditions in samples of griffon vultures (*Gyps fulvus*) exposed to carbamates, suggesting that it is a suitable biomarker for monitoring the exposure of this toxin.

Similarly, in aquatic environments, the enzymatic activity of cholinesterase has been studied as an indication of environmental contamination (Oliveira et al. 2007). Accordingly, some aquatic species can be considered sentinels. According to the results obtained by Coito et al. (2007), marine sponges belonging to the species *Spongia officinalis* and *Spongia agaricina* show cholinesterase activity and can be used as biomarkers of pesticide exposure. Omar-Ali et al. (2017) found a significant decrease in plasma acetylcholinesterase activity in *Atractosteus spatula* (alligator gar) under chronic exposure to organophosphate pesticide (diazinon). However, in our study, no evidence of environmental contamination by pesticides was measured.

However, plasma cholinesterase activity may vary as a function of exposure to other toxic compounds, depending on the exposure period. When studying the effect of exposure to iron on acetylcholinesterase (AChE) enzyme activity in fish brains and livers, Sant'Anna et al. (2011) observed an increase of this enzyme 24 hours after exposure. Carvalho et al. (2017) obtained similar results when they evaluated the activity of this enzyme in the brain of tadpoles after 48 hours of exposure to different types of heavy metals. However, after 16 days, a significant decrease of the enzymatic activity was demonstrated, which suggests that AChE can be used as a predictive biomarker in heavy metal-contaminated environments.

Environmental contamination by iron ore is an old, widely-discussed problem in the locality in which the G2 resides (Pinheiro 2013). In addition, as already mentioned, the locality of G2 also has an elevated water temperature and the presence of abundant organic matter of algae. In our study, animals from the G2 showed an increase in enzymatic activity, when compared to those of the G1. This difference may suggest an anthropogenic influence on the habitat in which the G2 inhabited. Thus, among some possibilities, it can be assumed that environmental contamination by iron ore, a problem experienced for years by the local population (Nassar et al. 2003), is a possible cause of the elevation of enzymatic activity. However, although analyses of the composition of the local water are carried out periodically, the results were not available in full. Further studies on the composition of the heavy metals present in this water and the time the animals are exposed to the toxics present in the effluent need to be performed.

Das (2007) and Silva et al. (2012) report that acetylcholinesterase and butyrylcholinesterase can be considered biomarkers of low-grade inflammation in various clinical conditions. This is because acetylcholinesterase and butyrylcholinesterase reduce the levels of acetylcholine, a molecule that plays an anti-inflammatory role locally and systemically by inhibiting the production of tumor necrosis factor, interleukin-1, inhibitory macrophage migration factor, and other proinflammatory cytokines. Thus, with increasing levels of acetylcholinesterase and butyrylcholinesterase, there is an increase in inflammatory events in the body.

Finally, according to Fonseca et al. (2015), because of the longevity of *C. mydas* sea turtles and their herbivorous feeding behavior, together with the fact that they have detectable plasma cholinesterase activity, this species can be proposed as a bioindicator of exposure to pollutants that influence the activity of this enzyme, especially in environments influenced by industrial activities. For this, it is necessary to evaluate enzymatic activity under different conditions and exposures, both *in situ* by comparison between sites and *ex situ* through bioassays. The possibility of using blood samples permits the development of studies that use minimally invasive techniques and minimize the ethical considerations of animal use in research.

CONCLUSIONS

In the present study, significant differences in plasma cholinesterase activity were observed among the evaluated populations.

Group 2 turtles showed higher activity of AChE in relation to Group 1; this could be attributed to an altered state caused by the anthropic effect on the habitat of these animals.

Establishing reference values for different sea turtle populations is necessary to interpret future sampling results and allows sea turtles to be used as sentinels of ecosystem health.

Future studies are necessary to evaluate other populations and the effect of different toxins on plasma cholinesterase activity in juvenile sea turtles.

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Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

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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.
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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".

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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.

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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.

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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.

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3. **Apresentação das Figuras:**

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

b) Enviar cada figura separadamente.

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

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

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

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

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

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

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

Exemplos de Referências

➢ Artigos publicados em periódicos:

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

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

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

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

- Livros:

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

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

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

- Capítulos de livros:

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

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

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

- Dissertações e Teses:

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

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

- Resumos publicados em eventos:

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

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

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

Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

GUIDE FOR AUTHORS

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

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

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

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

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

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

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

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

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

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

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

The author rights of the accepted papers are preserved.

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

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

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

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

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

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

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

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

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

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

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

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

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

m) **Conflict of interest or none** should be mentioned.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3. **Figure presentation:**

a) Save images at 300 dpi, TIF files.

b) Send each figure separately.

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

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

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

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

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

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

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

Exemples for References:

➢ Articles published in scientific journals:

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

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

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

➢ Books:

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

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

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

➢ Chapters of books:

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

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

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

➢ Dissertations and Theses:

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

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

➢ Abstracts published in Events:

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

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(Note: Consult entire papers instead of only Abstracts)

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