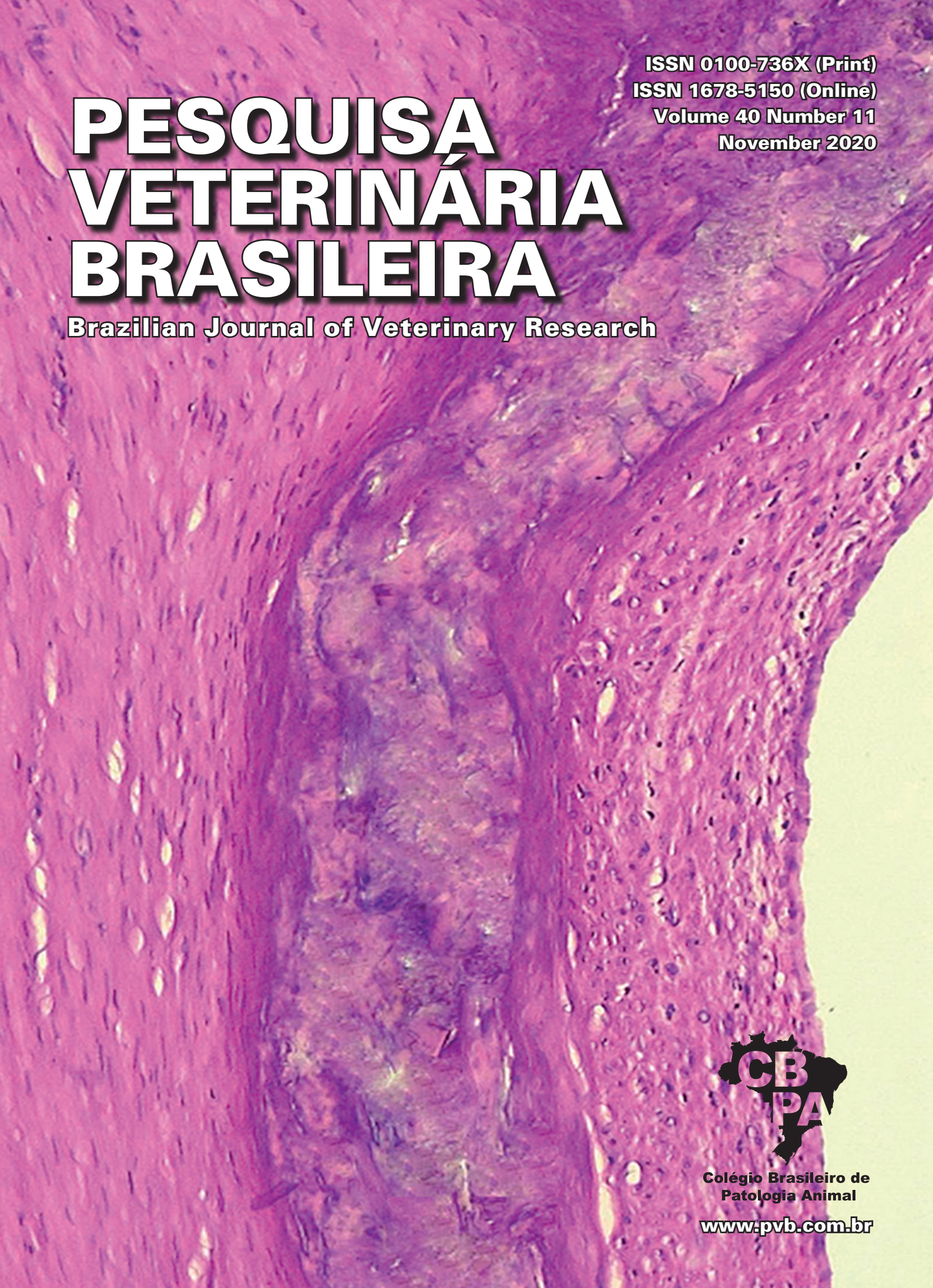
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Cover illustration: Tunica media mineralization and intimal hyperplasia in the carotid artery of a sheep poisoned by Nierembergia rivularis (Machado et al., pag. 834).

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Original Article
Livestock Diseases
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Enzootic calcinosis in sheep in Uruguay: a brief review and report of two outbreaks[1]

Mizael Machado[2], Carlos O. Schild[2], Marcela Preliasco[3], Aldana Balserini[4], Rosane M.T. Medeiros[5], Severo S. Barros[6] and Franklin Riet-Correa[2,7]*

ABSTRACT.- Machado M., Schild C.O., Preliasco M., Balserini A., Medeiros R.M.T., Barros S.S. & Riet-Correa F. 2020. **Enzootic calcinosis in sheep in Uruguay: a brief review and report of two outbreaks.** *Pesquisa Veterinária Brasileira 40(11):831-836*. Instituto Nacional de Investigación Agropecuaria, Plataforma de Salud Animal, Estación Experimental INIA Tacuarembó, Ruta 5 Km 386, Tacuarembó, Uruguay. E-mail: franklinrietcorrea@gmail.com

This study aimed to do a brief review of enzootic calcinosis in sheep and to report two outbreaks of *Nierembergia rivularis* poisoning in sheep in Uruguay. The outbreaks occurred in farms located on an island (Outbreak A) and on the border (Outbreak B) of the Rincón del Bonete lake. Sheep of all ages were affected, with the exception of suckling lambs. The first clinical signs occurred in early October, and deaths occurred from December to February. Outbreaks A and B had morbidity of 10%, and the mortality was 7.2% and 2.8% in Outbreaks A and B, respectively. The clinical signs included weight loss, retracted abdomen, stiff gait, and kyphosis. An autopsy was performed on one sheep from each outbreak. Pulmonary and arterial calcification, nephrocalcinosis, and osteopetrosis were observed in gross and microscopic examination in both sheep. Thyroid C-cell hyperplasia and carcinoma was observed in sheep A. Sheep B showed thyroid C-cell hyperplasia and parathyroid chief cell atrophy. The parathyroid was not examined in the sheep from Outbreak A. The differential diagnosis of enzootic calcinosis in southern South America should consider four toxic plants in the Solanaceae family: *Solanum glaucophyllum, Solanum stuckertii, Nierembergia veitchii*, and *Nierembergia rivularis*.

INDEX TERMS: Sheep diseases, enzootic calcinosis, *Nierembergia*, Uruguay.

RESUMO.- [**Calcinose enzoótica em ovinos no Uruguai: revisão de literatura e descrição de dois surtos.**] Este trabalho faz uma breve revisão da calcinose enzoótica em ovinos e descreve dois surtos de intoxicação por *Nierembergia rivularis* em ovinos no Uruguai. Os surtos ocorreram em propriedades localizadas em uma ilha (Surto A), e nas margens (Surto B) do lago do Rincón del Bonete. Foram afetados ovinos de todas as idades, exceto cordeiros lactentes. Os primeiros sinais clínicos ocorreram no início de outubro e as mortes de dezembro a fevereiro. Morbidade de 10% foi observada nos Surtos A e B. A mortalidade foi de 7,2% e 2,8% nos Surtos A e B, respectivamente. Os sinais clínicos incluíram perda de peso, abdômen retraído, marcha rígida e cifose. Foram necropsiados um animal de cada rebanho. Observou-se mineralização arterial e pulmonar, nefrocalcinose e osteopetrose no exame macroscópico e histológico dos dois ovinos. Hiperplasia e carcinoma de células C da tireoide foram observados no ovino A. O ovino B apresentou hiperplasia de células C da tireoide e atrofia das células principais da paratireoide. As paratireoides do ovino A não foram examinadas. O diagnóstico

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diferencial da calcinose enzoótica no Sul da América do Sul deve considerar quatro plantas calcinogênicas da família Solanaceae: *Solanum glaucophyllum, Solanum stuckertii, Nierembergia veitchii* e *Nierembergia rivularis*.

TERMOS DE INDEXAÇÃO: Doenças de ovinos, calcinose enzoótica, *Nierembergia*, Uruguai.

INTRODUCTION

Poisoning by calcinogenic plants produces systemic soft tissue calcification in different animal species. Plants that are known to be calcinogenic in South America (Argentina, Brazil, and Uruguay) are *Solanum glaucophyllum* (syn. *Solanum malacoxylon*), which affects cattle (Döbereiner et al. 1971, Riet-Correa et al. 1975, Gimeno 2001), sheep (García y Santos et al. 2007), buffaloes (Santos et al. 2011), and equine (Odriozola et al. 2017); *Solanum stukertii*, which affects goats in Argentina (Iglesias et al. 2008, Rossanigo et al. 2017); *Nierembergia veitchii* (Barros et al. 1970, Riet-Correa et al. 1987, Rissi et al. 2007, 2009) and *Nierembergia rivularis* (syn. *Nierembergia repens*) (García y Santos et al. 2012), which affect sheep and, more rarely, cattle in Rio Grande do Sul state, Brazil. Enzootic calcinosis (EC) of unknown cause has been reported in Central Brazil, affecting sheep, goats, and cattle (Guedes et al. 2011).

Poisoning of sheep by *N. rivularis* in wetlands in the region of Batovi, Department of Tacuarembo, Uruguay has been reported since 1989 (Mederos et al. 1991), as well as near flooded areas in the Department of Rivera (García y Santos et al. 2012), on the islands of the Rincon del Bonete Lake (department of Durazno), and along the borders of the same lake in the department of Tacuarembo (Preliasco et al. 2014). The poisoning occurs between October (spring) and March (summer) with a mortality of 5-24% (Table 1), affecting sheep of all ages, with the exception of suckling lambs (Mederos et al. 1991, García y Santos et al. 2012, Preliasco et al. 2014). Enzootic calcinosis was produced experimentally in sheep grazing for three months in a 625 m^2 area of pasture infested by *N. rivularis* (García y Santos et al. 2012).

Cases of EC in sheep in Rio Grande do Sul state were initially described by Barros et al. (1970); later, poisoning was reproduced by the experimental administration of *N. veitchii* to rabbits (Riet-Correa et al. 1981) and sheep (Riet-Correa et al. 1993). The disease has been described mainly in mountainous areas of the Planalto Medio and the south of Rio Grande do Sul state, Brazil (Riet-Correa et al. 1987, Barros et al. 1992,). In contrast, *N. rivularis* occurs mainly in low and humid areas in Uruguay (Fig.1) (García y Santos et al. 2012, Preliasco et al. 2014). Poisonings by *N. veitchii* and *N. rivularis* affect sheep of all ages from October to March, with the exception of suckling lambs. An increase in calcemia has been detected in October, reaching a peak in February, and decreasing from March (Riet-Correa et al. 1987). Despite the low lethality of EC caused by *N. veitchii* (1-25%), the morbidity can be in the range of 1-80%, which results in underestimated losses as most of the affected herd undergo weight loss that can be mistaken for malnutrition or parasitic infections (Riet-Correa et al. 1987, Barros et al. 1992).

In Uruguay, an outbreak of poisoning by *S. glaucophyllum*, which contains glycosides derivates of $1,25(OH)_2D_3$ (calcitriol), was reported in sheep near the streams of Solis Chico and Mosquito, department of Canelones, in which 15 ewes out of a flock of 100 sheep of different ages died (García y Santos et al. 2007). Poisoning by *S. glaucophyllum* has previously been described in cattle in the departments of Canelones, Colonia, Rocha (Riet-Correa et al. 1975) and Paysandú (Rivero et al. 1989). In Rocha, EC occurs between November and February in a wetland area of approximately 110,000 hectares, with morbidity ranging from 2-80% and averaging at 8-10% (Riet-Correa et al. 1975). However, since 1975, most of the wetland areas have been dried for rice farming, which has decreased

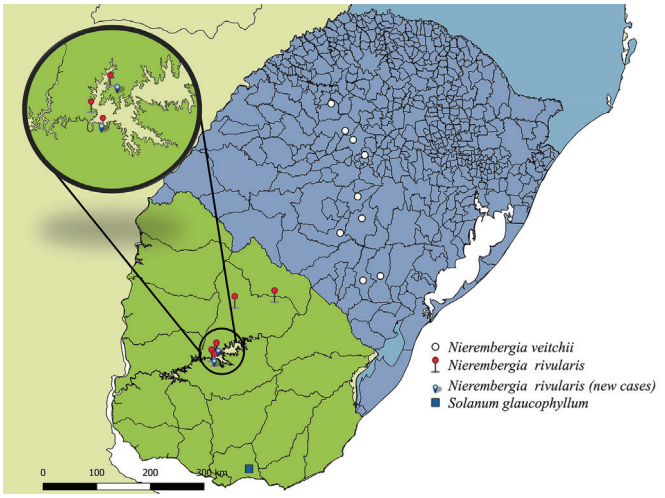


Fig.1. Location and geographic distribution of outbreaks of enzootic calcinosis in sheep poisoned by *Nierembergia rivularis* in Uruguay and *Nierembergia veitchii* in Rio Grande do Sul (RS), Brazil. The disease occurs mainly in humid areas in the departments of Tacuarembó, Rivera, and Durazno, Uruguay, and in high areas of the Planalto Medio/RS, Brazil. Inset of the borders of Rincon del Bonete lake, where the two outbreaks described in the present research and three others previously reported occurred.

Table 1: Outbreaks of enzootic calcinosis reported in sheep in Uruguay from 1989 to 2013

Departments	Period of outbreaks	Plants	Sheep		References
			Total	Mortality (%)	
Tacuarembó	Out/1989	*Nierembergia rivularis*	-	6	Mederos et al. 1991
Rivera	Dez/2005-Feb/2006	*N. rivularis*	200	6 (12/200)	García y Santos et al. 2012
Tacuarembó	Oct/2012-Mar/2013	*N. rivularis*	199	5 (10/199)	Preliasco et al. 2014
Tacuarembó	Oct/2012-Mar/2013	*N. rivularis*	400	12.5 (50/400)	Preliasco et al. 2014
Durazno	Oct/2012-Mar/2013	*N. rivularis*	250	24 (60/250)	Preliasco et al. 2014
Canelones	Aug-Dec/2006	*Solanum glaucophyllum*	100	15 (15/100)	García y Santos et al. 2007

the distribution and frequency of poisoning (Franklin Riet-Correa, unpublished data).

Enzootic calcinosis is a chronic disease in sheep that is clinically characterized by anorexia, weight loss, stiff gait, and kyphosis with hypercalcemia and hyperphosphatemia (Barros et al. 1970, Riet-Correa et al. 1987). Exercise intolerance and sudden death caused by acute pulmonary edema are often observed during management practices (bathing, shearing, and dosing) of the flock (Mederos et al. 1991, Rissi et al. 2007, 2009, Preliasco et al. 2014). Soft tissue calcification, thyroid C cell hyperplasia, parathyroid chief cell atrophy, osteonecrosis, and osteopetrosis are the main changes observed in sheep poisoned by *N. veitchii* (Riet-Correa et al. 1987, 1993) and *N. rivularis* (García y Santos et al. 2012). *N. veitchii* has a toxic principle with a biological activity similar to $1,25(OH)_2D_3$ (Riet-Correa et al. 1987).

This paper describes the epidemiological, clinical, and pathological findings of two outbreaks of EC in sheep associated with the spontaneous consumption of *N. rivularis* that occurred in Uruguay in the years 2016 and 2018.

MATERIALS AND METHODS

Epidemiological and clinical data were obtained from farmers and veterinarians in relation to two farms located in the departments of Durazno and Tacuarembó, Uruguay, in which EC outbreaks occurred. Clinical evaluation and blood sample collection for quantification of serum concentration of calcium and phosphorus were performed in one sheep from Outbreak A and 13 sheep from Outbreak B. One sheep from Outbreak A (Sheep A) that died spontaneously during herding and one from Outbreak B (Sheep B), which was euthanized, were autopsied. Organ samples including fragments of the femur cortex were collected, fixed in 10% buffered formalin (pH 7.0), processed routinely, cut at 4 µm, stained using the hematoxylin and eosin (HE) technique, and histologically evaluated. The samples of the femur were decalcified with 7% nitric acid. Samples of *Nierembergia* sp. were collected and identified as *Nierembergia rivularis* Miers (syn. *Nierembergia repens* Ruiz & Pav) (Cabrera 1979) and deposited under the number MVFA 34988 in the herbarium Ing. Agr. Bernardo Rosengurtt, at the Agronomy Faculty of the Republic University in Montevideo, Uruguay. A map showing the distribution and location of the cases of poisoning by *Nierembergia* spp. was constructed with Quantum Gis 2.14® software.

RESULTS

Epidemiology and clinical signs

Outbreak A was confirmed in February 2016 on a 50-hectare farm located on an island in the Rincon del Bonete lake, department of Durazno. In a flock of 110 sheep, 11 animals (10%) exhibited clinical signs and eight (7.27%) died. There were no cattle on the farm. Outbreak B was confirmed in January 2018 at a farm near the Rincon del Bonete lake, Cardozo region, Tacuarembó department. The disease occurred in a 90-hectare paddock on the border of the lake in which 220 sheep and 70 cattle were grazing. Twenty sheep (10%) and four cows (2.8%) had clinical signs. Sheep of different ages were affected, with the exception of suckling lambs. Seven sheep (3.1%) died from December to February (summer) two to five months after the onset of the disease in spring. Laboratory data showed hyperphosphatemia (8.5mg/dL - reference range: 5.0-7.3mg/dL) in the sheep that was necropsied. Normal calcium and phosphorus levels were observed in the other animals examined. The sample from Sheep A was not examined due to hemolysis.

The animals in both farms were raised in wetlands along the border of the Rincon del Bonete lake that was invaded by *Nierembergia rivularis* (Fig.2). The animals remained in this area all year round. The sheep exhibited clinical signs, such as progressive weight loss, retracted abdomen, kyphosis (Fig.3A), stiff gait, lameness, exercise intolerance, weakness, and recumbence. When managed, some sheep presented acute clinical manifestation with difficulty breathing, tachypnea, pulmonary edema, and recumbency that subsequently progressed to death. Annual outbreaks have been reported since 2012 on the farm located on the island and since 2011 on the farm located on the border of the lake.

Gross and histological lesions

The cadavers were emaciated and showed widespread soft tissue calcification. Muscular and elastic arteries were diffusely rigid, with marked loss of elasticity. The intimal surface was irregular with multiple white, prominent, and multifocal-to-coalescing intramural plaques (Fig.3B). The bicuspid and semilunar aortic valves exhibited similar plaques that were also identified in the tendinous and endocardial cords. The lungs did not collapse and showed multifocal calcification, mainly in the caudal lobe. The kidneys exhibited whitish, opaque, linear, and radially arranged striations in the cortical and medullary region (Fig.3C). Marked thickening of



Fig.2. Location of outbreaks of enzootic calcinosis in sheep caused by *Nierembergia rivularis* in Uruguay. (**A**) Outbreak A. The border of the Rincon del Bonete lake is invaded by the plant. (**B**) Natural pasture invaded by *Nierembergia rivularis*. (**C**) Details of the flowering plant.

cortical bone resulted in a reduction in the medullary space of the long bones.

Histologically, the intima of the carotid artery exhibited non-mineralized hyperplasia (Fig.3D) of variable intensity, with loss of undulation of the internal elastic lamina. Extensive areas of irregular deposition of basophilic material (calcification) expanded and disorganized the tunica media, sometimes with islands of bone and cartilaginous metaplasia, in addition to mild macrophage infiltrate. A thin layer of smooth muscle cells of the tunica media, adjacent to the adventitia, remained unchanged. Occasional calcification of the *vasa vasorum* wall was observed in the adventitia. The tunica media and intima of the aorta exhibited similar mineral deposits.

Mild to marked calcification of the alveolar septa occasionally replaced the lung parenchyma with dense areas of calcification with bone and chondroid metaplasia. Calcification was also verified on renal tissues, affecting the distal tubular lumen, arterioles, and interstitium. Thyroid C-cell hyperplasia and carcinoma were observed in Sheep A (Machado et al. 2020). Sheep B showed thyroid C-cell hyperplasia and parathyroid chief cell atrophy. The parathyroid was not examined in Sheep A. In Sheep B, the cortical bones were moderately thickened,



Fig.3. Enzootic calcinosis in sheep caused by *Nierembergia rivularis* in Uruguay. Outbreak B. (**A**) Sheep exhibiting kyphosis, retracted abdomen, poor body condition, and arched limbs. (**B**) Aorta. Diffusely irregular intimal surface with extensive calcified plates. (**C**) Kidney. Nephrocalcinosis characterized by white streaks radially arranged at the corticomedullary junction. (**D**) Carotid artery. Moderate intimal hyperplasia and tunica media calcification. HE, obj.10x.

with increased osteoid and decreased Haversian channels. The trabecular bone of the epiphysis exhibited thickened trabeculae lined by sparse osteoclasts and osteoblasts. The proximal joint surface of the femoral head showed multifocal, disorganized, degenerated, and calcified chondrocytes.

DISCUSSION

The diagnosis of poisoning by *Nierembergia rivularis* in sheep in both outbreaks was made based on epidemiological, clinical, and pathological findings, in addition to the presence of the plant in the pastures. These findings were similar to those previously described in cases of EC (Barros et al. 1970, 1992, Riet-Correa et al. 1987, Mello 2003). Both farmers reported the sudden death of some sheep while being managed due to heart failure caused by severe calcification of heart valves and arteries, resulting in acute pulmonary edema and death (Barros et al. 1992, Rissi et al. 2007, 2009).

The mortality observed in both outbreaks (3.1% and 7.1%), which was similar to previously reported rates (5-24%) (Mederos et al. 1991, García y Santos et al. 2012, Preliasco et al. 2014), caused significant losses for the farmers. Economic losses must be considered in sheep that did not present with clinical signs (Riet-Correa et al. 1987). Therefore, poisoning by *N. rivularis* causes significant economic losses in some farms with paddocks located along the borders of the islands of the Rincon del Bonete lake, as well as wetlands near streams in the Tacuarembo and Rivera departments (Mederos et al. 1991, García y Santos et al. 2012, Preliasco et al. 2014).

Only one of the sheep examined showed hyperphosphatemia, and the serum concentrations of Ca were within normal concentrations in all sheep. Hypercalcemia and hyperphosphatemia were the results of remarkable changes in vitamin D poisoning (Mello 2003); these conditions have also been reported in cattle spontaneously poisoned by *Trisetum flavescens* (Köhler & Libiseller 1970) and *Cestrum diurnum* (Durand et al. 1999), as well as in experimental poisoning by *Solanum glaucophyllum* in cattle (Döbereiner et al. 1971) and *N. veitchii* in sheep (Riet-Correa et al. 1993).

Hypercalcemia without hyperphosphatemia has been reported in horses (KrooK et al. 1975a) and cattle (Krook et al. 1975b) poisoned by *Cestrum diurnum* and in sheep poisoned by *N. veitchii* (Riet-Correa et al. 1987). Hyperphosphatemia without hypercalcemia has been reported in cattle poisoned by *Trisetum flavescens* (Dirksen et al. 1970) and sheep poisoned by *N. rivularis* (Preliasco et al. 2014). In contrast, cattle naturally poisoned by *S. glaucophyllum* have been found to exhibit no changes (Döbereiner et al. 1971). Research by Döbereiner et al. (1971) suggested that animals with EC with normal Ca and P serum levels had not consumed calcinogenic plants days before blood collection. Increased Ca and P concentrations are evident in animals soon after plant consumption and during experimental exposure (Döbereiner et al. 1971, Riet-Correa et al. 1993). The product of Ca x P values (>60) allows for a better evaluation of their metabolism (Gimeno 2001) and has been indicated for the diagnosis of subclinical cases of EC. Considered together, these reports demonstrate that an increase in Ca or P alone or an absence of changes in serum concentrations of these minerals does not exclude the diagnosis of EC.

To prevent and control poisoning, it should be noted that poisoning by *N. rivularis* and *N. veitchii* (Riet-Correa et al. 1987, Rissi et al. 2007, 2009) is seasonal and coincides with the vegetative growth of the plant, leading to the onset of clinical signs in the spring and higher mortality during the summer. There is no treatment for poisoning by calcinogenic plants, and the reversibility of the lesions is limited. When moved to pastures free of the plant, animals may exhibit slight recovery in weight gain, partially decreasing economic losses (Barros et al. 1992). Thus, it is recommended that immediately after the first signs or at the start of the spring, sheep should be moved to paddocks free from or with low amounts of *N. rivularis.* Disease seasonality and occurrence in low wetlands along borders of lakes and rivers allows for the use of rotative grazing, with a short-term stay on highly infested paddocks as a recommended measure of disease control.

As most of the *N. rivularis* plants on both farms were in areas surrounding water, the plants could be controlled using herbicides in restricted areas. Recently, various herbicides within the group of synthetic auxins and acetolactate synthase inhibitors were tested to control *N. rivularis*. In the short term the control of the aerial biomass was efficient (greater than 95%) with mixtures of the 2,4-D + Metsulfuron; 2,4-D + Starane; or 2,4-D + Lexus. However, in the following growing season, although to a lesser degree, the infestation by this species continued. Control in the medium and long term was much better when, in addition to the application of herbicides, continuous overgrazing of the treated area was avoided, in order to favor the competition of the forage species of interest. The chemical recommendation consists of the application of 2 liters of 2,4-D plus 200 cc of Tordon plus 10g Metsulfuron per hectare with the aim of achieving good control in the short term and maintaining a residual control in the medium term. However, to avoid the annual reapplication of herbicides, it is necessary to adjust grazing management (Alejandro Garcia, Instituto Nacional de Investigación Agropecuaria, INIA, Uruguay, unpublished data).

Cattle are less affected by *N. veitchii* poisoning than sheep due to the different feeding habits of both species and the morphological characteristics of the plant (Riet-Correa et al. 1987). In the present study, in Outbreak B, cattle grazing in the same paddock as sheep developed clinical signs that were suggestive of EC. Further studies in areas of cattle breeding invaded by *N. rivularis* are necessary to confirm the occurrence and importance of EC in this species.

The differential diagnosis of EC in sheep includes paratuberculosis (Johne's disease), which is a chronic debilitating disease affecting sheep that is characterized by progressive weight loss, exercise intolerance, and, occasionally, diarrhea (Giannitti et al. 2018). However, granulomatous enteritis observed histologically and the occurrence of diarrhea in some cases of paratuberculosis assist in the differentiation of the diseases. Gastrointestinal parasitosis, mainly haemonchosis, which occurs frequently in the spring and summer, should also be considered in the differential diagnosis.

CONCLUSIONS

Poisoning by *Nierembergia rivularis* in Uruguay is a chronic disease in sheep, sometimes causing sudden death due to acute pulmonary edema induced by heart failure. It causes losses in sheep farming in wetlands near rivers or streams in the departments of Tacuarembó and Rivera and in the islands in the Rincon del Bonete lake.

Like other calcinogenic plants, *N. rivularis* causes soft tissue calcification (enzootic calcinosis).

The only preventive measure is to avoid grazing sheep for long periods from October to February in paddocks invaded by the plant.

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Original Article
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Cobalt deficiency in cattle and its impact on production[1]

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ABSTRACT.- Silva W.J., Guizelini C.C., Franco G.L., Veiga R.C.P., Arruda W.G., Nascimento V.A., Melo E.S.P. & Lemos R.A.A. 2020. **Cobalt deficiency in cattle and its impact on production**. *Pesquisa Veterinária Brasileira 40(11):837-841*. Laboratório de Anatomia Patológica, Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul, Avenida Senador Filinto Muller 2443, Vila Ipiranga, Campo Grande, MS 79074-460, Brazil. E-mail: ricardo.lemos@ufms.br

Two outbreaks of cobalt deficiency in beef cattle were diagnosed in Midwestern Brazil. We discuss the clinical, epidemiological, pathological features, therapeutic measures, and impact aspects of the production system associated with these outbreaks occurring outbreaks in two farms of extensive cattle raising-system in the state of Mato Grosso do Sul. Seven affected cattle were euthanized and necropsied. Tissues for histopathology and microelements dosage were secured. At Farm A, 3100 cattle of all ages got sick, and 396 died; at Farm B, 148 were affected, and 110 died. In both farms, cattle were fed the same mineral supplement. The main clinical signs were weight loss and weakness, even though a good supply of forage was available in the paddocks. Many cattle stop grazing and chew at tree barks, wood chips from fence posts, and bones. In addition to the deaths, there was a compromised growth, and the reproductive rates fell sharply. The necropsied cattle were thin, with rough hair coat and pale mucous membranes. The liver was diffusely orange and showed a lobular pattern. The bone marrow was gelatinous and diffusely yellow. Histological changes included hemosiderosis in the liver and spleen, hepatocellular vacuolar degeneration, and myeloid and erythroid hypoplasia of the bone marrow. In the white matter of four cattle's brains, the myelin sheath was markedly distended (spongy degeneration). Proliferative parasitic abomasitis was observed in three cattle. The presumptive diagnosis was based on the association of the clinical picture, the necropsy findings, and the ruling out of other possible causes. The diagnosis was confirmed by the favorable response to treatment with cobalt and vitamin B12 orally and by mineral supplementation.

INDEX TERMS: Cattle diseases, deficiency diseases, mineral deficiencies, cobalt.

RESUMO. - [Deficiência de cobalto em bovinos e seu impacto na produção.] Dois surtos de deficiência de cobalto em bovinos de corte foram diagnosticados. Os aspectos clínicos, epidemiológicos, anatomopatológicos, terapêuticos e impactos no sistema de produção são descritos e discutidos. Os surtos ocorreram em duas fazendas de criação extensiva estado de Mato Grosso do Sul, Centro-Oeste brasileiro. Sete bovinos afetados foram eutanasiados e necropsiados. Na necropsia foram colhidas amostras para exames histopatológicos e dosagem de microelementos. Na Fazenda A, 3100 bovinos de todas as idades adoeceram e 396 morreram e na Fazenda B, 148 bovinos adoeceram e 110 morreram. Ambas as fazendas utilizavam o mesmo suplemento mineral. Os principais sinais clínicos observados foram emagrecimento e fraqueza, apesar da boa oferta de forragem nos piquetes, muitos bovinos deixaram de pastejar e comiam cascas de árvores, madeira das porteiras e ossos. Além das mortes, tiveram crescimento comprometido e os índices reprodutivos tiveram queda acentuada. Os bovinos necropsiados estavam magros, com os pelos arrepiados e

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mucosas pálidas. O fígado estava difusamente alaranjado e com evidenciação do padrão lobular. A medula óssea estava de consistência gelatinosa e difusamente amarelada. Alterações histológicas incluíam degeneração vacuolar hemossiderose que era moderada no fígado e marcada no baço. Hipoplasia mieloide e eritoide era vista na medula óssea. Na substância branca do encéfalo de quatro bovinos, a bainha de mielina estava marcadamente distendida (degeneração esponjosa). Abomasite parasitária proliferativa foi observada em três bovinos. O diagnóstico presuntivo baseou-se na associação do quadro clínico, nos achados de necropsia e na eliminação de outras possíveis causas. O diagnóstico foi confirmado pela resposta favorável ao tratamento com cobalto e vitamina B12 por via oral, e a suplementação mineral.

TERMOS DE INDEXAÇÃO: Doença de bovinos, doenças carenciais, deficiências minerais, cobalto, bovinos.

INTRODUCTION

Mineral elements are essential for animal nutrition, and their needs should be met through feeding practices, as the body cannot synthesize them. In balanced concentrations, they assist in the acid-base balance, forming structural components, such as enzymatic cofactors, and the transfer of energy (Riet-Correa 2007, NRBC 2016).

In farm animals, deficiency of mineral elements is due mainly to diets lacking the minimum requirements. Mild deficiencies lead to low fertility problems and low meat production, while severe forms can cause death (Tokarnia et al. 2000). Cobalt (Co) is an important microelement to ruminants' diet, as it is used by ruminal microorganisms for the synthesis of vitamin B12. In mammals, there are two vitamin B12-dependent enzymes: (1) methylmalonyl-CoA mutase, essential for the conversion of propionate to succinate, since it catalyzes the conversion of L-methylmalonyl-CoA into succinyl-CoA and (2) 5-methyltetrahydrofolate homocysteine-methyltransferase (methionine synthase), which catalyzes the transfer of methyl groups from 5-methyltetrahydrofolate to homocysteine, to form methionine and tetrahydrofolate. This reaction is crucial in recycling methionine after the transfer of its methyl group (NRBC 2016). The inability of ruminal microorganisms to convert succinate to propionate is the earliest manifestation of Co deficiency (Kennedy et al. 1991).

The first signs of Co deficiency are anorexia, moderate weight loss, and poor growth rates. When deficiency becomes severe, cattle exhibit rapid weight loss, fatty liver, and mucous pallor indicating anemia (NRBC 2016). The occurrence of outbreaks of Co deficiency only occurs in ruminants and most often affects animals held in pastures deficient in this microelement, which do not get a quality mineral supplement (Underwood & Suttle 1999, Tokarnia et al. 2010). The recommended Co level for beef cattle is 0.15mg/kg dry matter (DM) (NRBC 2016).

The goal of this study is to report two outbreaks of Co deficiency in beef cattle and discuss the clinical, epidemiological, pathological features, therapeutic measures, and impact aspects of the production system associated with these outbreaks.

MATERIALS AND METHODS

Our faculty team obtained the data on the outbreak during on-site visits to the two farms. We did three visits to each farm at 30-day intervals and constant interviews with the veterinarian practitioner tending the animal caretakers. Clinical examination was performed in Bovines 1, 3-7. The husbandry indexes were collected from the control sheets of each farm. In Farm A, groups of cattle were weighed to estimate weight loss. This property was monitored for a period of 12 months after our first visit to obtain reproductive data and calf weight at weaning. Bovines 1, 3-7 were euthanized due to their poor body, and health conditions. Bovine 2 was found dead. We necropsied these seven affected cattle, six of which (Bovines 1-6) were from Farm A, and one (Bovine 7) from Farm B. From the seven necropsied cattle, fragments were collected from all organs and fixed in 10% buffered formaldehyde, for subsequent routine histopathology. Liver fragments from Bovines 1-6 were also kept at -20°C to determine the copper (Cu) and Co levels employing the optical emission spectrometry technique with inductively coupled plasma (ICP-OES). These results were compared with the reference values of 100mg/kg for Cu and >0.12mg/kg for Co (Tokarnia et al. 2010).

Samples of the mineral supplement fed to cattle in both farms were analyzed for micro minerals in a laboratory accredited by the Brazilian "Ministério da Agricultura Pecuária e Abastecimento" (MAPA).

RESULTS

Epidemiology and clinical signs

Farm A, with extensive rearing of beef cattle, had sandy soil and a pasture predominantly composed of *Brachiaria brizantha* cv. Marandu. There grazed 8,327 cattle (of different ages, from calves to adults. Most of the cattle population were of Nelore (*Bos indicus*), although a small proportion of crossbreds (*Bos Taurus* x *Bos indicus*) existed. At the end of the outbreak, a total of 3100 cattle of all ages were sick, and 396 had died. Ten months before our first inspection of this farm on June 6, 2019, the owner had changed the suppliers of the mineral supplement; however, the new product supposedly contained adequate mineral levels similar to those used previously. There was no change in *ad libitum* supply practices, with daily estimates consumption of 120g/bovine. In April 2019, cattle of various ages and categories began to show weight loss and weakness. Despite the good of plenty of grass pasture available, several cattle did not eat but chew on tree bark, wood chips from fence posts, and, occasionally, bones. Once affected cattle started to lose weight, they oud die within 30-60 days. In the terminal phase of cachexia, affected cattle were markedly apathetic and had difficulty standing up and moving around. They were easily pushed by other cattle, fell several times, and finally remained in decubitus until death. Deaths occurred in April (17.5%), May (12%), June (52.5%) and July (18%). In June, deaths occurred within a week after the completion of a new treatment protocol (detailed below) and ceased on July 11. Part of the deaths recorded in July may have occurred in the previous month, as only the carcasses were detected in July and not sick animals. Deaths by animal category were as follows: 52.5% heifers, 25.5% suckling calves, 9% yearlings, 7.5% weaned calves and 4.5% bulls.

On the date of the first inspection of Farm A, five hundred sick cattle of various categories had been treated with Co (10ml/bovine, intravenously [IV]) and dewormed with 1% ivermectin containing vitamins A, D, and E (1ml/50kg/body weight (bw), IV). There was no regression of clinical signs. We then recommended a new treatment protocol consisting of albendazole and cobalt sulfate (Valbazen 10 Cobalto®, 30ml/cow, 20ml/heifer and 10ml/calves, orally) associated with

vitamin B12, chloride cobalt, copper sulfate and iron citrate (Cobalzan®, 60ml/cow and 20ml/calf, orally) and medicine containing only vitamin B12 (Monovin B12®, 5ml/cow and 2ml/calf, IV). The mineral mixture supplied to the cattle had been changed on June 3, 2019. A total of 3,040 cattle identified with clinical signs received the new therapy protocol. The treatment started on June 12, 2019, and was concluded on June 20, 2019. Several cattle were feeble and, during handling, for medication, fell and were trampled by the others. Others lay down on the containment corral during medication and needed assistance to stand up and be removed from there. Some cattle were unable to walk to the corral and had to be treated in the pasture.

Surviving cattle showed a marked drop in weight gain, which was estimated by weighing 10 cows that weighed 300kg, compared to the normal average weight of 450kg. Ten suckling calves weighed an average of 115kg, while the expected weight for that age group would be 170kg according to the farm records of previous years. Weaned calves also showed a marked reduction in weight gain. In 193 males and 192 females from Angus crosses (½ Nellore + ½ Angus), the mean weights ranged from 193 and 190kg, respectively. The normal weight would be over 220kg for both sexes. Twenty-two Nelore males calves, and twenty females calves were weighed, and the respective average weights were 156kg and 160kg. However, the expected weights were over 200kg. Ten healthy Nelore calves weighed 192kg.

The annual pregnancy rate, which previously to the outbark was 80%, was reduced to 50.26%, and the ratios of morbidity, mortality, and lethality were, respectively, 36%, 4.75%, and 13.2%. In 2020, weaned calves' average weight was 237kg for male Angus crosses, and 223.9kg for female Angus crosses. Nelore cattle weighed 220kg (females) and 231kg (males). The pregnancy coefficient in 2020 was 83.9%.

In Farm B, the pasture also consisted mainly of *Brachiaria brizantha* cv. Marandu, and a total of 1,195 Nellore cattle of different categories. A hundred and forty-eight cattle of all categories got sick, of which 110 died. Eighteen months before the outbreak, cattle were offered, ad libitum, the same mineral supplement used lately in Farm A. Daily consumption was estimated at 100g/bovine. Affected cattle lost weight and, despite the abundant pasture. They had difficulty defecating, and the feces were dry. Animals became extremely feeble and died within six months. When deaths started, the mineral supplement was changed and, although there were no new cases, thirty-eight bovines that showed severe signs did not recover and were treated with the same therapy protocol used property A. Despite the treatment, still two calves died. The rates of morbidity, mortality, and lethality in Farm B were 12.4%, 9.2%, and 74.32%. In addition to the deaths, calves had compromised growth, with weight deficits of up to 140kg at weaning, when compared to the weights observed in the previous year. In the cows, there was a reduction from 80% to 60% in the pregnancy rate.

The levels of phosphorus (0.25g/kg), Co (13.06mg/kg), iron (981mg/kg) and sodium (45.85g/kg) in the mineral supplement used in both farms were well below those indicated by the manufacturer and Cu, manganese and zinc were not quantified, as they were below the minimum detection range.

Necropsy findings and histopathology

The seven necropsied cattle were thin to cachectic, with rough and opaque hair coat; the ocular and oral mucosae were pale. Consistently, ruminal and intestinal contents were scant, liquid to slightly pasty, green with few bulky components, and occasional fragments of wood admixed with sand. The liver was diffusely orange, with evidence of the lobular pattern, and bone marrow was gelatinous and diffusely yellow.

Histological changes were similar in all necropsied cattle and included hepatocellular vacuolar degeneration and hemosiderosis that was moderate in the liver and marked in the spleen. Myeloid and erythroid hypoplasia was seen in the bone marrow. In the white matter of the brain of four cattle, the myelin sheath was markedly distended, forming a large clear space around the axon (spongy degeneration). The spongy degeneration was marked in the subcortical white matter of the frontal lobes, basal nuclei, midbrain, and medulla and moderate in the subcortical white matter of the parietal and occipital lobes, hippocampus, thalamus, cerebellum, and pons.

Three cattle had had to moderate proliferation abomasal glands associated with numerous nematodes (*Trichostrongylus* sp.). Nematodes were seen in the lumen of the glands and free in the lamina propria.

Dosages of trace elements

Levels of Co were low in the liver of two cattle (0.06 and 0.09 mg/kg), and those of Cu were normal (from 190.99 to 380.24mg/kg).

DISCUSSION

The presumptive diagnosis of Co deficiency was made associating the clinical signs and epidemiological and by the elimination of other possible causes with similar clinical manifestations (Underwood & Suttle 1999, Tokarnia et al. 2010, Constable et al. 2017). The diagnosis was confirmed by the positive response to treatment with Co and vitamin B12, orally, and mineral supplementation. The response to supplementation is the most conclusive means of determining whether a ruminant in production is affected by the deficiency of a trace element. The criteria to be evaluated are weight gain, milk production, and reproductive performance after introducing the treatment (Constable et al. 2017). Since we were dealing with a herd of beef cattle in this report, the response to weight gain and reproductive indices was evaluated. In both farms studied, cattle recovered weight gain. In Farm A, the reproductive indices were followed for a period of 12 months; after treatment, they returned to the indices recorded prior to the outbreak. The reproductive indices in Farm B, were not followed; however, the recovery in weight gain after the treatment was recorded.

The majority of deaths occurred in young cattle, which is a category considered more sensitive to Co deficiency (Tokarnia et al. 2010, Constable et al. 2017). However, despite the low mortality, breeding cows lost weight and were severely affected in their reproductive capacity. Low mortality with weight loss and reduced fertility is described in beef cows (Judson et al. 1997).

The clinical signs observed in cattle of Farms A and B are characteristic of Co deficiency (Tokarnia et al. 2000, Constable et al. 2017). In the outbreaks described here, weight loss and

general weakness are attributing to the lack of food intake due to anorexia. Pica was a clinical sign seen in the first outbreak and has been described in association with Co deficiency in Brazil (Tokarnia et al. 2010, Riet-Correa 2007). However, no explanation is offered to why it develops.

The Co demands of cattle were not met by the mineral supplement and triggered the clinical condition described here. Co is essential in the diet of ruminants, as ruminal bacteria use it to synthesize cyanocobalamin (vitamin B12). It influences energy metabolism, facilitating the formation of glucose through methylmalonyl-CoA mutase, forming succinate from propionate (Underwood & Suttle 1999, NRBC 2016). The clinical signs start when depletion of available Co reserves for the rumen microbiota occurs, blocking the production of vitamin B12 and resulting in the accumulation of propionate in the blood. Propionate activates receptors that control food intake (Underwood & Suttle 1999). With low food intake, there is a further decrease in the ruminal microbiota, which also uses vitamin B12 for the conversion of succinate to propionate; consequently, there is a loss in the fermentation of forage consumed by Co deficient cattle, which causes a deficit in energy supply and, therefore, a marked loss of weight.

The necropsy and histopathological findings of necropsied cattle in the present outbreak are similar to previous reports of Co deficiency (Vellema et al. 1999, Tokarnia et al. 2000) as they are shared changes by severe malnutrition and anemia. The hypoplasia erythroid hypoplasia observed in the bone marrow indicates of anemia (Valli et al. 2016). It is known that low levels of vitamin B12 cause a reduction in the heme group's elements, resulting in anemia, a drop in the erythrocyte, hemoglobin, and hematocrit count (Stangl et al. 1999). The spongy degeneration in the white matter of the brain of four cattle has already been described in Co deficiency in sheep (Richards & Harrison 1981, Constable et al. 2017) and was attributed to hepatic encephalopathy, since the liver, in that animal species, is the most affected organ. However, in cattle, it is not clear why this change occurs. One possible explanation is that, since Co is essential to trigger the chain of biochemical reactions necessary to glucose production, hypoglycemia leads to spongy degeneration (Suttle 1998, Barros 2016).

Two bovines presented Co levels below reference values (0.5mg/kg indicate a deficiency, values between 0.05-0.12mg/kg marginal deficiency and values above 0.12mg/kg are considered normal (Tokarnia et al. 2010). The Co levels of Bovines 2-4 and 5 were within normal reference levels. However, these four cattle were treated IV with Co by before blood sampling for analysis; possibly, this masked the results: after Co-administration, there is a rapid increase in the element in body tissues (Constable et al. 2017). Together with the failure responding to IV treatment, these results reinforce the importance of the administration of Co by the oral route. In severe cases, vitamin B12 should concomitantly be used parenterally. Several authors describe that the most efficient treatment is the oral supply of sources of Co at doses of 5-10mg/Co/bovine/day (Tokarnia et al. 2010). The application of vitamin B12 is recommended because it has efficiency similar to that of the supply of Co. Its only disadvantage is being costly (Underwood & Suttle 1999, Constable et al. 2017). The parenteral administration of Co is inefficient since it does not reach the rumen in a significant amount to be used by ruminal bacteria in the synthesis of vitamin B12 (Underwood & Suttle 1999). Normal levels of Co in cattle treated by parenteral route indicate that the dosage of the element in the liver would not then be a good criterion for the diagnosis of Co deficiency. Cattle deficient in Co are more susceptible to gastrointestinal parasites. Thus, the administration of deworming drugs containing Co is indicated (Constable et al. 2017). Mineral supplementation with mixtures containing proper levels of Co, although effective for preventing deficiency, is insufficient to reverse severe cases of the established disease, as demonstrated in this study.

Co deficiency must be distinguished from food deprivation, which is the most common cause of cattle wasting. Food deprivation is due mainly to a lack of planning for forage supply during the dry season when the amount and nutritional value of the forage mass decrease (Euclides Filho et al. 2002). In the outbreaks reported here, this was ruled out, since, in both farms, there was a good supply of quality forage. Although other mineral deficiencies, such as copper and phosphorus, can cause weight loss, they have distinct clinical characteristics. In copper deficiency, the most frequent clinical sign is acromotrichia, defined as hair discoloration, observed mainly around the eyes. In severe phosphorus deficiency, osteophagia is a characteristic clinical manifestation, and the occurrence of spontaneous fractures is frequent. Bovines can also have arched limbs, lordosis, narrow chest, small pelvis, and thin bones (Riet-Correa 2007). Although severe phosphorus deficiency as the cause of death in the present study can be ruled out, the possibility of marginal deficiency of this element cannot be completely discarded, as phosphorus was not properly represented in the mineral mix.

CONCLUSION

Cobalt deficiency occurs in the state of Mato Grosso do Sul, and the inadequate mineral supplementation of Co is a potential risk for the occurrence of outbreaks that cause losses of livestock business in the short and long term. Early recognition of this deficiency is essential for decision making in order to mitigate the losses.

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Bovine digital dermatitis in the Brazilian Amazon biome and topical treatment with *Copaifera reticulata* oil[1]

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ABSTRACT.- Bomjardim H.A., Oliveira M.C., Brito M.F., Oliveira C.M.C., Monteiro B.M., Silveira N.S.S. & Barbosa J.D. 2020. **Bovine digital dermatitis in the Brazilian Amazon biome and topic treatment with *Copaifera reticulata* oil.** *Pesquisa Veterinária Brasileira 40(11):842-851*. Graduate Program in Animal Science, Instituto de Medicina Veterinária, Campus de Castanhal, Universidade Federal do Pará, Rodovia BR-316 Km 61, Castanhal, PA 68741-740, Brazil. E-mail: henriquebomjardim@unifesspa.edu.br

Bovine Digital Dermatitis (BDD) was studied in crossbred dairy cows grazing in Rondon do Pará, in the state of Pará, as well as in Açailândia and Cidelândia, in the state of Maranhão, Brazilian Amazon biome. The digits inspection from the dairy cows during milking was performed in ten farms comprising four visits (August and November 2016; April and July 2017). The cows were kept all year in pastures, and were mechanically milked on concrete floors and the animals were protected against the rains in eight farms, maintaining a daily cleaning, however, it could not be found a concrete floor in pre- or post-milking to ensure milking parlor on three farms. Manual milking on no concrete floors was performed in two farms. No preventive measures against hoof lesions were adopted. The BDD prevalence was 1.3% (22/1664), and no statistical difference among rainy or no rainy season was obtained (p = 0.72). The BDD lesions were classified according to "M system" (M0 = no lesion, M1 = active ulceration <2cm, M2 = active ulceration >2cm, M3 = healing stage, M4 = chronic stage, M4.1 = M4 with active ulceration). Regarding the 22 BDD lesions observed, 22.7% (5/22) were M1 stage, 36.4% (8/22) M2, 22.7% (5/22) M3, 13.6% (3/22) M4 and 4.5 (1/22) M4.1. Hypertrophic hairs at the edges of the lesions caused by fly larvae of genus *Cochliomyia* spp. as well as alterations on the hoof were also observed. Topical treatment was performed in six BDD lesions with a raw extract from trees of the genus *Copaifera reticulata* (Copaiba oil) and compared with the treatment of salicylic acid paste in five BDD lesions. The lesions were protected with a bandage for seven days and followed weekly until recovery. The complete therapeutic responses were 83.4% (5/6) and 75% (3/4), respectively, with an average time of seven weeks. The BDD in the Amazon biome occurs in low prevalence, not seasonal, and presents macro and microscopic features similar to BDD lesions from dairy cattle kept in free-stall housing. The treatment with copaiba oil showed similar results to the treatment of salicylic acid paste and can be used in control measures to BDD in the Amazon biome.

INDEX TERMS: Bovine digital dermatitis, Amazon region, Brazil, topical treatment, *Copaifera reticulata,* copaiba oil, Mortellaro's disease, grazing system.

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RESUMO.- [**Dermatite digital bovina no bioma amazônico brasileiro e tratamento tópico com óleo de *Copaifera reticulata*.**] A dermatite digital bovina (DDB) foi estudada em vacas mestiças leiteiras nos municípios de Rondon do Pará no Pará, Açailândia e Cidelândia no Maranhão, bioma amazônico brasileiro. Para tanto, foram realizadas quatro visitas a 10 propriedades, nos meses de agosto e novembro em 2016 e abril e julho em 2017, e realizada a inspeção dos dígitos de 1.664 vacas em lactação durante a ordenha. Nestas propriedades as vacas eram criadas a pasto durante todo o ano e ordenhadas mecanicamente em oito propriedades. As quais,

as salas de ordenha tinham piso concretado, com proteção contra as chuvas e era realizada a limpeza diariamente. No entanto, em três dessas propriedades, as salas de pré ou pós ordenha tinham piso não concretado e em duas, a ordenha era manual em piso de chão batido. Em todas elas não havia medidas profiláticas para afecções podais. A prevalência de DDB foi de 1,3% (22/1.664) e não se obteve diferença estatística entre os períodos chuvoso e não chuvoso (P = 0,72). As lesões observadas foram classificadas de acordo com o sistema M (M0-sem lesão; M1-lesão ulcerada <2cm; M2-lesão ulcerada >2cm; M3-lesão em cicatrização; M4-lesão crônica; M4.1- M4 com área ulcerada). De 22 lesões observadas, 22,7% (5/22) apresentavam-se em estágio M1, 36,4% (8/22) em M2, 22,7% (5/22) em M3, 13,6% (3/22) em M4 e 4,5% (1/22) em M4.1. Pelos hipertrofiados nos bordos das lesões, larvas de moscas do gênero *Cochliomyia* spp. e alterações no tecido córneo também foram observados. Nas lesões de 11 bovinos, em seis, foi realizado o tratamento tópico com extrato bruto de *Copaifera reticulata* (óleo de copaíba) e em cinco, com a pasta de ácido salicílico a 660mg/g e ambos os tratamentos foram protegidos com bandagem por sete dias. Após, as lesões foram acompanhadas semanalmente até a cura e obteve-se um índice de 83,4% (5/6) e 75% (3/4) de cura com esses tratamentos, respectivamente, com uma média de 7 semanas. Conclui-se que no bioma amazônico a DDB ocorre em baixa prevalência, não sazonal e com características macroscópicas semelhantes às lesões de vacas leiteiras criadas em sistema *free stall*. O tratamento com o óleo da copaíba apresentou resultados semelhante ao ácido salicílico e pode ser uma alternativa como medida de controle da DDB no bioma amazônico.

TERMOS DE INDEXAÇÃO: Dermatite digital bovina, região Amazônica, Brasil, tratamento tópico, *Copaifera reticulata,* óleo de copaíba, doença de Mortellaro, criação a pasto.

INTRODUCTION

Bovine digital dermatitis (BDD) is a contagious inflammatory lesion of the skin of bovine digits (Cheli & Mortellaro 1974). It has a multifactorial etiology with the interaction between environment, host, and bacterial agents, especially spirochetes of the genus *Treponema* sp. (Blowey & Sharp 1988, Rodríguez-Lainz et al. 1996, Scholey et al. 2010, Krull et al. 2014). It is a disease that may be considered endemic when dairy cows are kept in free-stall stables (Wilson-Welder et al. 2015) and being of low prevalence in beef cattle and crossbred dairy cows kept in a pasture (Wells et al. 1999, Brown et al. 2000, Silveira et al. 2009, 2018, Sullivan et al. 2013).

This disease was firstly described in Italy in 1974, and in the following years also in other European countries and North America, however, nowadays, the BDD is a worldwide disease (Blowey & Sharp 1988, Murray et al. 1996, Rodríguez-Lainz et al. 1999, Manske et al. 2002a, Sogstad et al. 2005). Among foot disorders, it stands out as the primary infectious disease that affects cloven-hoofed animals (Cramer et al. 2008). In Brazil, BDD has already been reported in dairy cattle in the states of Minas Gerais, Rio Grande do Sul, Goiás, Maranhão, and Pará (Molina et al. 1999, Cruz et al. 2001, Silva et al. 2001, Machado et al. 2008, Silveira et al. 2009, Moreira et al. 2018) and also in beef cattle in the states of Rio Grande do Sul and Pará (Cruz et al. 2005, Silveira et al. 2018).

Thus, the BDD injuries regarding the acute phase are painful and cause the lameness, notwithstanding the evidence, the main clinical consequence has severe implications for animal welfare (Sullivan et al. 2013). Therefore, economic losses related to the productive sector are associated with less milk production, low reproductive performance and treatment expenses (Barkema et al. 1994, Argáez-Rodríguez et al. 1997, Somers et al. 2005, Losinger 2006, Relun et al. 2013a).

Commonly, the control of BDD has been carried out with the topical and systemic application of antibiotics. Among these, oxytetracycline is often used with significant results (Manske et al. 2002b). On the other hand, solutions containing formaldehyde, copper, or zinc sulfate in footbaths, have also been used, but fell into disuse (Laven & Logue 2006). However, other alternatives have been tested owing to bacterial resistance related to overuse or inappropriate use of antibiotic therapy, as well as the presence of residues in milk or meat, and the high costs and toxicity in the use of formaldehyde or copper sulfate (Moore et al. 2001, Holzhauer et al. 2011, Evans et al. 2016, Yamamoto et al. 2018). As alternative treatments, there are raising incentives to invest in non-antibiotic products, i.e., having good efficacy, low cost, and low toxicity, which do not generate residues in the milk and are relatively easy to use.

Among the alternatives for using antibiotics, the salicylic acid (2-hydroxybenzoic), protected by bandages, has been shown efficacy similar to the treatment with oxytetracycline in dairy cows (Schultz & Capion 2013). This salicylic acid has antimicrobial, keratinolytic, and keratoplastic effects; i.e., in addition to "fighting" bacteria, it can dissolve the superficial layer of the epidermis and act in deeper layers and promote tissue repair (Lodén et al. 1995, Monte et al. 2014). Salicylic acid, seeing that it is a non-antibiotic product, and shall be allowed a 24-hour grace period for meat and milk, may present positive results for the treatment of BDD (Schultz & Capion 2013).

In Brazil, a natural substance extracted from the trunk of trees of the genus *Copaifera* L., commonly known as copaiba oil, is used in traditional folk medicine to treat various diseases in humans and animals (Pieri et al. 2009). In the Amazon region, this oil is well known and also available at low cost in open markets and markets in general. Copaiba oil is a substance rich in diterpenic acids and sesquiterpene hydrocarbons that have, among other properties, antimicrobial activities, anti-inflammatory effects, analgesic, and healing actions (Cascon & Gilbert 2000, Carvalho et al. 2005, Pacheco et al. 2006, Veiga Júnior et al. 2007, Santos et al. 2008, Barbosa et al. 2019). This plant may be used due their properties, and it could be a useful and ecologically sustainable alternative in treating BDD in the Brazilian Amazon region.

In this region, where animals are allowed to graze throughout the year, and therefore there is a dairy cattle breed predilections, seeing that these animals are more resistant to high temperatures and humidity. On the other hand, the BDD's epidemiological situation is not well studied, and alternative treatments for using of antibiotics should be proposed. Therefore, the objective of this study was aimed to investigate the BDD in crossbred dairy cows reared under pasture in the Brazilian Amazon biome, as well as testing the topical treatment for skin lesions with the crude extract of *Copaifera reticulata* and comparing the data with the treatment with salicylic acid paste.

MATERIALS AND METHODS

Study area and period. The BDD was evaluated in crossbred dairy cows in 10 properties located in the municipalities of Rondon do Pará/PA, Açailândia/MA and Cidelândia/MA, in the Brazilian Amazon biome. The climate of this region is related to the category of super humid equatorial, type Am, according to the Köppen classification, also being at the limit of transition to type Aw. It has average annual temperatures of 26ºC (maximum of 32ºC and minimum of 22ºC). The relative humidity is high, between climate oscillations in the rainy season (from November to May) and the dry season (from June to October), from 100 to 52%, and an average of 78%. The index of annual rainfall has an average of 2,000mm (Rondon do Pará 2018). Four visits were made in 2016 and 2017 – the first visit was in August 2016, the second one in November 2016, the third in April 2017, and the forth in July 2017.

Previously, and when visiting the properties, the cumulative rainfall indexes of the region were obtained from the database of the "Instituto Nacional de Meteorologia" (INMET) of the meteorological station in the municipality of Rondon do Pará, from January 2016 to July 2017 (INMET 2018), in order to assess possible seasonality in the occurrence of the disease.

The study was carried out according to the ethical principles of animal experimentation recommended by the "Colégio Brasileiro de Experimentação Animal" (COBEA).

Studied farms. The farms were selected owing to their prior knowledge regarding foot disorders as well as the producers' acceptance to participate in the study. The dairy cows were kept on pasture, in which the vegetation consists of *Urochloa brizantha* (synonym *Brachiaria*) (about 95%) and grasses of the genus *Panicum* spp. cv Mombaça and being added commercial mineral supplementation. Supplementation with cornmeal concentrate and soybean meal, corn silage, or sugar cane was offered to the animals in 50% (5/10) of the properties, mainly in the dry period. In 80% (8/10) of the properties, milking was mechanized and carried out in rooms with concrete floors and the animals were protected against rain, including pre- and/or post-milking rooms on concrete floors or beaten floors. In 20% (2/10) of the properties, milking was manual and carried out in a place with a beaten floor. In the properties in which mechanical milking was carried out, and the milking rooms was cleaned daily and at the end of each milking session. This cleaning could be done by scraping the organic matter and washing with water. All properties did not adopt measures to prevent foot infections or control the animals that entry into the herd (Table 1).

Animals. The crossbreeding had herds that were composed from the Holstein breed's crosses between the Zebu breeds (Gir, Guzerá, Tabapuã, and Nelore) in different blood grades, from three to 12 years of age and at different stages of the lactation period.

Diagnosis. The BDD diagnosis was performed during the milking of dairy cows by inspecting all bovine distal limbs with a flashlight. When the organic matter adhered to the distal limbs, they were superficially scraped (manual milking) or cleaned with water jets (mechanical milking).

The bovine distal limbs of 1,664 dairy cows were inspected. Concerning the four visits in order to test all dairy cows, it was not possible to identify each animal, and some bovine distal limbs were inspected more than once. Indeed, some cows in lactation moved to the dry cow group after the first visit, and others belonging to the dry cow group moved to the lactating cow group since there were cows in different third lactation.

At the end of milking, it was performed the scores of claudication symptoms of dairy cows identified with BDD, on a scale from 0 (without claudication) to 5 (severe claudication), according to Flower & Weary (2006). Afterward, physical restraint was performed with ropes and kept in the lateral position for cleaning, inspection, and treatment. In dairy cows highly agitated, 2% xylazine at a dose of 0.1mg/kg body weight was previously administered intramuscularly. The distal extremities were then washed with water, and the lesions measured in centimeters in the vertical and horizontal directions. The anatomical location and macroscopic characteristics were described, as well as the photographic record.

Classification. The lesions were classified into four stages, according to their macro and microscopic characteristics, such as, M1 (first stage of ulcer, <2cm in diameter), M2 (stage of painful ulcer, >2cm), M3 (healing stage, covered in a crust), M4 (chronic stage with hyperkeratotic surface), M4.1 (stage M4 with ulcerated area), according to the score of Döpfer et al. (1997) and Berry et al. (2012).

Table 1. Characteristics of the 10 properties studied regarding the size of the dairy cows lots, supplemented with concentrate and corn or sugarcane silage, manual or mechanical milking and the type of flooring material of the milking pens in the Brazilian biome

Municipality	Property	Dairy cows*	Concentrate		Silage**		Milking***	Concrete flooring		
			kg/animal/day	Offer period	Animals quantity	kg/animal		Pre-milking	Milking	Post-milking
Rondon do Pará /PA	I	70	3	Dry	All	-	ME	X	X	-
	II	55	4	Dry and rainy	20 cows	X	ME	X	X	X
	III	50	4.5	Dry and rainy	20 cows	X	ME	X	X	-
	IV	20	2	Dry	All	-	ME	-	X	-
	V	25	-	-	-	-	ME	-	X	-
	VI	15	4	Dry and rainy	-	X	ME	X	X	X
Açailândia/MA	VII	60	-	-	-	-	MAM	-	-	-
	VIII	40	-	-	-	-	MAM	-	-	-
	IX	35	6	Dry and rainy	14 cows	-	ME	X	X	-
Cidelândia/MA	X	60	-	-	-	X	ME	-	X	-

* Average dairy cows between the four visits made, ** about 10 to 15kg/animal/day of corn silage was offered; on property II, cows received about 25 to 30kg/animal/day of corn or sugarcane, *** ME = mechanical milking, MAM = manual milking; PA = Pará, MA = Maranhão.

Treatment. Topical treatment was tested, with approximately 7ml of crude extract of *Copaifera reticulata*, in six BDD lesions of six cattle. After the biopsy, histopathological study and molecular biology were performed, described by Bomjardim et al. (2020). The extract used was obtained by drilling the tree trunk in August 2016 in Rondon do Pará. The species was determined by the "Empresa Brasileira de Pesquisa Agropecuária" (Embrapa) Amazônia Oriental. For comparing the two groups, topical treatment with 10g of a commercial paste based on 660mg/g salicylic acid (Novaderma®, WDT, Garbsen, Germany) was also carried out on five BDD lesions of five cattle. Both products were applied with a bandage composed of gauze, orthopedic cotton, crepon bandage, and adhesive tape. The bandage was removed after seven days, and a macroscopic description of the lesions was performed. All treated cattle were followed up, and the lesions were described weekly until healing.

Statistical analysis. The binomial variables were presented in percentage (%) for the frequency of occurrence. The comparison between the variables according to the visits (1, 2, 3, and 4) was carried out by analysis of variance (ANOVA), using the GLASMIX procedure of SAS® version 9.3 (SAS/STAT, SAS Institute Inc., Cary, NC, USA).

The statistical models were based on the linear effects in classification method regarding variable visit and farms (I to X). The binary response variables consisted of the occurrences of BDD and lesion stages (dist = binomial).

Correlation analyzes were performed using the SAS CORR RANK procedure. The continuous response variables were submitted to the normality of residues and homogeneity of variances tests through the Guided Data Analysis Solutions of SAS. The variables that did not meet the statistical assumptions were converted into it. The tables were made using Microsoft Excel version 2010 for Windows 7.0. Significant difference was considered when $p \leq 0.05$.

RESULTS

Prevalence

According to the four visits in 10 properties, the BDD was diagnosed in 90% (9/10) of the properties, being detected in, at least, one of the four visits made, with a prevalence of 1.3% (22/1,664). Between visits, there was no significant difference in the occurrence of BDD (p=0.72) (Table 2) and no statistical correlations were observed between the occurrence of BDD and the rainfall index (r=0.02, p=0.38) (Fig.1).

Clinical characteristics

The BDD was observed mainly in the plantar region and affected the commissure of the inter digital space and/or in the coronary bands, but also in the palmar region, as well as in the medial portion of the quartile and cranial of the commissure of inter digital space. By evaluating these animals, the claudication was a discrete clinical sign and scores ranged from 1 to 2, including the absent one (Table 3).

The lesions were diagnosed in the first stage of ulcer (M1 and M2), healing stage (M3), chronic stage (M4), and chronic stage with an area of ulceration (M4.1). Regarding the forms of ulcers, the lesion sizes ranged from 1.0 to 5.5cm, being spherical to oval in shape, with concave, alopecic, moist, reddish surfaces, as well as with a granular surface, being painful to touch the high edges. The healing lesion sizes ranged from 0.3 to 4.5cm and covered with grayish crusty material. In three

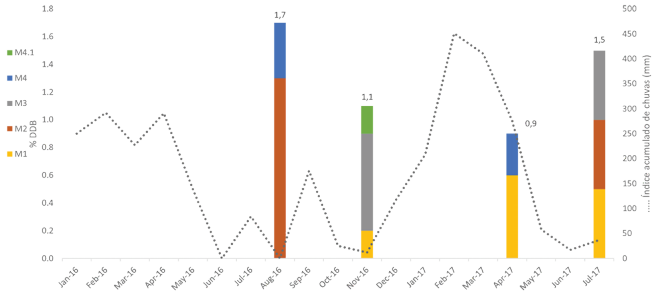


Fig.1. Graphical representation of the index of rainfall accumulation from January 2016 to July 2017 and the prevalence of bovine digital dermatitis (BDD) and scores distributed through the four visits (1st visit: Aug./16, 2nd visit = Nov./16, 3rd visit: Apr./17, 4th visit: Jul./17) that were conducted within 10 properties in Rondon do Pará/PA, Açailândia/MA and Cidelândia/MA in the Brazilian Amazon biome.

Table 2. Quantities of dairy cows inspected by property and number of animals with bovine digital dermatitis (BDD), as well as the percentage in the four visits carried out from August 2016 to July 2017

Property	Visits											
	1st - Aug/16 Animals			2nd - Nov/16 Animals			3rd - Apr/17 Animals			4th - Jul/17 Animals		
	n	BDD	%	n	BDD	%	n	BDD	%	n	BDD	%
I	81	2	2.5	73	-	-	55	1	1.8	61	-	-
II	78	1	1.3	74	-	-	41	-	-	30	1	3.3
III	52	1	1.9	53	1	1.9	42	-	-	61	1	1.6
IV	22	-	-	19	1	5.3	10	-	-	23	-	-
V	27	1	3.7	29	2	6.9	22	1	4.6	19	1	5.3
VI	14	1	7.1	15	-	-	12	-	-	18	-	-
VII	69	1	1.5	69	-	-	43	-	-	50	-	-
VIII	49	1	2.0	31	1	3.2	33	-	-	*	*	*
IX	23	-	-	28	-	-	34	-	-	53	-	-
X	61	-	-	47	-	-	61	1	1.6	82	3	3.7
Total	476	8	1.7[a]	438	5	1.1[a]	353	3	0.9[a]	397	6	1.5[a]

* Property not evaluated in the period, as the cows were not milked; The letters present on the same line do not differ statistically (p>0.05).

cows, there was a history of topical treatment of lesions with unspecified antibiotics.

In the chronic stage (stage M4), the lesion sizes ranged from 2.0 to 9.2cm, consisting of irregular shapes with a convex surface, yellowish-white to blackish-brown hyperkeratotic projections, and painless to touch. In two cows, the lesion extended to the coronary bands, and the commissure of the inter digital space assumed the shape of the letter "W". In an M4 stage, the area of ulceration was reddish, moist, and painful to touch (M4.1).

It was also observed hypertrophy at the edges of the lesions, changes in the corneal tissue of the digits, such as retinal detachment, accumulation of putrid organic matter in the cornea crack formed by the retinal detachment, double sole, and larvae of *Cochliomyia* spp. (Table 3).

Treatment

A cure rate of 83.4% (5/6) was obtained after topical treatment with copaiba oil and 75% (3/4) after topical treatment with salicylic acid paste. It was necessary to have follow-up of the animals until complete skin recovery in an average time of seven weeks (Table 4). After removing the bandage, in seven days, the lesions treated with copaiba oil presented an area of reduced ulcer, as well as in the active lesions and the hyperkeratotic lesions in the chronic stage, covered by whitish crusty material on the lesion and presence of characteristic odor of copaiba oil (Fig.2). A second topical application of copaiba oil with a bandage was carried out on a bovine for seven days. When the bandage was removed, the lesion had a reddish, moist area, painful to the touch, and a putrid odor.

In the treatment with salicylic acid paste, the lesions, when removing the bandage, were covered with a yellowish to whitish crusty material with a characteristic acid odor (Fig.3). In a bovine, the skin adjacent to the lesion was necrotic.

Before topical application of the products and bandages, the manual removal of fly larvae from the lesions was performed with anatomical forceps. By removing the bandage, after seven days, excision of the cracks formed in the corneal tissue, and the double sole was performed with the aid of "rinetas". After removing the bandage until the normal skin recovered, the presence of eggs or fly larvae was not observed.

Table 3. Classification of scores* regarding in bovine digital dermatitis (BDD), clinical signs, lesion location, macroscopic characterization and other changes in the corneal tissue in crossbred dairy cows raised on pasture in the Brazilian Amazon biome

Stage of BDD	Bovine	Member**	Degree of lameness	Anatomical region***	Diameter (cm)	HH	Fly larvae	Alterations in corneal tissue		
								D	OM	DS
M1	01	LP	0	IC, PL	1.0x2.0	-	-	-	-	-
	02	LP	0	RMQ, PL	1.6x1.5	-	-	-	-	-
	03	LP	0	CB/LD, PL	1.5x1.7	x	-	-	-	-
	04	LP	0	IC, PL	1.3x2.0	-	-	x	-	-
	05	LP	0	RMQ, PL	1.2x2.4	x	-	-	-	-
M2	06	LP	0	IC, PL	2.0x2.0	x	-	-	-	-
	07	LP	1	CB/LD, ET, PL	3.2x1.5	-	x	x	x	-
	08	RP	1	IC, CB/MD, PL	2.5x2.5	-	-	x	x	-
	09	RP	2	CB/LD, ET, PL	2.5x4.0	-	-	x	x	x
	10	LP	2	CB/LD, ET, PL	3.7x2.4	-	x	x	x	-
	11	RT	1	IC, C	4.5x3.6	x	-	x	-	-
	12	RP	1	IC, CB/LD e MD, PL	4.3x5.5	x	x	x	-	-
	13	LP	2	IC, CB/LD e MD, ET/MD, PL	5.0x5.0	x	x	x	x	x
M3	14	LP	0	CB/LD, PL	0.3x0.4	-	-	x	-	-
	15	LP	0	IC, PL	0.6x0.9	-	-	x	x	-
	16	RP	0	IC, PL	1.3x2.0	-	-	x	-	-
	17	LP	0	IC, PL	1.9x4.8	-	-	x	-	-
	18	LP	1	CB/LD, ET, PL	4.5x3.7	x	-	x	-	-
M4	19	LP	0	CB/LD e MD, PL	2.8x2.0	x	-	-	-	-
	20	LP	1	IC, CB/LD e MD, PL	6.4x5.5	-	-	x	x	-
	21	LP	0	IC, CB/LD e MD, PL	9.2x7x4	x	-	x	x	-
M4.1	22	RT	2	CB/LD e MD, PA	6.0x5.0	x	x	x	x	-

* According to Döpfer et al. (1997) and Berry et al. (2012); ** Affected member: RT = right-sided thoracic, LT = left-sided thoracic, RP = right-sided pelvic, LP = left-sided pelvic; *** Affected region: IC = Commissure of the inter digital space , CB = coronary band, PA = palmar, C = cranial, LD = lateral digit, MD = medial digit, ET = extent of injury to the talon, medial region of the quartile; HH = hypertrophic hair, D = retinal detachment, OM = organic matter in dark color with putrid odor, DS = double sole.

Table 4. Topical treatment with salicylic acid paste and crude extract of *Copaifera reticulata* (Copaiba oil) from stages M1, M2, M4 and M4.1 of bovine digital dermatitis (BDD) in the Brazilian Amazon biome

Topical treatment	Bovine	BDD	Macroscopic characteristic of lesions by removing bandage	Healing process (weeks)	Result
Salicylic acid paste (Novaderma®)	6	M2	Surface covered by salicylic acid paste	8	Treated
	7	M2	Covered by flaky white material	3	- *
	8	M2	Off-white yellow surface	10	NT **
	12	M2	Yellow surface and skin adjacent to the necrotic lesion	10	Treated
	19	M4	Brownish-yellow surface	4	Treated
Crude extract of *Copaifera reticulata*	1	M1	Covered by flaky white material	9	Treated
	10	M2	Wet and reddish surface	8	Treated
	13	M2	Reddish, granular surface and covered with yellowish crusty material	9	NT **
	20	M4	Covered by flaky white material	10	Treated
	21	M4	Covered by flaky white material	7	Treated
	22	M4.1	Circumscribed area with a wet and reddish surface, surrounded by yellowish material	5	Treated

* Treatment accompanied by only 21 days, as the animal died on the property of unclear cause, ** NT = non treated.



Fig.2. Topical treatment of bovine digital dermatitis with crude extract of *Copaifera reticulata* (copaiba oil). Bovine 21 referred in Table 4. (**A**) chronic bovine digital dermatitis (M4) on the day 0; (**B**) bovine digital dermatitis when the bandage are removed, covered with a whitish to pale yellowish crusty material, day 7.



Fig.3. Topical treatment of bovine digital dermatitis with a salicylic acid paste (Novaderma®). Bovine 7 referred in Table 4. (**A**) Digital dermatitis in the first stage of ulcer (M2) on the day 0; (**B**) Bovine digital dermatitis is shown when bandage are removed, without an ulceration area and covered with a whitish to pale yellowish crusty material, day 7.

DISCUSSION

The BDD is widespread among cattle herds from the studied properties, with a prevalence of 1.3% (22/1,664). This result was superior to that one observed by Silveira et al. (2009) in 2008, being performed in the same study region, in which an occurrence of 0.48% of BDD was observed (6/1,236). However, the study conducted by Silveira et al. (2009) paid special attention to claudicating animals. Non-claudicating animals, suffering from initial lesions, as observed in this study, were not detected, which could increase the prevalence rate of BDD in 2008. However, this higher prevalence of BDD in this region may be related to the absence of prophylactic and therapeutic measures for the contagious disease of the foot.

The low prevalence of BDD in cows destined for dairy production, in the Amazon biome, observed in this study and also observed by Machado et al. (2008) and Silveira et al. (2009) was similar to the prevalence of the disease in beef cattle from the same biome, as reported by Silveira et al. (2018), in dairy cows reared under a semi-intensive manner in the Brazilian Cerrado (Leão et al. 2009, Tomasella et al. 2014) and the Atlantic Forest (Souza et al. 2007). However, the prevalence of BDD was at a lowest level, i.e., a minimum of 30%, observed in dairy cows intensively reared and housed in a free-stall system in Brazil (Cruz et al. 2001, Souza et al. 2015), in Canada (Cramer et al. 2008), the Netherlands (Somers et al. 2005), Denmark (Klitgaard et al. 2008), Germany (Nordhoff et al. 2008) and Japan (Yano et al. 2010). These studies emphasize the close relationship between BDD and the type of breeding animals.

Regarding the extensive breeding system, cattle are kept in extensive pasture areas, areas of lower humidity. The bovine digits are less exposed to feces, and there is less contact between animals, as the concentration of animals per area is low. Breeding conditions opposite to what is observed in stable dairy cows, where digits are exposed continuously to feces and urine, which promote the skin of bovine digits to high humidity, mostly in stables where feces are not scraping frequently, as pointed out by Somers et al. (2005).

The prevalence of BDD in the properties in this study was constant over this time study, with no statistical correlation between the rainfall rate and the occurrence of the disease (r=0.02, *p*=0.38), which indicates that the disease can occur at any time of year in the Amazon biome, regardless of whether rains or not (Fig.1). However, a close relationship between a high prevalence of foot infections and poor hygiene conditions is pointed out by Van Metre (2017) or problems with muddy pens by Rodríguez-Lainz et al. (1996). For this reason, we believe that the rainy season may have a significant influence on the occurrence of BDD in the Amazon biome. In this season, the animals remain in pens from two to four hours, i.e., before, during, or after milking with muddy pens, feces, and urine, as reported by Silveira et al. (2009). The influence of the rainy season and the BDD occurrence is shown in Figure 1, where there is an increase in the disease occurrence from December to May.

Relun et al. (2013b) and Krull et al. (2016) observed an average of 140 days (5 months) for normal digit skin to present an ulcerated lesion in stable dairy cows. This time of development of the lesions may explain the increase in the prevalence of BDD, although not statistically significant, after the rainy season.

According to Sullivan et al. (2013), lameness is the first clinical sign observed in BDD. In this study, lameness was a clinical sign absent in the initial lesions (M1), in those in the healing stage (M3), and in the chronic (M4) and mild stages, grades form 1 to 2, in active lesions. For Frankena et al. (2009), the degree of claudication in cattle increases in parallel with the stage or severity of BDD lesions, when deeper and more extensive structures of the skin and or structures of the corneal tissue are reached. Thus, it is necessary to inspect the distal extremities during milking periodically. According to Orsel et al. (2017), the bovine digital space must be inspected during milking, i.e., being an easy practice to be performed and allowed the identification of BDD lesions in the initial stages, for immediate treatment, and monitoring of the herd.

The BDD lesions in the present study occurred preferentially on the skin of the plantar region of the caudal commissure and coronary bands, the preferred location for the formation of the lesions, as was also reported by Read & Walker (1998) and Cruz et al. (2001). Sogstad et al. (2005) related this preference of lesions to the limbs with more contact with feces and urine. In beef cattle, foot lesions preferentially occur in the pelvic limbs. These are also due to abrasions on the bovine digital skin with the non-castrated cattle, as observed by Silveira et al. (2018). Despite this preference, the thoracic digits and the pelvic digits of dorsal region should also be inspected.

The BDD lesions that formed or reached the cell crown led to the retinal detachment and the formation of a cornea crack. This change, already reported in dairy cows by Cruz et al. (2001) and in beef cattle by Sullivan et al. (2013), is probably related to the interruption or decrease in the supply of nutrients and oxygen to the precursor cells of the corneal tissue, located in the cell crown, due to the inflammatory response triggered. The accumulation of organic matter in these bovine dermatitis accompanied by cracked between the digits, associated with low oxygenation conditions, probably promotes an environment favorable to the colonization of anaerobic bacteria that lead to the destruction of healthy corneal tissue and the occurrence of other changes in the digits such as septic pododermatitis and double sole in cattle. Therefore, BDD can lead to the development of more secondary severe lesions in the digits of cattle. Cruz et al. (2001) also described angular deformations and increased sole thickness as changes secondary to BDD. In treating BDD lesions, it is necessary to remove these secondary changes in the corneal tissue. The development of larvae of flies of the genus *Cochliomyia* spp. in lesions of BDD, species of Diptera are more common in tropical areas, they can aggravate the lesions and lead cattle to a more severe clinical lameness, as they produce proteolytic enzymes that degrade healthy tissue.

Topical treatment with copaiba oil, protected by bandaging for seven days, proved to have similar therapeutic efficacy when compared to salicylic acid paste. Both showed similar results to the topical treatment with oxytetracycline reported by Manske et al. (2002b). When removing the bandage, both treatments showed improvement in the macroscopic aspects of the lesions, reducing putrid odor and painful to touch. However, among the treatments with copaiba oil, bovine 13 had the area of inflammation of the lesion reduced in the first bandage. However, it presented with large area of ulcers, very painful to touch, and with a putrid odor. The amount of time of contact of the product with the wound was probably not sufficient to treat the injury. In the treatment with salicylic acid paste, it was observed, in a bovine, that the tissue adjacent to the lesion was necrotic. Salicylic acid paste, when used in excess, can react with normal tissue and promote areas of necrosis. Therefore, using this product, an amount of acid sufficient to cover the lesion surface is recommended.

Copaiba oil reduced partial and total ulceration in lesions in the active stage, undid the hyperkeratotic surface, and promoted tissue repair in lesions in the chronic stage. This improvement in the macroscopic characteristic of the lesions, by reducing inflammation, in the keratolytic effect, and in tissue repair, may have resulted from the joint action formula of different compounds present in the copaiba oil described by Cascon & Gilbert (2000). According to Veiga Júnior et al. (2007), among these compounds, β-caryophyllene has a potent anti-inflammatory action, and the fractionated diterpenic acids of *Copaifera reticulata* (*ent*-kaurenoic acids, kovalenic, (13*E*)-*ent*-labda-7,13-dien-15-oic) have high antimicrobial activity, according to Barbosa et al. (2019). Copaiba oil was effective in treating human periodontitis, described by Bardají et al. (2016) and in dogs, by Pieri et al. (2010).

In both treatments, one in each, lesion recurrence was observed. These results pointed out to two possibilities: i) the treatment effect was not significant, since bacteria are found in deep layers of the epidermis, as shown by Zinicola et al. (2015); ii) the treatments were effective, but after a period, the lesions were reinfected, as pointed out by Berry et al. (2010) in lesions of BDD showing clinical improvement at the beginning and then returns to the active stage. In the

case of ineffective treatments or recurrence of the lesions, it is recommended to perform a second bandage with the same product or alternate the tested topical products.

With the low prevalence of the disease in the region regarding this study and difficulties to reach properties, due to long distances, it was not possible to evaluate both treatments in a larger number of cattle and to compare it with a control group, only being compared with the bandage. According to Manske et al. (2002b), the bandage may promote a longer time of action of the product with the lesion by keeping the bovine digits clean, without contact with mud or feces. Previous studies by Thomsen et al. (2012) pointed out that cleaning the digits periodically, the BDD prevalence can decrease. The results obtained regarding the animals treated in this study were probably related to a joint action formula of the product and the application of the bandage.

CONCLUSIONS

The BDD is a disease of low prevalence and not seasonal occurrence in crossbred dairy cows raised on pasture in the Brazilian Amazon biome. It is widespread among herds and has macroscopic characteristics similar to the BDD observed in dairy cows from farms housed in a free-stall system.

Copaiba oil has shown positive results regarding the treatment of BDD and may be an alternative to disease control measures in the Brazilian Amazon biome.

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Pathological features of cloned calves that died in the neonatal period[1]

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ABSTRACT.- Pereira L.S., Porto M.R., Reis Júnior J.L., Rumpf R., Silva Júnior E.R., Soto-Blanco B., Câmara A.C.L. & Borges J.R.J. 2020. **Pathological features of cloned calves that died in the neonatal period.** *Pesquisa Veterinária Brasileira 40(11):852-862*. Hospital Escola de Grandes Animais, Universidade de Brasília, Área Especial SRB, Galpão 4, Granja do Torto, DF 70636-200, Brazil. E-mail: aclcamara@yahoo.com.br

Somatic-cell nuclear transfer is a cloning technique that enables the creation of a viable embryo from a donor adult to produce a genetically identical individual. This technique opens numerous potential possibilities for medicine and animal reproduction. However, several reports have documented cloning-related issues. Embryo and fetal losses remain significantly higher than in other techniques, and there is a high incidence of dystocia and hydrops, which decreases efficiency and increases costs. Animals delivered at term often exhibit a syndrome known as macrosomia and experience difficulties in adapting to life outside the uterus, and death is a common outcome. In the present study, 41 cloned calves that died in the neonatal period were subjected to gross and histopathological examination. Most important gross lesions were found in the liver (enlargement, congestion, yellowish color), kidneys (brownish color at surface and cut, and cysts), lungs (atelectasis, parenchymal consolidation, and secretions in bronchi and bronchioles), and heart (concentric and eccentric hypertrophy, hematic cysts, persistence of ductus arteriosus). Primary microscopic findings were seen in the liver, kidneys, and lungs from neonatal calves. In the liver, 85% of the animals exhibited hepatic degeneration. The presence of a brownish pigment within the cortical tubules of the kidneys was found in approximately 90% of the samples; the presence of this pigment has not been previously reported in cloned calves. In the lungs, a large number of animals exhibiting lesions characteristic of pneumonia (55%). These changes were the pivotal causes of death, mainly due to problems in adapting to life outside the uterus and opportunistic infections in the neonatal period. Further investigation focusing on pathological anatomical changes is necessary to map these abnormalities in cloned animals.

INDEX TERMS: Neonatology, cattle, calf, clone, necropsy, histopathology.

RESUMO.- [**Aspectos patológicos de bezerros clonados com óbito no período neonatal.**] A transferência nuclear de células somáticas ou clonagem é uma técnica que permite produzir um indivíduo geneticamente igual a um outro indivíduo adulto. Esta técnica abre inúmeras possibilidades para a medicina e para a reprodução animal. Porém, existem inúmeros relatos de problemas associados à clonagem. A taxa de perda nos períodos embrionário e fetal ainda é muito alta quando comparada a outras biotécnicas; além disso, há uma maior incidência de hidropsias e distocias, diminuindo a eficiência e aumentando o custo da técnica. Os animais que vem a termo frequentemente apresentam

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uma síndrome chamada de macrossomia, e apresentam dificuldades de adaptação à vida extrauterina e, por isso, o óbito é um desfecho comum. No presente trabalho realizou-se necropsia e coleta de fragmentos de órgãos para avaliação histopatológica de 41 bezerros com óbito neonatal. As lesões macroscópicas mais importantes foram encontradas no fígado (hepatomegalia, congestão e coloração amarelada), rins (coloração amarronzada na superfície e ao corte, e cistos), pulmões (atelectasia, parênquima consolidado, e secreções nos brônquios e bronquíolos), e coração (hipertrofia concêntrica e excêntrica, cistos hemáticos e persistência de ducto arterioso). As principais alterações microscópicas observadas foram presença de pigmento acastanhado no interior dos túbulos corticais renais (aproximadamente 90% dos animais), degeneração hepática (85% das amostras avaliadas) e lesões características de pneumonia (55% dos animais). A pigmentação acastanhada no interior dos túbulos corticais é uma alteração que ainda não havia sido relatada anteriormente em animais clonados. As alterações observadas nestes órgãos foram determinantes para o óbito, e devem ter ocorrido sobretudo devido a problemas na adaptação ao ambiente extrauterino e em decorrência de infecções adquiridas no período neonatal. Os achados encontrados no presente trabalho denotam a necessidade de investigação anatomopatológica detalhada de animais clonados inviáveis, na tentativa de mapear as anormalidades apresentadas por eles.

TERMOS DE INDEXAÇÃO: Neonatologia, bovinos, bezerro, clone, necropsia, histopatologia.

INTRODUCTION

Despite advances in the field of somatic cell nuclear transfer (SCNT), especially in cattle, market growth continues to stumble due to the low efficiency of the cloning technique in producing viable offspring. Only 0.5% to 5% of reconstructed embryos produce term animals; this low success rate results in losses at the embryonic, fetal, and perinatal stages (Palmieri et al. 2008, Bressan et al. 2015, Ibtisham et al. 2017). It has been estimated that up to 80% of losses occur in the second month of pregnancies in SCNT animals; in contrast, such losses occur in only one-quarter of pregnancies from in vitro fertilization (Wiltbank et al. 2016).

It is believed that problems with placentation (placentomegaly, reduced vascularization of the chorioallantoid membrane, hypoplasia of the trophoblastic epithelium, and changes in the basement membrane) can result in significant losses during any gestational period (Lee et al. 2004, Palmieri et al. 2008, Barreto et al. 2009, Maiorka et al. 2015, Ao et al. 2019). Additionally, epigenetic reprogramming failures can lead to abnormal transcription of genes regulating implantation mechanisms, initial post-implantation development, and fetal growth. Such failures lead to a higher rate of late fetal losses, abnormalities observed in cloned calves (macrosomia, pulmonary hypertension, and neonatal asphyxia), and the highest rate of deaths in the neonatal period (Chavatte-Palmer et al. 2002, Lee et al. 2004, Kiefer et al. 2016).

Necropsy reports addressing cloned cattle are scarce, and those that are available only describe specific abnormalities (Birgel Junior et al. 2011, Maiorka et al. 2015). Moreover, the literature addressing the normal morphophysiology of newborn cattle is similarly scarce. Therefore, the present study aimed to describe pathological features of cloned (i.e., SCNT) calves that died in the neonatal period and to compare these with neonatal cattle of the same age group.

MATERIALS AND METHODS

Animals. This work was approved by the Ethics Committee on the Use of Animals (CEUA) of the Faculty of Agronomy and Veterinary Medicine of the University of Brasília (Brasília, Brazil), under process No. 2312/2013. Information regarding gestational length, calf age, and weight at death was collected. A total of 45 calves were evaluated, of which 41 were cloned that died in the neonatal period, and these were separated into two groups: Immediate Neonatal Group (ING), consisting of 25 cloned calves that died within the first 24 hours of life; and Late Neonatal Group (LNG), comprising 16 cloned calves that died between 2 and 28 days of life. All cloned calves are originated from Zebu breeds (subspecies *Bos taurus indicus*), from a commercial cloning company. The Control Group (CG) consisted of four male Girolando calves with age ranging from two to four days old. These animals were humanely euthanized using xylazine and thiopental (CFMV 2013) to compare pathological findings and verify whether such alterations are common to newborn cattle or are exclusive to cloned animals.

Necropsy and histological samples. The calves were subjected to necropsy immediately after spontaneous death (i.e., ING and LNG) or euthanasia (CG). Samples of the thoracic (heart and lungs) and abdominal (spleen, liver, and kidney) organs were fixed in 10% buffered formaldehyde and subsequently subjected to routine histological processing. Hematoxylin and eosin (HE) standard staining was initially used for screening, and the slides were evaluated under a light microscope. After initial screening, liver and kidney samples were stained with Schiff's periodic acid (PAS). Kidney samples were also submitted to Hall staining to reveal bile pigments, Perls, for iron analysis; PAS staining for glycogen and lipofuscin; and Ziehl-Neelsen staining for lipofuscin. A comparative analysis of histological characteristics was performed among the three groups (i.e., ING, LNG, and CG).

RESULTS

The mean gestational age of the cloned calves was 290.5 days, being 289.9 days in ING and 291.5 days in LNG. The mean weight was 45.5 ± 10.4 kg, achieving 43.4 kg and 48.8 kg in ING and LNG, respectively (Table 1).

Main macroscopic findings are summarized in Table 2. Primary lesions were found in the liver (enlargement, congestion, yellowish color) (Fig.1A-B), kidneys (brownish color at surface and cut, and presence of cysts associated with cortical spinal rarefaction) (Fig.2A-B), lungs (atelectasis, parenchymal consolidation, and presence of varied secretions in bronchi and bronchioles) (Fig.3A-B), and heart (concentric and eccentric hypertrophy, presence of hematic cysts, and persistence of ductus arteriosus) (Fig.4A-B).

Main microscopic findings of the abdominal and thoracic organs are summarized in Table 3 and 4, respectively. In the liver, the most important changes included well-defined lobular pattern, virtually imperceptible sinusoids (both in 73% of cloned calves), closed distance between portal spaces (83%), cytoplasmic vacuolization of hepatocytes (85%) (Fig.1C-D), accumulation of glycogen (confirmed by PAS staining; 41%), centrilobular necrosis with fibrin deposition, and inflammatory infiltrate (6%). In the spleen,

Table 1. Minimum and maximum values, mean and standard deviation of gestation length (days), age (days), and weight (kg) of cloned calves that died in the neonatal period

Group	Gestation length (days)			Age (days)			Weight (kg)		
	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD
ING (n=25)	272	308	289.9 ± 8.69	0	2	0.75 ± 0.76	23	59	43.4 ± 9.92
LNG (n=16)	278	303	291.5 ± 8.39	4.5	20	9.41 ± 5.38	23	67	48.8 ± 10.5
All calves (n=41)	272	308	290.5 ± 8.51	0	20	4.12 ± 5.43	23	67	45.5 ± 10.4

ING= immediate neonatal group, LNG = late neonatal group, Min = minimum, Max = maximum, SD = standard deviation.

Table 2. Gross pathology findings in cloned and control calves that died in the neonatal period

Organ	Cloned calves		Control
	ING	LNG	
Liver	Fibrosis Necrosis Hepatomegaly Irregular surface Opaque thickened capsule Subcapsular hematoma Pale or yellowish coloring on the surface and cut Reddish spots on the surface and cut Congestion	Hepatomegaly Pale or yellowish coloring on the surface and cut Congestion	Brownish color, on the surface and cut Regular edges Smooth and shiny surface
Spleen	Thickened and whitish capsule Mild splenomegaly	White pulp hyperplasia	No lesion
Kidneys	Hydronephrosis Congestion Fat accumulation at perirenal, pelvis, goblets, and papillae Translucent gelatinous material in pelvis Blackish brown color on the surface and cut Whitish to yellowish streaks in cortex and medulla Cystic dilations in the region of renal or medullary calyxes, with associated medullary cortication	Congestion Friable Fat accumulation at perirenal, pelvis, goblets, and papillae Blackish brown color on the surface and cut Whitish to yellowish streaks in cortex and medulla Cystic dilations in the region of renal or medullary calyxes, with associated medullary cortication	Brownish color on the surface (cortical and pinkish medullary region) Moderate amount of perirenal fat reaching the pelvis
Respiratory system	Pulmonary edema Congestion Aspirative pneumonia Tracheal hemorrhage Pulmonary emphysema Areas of atelectasis Consolidation areas Irregular surface Variable amount of whitish foam and liquid from the beginning of the trachea to secondary bronchi Yellowish or greenish material from lumpy to pasty consistency Petechiae and red striations in the larynx, trachea, and bronchi	Pulmonary edema Pneumonia Consolidation areas Irregular surface Moderate amount of whitish, yellowish or greenish catarrhal material in bronchi and bronchioles	Pink coloring Uniform appearance Fully expanded
Cardiovascular system	Cardiomegaly Umbilical veins and caudal vena cava rupture Fat accumulation in epicardium Identifiable oval foramen and ductus arteriosus Persistence of the ductus arteriosus Hematic cysts adhered to the endocardium (mainly in the atrioventricular valves) Bright, slightly firm nodules adhered to atrioventricular valves, suggestive of endocardiosis Concentric hypertrophy of the right ventricle (RV) and interventricular septum, and more rarely affecting the left ventricle (LV) RV eccentric hypertrophy	Cardiomegaly Pericardial petechiae Valvar hemorrhage Fibrillary adhesions between pericardial sac and epicardium Hydropericardium Hematic cysts adhered to the endocardium (mainly in the atrioventricular valves) Fat accumulation in epicardium Iliac arteries thrombosis	Fat accumulation in epicardium, (interventricular septum and base) RV and inter-ventricular septum free wall slightly thickened Oval foramen and arterial duct identifiable, but non functional
Other findings	Hemothorax Macrosomia Enteritis Omphalitis Fetal monster (Dexter)	Peritonitis Omphalitis Macrosomia Enteritis Ruminal drinker Abomasum rupture Bladder rupture	No lesion

ING = Immediate neonatal group, LNG = late neonatal group.

the most common finding was reduction in white pulp (48% of cases), evidenced by cellular rarefaction in lymphoid follicles. The kidneys exhibited smaller, hypercellular, and closer glomeruli (glomeruli with an embryonic aspect; 88%) and brownish granules within the cortical tubule cells (90%) (Fig.2C). After applying special stains (Hall, Perls, PAS, and Ziehl-Neelsen), only the lipofuscin stain labeled the observed granules, but not as characteristically as control fragments (Fig.2D). Inflammatory cell infiltration (55% of cloned calves) (Fig.3C-D), atelectasis (45%), and pulmonary edema (35%) (Fig.3E) were the most common findings in the lungs. A yellowish amorphous substance (suggestive of meconium aspiration; 28% of cases) (Fig.3C), and pinkish amorphous substance (suggestive of milk aspiration; 18%) were also observed, most often associated with severe inflammatory infiltrate, indicating aspiration pneumonia. In the heart, there was a slight to moderate vacuolization of cardiac fibers (28%), which appeared as round vacuoles that left the cell swollen (Fig.4C). Greater spacing between fiber bundles was also observed (45%). In the CG, the same spacing of cardiac fiber bundles was observed, but at lower intensity (Fig.4D).

DISCUSSION

Dead cloned calves in the present study exhibited pathological changes in several organs. Main changes observed were the presence of a brownish pigment inside the renal cortical tubules (approximately 90% of cases), liver degeneration (85% of evaluated samples), and lesions characteristic of pneumonia (55% of animals). In fact, several reports have described congenital and acquired changes in cloned animals (Garry et al. 1996, Taneja et al. 2001, Rhind et al. 2003, Panarace et al. 2007, Santos et al. 2010, Maiorka et al. 2015). On the other hand, some studies did not find any alteration(s) at necropsy of cloned animals that died in the neonatal period (Wilmut et al. 1997, Kato et al. 1998, Lee et al. 2004). This absence of changes may be related to acute anoxia, which may explain the apparent lack of findings in many necropsies (Mee 2011). Additionally, neonatal deaths are often associated with respiratory problems, especially in depressed and long-standing calves (Wilson et al. 1995, Garry et al. 1996). Respiratory distress is believed to be the most common sign in calves that died without any other abnormalities (Panarace et al. 2007).

In the present study, the livers of cloned calves exhibited an irregular surface and a thickened and opaque capsule, which have also been previously described (Hill et al. 1999).

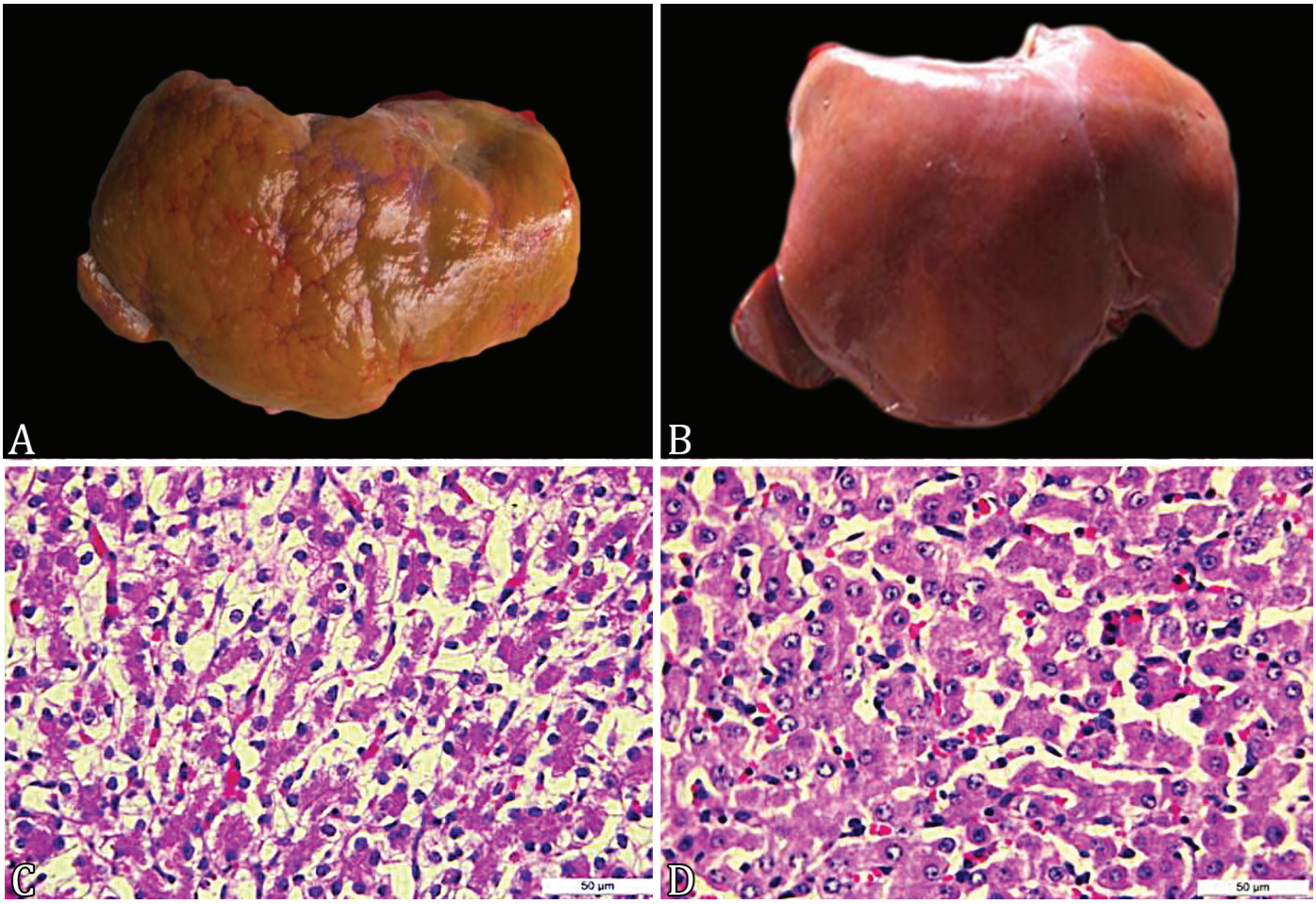


Fig.1. Changes observed in the liver of cloned and control neonates. (**A**) Irregular liver surface, cloned calf. (**B**) Normal liver, control animal. (**C**) Marked hepatocyte vacuolization, cloned bovine. HE, obj.40x. (**D**) Discrete bovine hepatocyte vacuolization, control calf. HE, obj.40x.

Subcapsular hematoma was observed in only one calf; however, there was no apparent explanation for its appearance and no correlation with other specific changes was found. Congestion and hepatic hemorrhage were observed in four animals under microscopy. The occurrence of hepatic congestion in cloned bovine neonates has been previously reported (Santos et al. 2010, Maiorka et al. 2015), indicating that this is a common finding in these animals. In control calves, the occurrence of hepatic congestion can be attributed to the agent used for euthanasia (thiopental) (Thomson 1998). The observation of hepatomegaly and hepatic steatosis, together with microscopic findings, revealed that vacuolated hepatocytes to varying degrees in cloned calves, however, more accentuated in ING animals, can be interpreted as fatty degeneration (due to the morphology of the vacuoles and macroscopic findings). Fatty degeneration has been attributed to the contribution of fat greater than the metabolism or the reduction in synthesis of proteins and lipoproteins involved in the transport of lipids out of the cell. It occurs in animals in starvation or severe food restriction, and also in other situations such as poisonings, ketosis in ruminants, and those with diabetes mellitus (Thomson 1998).

In the CG, some degree of hepatic fatty degeneration and areas of central-lobular necrosis were also observed; however, the intensity of injury was less severe compared with cloned calves. Fatty degeneration, enhancement of the lobular pattern, and central-lobular necrosis can be associated with hypoxia and death (Thomson 1998). In cloned sheep fetuses, hepatic degeneration suggests that the cause of such abnormalities is due to the lack of placental nutrition (De Sousa et al. 2001), as observed in several cloned calves, mainly from the ING. These changes were prominent in clones of advanced age, which may indicate liver regeneration in response to enteral feeding and independence from placental nutrition. The presence of hepatocyte vacuolization in the liver of bovine fetuses (Okano et al. 2003) and bovine neonates from in vitro fertilization (Prestes et al. 2009) has also been reported. It can be inferred, therefore, that some degree of hepatocyte vacuolization is normal in newborn animals. However, moderate to severe degeneration(s) can be interpreted as abnormal findings.

Okano et al. (2003) attributed hepatic vacuolization to the accumulation of glycogen in aborted animals, confirmed by PAS staining. Although hepatic glycogen was found in some animals, which is considered to be normal in mild to moderate amounts (Thomson 1998), a pathological finding (i.e., significant amount)

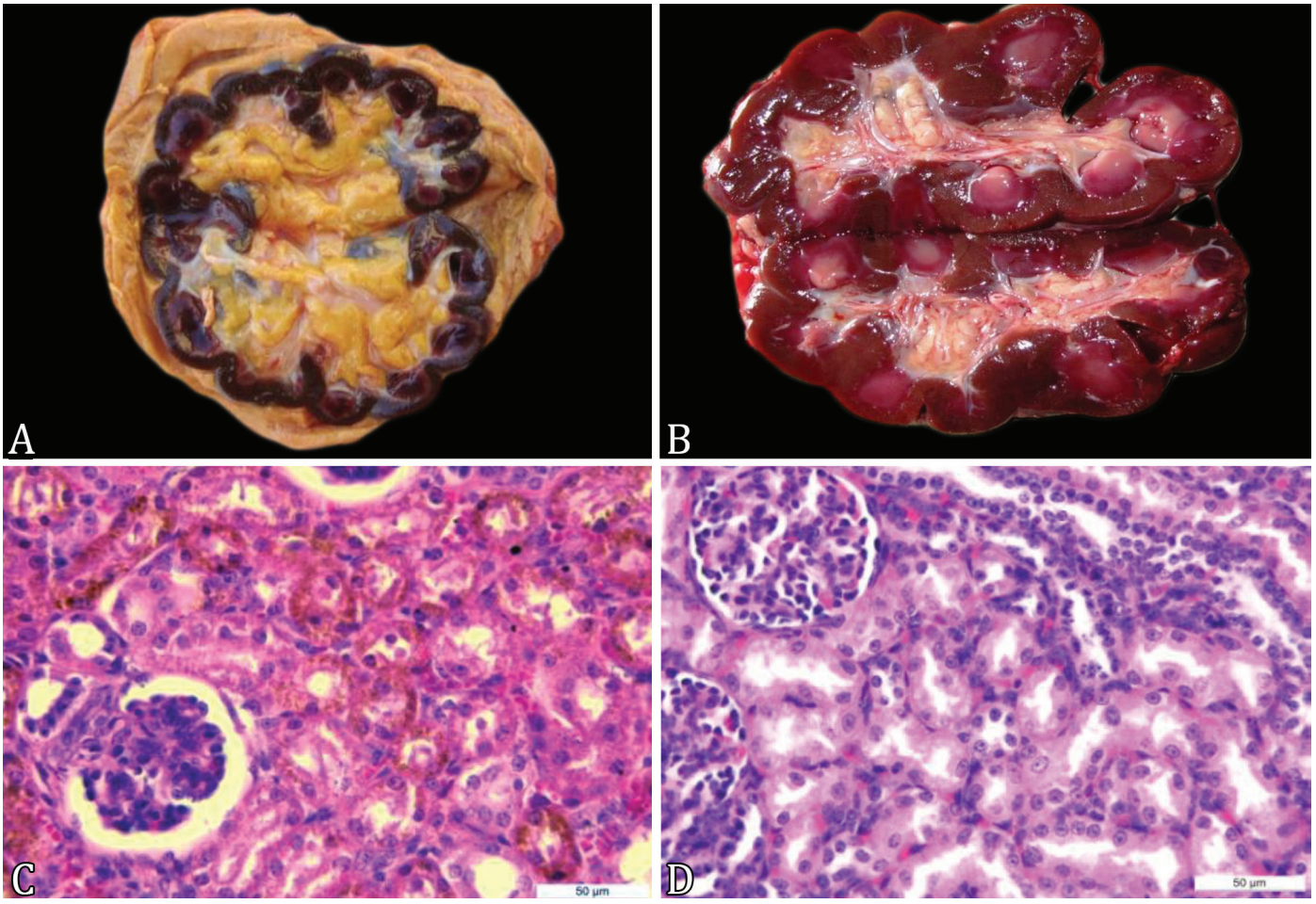


Fig.2. Changes observed in the kidneys of cloned neonates and controls. (**A**) Marked amount of perirenal fat, as well as in the pelvis, goblets, and renal papillae, brownish-black color of the renal cortex, cloned calf. (**B**) Kidney without changes, control calf. (**C**) Presence of brownish granules in the cytoplasm of tubules of the renal cortex, cloned calf. HE, obj.40x. (**D**) Normal appearance of the renal cortical tubules, control calf. HE, obj.40x.

was present in 8 of 41 cloned and 1 of 4 control calves. It was found that changes in DNA methylation patterns in cloned cattle can interfere with hepatic metabolism, leading to changes in glycogen storage and fatty acid composition (Kiefer et al. 2016). It is mainly due to changes in genes, such as *TCF7L2*, in which polymorphisms have been associated with the development of type 2 diabetes. In fact, in the present investigation and several previous studies, moderate to severe fat deposition was observed in various tissues (Chavatte-Palmer et al. 2002, Santos et al. 2010, Maiorka et al. 2015).

In the spleen of cloned animals, macroscopic findings included mild splenomegaly and thickening of the capsule. Regarding

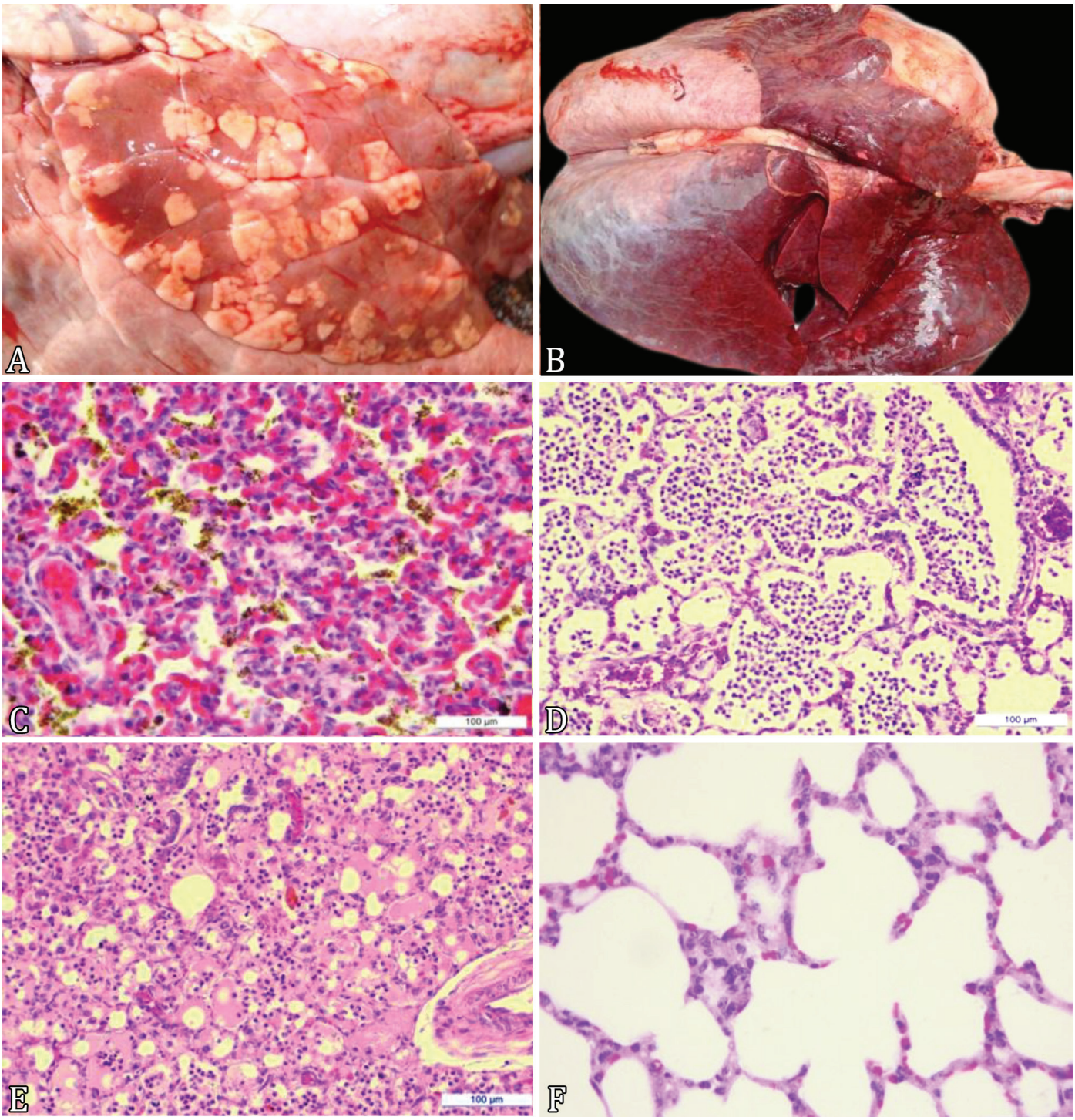


Fig.3. Changes observed in the lungs of cloned and control neonates. (**A**) Areas of atelectasis, cloned calf. (**B**) Pneumonia, areas of consolidation, note the reduced functional pulmonary area. (**C**) Marked infiltration of inflammatory cells, presence of meconium inside the alveoli, cloned calf. HE, obj.10x. (**D**) Marked infiltration of inflammatory cells, cloned calf. HE, obj.10x. (**E**) Marked pulmonary edema, cloned calf. HE, obj.10x. (**F**) Normal appearance of the pulmonary alveoli, control calf. HE, obj.10x.

microscopic findings, congestion was observed because it is related to congestive heart failure, liver cirrhosis, or other causes of increased retrograde pressure (Thomson 1998). This fact is consistent with that observed in the present study, which sometimes revealed thickening of the right ventricle and liver degeneration. Hemorrhage and hemosiderosis are likely due to prolonged congestion (Thomson 1998). Little evidence of white pulp was also observed in the spleens of cloned animals; and previous reports showed marked follicular rarefaction in a 50-day-old calf (Renard et al. 1999), in addition to white pulp hypoplasia and changes in other lymphoid organs in cloned calves (Santos et al. 2010).

In the kidneys of cloned calves, brown granules, proliferation of connective tissue, fibrosis, tubular degeneration, necrosis, and lymphocytic and plasmacytic infiltrates were observed. Several renal lesions have been reported in cloned animals, including cystic formations associated with dilated tubules (Santos et al. 2010), and severe bilateral hydronephrosis associated with obstructive lesions in the urinary system (Wells et al. 1997, Rhind et al. 2003). The presence of brownish granules inside the cells of the cortical and medullary tubules was observed only in the kidneys of cloned calves. It was not possible to accurately identify this pigmentation; however, it is known that the observed pigment does not originate from the accumulation of bile pigments, iron, hemosiderin or glycogen, as no characteristic coloring was evident with the techniques used (i.e., Hall, Perls, and PAS, respectively). Okano et al. (2003) described vacuolization of the renal tubular epithelium in aborted calves that exhibited positive staining using the PAS method. In the present study, PAS staining did not characterize the granules observed in the tubular epithelium. However, there was positive staining in the collecting tubules of the renal cortex using this method. The granules observed exhibited many characteristics similar to those of lipofuscin; however, due to the techniques used in this study, such confirmation was not possible. Although staining for lipofuscin identified the granules, it was not with the expected intensity. Lipofuscin can accumulate within epithelial cells of the renal tubules in older cattle in the form of fine golden granules (Zachary & McGavin 2012). Macroscopically, lipofuscin is evident as brown streaks in the renal cortex, similar to the macroscopic findings reported here. Akagi et al. (2003) described a kidney injury as a deposit of lipofuscin in the tubular epithelium of a cloned calf that died at 13 days of age. It is believed that the presence of such granules warrant further analysis in attempts to determine the origin of the pigment and its relationship with observed renal changes.

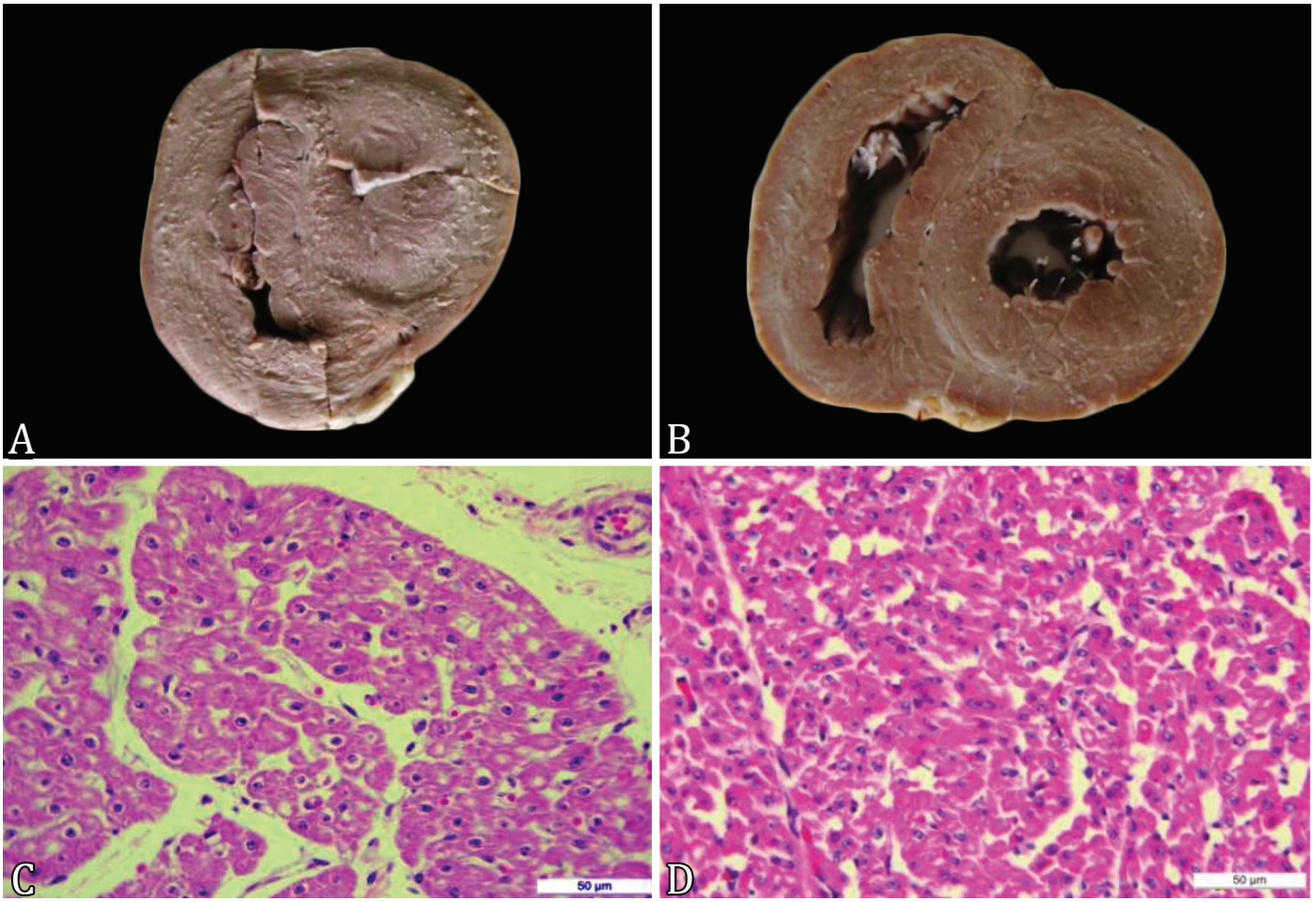


Fig.4. Changes observed in the heart of cloned neonates and controls. (**A**) Cardiac muscle hypertrophy, cloned calf. (**B**) Normal organ characteristic, control calf. (**C**) Vacuolization of cardiomyocytes, cloned calf. HE, obj.40x. (**D**) Note less vacuolization and spacing of cardiomyocytes, control calf. HE, obj.40x.

The lungs were the organs with the highest number of alterations (virtually all cloned calves exhibited some finding in the lungs). In the ING, the most common finding was atelectasis, which is consistent with previous studies (Santos et al. 2010, Maiorka et al. 2015). Atelectasis is characterized by incomplete expansion of the alveoli and can be caused by several factors, including neonatal asphyxia, pulmonary hypertension, and aspiration of amniotic fluid or meconium (Dungworth 1993). The occurrence of atelectasis was found to be determinant of the animals' survival beyond the immediate

Table 3. Microscopic findings in spleen, liver and kidneys of cloned and control calves that died in the neonatal period

Organ/Findings	Clones (n=41)			Control (n=4)
	ING (n=25)	LNG (n=16)	Total	
Spleen				
Reduction in white pulp	14 (58%)	5 (31%)	19 (48%)	0
Hemorrhage	11 (46%)	4 (25%)	15 (38%)	0
Congestion	1 (4%)	8 (50%)	9 (23%)	0
Hemosiderosis	0	2 (13%)	2 (5%)	0
Histiocytic infiltrate	0	1 (6%)	1 (3%)	0
No lesion	7 (29%)	4 (25%)	11 (28%)	4 (100%)
Liver				
Well-defined lobular pattern	25 (100%)	5 (31%)	30 (73%)	4 (100%)
Virtually imperceptible sinusoids	25 (100%)	5 (31%)	30 (73%)	4 (100%)
Closed distance between portal spaces	25 (100%)	9 (56%)	34 (83%)	4 (100%)
Cytoplasmic vacuolization of hepatocytes	23 (92%)	12 (75%)	35 (85%)	3 (75%)
Mild	6 (24%)	9 (56%)	15 (37%)	2 (50%)
Moderate	7 (28%)	1 (6%)	8 (20%)	0
Severe	10 (40%)	2 (13%)	12 (29%)	1 (25%)
PAS negative	10 (40%)	14 (88%)	24 (59%)	1 (25%)
PAS positive (accumulation of glycogen)	15 (60%)	2 (12%)	17 (41%)	3 (75%)
Mild	3 (12%)	2 (13%)	4 (10%)	1 (25%)
Moderate	4 (16%)	0	3 (7%)	1 (25%)
Severe	8 (32%)	0	8 (20%)	1 (25%)
Extramedullary hematopoiesis	4 (16%)	4 (25%)	8 (20%)	0
Congestion	2 (8%)	2 (13%)	4 (10%)	0
Hemorrhage	5 (20%)	0	5 (12%)	0
Necrosis	4 (16%)	1 (6%)	5 (12%)	0
Fibrin	1 (4%)	1 (6%)	2 (5%)	0
Histiocytic infiltrate	1 (4%)	1 (6%)	2 (5%)	0
Neutrophilic infiltrate	1 (4%)	1 (6%)	2 (5%)	0
Duct proliferation	0	1 (6%)	1 (2%)	0
Connective tissue proliferation	1 (4%)	0	1 (2%)	0
Cholestasis	0	1 (6%)	1 (2%)	0
No lesion	0	3 (19%)	3 (7%)	0
Kidneys				
Glomeruli with an embryonic aspect	25 (100%)	11 (69%)	36 (88%)	4 (100%)
Brownish granules within cortical tubule cells	25 (100%)	12 (75%)	37 (90%)	0
Mild	8 (32%)	5 (31%)	13 (32%)	0
Moderate	6 (24%)	5 (31%)	11 (27%)	0
Severe	11 (44%)	2 (13%)	13 (32%)	0
Congestion	8 (32%)	8 (50%)	16 (39%)	0
Hemorrhage	1 (4%)	0	1 (2%)	0
Tubular dilation	2 (8%)	3 (19%)	5 (12%)	0
Connective tissue proliferation	2 (8%)	0	2 (5%)	0
Tubular degeneration	0	2 (13%)	2 (5%)	0
Lymphocytic infiltrate	0	2 (13%)	2 (5%)	0
Fibrosis	0	1 (6%)	1 (2%)	0
Necrosis	0	1 (6%)	1 (2%)	0
Plasmacytic infiltrate	0	1 (6%)	1 (2%)	0

ING = immediate neonatal group, LNG = late neonatal group.

neonatal period. The atelectasis observed in cloned animals could often be associated with pulmonary immaturity and deficiency in surfactant production (Prestes et al. 2009), which leads to chronic pulmonary hypertension. Meconium aspiration observed in several ING animals, and also previously reported in cloned calves (Maiorka et al. 2015), is another factor that may have contributed to the observed atelectasis. Meconium reacts with phospholipids present in the surfactant, which causes failures in this layer and, thus, leads to atelectasis (Bleul 2009). In LNG animals, the most striking finding in the lungs was pneumonia. Many animals that died in the late neonatal period presented with aspiration pneumonia (meconium or milk aspiration), diffuse peritonitis due to ruptured abomasal ulcer(s), umbilical conditions, and passive immunity transfer failure. Therefore, the occurrence of such pulmonary changes is often secondary to other conditions. In septicemia, it is common detachment of septic emboli, which may migrate to the lungs, causing severe pneumonia (Dungworth 1993). Other changes observed in the lungs, such as fibrin deposition, proliferation of type II pneumocytes, thickening of the septum, hyperplasia of type I pneumocytes, proliferation of connective tissue, and necrosis, are common findings in pneumonia and have also been previously reported (Hill et al. 2000, Li et al. 2005, Santos et al. 2010, Maiorka et al. 2015).

In the heart, the fibrillar adhesions observed between the pericardial sac and the epicardium were consistent with an initial condition of fibrinous pericarditis, which, in neonates, may be due to coliform infection acquired by umbilical route (Robinson & Maxie 1993). Lesions suggestive of endocardiosis were observed only in the hearts of cloned animals. Endocardiosis refers to a proliferation of loose connective tissue in the valve(s), accompanied by deposition of glycosaminoglycan, with concomitant collagen degeneration in the fibrous valve (Robinson & Maxie 1993). Lesions related to endocardiosis have not yet been described in cloned animals. A case of hydropericardium was also identified, a lesion previously reported in cloned calves (Maiorka et al. 2015). This change may be related to congestive heart failure; other common causes of hydropericardium include anasarca, emaciating diseases, and neoplasms (Robinson & Maxie 1993). Hypertrophy of the right ventricle, previously described in cloned animals (Garry et al. 1996, Hill et al. 1999, Rhind et al. 2003, Santos et al. 2010), is attributed to congenital pulmonary hypertension (Hill et al. 1999, Rhind et al. 2003). An additional finding in the hearts of cloned calves in the present study was the persistence of foramen ovale and ductus arteriosus, which was also reported (Maiorka et al. 2015). Hematic cysts are generally not attributed to any specific lesion. However, in

Table 4. Microscopic findings in lungs and heart of cloned and control calves that died in the neonatal period

Organ/Findings	Clones (n=41)			Control (n=4)
	ING (n=25)	LNG (n=16)	Total	
Lungs				
Inflammatory cell infiltration	7 (29%)	15 (44%)	22 (55%)	3 (75%)
Histiocytic infiltration	6 (25%)	10 (63%)	16 (40%)	0
Neutrophilic infiltration	3 (13%)	13 (81%)	16 (40%)	2 (50%)
Lymphocytic infiltration	1 (4%)	1 (6%)	2 (5%)	1 (25%)
Atelectasis	16 (67%)	2 (13%)	18 (45%)	0
Pulmonary edema	8 (33%)	6 (38%)	14 (35%)	0
Congestion	6 (25%)	3 (19%)	9 (23%)	4 (100%)
Hemorrhage	4 (17%)	3 (19%)	7 (18%)	0
Meconium	10 (42%)	1 (6%)	11 (28%)	0
Milk	6 (25%)	1 (6%)	7 (18%)	1 (25%)
Fibrin	2 (8%)	3 (19%)	5 (13%)	0
Type II pneumocyte Proliferation	0	3 (19%)	3 (8%)	0
Septal thickening	0	3 (19%)	3 (8%)	0
Thrombus	1 (4%)	2 (13%)	3 (8%)	0
Type I pneumocyte hyperplasia	0	1 (6%)	1 (3%)	1 (25%)
Connective tissue proliferation	0	1 (6%)	1 (3%)	0
Necrosis	0	1 (6%)	1 (3%)	0
Heart				
Vacuolization of cardiac fibers	6 (25%)	5 (31%)	11 (28%)	0
Same spacing of cardiac fiber bundles	10 (42%)	8 (50%)	18 (45%)	0
Histiocytic infiltration	1 (4%)	3 (19%)	4 (10%)	0
Lymphocytic infiltration	3 (13%)	3 (19%)	3 (8%)	1 (25%)
Neutrophilic infiltration	1 (4%)	0	1 (3%)	0
Fibrin	1 (4%)	1 (6%)	2 (5%)	0
Congestion	0	1 (6%)	1 (3%)	0
Hemorrhage	0	1 (6%)	1 (3%)	0
Adherence	0	1 (6%)	1 (3%)	0
No lesion	8 (33%)	7 (44%)	15 (38%)	3 (75%)

ING = immediate neonatal group, LNG = late neonatal group.

the present study, they were observed only in cloned calves. As frequently observed in cloned calves (Santos et al. 2010, Maiorka et al. 2015), especially in animals with heart disease, it is likely that cloned animals develop heart disease with the formation of hematic cysts and persistence of the foramen ovale and ductus arteriosus.

Vacuolization of cardiomyocytes was observed in the present study. However, it was not possible to identify the origin of the vacuoles due to the preparation/storage method of the sample material, which was stored in formaldehyde, thus making specific staining (i.e., Sudan staining) impossible, which requires the material to be frozen. It is probably related to fatty degeneration, similar to the degeneration seen in the liver (Thomson 1998), and analogous to cardiac degeneration in animals from in vitro fertilization (Prestes et al. 2009). The greater spacing of cardiac fibers was an alteration observed in cloned animals to a higher degree than in the CG and is often associated with myocyte vacuolization. The implications of this greater spacing on the health of cloned animals remain unclear, and such reports were not found in the literature.

The increased mortality of newborn cloned calves might be due to an abnormal immunity, increasing the susceptibility to infections. In health calves, the immune system's development is complete at birth, although it is still immature (Cortese 2009). In fact, about half of the cloned calves evaluated here showed reduced spleen white pulp. This fact may had increased the risk for pneumonia and umbilical infection. Additionally, most cloned calves present failures during adaptation to extra-uterine life, being commonly observed prostration, lethargy, absence of the suction reflex and meconium elimination (Birgel Junior et al. 2011). Furthermore, vascular disorders were reported in cloned calves (Maiorka et al. 2015), which was also evident herein. Another reason contributing for the high mortality rate in cloned animals in the present study is impaired kidney function. Most common lesions included presence of brown granules (probably lipofuscin), proliferation of connective tissue, fibrosis, tubular degeneration, necrosis, and lymphocytic and plasmacytic infiltrates.

CONCLUSIONS

The main causes of death among cloned calves that died in the immediate neonatal period were liver damage, characterized mainly by marked hepatic degeneration, and difficulties in adapting to extrauterine life, clinically demonstrated as respiratory difficulty and pathologically as pulmonary disease (more specifically atelectasis), which circumstantially contributed to the death of these animals.

In calves that experienced late neonatal death (i.e., LNG), the most notable finding was severe pneumonia, which in many cases was secondary to other concurrent diseases. It is believed that this type of pneumonia is a significant contributor to death.

Findings in the kidneys of cloned animals warrant further investigations to determine the origin of the granules inside the cortical tubules and their relationship with other abnormalities in these animals. These changes may be caused by changes in DNA methylation pattern(s), resulting in epigenetic changes in animal metabolism.

Conflict of interest statement.- There are no conflict of interests.

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Isolation and genotyping of *Mycobacterium bovis* in suggestive lesions of tuberculosis in cattle slaughtered in the state of Ceará, Brazil[1]

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ABSTRACT.- Ferreira F.F., Oliveira M.L.M., Alzamora Filho F., Santos B.R., Araújo L.C.S.S.C., Santos E.S.V., Gomes H.M. & Costa J.N. 2020. **Isolation and genotyping of *Mycobacterium bovis* in suggestive lesions of tuberculosis in cattle slaughtered in the state of Ceará, Brazil**. *Pesquisa Veterinária Brasileira 40(11):863-870*. Agência de Defesa Agropecuária do Estado do Ceará, Avenida Dr. José Martins Rodrigues 150, Edson Queiroz, Fortaleza, CE 60811-520, Brazil. E-mail: felipefrancelinovet@gmail.com

Bovine tuberculosis (BTB) is a zoonosis caused by the bacterium *Mycobacterium bovis*, which induces the development of nodular and granulomatous lesions in various animal tissues. The recognition of these suggestive gross lesions during *postmortem* sanitary inspection in slaughterhouses provides a presumptive diagnosis, which requires the use of complementary tests to confirm the disease. This study aimed to verify the occurrence of BTB in cattle slaughtered in slaughterhouses in the state of Ceará, Brazil, using bacteriological and molecular methods. To this end, suggestive lesions were analyzed on carcasses condemned by the "Serviço de Inspeção Estadual" (SIE). The samples were submitted to microbiological analysis using culture media and specific staining followed by spoligotyping molecular technique for identification and genotyping of the mycobacteria. Occurrence of lesions suggestive of BTB was verified in bovine carcasses (0.071%) from different municipalities of the state. These lesions were located mainly in the lung (95.12%), lymph nodes (58.53%), and liver (36.58%). Microbiological culture showed bacterial isolation (17.94%), with the growth of colonies showing morphological and tannic characteristics belonging to genus *Mycobacterium* spp. Genetic polymorphism analysis identified *M. bovis* in all isolates, which were discriminated into six spoligotypes (SB0121, SB0295, SB1064, SB0120, SB0870, and SB0852). These profiles have been described in Brazil and several areas of the world, except for profiles SB1064 and SB0852, which were described in the country for the first time. The results show that the association of the diagnostic methods used was the basis for the first study on identification of mycobacteria found in the state, which may provide a database for the epidemiological study of BTB in the state of Ceará.

INDEX TERM: Genotyping, *Mycobacterium bovis*, lesions, tuberculosis, cattle, slaughter, Brazil, sanitary inspection, zoonosis, bovine tuberculosis, spoligotyping.

RESUMO.- [**Isolamento e tipificação genética de *Mycobacterium bovis* em lesões sugestivas de tuberculose em bovinos abatidos no estado do Ceará.**] A tuberculose bovina (TB) é uma zoonose causada pelo *Mycobacterium bovis*, o qual induz ao desenvolvimento de lesões nodulares e granulomatosas em vários tecidos do animal. O reconhecimento dessas lesões macroscópicas sugestivas durante a inspeção sanitária *post mortem* em matadouros fornece um diagnóstico presuntivo, sendo necessário a utilização de testes complementares para confirmação da doença. O objetivo deste trabalho foi verificar

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a ocorrência da TB em animais abatidos em matadouros-frigoríficos no estado do Ceará através da utilização de métodos bacteriológicos e moleculares. Para tanto, foram analisadas lesões sugestivas de TB em carcaças condenadas pelo Serviço de Inspeção Estadual (SIE). As amostras foram submetidas à análise microbiológica, utilizando meios de cultivo e de coloração específicos, seguida pela técnica molecular *spoligotyping* para identificação e tipificação genética da micobactéria. Verificou-se a ocorrência de lesões sugestivas de TB em carcaças bovinas (0,071%) oriundas de diferentes municípios do estado do Ceará. Essas lesões estavam localizadas principalmente no pulmão (95,12%), linfonodos (58,53%) e fígado (36,58%). O cultivo microbiológico obteve isolamento bacteriano (17,94%), com o crescimento de colônias apresentando características morfológicas e tintoriais pertencentes ao gênero *Mycobacterium* spp. A análise do polimorfismo genético identificou a presença de *M. bovis* em todos os isolados, que foram discriminados em seis espoligotipos (SB0121, SB0295, SB1064, SB0120, SB0870 e SB0852), descritos no Brasil e em diversas áreas do mundo, exceto os perfis SB1064 e SB0852 que foram descritos pela primeira vez no país. Os resultados obtidos demonstram que a associação dos métodos diagnósticos utilizados foram a base do primeiro estudo de identificação das micobactérias encontradas no estado do Ceará, o que pode contribuir para a criação de um banco de dados para o estudo epidemiológico da TB no estado.

TERMOS DE INDEXAÇÃO: Tipificação genética, *Mycobacterium bovis*, lesões, tuberculose, bovinos, abatedouro, Brazil, inspeção sanitária, zoonose, tuberculose bovina, spoligotyping.

INTRODUCTION

Bovine tuberculosis (BTB) is an infectious and contagious disease of progressive character and chronic evolution caused mainly by the bacterium *Mycobacterium bovis*. It is characterized by development of nodular and granulomatous lesions in different organs, mainly in the lung, since the main form of transmission occurs through inhalation of aerosols (Paes & Franco 2016). BT is of great importance in public and animal health due to its zoonotic potential, determining a situation of high morbidity and mortality in humans (Pérez-Lago et al. 2014). In addition, it causes marked economic losses in cattle herds as a result of animal death, reduced milk production and weight gain, reproductive changes, animal disposal, and carcass condemnation (Pacheco et al. 2009, Paes & Franco 2016).

Brazil has the largest commercial cattle herd globally, with approximately 214.89 million head (IBGE 2017). Consequently, animal products destined for the domestic and foreign markets must present high quality and low health risk.

onducting epidemiological surveys in slaughterhouses is an indicator of BT prevalence in cattle destined for slaughter and, therefore, of the probability of human exposure through infected meat consumption (Biffa et al. 2010). In the state of Ceará, surveillance for BT in slaughterhouses has not been reported. Only one epidemiological survey on BT has been identified in the state of Ceará. This survey was carried out in rural settlements in the municipality of Quixerambim, in which 248 cattle were submitted to tuberculization and no positive animals were identified. However, it cannot be said that there is no tuberculosis in cattle in the surveyed region, as other factors may have contributed to test negativity. These factors include the voluntary adherence of the participating owners and the drought that spread in the region, which may have contributed to the death of debilitated and sick animals, limiting the spread of the disease (Nascimento 2014).

It is known that results obtained from surveillance in slaughterhouses can indicate BT health status in the herd. However, due to the similarity between the disease's lesions and other nodular and granulomatous inflammatory processes, it is often impossible to identify all sick animals (Roxo 1997, Mendes et al. 2013). In general, recognition of gross lesions associated with BT during routine health inspection in slaughterhouses has been the only measure used to diagnose the disease, which may incur errors in its detection, especially in paucibacillary lesions. It is recommended that complementary diagnostic tests be used in these lesions to minimize these misunderstandings (Furlanetto et al. 2012).

Microbiological culture of the etiologic agent is the certified method for direct diagnosis. However, it presents low sensitivity and prolonged time between primary isolation and final identification of the species (Corner 1994). In contrast, an alternative that enables rapid identification of mycobacteria is the application molecular techniques, which can distinguish between different strains, allowing increased sensitivity and specificity and a decrease in the number of diagnoses of this infection (Ramos et al. 2015). Some studies have demonstrated the possibility of using polymerase chain reaction (PCR) and spoligotyping techniques to detect and type *M. bovis* in fragments of lesions collected in slaughterhouses (Rodriguez et al. 2004, Costa et al. 2012, Parreiras et al. 2012, Alzamora Filho et al. 2014b).

This study aimed to analyze the occurrence of BTB in the state of Ceará through the isolation, identification, and genetic characterization of *M. bovis* from gross lesions suggestive of tuberculosis in cattle slaughtered in slaughterhouses under the "Serviço de Inspeção Estadual".

MATERIALS AND METHODS

Between August 2017 and September 2018, slaughter and *postmortem* inspection of cattle were monitored by the "Serviço de Inspeção Estadual" (SIE) of the state of Ceará in three slaughterhouses located in the municipalities of Iguatu and Juazeiro do Norte and of Maracanaú in the metropolitan region of Fortaleza, south and center-south of the state. Data on the animals' origin were obtained from the "Guia de Trânsito Animal" (GTA). An additional assessment of cattle traceability was carried out by analyzing the health records of producers and rural properties obtained from the "Agência de Defesa Agropecuária do Estado do Ceará", Ceará state (ADAGRI 2019).

According to methods recommended by Ministério da Agricultura, Pecuária e Abastecimento (MAPA) through the "Regulamento da Inspeção Industrial e Sanitária de Produtos de Origem Animal" - RIISPOA (Brasil 2017), samples of lesions suggestive of BTB were collected by veterinary medical inspectors during routine health inspection of beef. Data on the characteristics and location of these lesions were recorded on slaughter follow-up forms.

For bacteriological exams, the collected samples were placed in sterile universal type collectors, identified, and stored at -20°C or in saturated sodium borate decahydrate solution ($Na_2B_4O_7 \cdot 10H_2O$, 140g/L), which preserves mycobacteria for up to 60 days at room temperature (Richards & Wright 1983). Subsequently,

the samples were sent to the "Laboratório de Micobacterioses" of the "Universidade Estadual de Santa Cruz" (LAMVET-UESC), Ihéus, state of Bahia, for processing and bacteriological analysis. Initially, the tissue fragments were decontaminated using 1.5% 1-hexadecylpyridinium chloride (HPC) for 30 min. Then they were inoculated in Stonebrink-Leslie and Lowenstein-Jensen culture media and incubated at 37 °C for up to 90 days. After the cultivation period, the isolated colonies were stained using the Ziehl-Neelsen (ZN) method for staining characterization of alcohol-acid resistant bacilli (AARB) (Rodriguez 2005, Brasil 2008).

From the isolates characterized as AARB, a bacterial suspension was performed in 200µL of Tris-EDTA (TE) buffer solution (10mM Tris-HCl; 1mM EDTA; pH 8.0) to inactivate the bacterium, and later DNA extraction was obtained by thermal lysis through incubation at 90 °C for 30 min, followed by centrifugation (Shi et al. 2018). The resulting DNA was taken to the "Laboratório de Biologia Molecular" applied to mycobacteria of the "Fundação Oswaldo Cruz" (FIOCRUZ - Rio de Janeiro), where molecular analyses were carried out.

Identification of *Mycobacterium bovis* was confirmed through molecular typing, and analysis of genetic polymorphism was performed using the Spoligotyping technique, as described by Kamerbeek et al. (1997). Direct region amplification was performed in a final volume of 50 µL with 0.2 mM containing dNTP (10mM Tris-HCl, pH 8.0; 50mM KCl), 1.5mM MgCl2, 20 pmol of primers for each primer (biotinylated DRa and DRb), and 1U of Taq DNA polymerase and 10ng of DNA. The polymerase chain reaction (PCR) consisted of an initial denaturation at 96 °C for 3 min., 20 cycles of amplification at 96 °C for 1 min., 55 °C for 1 min, and 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. *M. bovis* BCG DNA was used as a positive control, whereas ultrapure water was the negative control. The PCR products were membrane hybridized containing 43 oligonucleotides of known spacer sequences. After incubation with streptavidin-peroxidase, the spacers were detected by chemiluminescence. The polymorphic patterns obtained in hybridization were compared with an international database for *M. bovis* available in the literature (Kremer et al. 1999, Van Embden et al. 2000, Haddad et al. 2001). The nomenclature adopted for the spoligotypes obtained was the same as that available in the Sitvit Web database (Machado et al. 2014) and on the website[8].

RESULTS

In the study period, 57,347 cattle were evaluated during the inspection routine for health on *ante-mortem* examination, but did not undergo tuberculosis screening tests. Of these, 41 animals (0.071%) presented lesions suggestive of tuberculosis at *postmortem* inspection and had their carcasses condemned in whole or in part by the SIE inspectors. Considering the gross findings of the official service, the percentage of condemnation (slaughtered cattle condemned because of lesions suggestive of BTB) was 0.32% (24/7374), 0.06% (15/25694), and 0.01% (2/24279) in the three slaughterhouses under the SIE of the state of Ceará (Table 1). The analysis of the mandatory animal health documents (GTA) showed that the sampled cattle belonged to herds from 13 municipalities located in different regions of the state of Ceará: Fortaleza, Caucaia, Cascavel, Ocara, Tauá, Saboeiro, Quixelô, Jucás, Iguatu, Icó, Cedro, Cariús, and Crato, with a positive diagnosis of BTB confirmed in three of these municipalities (Jucás, Iguatu, and Quixelô), as shown in Figure 1.

The condemned animals presented firm, nodular lesions, focal to multifocal, with a purulent or caseous aspect, presence of fibrous capsule, in some cases showing areas of calcification in the center of the lesion, evidenced by knife creaking at cut. These lesions, present in one or more tissues of the same animal, were located mainly in the lungs (95.12%) and lymph nodes (58.53%), as well as on the head, neck, thoracic lymph nodes, and liver (36.58%). In addition, two cattle were identified with suggestive nodules in the spleen and kidney, each associated with liver and lung injuries (Table 2). At the gross examination

Table 1. Occurrence of cattle with lesions suggestive of bovine tuberculosis (BTB) in slaughterhouses under the "Serviço de Inspeção Estadual" (SIE) in the state of Ceará, Brazil

Slaughterhouses SIE	Number of cattle		Condemnation (%)
	Slaughtered	Suggestive BTB lesions	
A	24,279	2	0.008
B	7374	24	0.325
C	25,694	15	0.058
Total	57,347	41	0.071

Table 2. Gross lesions suggestive of bovine tuberculosis (BTB) in animals condemned by the "Serviço de Inspeção Estadual" (SIE) in the state of Ceará, Brazil

Lesion location	Number of lesions*	Occurrence of lesions in condemned animals (%)
Lung	39	95.12
Lymph nodes	24	58.53
Liver	15	36.58
Spleen	01	2.43
Kidney	01	2.43

*N = 41 bovines; in total, 80 fragments of lesions located in different organs were collected, and one or more lesions suggestive of BTB were collected from the same animal (sample).

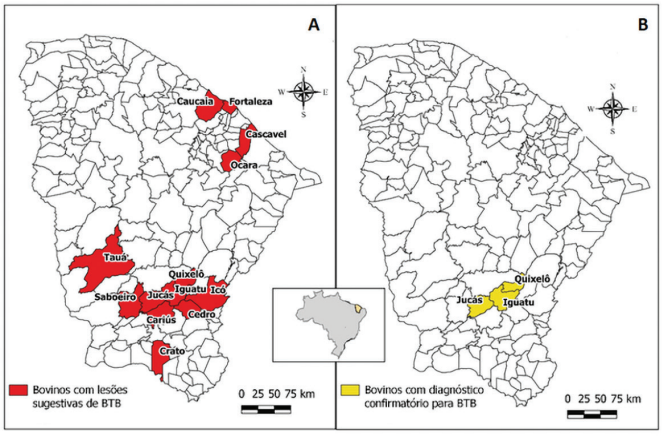


Fig.1. Map of the state of Ceará, Brazil, highlighting (**A**) origin municipalities of the cattle with lesions suggestive of bovine tuberculosis (BTB) condemned by the Serviço de Inspeção Estadual (SIE), (**B**) municipalities where the bovines came from, whose suggestive lesions had a confirmatory diagnosis for *Mycobacterium bovis*.

[8] Available at <www.mbovis.org>

of the lung lesions, the initially noticeable nodules on the lobes surface were deepened by cutting the parenchyma. Bovine carcasses that showed lesions not suggestive of BTB, considered only as common or nonspecific lymphadenitis by the SIE evaluation, did not have their samples collected.

Of the samples of presumptive BTB lesions collected by the official service, two were discarded due to tissue autolysis, and 39 were processed and cultured. The bacteriological analysis revealed bacterial isolation in 17.94% (7/39) of the samples, with growth of small, rounded, cream-yellowish colonies with irregular edges and granular surface in the Stonebrink-Leslie culture medium, with average observed time for the appearance of colonies of 28 days- minimum of 16 days and maximum of 32 days. Of the seven positive samples in the Stonebrink-Leslie medium, growth of colonies was observed in three samples in the Lowenstein-Jensen culture medium, with an average time observed for the appearance of colonies of 45 days (minimum 32 days and maximum 64 days). All smears from the 10 isolated colonies presented alcohol-acid resistant bacilli ($AARB^{+}$) when subjected to Ziehl-Neelsen (ZN) staining.

Considering the stratification by sex and age group of cattle with positive bacteriological diagnosis for *Mycobacterium* spp., it was found that 85.71% (6/7) of the animals were female, and only 14.29% (1/7) were male. The age group most affected was over 36 months, with 83.33% (5/6) of positive females having this age. Of the positive animals, only one young female (16.67%), aged 13-24 months, was affected by the disease. The municipality of Iguatu stood out as the most affected by BTB, representing the origin of 71.42% (5/7) of the positive cattle diagnosed in the study, and the positive animals were all from different herds and properties (Table 3).

In the genetic material of the isolates submitted to the spoligotyping technique, six spoligotypes were identified as *Mycobacterium bovis*. The studied profiles showed deletions in spacers 3, 9, 16, and 39-43, characterizing the pathogen. Of the seven samples, three showed two different spoligotypes each, which justifies seven positive animals and 10 isolates. Six spoligotypes previously identified in the databases were observed in the 10 AARB isolates. Three of them formed clusters of genotypes and three had a single profile. Three AARB isolates had spoligotype profile SB0121, two presented SB0295, two showed SB1064, and the single profiles SB0120, SB0852, and SB0870 were found in one isolate (Table 4).

DISCUSSION

This study found that, according to the SIE, based on the occurrence of gross lesions suggestive of BTB (0.071%), this disease is present in the cattle herd of the state of Ceará. Condemnation of cattle carcasses with suspicion of BTB were previously reported by Baptista et al. (2004), who found a prevalence of 0.07% of suggestive lesions in animals slaughtered in Minas Gerais. There is a wide variation in BTB prevalence from *postmortem* inspection of cattle in Brazil (0.17 to 5.16%). This difference may be related to the degree of regional development, especially concerning sanitary measures in the herds (Araújo 2004).

In this study, the carcass condemnation rate (CCR) per establishment ranged from 0.01 to 0.32%. These data are probably related to the slaughtering particularities in each establishment, such as type of exploitation, density, and health practices. In the slaughterhouse with the lowest CCR (SIE - A), in addition to having a higher slaughtering volume, most of the animals were originated from extensively reared beef herds, a category in which BTB presents sporadic occurrence

Table 3. Origin and stratification by sex and age group of cattle with confirmatory diagnosis for bovine tuberculosis (BTB) in the state of Ceará, Brazil

Animal	Sex		Age group (in months)				Municipality of origin
	Female	Male	0 to 12	13 to 24	25 to 36	>36	
Bovine 1	1	-	-	-	-	1	Iguatu
Bovine 2	1	-	-	-	-	1	Iguatu
Bovine 3	1	-	-	-	-	1	Iguatu
Bovine 4	-	1	-	-	1	-	Jucás
Bovine 5	1	-	-	-	-	1	Quixelô
Bovine 6	1	-	-	1	-	-	Iguatu
Bovine 7	1	-	-	-	-	1	Iguatu
TOTAL	6	1	-	1	1	5	-

Table 4. Spoligotyping of *Mycobacterium bovis* strains isolated from cattle slaughtered in slaughterhouses under the "Serviço de Inspeção Estadual" (SIE) in the state of Ceará, Brazil

Spoligotype pattern*	Spoligotype profile**	Isolates (n)	Municipality of origin
SB0121	■■□■■■■■□■■■■■■□■■■■□■■■■■■■■■■■■■■■■□□□□□	3	Iguatu
SB0295	■■□■■■■■□■■■■■■□■■■■□■■■■■■■■■■■■■■■□■□□□□□	2	Quixelô
SB1064	■■□■■■■■□■■■■■■□■■■□■□■■■■■■■■■■■■■■■□□□□□	2	Iguatu, Jucás
SB0120	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	1	Iguatu
SB0852	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■□■□□□□□	1	Iguatu
SB0870	■■□■■■■■□■■■■■■□■■■□■■■■■■■■■■■■■■■■■■□□□□□	1	Iguatu

* International name given by Mbovis.org. and SitVitWeb, ** Black and white boxes indicate the presence and absence, respectively, of the specific spacer in position 1-43 in the direct repeat (DR) locus.

(Rodriguez 2005). In contrast, in the establishment with the highest CCR (SIE - B), this value is possibly related to the increased slaughter of animals discarded from dairy or mixed herds concentrated in the region, cases in which the probability of BTB occurrence increases (Costa et al. 2012). Moreover, detection of lesions suggestive of BTB depends on the time available to scrutinize the carcass. A short time may be insufficient to identify discreet lesions, and this identification may also be hindered by lack of inspection experience. In this way, the slaughter inspection accuracy probably varies between regions and slaughterhouses (Perrez et al. 2002, Alzamora Filho et al. 2014a).

In the present study, it was observed that the organ most affected by lesions suggestive of BTB was the lung, followed by the lymph node chain, and the liver, which is in agreement with results obtained in other studies (Alzamora Filho et al. 2014a, Silva et al. 2014). The more significant occurrence of lung lesions may be directly related to inhalation of contaminated aerosols, which are eliminated through the cough or nasal discharge of infected animals, occurring mainly in dairy herds as a result of high population density and confinement (Paes & Franco 2016). The predominant lesions in this study are in line with the description of focal, multifocal, or diffuse nodular lesions in affected organs (Costa et al. 2013), with a high occurrence of caseous and calcified lung injuries (Silva et al. 2014). In the generalized form of BTB, dissemination of the bacillus reaches several organs such as the spleen, udders, and kidneys (Oliveira et al. 2012), corroborating the gross findings of detected renal and splenic lesions.

Regarding the bacteriological analysis, considering the characteristics of the colonies described by Corner (1994), colonies compatible with *Mycobacterium bovis* were isolated in only seven samples, which reflected a sensitivity of 17.94% for this method. Due to the characteristics of the lesions obtained in slaughterhouses, many isolations of the pathogen were expected. As verified by Fráguas et al. (2008), in the present study, the low sensitivity of the bacteriological culture may be associated with the decontamination techniques used in the processing of samples. These techniques may have contributed to death of the bacilli and the consequent low index of mycobacterial isolation, especially in paucibacillary samples. Low rates of mycobacterial isolation have been reported in previous studies addressing lesions suggestive of BTB (Pinto et al. 2002, Araújo et al. 2005). It can also be explained by the occurrence of nodular and granulomatous inflammatory processes determined by other etiologies, with specific characteristics, and undifferentiated gross features of BTB, such as lymphosarcoma, actinomycosis, actinobacillosis, and nocardiosis (Roxo 1997, Mendes et al. 2013, Paes & Franco 2016). The absence or deficiency of viable pathogens in suggestive gross lesions may also be associated with the difficulty of the pathogen to multiply in culture, because the lesions present a bacillary concentration below 10,000 CFU/mL (Zanini et al. 2001), and the death of mycobacteria after promoting the lesion by defending the organism from the chronic nature of the disease (Pinto et al. 2002, Fráguas et al. 2008). A reduced number of *M. bovis* isolates have also been observed in studies that bacteriologically evaluated both lesions suggestive of BTB and nonspecific lymphadenitis during *postmortem* examination (Furlanetto et al. 2012, Alzamora Filho et al. 2014a). Considered the gold standard for BTB diagnosis, higher isolation rates are expected in bacteriological culture, with mycobacterial growth rates that can vary from 60.0 (Nassar et al. 2007) to 68.75% (Silva et al. 2018) of the suggestive lesions. In contrast, despite the low frequency of positive samples verified in the present study, ZN staining revealed $AARB^{+}$ in 100% of the isolates, confirming the high identification specificity of the mycobacterial method (Corner 1994).

In Brazil, BTB is endemic and affects many dairy properties, since animals are subjected to confinement and high metabolic demand (Brasil 2006). The occurrence of lesions suggestive of BTB had a confirmatory diagnosis for *M. bovis* in municipalities in the center-south region of Ceará (Fig.1B), a critical dairy basin in the state, with emphasis on the municipality of Iguatu, which is among the 10 municipalities with highest production (IPECE 2018). Among the positive cattle, females aged >36 months were the most affected, suggesting that they were culling cows with advanced age for reproduction, probably from dairy or mixed herds located in the affected region. Similar to our results, Grisi Filho et al. (2011), when evaluating animals condemned for BTB according to the isolation of *M. bovis* in the state of São Paulo, reported greater positivity in females (58.89%). Other studies conducted with female bovines slaughtered at old age, which demonstrated a high occurrence of lesions suggestive of BTB followed by significant positivity rates in bacterial isolation, have also been found in the literature (Damina et al. 2011, Okeke et al. 2014). Those authors suggested that the findings may be related to the chronic character of the disease and the increased exposure of the animals to mycobacteria over time, considering that females have a longer productive life.

In association with classic bacteriological isolation, the use of complementary molecular methods to identify mycobacteria is more sensitive and efficient for a definitive diagnosis of BTB. Spoligotyping is a molecular technique that allows simultaneous detection and typification of the *M. tuberculosis* complex (Kamerbeek et al. 1997). It is referred as the technique of choice for comparing the strain with few copies of IS 6110. Another advantage is the differentiation between *M. bovis* and *M. tuberculosis* due to the absence and presence, respectively, of spacers 39 to 43 (Rodriguez et al. 2004). Research on spoligotypes was a vital tool to identify and typify the genotypes of *M. bovis* present in cattle herds in the state of Ceará. In the present study, six spoligotypes were identified: SB0121, SB0295, SB1064, SB0120, SB0852, and SB0870 with deletions in spacers 3, 9, 16, and 39-43, which is characteristic of *M. bovis* strains (Kamerbeek et al. 1997, Haddad et al. 2004).

Spoligotype SB0121 was the most frequent in the present study (30%), with a cluster in three isolates registered in the municipality of Iguatu. This predominant genetic profile is in line with the results obtained in other studies in Brazil (Parreira et al. 2012, Rocha et al. 2013, Alzamora Filho et al. 2014b, Ramos et al. 2014, Cazola et al. 2015, Carvalho et al. 2016). In addition to being described as the most frequent spoligotype in Brazil, Mexico (Zumárraga et al. 2013), and Portugal (Ghavidel et al. 2018), it has also been reported in countries such as Argentina, Belgium, Germany, Spain, France, United Kingdom, Italy, Sweden, Tunisia, Uruguay, the United States, and South Africa, as verified in the Sitvit Web database (Machado et al. 2014).

Spoligotypes SB0295 and SB1064 formed the other two clusters, with two isolates each. SB0295 had a prevalence of 24% (Zumárraga et al. 2013), and has been described in the states of Mato Grosso, Goiás (Carvalho et al. 2016) Paraíba, Federal District, Minas Gerais, Mato Grosso do Sul and Santa Catarina (Parreiras et al. 2012), São Paulo (Rocha et al. 2013), and Bahia (Alzamora Filho et al. 2014b). This spoligotype has also been reported in Argentina, Belgium, Costa Rica, Spain, Portugal, France, Mexico, and the United States (Sitvit Web). In contrast, spoligotype SB1064 has had its pattern isolated in Portugal (Matos et al. 2010) and Mexico (Sitvit Web).

The profiles SB0120, SB0870, and SB0852 were isolated, each in different samples from the municipality of Iguatu. Spoligotype SB0120 was the most common circulating type on several continents (Ghavidel et al. 2018). In Brazil, it was identified in the states of Paraíba and the Federal District (Parreiras et al. 2012), Bahia (Costa et al. 2012), and São Paulo (Rocha et al. 2013), having importance in other regions of the world. This spoligotype is widespread in several European countries, being mostly described in France with a prevalence of 26% of the isolates (Haddad et al. 2001). However, it has also been found in Africa and Asia (Sitvit Web). In addition, cases of human tuberculosis caused by *M. bovis* (spoligotype SB0120) have been reported in the United Kingdom (Gibson et al. 2004), indicating the zoonotic potential of this profile. Spoligotype SB0870, in turn, was reported in a single isolate identified in a sample obtained in the state of São Paulo (Rocha 2013), as well as in some European countries such as Germany, Denmark, and France (Sitvit Web). Spoligotype SB0852 has been reported only in France and the United States (Sitvit Web).

With the molecular discrimination of spoligotypes SB1064 and SB0852, the present study seems to have generated unprecedented results in identifying the polymorphism of genetically typified *M. bovis* isolates in Brazil. This study seems to be the first report of these spoligotype profiles in the country.

The identification of *M. bovis* through bacteriological and molecular methods confirmed the existence of BTB focus properties in the state of Ceará. The tracking of the bovines' origin with confirmatory diagnosis, associated with the evaluation of the transport history of the focus properties, allowed us to identify that the animals of these herds were entered or discharged from properties from 17 different municipalities in the state of Ceará. Bovines with lesions suggestive of BTB originating from six of these municipalities were identified (Fig.1A).

The focus properties showed several cattle, between 52 and 457 animals, with characteristics of mixed or dairy herds. In general, these properties acquired cattle from other rural properties through intra and/or inter-municipal transport, and were characterized by intense movement of animals due to the accentuated inter-municipal cattle trade. In this internal traffic, it is not mandatory to carry out screening exams for GTA issuance. In addition, there was interstate transport of cattle, including the egress of animals to the states of Paraíba and Bahia, and admission from the state of São Paulo - this official transport with exams and GTA issuance. However, there may be unrecorded irregular transport of animals without compliance with sanitary requirements. Previous studies pointed out BTB prevalence or occurrence with a confirmatory diagnosis of *M. bovis* in these states (Rocha et al. 2013, Alzamora Filho et al. 2014a, Ramos et al. 2018). This suggests a possible route for the movement of infected animals between the state of Ceará and these geographic regions, especially with the states of Bahia (Costa et al. 2012, Alzamora Filho et al. 2014a) and São Paulo (Rocha et al. 2013), where the presence of *M. bovis* strains similar to those identified in this work were previously identified (SB0120, SB 0121, and SB0295).

The transit of cattle with BTB between properties, regions, or countries represents a potentially critical factor for the transmission of this disease (Skuce et al. 2012), which can be confirmed by the genetic diversity of *M. bovis* isolates from bovine carcasses in the state of Ceará. The risk factors frequently identified and described for the disease include free movement of possibly infected animals, occurrence of the disease on the property and in neighboring areas, number of animals, type of exploration, and use of the intensive system, in which herds are kept together for long periods, and entry of animals from herds with a history of BTB (Skuce et al. 2012, Bahiense et al. 2016, Barbieri et al. 2016, Belchior et al. 2016, Dias et al. 2016). Additionally, the history of cattle transported to local livestock events, such as livestock shows and events (*vaquejadas*), was also verified in focus properties of the present study, which are characterized as important areas of risk for BTB due to the agglomeration of animals of different origins (Brasil 2006).

CONCLUSIONS

The association of *post mortem* BTB diagnosis techniques with molecular genotyping techniques were the basis of the first study to identify mycobacteria in the state of Ceará, and this work was the starting point for the creation of a database of great relevance to the epidemiological study of this disease in the state.

In addition, among the genetically typified *Mycobacterium bovis* isolates, two spoligotypes that had not yet been reported in Brazil were identified (SB1064 and SB0852). Therefore, this study is the first report on these profiles in the country.

Conflict of interest statement.- The authors declare having no conflict of interest concerning the publication of this article.

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Eimeria spp. infection in lambs from southern Brazil[1]

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ABSTRACT.- Martins N.S., Motta S.P., Santos C.C., Moreira A.S., Farias N.A.R. & Ruas J.L. 2020. ***Eimeria* spp. infection in lambs from southern Brazil**. *Pesquisa Veterinária Brasileira 40(11):871-874*. Departamento de Microbiologia e Parasitologia, Instituto de Biologia, Universidade Federal de Pelotas, Campus Universitário, Travessa André Dreyfus s/n, Capão do Leão, RS 96010-900, Brazil. E-mail: jeronimo.ruas@gmail.com

Eimeria infections are common in the sheep industry worldwide. Lambs are more susceptible to coccidiosis, especially in stressful conditions, being infected by different species of the parasite. *Eimeria crandallis* and *Eimeria ovinoidalis* are considered the most pathogenic, causing reduced growth, dehydration, anorexia, and death. In this study, the frequency of *Eimeria* species was evaluated in lambs from the southern region of the Rio Grande do Sul state, Brazil. Fecal samples from 248 lambs, from 19 farms, were tested for the presence of oocysts. The positive samples were re-examined and the sporulated oocysts analyzed morphometrically to identify the presence of *Eimeria* species. In 100% of the evaluated farms, there were animals positive for the protozoan. The frequency of *Eimeria* species was: *E. ovinoidalis* (94.74%), *E. crandallis* (89.47%), *E. granulosa* (78.95%), *E. parva* (68.42%), *E. ahsata* (63.13%), *E. punctata* (42.11%), *E. bakuensis* (36.84%), *E. faurei* (10.53%), and *E. pallida* (5.26%). Mixed infection was found in 94.74% of the samples. This research describes, for the first time, the occurrence of *E. crandallis* and *E. ovinoidalis* infecting lambs in the study area. The wide distribution of this protozoan and the high frequency of pathogenic species show the importance and potential damage of sheep coccidiosis in herds from Rio Grande do Sul.

INDEX TERMS: Infection, lambs, Brazil, sheep industry, coccidiosis, *Eimeria crandallis*, *Eimeria ovinoidalis*.

RESUMO.- [**Infecção por *Eimeria* spp. em cordeiros do sul do Brasil.**] As infecções por *Eimeria* são comuns na ovinocultura mundial. Cordeiros são mais suscetíveis a coccidiose, especialmente em condições estressantes, sendo infectados por diferentes espécies do parasito. *Eimeria crandallis* e *Eimeria ovinoidalis* são consideradas as mais patogênicas, causando redução do crescimento, desidratação, anorexia e morte. Neste estudo, a prevalência de *Eimeria* spp. foi avaliada em cordeiros da região sul do Estado do Rio Grande do Sul, Brasil. Amostras fecais de 248 cordeiros, provenientes de 19 fazendas, foram testadas quanto à presença de oocistos. As amostras positivas foram reexaminadas e os oocistos esporulados analisados morfometricamente para identificação das espécies de *Eimeria* presentes. Em 100% das fazendas avaliadas houve animais positivos para o protozoário. A frequência das espécies de *Eimeria* foi: *E. ovinoidalis* (94.74%), *E. crandallis* (89.47%), *E. granulosa* (78.95%), *E. parva* (68.42%), *E. ahsata* (63.13%), *E. punctata* (42.11%), *E. bakuensis* (36.84%), *E. faurei* (10.53%) e *E. pallida* (5.26). Infecção mista foi encontrada em 94.74% das amostras. Este trabalho descreve pela primeira vez a ocorrência de *E. crandallis* e *E. ovinoidalis* infectando cordeiros na área de estudo. Este trabalho descreve pela primeira vez a ocorrência de *E. crandallis* e *E. ovinoidalis* infectando cordeiros na área de estudo. A ampla distribuição desse protozoário e a alta frequência das espécies patogênicas evidenciam a importância da coccidiose ovina e os danos potenciais nos rebanhos do Rio Grande do Sul.

TERMOS DE INDEXAÇÃO: Infecção, cordeiros, Brasil, ovinocultura, coccidiose, *Eimeria crandallis*, *Eimeria ovinoidalis*, ovinos.

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INTRODUCTION

Traditionally, the sheep industry has always been relevant to the economy of the Rio Grande do Sul state. Even with the crises that occurred in the 80s and 90s, the herd remains one of the largest in Brazil, and its distribution remains concentrated in the southern state region (Silva et al. 2013, IBGE 2017). This industry still faces some obstacles; parasitic infections being one of the factors that influences a reduction in sheep production performance. Among such diseases, coccidiosis causes significant economic losses as a result of clinical (diarrhea) and subclinical disease (poor weight gain) (Chartier & Paraud 2012).

Coccidiosis of farm animals is caused by coccidial parasites of the genus *Eimeria* which develop in the small and the large intestines. Coccidiosis is of great economic importance, not only in terms of its effect on the animal (poor weight gain in particular) but also in terms of the required treatment costs. Many *Eimeria* infections in sheep are asymptomatic; however, more frequently in lambs, some species have been associated with diarrhea and stunted growth (Chartier & Paraud 2012). Infections occur due to the ingestion of sporulated oocysts, and the hosts can be simultaneously infected by different species of the parasite. Currently, eleven *Eimeria* species are recognized as causing sheep infections, with *E. crandallis* and *E. ovinoidalis* being the most pathogenic (Andrews 2013). Species differentiation is still a challenge, requiring coprological examination with a precise morphometric analysis of sporulated oocysts (Macedo et al. 2019).

Clinical signs are unapparent in most *Eimeria* infected animals. However, it is common to have some degree of inappetence or anorexia, weight loss and growth retardation (Andrews 2013). Infection leads to underdevelopment due to parasite multiplication occurring within enterocytes, which causes villous atrophy, leading to malabsorption of nutrients (Martins et al. 2020). Fecal staining around the hindquarters, due to diarrhea, can occur. As the clinical conditions worsen, lambs develop profuse watery diarrhea that may contain blood-streaks (Keeton & Navarre 2017). If treatment is not performed, intense dehydration can lead to death.

Subclinical disease and the non-specificity of clinical signs are obstacles to the detection of coccidiosis by farmers and veterinarians. Thus, the diagnosis must be based on the analysis of clinical signs, coprological examinations, animal management, age, and climatic factors (Martins et al. 2020).

Coccidiosis is a substantial problem, causing an important economic impact on livestock and, although the sheep industry is a traditional activity in Rio Grande do Sul, the epidemiology data of this parasitosis in the state are scarce, especially in the southern region.

Given that, the aim of this study was to estimate the frequency of *Eimeria* species in lambs from the southern Rio Grande do Sul state, Brazil.

MATERIALS AND METHODS

All procedures performed in this study were approved by the Ethics Commission on Animal Experimentation of the Federal University of Pelotas under protocol number 15450/2018.

A cross-sectional epidemiological study was carried out on 19 ruminant farms from 8 cities of the southern Rio Grande do Sul state, Brazil (Fig.1). The minimum sample size (n=246) was estimated, with EpiInfoTM epidemiological calculators, based on the sheep population (n=499,898) of the study area (IBGE 2019). A confidence level of 95% and statistical error of 5% were considered.

From August 2018 to September 2019, stool samples were collected directly from the rectum of 248 animals, which represented at least 10% of the total lambs from each farm. Analyzed farms adopted an intensive, extensive or semi-extensive production system. Animals enrolled in this study were observed for the presence of clinical signs related to coccidiosis (low body condition, diarrhea, apathy, and emaciation).

The samples were examined for the presence and number of oocysts per gram (OPG) of feces using the modified Gordon & Whitlock technique (Ueno & Gonçalves 1998). Subsequently, positive samples

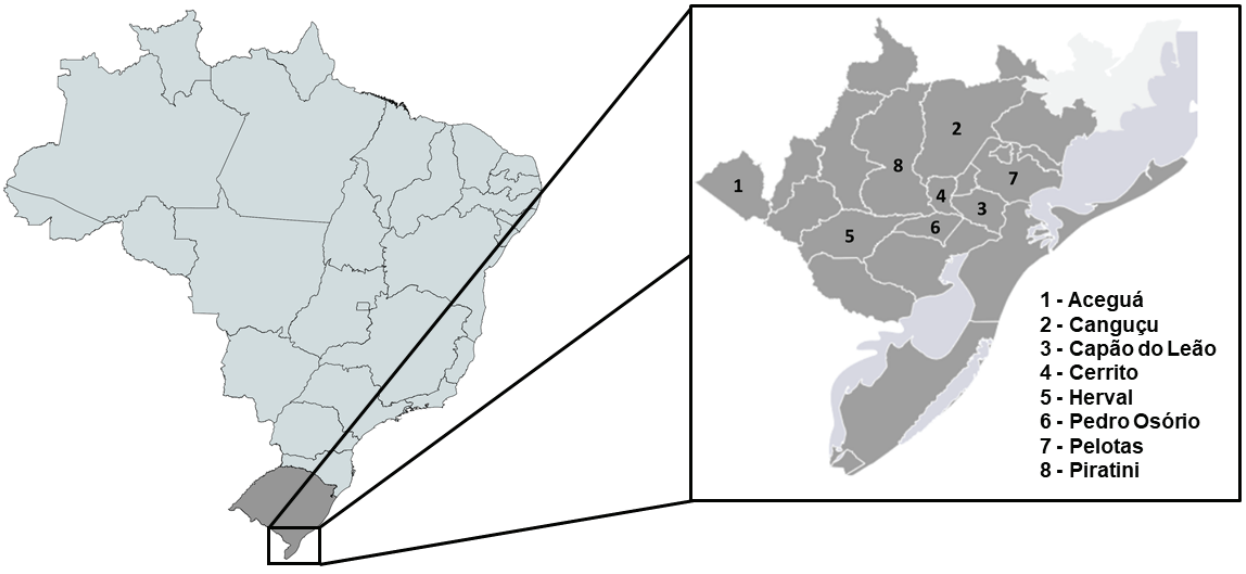


Fig.1. Municipalities from the southern Rio Grande do Sul state where lamb stool samples were collected for the detection of *Eimeria* in 2018 and 2019.

from each farm were pooled and stored in Falcon tubes containing 2.5% potassium dichromate ($K_2Cr_2O_7$), which were regularly moved in order to allow oxygenation and kept at room temperature for ten days until sporulation of *Eimeria* oocysts was completed (Carrau et al. 2018). Sporulated oocysts were separated from the fecal mixture and potassium dichromate using a modified centrifugal flotation technique (Duszynski & Wiiber 1997). Ultimately, 100 oocysts from each sample were randomly selected in the optical microscopy examination. In samples with less than 100 oocysts, all were analyzed. Species differentiation was based on morphometric and morphological characteristics, such as: shape, color, and presence or absence of micropyle and micropylar caps, following Souza et al. (2015) and Macedo et al. (2019).

For descriptive analysis, the Excel® 2019 software package was used. True prevalence, and the Chi-square test, was performed to compare the occurrence of *Eimeria* species in different breeding systems, with a 5% significance level, using EpiTools epidemiological calculators (Sergeant 2018).

RESULTS

Eimeria oocysts were found in 85.98% of the fecal samples with a mean of 5,034 oocysts per gram (OPG) of feces (0 a 120,100 OPG). Furthermore, at least one animal showed *Eimeria* infection in all farms (100%). The overall results of positivity for any *Eimeria* species are shown in Table 1. Overall, 9 *Eimeria* species were identified in fecal samples with the following prevalence: *E. ovinoidalis* (94.74%), *E. crandallis* (89.47%), *E. granulosa* (78.95%), *E. parva* (68.42%), *E. ahsata* (63.13%), *E. punctata* (42.11%), *E. bakuensis* (36.84%), *E. faurei* (10.53%) and *E. pallida* (5.26%). Concomitant infections were found in 94.75% of analyzed farms, with 3 to 8 different *Eimeria* species present in the same pooled sample (Table 1). No significant changes of color, odor or consistency were identified in the samples. Diarrhea was not observed in the analyzed lambs although, in some farms, poor body condition and apathy was noted.

DISCUSSION

The results obtained in this research showed that the *Eimeria* species has a wide distribution in the study area. Brazilian and worldwide studies have found a variable prevalence of *Eimeria* species in sheep; however, in the majority of cases, young animals had higher rates of positivity for the parasite (Lopes et al. 2013, Souza et al. 2015, Carrau et al. 2018). The high prevalence of positive samples found in the present study (85.98%) probably occurred due to the focus on young lambs, the age group most susceptible to coccidiosis.

Table 1. Presence of *Eimeria* oocysts in lambs from southern Rio Grande do Sul state, Brazil

	Mean (OPG)	Median (OPG)	Frequency (%)
Farming systems			
Intensive	5,971	1,000	95.24%[a]
Semi-extensive	8,916	1,200	76%[bc]
Extensive	4,250	1,100	87.8%[ac]
P value	-	-	0.0281
Co-infections			
Triple	7,805	3,600	10.54%[a]
Quadruple	2,568	200	42.11%[b]
Quintuple	4,561	1,100	31.58%[ab]
> Six	13,795	2,300	10.52%[a]
P value	-	-	0.0193

OPG = Oocysts per gram; [a,b,c] Different lowercase letters on the same column indicate a statistically significant difference (Chi-square P<0.05).

The pathogenic species for sheep, *E. crandallis* and *E. ovinoidalis* (Andrews 2013), were the most prevalent in the present study, which indicates that the evaluated farms have considerable risk for losses associated with coccidiosis. This is the first description of these species in the study area. The results are similar to the data found by Carrau et al. (2018), who obtained prevalence for *E. ovinoidalis* and *E. crandallis* of 97% and 89.4%, respectively. These two species are usually associated with reduced fluid absorption and consequent diarrhea, but are also known to induce strong protective immunity without clinical signs (Carrau et al. 2018).

E. ahsata, E. bakuensis, E. granulosa, E. faurei, E. parva, *E. pallida,* and *E. punctata* are considered nonpathogenic but can cause impairment of growth due to nutrient malabsorption (Keeton & Navarre 2017, Taylor et al. 2017), which might trigger subclinical coccidiosis in animals. Investigations carried out in different regions of Brazil already describe the presence of these species infecting sheep with variable prevalence (Tembue et al. 2009, Silva et al. 2011, Lopes et al. 2013, Souza et al. 2015, Macedo et al. 2019.

Previously studies demonstrated that the intensive and semi-intensive farming systems could facilitate the dissemination of *Eimeria* species (Lopes et al. 2013, Tomczuk et al. 2015). In this study, there were statistical differences (p=0.028) between animal infection and different breeding systems. Lambs raised under the intensive system had a higher frequency of parasitism by *Eimeria* (Table 1), with statistical difference in relation to the semi-extensive regime (p=0.010), which agrees with previous reports (Lopes et al. 2013, Macedo et al. 2019). In intensive breeding conditions accompanied by a high animal density, coccidiosis can become an infection of significant economic importance (Chartier & Paraud 2012). Lower frequency was found in the semi-extensive system, however the lambs raised in this regime has greater excretion of oocysts (Table 1). This fact could be related with the poor hygienic sanitary conditions and high stocking rates observed on some of these farms, which may be considered an aggravating factor for the spread of coccidiosis (Macedo et al 2019). Chartier & Paraud (2012) suggested that the economic impact of coccidiosis in small ruminants from tropical regions is probably not of a significant impact compared with other infections; however, coccidiosis was considered a potential risk for sheep raised under extensive systems in Brazil (Silva et al 2011, Souza et al 2015). The high frequency of *Eimeria* infections and oocysts shedding found in extensive breeding, indicating that the presence of this parasite cannot be underestimated.

For Keeton & Navarre (2017), mixed infections with multiple pathogenic and nonpathogenic species are common. This was observed in almost all positive lambs and suggests that these infections usually occur by more than one species in the study region. In fact, field-acquired infections are generally mixed, as the animals are more exposed to the existing environmental biodiversity (Silva et al. 2011).

Despite the high occurrence and the mixed infection of *Eimeria* species, no significant changes in stool characteristics,

such as diarrhea, were observed. However, some animals presented at least one suggestive clinical sign of coccidiosis, such as poor body condition, rough hair coat, and apathy. A poor growth rate, in an appropriate epidemiological setting, should indicate a diagnosis of subclinical coccidiosis (Chartier & Paraud 2012), with consequent economic losses for farmers. The non-specificity or nonexistence of clinical signs makes coprological monitoring essential to identify losses in livestock production, as is the development of partnerships between veterinarians and farmers.

So far, little is known about ovine coccidiosis in the southern Rio Grande do Sul region. This study on natural infections of *Eimeria* species is considered to contribute to the better understanding of coccidiosis epidemiology in this study area. New studies should be encouraged to elucidate the risk factors of these parasites, emphasizing the need to develop diagnostic and control programs, contributing to the progress of the sheep industry.

CONCLUSIONS

The results obtained in this research showed that *Eimeria* species constitute an endemic infection on the evaluated farms, with a wide distribution of the parasite. *Eimeria ovinoidalis* and *Eimeria crandallis* was the most frequent species, followed by *Eimeria granulosa*, *Eimeria parva*, *Eimeria ahsata*, *Eimeria punctata*, *Eimeria bakuensis*, *Eimeria faurei,* and *Eimeria pallida*.

Higher levels of parasitism were diagnosed in animals that were raised in an intensive breeding system (P≤0.05).

Concomitant infections were found in almost all analyzed farms.

The high frequency of this protozoan, and the description of the pathogenic species *E. crandallis* and *E. ovinoidalis* for the first time in the region, highlight the importance of sheep coccidiosis in the southern Rio Grande do Sul state, Brazil.

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Original Article
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Prepartum anionic diet induces hyperchloremic acidosis in high-producing dairy cows without preventing subclinical hypocalcemia[1]

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ABSTRACT.- Ramella K.D.C.L, Cucunubo Santos L.G., Patelli T.H.C., Flaiban K.K.M.C. & Lisbôa J.A.N. 2020. **Prepartum anionic diet induces hyperchloremic acidosis in high-producing dairy cows without preventing subclinical hypocalcemia**. *Pesquisa Veterinária Brasileira 40(11):875-881*. Departamento de Clínicas Veterinárias, Centro de Ciências Agrárias, Universidade Estadual de Londrina, Campus Universitário, Cx. Postal 10011, Londrina, PR 86057-970, Brazil. E-mail: janlisboa@uel.br

In this study we evaluated the effects of the prepartum anionic diet on the electrolyte balance and calcemia of high producing dairy cows in the first days of lactation, and investigated the impact on the frequency of subclinical hypocalcemia (SCH). Sixty healthy Holstein cows, producing 30 kg of milk/day, handled in intensive system (compost barn), were distributed in groups (n=15) according to lactation order: first, second, third, and fourth to sixth. In the last three weeks before calving they received a diet with negative DCAD (-6mEq/100g DM) and high chloride content. After calving, they received a diet with positive DCAD (18mEq/100g DM). Urine pH was measured before calving. Serum Na^+, Cl^-, K^+, and total Ca concentrations, and the strong ion difference (SID_3) were determined in samples taken soon after calving (0h), 24, 48, 72 and 96h after. The frequencies of SCH were determined considering the critical value of 2.125mmol/L (8.5mg/dL). Two-way repeated measures ANOVA and chi-square test were used for comparisons. The cows eliminated acidic urine before calving. Na^+, K^+, Cl^-, and SID_3 values did not differ between groups. Na^+ and K^+ did not vary between days; Cl^- was elevated at calving and decreased until 72h; and SID_3 was reduced at calving and increased up to 48h. The Ca levels were reduced until 24h and increased up to 72h. Cows of third and fourth to sixth lactations presented lower values up to 24h. SCH was observed in almost half of the cows (43.3% to 55%) until 48h. The maintenance of hypocalcemia for three or more consecutive days occurred in 53.3% of third and fourth to sixth lactations cows. Ingestion of a high chloride prepartum anionic diet led to hyperchloremic acidosis and this imbalance was reversed on the second postpartum day. The induced effects on electrolyte and acid-base balances were not able to prevent the occurrence of SCH in the first days of lactation.

INDEX TERMS: Hyperchloremic acidosis, hypocalcemia, transition period, serum electrolytes, ammonium chloride, calcium metabolism, dietary cation-anion difference, dairy cattle, prepartum diet, cattle.

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RESUMO.- [**Dieta aniônica pré-parto provoca acidose hiperclorêmica em vacas leiteiras de alta produção mas não evita a hipocalcemia subclínica.**] Os objetivos do estudo foram avaliar os efeitos que a dieta aniônica pré-parto provoca sobre o equilíbrio eletrolítico e sobre a calcemia de vacas leiteiras de alta produção nos primeiros dias de lactação, e verificar o impacto sobre a frequência da hipocalcemia subclínica (HSC). Sessenta fêmeas hígidas HPB, com produção de 30 kg de leite/dia, manejadas em sistema intensivo (*compost barn*), foram distribuídas por grupos (n=15) de acordo com a ordem de lactação: primeira, segunda, terceira e quarta a sexta. Nas três semanas pré-parto receberam dieta com DCAD negativa (-6mEq/100g MS) e teor de cloreto elevado. Após o parto receberam dieta com DCAD positiva (18mEq/100g MS). O pH da urina foi mensurado antes do parto. As concentrações séricas de Na^+, Cl^-, K^+ e Ca total e a diferença de íons fortes (SID_3) foram determinadas em amostras colhidas ao parto (0h), 24, 48, 72 e 96h após. As frequências de HSC foram determinadas considerando-se o valor crítico de 2,125mmol/L (8,5mg/dL). ANOVA de medidas repetidas e teste de qui-quadrado foram empregados para as comparações. As vacas eliminavam urina ácida antes do parto. Os valores de Na^+, K^+, Cl^- e SID_3 não diferiram entre os grupos. Na^+ e K^+ não variaram entre os dias; Cl^- era elevado ao parto e diminuiu até 72h; e SID_3 era reduzida ao parto e aumentou até 48h. A calcemia era reduzida até 24h e se elevou até 72h. Vacas de terceira e de quarta a sexta lactações apresentaram valores mais baixos até 24h. A HSC foi observada em quase metade das vacas (43,3% a 55%) até 48h. A manutenção de hipocalcemia por três ou mais dias seguidos ocorreu em 53,3% das vacas de terceira e de quarta a sexta lactações. A ingestão de dieta aniônica pré-parto com alto teor de cloreto provocou acidose hiperclorêmica e este desequilíbrio se reverteu no segundo dia pós-parto. Os efeitos induzidos sobre os equilíbrios eletrolítico e ácido base não foram capazes de prevenir a ocorrência de HSC nos primeiros dias da lactação.

TERMOS DE INDEXAÇÃO: Acidose hiperclorêmica, hipocalcemia, período de transição, eletrólitos séricos, cloreto de amônio, metabolismo do cálcio, diferença entre cátions e ânions na dieta, bovinos leiteiros, dieta pré-parto.

INTRODUCTION

High-yielding dairy cows, notably those with a higher number of lactations, have difficulty to maintain calcium (Ca) homeostasis in the early days of lactation because of the sudden and excessive loss of Ca by colostrum and the slow and insufficient response of the mechanisms responsible for maintaining calcemia in physiological levels (Oetzel 2013, Goff 2014). The resulting drop in blood Ca concentrations may be accentuated, causing hypocalcemic puerperal paresis (HPP), a disease also known as milk fever or clinical hypocalcemia, or it may be mild, characterizing subclinical hypocalcemia (SCH) (Lean et al. 2013). SCH is much more frequent than HPP, and can occur in around 50% of fresh multiparous cows (Reinhardt et al. 2011, Caixeta et al. 2015), and causes much greater economic loss because it is associated with the appearance of other diseases in the postpartum transition period (Kimura et al. 2006, DeGaris & Lean 2009, Martinez et al. 2012).

Among the preventive measures for hypocalcemia, it should be emphasized the intake of anionic diet in the prepartum transition period, that is, in the last three weeks of pregnancy. This type of diet has a low value for the dietary cation-anion difference (DCAD), calculated by the equation DCAD = (Na + K) – (Cl + S). Unlike the conventional diet, in which cations predominate, the anionic diet has a higher concentration of anions and therefore, negative DCAD value (Constable 1999, 2014, DeGaris & Lean 2009). Cows that eat a low DCAD diet develop metabolic acidosis which activates the mechanisms responsible for maintaining calcemia at a time when the metabolic demand for Ca is reduced. The previous activation of these mechanisms allows the cow to face the marked demand for Ca at the beginning of lactation in a reasonably balanced manner (Goff 2008, 2014).

The supply of diets with low DCAD at the end of pregnancy has been in use for four decades and is widespread, including in Brazilian dairy herds. Its effectiveness in reducing the incidence of HPP is proven (Charbonneau et al. 2006, Lean et al. 2013, Hassan et al. 2018). In the case of SCH, it is estimated that the intake of anionic diet can halve the incidence of this condition (Goff 2008, Oetzel 2013), however, there is a lack of scientific evidence to support this statement.

Metabolic acidosis resulting from low DCAD diets intake is well documented in dairy cows (Joyce et al. 1997, Gelfert et al. 2007, 2010, Zimpel et al. 2018). On the other hand, the influence of this type of diet on the variation of serum electrolytes before and after calving was presented in a single report (Grünberg et al. 2011). The study presented here aimed to evaluate the effects that the prepartum anionic diet has on the electrolyte balance and on the calcemia of high-producing, primiparous and multiparous dairy cows, during the first days of lactation, and to verify the impact on the frequency of SCH.

MATERIALS AND METHODS

The experimental protocol was approved by the Ethics Commission on the Use of Animals of the "Universidade Estadual de Londrina" (CEUA-UEL), registered as process number 13822.2017.94.

Animals and management. The present study was carried out on a dairy farm in the municipality of Toledo, Paraná, Brazil (latitude 24º42'49" S, longitude 53º44'35" W, and average altitude of 560m) from November 2017 to June 2018. Sixty apparently healthy Holstein cows, with an average milk yield of 30kg/day, were included in the study. The cows belonged to a single farm, where they remained throughout the experimental period. The following groups (15 animals each) were composed according to the lactation order: first, second, third, and fourth to sixth lactations. The cows remained confined in facilities of compost barn type with efficient ventilation system and bed of shavings. At 60 days prepartum, the cows were dried and housed together forming a single batch. At 21 days prepartum, the cows were relocated to form a transition period group, remaining in this batch until calving. After calving, the calves were immediately separated and the cows were transferred to the lactation batch. Milking was performed three times a day (5:00 a.m., 1:30 p.m. and 9:00 p.m.) using a conventional mechanical milking system. In the newly calved cows, the first milking of the colostrum was performed at the next time of the milking routine, after milking all cows. Most of the cows included in this study calved at night and were milked at the morning milking time.

For inclusion in the study, cows should have a body condition score (BCS) between 3.0 and 3.5 and urinary pH with a range of 6.2 to 6.8 (Goff 2008, 2014) in the period comprising the last two weeks

before calving. Dystocic or assisted calvings and twin pregnancies were considered as exclusion criteria in the study.

Formulation and composition of diets. The diet was offered to the animals twice a day in the form of total mixed ration (TMR) and water was freely available. TMR offers took place at 8:00 a.m. and 3:00 p.m. The diet offered from 21 days before calving was formulated to achieve a negative DCAD and calculated for high-producing cows with an estimated weight of 650kg. The diet offered after calving was calculated considering the estimated weight of 580 kg, and the expectation that the milk produced would contain concentrations of 3.7% and 3% for fat and proteins, respectively. A sample of each type of diet, anionic in the prepartum and cationic in lactation, was taken directly from the trough and subjected to bromatological and mineral analysis. The characteristics of the diets offered before and after calving are shown in Table 1.

DCAD was calculated using the equation DCAD (mEq/100g DM) = $[(Na^+ + K^+) - (Cl^- + S^{2-})]$ (DeGaris & Lean 2009). The conversion to a thousandth part of the Equivalent (mEq), was obtained from the atomic weight and valence of each cation or anion, using the formula DCAD (mEq/100g DM) = $[(\% Na^+/0.023 + \% K^+/0.039) - (\% Cl^-/0.0355 + \% S^{2-}/0.016)]$, and considering the percentage of cations and anions in DM.

Sample collection and laboratory analysis. The BCS and urine pH measurements were performed only once and on the same day, between seven and ten days before calving. The BCS was assessed using a scale from 1 to 5 (Edmonson et al. 1989). To minimize the subjectivity of the assessment, the BCS Cowdition app (Bayer Animal Health, Germany), available for smartphones, was used. Urine collection was performed by micturition induced by massage on the vulva and perineum and the pH was measured right after collection using an AK90 portable device (AKSO; São Leopoldo, RS).

Individual milk yield was measured on the 20th and 21st days of lactation, using milking controllers (Metatron 52; GEA Westfalia;

Table 1. Composition of diets offered in the prepartum and postpartum periods, and values recommended by the NRC (2001) for high-producing Holstein cows

	Prepartum		Postpartum	
	(kg DM/d)	% DM	(kg DM/d)	% DM
Components				
Corn silagem	7.00	66.13	5.95	41.36
Tifton hay	0.65	6.14	1.02	7.09
Roasted soybeans	-	-	0.64	4.43
Corn meal	1.06	9.98	2.64	18.37
Soybean meal	0.88	8.33	1.86	12.93
Extruded soy 46%	-	-	0.72	4.98
Soybean husk	0.46	4.32	0.48	3.34
Sugar	-	-	0.24	1.67
Protected fat	-	-	0.14	1.00
Anionic mineral core [a]	0.50	4.72	-	-
Mycotoxin adsorbent [b]	0.04	0.38	0.03	0.21
Mineral core [c]	-	-	0.48	3.30
Nacl	-	-	0.04	0.24
Urea	-	-	0.06	0.44
Buffering [d]	-	-	0.09	0.60
Chemical composition		Recommended*		Recommended*
Dry matter (%)	44.56	42.40	43.56	53.20
Net energy (Mcal/kg)	1.93	1.54 - 1.62	1.90	2.06
Crude protein (%)	15.56	13.5 - 15.0	18.99	17.5
NDF (%)	37.00	25.0 - 33.0	39.58	25.0 - 33.0
ADF (%)	15.61	17.0 - 21.0	14.99	17.0 - 21.0
Ethereal extract (%)	2.42	3.1	3.67	4.8 - 5.1
Ca (%)	0.72	0.40 - 0.44	0.68	0.74
P (%)	0.36	0.23 - 0.42	0.31	0.38
Mg (%)	0.35	0.40	0.22	0.27
K (%)	1.29	1.32 - 1.35	1.32	1.19
Na (%)	0.30	0.12 - 0.13	0.29	0.34
S (%)	0.26	0.20 - 0.40	0.17	0.20
Cl (%)	1.28	0.42 - 0.89	0.27	0.36
DCAD (mEq/100g DM)	-6.11		18.27	

Consumption time: 21 days; DM = dry matter; [a] Núcleo Pré-parto Aniônico Salus® (Salus; Santo Antônio da Posse/SP), [b] Safetox Plus® (Safeeds; Cascavel/PR), [c] Núcleo Salus Lactação® (Salus; Santo Antônio da Posse/SP), [d] Rumox® (Safeeds; Cascavel/PR); * Diets formulated according to the NRC (2001) for high-producing Holstein cows weighing approximately 650kg before calving and 580kg at the beginning of lactation; NDF = neutral detergent fiber, ADF = acid detergent fiber, DCAD = dietary cation-anion difference.

Germany). The average value of the milk volumes produced in the two days was accepted as the yield value of each cow.

Venous blood samples were taken at five defined times: after calving (just after the first milking of colostrum and up to 5 hours after calving), 24, 48, 72 and 96 hours later. Blood samples were obtained by puncture of the coccygeal vein with 21G needles (25 x 0.8mm) from the vacuum collection system, using bottles without anticoagulant. Blood serum was obtained by centrifugation after clot retraction and preserved by freezing (-20°C) until the time of the analyzes, performed up to 10 months after collection.

To determine serum total Ca concentrations, a colorimetric method and spectrophotometric reading (Dimension Xpand Plus®; Siemens; São Paulo/SP) were used, using a specific commercial reagent (CA Flex® reagent cartridge; Siemens). The concentrations of sodium (Na^+), potassium (K^+) and chlorides (Cl^-) were measured using the ion selective electrode method (RAPIDPoint 500 System; Siemens Healthcare Diagnostics Inc.; USA). The strong ion difference (SID_3) was calculated using the following formula: $SID_3 = (Na^+ + K^+) - (Cl^-)$ (Constable 2014).

Statistical analysis. One-way analysis of variance was used to compare milk production and urine pH between groups. For serum variables (total Ca, electrolytes and SID3), two-way repeated measures analysis of variance was used, testing the effect of time (days in milk - DIM), the effect of the lactation order and the interaction between these two factors. When the F statistic was significant, Tukey test was used for multiple comparisons.

The blood serum total Ca concentration of 2.125mmol/L (8.5mg/dL) was admitted as the critical value indicative of the equilibrium condition (Oetzel 2013, Farnia et al. 2018). The frequency distribution of cows with hypocalcemia was established on each of the first five DIM, and on each individual group, considering the maintenance of hypocalcemia for two or more consecutive days. The chi-square test was used to verify whether the occurrence of hypocalcemia was associated with DIM or lactation orders of cows.

Relationships between the studied variables were verified using Pearson's correlation test. For all statistical methods used, the probability of error of 5% was admitted. All analyzes were performed in SigmaStat for Windows 3.1.

RESULTS

The studied cows showed milk yield of 30.99±7.32kg at the end of the third week of lactation. According to the lactation order, the yield means were 28.99±6.48kg, 32.99±7.67kg, 31.48±6.06kg and 30.51±8.92kg in first, second, third and fourth to sixth lactation cows, respectively. There was no difference between groups (P=0.511).

The urine pH, measured 7 to 10 days before calving, did not differ between groups (P=0.062) and showed an overall value of 6.65±0.30. The values were 6.46±0.29 in the first lactation cows, 6.71±0.25 in the second lactation, 6.74±0.34 in the third lactation and 6.70±0.24 in the fourth to sixth lactations.

Unlike calcemia, the serum electrolyte concentrations in the first days of lactation were not influenced by the lactation order of the cows (Table 2). Considering the variations between DIM, on the other hand, the concentrations of Na^+ and K^+ remained unchanged, while chloremia and SID_3 showed an opposite behavior, with reduction and increase, respectively. The decrease of chloremia was completed within 72 hours and the SID_3 elevation was complete within 48 hours postpartum.

The serum concentration of total Ca differed both between groups and between the first DIM, although there was no interaction between these factors (Table 2). The calcemia, lower at birth and at 24 hours, increased and maintained higher values after 72 hours. The influence of the groups was proven up to 24 hours postpartum and, after 48 hours, total Ca concentrations no longer differed between cows of different lactation orders (Fig.1). At calving, first and second

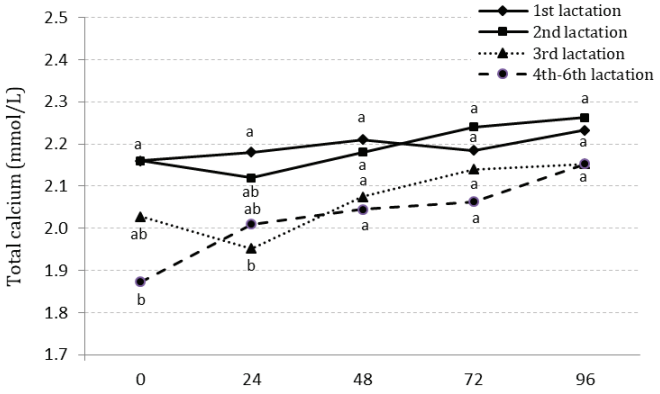


Fig.1. Variation of total calcium concentration in the blood serum, during the first 96 hours of lactation, of high-producing Holstein cows divided according to the lactation order (n=15 each) and that received anionic diet before calving.

Table 2. Global mean values of sodium (Na^+), potassium (K^+), chloride (Cl^-) and total calcium (Ca) concentrations and the strong ion difference (SID_3) in the blood serum of high-producing Holstein cows during the first five days in milk and who received anionic diet before calving. Effects of the lactation order and the day in milk, and interaction between the two factors

	Calving	24 h	48 h	72 h	96 h	SEM	LO *P* value	DIM *P* value	LO x DIM *P* value
Na^+ (mmol/L)	143.07	141.29	149.21	140.81	140.00	0.832	0.297	0.108	0.687
K^+ (mmol/L)	4.65	4.77	4.70	4.63	4.58	0.070	0.282	0.291	0.342
Cl^- (mmol/L)	111.21^{a}	108.30^{b}	106.58^{bc}	105.96^{c}	105.33^{c}	0.513	0.229	<0.001	0.117
SID_3 (mmol/L)	36.51^{b}	37.76^{ab}	39.32^{a}	39.48^{a}	39.25^{a}	0.693	0.275	0.005	0.532
Ca (mmol/L)	2.055^{b}	2.065^{b}	2.127^{ab}	2.155^{a}	2.200^{a}	0.028	0.007	<0.001	0.396

SEM = standard error of mean, LO = lactation orders: first lactation, second lactation, third lactation and fourth to sixth lactation (n=15 for each group), DIM = day in milk; ^{a,b,c} different letters on the same line represent difference between days (P<0.05).

Table 3. Occurrence of subclinical hypocalcemia (total calcium concentration in blood serum <2.125mmol/L) in high-producing Holstein cows in the first five days in milk and who received an anionic diet before calving

Lactation order	Calving	24 h	48 h	72 h	96 h
1st (n=15)	2	4	3	6	2
2nd (n=15)	5	9	6	4	3
3rd (n=15)	10	12	6	5	4
4th to 6th (n=15)	12	8	11	5	5
TOTAL (n=60)	29 (48.3%)	33 (55%)	26 (43.3%)	20 (33.3%)	14 (23.3%)

lactation cows showed higher values than cows with four to six lactations, and 24 hours postpartum, first lactation cows had higher concentrations than third lactation cows.

Hypocalcemia was observed in practically half of the cows up to 48 hours after calving (Table 3) and the frequency of hypocalcemic cows decreased in the following days (P=0.004). The problem was less pronounced in cows with fewer lactations, especially in the first 48 hours in milk. Considering the maintenance of hypocalcemia for two or more consecutive days, this type of occurrence was observed in 58.3% (35/60) of the cows, with no difference between groups (P=0.257): 40% (6/15) in heifers, and 53.3% (8/15), 73.3% (11/15) and 66.7% (10/15) in second, third and fourth to sixth lactation cows, respectively. When the hypocalcemia maintenance period was extended to three or more consecutive days, the occurrence dropped to 31.7% (19/60) of the cows and the difference between groups became marked (P=0.004); more frequently in the third and fourth to sixth lactation cows (53.3%; 8/15 in both) and less frequently in primiparous (6.7%; 1/15) and in second lactation cows (13.3%; 2/15).

The concentrations of serum Ca at calving and at 24 hours of lactation did not correlate with those of Na^+ and K^+, and showed a weak correlation with those of Cl^- (r=-0.21; P=0.021) and with SID_3 (r=0.27; P=0.002).

DISCUSSION

The critical value of total serum Ca concentration below which SCH is defined is variable among the authors. The concentration of 2.0mmol/L (8.0mg/dL) has been classically admitted for a long time (Goff 2008, DeGaris & Lean 2009, Reinhardt et al. 2011) and is still accepted (Goff 2014, Caixeta et al. 2015). However, based on the study of Martinez et al. (2012), proving that serum Ca concentrations below 2.15mmol/L (8.6mg/dL) were related to decreased neutrophil activity, the value of 2.125mmol/L (8.5mg/dL) came to be considered critical (Oetzel 2013, Farnia et al. 2018). Following the most current trend, SCH was admitted in the study presented here as a concentration below 2.125mmol/L. Values close to, but above these limits, could be assumed to be indicative of a physiological drop in calcemia near calving (Goff 2008, 2014, Oetzel 2013).

The percentages of hypocalcemic cows observed (Table 3) are consistent with those indicated in epidemiological surveys carried out in herds of Holstein cows (Reinhardt et al. 2011, Caixeta et al. 2015). This indicates that, with respect to the maintenance of calcemia in the first days of lactation, the studied cows behave similarly to North American high-producing cows. The difficulty in maintaining calcemia is more pronounced in cows with a higher number of lactations (Reinhardt et al. 2011), especially in the first 48 hours postpartum (Caixeta et al. 2015). As estimated by the results of a meta-analysis, the risk of hypocalcaemia is 9% higher with each subsequent lactation (Lean et al. 2013).

A result that must be highlighted is that SCH remained present for two or more consecutive days in a significant number of the studied cows. Half of the third and fourth to sixth lactation cows remained hypocalcemic for three or more days, unlike the younger cows. This discrepancy observed in relation to lactation orders clearly characterizes that the younger cows were able to recover their balance quickly, while the older cows exhibited more difficulty in reversing the metabolic imbalance. The increased risk of hypocalcemia with advancing age can be explained by different reasons: decreased number of receptors for calcitriol (active vitamin D) in enterocytes, decreased number of osteoblasts and active osteoclasts at bone remodeling sites, and probably a decrease in the number of receptors for parathyroid hormone (PTH) in renal cells (DeGaris & Lean 2009, Goff 2014). The responses to positive stimuli for maintaining calcemia (PTH and calcitriol) are, therefore, slower and of lesser magnitude in cows with third or more lactations, highlighting the lower capacities of intestinal Ca absorption and bone Ca mobilization.

In the case of studied cows with three or more lactations, the greatest concern is not due to the magnitude of the hypocalcaemia itself, but rather to its prolonged duration. In average values, third lactation cows corrected calcemia 72 hours after calving and those from fourth to sixth lactations only 24 hours after this (Fig.1). The variation curve for serum Ca concentration in older cows is compatible with that obtained by Kimura et al. (2006) in Jersey cows, proven to be more susceptible than Holstein cows to developing postpartum hypocalcaemia (Goff 2014). The lowest concentrations of Ca were found at birth and 24 hours later, and the concentration of 2.125 mmol/L was only achieved with 96 hours of lactation (Kimura et al. 2006). The persistence of SCH during the first four days of lactation may increase the risk of older cows developing other related diseases in the postpartum transition period (Goff 2008, 2014, DeGaris & Lean 2009, Oetzel 2013), in addition to having impaired reproductive function (Caixeta et al. 2017), which causes economic loss. The occurrence of diseases was not evaluated in the studied cows, but milk yield did not differ between groups at the end of the third week of lactation.

Even if we considered the critical value of 2.0mmol/L for the concentration of Ca in blood serum, the prevalence of SCH would remain high at the beginning of the observation period: 35% of cows at calving, 40% at 24 hours, 20% at 48 hours, 16.7% at 72 hours and 13.3% at 96 hours postpartum. Half of the third (8/15, 53.3%) and fourth and sixth lactation cows (8/15, 53.3%) maintained hypocalcemia for two or more consecutive days. SCH must therefore be understood as a relevant problem in the studied herd, and it is clear that the intake of anionic diet in the prepartum transition period did not contribute to preventing this imbalance.

The diet eaten in the last three weeks of pregnancy has the characteristics of an anionic diet. The DCAD is negative (-6.11mEq/100g DM) because the chloride concentration is very high (Table 1) and the sum of anions (Cl^- and S^{2-}) exceeds the sum of cations (Na^+ and K^+). The anionic mineral core

used in the preparation of TMR contained NaCl and NH_4Cl as sources of chloride and $MgSO_4$ as the main source of sulfur. Due to the markedly high chloride content in the diet, it is correct to say, therefore, that NH_4Cl was the main anionic salt ingested by cows. After calving, the cows began to receive the typically cationic lactation diet (DCAD of 18.27mEq/100g DM), with reduced chloride content due to the removal of NH_4Cl from the composition.

The effects of the ingestion of anionic diet before calving and the sudden change to the cationic lactation diet on the electrolyte balance of the studied cows are clearly demonstrated in Table 2. While the concentrations of Na^+ and K^+ remained unchanged, chloremia, elevated at calving, decreased rapidly and plasma SID_3, decreased at calving, increased. This proves that the anionic diet caused hyperchloremia in the cows and that the electrolyte imbalance was promptly reversed when they started to receive the lactation diet.

Although the blood gas analysis was not performed, it is possible to state that the studied cows developed the state of metabolic acidosis based on the fact that they eliminate acidic urine (Constable et al. 2009). According to the strong ion theory, hyperchloremia, determining the decrease of SID_3 in plasma, is the primary imbalance caused by the anionic diet and metabolic acidosis is installed as a mandatory consequence (Constable 1999, 2014). It can be concluded, therefore, that the ingestion of the anionic supplement rich in NH_4Cl caused hyperchloremic acidosis in cows in late gestation.

These same types of electrolyte and acid-base imbalances have been proven in goats (Singh et al. 2007) and in sheep (Ferreira et al. 2014) that ingested NH_4Cl for the purpose of acidifying urine to prevent urolithiasis. The excess of ingested chloride causes an increase in the urinary fractional excretion of chlorides (Stratton-Phelps & House 2004, Mavangira et al. 2010, Ferreira et al. 2018) and this explains the reduction in the urine pH, since the higher concentration of chlorides reduces urinary SID_3 (Constable et al. 2009). Other studies using sheep have shown that hyperchloremic acidosis also occurs with the ingestion of other types of anionic salts based on chlorides, such as $CaCl_2$ (Espino et al. 2003), HCl (Las et al. 2007) and $MgCl_2$ (Wilkens et al. 2016).

Variations in serum electrolytes in newly calved cows that received a chloride-rich anion diet in the prepartum transition period were previously presented in a single record (Grünberg et al. 2011). The results obtained were similar to those of the study presented here, however, unlike the studied cows, serum Na^+ concentrations were reduced after calving. Hyperchloremic metabolic acidosis has also been proven, as well as urine acidification with increased Cl^- excretion, and these imbalances were quickly reversed after calving by switching to a lactation diet containing low chloride content. More recently, the results obtained by Zimpel et al. (2018) in cows at the end of gestation reinforce the evidence that hyperchloremic acidosis is, in fact, the imbalance caused by the anionic diet.

The acidogenic effect of the anionic diet is expected and desirable so that it can play its role in helping to maintain Ca homeostasis in high-producing dairy cows that begin lactation (DeGaris & Lean 2009, Goff 2014). Among the suggested mechanisms, currently stands out the evidence that metabolic acidosis induces hypercalciuria by decreasing tubular calcium reabsorption when urine is acidic (Gelfert et al. 2007, 2010, Grünberg et al. 2011, Farnia et al 2018, Lopera et al. 2018, Zimpel et al. 2018). The continuous loss of calcium through the urine in the last weeks of pregnancy activates the mechanisms involved in the maintenance of calcemia, and mediated by PTH and calcitriol. After calving, with the suspension of anionic diet intake, the metabolic acidosis is quickly reversed (Joyce et al. 1997), the urine returns to alkaline (Grünberg et al. 2011) and the elimination of Ca by the urine ceases promptly (Farnia et al. 2018), contributing to increase blood Ca concentration. The previous activation of the mechanisms of bone Ca mobilization, calcitriol synthesis and active intestinal Ca absorption, enables the cow kept on anionic diet in the prepartum transition period to respond efficiently to the great challenge imposed by the sudden loss of Ca for colostrum (Goff 2008, 2014).

Ingestion of anionic diet at the end of pregnancy is proven to be effective in preventing the occurrence of HPP (Joyce et al. 1997, Charbonneau et al. 2006, DeGaris & Lean 2009, Lean et al. 2013, Hassan et al. 2018), also called clinical hypocalcemia, certainly because it prevents the fall of calcemia from being very accentuated at the beginning of lactation. In fact, during the study period, the frequency of HPP cases was very low in the herd (9/591; 1.5%). Reducing DCAD in the prepartum transition period has other advantages for multiparous cows in the beginning of lactation, such as increased dry matter intake and milk yield, and reduced risk of developing diseases, notably retained placenta and metritis (Santos et al. 2019). It is, therefore, a preventive management measure that is fully justified in high-producing dairy cows.

However, its effectiveness in preventing the occurrence of SCH is doubtful (Joyce et al. 1997, Farnia et al. 2018, Lopera et al. 2018) and the obtained results reinforce this. It is consistent to assume that the improvement produced in Ca metabolism is accompanied by an increase in blood Ca concentration sufficient to prevent the occurrence of HPP, but not always sufficient to prevent the occurrence of SCH. The ability of the anionic diet to decrease the frequency and duration of SCH in dairy herds is a relevant issue that needs to be further investigated.

CONCLUSION

The ingestion of a prepartum anionic diet with a high content of ammonium chloride causes hyperchloremic acidosis, an imbalance that is reversed on the second postpartum day. These effects on electrolyte and acid-base balances did not prevent the occurrence of subclinical hypocalcemia in high-yielding cows in the early days in milk.

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

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Haematological variables of Santa Ines and Ile de France suckling lambs: Influence of *Haemonchus contortus* infection[1]

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ABSTRACT.- Lins J.G.G., Almeida F.A. & Amarante A.F.T. 2020. **Haematological variables of Santa Ines and Ile de France suckling lambs: influence of *Haemonchus contortus* infection.** *Pesquisa Veterinária Brasileira 40(11):882-891.* Graduate Program in Veterinary Medicine, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rua Prof. Doutor Walter Mauricio Correa s/n, Botucatu, SP 18618-681, Brazil. E-mail: josegabriel_lins@hotmail.com

Haemonchus contortus is the major gastrointestinal parasite of sheep raised in tropical and subtropical areas worldwide. This trial aimed to evaluate the influence of *H. contortus* infection on the bone marrow response of Santa Ines (SI) and Ile de France (IF) suckling lambs experimentally infected with *H. contortus*. Fourteen SI lambs and 12 IF lambs were randomized in four groups: infected SI (n=8), non-infected SI (n=6), infected IF (n=8) and non-infected IF (n=4). Lambs of infected groups were submitted to 27 infections, conducted every two days, from 14 to 68 days of age, and each lamb received a total of 5400 *H. contortus* infective larvae (L3). Ten blood samples were obtained during the experimental period to be used for erythrocyte and leukocyte counts, packed cell volume and total plasma protein estimation. Additionally, it was carried out a differential leukocyte count. Lambs from control groups did not shed eggs in faeces all over the experiment, while infected Santa Ines and Ile de France lambs presented means of 2963 EPG (Eggs Per Gram of faeces) and 8175 EPG in the last sampling ($P<0.05$), respectively. Infected Santa Ines lambs had an increase in eosinophil release, however differences ($P<0.05$) on circulation number in comparison with infected Ile de France lambs were identified only in the last sampling (54 days post first infection). The mild *H. cortortus* infection did not produce significant changes in the blood variables of the Ile de France and Santa Ines suckling lambs.

INDEX TERMS: Haemathology, sheep, Santa Ines, Ile de France, lamb, *Haemonchus contortus*, infection, *Ovis aries*, gastrointestinal nematodes, eosinophils.

RESUMO.- [Variáveis hematológicas de cordeiros lactentes Santa Inês e Ile de France: influência da infecção por *Haemonchus contortus*.] *Haemonchus contortus* é o principal parasita gastrintestinal de ovinos criados em áreas tropicais e subtropicais. Este estudo objetivou avaliar a influência da infecção por *H. contortus* na resposta medular de cordeiros lactentes das raças Santa Inês (SI) e Ile de France (IF) infectados experimentalmente com *H. contortus*. Quatorze cordeiros SI e 12 cordeiros IF, foram distribuídos em quatro grupos: SI infectado (n=8), SI não infectado (n=6), IF infectado (n=8) e IF não infectado (n=4). Cordeiros dos grupos infectados foram submetidos a 27 infecções, realizadas a cada dois dias, do 14º até 68º dia de vida, com um total de 5400 larvas infectantes (L3) de *H. contortus* por animal. Foram realizadas 10 coletas de sangue ao longo do período experimental para determinação do volume globular, proteínas plasmáticas totais, contagem de eritrócitos e leucócitos, além do diferencial de leucócitos. Cordeiros controles não infectados não eliminaram ovos nas fezes durante todo o experimento, enquanto que os cordeiros Santa Ines e Ile de France infectados apresentaram em média 2963 ovos por grama de fezes (OPG) e 8175 OPG na última coleta ($P<0,05$), respectivamente. Cordeiros Santa Inês infectados tiveram um aumento na produção de eosinófilos,

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mas diferenças (P<0.05) no número de eosinófilos sanguíneos em comparação a cordeiros Ile de France infectados foram detectadas somente na última coleta (54 dias após a primeira infecção). A infecção leve por *H. cortortus* não induziu alterações significativas nas variáveis sanguíneas de cordeiros lactentes Ile de France e Santa Inês.

TERMOS DE INDEXAÇÃO: Hematologia, ovino, Santa Inês, Ile de France, cordeiro, *Haemonchus contortus*, infecção, *Ovis aries*, nematódeos gastrintestinais, eosinófilos.

INTRODUCTION

Occurrence of parasitic diseases in sheep is common, being an important cause of mortality, especially in young animals (Amarante & Amarante 2016). *Haemonchus contortus* is an abomasal blood-sucking nematode considered one of the major gastrointestinal parasite of small ruminants in tropical and subtropical areas worldwide (Amarante 2014), mainly due its high pathogenicity and multiple resistance to anthelmintics (Almeida et al. 2010).

Haematological variables are sensitive indicators for the degree of parasitic infection severity, and animals presenting variables within the reference interval tend to have a better performance than those presenting any parasitic infection (Kumar et al. 2015). Heavy *H. contortus* infections might cause reduction in packed cell volume, and also in the total plasma protein concentration (Amarante et al. 2004), resulting consequently in the development of anaemia, hypoproteinemia and subsequent death even before the pre-patent period of the infection (Emery et al. 2016).

According to Weiss & Wardrop (2010), the degree of anaemia varies according to age and resistance of infected hosts. Animal immunity plays an important role in resistance to worms, and individual factors such as age, breed and physiological condition regulate host response against parasites (Amarante 2014). Differences between sheep breeds regarding their susceptibility to infections by gastrointestinal nematodes have been reported (Amarante et al. 2004, Shakya et al. 2009). This is the case of Santa Ines sheep, a native breed from northeastern Brazil, considered more resistant to *H. contortus* infection when compared to Ile de France sheep (Amarante et al. 2004, Rocha et al. 2005, Amarante et al. 2009). Santa Ines lambs, after weaning, showed a higher resistance against *H. contortus* infection when compared to lambs of European breeds (Amarante et al. 2004, Albuquerque et al. 2019).

There are few studies that evaluated the immune response of suckling lambs against parasitic infections by gastrointestinal nematodes. Rocha et al. (2005) observed that Santa Ines lambs had higher eosinophil counts than Ile de France suckling lambs. Bahirathan et al. (1996) observed that Gulf Coast Native lambs had the ability to limit *H. contortus* infections by eliminating parasites, while Suffolk lambs developed haemonchosis and required anthelmintic treatment.

As haematological studies in suckling lambs infected with gastrointestinal nematodes in the first few days of life are limited, we carried out an evaluation on the haematological variables to assess the influence of *H. contortus* experimental infection on the bone marrow response of Santa Ines and Ile de France suckling lambs.

MATERIALS AND METHODS

Study area. The trial was conducted in the experimental area of the "Departamento de Bioestatística, Biologia Vegetal, Parasitologia e Zoologia", Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho" (Unesp), Botucatu/SP, Brazil. All the procedures involving animals in this study were conducted in accordance with the local Ethics Committee on Animal Use (protocol number 0118/2018, FMVZ-Unesp).

Production of infective larvae (L3) of *Haemonchus contortus.* An *H. contortus* isolate susceptible to anthelmintics (Echevarria et al. 1991) stored in liquid nitrogen, and maintained at the "Departamento de Bioestatística, Biologia Vegetal, Parasitologia e Zoologia" of IBB, was thawed to infect two donor lambs. The worm-free status of donors was confirmed by several faecal examinations. Each lamb was infected orally with 5500 *H. contortus* L3 in a single dose.

After pre-patent period of the parasite, donor lambs faeces were collected individually into collecting plastic bags twice a day, and then, each sample was used to perform faecal cultures, according to Ueno & Gonçalves (1998), for the production of L3 that was used in the infection of the experimental lambs.

Study design. Ile de France and Santa Ines pregnant ewes in the last third of gestation were acquired from two farms located in the rural area of São Manuel and Pardinho, respectively, both of them in São Paulo state, Brazil. After the naïve lambs were born, each ewe and its respective lamb were allocated into individual concrete floor pens with feeders and water fountains. Each pen was cleaned daily with water using a high-pressure pump in order to prevent undesired parasitic infections.

Fourteen Santa Ines lambs and 12 Ile de France lambs were randomized in four groups: infected Santa Ines (n=8), non-infected Santa Ines (n=6), infected Ile de France (n=8) and non-infected Ile de France (n=4). The criterion for distribution of the lambs into the groups was the order of birth. The first male or female born of each breed was allocated in the infected group; the second female or male born was allotted in the control group, and then consecutively until all groups were composed. It was used a 2 x 2 factorial design, with two infection status (Infected and Control) and two sheep breeds (Santa Ines and Ile de France).

Food and sanitary management. Ewes and their lambs were fed with *Cynodon* spp. hay, free of nematode-infective larvae, and received a daily dietary supplement with 18% of crude protein (CP) (Nutrição Animal Coopermota®). Additionally, the animals had free access to tap water and mineral salt (OvinoFós Tortuga®).

Experimental infections. Naïve lambs of the infected groups were infected, from 14 days until 66 days old, with *H. contortus* L3 through a serial infection protocol with a progressive increase in L3 numbers. Experimental infections were carried out each two days, being divided into three steps, differing in the number of L3 administered at each stage. In total, it was conducted 27 direct artificial infections (Fig.1). Infective larvae were administered directly into the lamb oral cavities. At the end of the infections (at 66 days of life), each lamb had received a total of 5400 L3. Control groups were kept worm-free during the whole experiment.

Data collection. Blood samples were obtained by jugular vein puncture into plain tubes containing anticoagulant (Vacutainer® K2 EDTA 7.2mg, BD, Brazil). Haematological exams performed were packed cell volume (PCV), total plasma protein estimation (TPP), red blood cell count (RBC), white blood cell count (WBC) and blood smear for differential leukocyte count (Jain 1986, Weiss & Wardrop 2010, Weiser 2012).

Table 1. Packed cell volume in percentage (%) means (± standard error) of Ile de France and Santa Ines suckling lambs experimentally infected with *Haemonchus contortus* and non-infected control

DPI	Ile de France		Santa Ines		Effects (*P*-value)	
	Infected (n=8)	Control (n=4)	Infected (n=8)	Control (n=6)	Breed	Infection status
0	36 (±1.11) a	35 (±1.03) ab	30 (±1.87) ab	29 (±1.94) b	0.0071	ns
6	34 (±1.12) a	34 (±0.33) a	28 (±1.87) bc	25 (±1.61) c	0.0002	ns
12	32 (±1.05) a	34 (±1.49) ab	27 (±1.82) bc	25 (±1.15) c	0.0007	ns
18	32 (±1.32) ab	35 (±0.96) b	27 (±1.1) ac	26 (±1.08) c	0.0002	ns
24	32 (±1.03)	37 (±0.25)	30 (±1.85)	30 (±1.33)	0.0153	ns
30	33 (±0.74) ab	36 (±0.91) a	31 (±1.16) b	32 (±1.33) ab	0.01	ns
36	33 (±0.53)	36 (±2.29)	33 (±1.0)	35 (±1.77)	ns	ns
42	34 (±0.81)	38 (±1.35)	34 (±0.85)	36 (±2.19)	ns	0.0293
48	34 (±1.32)	37 (±2.04)	34 (±1.13)	35 (±2.29)	ns	ns
54	36 (±1.13)	39 (±1.03)	35 (±1.43)	38 (±2.25)	ns	ns

DPI = Days post first infection; In each row, arithmetic means with different lower case letters are significantly different by Tukey test ($P<0.05$); ns = not significant ($P>0.05$); There was no significant interaction between breed x infection status ($P>0.05$).

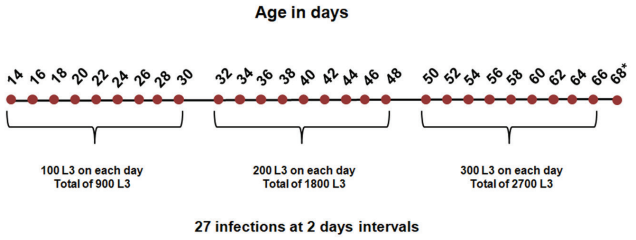


Fig.1. Timeline with the protocol of the experimental infections (27 at two days intervals) of the lambs with 5400 infective larvae (L3) of *Haemonchus contortus.* The last sampling was conducted at 68 days of age (asterisk).

Packed cell volume was determined by microhematocrit centrifugation (5 min/15000g, Fanem 211 microhematocrit centrifuge), and TPP concentrations were estimated using a hand held refractometer (Refractometer SPR-N, Atago).

Red blood cell count was performed manually in a Neubauer chamber using a 1:201 of blood (20µL of blood and 4000µL of saline solution 0.9% NaCl. The dilution was mixed continuously for two minutes by mechanical shaker. White blood cell count was performed manually in a Neubauer chamber using a 1:20 dilution of blood (20µL of blood and 380µL of Turk`s solution) (Jain 1986).

For differential leukocyte count, blood smear was stained using a Diff Quick stain (Panótico Rápido, Laborclin/PR, Brazil). Additionally, 100 leukocytes were differentiated and results were presented in absolute values (Jain 1986).

Faecal samples were collected directly from the rectum of animals. Faecal egg counts determination was performed using a modified McMaster technique in which each worm egg counted represented 100 eggs per gram (EPG) of faeces (Ueno & Gonçalves 1998).

Finally, it was conducted 10 sampling data collection in total, every six days, from 14 to 68 days old.

Statistical analysis. Data were evaluated in a normality test (Shapiro-Wilk test), and only PCV and TPP had normal distribution. Cell count results were non-normally distributed, and were transformed to log10(x + 1) prior to analysis. Furthermore, data were analyzed by analysis of variance through the General Linear Model (GLM) for variables determined at several time points, using the Statistical Analysis System, version 9.2 (SAS Institute, Inc., Cary/NC, USA). Means were compared by Tukey's test at a 5% significance level, and only significant interactions were reported in the results. Means are presented in the results as the arithmetic means (± standard error).

Table 2. Total plasma protein (TPP, g/dL) means (± standard error) of Ile de France and Santa Ines suckling lambs experimentally infected with *Haemonchus contortus* and non-infected control

DPI	Ile de France		Santa Ines	
	Infected (n=8)	Control (n=4)	Infected (n=8)	Control (n=6)
0	5.6 (±0.12)	5.9 (±0.24)	5.8 (±0.19)	6.1 (±0.34)
6	5.6 (±0.15)	5.9 (±0.18)	5.8 (±0.23)	5.9 (±0.35)
12	5.6 (±0.17)	5.8 (±0.13)	5.7 (±0.14)	5.7 (±0.37)
18	5.8 (±0.17)	5.7 (±0.22)	5.5 (±0.15)	5.5 (±0.36)
24	5.6 (±0.20)	5.9 (±0.13)	5.5 (±0.19)	5.6 (±0.29)
30	5.5 (±0.24)	5.5 (±0.15)	5.7 (±0.17)	5.6 (±0.35)
36	5.7 (±0.29)	5.9 (±0.05)	5.5 (±0.18)	5.7 (±0.32)
42	5.7 (±0.28)	6.0 (±0.15)	5.8 (±0.17)	5.8 (±0.26)
48	5.8 (±0.24)	6.0 (±0.14)	5.9 (±0.22)	5.9 (±0.26)
54	5.8 (±0.23)	6.2 (±0.14)	6.0 (±0.21)	6.0 (±0.23)

DPI = Days post first infection; There were no breed and infection status effects, and breed x infection status interaction on TPP values ($P>0.05$).

RESULTS

Lambs from control groups did not shed egg in faeces all over the experiment, while infected Santa Ines and Ile de France lambs presented in average 2963 EPG and 8175 EPG in the last sampling ($P<0.05$), respectively.

There was a time effect ($P<0.0001$) on PCV values (Table 1). Breed had a significant effect on PCV values from day 14 to 44 days of age, with Santa Ines lambs showing the lowest PCV values. In addition, there was an infection status effect ($P<0.05$) at 56 days of age (42 days post first infection - DPI) with control lambs showing the highest PCV means.

Furthermore, there was time effect ($P=0.0153$) on the TPP concentration (Table 2). Over the experiment, most of the TPP means remained below the reference interval for sheep.

In relation to RBC counting, it was noted a breed effect at 20, 44, 50, 56, 62 and 68 days old (6, 30, 36, 42, 48 and 54 DPI, respectively), with Santa Ines lambs presenting the highest RBC means from 44 to 68 days of age. Red blood cell trend was similar in both breeds, and the counting increased slightly as the suckling lambs got older (Table 3). There was time effect ($P<0.0001$) and a significant Time x Breed interaction ($P<0.0001$) on the RBC counting with Santa Ines lambs showing higher means in the last five samplings.

Infected Santa Ines lambs presented a higher number of RBC when compared to infected Ile de France lambs at 44 (P=0.017) and 56 (P=0.0346) days old (30 and 42 DPI, respectively). However RBC values of infected groups were within the reference interval.

White blood cell counts of infected and non-infected (control) groups are shown in Table 4. There was no significant difference among the groups all over the experiment. Breed influence (P=0.0182) occurred only at 32 days old (18 DPI) when Ile de France lambs had the highest counts. All values

Table 3. Red blood cell (x 10^6/µL) means (minimum and maximum values) of Ile de France and Santa Ines suckling lambs experimentally infected with *Haemonchus contortus* and non-infected control

DPI	Ile de France		Santa Ines		Effect (P-value)
	Infected (n=8)	Control (n=4)	Infected (n=8)	Control (n=6)	Breed
0	8.7 (7.6-9.9) #0.98 (±0.01)	7.9 (7.4-8.6) 0.95 (±0.01)	8.0 (5.8-9.82) 0.95 (±0.02)	8.2 (6.8-9.7) 0.96 (±0.02)	ns
6	9.5 (8.2-11) 1.02 (±0.01) a	9.16 (8.3-10.0) 0.98 (±0.03) ab	8.3 (7.11-9.97) 0.97 (±0.02) ab	7.8 (6.8-9.0) 0.94 (±0.01) b	0.0173
12	9.4 (8.4-11.3) 1.01 (±0.01)	9.1 (8.1-9.9) 1.0 (±0.02)	8.8 (7.63-10.5) 0.99 (±0.01)	8.3 (7.7-9.5) 0.97 (±0.01)	ns
18	9.4 (7.3-10.4) 1.02 (±0.01)	9.6 (8.7-10.5) 1.03 (±0.02)	9.4 (8.25-10.2) 1.02 (±0.01)	8.4 (7.4-9.7) 0.97 (±0.02)	ns
24	9.1 (6.9-10.6) 1.0 (±0.02)	10.3 (9.5-10.8) 1.05 (±0.01)	9.8 (9.1-12.6) 1.03 (±0.01)	10.0 (9.6-10.6) 1.04 (±0.006)	ns
30	9.3 (7.5-10.6) 1.01 (±0.01) a	10.0 (9.7-10.4) 1.04 (±0.007) ab	10.9 (9.1-12.6) 1.07 (±0.02) b	11.1 (9.4-12.6) 1.08 (±0.02) b	0.0074
36	9.6 (7.3-10.6) 1.02 (±0.02) a	9.8 (8.6-10.4) 1.03 (±0.02) ab	11 (8.7-14.1) 1.08 (±0.02) ab	11.9 (10.1-13.7) 1.11 (±0.02) b	0.0052
42	9.4 (5-12.5) 1.01 (±0.04) a	10.1 (8.3-12.0) 1.04 (±0.03) ab	12.3 (9.7-15.1) 1.12 (±0.02) b	12.1 (8.9-14.0) 1.11 (±0.03) ab	0.0094
48	10.2 (8.7-13.0) 1.05 (±0.02)	10.5 (9.0-12.2) 1.06 (±0.02)	12.0 (10-14.5) 1.11 (±0.02)	12.0 (9.7-17.2) 1.11 (±0.03)	0.0329
54	10.3 (8.4-14.4) 1.05 (±0.02) a	11.7 (10.4-12.4) 1.1 (±0.02) ab	12.9 (9.9-15.7) 1.14 (±0.03) ab	13.8 (10.3-16.2) 1.16 (±0.03) b	0.0129

DPI = Days post first infection; # means (± standard error) of data transformed using log (x + 1); In each row, arithmetic means with different lower case letters are significantly different by Tukey test (P<0.05); ns = not significant (P>0.05); There were no infection status effects, and breed x infection status interaction on red blood cell counts (P>0.05).

Table 4. White blood cell (cells/µL) means (minimum and maximum values) of Ile de France and Santa Ines suckling lambs experimentally infected with *Haemonchus contortus* and non-infected control

DPI	Ile de France		Santa Ines		Effects (P-value)
	Infected (n=8)	Control (n=4)	Infected (n=8)	Control (n=6)	Breed
0	6544 (4200-8700) #3.8 (±0.04)	7025 (5550-8450) 3.84 (±0.05)	6175 (3850-10700) 3.76 (±0.06)	6025 (4500-8000) 3.77 (±0.05)	ns
6	7481 (4300-10200) 3.9 (±0.04)	6500 (5400-8550) 3.8 (±0.06)	6238 (4050-8100) 3.78 (±0.04)	9458 (5300-13500) 3.95 (±0.07)	ns
12	7109 (4875-10200) 3.84 (±0.04)	7363 (5800-8950) 3.86 (±0.04)	6225 (3300-11500) 3.76 (±0.06)	6592 (3950-9950) 3.8 (±0.06)	ns
18	7188 (5250-8600) 3.85 (±0.03)	9738 (6100-11550) 3.98 (±0.06)	6613 (3950-11650) 3.8 (±0.05)	5792 (4600-6350) 3.76 (±0.02)	0.0182
24	7863 (6300-11250) 3.89 (±0.03)	9663 (7300-11200) 3.98 (±0.04)	7625 (3500-10850) 3.85 (±0.06)	6525 (4950-9500) 3.81 (±0.04)	ns
30	8344 (6700-11750) 3.92 (±0.03)	9213 (8000-10400) 3.96 (±0.02)	7338 (4750-10450) 3.85 (±0.05)	7333 (5250-9050) 3.86 (±0.04)	ns
36	9581 (5050-16800) 3.96 (±0.05)	9375 (7000-11200) 3.97 (±0.05)	7756 (5650-10750) 3.88 (±0.03)	8175 (6200-9800) 3.91 (±0.03)	ns
42	9525 (6600-12150) 3.97 (±0.04)	9650 (8300-10950) 3.98 (±0.03)	8631 (4800-12850) 3.92 (±0.05)	8700 (6700-10150) 3.93 (±0.03)	ns
48	10000 (7600-17300) 3.98 (±0.04)	9363 (8500-10600) 3.97 (±0.02)	9969 (5500-15600) 3.98 (±0.05)	8483 (6200-11950) 3.92 (±0.04)	ns
54	8244 (6300-12350) 3.9 (±0.04)	9275 (8400-10500) 3.97 (±0.02)	10131 (6450-16000) 3.99 (±0.05)	9358 (6000-12050) 3.96 (±0.05)	ns

DPI = Days post first infection; # means (± standard error) of data transformed using log (x + 1); ns = not significant (p>0.05); There were no infection status effects, and breed x infection status interaction on white blood cell counts (P>0.05).

found for WBC were within the reference interval for the species. There was a significant time effect (P<0.0001), Time x Breed (P=0.0479) and Time x Breed x Infection status (P=0.0326) interactions on the WBC counting.

Differential leukocyte counting is presented in absolute numbers. With regards to eosinophils means, there was a breed effect (P=0.0037) on eosinophils counting at 20 days old (6 DPI), with a significant difference only between infected and non-infected Santa Ines lambs (P=0.0037). A similar result was observed at 42 DPI (56 days old), when infected Santa Ines lambs had higher number of eosinophils than non-infected Santa Ines counterparts (P=0.0242). There was a significant Breed x Infection status interaction on eosinophils counting at 24, 36, 42 and 54 DPI (38, 50, 56 and 68 days old). Additionally, infected Santa Ines lambs had significantly higher number (P=0.0256) of eosinophils at 68 days old, when compared to infected Ile de France lambs (Table 5).

There was an infection status effect on neutrophils counting (P=0.0481) at 6 DPI (20 days old) (Table 6). Infected Santa Ines lambs had lower neutrophil count than non-infected

Table 5. Eosinophil (cells/μL) means (minimum and maximum values) of Ile de France and Santa Ines suckling lambs experimentally infected with *Haemonchus contortus* and non-infected control

DPI	Ile de France		Santa Ines		Effects (P-value)		
	Infected (n=8)	Control (n=4)	Infected (n=8)	Control (n=6)	Breed	Infection status	Breed x Infection status
0	398 (49-959)	355 (111-761)	323 (52-1071)	254 (0-576)	ns	ns	ns
	#2.42 (±0.16)	2.42 (±0.18)	2.31 (±0.16)	1.71 (±0.54)			
6	492 (61-918)	311 (171-540)	216 (41-568)	80 (0-212)	0.0037	ns	ns
	2.54 (±0.17) a	2.55 (±0.15) a	2.18 (±0.14) a	1.1 (±0.49) b			
12	286 (0-918)	484 (116-1074)	169 (48-504)	199 (0-624)	ns	ns	ns
	1.85 (±0.42)	2.54 (±0.21)	2.11 (±0.12)	1.52 (±0.51)			
18	139 (0-453)	423 (217-941)	191 (0-557)	139 (46-315)	ns	ns	ns
	1.82 (±0.28)	2.54 (±0.15)	1.95 (±0.3)	2.06 (±0.12)			
24	105 (0-258)	469 (91-1232)	247 (70-508)	95 (0-285)	ns	ns	0.0064
	1.16 (±0.44)	2.48 (±0.24)	2.3 (±0.11)	1.39 (±0.45)			
30	220 (0-668)	474 (80-1128)	433 (95-1012)	223 (67-496)	ns	ns	ns
	1.79 (±0.4)	2.5 (±0.24)	2.52 (0.12)	2.25 (±0.14)			
36	133 (0-344)	405 (87-959)	452 (170-1656)	198 (0-496)	ns	ns	0.0240
	1.62 (±0.37)	2.46 (±0.22)	2.52 (±0.11)	1.63 (±0.52)			
42	210 (0-495)	350 (83-657)	443 (120-994)	108 (0-335)	ns	ns	0.0126
	2.0 (±0.30) ab	2.37 (±0.24) ab	2.57 (±0.10) a	1.14 (±0.52) b			
48	244 (79-519)	225 (106-425)	500 (96-1212)	228 (91-372)	ns	ns	ns
	2.31 (±0.1)	2.30 (±0.12)	2.59 (±0.13)	2.31 (±0.1)			
54	138 (0-247)	458 (281-672)	508 (160-867)	148 (97-279)	ns	ns	0.0095
	1.69 (±0.37) a	2.63 (±0.08) ab	2.63 (±0.10) b	2.14 (±0.07) ab			

DPI = Days post first infection; # means (± standard error) of data transformed using log (x + 1); In each row, arithmetic means with different lower case letters are significantly different by Tukey test (P<0.05); ns = not significant (P>0.05).

Table 6. Neutrophil (cells/μL) means (minimum and maximum values) of Ile de France and Santa Ines suckling lambs experimentally infected with *Haemonchus contortus* and non-infected control

DPI	Ile de France		Santa Ines		Effects (P-value)
	Infected (n=8)	Control (n=4)	Infected (n=8)	Control (n=6)	Infection status
0	2769 (1308-4300)	3337 (2498-4452)	3055 (1519-5243)	3306 (1786-5076)	ns
	#3.41 (±0.06)	3.51 (±0.06)	3.44 (±0.07)	3.49 (±0.07)	
6	2584 (1333-3672)	3311 (1404-5643)	2617 (1134-4350)	6396 (2096-10004)	0.0481
	3.39 (±0.06) a	3.45 (±0.3) ab	3.39 (±0.06) a	3.73 (±0.12) b	
12	2334 (975-3978)	2797 (2280-3491)	2869 (825-6670)	3606 (2054-6048)	ns
	3.32 (±0.08)	3.44 (±0.17)	3.39 (±0.09)	3.51 (±0.09)	
18	2385 (1304-4043)	4919 (2074-8138)	2480 (672-6058)	2332 (1260-3422)	ns
	3.35 (±0.06)	3.63 (±0.04)	3.31 (±0.1)	3.33 (±0.08)	
24	2936 (1935-5063)	3815 (2628-5159)	2981 (945-4557)	2519 (1040-4275)	ns
	3.45 (±0.05)	3.57 (±0.13)	3.42 (±0.09)	3.35 (±0.09)	
30	2249 (1192-3525)	3375 (3008-3620)	2921 (1140-5225)	3108 (1628-4446)	ns
	3.32 (±0.06)	3.53 (±0.06)	3.42 (±0.07)	3.47 (±0.07)	
36	3985 (1414-12432)	2819 (2450-3136)	2858 (1541-4623)	3671 (2146-6174)	ns
	3.5 (±0.1)	3.45 (±0.02)	3.44 (±0.05)	3.54 (±0.06)	
42	3159 (1188-5429)	3602 (2883-5037)	3570 (1440-6570)	4167 (3082-5887)	ns
	3.45 (±0.08)	3.55 (±0.05)	3.5 (±0.07)	3.61 (±0.04)	
48	3434 (1238-9688)	3548 (2635-4346)	3927 (1540-9204)	4661 (2604-7768)	ns
	3.44 (±0.1)	3.54 (±0.06)	3.53 (±0.08)	3.64 (±0.07)	
54	2667 (704-4940)	3172 (2415-4602)	3762 (1812-9760)	4718 (2280-8556)	ns
	3.35 (±0.1)	3.49 (±0.06)	3.51 (±0.08)	3.64 (±0.08)	

DPI = Days post first infection; # means (± standard error) of data transformed using log (x + 1); In each row, arithmetic means with different lower case letters are significantly different by Tukey test (P<0.05); ns = not significant (P>0.05); There were not breed effect and breed x infection status interaction on Neutrophil count (P > 0.05).

Santa Ines (P=0.0376). There was a significant Time x Breed interaction (P=0.0125) on the neutrophil counting.

Regarding to basophils counting (Table 7), infected Santa Ines lambs had higher values when compared to infected Ile de France, furthermore there was a significant difference between infected groups only at 6 DPI (P=0.0001).

It was observed a time effect (P<0.0001) on the lymphocytes number (Table 8). Infected Ile de France lambs had higher lymphocytes numbers than infected Santa Ines at 12 and 30 DPI (26 and 44 days old) (P=0.0117 and P=0.0043, respectively). There was breed effect on lymphocyte counting at 12, 30, 36, 42 and 48 DPI (26, 44, 50, 56 and 62 days old), and infection

Table 7. Basophil (cells/µL) means (minimum and maximum values) of Ile de France and Santa Ines suckling lambs experimentally infected with *Haemonchus contortus* and non-infected control

DPI	Ile de France		Santa Ines		Effects (*P*-value)
	Infected (n=8)	Control (n=4)	Infected (n=8)	Control (n=6)	Breed
0	49 (0-137) #0.99 (±0.38)	77 (0-168) 1.48 (±0.5)	113 (0-230) 1.81 (±0.27)	82 (0-144) 1.64 (±0.33)	ns
6	25 (0-126) 0.5 (±0.33) a	37 (0-56) 1.16 (±0.58) ab	198 (52-355) 2.24 (±0.09) b	144 (64-368) 2.08 (±0.11) b	0.0001
12	71 (0-146) 1.27 (±0.38)	74 (58-90) 1.87 (±0.04)	103 (0-220) 1.56 (±0.35)	56 (0-126) 1.27 (±0.4)	ns
18	39 (0-125) 0.93 (±0.36)	86 (0-122) 1.54 (±0.52)	102 (0-375) 1.34 (±0.4)	95 (0-173) 1.69 (±0.34)	ns
24	80 (0-210) 1.49 (±0.33)	64 (0-146) 1.05 (±0.61)	178 (0-305 1.99 (±0.29)	47 (0-118) 0.98 (±0.44)	ns
30	45 (0-118) 0.97 (±0.37)	95 (0-181) 1.56 (±0.52)	95 (0-244) 1.53 (±0.34)	30 (0-67) 0.89 (±0.39)	ns
36	65 (0-157) 1.24 (±0.37)	75 (0-213) 1.07 (±0.62)	139 (0-335) 1.68 (±0.37)	71 (0-166) 1.34 (±0.43)	ns
42	69 (0-122) 1.47 (±0.32)	72 (0-110) 1.48 (±0.49)	113 (0-257) 1.8 (±0.27)	70 (0-185) 1.07 (±0.48)	ns
48	77 (0-210) 1.29 (±0.38)	51 (0-106) 1.0 (±0.58)	125 (0-312) 1.62 (±0.36)	35 (0-120) 0.67 (±0.43)	ns
54	63 (0-247) 1.02 (±0.39)	96 (0-210) 1.55 (±0.52)	111 (0-269) 1.39 (±0.41)	75 (0-241) 1.07 (±0.48)	ns

DPI = Days post first infection; # means (± standard error) of data transformed using log (x + 1); In each row, arithmetic means with different lower case letters are significantly different by Tukey test (P<0.05); ns = not significant (P>0.05); There were no infection status effect and breed x infection status interaction on Basophil count (P>0.05).

Table 8. Lymphocyte (cells/µL) means (minimum and maximum values) of Ile de France and Santa Ines suckling lambs experimentally infected with *Haemonchus contortus* and non-infected control

DPI	Ile de France		Santa Ines		Effects (P-value)		
	Infected (n=8)	Control (n=4)	Infected (n=8)	Control (n=6)	Breed	Infection status	Breed x Infection status
0	3299 (2040-4611) #3.5 (±0.05)	3194 (2280-3972) 3.5 (±0.05)	2593 (1360-5136) 3.38 (±0.06)	2310 (1350-3360) 3.34 (±0.06)	ns	ns	ns
6	4297 (2623-5765) 3.61 (±0.05) a	2787 (2331-3294) 3.44 (±0.04) ab	3123 (2273-4131) 3.49 (±0.03) ab	2728 (1960-4001) 3.42 (±0.06) b	ns	0.0377	ns
12	4328 (3045-6624) 3.62 (±0.04) a	3912 (3190-4560) 3.59 (±0.03) ab	2996 (2138-4715) 3.46 (±0.05) b	2668 (1857-3980) 3.41 (±0.05) b	0.004	ns	ns
18	4476 (3379-6439) 3.64 (±0.03)	4253 (2496-5957) 3.6 (±0.08)	3780 (2491-5476) 3.56 (±0.04)	3185 (2204-4725) 3.49 (±0.05)	ns	ns	ns
24	4640 (3577-6586) 3.66 (±0.03)	5130 (3620-6882) 3.7 (±0.06)	4172 (2485-6192) 3.6 (±0.05)	3814 (2596-5320) 3.56 (±0.05)	ns	ns	ns
30	5757 (4416-7638) 3.75 (±0.03) a	5095 (4480-6032) 3.7 (±0.03) ab	3801 (2744-5638) 3.57 (±0.04) b	3880 (2747-6210) 3.57 (±0.06) b	0.0016	ns	ns
36	5272 (3535-6870) 3.71 (±0.04)	6076 (4200-7840) 3.77 (±0.06)	4183 (3221-5698) 3.62 (±0.03)	4101 (2542-5307) 3.59 (±0.05)	0.0110	ns	ns
42	6034 (4123-8505) 3.77 (±0.04)	5757 (4897-7437) 3.75 (±0.04)	4391 (2976-6682) 3.63 (±0.04)	4277 (3948-5700) 3.62 (±0.04)	0.0048	ns	ns
48	6169 (4582-8362) 3.78 (±0.03) a	5470 (4947-6042) 3.74 (±0.02) a	5162 (3300-7028) 3.7 (±0.04) a	3452 (2730-4160) 3.53 (±0.02) b	0.0012	0.0105	ns
54	5224 (3564-6669) 3.71 (±0.03)	5969 (4872-7350) 3.77 (±0.04)	5681 (3999-9104) 3.7 (±0.04)	4373 (3133-6612) 3.63 (±0.05)	ns	ns	0.0319

DPI = Days post first infection; # means (± standard error) of data transformed using log (x + 1); In each row, arithmetic means with different lower case letters are significantly different by Tukey test (P<0.05); ns = not significant (P>0.05).

status effect at 6 DPI and 48 DPI. Significant Breed x Infection status effect was found only at 54 DPI (68 days old). Infected Santa Ines had a higher lymphocyte count than non-infected Santa Ines lambs (P=0.0001) at 48 DPI (62 days old).

With regards to monocytes means (Table 9), there was an infection status effect at 18 DPI (32 days old) (P=0.0321). A significant Breed x Infection interaction, and a breed effect (P=0.002) were observed at 36 DPI (50 days old). Infected Ile de France had higher number of monocytes than non-infected Ile de France lambs at 36 DPI (50 days old) (P=0.0051).

DISCUSSION

Clinical signs caused by *Haemonchus contortus* infection, such as anaemia, apathy and submandibular oedema were absent in the Santa Ines and Ile de France suckling lambs of the present study. The number of *H. contortus* L3 for conducting experimental infection did not cause significant reduction in PCV, TPP and RBC means over the experiment.

According to Jain (1986), PCV and RBC for healthy sheep are 27-45%, and 8-16 x 10^6/µL, respectively. Santa Ines and Ile de France suckling lambs from infected and control groups presented PCV means within reference interval for healthy sheep almost all over the trial, excepting for non-infected Santa Ines group in the first days of age. By the age of 38 days old (24 DPI), Santa Ines lambs (infected and control) presented PCV means over 30%. In addition, Ile de France lambs had RBC counts under reference interval at 14 days old, and Santa Ines lambs from infected and non-infected groups presented RBC below reference values until 26 days of age (12 DPI).

Pereira et al. (2015) observed that healthy Santa Ines lambs under 75 days old had PVC and RBC means of 29.15% and 11.72 x 10^6/µL, respectively. We observed that Santa Ines suckling lambs up to 68 days of age had, in some time points, PCV means and RBC count approximated of those found by Pereira et al. (2015). In a survey conducted by Meneghini et al. (2016) healthy Santa Ines lambs up to 6 months old presented RBC and PCV means of 11.98 x 10^6/µL and 32.59%, respectively.

Our results showed that for Santa Ines suckling lambs (infected and non-infected) between 14 and 32 days old, while RBC count increased, PCV values decreased. Considering only non-infected groups of both breeds as control reference groups, low PCV and RBC values appears to be a breed characteristic of Santa Ines lambs during the first few days of life, once both variables for Ile de France lambs remained stable.

Our results indicated that healthy Santa Ines and Ile de France suckling lambs up to 68 days old, had TPP means between 5.0 and 6.2g/dL. These results of Ile de France and Santa Ines of both groups were below the reference intervals at least until 50 days old (36 DPI) for Ile de France lambs, and 68 days old (54 DPI) for Santa Ines lambs. Our results for non-infected Santa Ines lambs were in disagreement with those found by Pereira et al. (2015) that noted 7.09g/dL as TPP mean in Santa Ines lambs up to 75 days old. Healthy Manchega Spanish lambs up to 30 days of age had TPP mean of 6.4g/dL, while in lambs aged between 30 and 70 days old, mean was 5.7g/dL (Bórnez et al. 2009).

Comparing to their respective control groups (non-infected), infected Santa Ines lambs had higher circulating WBC than infected Ile de France lambs, but such differences were not significant. In studies focusing on differences between susceptible and resistant breeds, Bowdridge et al. (2015) reported similar results in St. Croix (resistant) that had higher WBC than wool lambs (susceptible) after experimental infection with *H. contortus*.

Regarding to lymphocyte counts, infected groups presented higher means than their respective non-infected control

Table 9. Monocyte (cells/µL) means (minimum and maximum values) of Ile de France and Santa Ines suckling lambs experimentally infected with *Haemonchus contortus* and non-infected control

DPI	Ile de France		Santa Ines		Effects (P-value)		
	Infected (n=8)	Control (n=4)	Infected (n=8)	Control (n=6)	Breed	Infection status	Breed x Infection status
0	28 (0-87) #0.7 (±0.34)	127 (0-252) 1.64 (±0.55)	80 (0-180) 1.47 (±0.33)	73 (0-160) 1.33 (±0.43)	ns	ns	ns
6	83 (0-378) 1.1 (±0.41)	55 (0-108) 1.26 (±0.64)	92 (0-303) 1.1 (±0.42)	110 (0-350) 1.42 (±0.46)	ns	ns	ns
12	91 (0-210) 1.52 (±0.34)	96 (0-152) 1.57 (±0.53)	89 (0-208) 1.53 (±0.34)	63 (0-199) 1.03 (±0.46)	ns	ns	ns
18	148 (0-344) 1.87 (±0.29)	57 (0-122) 1.03 (±0.59)	74 (0-239) 1.43 (±0.32)	41 (0-127) 0.7 (±0.44)	ns	0.0321	ns
24	102 (0-225) 1.76 (±0.26)	185 (0-333) 1.79 (±0.6)	61 (0-124) 1.24 (±0.36)	49 (0-70) 1.48 (±0.3)	ns	ns	ns
30	74 (0-173) 1.48 (±0.33)	151 (0-416) 1.22 (±0.71)	89 (0-276) 1.47 (±0.34)	92 (0-201) 1.41 (±0.45)	ns	ns	ns
36	128 (0-336) 1.61 (±0.36) a	0 (0-0) 0 (±0.0) b	108 (0-201) 1.79 (±0.27) a	136 (74-261) 2.09 (±0.08) a	0.002	ns	0.0071
42	54 (0-363) 0.55 (±36)	121 (0-219) 1.64 (±0.55)	129 (0-359) 1.82 (±0.28)	78 (0-201) 1.36 (±0.43)	ns	ns	ns
48	152 (0-865) 1.34 (±0.41)	70 (0-194) 1.06 (±0.55)	254 (126-468) 2.36 (±0.07)	108 (0-239) 1.71 (±0.35)	ns	ns	ns
54	91 (0-247) 1.51 (±0.34)	23 (0-94) 1.06 (±0.61)	68 (0-227) 1.05 (±0.4)	45 (0-97) 0.98 (±0.44)	ns	ns	ns

DPI = Days post first infection; # means (± standard error) of data transformed using log (x + 1); In each row, arithmetic means with different lower case letters are significantly different by Tukey test (P<0.05); ns = not significant (P>0.05).

groups, however, infected Santa Ines lambs had significantly higher number of cells than non-infected Santa Ines only at 62 days old (48 DPI). Increases in lymphocyte number and WBC count were related to the recruitment of defence Th2 cells to the parasite-host interaction site (Balic et al. 2000, Bowdridge et al. 2015).

Significant differences in blood eosinophil count between Santa Ines groups (infected and uninfected control) were observed in two time points. Comparing infected groups only, there was a significant difference between breeds in circulating eosinophil count at 54 DPI (68 days old), with infected Santa Ines lambs presenting the highest mean. However, the number of eosinophils was within the reference interval for sheep in both breeds. Rocha et al. (2005) in study with Santa Ines and Ile the France suckling lambs naturally infected by gastrointestinal nematodes, observed no significant differences between breeds regarding to eosinophil counts, although Santa Ines lambs had higher means of eosinophils than Ile the France lambs over the trial. In a *H. contortus* chronic infection, naturally infected Santa Ines lambs (8 months old) had higher mean blood eosinophil counts than Ile de France lambs (Albuquerque et al. 2019).

Eosinophils are important in the defence against infective larvae of gastrointestinal nematodes (Meeusen & Balic 2000, Tizard 2014), and eosinophilia is reported as a protective response against *H. contortus* (Terefe et al. 2005). Additionally, they have a variety of receptors (including those for cytokines, and immunoglobulins) that enable them to be recruited into affected tissues (Makepeace et al. 2012). *In vitro* studies have shown that eosinophils are able of reducing *H. contortus* L3 motility, and decrease infectivity of L3 in the host (Terefe et al. 2005, Holt et al. 2015). On tissue, eosinophils can limit parasite growth, decrease fecundity of the parasite, and also damaging it morphologically (Balic et al. 2000, McRae et al. 2015, Huang & Appleton 2016).

A type 2 immune (Th2) response plays a major role in host protection against gastrointestinal nematodes (GIN) infections, through the induction of cytokines, antibody production, and recruitment of multiple inflammatory cells (McRae et al. 2015, Sorobetea et al. 2018). After GIN getting the site of infection, their antigens are presented by antigen-presenting cells to naïve T cells, that result in the release of Th2-associated cytokines (Makepeace et al. 2012, McRae et al. 2015). Interleukins secreted by Th2 cells, such as interleukin (IL) 5 and IL-13, can stimulate stem cell of bone morrow into eosinophil differentiation (Park & Bochner 2010), and to their recruitment to the site of the infection. For this reason, eosinophil number increases significantly in the blood (Meeusen et al. 2005, Tizard 2014) and after getting into circulation, eosinophils rapidly migrate into tissues to the site of infection (Anthony et al. 2007). Resistant sheep breeds may present a higher production of interleukins related to Th2 response, especially IL-4, IL-5 and IL-13 (Terefe et al. 2007).

Several studies have shown that Santa Ines sheep is considered more resistant to GIN infections compared to Ile de France sheep at different age categories (Amarante et al. 2004, 2009, Rocha et al. 2005, 2011), mainly because of Santa Ines sheep present an earlier and robust initial Th2 response (Albuquerque et al. 2019).

Absence of difference between susceptible and resistant sheep breeds in circulating eosinophil count may thus reflect immune responses to different stages of GIN infection (Bowdridge et al. 2015). As supposed by Albuquerque et al. (2019), our results suggest that Santa Ines lambs had an increase in eosinophil production, however differences on circulation number were not identified, because they also promote a rapid migration of such cells to the site of parasite infection.

Neuthrophil and monocyte counts were similar in both breeds. Different results were reported in older lambs, previously infected and then artificially challenged with *H. contortus*. In this study, St. Croix hair (resistant) had higher WBC and neutrophil counts than wool lambs (susceptible) (Bowdridge et al. 2015). *In vitro,* neutrophils from these resistant St. Croix lambs, bound to *H. contortus* L3 after reinfection, in a higher percentage than neutrophils from susceptible Suffolk lambs (Garza et al. 2018). Neutrophils are important cells that guide and support the innate and adaptive immune response (Mantovani et al. 2011). Such cells secrete a variety of pro-inflammatory mediators that amplify the inflammatory process, and their counts indicate an important role in modulating immune response (Bowdridge et al. 2015, Weiss & Wardrop 2010) and as indicator of effective host response against helminthiasis (Ortolani et al. 2013). After a brief period in circulation, neutrophils may transmigrate into site of parasitic helminth invasion, and working in coordination with other cell populations, including eosinophils and macrophages, prevent larval establishment through damage tissue-dwelling helminths (Anthony et al. 2007).

Even within the reference range for sheep and without statistical differences, infected Santa Ines lambs presented highest means of basophils, excepting at 30 DPI (44 days old). Such increased in basophils number coincided with the increase in eosinophils values at 20 days old (6 DPI) as well. In accordance with our results, Bowdridge et al. (2015) observed that St. Croix hair (resistant) presented higher means of basophils than wool (susceptible) lambs. Basophils are relatively rare and short-lived cell type, however they play an important role in amplifying type 2 immune response to helminth infections through the production in large quantities of effector molecules including histamine, cytokines, chemokines, and lipid mediators (Mitre & Nutman 2006, Eberle & Voehringer 2016).

CONCLUSION

The mild *Haemonchus cortortus* infection did not induce significant changes in the blood variables of the Ile de France and Santa Ines suckling lambs, although infected Santa Ines lambs had higher counts of eosinophils. Healthy Santa Ines and Ile de France suckling lambs up to 68 days old, had total plasma protein (TPP) means between 5.0 and 6.2g/dL. Low packed cell volume (PCV) and red blood cell count (RBC) count could be a breed characteristic of Santa Ines lambs during the first days of life.

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Black queen cell virus and *Nosema ceranae* coinfection in Africanized honey bees from southern Brazil[1]

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ABSTRACT.- Chagas D.B., Monteiro F.L., Barcelos L.S., Frühauf M.I., Ribeiro L.C., Lima M., Hübner S. & Fischer G. 2020. **Black queen cell virus and *Nosema ceranae* coinfection in Africanized honey bees from southern Brazil.** *Pesquisa Veterinária Brasileira 40(11):892-897.* Laboratório de Virologia e Imunologia, Departamento de Veterinária Preventiva, Faculdade de Veterinária, Universidade Federal de Pelotas, Campus Capão do Leão, Pelotas, RS 96010-900, Brazil. E-mail: geferson.fischer@gmail.com

Bees are fundamental in several aspects, especially in relation to plant biodiversity and pollination. Recently, immense losses are being faced in the number of Brazilian colonies, mainly in southern states of the country, which has a strong beekeeping activity. There are indications that, among the reasons for the losses, pathogens that affect the health of bees may be involved. Among them, the microsporidium *Nosema* and the black queen cell virus (BQCV) stand out for their prevalence. In this study, 92 colonies of 17 apiaries from southern Brazil were evaluated for infection by *Nosema ceranae*, *Nosema apis* and BQCV. Nucleic acid extractions and cDNA synthesis were performed from adult bee samples, followed by Reverse Transcription Polymerase Chain Reaction (RT-PCR) and multiplex PCR. Eight BQCV positive samples were subjected to sequencing. The results showed that *N. ceranae* and BQCV are circulating in the Southern region of the country, which may be the reason for the loss of colonies. *N. apis* was not found. *N. ceranae* was found in 57.6% (53/92) of the colonies and BQCV in 32.6% (30/92). Co-infection was found in 25% (23/92) of the colonies studied, a factor that is suggested to be reducing the hosts' longevity due to the synergistic action of the pathogens. The samples submitted to sequencing indicated similarity of 96.8 to 100% between them, in addition to strong similarity with sequences from Asia, United States, Germany and Peru. This study reports the circulation of *N. ceranae* and BQCV in apiaries in southern Brazil, in addition to being the first phylogenetic analysis of the Brazilian BQCV sequence.

INDEX TERMS: Black queen cell virus, *Nosema ceranae*, coinfection, Africanized honey bees, Brazil, *Apis mellifera*, BQCV, RT-PCR, phylogeny.

RESUMO.- [**Coinfecção por vírus da realeira negra (BQCV) e *Nosema ceranae* em abelhas africanizadas no sul do Brasil.**] As abelhas mostram-se fundamentais em diversos aspectos, especialmente com relação à biodiversidade de plantas e polinização. Recentemente, estão sendo enfrentadas imensas perdas no número de colônias brasileiras, principalmente nos estados do sul do país, com forte atividade apícola. Há indicativos de que, dentre as razões para as perdas, possam estar envolvidos patógenos que afetam a saúde das abelhas. Dentre eles, o microsporídio *Nosema* e o vírus da realeira negra (BQCV) destacam-se pela prevalência. Neste estudo, foram avaliadas 92 colônias, de 17 apiários do sul do Brasil, a respeito da infecção por *Nosema ceranae, Nosema apis* e BQCV. Foram realizadas extrações de ácidos nucleicos e síntese de cDNA a partir de amostras de abelhas adultas, seguidos de Reação em Cadeia da Polimerase-Transcriptase Reversa (RT-PCR). Oito amostras positivas para BQCV foram submetidas a sequenciamento. Os resultados mostraram que *N. ceranae* e BQCV estão circulando na região sul do país, podendo ser a razão para as perdas de colônias. *N. apis* não foi encontrado. *N. ceranae* foi encontrado em 57.6% (53/92) das colônias e BQCV em 32.6% (30/92). Foi encontrada coinfecção por ambos

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em 25% (23/92) das colônias estudadas, fator que sugere a diminuição da longevidade do hospedeiro por ação sinérgica dos patógenos. As amostras submetidas ao sequenciamento indicaram similaridade de 96.8 a 100% entre elas, além de forte similaridade com sequências da Ásia, Estados Unidos, Alemanha e Peru. Este estudo relata a circulação de *N. ceranae* e BQCV nos apiários do sul do Brasil, além de ser a primeira análise filogenética da sequência do BQCV brasileiro.

TERMOS DE INDEXAÇÃO: Coinfecção, vírus da realeira negra, *Nosema ceranae*, abelhas africanizadas, Brasil, *Apis mellifera*, BQCV, RT-PCR, filogenia.

INTRODUCTION

The honey bee plays a vital role in world agriculture by providing pollination services to numerous wild flowering plants, there by supporting biodiversity (Allsopp et al. 2008, Smith et al. 2013). In Brazil, almost 40 thousand tons of honey are produced annually from 2.5 to 3 million hives spread throughout the national territory (Wolff et al 2018). In recent years, extensive colony losses and declining bee populations have been observed in Brazil, especially in the southern region. In three months, more than 500 million bees were found dead by beekeepers in only four Brazilian states, with Rio Grande do Sul responsible for 400 million deaths and Santa Catarina for 50 million deaths (Wolff et al 2018).

While the exact cause of colony losses remains unidentified, a growing number of evidences in various countries indicates that pathogens are associated in population declines of honey bees (Higes et al. 2008, Carreck et al. 2010). Environmental changes due to new agricultural practices and globalization may facilitate the spread of pathogens. Among the multiple pathogens infecting honey bees (Chagas et al. 2019), the microsporidia *Nosema ceranae* is one of the most prevalent parasite of these insects. Besides this, honey bees are also infected by several RNA viruses, and one of the most prevalent is the black queen cell virus (BQCV) (Yang et al. 2013, Porrini et al. 2016).

The microsporidian *Nosema* infects insects such as honey bees, bumble bees and silkworms. In honey bees has been considered one of the most serious pathogens (Higes et al. 2008). Two species can affect honey bees: *N. ceranae,* originally from Asia, and *Nosema apis*, which infects the Western honey bee (Fries et al. 1996). *N. ceranae* has been infecting Africanized *A. mellifera* bees in Brazil for at least the past 34 years (Teixeira et al. 2013). The Nosemosis caused by these parasites has spread worldwide and is currently responsible for considerable economic losses in apiculture (Fries 2010). The microsporidian invades the mid gut epithelial cells of the honey bees, can affect the productivity and survival of colonies (Kang et al. 1976).

The BQCV is a non-enveloped virus with a linear single positive-strand RNA genome of 9.5 kb, belonging to the genus *Triatovirus,* family *Dicistroviridae* and to the order *Picornavirales*, the same order of the human hepatitis A virus (Leat et al. 2000, Spurny et al. 2017). BQCV was originally isolated in samples of decomposed queens with black spots, giving it the name of "black queen". At the initial stage of infection, larvae have a yellow appearance and a hard and rough tegument, which are clinical signs of the virus. BQCV cause mortality in queen bee pupae (Leat et al. 2000). Although only the larvae are visibly affected by this disease, adults can also be infected, but in an asymptomatic form (De Miranda et al. 2013, Muz & Muz 2017). The transmission of BQCV occurs mainly through the fecal-oral route. It has been reported the active replication of virus in drones and the transmission through semen (Alger et al. 2019, Prodělalová et al. 2019). BQCV has already been detected in flowers collected from the apiaries (Alger et al. 2019), and in samples of honey (Milićević et al. 2018).

The BQCV and *N. ceranae* have been shown to interact synergistically in honey bee workers, with coinfection significantly decreasing host survival (Bacandritsos et al. 2010, Dainat et al. 2012, Doublet et al. 2016). In addition, an interaction was observed between *N. ceranae* and triaclopride, an insecticide, increasing individual bee mortality. Common microbial pathogens appear to be major threats to honey bees, while sublethal doses of pesticide may enhance their deleterious effects on honey bee larvae and adults (Doublet et al. 2015).

In this study, we report detection of BQCV and *N. ceranae* and coinfection by two agents in apiaries from southern Brazil. We also performed the first phylogenetic analysis with Brazilian sequences of BQCV.

MATERIALS AND METHODS

Sample collection. Adult bees (≅30) were collected from 17 apiaries (92 colonies) of *Apis mellifera* (Africanized honey bee), located in nine municipalities of southern Brazil: Piratini/RS (31°26'49.3"S 53°05'52.7"W) - three apiaries, Arroio do Padre/RS (31°26'31.7"S 52°25'22.2"W) - four apiaries, São Carlos/SC (27°05'00.9"S 53°00'20.5"W) - one apiary, Saudades/SC (26°55'30.7"S 53°00'17.4"W) - two apiaries, Formosa do Sul/SC (26°38'49.1"S 52°47'36.2"W) - one apiary, Maravilha/SC (26°45'47.6"S 53°10'31.9"W) - one apiary, São Miguel do Oeste/SC (26°44'01.4"S 53°31'12.0"W) - two apiaries, Capão do Leão/RS (31°45'21.8"S 52°29'22.3"W) - one apiary, and Pelotas/RS (31°37'14.8"S 52°18'14.8"W) - two apiaries. Samples were collected from April to October 2019. All samples were kept refrigerated during transport and immediately frozen at -70°C.

Nucleic acid extraction and cDNA. The total RNA was extracted from six abdomen (*pool*) of adult bees by colony using the TRIzol® Reagent (Life Technologies, Carlsbad, CA). After RNA extraction, complementary DNA (cDNA) was synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad). For DNA extraction the kit DNA Mini Spin (Kasvi) was used, by collecting from 100 to 200µl of liquid of twenty abdomen (*pool*) of adult bees by colony. This liquid was obtained by macerating 20 adult bees.

PCR and RT-PCR. The cDNA was submitted to Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) assay for capsid protein gene of BQCV (Tsevegmid et al. 2016) and the DNA was submitted to multiplex PCR assay for small subunit ribosomal RNA gene of *Nosema ceranae* and *Nosema apis* (Martín-Hernández et al. 2007). All samples were tested with the endogenous control (glyceraldehyde 3-phosphate dehydrogenase - GAPDH) (Scharlaken et al. 2008) to verify the efficiency of the whole process. Primers used for amplification are described in Table 1 and PCR conditions were according to the reference. The PCR's was performed in a 25µL volume, using 100-200ng of template, 1x GoTaq® Colorless Master Mix (Promega) and 0.4µM of each primer. PCR products were resolved in a 1.5% agarose gel stained and visualized under UV light after electrophoresis (80V, 40min). Bees's *pools* were used as control and gBlock® Gene Fragment (Integrated DNA Technologies) was used as control of BQCV.

Table 1. Primers used for detection of endogenous control (GAPDH), *black queen cell virus* (BQCV), and *Nosema ceranae* and *Nosema apis* in multiplex PCR

Primer	Sequence[a] (5'-3')	PCR product size (pb)	Specificity
GAPDH_F	GATGCACCCATGTTTGTTTG	203	GAPDH
GAPDH_R	TTTGCAGAAGGTGCATCAAC		
BQCV_11	AGTGGCGGAGATGTATGC	294	BQCV
BQCV_12	GGAGGTGAAGTGGCTATATC		
218MITOC-FOR	CGGCGACGATGTGATATGAAAATATTAA	218-219b	N. ceranae
218MITOC-REV	CCCGGTCATTCTCAAACAAAAAACCG		
321APIS-FOR	GGGGGCATGTCTTTGACGTACTATGTA	321	N. apis
321APIS-REV	GGGGGGCGTTTAAAATGTGAAACAACTATG		

[a] CG tails added to primers are underlined, [b] there is a 1-bp difference in the *N. ceranae* amplicon size depending on the sequences for *N. ceranae* available in GenBank (http://www.ncbi.nlm.nih.gov) (Martín-Hernández et al. 2007).

Sequencing analysis. One positive sample of *N. ceranae* was sequenced in duplicate using BigDye Terminator Cycle Sequencing kit (Thermo Fischer) to confirm sequences identity. Results were analyzed by the Staden program (Staden 1996) to obtain a consensus sequence from each duplicate. The alignment of sequences with those from GenBank was performed using the BioEdit version 7.0.5.3. (Hall 1999), positive samples were also sequenced. Eight samples of BQCV were sequenced in duplicate, analyzed in software's and used for phylogeny. Phylogenetic analysis was conducted by the Molecular Evolutionary Genetics Analysis (MEGA) 6.0 (Tamura et al. 2013). The evolutionary distances were inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura et al. 1980).

RESULTS

Ninety-two Africanized honey bee colonies were analyzed, covering to 17 apiaries in the southern region of Brazil. *Nosema ceranae* was detected in 57.6% (53/92) and BQCV was detected in 32.6% (30/92) of colonies, and *Nosema apis* was not detected in these samples. BQCV and *N. ceranae* coinfection was observed in 25% (23/92) of colonies from this study.

Nucleotide sequences from eight positive samples of BQCV (8/30) were analyzed and the sequences have been deposited in GenBank. The identity obtained among all samples analyzed was 96.8 to 100%. In the phylogenetic analysis of partial capsid protein gene (Fig.1), the sequences of this study (BR-BQCV) showed greater similarity with Asian sequences (China, Japan, South Korea, Thailand and Mongolia) and sequences from the United States (USA), Germany and Peru. The sequence from Brazil D6 (EU292211), collected in 2007 in the Southeast region, was grouped in a different cluster when compared with the samples from this study. There was only one BQCV sequence from Brazil corresponding to the capsid protein gene deposited in GenBank and, for this reason, only this sequence was used for comparison (Teixeira et al. 2008).

DISCUSSION

We report the first survey of the occurrence of *Nosema ceranae* and BQCV in Africanized honey bees from southern Brazil. Our findings indicate that these two agents, that have been implicated in population declines of these insects in several countries, are widespread in populations of Africanized honey bees in southern Brazil (Ryba et al. 2012, Mendoza et al. 2013). However, the occurrence and infection profiles of *N. ceranae* and BQCV differs significantly across different counties in southern Brazil. We emphasize that, in this research, the *Varroa destructor* mite, which has been related to the occurrence of both BQCV and Nosema spp., was not counted.

In this study, *N. ceranae* was detected in 57% (53/92) of the Africanized honey bee colonies from southern Brazil. *Nosema apis* was not detected in the samples. The microsporidian *Nosema* has been considered one of the most serious pathogens of honey bees (Higes et al. 2008), once invades the mid gut epithelial cells of the honey bees, can affect the productivity and survival of colonies (Kang et al. 1976). *N. ceranae*, which has been infecting Africanized *A. mellifera* bees in Brazil for at least the past 34 years (Teixeira et al. 2013), is considered more common and virulent than *N. apis* (Huang et al. 2008, Chaimanee et al. 2011), corroborating our results.

BQCV was detected in 32.6% (30/92) of the Africanized honey bee colonies. Studies in other countries have demonstrated the high occurrence of BQCV in adult bees, 29.3% in Croatia (Gajger et al. 2014), 65% in Australia (Roberts et al. 2017) and 25.6% in Iran (Moharrami & Modirrousta 2018). In Southeast Brazil, in 2007, BQCV was detected in 37% of honey bees collected in 10 apiaries (Teixeira et al. 2008). Our results corroborate the high rates found in these places. However, differing from data commonly observed in the literature, such Shumkova et al. (2018), that detected only one case of BQCV in Bulgaria. In Argentina, only 8% of the tested samples were positive for the virus (Molineri et al. 2017). These data demonstrate that despite the high occurrence of BQCV, the presence of this virus may be lower in some regions, which reinforces the importance of specific studies in different regions of the world, especially in Brazil, where information regarding viral infections of honey bees are scarce (Teixeira et al. 2008).

Previous studies have reported the association between the BQCV with microsporidia *Nosema* spp., suggesting that these pathogens can act synergistically, with coinfection significantly decreasing host survival (Bacandritsos et al. 2010, Dainat et al. 2012, Doublet et al. 2015, 2016). BQCV and *N. ceranae* coinfection was observed in 25% (23/92) of Africanized honey bee colonies from southern Brazil. *N. ceranae* is able to cause depression of the immune system which could favor the infection by viruses (Antúnez et al. 2006). Studies describe changes in the intestinal microbiota in bees infected with *N. ceranae*, with quantities being lower of *Bifidobacterium* and Pasteurellaceae. This result can indicate that *Nosema* infection could result in the reduction of these microbiota components

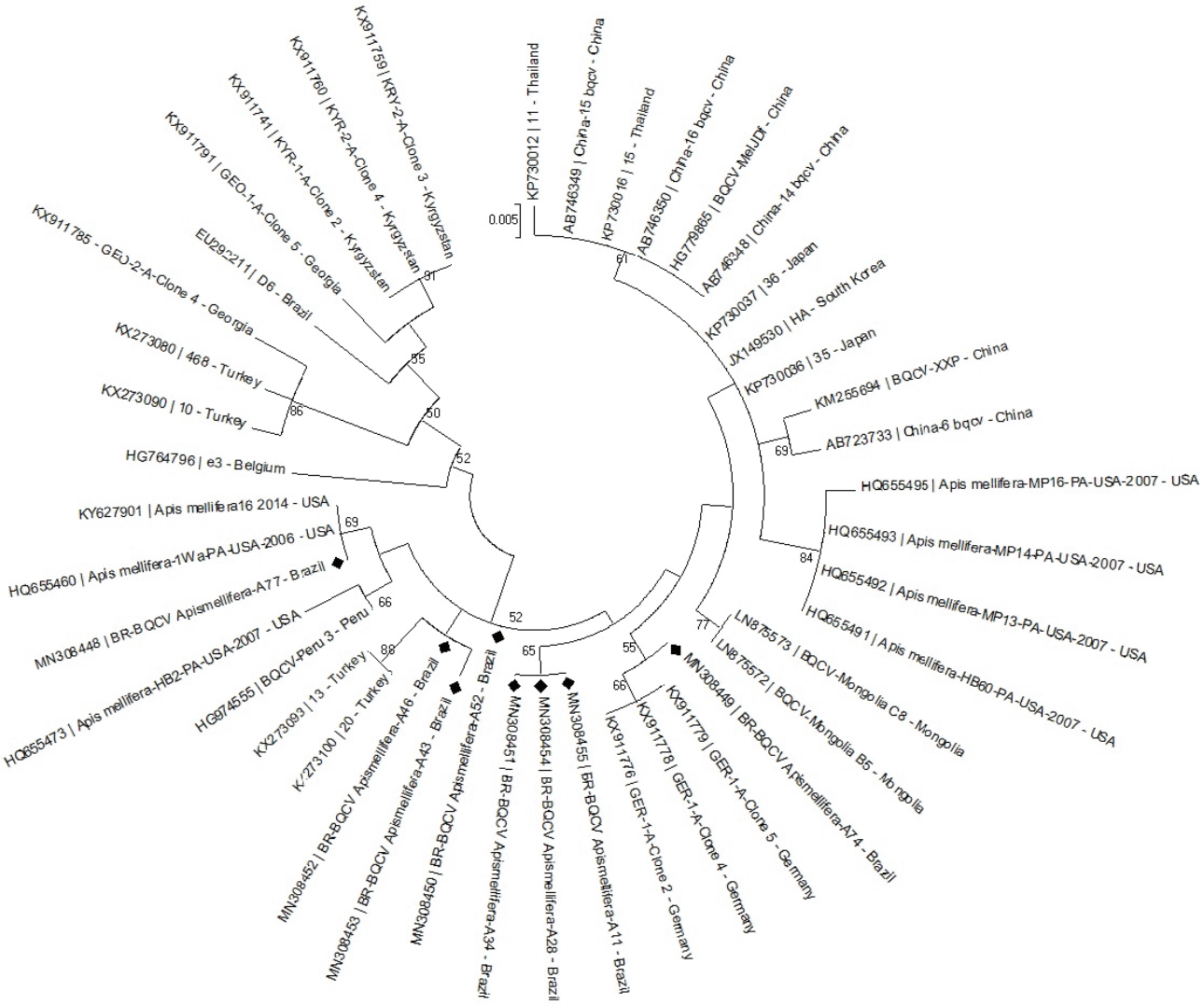


Fig.1. Phylogenetic analysis of partial capsid gene region of BQCV nucleotide sequences isolated from southern Rio Grande do Sul, Brazil. The tree was built using the *Maximum Likelihood* method based on the Kimura 2-parameter model and 1000 replicates bootstrap value (only node value above 50% are shown). The identified viruses are indicated by diamonds.

or conversely the microbiota composition could determine the susceptibility to infection (Li et al. 2012).

In this study, eight samples of BQCV were analyzed and deposited in GenBank (Fig.1). The sequences MN308455 (Piratini) and MN308454 (Canguçu) showed 100% nucleotide identity, suggesting that the same virus could be circulating in both municipalities. However, the identity obtained among all samples analyzed was 96.8 to 100%, also demonstrating that there are differences between viruses detected in different municipalities and within the same municipality. In the phylogenetic analysis of partial capsid protein gene (Fig.1), the sequences of this study (BR-BQCV) showed greater similarity with Asian sequences and sequences from the United States (USA), Germany and Peru. The sequences from Belgium, Kyrgyzstan and Georgia are grouped into a distinct cluster, suggesting that geographic origins of sequences can be decisive for their genetic divergence (Roberts et al. 2017, Milićević et al. 2018, Prodělalová et al. 2019). The sequence from Brazil D6 (EU292211), collected in 2007 in the Southeast region, was grouped in a different cluster when compared with the samples from this study. BQCV transmission can occur from the queen bee to its progeny (Chen et al. 2006) and, the purchase of queen bees from other places may favor the genetic diversity found in studies around Brazil.

CONCLUSIONS

The microsporidian parasite *Nosema ceranae* and BQCV were detected separately and in coinfections in Africanized honey bees from southern Brazil.

Although more studies are needed to determine the distribution and prevalence of *Nosema* and BQCV in southern Brazil, our results demonstrate that both agents are circulating in Africanized bees and may be related to the population decline of bees in the region. Also, our results include what seems to be the first phylogenetic analysis with Brazilian sequences of BQCV.

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Immunomodulatory effect of *Bacillus toyonensis* BCT-7112 supplementation in puppies vaccinated against canine parvovirus[1]

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ABSTRACT.- Franz H.C., Conrad N.L., Santos F.D.S., Gonçalves V.S., Fonseca R.N., Brasil C.L., Hübner S.O. & Leite F.L.P. 2020. **Immunomodulatory effect of *Bacillus toyonensis* BCT-7112 supplementation in puppies vaccinated against canine parvovirus.** *Pesquisa Veterinária Brasileira 40(11):898-902*. Núcleo de Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Campus Capão do Leão, Pelotas, RS 96010-900, Brazil. E-mail: fleivasleite@gmail.com, fabio_leite@ufpel.edu.br

Bacillus toyonensis is a probiotic microorganism that for decades has been used in animal nutrition around the world. The objective of this work was to evaluate the immunomodulatory effect of oral *B. toyonensis* supplementation in dogs vaccinated against canine parvovirus. Puppies were randomly selected and divided in two groups, one received *B. toyonensis* at a concentration of $2x10^8$ viable spores per day and another group without supplementation was left as control. The puppies were vaccinated against canine parvovirus type 2. *B. toyonensis* supplementation was efficient in stimulating specific IgG for parvovirus with titers of 2, 3, and 2.5-fold higher than controls at 7, 21, and 35 pos-vaccination days respectively. Peripheral blood mononuclear cells (PBMCs) from dogs were cultured and stimulated with *B. toyonensis* DNA, vegetative cell and spores. The mRNA transcription of cytokines IL-4, IL-17, and IFN-γ up modulated by the stimuli. Thus, we conclude in this study that *B. toyonensis* supplementation may amplify the vaccine immune response against canine parvovirus.

INDEX TERMS: Immunomodulatory effect, *Bacillus toyonensis*, BCT-7112, supplementation, puppies, vaccine, canine parvovirus, probiotic, adjuvancity, dogs.

RESUMO.- [**Efeito imunomodulador da suplementação de *Bacillus toyonensis* BCT-7112 em cachorros vacinados contra o parvovírus canino.**] *Bacillus toyonensis* é um micro-organismo probiótico que há décadas é utilizado na nutrição animal em todo o mundo. O objetivo deste trabalho foi avaliar o efeito imunomodulador da suplementação oral de *B. toyonensis* em cães vacinados contra o parvovírus canino. Os filhotes foram selecionados aleatoriamente e divididos em dois grupos, um recebeu *B. toyonensis* na concentração de 2×10^8 esporos viáveis por dia e outro grupo sem suplementação como controle. Os filhotes foram vacinados contra o parvovírus canino tipo 2. A suplementação com *B. toyonensis* foi eficiente em estimular IgG específica para parvovírus com títulos de 2, 3 e 2,5 vezes maior que os controles aos 7, 21 e 35 dias pós-vacinação, respectivamente. Células mononucleares do sangue periférico (PBMCs) de cães foram cultivadas e estimuladas com DNA de *B. toyonensis*, células vegetativas e esporos. A transcrição do mRNA das citocinas IL-4, IL-17 e IFN-γ foi modulada pelos estímulos. Assim, concluímos neste estudo que a suplementação com *B. toyonensis* pode amplificar a resposta imune da vacina contra o parvovírus canino.

TERMOS DE INDEXAÇÃO: Efeito imunomodulador, suplementação, *Bacillus toyonensis*, BCT-7112, cachorros, vacina, parvovírus canino, probiótico, adjuvanticidade, caninos.

INTRODUCTION

Vaccination of dogs has been regarded as one of the major success interventions in veterinary medicine and has proven successful by preventing future infections. Vaccination stimulates both humoral and cellular response (Greene et al. 2001), been

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the duration of immunity depends on the immunological memory established (Pardo et al. 1997, Tizard 2004). Puppies can vary its ability to respond to vaccines, been the principal cause, levels of persistent maternal immunity. The first prime immune response to the vaccine antigen needs to be effective in order to subsequent exposures to the same antigen eliciting a stronger and faster response (Davis-Wurzler 2006). One possible way to improve vaccine initial prime immune response is by modulating the immune system with the supplementation of probiotics (Roos et al. 2010, 2012, 2018, Santos et al. 2018).

Probiotics are microorganisms that are added to the diet to exert beneficial effects on the host. Probiotics have the potential to stimulate innate immune responses without inducing inflammation; initiate responses mediated by interacting with follicle-associated epithelial cells, macrophages, T- and B-lymphocytes and dendritic cells (DCs) (Matsumoto et al. 2011). DCs activated by probiotic, stimulate naïve T lymphocytes, which migrate to the mesenteric lymph nodes, then systemic circulation where they can mount an immune response far from the location of their original activation (Lebeer et al. 2010).

Bacillus toyonensis is a non-pathogenic Gram-positive bacterium that has been used as a probiotic in animal feed (Gil-Turnes et al. 2007, Williams et al. 2009). Supplementation with *B. toyonensis* exerts immunomodulatory effects capable of enhancing the effectiveness of vaccines in sheep, pigs and mice (Coppola et al. 2005, Roos et al. 2010, 2012).

Canine parvovirus (CPV) is an important enteric pathogen because of the associated high morbidity and mortality rates (Greene 2012). CPV, caused by three variants of canine parvovirus type 2 (CPV-2; family Parvoviridae, genus *Parvovirus*), is a leading cause of morbidity and mortality in dogs globally. CPV-2 evolved into two variants (CPV-2a and CPV-2b) (Carmichael 2005, Martella et al. 2006) while in 2000, a third variant (CPV-2c) has been reported (Buonavoglia et al. 2001, Decaro et al. 2006). It can affect dogs of any age, but severe infections are most likely to occur from 6 weeks to 6 months after birth (Miranda & Thompson 2016). Vaccination has proven successful to prevent CPV infections (Thiry & Horzinek 2007, Greene & Vandevelde 2012). Current recommendations suggest that puppies should have the first (or primary) vaccination scheduled between the first 6 and 8 weeks of age, followed by a booster shot. The number of vaccine boosts required might vary with the vaccination protocol, but principally with the prime vaccine effective response (Day et al. 2016).

Thus, the main goal of this study was to investigate the *B. toyonensis* immune modulation in puppies undergoing CPV vaccination.

MATERIALS AND METHODS

Probiotics. The probiotic used in this study, *Bacillus toyonensis* BCT-7112^T, was sourced from the "Laboratório de Microbiologia", "CDTec-Núcleo de Biotecnologia", "Universidade Federal de Pelotas" (UFPel), Brazil. *B. toyonensis* preparations were made as previously described by Santos et al. (2018). Briefly, the bacteria were cultured in a bioreactor (BIOSTAT® B; Braun Biotech International, Melsungen, Germany) containing 3.5L of Nutrient Yeast Extract Salt medium (NYSM; 0.5% meat peptone, 0.5% meat extract, 0.1% yeast extract, 0.5mM KH_2PO_4, 0.8mM $MgSO_4$, 0.06mM $MnSO_4$, 0.06mM $ZnSO_4$, 0.06mM $FeSO_4$, 0.01mM $CaCO_3$), maintained under shaking, at 37°C for 96h. The resultant culture was spun down in a Sorvall™ RC 6 Plus centrifuge (Thermo Scientific, Langenselbold, Germany) at 5,000 × *g*, for 20 min, at 4°C and the pellet was resuspended in 500ml of phosphate buffered saline (PBS; 137mM sodium chloride, 10mM sodium phosphate, 2.7mM potassium chloride; pH 7.4). Gram staining and culturing in sheep blood agar were used to ensure the purity of the strains at all stages of culture.

Ethical standards. All dogs were maintained and handled at the owner house, with no food or water restrictions. All procedures were performed in accordance with the Brazilian Committee for animal care and use (COBEA) guidelines and were approved by the UFPel Ethics Committee for animal research (project number 3246).

Supplementation and vaccination of the dogs. Ten Australian Cattle Dog, seven males and 3 females, 11 weeks old, were randomly divided in two groups, 5 animals each, supplemented and control. The supplemented group received 2×10^8 *B. toyonensis* viable spores (on 1ml) orally once a day, whereas the control group received 1 ml of PBS. The probiotic supplementation starts 7 days prior the prime vaccination and was maintained until the end of the experimental period (day 35). The vaccination follows the owner protocol, using two shots of Vanguard® HTLP 5/CV-L[4] with a 21-day interval, days 7 and 28 of the experimental period. Whole blood was collected on day 0, 7, 21 e 35 using a standard jugular venipuncture approach and evacuated into sterile tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant.

Clinical and hematological parameters. All dogs were monitored to respiratory and cardiac rate, body temperature during all experiment. Hematological parameters including red blood cells (RBCs) and white blood cells (WBCs) count and hemoglobin concentration (Hb) were analyzed using a PocH - 100iVDiff®5. Blood smears were immediately prepared from EDTA blood samples and were stained with Diff Quick stain (EMD Chemicals Inc., Gibbstown/New Jersey 08027, USA) and 200 leukocytes were differentiated in smears prepared from each animal.

Canine parvovirus antigen. The strain Cornell of canine parvovirus, used as antigen in ELISA, was kindly supplied by the "Laboratório de Virologia e Imunologia Veterinária" of the "Faculdade de Veterinária" of UFPel. The virus was inoculated in CRFK cells (Crandell feline kidney cells ATCC® CCL-94™) maintained in minimal essential media (MEM, Sigma-Aldrich, St. Louis/MO, USA at 37°C, until observation of cytophatic effect on 90% of cells (~72 hours). The virus suspension was titrated by hemagglutination using porcine erythrocytes according to (Pratelli et al. 2001). The titer of the virus was adjusted for 128 hemagglutinating units (UHA).

Indirect ELISA. The estimation of total specific IgG levels was carried out by indirect ELISA. Briefly, 96-well microplates (Corning®, New York, USA) were coated with 50µl per well of CPV suspension containing 128 UHA diluted in coating buffer (0.1M carbonate bicarbonate buffer, pH 9.6) at 4°C overnight. The response throughout the experiment was verified by analyzing the sera collected on days 0, 7, 21 and 35, diluted 1:1000 in PBS-T. To determine the antibody titer serum samples were serially two-fold diluted starting at 1:100 to 1:51200 and added to the plates in triplicate. After incubation at 37°C for 60 min, the plates were washed three times with PBS-T, followed by addition of 100µl of horseradish peroxidase (HRP)-conjugated rabbit anti-dog IgG whole molecule antibodies (1:4000 dilution, Sigma-Aldrich, St. Louis/MO, USA). Finally, 100µl of substrate solution (10mg ortho-phenylenodiamine (OPD, Sigma-Aldrich) in 10ml of 0.1M phosphate citrate buffer and 10µl of 30% H_2O_2) was added to each well and the plates were incubated in the dark at

room temperature for 15 min. Further, 50µl of 2N H_2SO_4 was added to each well to stop the reaction.

Culture of peripheral blood mononuclear cells and RNA extraction. Blood was collected from healthy adult dogs and Peripheral Blood Mononuclear Cells (PBMCs) isolated as previously described by Leite et al. (2004). Approximately 5 × 10^7 cells were cultured in 24-well plates (Kasvi, China) containing 1ml of RPMI 1640 medium (Gibco, USA) with 10% fetal bovine serum (FBS; Gibco), 10,000IU/ml penicillin, 10mg/ml streptomycin, and 25mg/ml amphotericin B (Gibco) for 24h at 37°C in 5% CO_2 atmosphere. PBMCs were stimulated with *B. toyonensis* DNA (5µg); vegetative cells (10^6 UFC) and spores (10^6 UFC). Were also stimulated with 10µg of concanavalin A (ConA) (Sigma-Aldrich) as a positive control, whereas RPMI 1640 as a negative control. The plates were incubated for 18 h under the same conditions. The supernatants were then discarded, and the cells were resuspended in TRIzol® Reagent (Life Technologies, Carlsbad/CA, USA) for RNA extraction following the manufacturer's instructions.

cDNA synthesis and qPCR. Reverse transcription was carried out using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA) using approximately 400 ng of RNA. Relative mRNA expression levels for interleukin (IL)-4, interleukin (IL)-17 and interferon (IFN)-γ were determined using quantitative polymerase chain reaction (qPCR) with β-actin and GAPDH used as endogenous reference genes (controls); β-actin was standardized as an internal reference gene based on its efficiency (M-value of 1.98). qPCR reactions were performed on a Stratagene Mx3005P qPCR system (Agilent Technologies, Santa Clara, USA), as described previously (De Avila et al. 2016). The primers used were described elsewhere (Hassanpour et al. 2017). All samples were analyzed in triplicate using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method to determine the relative mRNA expression compared to β-actin as the reference gene. The Ct values obtained by the qPCR were analyzed using the following equation (Livak & Schmitted 2001): ΔΔCt = (CtTarget-CtHousekeping)-(CtTarget-CtHousekeping).

Statistical analysis. The data were analyzed using GraphPad Prism version 7 (USA). The differences in antibody titers between groups were analyzed using ELISA values, where positive were considered values of + 3 SD above the mean of the negative controls. The results were subjected to two-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparisons test. A one-way ANOVA followed by Dunnett's test was used to analyze differences between cytokine mRNA transcription.

RESULTS

No significant difference was detected in the respiratory rate, pulse rate, body temperature between groups. Also, the hematological parameters were the expected for the dogs age. These results suggest that the *Bacillus toyonensis* supplementation did not cause any observable adverse physiological effects to the dogs and that it was safe for use in dogs.

All animals of experimental groups had similar maternal antibody levels against CPV on day 0 of the experiment. Total specific IgG levels were increased in both groups in response to vaccination. Significantly higher IgG titers were observed for *B. toyonensis* supplemented dogs (P<0.05) as compared with the control group. At seven days after the primer immunization, the supplemented group had significant (P<0.05) high levels of specific total IgG anti-CPV comparing with the control group (Fig.1A). Noteworthy, that the IgG levels in the supplemented group continue increasing, on day 21 there was, almost 3-fold increase comparing with the control group. After the boost, the both groups responded following the same trend (Fig.1A). At day 35 of the experiment, the levels of specific IgG in the supplemented group also were significantly higher than the control group reaching up to 1:51200, 8-fold higher than the controls (Fig.1B).

In order to evaluate the cytokines mRNA transcription of PBMCs stimulated with *B. toyonensis*, PBMCs from healthy adult dogs were stimulated with *B. toyonensis* DNA, vegetative cells and spores. PBMCs stimulated with *B. toyonensis* DNA were able to induce significant transcription levels of IL-4 by 4.7, IL-17 by 3.5, and IFN-γ by 2-fold (Fig.2A). Stimulus with vegetative cells showed an increase transcription level for IL-4, IL-17 and IFN-γ of 1.7, 3.15 and 1.2-fold, respectively (Fig.2B). Then, when stimulated with *B. toyonensis* spores had an increase in cytokines transcription levels of 4.4, 1.3, and 1.6-fold, for IL-4, IL-17 and IFN-γ respectively (Fig.2C).

DISCUSSION

Vaccination is the most effective and cost-benefice method of controlling infectious diseases in domestic animals (Loots et al. 2017, Thiry & Horzinek 2007). Vaccine efficacy can be measure by the ability to induce specific serum antibody against the pathogen. The level of specific antibodies elicited

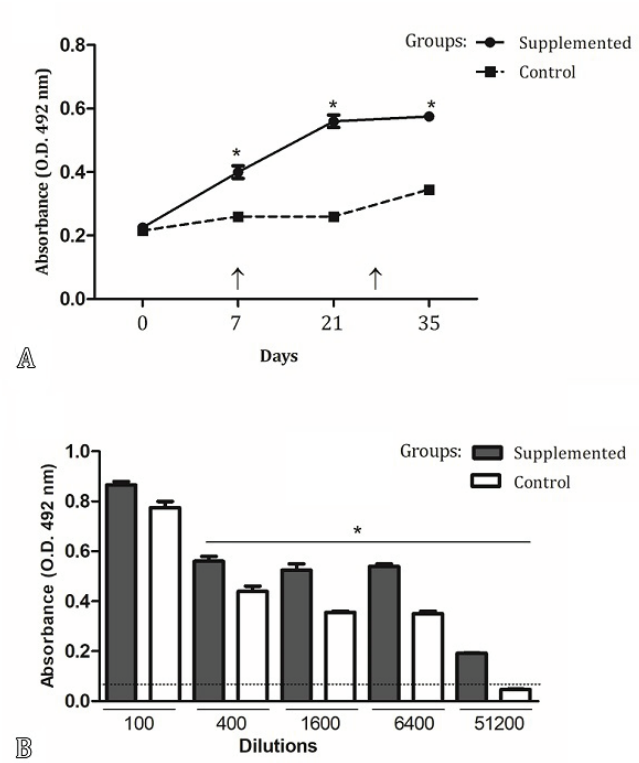


Fig.1. Dynamics of total IgG against canine parvovirus. The data represent the mean of ELISA values of specific total IgG against canine parvovirus (CPV) in the *Bacillus toyonensis* supplemented and control dogs. (**A**) Antibody response in different time points of the experiment. Arrows indicate vaccination days. (**B**) Specific CPV serum IgG titer of vaccinated dogs at 35th day of experiment. The dotted line represents the cut-off (the mean of the negatives + 3 SD). Asterisks mean significant difference (P<0.05) between the *B. toyonensis* supplemented and control group.

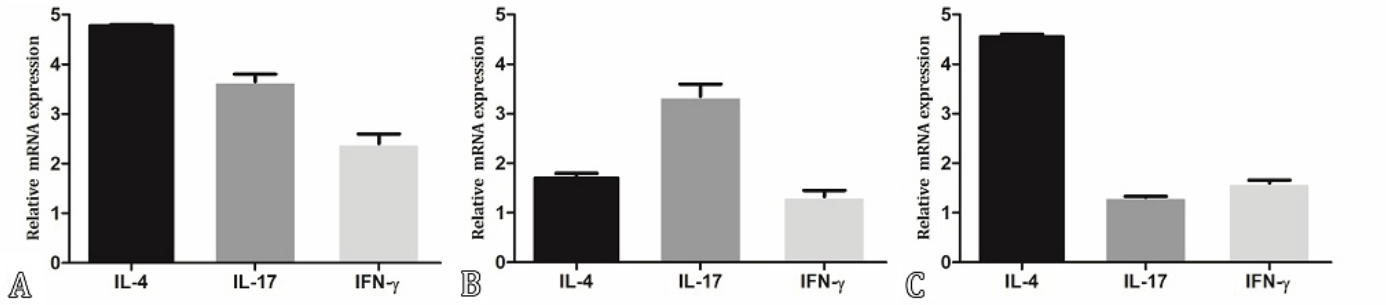


Fig.2. Quantitative polymerase chain reaction (qPCR) of cytokine mRNA transcription for IL-4, IL-17, and IFN-y. The data represent the mean (± standard error) of cytokine mRNA transcription of canine PBMCs stimulated with *Bacillus toyonensis* (**A**) DNA; (**B**) vegetative cells and (**C**) spores. The relative mRNA expression was determined by the comparative threshold cycle ($2^{-\Delta\Delta C}{}_{T}$).

by a vaccine against CPV usually correlates with protection (Jensen et al. 2015). Consequently, studying vaccine antibody responses in animals supplemented with probiotic one may consider a good way to determine the probiotics modulation of the immune responses to CPV.

In this study, we observed that dogs vaccinated and supplemented with *B. toyonensis*, presented significant higher specific anti-CPV IgG levels when compared to with the control group (Fig.1). Remarkable, the significant higher levels of specific CPV IgG antibodies in supplemented dogs were developed after prime vaccination, with an increase of approximately 2 and 3-folds at 7 and 21 days, respectively, comparing with the control group. After the boost (day 28), although both groups responded, the control group do not reach the IgG levels of the supplemented group, showing a level of antibodies significantly lower than the supplemented group. These findings are quite interesting, since one can assume that the maturity of immune system, as well as, the level of maternal antibodies of the dogs were similar, suggesting that the *B. toyonensis* immune modulation was the responsible to induce higher levels of antibodies anti-CPV.

One mechanism of probiotic immune modulation is by change the cytokine profile produced in the environment where the vaccine antigens will interact with the immune cells (Shida et al. 2011). The higher levels of IL-4, IL-17 and IFN-γ mRNA transcription observed when PBMCs were stimulated with *B. toyonensis* (DNA, vegetative cell and spore), suggest that these cytokines might play a role in the *B. toyonensis* modulation of vaccine response to CPV (Roos et al. 2012, Santos et al. 2018).

We observed an up regulation for IL-4, IL-17 and IFN-y mRNA transcription on PBMCs ($p<0.05$). The cytokine IL-4 is mainly produced by Th2 lymphocytes and promotes B lymphocyte response and isotype switching (Paul & Zhu 2010). The presence of IL-4 improves antigen presentation through increased expression of major histocompatibility complex II molecules, maturation of DCs and T lymphocytes proliferation (Finkelman et al. 1990, Lutz et al. 1996, Wells et al. 2005). Interleukin-17 (IL-17) is a pro-inflammatory cytokine produced by a subset of T helper cells (Th17). These cells act as physiological mediators of inflammation and are characterized by a unique expression pattern of transcription factors and cytokines. It was demonstrated that IL-17 not only trigger B-lymphocyte proliferation, but also promote the formation of GCs (Germinal Centers) together with IgG isotype switching (Mitsdoerffera et al. 2010). Our experiment revealed an increase IL-17 mRNA transcription on PBMCs with different *B. toyonensis* stimuli, compared with control group. The significant higher levels of IFN-y mRNA transcription observed on canine PBMCs suggest the stimulation of a Th1 response, an effect of great importance on the vaccine immune response, principally for anti-viral vaccines. Previously, it was reported a similar effect on IFN-y mRNA transcription in mice supplemented with *B. toyonensis* and vaccinated against BoHV-5 (Roos et al. 2012, Santos et al. 2018). The increase in mRNA transcription of the cytokines IL-4, IL-17, and IFN-γ due probiotic modulation may have resulted in significantly greater levels of total specific CPV IgG, as detected in the sera of the probiotic-supplemented animals. Therefore, it is possible to suggest that part of the probiotic immune modulation effect on the antibody dynamics, observed in this study, may be due to the cytokines.

CONCLUSION

The data obtained in this study allow us to conclude that the supplementation of dogs with *Bacillus toyonensis* have a modulatory effect on immune response against canine parvovirus vaccine.

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Clinical and microbiological characteristics of dogs in sepsis in an academic veterinary hospital in the north of Paraná[1]

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ABSTRACT.- Camargo Junior V.E., Bahr Arias M.V. & Perugini M.R.E. 2020. **Clinical and microbiological characteristics of dogs in sepsis in an academic veterinary hospital in the north of Paraná.** *Pesquisa Veterinária Brasileira 40(11):903-913.* Departamento de Clínicas Veterinárias, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid Km 380, Londrina, PR 86057-970, Brazil. E-mail: vicky@uel.br

Sepsis is a life-threatening organ dysfunction caused by a patient's unregulated response to an infectious process. In veterinary medicine, the exact incidence of sepsis is unknown. Early recognition of sepsis in critically ill patients is essential for rapid and effective therapeutic intervention. The present study aimed to apply the criteria of an adapted sepsis assessment protocol based on the Second International Consensus Definition for Sepsis and Septic Shock or Sepsis-2 of human medicine, in canine patients with suspected systemic inflammatory response syndrome (SIRS) and/or organ dysfunction, and to identify infectious agents as well as their antimicrobial resistance profile in the focus of infection, in the bloodstream and colonizing the rectal mucosa. Patients were evaluated for survival and severity of sepsis. Of the 37/42 dogs that met the sepsis criteria, six presented septic shock, 26 (70.2%) had at least two signs of SIRS, and sepsis with organ dysfunction was diagnosed in 27 (73%) dogs. The primary dysfunctions observed were decreased level of consciousness in 21/37 (56.8%), hyperlactatemia in 19/37 (51.4%), and hypoalbuminemia in 18/37 (48.6%). Two or more SIRS signs associated with hypotension and hypoalbuminemia were related to more than half of the deaths. The most frequent infectious focus was skin and soft tissue in 20/37 (54%), followed by organs and cavities in 8/37 (21.6%). The survival rate was 56.7%. Blood culture confirmed bacteremia in nine patients (24.3%), with a predominance of Gram-positive microorganisms (*Staphylococcus intermedius*, *Streptococcus* spp.) in 66.6% of dogs and one yeast (*Candida glabrata*). The most frequent bacteria in the focus of infection were gram-negative bacteria (46.2%), mainly *Escherichia coli, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, in 19.5%, 14.6%, and 12.1%, respectively. We observed colonization by gram-negative bacteria such as *E. coli*-ESBL (31.5%), *K. pneumoniae*-ESBL (15.7%), and *P. aeruginosa* (15.7%), and the presence of ESBL bacteria was more associated with death when compared with other microorganisms. Vancomycin-resistant Enterococcus (VRE) were isolated from rectal mucosa in four dogs. Gram-negative microorganisms were the most frequent in both infections and colonization, and most of them were resistant to fluoroquinolones, sulfonamides, tetracyclines, and cephalosporins. Based on this information, it can be concluded that mortality due to sepsis in dogs was high. Due to the presence of multi-resistant bacteria, the use of antimicrobials should be judicious, suggesting the implementation of the same precautions used in human hospitals to prevent the spread of multi-resistant microorganisms.

INDEX TERMS: Clinics, microbiology, sepsis, dogs, bacteria, blood culture, bacteremia, Brazil.

RESUMO.- [Características clínicas e microbiológicas de cães em sepse de um hospital veterinário escola do norte do Paraná.] A sepse é uma disfunção orgânica ameaçadora à vida, causada por uma resposta desregulada do hospedeiro à infecção e na medicina veterinária sua incidência exata é desconhecida. O reconhecimento precoce da sepse nos pacientes críticos é essencial para que a intervenção terapêutica seja rápida e eficaz. Assim, os objetivos do

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presente estudo foram aplicar os critérios de um protocolo de avaliação da sepse adaptado com base no Segundo Consenso Internacional para Sepse e Choque Séptico, ou Sepse-2, da medicina humana, em pacientes caninos com suspeita de infecção e/ou Síndrome da Resposta Inflamatória Sistêmica e/ou disfunção orgânica e identificar os agentes infecciosos bem como seu perfil de resistência a antimicrobianos no foco de infecção, na corrente sanguínea e colonizando a mucosa retal. Os pacientes foram avaliados quanto à sobrevivência e severidade da sepse. Dos 37/42 cães que se enquadraram nos critérios de sepse, seis estavam em choque séptico, 26 (70,2%) apresentaram pelo menos dois sinais de SIRS, e a sepse com disfunção orgânica foi diagnosticada em 27 (73%) cães. As principais disfunções verificadas foram diminuição do nível de consciência em 21/37 (56,8%), hiperlactatemia em 19/37 (51,4%) e hipoalbuminemia em 18/37 (48,6%). A presença de dois ou mais sinais de SIRS associados com hipotensão e hipoalbuminemia estiveram relacionadas com mais da metade dos óbitos. O foco infeccioso mais frequente foi pele e partes moles em 20/37 (54%) seguido por órgãos e cavidades em 8/37 (21,6%). A taxa de sobrevivência foi de 56,7%. Na hemocultura confirmou-se bacteremia em nove pacientes (24,3%), com predominância de microrganismos gram-positivos (*Staphylococcus intermedius*, *Streptococcus spp*.) em 66,6% dos cães e uma levedura (*Candida glabrata*). As bactérias mais frequentes no foco de infecção foram as gram-negativas (46,2%) principalmente *Escherichia coli*, *Klebsiella pneumoniae* e *Pseudomonas aeruginosa*, em 19,5%, 14,6% e 12,1% respectivamente. Foi constatada colonização por bactérias gram-negativas como *E. coli*-ESBL (31,5%), *K. pneumoniae*-ESBL (15,7%) e *P. aeruginosa* (15,7%), sendo que a colonização de cães por bactérias ESBL foi associada ao óbito quando comparada com outros microrganismos. Foram também isolados da mucosa retal *Enterococcus* resistentes à vancomicina (VRE) em quatro cães. Os microrganismos gram-negativos foram os mais frequentes, tanto nas infecções quanto nas colonizações e a maioria apresentava resistência à fluorquinolonas, sulfonamidas, tetraciclinas e cefalosporinas. Com base nestas informações, conclui-se que a mortalidade em decorrência da sepse em cães foi alta, e devido à presença de bactérias multirresistentes, o uso de antimicrobianos deve ser criterioso, sugerindo-se ainda a implantação das mesmas precauções utilizadas em hospitais humanos para evitar disseminação de microrganismos multirresistentes.

TERMOS DE INDEXAÇÃO: Clínica, microbiologia, sepse, cães, bactérias, hemocultura, bacteremia, caninos, Brasil.

INTRODUCTION

Sepsis is life-threatening organ dysfunction caused by a host's unregulated response to infection (Singer et al. 2016). Septic shock is a subset of sepsis. There are adjacent abnormalities of circulatory and cellular metabolism and persistent hyperlactatemia after adequate resuscitation with fluids. Vasopressor therapy is necessary to maintain mean arterial pressure (MAP) above 65mmHg. In veterinary medicine, the exact incidence of sepsis is unknown, although dogs have been used as experimental models for human sepsis for many years (Otto & Silverstein 2015). Veterinary mortality varies from 21 to 70% (de Laforcade et al. 2003, Rabelo 2012, Hardy et al. 2018). The main etiologies of sepsis in this species include peritonitis of gastrointestinal or urogenital origin, pneumonia, immunosuppression (Otto & Silverstein 2015), pyometra (Conti-Patara et al. 2012), necrotizing fasciitis (Troia et al. 2018) and wounds (de Laforcade et al. 2003, Bahr Arias et al. 2017). Gram-negative bacteria, members of the Enterobacteriaceae family, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* spp., *Pseudomonas* spp. and *Proteus* spp., are primarily responsible for many of the severe infections in humans and animals (Otto & Silverstein 2015). Gram-positive bacteria like *Staphylococcus* spp. and *Streptococcus* spp. also occur, albeit less frequently, in surgical site infections, pyoderma, otitis, urinary tract infection (Weese & van Duijkeren 2010), and traumatic wounds (Bahr Arias et al. 2017).

Early recognition of sepsis in critically ill patients is essential for quick and effective therapeutic intervention (Otto 2007, Otto & Silverstein 2015). In human medicine, since 1991, several consensuses have been reached to establish definitions regarding the diagnosis and treatment of sepsis. In 2001, the Sepsis-2 consensus was launched, containing a list of several signs of Systemic Inflammatory Response Syndrome (SIRS) in response to infection, which could be used to check whether the patient was septic (Levy et al. 2003). In 2004, the Surviving Sepsis Campaign's initial guidelines were launched, which were revised in 2008 and 2012. Several studies were incorporated into the manuscript, which in 2016 was called the Third International Consensus Definition for Sepsis and Septic Shock or Sepsis-3 (Singer et al. 2016, Rhodes et al. 2017). In this consensus, the concept of severe sepsis was removed, as well as the use of SIRS criteria for the diagnosis of sepsis (Singer et al. 2016, Castro & Rabelo 2017), with problems in its acceptance by the medical community, mainly due to the possible occurrence of failure to identify septic patients (Carneiro et al. 2017).

There is no similar document in veterinary medicine, so several clinical and laboratory criteria help identify the infectious process and patients in sepsis (Castro & Rabelo 2017, Troia et al. 2018). There is a lack of protocols and knowledge of prognostic indexes. There was no validation of the criteria proposed in the the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3), making its direct application impractical (Castro & Rabelo 2017). The human consensus Sepsis-3 started to use the Sequential Organ Failure Assessment (SOFA) and Quick SOFA (in the first aid) scores to help diagnose sepsis. However, these have not been validated in veterinarians yet; the terms infection, sepsis, and septic shock are accepted (Castro & Rabelo 2017). In most recent studies with dogs in sepsis, the parameters used for patient inclusion were the presence of two or more signs of SIRS and confirmation of infection by laboratory methods (Hardy et al. 2018, Troia et al. 2018, Letendre & Goggs 2018).

This study aimed to use a protocol adapted for dogs, based on the Second International Consensus Definitions for Sepsis and Septic Shock (Sepsis-2), to identify sepsis patients and characterize their epidemiological, clinical, and laboratory profile concerning the clinical outcome in the "Hospital Veterinário Escola" of the North region of Paraná state, from 2016 to 2017.

MATERIALS AND METHODS

This study is observational, descriptive, and prospective with sampling for convenience, conducted in the sector of "Clínica Cirúrgica de Animais de Companhia" of the "Hospital Veterinário" of "Universidade Estadual

de Londrina" (HV-UEL) from March 2016 to April 2017. Forty-two dogs with suspected sepsis were evaluated. The Animal Use Ethics Committee of the "Universidade Estadual de Londrina" (CEUA-UEL) approved this study, under process number 7274.2016.86.

Sepsis protocol. The protocol (Fig.1) was specially developed for this study based on the Second International Consensus Definition for Sepsis and Septic Shock or Sepsis-2 (Levy et al. 2003) adapted to the veterinary based on authors and articles published on the subject (Otto 2007, Rabelo 2012, ILAS 2015, Otto & Silverstein 2015). The identification was carried out according to the following criteria. I) SIRS - animal with at least two of the following abnormal parameters: heart rate greater than 150 beats per minute; respiratory rate greater than 40 breaths per minute; temperature above 39.4°C or below 37.2°C; leukocyte count above 19,000 or below 5,000/mm^3 or left shift greater than 3%. II) Sepsis - presence of a proven or suspected focus of infection associated with at least two more signs of SIRS; III) Sepsis with organ dysfunction - presence of sepsis associated or not with signs of SIRS with the presence of one of the following organic disorders: systolic pressure less than 90mmHg or mean arterial pressure less than 65 mmHg; oliguria; breathing difficulty; hyperlactatemia greater than 3.2mmol/dL; hypoalbuminemia less than 2.5g/dL; decreased level of consciousness (<17) according to an adapted Glasgow scale; creatinine greater than 2.0 mg/dL; total bilirubin greater than 0.5mg/dL and platelet count less than 50 thousand/mm^3. IV) Septic shock - presence of hypotension associated with sepsis not responsive to fluid resuscitation.

Specimen Collection. After identifying the septic patient, through the protocol mentioned above, blood was collected for blood culture from two different sites, material from the infectious focus and rectal swab. Blood cultures were duplicated to increase the test's sensitivity, which is considered positive only when there was growth in the two samples. The blood was collected with a syringe and needle after trichotomy and surgical antisepsis. In dogs weighing less than 10 kg, between 1 and 3ml of blood were collected. In comparison, from dogs weighing more than 10kg were collected between 3 and 5mL of blood, which were inoculated into aerobic blood culture flasks at room temperature.

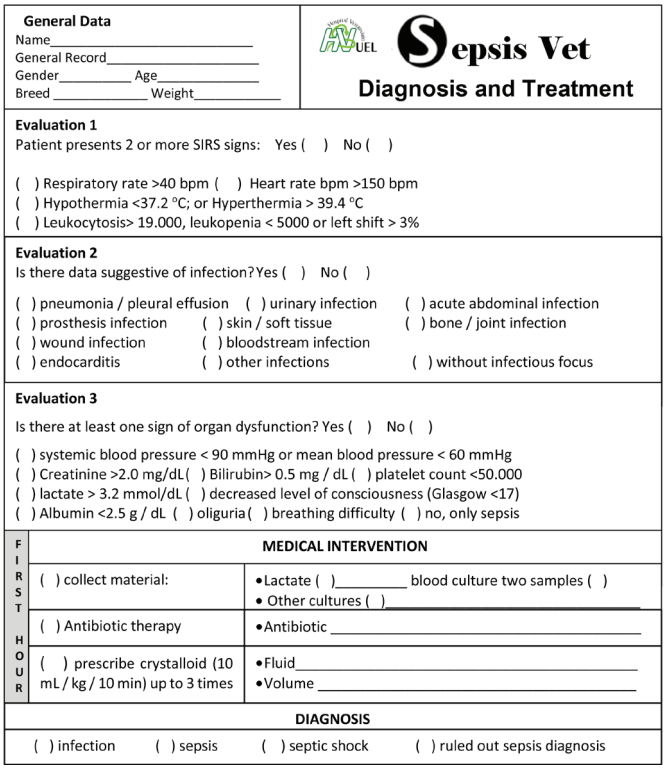


Fig.1. Protocol for the diagnosis of sepsis applied to dogs from the "Clínica Cirúrgica de Animais de Companhia" sector of the "Hospital Veterinário" of the "Universidade Estadual de Londrina" (HV-UEL) from March 2016 to April 2017.

The sources of infection were selected according to the frequency and classified into pneumonia/pleural effusion, urinary tract infection, acute abdominal infection, skin/soft tissues (traumatic wounds), bone/joint infection, wound infection, bloodstream infection, endocarditis, prosthesis infection, with no defined focus or other infections (ILAS 2015)

When the material of the infectious focus for culture was a tissue fragment (example - wound), it was collected with a sterile scalpel and scissors and placed in a sterile flask containing sterile saline solution. When the material consisted of liquid or secretions, it was sent in the collection syringe itself to the laboratory.

Microbiological analysis. At the "Laboratório de Habilidades Farmacêuticas" of the "Centro de Ciências da Saúde" (CCS), commercial blood culture bottles (BD-Bactec® Peds PlusTM and BD Bactec® Plus) were placed in a BD BactecTM 9000® automated blood culture system (BD Diagnostics, Spars, MD, USA), which detects bacterial growth up to five days. For flasks that showed positive growth, an aliquot of the material was collected, which was sown on blood agar for later identification of the microbial agent, and analysis of sensitivity to antimicrobials.

Other collected materials were also inoculated in blood agar, chromogenic agar, and MacConkey agar and incubated at 37°C. Afterward, the colonies that developed were identified according to Versalovic et al. (2011).

Rectal swab. The swabs were inoculated in two tubes containing Soy Broth Tripticaseína (Tryptic Soy Broth-TSB®), one containing 8µg/ml of cefotaxime and another containing 6µg/ml of vancomycin. After overnight incubation at 37°C, the broth from the first tube was peaked on a plate containing MacConkey® agar (Merck Sharp & Dohme) supplemented with 8µg/mL cefotaxime and the second on VRE® agar (OXOID) supplemented with 6µg/mL vancomycin. After incubation for 24 hours, colonies that developed were identified using methodology standardized by Versalovic et al. (2011). Antimicrobial sensitivity was determined by disk diffusion according to the Clinical and Laboratory Standards Institute (CLSI 2008).

Antimicrobial sensitivity test (AST). The tests were performed using Muller Hinton agar. A bacterial concentration corresponding to 0.5 turbidity was used on the Mc Farland scale. Petri dishes and flasks containing paper discs impregnated with antibiotics were kept at room temperature for 20 to 30 minutes before use. The paper discs impregnated with antibiotics were selected based on the microbial agents identified in the samples, following the recommendations of CLSI (2008). They were added 15 minutes after sowing the organisms in the test. A maximum of five paper discs impregnated with antibiotics was used per 100mm Petri dish, and they were incubated at 35-37°C. The test was interpreted between 18 to 24 hours after incubation. The sensitivity profile was determined by measuring the growth inhibition zone, and the results were reported as sensitive, intermediate, or resistant.

Statistical analysis. Analysis of contingency tables ($\chi 2$ test or Fisher's exact test) was used to verify the associations between categorical variables and clinical outcome of death or survival. Continuous variables were assessed using the Student's t-test. The Kolmogorov-Smirnov and Levene test was used to assess the

normality of the distribution and the variances' homogeneity. Odds ratio (OR) and 95% confidence interval (CI) were calculated. Categorical variables were expressed as absolute number (n), and percentage (%) and continuous variables were expressed as mean ± standard deviation. A binary logistic regression was performed using the Forward Stepwise selection method to outline the best predictors for the outcome of death. All analyzes were performed using IBM SPSS Statistics 24®. The tests were bilateral with a 0.05 significance level.

RESULTS

After applying the adapted protocol to 42 patients in the period described, five animals had an infection, and 37 dogs met the sepsis classification criteria. Of these 37 dogs, 31 were in sepsis and six in septic shock. Table 1 shows the clinical and laboratory characteristics of the dogs in the study. Among the 37 dogs in sepsis, 51.4% were females with an average weight of 13.8 kg (3-30 kg). Two or more signs of SIRS were

Table 1. Epidemiological, clinical and laboratory characteristics in relation to the total number of dogs treated with sepsis, to verify associations between variables and clinical outcome, in the "Clínica Cirúrgica de Animais de Companhia" sector at the "Hospital Veterinário" of the "Universidade Estadual de Londrina" (HV-UEL) from March 2016 to April 2017

Variables	Total dogs (n=37)	Discharge (n=21)	Death (n=16)	p-value	OR (CI 95%)
Sex					
Male	18 (48.6)	11 (52.4)	7 (43.8)	0.603	-
Female	19 (51.4)	10 (47.6)	9 (56.3)		
Castration					
Yes	8 (21.6)	6 (28.8)	2 (12.5)	0.423	-
No	29 (78.4)	15 (71.4)	14 (87.5)		
Weight (kg)	13.81 (±8.67)	12.62 (±7.83)	15.37 (±9.69)	0.345	-
Clinical signs					
Two or more SIRS signs	26 (70.2)	13 (61.9)	13 (81.3)	0.285	-
Leukogram x10^3; <6>16;>3%	27 (72.9)	14 (66.7)	13 (86.7)	0.252	-
Hypothermia <37.2 °C; or Hyperthermia > 39.4	20 (54.0)	10 (47.6)	10 (62.5)	0.368	-
Respiratory rate >40 bpm	19 (51.3)	9 (42.9)	10 (62.5)	0.236	-
Heart rate bpm >150 bpm	19 (51.3)	10 (50.0)	9 (56.3)	0.709	-
Respiratory difficulty	8 (21.6)	1 (4.8)	7 (43.8)	0.007	15.55 (1.66-145.84)
Dysfunctions					
Organic dysfunction	27 (73.0)	12 (57.1)	15 (93.8)	0.023	11.25 (1.24-101.63)
Decreased level of consciousness	21 (56.8)	9 (42.9)	12 (75.0)	0.093	-
Hyperlactatemia >3.2mmol/dL	19 (51.4)	11 (52.4)	8 (50.0)	1.000	-
Hypoalbuminemia <2.5 g/dL	18 (48.6)	7 (33.3)	11 (68.8)	0.049	4.40 (1.09-17.7)
Creatinine >2.0 mg/dL	9 (24.0)	4 (19.0)	5 (31.3)	0.458	-
Hypotension (SP<90; MBP<65)	9 (24.3)	1 (4.8)	8 (50.0)	0.002	20.00 (2.14-186.88)
Oliguria 0.5 ml/kg/hr	6 (16.2)	2 (9.5)	4 (25.0)	0.371	-
Diagnosis					
Sepsis	31 (83.8)	21 (100)	10 (62.6)		30.33 (1.96-960.0)
Septic shock	6 (16.2)	0 (0.0)	6 (37.5)	0.009	25.32 (1.28-499.0)
Infectious focus					
Skin and soft tissue	20 (54.1)	11 (52.4)	9 (56.3)	0.565	-
Acute abdominal infection	9 (24.3)	4 (19.1)	5 (31.3)		
Urinary tract	5 (13.5)	4 (19.0)	1 (6.3)		
Bloodstream	2 (5.4)	1 (4.8)	1 (6.3)		
Bone and joint	1 (2.7)	1 (4.8)	1 (6.3)		
Empirical antibiotic therapy					
Enrofloxacin	9 (24.3)	6 (28.6)	3 (18.8)	0.278	-
Enrofloxacin + Metronidazole	6 (16.2)	3 (14.3)	3 (18.8)	NA	
Ampicillin + Gentamicin	5 (13.5)	5 (23.3)	0 (0.0)		
Ceftriaxone	4 (10.8)	2 (9.5)	2 (12.5)		

Variables	Total dogs (n=37)	Discharge (n=21)	Death (n=16)	p-value	OR (CI 95%)
Enrofloxacin + Gentamicin	3 (8.1)	1 (4.8)	2 (12.5)		
Gentamicin	3 (8.1)	1 (4.8)	2 (12.5)		
Enrofloxacin + Ceftriaxone	2 (5.4)	0 (0.0)	2 (12.5)		
Gentamicin + Ceftriaxone	1 (2.7)	1 (4.8)	0 (0.0)		
Enrofloxacin + Doxycycline	1 (2.7)	1 (4.8)	0 (0.0)		
Cefazolin	1 (2.7)	0 (0.0)	1 (6.3)		
Amoxicillin/Clavulanate	1 (2.7)	0 (0.0)	1 (6.3)		
Sulfamethoxazole-Trimethoprim	1 (2.7)	1 (4.8)	0 (0.0)		
Cultures					
Positive blood culture	9 (25)	4 (19.0)	5 (33.3)	0.443	-
Positive infectious focus	26 (70.3)	15 (71.4)	11 (68.8)	0.859	-
Positive rectal swab	29 (78.4)	17 (81.0)	12 (75.0)	0.705	-

OR = Odds ratio, CI = confidence interval, SP = systolic pressure, MBP = mean blood pressure, NA = not applicable.

found in 70.2% of cases. More than half had tachypnea and tachycardia (51.3%). The presence of organic dysfunction was found in 73% of patients, decreased consciousness level in 56.8%, and increased serum lactate in 51.4%. The predominant infectious foci were skin and soft tissues (54.1%), followed by acute abdominal and urinary tract infections. The empirical antimicrobials used at the first evaluation were enrofloxacin (24.3%) and enrofloxacin in combination with metronidazole (16.2%). Gram-negative bacteria predominated in 64% of cultures. The survival outcome was obtained in more than half of the dogs (56.7%).

Table 2 shows the results of the microbiological analyzes. Bacterial growth occurred in 24.3% (9/37) of blood cultures, with gram-positive microorganisms being the most frequent (66.6%), mainly *Staphylococcus pseudointermedius* (33.3%), *Streptococcus canis* (22.2%), and *Enterococcus* spp. (11.1%). In the blood culture of a dog, *Candida glabrata* was isolated. In infectious foci, Gram-negative bacteria such as *Escherichia coli* (19.5%), *Klebsiella pneumoniae* (14.6%), and *Pseudomonas aeruginosa* (12.1%) were the most frequent, as well as in the rectal mucosa. However, in this location, several ESBL bacteria were identified. *Enterococcus* spp. resistant to vancomycin (VRE) in 10.5% (4/38) and methicillin-resistant *S. pseudointermedius* (MRSP) in one case (Table 2).

Table 3 shows the general in vitro sensitivity profile of the isolated microorganisms. Aminoglycosides and amoxicillin/clavulanate showed the lowest percentage of resistance. Ampicillin, sulfamethoxazole, and enrofloxacin were the antimicrobials with the highest resistance rates, reaching 90%, 89%, and 87%, respectively.

When the discharge/death outcome was interpreted by univariate analysis, dogs in septic shock were 29.33 times more likely to die than animals in sepsis (p=0.009). However, no difference was observed when the sepsis and sepsis with organ dysfunction were compared (p=0.739, OR: 1.5/0.135-16.55). The presence of respiratory difficulty (p=0.007, OR: 15.55), organ dysfunction (p=0.023, OR: 11.25), hypoalbuminemia (p=0.049, OR: 4.40) and hypotension (p=0.007, OR: 20.0) were associated with death during hospitalization. The other variables were not associated with this outcome (p> 0.05) (Table 1).

Although the positivity of the infection focus cultures is not associated with the outcome, among the rectal swab isolates, ESBL bacteria's presence was associated with death compared to the *P. aeruginosa* and Gram-positive isolates (p=0.039). The isolated Gram-negative bacilli (GNB), Gram-positive cocci (GPC), and polymicrobial samples obtained from cultures of the infectious focus did not differ in terms of the chance of death (p=0.789). It was impossible to analyze the isolates found in blood cultures with the outcome due to the small number of positive cultures (n=9) (Table 4).

Regarding the best predictors for death, after performing binary logistic regression with the forward stepwise input method, the discharge/death outcome was classified as a dependent variable. All clinical and laboratory variables were analyzed in the present study as explanatory variables (Table 5). The presence of two or more signs of SIRS (p=0.045, OR: 8.78), hypotension (p=0.013, OR: 39.34), and hypoalbuminemia (p=0.014, OR: 16.82) were independently associated with death. The present model correctly classified 82.4% of the cases, with a sensitivity of 90.0% and specificity of 71.4%. In addition, the presence of two or more signs of SIRS together with hypotension and hypoalbuminemia explain 53.7% of the death outcome (Nagelkerke's R^2). The other variables, such as sex, reproductive status, clinical signs, dysfunctions, diagnosis, infectious focus, treatment, presence of positive blood culture, culture of positive infectious focus, or positive rectal swab, were not independent predictors for death (p>0.05).

DISCUSSION

In the prospective observational study described here, it was found that the application of a protocol allowed the identification of 37 dogs in sepsis, with six patients showing signs of septic shock. The primary dysfunctions observed were decreased level of consciousness, hyperlactatemia, and hypoalbuminemia. The presence of two or more SIRS signs associated with hypotension and hypoalbuminemia was related to more than half of the deaths. The most frequent infectious focus was skin and soft tissue, and gram-negative bacteria were mainly responsible for these conditions. Early recognition and intervention favored a survival rate in just over half of the patients.

More than 70% of patients had at least two signs of SIRS associated with the infection, and approximately 30% showed no signs or expressed only one parameter. It demonstrates that the absence of these signs does not rule out sepsis since organic dysfunctions may be present in the septic patient even without signs of SIRS, as proposed by the Sepsis-3 consensus (Rhodes et al. 2017). On the other hand, the SIRS criteria are predictive of exaggerated inflammation and would not necessarily indicate the presence of infection or an unregulated response (Castro & Rabelo 2017). The diagnosis of sepsis performed only based on the list of organ dysfunctions, as proposed according to the human consensus Sepsis-3 may have limitations, since organic dysfunctions do not always result from an infectious process, thus causing a delay in the diagnosis and treatment of the infection (Castro & Rabelo 2017). Thus, the balance between sensitivity of the SIRS criteria and specificity of organ dysfunctions in identifying canine sepsis patients can vary according to several factors, such as evaluated parameters, type, location, and extent of infection (Otto 2007).

The presence of two or more signs of SIRS, together with dysfunctions hypotension and hypoalbuminemia, represented the cause of more than half of deaths, portraying how organic dysfunctions worsen the prognosis, as they indicate greater severity of the condition. However, according to the Sepsis-3 Consensus these dysfunctions would no longer be classified as severe sepsis (Castro & Rabelo 2017, Hardy et al. 2018). The focus of sepsis was traumatic wounds, but there was no relationship between this etiology and death. In humans, sepsis's

Table 2. Frequency of microorganisms isolated from blood, infectious focus and rectal swab of 37 dogs characterized with sepsis

Microorganism	Blood	Infectious focus	Rectal swab
Enterobacteria			
Escherichia coli	-	8/41 (19.5%)	12/38 (31.5%)**
Klebsiella pneumoniae	1/37 (2.7%)	6/41 (14.6%)	6/38 (15.7%)**
Enterobacter cloacae	-	2/41 (4.8%)	3/38 (7.8%)**
Morganela spp.	-	-	1/38 (2.6%)**
Proteus mirabilis	-	3/41(7.3%)	1/38 (2.6%)**
Citrobacter spp.	-	1/41 (2.4%)	3/38 (7.8%)**
Gram-positive coccus			
Staphylococcus intermedius (group)	3/37 (8.1%)††	4/41 (9.7%)	-
MRSP	-	-	1/38(2.6%)
Streptococcus sp.	2/37 (5.4%)††	2/41 (4.8%)	-
Enterococcus spp.	1/37 (2.7%)	4/41 (9.7%)	1/38 (2.6%)
VRE			4(10.5%)
CNE	-	1/41 (2.4%)	-
Other GNB	-	-	-
Acinetobacter sp.	-	2/41 (4.8%)	-
Acromobacter sp.	-	1/41 (2.4%)	-
Pseudomonas aeruginosa	-	5/41 (12.1%)	6/38 (15.7%)
Stenotrophomonas sp.	-	2/41 (4.8%)	-
Pasteurela multocida	1/37 (2.7%)†	-	-
Fungi			
Candida glabrata	1/37 (2.7%)	-	-
TOTAL	9/37 (24.3%)	41*(100%)	38*(100%)

* Total number of isolated bacteria, ** ESBL = extended-spectrum beta-lactamases; MRSP = methicillin resistant *Staphylococcus intermedius*, CNE = coagulase negative *Staphylococcus*, VRE = vancomycin resistant *Enterococcus*, GNB = Gram-negative bacilli, † number of dogs that died.

Table 3. General sensitivity profile of microorganisms isolated from dogs with sepsis seen at the "Clínica Cirúrgica de Animais de Companhia" of the "Hospital Veterinário" of the "Universidade Estadual de Londrina" (HV-UEL) from March 2016 to April 2017

Antimicrobial	% resistant
Amikacin	15
Amoxicillin/clavulanic acid	15
Ampicillin	90
Cefepime	85
Enrofloxacin	87
Gentamycin	59
Sulfamethoxazole	89
Tetracycline	87
Imipenem	0

Table 4. Blood culture isolates, culture of the infectious focus and rectal swab and statistical analysis to verify associations between culture results and clinical outcome, in dogs treated with sepsis in the "Clínica Cirúrgica de Animais de Companhia" sector of the "Hospital Veterinário" of the "Universidade Estadual de Londrina" (HV-UEL) from March 2016 to April 2017

Culture	Total dogs n and %	Discharge n and %	Death n and %	p-value
Blood culture (positive) (n=9)				
GPC	6 (66.6)	2 (50.0)	4 (80.0)	NA
GNB	2 (22.6)	1 (25.0)	1 (20.0)	
Candida glabrata	1 (16.6)	1 (25.0)	0 (0.0)	
Infectious focus (positive) (n=26)				
GNB	4 (15.4)	2 (13.3)	2 (18.2)	0.789
GPC	9 (34.6)	6 (40.0)	3 (27.3)	
Polymicrobial	13(50.0)	7 (46.6)	6 (54.5)	
Rectal swab (positive) (n=29)				
ESBL	21 (72.4)	9 (53.0)	12 (100.0)	0.039
Gram-positive	4 (13.8)	4 (23.5)	0 (0.0)	
Pseudomonas aeruginosa	4 (13.8)	4 (23.5)	0 (0.0)	

Chi-square test; NA = not applicable, GNB = Gram-negative bacilli, GPC = Gram-positive cocci, ESBL = extended-spectrum beta-lactamases bacteria, F = frequency.

Table 5. Binary logistic regression (Forward Stepwise) with the outcome discharge/death as a dependent variable and clinical and laboratory data as explanatory variables.

Explanatory variable	B	Wald	DF	p-value	OR (CI 95%)
Two or more SIRS signals	2.173	3.668	1	0.045	8.78 (1.95-18.14)
Hypotension	3.672	6.148	1	0.013	39.34 (2.16-71.70)
Hypoalbuminemia	2.823	6.316	1	0.014	16.82 (1.86-25.04)

DF = degree of freedom, OR = odds ratio, CI = confidence interval

main etiologies are pneumonia, urinary tract infection, and intra-abdominal infections, with pneumonia being the most related to death (ILAS 2015). Respiratory infections were not observed in the present study; however, urinary, and intra-abdominal infections were frequent and not related to death.

Although in The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) held in 2016, the concept of severe sepsis was removed from the definitions due to the presence of life-threatening organ dysfunction since the beginning of the septic process, making the term "severe sepsis" redundant, this has not been validated in the veterinary medicine (Castro & Rabelo 2017). The term severe sepsis was used in a recent article on sepsis that measured the procalcitonin biomarker in 53 dogs (Troia et al. 2018). There is controversy about accepting these terms in human medicine, as there is also disagreement about whether the Second or Third Consensus better identifies the critically ill patient (Carneiro et al. 2017). In addition, the Sequential Organ Failure Assessment (SOFA) and SOFA scores, from the Sepsis-3 Consensus, which allow the identification of critical patients, have not been validated in the veterinary. However, the terminology sepsis, septic shock, and sepsis are still used, considering that sepsis is an infection with a new organic dysfunction, as recommended by the "Instituto Latino Americano de Sepse" (Castro & Rabelo 2017). Although 70% of the animals were classified as having severe sepsis in the present study, survival was greater than the death rate. It demonstrates that despite the presence of organic dysfunction is associated with increased mortality, early recognition and treatment, through the application of the protocol developed to identify sepsis, allowed the identification of significant clinical and laboratory parameters associated with a greater chance of death. The parameters include the presence of two or more signs of SIRS, respiratory distress, hypotension, and hypoalbuminemia.

All dogs in the present study classified as being in septic shock died. Hypotension in sepsis is a consequence of decreased cardiac output due to the excess of inflammatory factors, causing other cardiovascular changes, common in both humans and animals in sepsis, with a low prognostic factor (Kumar et al. 2000). When hypotension does not respond to vasopressor treatment, patients are classified as in septic shock, and patients who met this classification had a worse outcome. In a study with bitches with pyometra, the presence of hypotension and septic shock were also related to a worse prognosis and death (Conti-Patara et al. 2012).

In assessing central nervous system dysfunction using the modified coma scale, it was possible to observe changes in the level of consciousness in more than half of the patients. Thus, it can be said that this neurological alteration is an important organ dysfunction to be considered in patients with sepsis, although it is not related to mortality. This alteration's pathophysiology is not yet fully understood. However, it is believed that decreased cerebral perfusion due to hypotension, hypovolemia, or arterial obstruction by microthrombi is involved (Johnson et al. 2004).

In the present study, just over half of the dogs showed an increase in lactate, showing impairment of tissue perfusion; however, this change was not correlated with mortality. In a study with bitches with pyometra and sepsis, diagnosed with at least two signs of SIRS and more than one sign of organ dysfunction, several tissue perfusion parameters were evaluated. It was concluded that the lactate concentration was also not a good mortality predictor when compared to the assessment of base deficit and central venous oxygen saturation, which discriminated better between survivors and non-survivors (Conti-Patara et al. 2012). One of the factors that may have contributed to the result was the number of cases, which was considered small, similar to the number of patients included in the present study. In a study with several veterinary centers, with a number of cases 14 times greater than that of the present study, lactate was a significant predictor of survival (Rabelo 2008). In human medicine, serum lactate levels have a better relationship with prognosis and assessment of response to treatment (Villar et al. 2019, Velissaris et al. 2019).

Patients with hypoalbuminemia at the time of inclusion in the study were more likely to die. One of the study's limitations was that the albumin value was not monitored after the dogs' inclusion in the study. However, all were in sepsis, and the majority had several organ dysfunctions. The progression of SIRS/Sepsis is characterized by rapid and unspecified clinical signs and serum concentration of acute-phase proteins. Septic patients have increased serum C-reactive protein (CRP), haptoglobin, ceruloplasmin, and fibrinogen in the blood. Other proteins, such as albumin and transferrin, gradually decrease during the inflammatory processes, being called negative proteins of the acute phase (Ceron et al. 2005). In a study of 33 dogs in SIRS, that was due or not to sepsis, hypoalbuminemia was observed, and the dogs that survived had higher values of total protein and albumin (Giunti et al. 2015). In another study, with 43 dogs with SIRS and sepsis, it was also observed that the gradual decrease in albumin values during hospitalization was related to a worse prognosis and that the plasma albumin concentration was inversely correlated with the severity of inflammation (Torrente et al. 2015). Albumin has several functions, such as maintaining oncotic pressure, transporting substances, mediating coagulation, inflammation, and healing, among others (Mazzaferro et al. 2002, Grimes et al. 2011). Although its measurement is cheap and easy to perform, it is considered a non-specific inflammatory biomarker. It can decrease in liver failure cases, enteropathies, nephrotic syndrome, malnutrition, and burns, among other conditions (Mazzaferro et al. 2002).

Acute kidney injury (AKI) in sepsis is multifactorial. It can result from hypovolemia, hypotension, and direct kidney injury. It is characterized by increased serum creatinine levels and decreased urinary output (Hoareau et al. 2014). In humans,

AKI has been documented in 47% of patients with a clinical diagnosis of sepsis, being a public health concern. It affects not only survival but also worsens chronic kidney disease that many patients already have. In a study of dogs with sepsis due to abdominal disorders, AKI was seen in 12 to 16% of cases, and only 14% of affected dogs survived. The canine population's survival rate in general, with AKI, depends on the etiology and varies from 12 to 76%. There are not many publications on kidney damage due to sepsis in veterinary medicine, as most articles focus on kidney damage due to nephrotoxins or renal ischemia (Keir & Kellum 2015).

In the present study, both the frequency of the organic dysfunction and the mortality rate were high. The respiratory difficulty was the dysfunction with the highest association with death, as observed in a study in which survival was assessed concerning the presence of Multiple Organ Dysfunction Syndrome (MODS) in 114 dogs with sepsis (Kenney et al. 2010). In humans, the early identification of organ dysfunctions is essential to prevent MODS, as the mortality rate is affected by the severity of the underlying disease and the number of dysfunctional organs (Osterbur et al. 2014, Singer et al. 2016). The mortality rate in humans with MODS ranges from 20% to 100%, and this value is affected by the number, severity, duration, type, and combination of compromised organ systems (Kenney et al. 2010, Osterbur et al. 2014). In veterinary medicine, although there are few studies on MODS (Osterbur et al. 2014, Hardy et al. 2018, Troia et al. 2018), the mortality rate of septic dogs has also increased in relation to the number of affected organs (Kenney et al. 2010).

Escherichia coli, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus intermedius*, and *Enterococcus* spp. were the most commonly isolated bacteria from infectious foci, mainly in skin soft tissue infections. In most studies on dogs in SIRS/sepsis, the infectious agents observed were not discriminated, as the articles focused on biomarkers and prognostic factors (Kenney et al. 2010, Grimes et al. 2011, Conti-Patara et al. 2012, Giunti et al. 2015, Torrente et al. 2015, Walters et al. 2017, Hardy et al. 2018, Troia et al. 2018, Letendre & Goggs 2018). In a study of dogs and cats with septic peritonitis, the most common bacteria in decreasing order were *E. coli*, *Enterococcus* spp., *Staphylococcus* spp. *Enterobacter* spp., *Streptococcus* spp., *Proteus* spp. and *Klebsiella* spp. In the same place where this study was carried out, in dogs with pyometra and SIRS, the bacteria most frequently isolated in two different periods were *E. coli*, *Streptococcus* spp., *Klebsiella* spp., *Pseudomonas* spp. and *Staphylococcus* spp. (Sant'Anna et al. 2014) and *E. coli* and *Streptococcus* spp. (Trautwein et al. 2017). In dogs with sepsis due to wounds, from 2012 to 2013, in the same place as the present study, the primary agents were *P. aeruginosa*, *Streptococcus* sp. and *Enterococcus* spp. Due to the significant problem of antimicrobial resistance in animals and humans, it is a consensus that the collection of material from the infection points for culture and antibiogram is carried out whenever possible to facilitate the early initiation of adequate treatment. Also, laboratories must use appropriate standards in examinations, such as those recommended by the Clinical and Laboratory Standards Institute (CLSI 2008) for the veterinary (Weese et al. 2015), as performed in the present study.

The choice of the antimicrobial used in septic patients was not related to death. However, in the cultures of the infectious foci, there was a high frequency of resistance to quinolones, ampicillin, sulfamethoxazole, and cefepime, a 4th generation cephalosporin. In the same place where this study was carried out, two studies were carried out evaluating the resistance of bacteria isolated from dogs with wounds (Arias et al. 2008) and dogs with wounds and sepsis (Bahr Arias et al. 2017). Another study evaluated the resistance of bacteria isolated from wounds, ear infections, and urinary system disorders in dogs and cats (Ishii et al. 2011). There was a trend of increasing resistance to antibiotics in the class of quinolones, aminoglycosides, and sulfonamides in the period between each study. It is worrisome because, in addition to the antibiotic options used in pets are decreasing, several genera of bacteria can be reservoirs of antibiotic resistance (Prescott 2014). *E. coli*, for example, is a commensal of humans and animals, widely disseminated in the environment. It has resistance genes that can be easily transferred to other pathogenic bacteria. The production of extended-spectrum beta-lactamases (ESBLs) and the development of resistance to quinolones are common in strains of E. coli and are a severe threat to humans and animals (Meireles et al. 2015). Still, concerning the comparison of antibiotic resistance indexes in the different periods, the amoxicillin/clavulanate association showed a critical reduction in resistance frequency. There was no change in the resistance to aminoglycosides and cephalosporins from the first to the fourth generation. However, the resistance to the latter remains high. Even though there is 100% sensitivity to carbapenem antimicrobials, their use should be restricted in the veterinary medicine to avoid the selection of multi-resistant bacteria that can be a risk to human health (Prescott 2014).

The collection and culture of material obtained from the rectal mucosa were performed in all dogs due to the importance of this monitoring in critically ill patients, since colonization by resistant bacteria can affect not only the result of the treatment but also the veterinary team and tutors of the animals, through the transmission of opportunistic bacteria (So et al. 2012, Hamilton et al. 2013). It is worrying that ESBL bacteria as well VRE have been isolated in the dogs in the present study. The presence of ESBL bacteria was more associated with death than *P. aeruginosa* and Gram-positive cocci. Simultaneously, in VREs it was not possible to correlate the presence of this bacterium with the outcome of death due to the small number of positive samples. Cases of ESBL enterobacterial infections were first reported in the late 1980s in humans. It is associated with increased mortality, length of stay, and costs (Razazi et al. 2012). There are already reports of infections caused by VRE bacteria (Abbott et al. 2009) in veterinary medicine. In a study in Korea, *E. coli* ESBL was identified in 33.3% of dogs admitted to Veterinary Hospitals (So et al. 2012).

According to the Surviving Sepsis Campaign (Rhodes et al. 2017), blood culture collection is indicated in all suspected sepsis cases. Bacteremia is a severe clinical complication with a critical role in the prognosis of sepsis. Positive blood cultures are found in approximately one-third of cases in human patients with severe sepsis (Hodgin & Moss 2008). In Veterinary Medicine, information on the prevalence of bacteremia in dogs and cats is still scarce (Dow et al. 1989, Greiner et al. 2008, Tsuyuki et al. 2018), but it was estimated between 30-50% (Calvert & Thomason 2011). In this study, almost 25% of blood cultures were positive, of which more

than half of the patients died. In a study of 100 critically ill dogs and cats, which 49 were in bacteremia, patients with severe disease and bacteremia were 15.6 times more likely to die than those with non-serious disease and negative blood culture results. However, Gram-negative bacteria results were the most frequent (Dow et al. 1989). In bitches with pyometra and sepsis, *E. coli* was the most common bacterium in blood culture (Kalenski et al. 2012). In the present study, Gram-positive bacteria were the leading cause of bloodstream infection as well as in a study with 140 dogs and 39 cats, in which *Staphylococcus* spp. and *Streptococcus* spp. were the main microorganisms (Greiner et al. 2008).

Candida glabrata was identified in a dog's blood culture that had been using quinolone for a long time and had diarrhea, abdominal effusion, presence of multi-resistant *E. coli* in peritoneal fluid, and candiduria. *Candida* spp. is a fungus that inhabits oral, gastrointestinal, upper respiratory, and urogenital mucosa of mammals, and several factors may be related to candidemia. These factors include prolonged hospitalization, use of antibiotics, alteration of the skin or mucous barriers due to trauma, surgery, catheter presence, urinary and vascular diseases, among others (Rogers et al. 2009). In this case, it cannot be said that the patient was in fungal sepsis, as not all inclusion criteria were met, such as the presence of two of the following three criteria: two positive blood cultures, detection of fungi at the tip of the catheter, severe septic clinical picture, and fungal septic metastases (Rogers et al. 2009). However, this fact is a warning since candidemia is already well known in human medicine in immunocompromised patients.

Sepsis is a clinical, multifactorial syndrome associated with several changes in physical and laboratory examination, and the absence of signs of a systemic inflammatory response does not rule out the possibility of dogs having sepsis. Due to the severity of the condition and lack of consensus on the diagnosis in veterinary medicine, there is great interest in the development of biomarkers and parameters to facilitate diagnosis and prognosis (de Laforcade et al. 2003, Conti-Patara et al. 2012, Giunti et al. 2015, Torrente et al. 2015, Hardy et al. 2018, Troia, et al. 2018). The protocol adapted based on the Second Human Consensus proved to be useful in identifying the septic patient, being essential the knowledge of the several parameters of identification of the sepsis phases so that it is recognized early, reducing costs and mortality. The collection of material for culture should be carried out, if possible, in all cases, to initiate the correct, fast, and effective treatment.

CONCLUSIONS

The results showed that the protocol adapted based on the Second International Consensus Definition for Sepsis and Septic Shock or Sepsis-2 used in the study allowed to verify the best parameters related to the early identification of the patient in sepsis and relate them to death. Most dogs showed two or more clinical signs of SIRS and/or changes in white blood cell count, temperature, respiratory rate, heart rate, and organ dysfunctions, including hypoalbuminemia, hypotension, and breathing difficulties. The best predictors for death were two or more SIRS parameters, hypotension, and hypoalbuminemia. Breathing difficulties and the presence of organ dysfunction were associated with mortality. ESBL bacteria colonizing the rectal mucosa also had a greater relationship with the death outcome.

The most frequent microorganisms found both as a cause of infection and colonizing the rectal mucosa were Gram-negative bacteria, *E. coli*, *K. pneumoniae* and *P. aeruginosa*. VRE, an important zoonotic agent, also colonized some patients, which is of great concern, suggesting that in Veterinary Medicine, the same precautions used in human ICUs should be implemented to prevent the spread of multi-resistant microorganisms. Blood culture was positive in approximately a quarter of the dogs and showed a prevalence of Gram-positive, including *S. intermedius* and *Streptococcus* spp. There was a high rate of antimicrobial resistance, mainly to quinolones, cephalosporins, ampicillin, and sulfonamides, emphasizing the need for the more careful use of antimicrobials. Aminoglycosides and amoxicillin/clavulanic acid still have the highest sensitivity rate.

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Original Article
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Ozonized solutions favor the repair of experimentally induced skin wounds in rats[1]

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ABSTRACT.- Sanguanini R.C., Bento M.F., Oliveira E., Arnhold E., Faleiro M.B.R., Franco L.G., Matos M.P.C. & Moura V.M.B.D. 2020. **Ozonized solutions favor the repair of experimentally induced skin wounds in rats**. *Pesquisa Veterinária Brasileira 40(11):914-921.* Setor de Patologia Animal, Escola de Veterinária e Zootecnia, Universidade Federal de Goiás, Avenida Esperança s/n, Campus Universitário Samambaia, Goiânia, GO 74690-900, Brazil. E-mail: vdmoura@ufg.br

This study aimed to evaluate and compare the effects of ozonized solutions on tissue wound repair in rats. Treatments consisted of ozonized water (GA), 0.9% sodium chloride (GCL), ozonized oil (GO), and 0.2% allantoin cream (GAL). The morphometric evaluation showed that wounds of the GA group presented a higher degree of retraction ($p<0.05$) at three and eight days of treatment (37.96 and 84.81%, respectively). Picrosirius red staining showed that groups GA and GO presented higher deposition ($p<0.05$) of type I collagen at 15 and 22 days of treatment, respectively. The neovascularization was higher in wounds of group GO on days 3, 8, and 15 ($p<0.05$), with higher VEGF immunostaining. ($p<0.05$). Thus, ozonized water enhances wound retraction and assists in the maturation and remodeling phase, while ozonized oil promotes higher neovascularization during tissue repair and higher deposition of type I collagen from the third week of treatment.

INDEX TERMS: Ozonized water, wound healing, ozonized oil, ozone therapy, skin wounds, rats.

RESUMO.- [**Soluções ozonizadas favorecem o reparo de feridas cutâneas experimentalmente induzidas em ratos.**] O objetivo deste estudo foi avaliar e comparar os efeitos de soluções ozonizadas sobre o reparo tecidual de feridas em ratos. Foram realizados os tratamentos: água ozonizada (GA), cloreto de sódio 0,9% (GCL), óleo ozonizado (GO) e creme de alantoína 0,2% (GAL). À avaliação morfométrica, as feridas do grupo GA apresentaram maior grau de retração ($p<0,05$) aos três e oito dias de tratamento (37,96% e 84,81%, respectivamente). A coloração de *picrosirius red* mostrou que os grupos GA e GO apresentaram maior deposição ($p<0,05$) de colágeno do tipo I aos 15 e aos 22 dias de tratamento, respectivamente. Já a variável neovascularização foi maior ($p<0,05$) nas feridas do grupo GO nos dias três, oito e 15, o que fora ratificado à imuno-histoquímica, com maior imunomarcação de VEGF nas feridas do grupo GO ($p<0,05$). Conclui-se que a água ozonizada potencializa a retração da ferida e auxilia na fase de maturação e remodelamento, enquanto o óleo ozonizado promove maior neovascularização durante o reparo tecidual e maior deposição de colágeno do tipo I a partir da terceira semana de tratamento.

TERMOS DE INDEXAÇÃO: Água ozonizada, cicatrização, óleo ozonizado, ozonioterapia, feridas cutâneas, ratos.

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INTRODUCTION

Ozone therapy has been used in the adjunctive treatment of wounds, assisting tissue repair in an economically viable, non-invasive way without side effects (Haddad et al. 2009). Ozone is an unstable molecule with a strong oxidizing effect that eliminates microorganisms without causing resistance (Yucesoy et al. 2017) and improves local inflammatory response by stimulating the synthesis and release of cytokine and growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF), and vascular endothelial growth factor (VEGF) (Araneda et al. 2008, Valacchi et al. 2011).

Ozone is used to treat skin wounds in gaseous form or dissolved in water or oil (ozonized solutions). The use of ozonized solutions has the advantage of reducing the risks associated with ozone inhalation, besides establishing the ideal humid environment to facilitate the interaction between ozone and biomolecules. Ozonized water is easy to handle and apply in hard-to-reach places, but dissolved ozone degrades rapidly, implying immediate use of water after ozonation. Oil diffuses ozone molecules slowly to the tissue due to its ability to stabilize the gas (Sechi et al. 2011, Valacchi et al. 2011).

Although ozonized solutions aid tissue repair, there is no standardized protocol for the treatment of skin wounds or even comparative studies to evaluate the advantages and disadvantages of these solutions. Therefore, this study aimed to verify the effects of ozonized water and oil on experimentally induced skin wounds in rats to establish a viable experimental protocol for clinical application, elucidating which aspects of tissue repair it is advantageous to use each of the ozonized solutions.

MATERIALS AND METHODS

This research was authorized by the Animal Use Ethics Committee (CEUA) of the "Universidade Federal de Goiás" (UFG) under protocol 079/2017. Forty-eight female Wistar albino rats (*Rattus norvegicus*) weighing from 250 to 300g, provided by the UFG Central Vivarium, were used. Two animals were placed per box, being separated by a transparent acrylic plate, in a sanitized, air-conditioned environment (23°C), with a 12-hour light cycle (light/dark) and both water and commercial feed *ad libitum*. The animals remained in the adaptation period for ten days before the beginning of the experiment.

Animals were randomly placed into four groups of 12 individuals each, according to the following treatments: ozonized water (GA), ozonized sunflower oil (GO), 0.9% sodium chloride (GCL), and 0.2% allantoin cream (GAL). Three animals from each group were euthanized on days 3, 8, 15, and 22 after wound induction for sample collection and microscopic analysis.

Ozonized water was produced with an O&L 1.5RM ozone generator (Ozone & Life, São José dos Campos, São Paulo, Brazil). For this, a 100mL beaker was filled with 50mL of deionized bidistilled water at 7°C, which was ozonized by bubbling for 15 minutes with the equipment adjusted to produce 62mcg/mL of ozone. The protocol used allowed reaching the final concentration of 4.5mcg of ozone per mL of water (Bocci 2005), as verified by the Vacu-Vials Ozone 0-5ppm test (CHEMetrics Inc., Midland, Virginia, USA).

Ozonized sunflower oil was purchased commercially (Ozone & Life, São Jose dos Campos, São Paulo, Brazil), with a peroxide index of 418.48mEq/kg. The 0.2% allantoin cream used in the wound of the positive control animals was also commercially purchased (Interdrogas, Goiânia, Goiás, Brazil), as well as the 0.9% sodium chloride (Laboratório Arboreto, Juiz de Fora, Minas Gerais, Brazil) used in the wound of animals of the negative control group.

Animals underwent isoflurane inhalation anesthetic protocol for wound induction. After trichotomy and antisepsis, a single wound was made using a 9mm diameter metal punch on the dorsal region of the animals, between the two scapulae, from which a skin fragment plus the epidermis, dermis, and subcutaneous tissue were removed.

Treatments were performed once a day until the animals were euthanized (3, 8, 15, and 22 days after wound induction). The wound of GA group animals was irrigated with 3mL of ozonized water for 30 seconds immediately after its ozonation. A new ozonation protocol was performed every three animals treated in order to lessen the impact of ozone depletion on water. The wound of GCL group animals was irrigated with 0.9% sodium chloride, following the same procedure as those used in the groups treated with ozonized water. The wound of GO group animals was treated with 50µL of ozonized oil, and that of GAL group animals received 150mg of 0.2% allantoin cream on the wound area.

Wound retraction was evaluated on days zero (surgery day), 3, 8, 15, and 22 days after wound induction. Wounds were photographed with a camera (Sony® SLT-A35), and the images were exported to the image-processing program Image J (NIH) version 1.3.1, which allowed demarcating wound boundaries were demarcated and measuring wound area (mm^2). The degree of wound retraction was calculated from values transformed into a percentage according to the equation:

$$DWR = \frac{(IWA - WA^N)}{IWA} \times 100$$

Where DWR is the degree of wound retraction, IWA is the initial wound area, and WA^N is the wound area on the day the photograph was taken (N = days 3, 8, 15, or 22).

Microscopic evaluation was performed by taken a rectangular skin fragment of each animal, including the entire lesion area and the edges of the adjacent normal tissue (1cm of the border). These fragments were fixed in 10% buffered formalin for 48 hours and then transferred to 70% alcohol solution, where they remained until the histological processing and paraffin inclusion. Histological sections of 5µm were distended on glass slides and stained with hematoxylin and eosin (HE).

The analysis of histomorphological variables was performed observing parameters that included reepithelialization, type of extracellular matrix (ECM), neovascularization, acanthosis, polymorphonuclear infiltrate, mononuclear infiltrate, and edema. Scores from zero to 3 were assigned to the reepithelization, as described by Silva et al. (2016). Scores from 1 to 3 were assigned for the type of ECM, where score one represented a vascular ECM with high number of newly formed vessels and immature fibroblasts and discrete deposition of weakly stained, sparse collagen fibers with a wavy appearance; score two represented the fibrovascular ECM with newly formed vessels, immature and mature fibroblasts, moderate deposition of more clustered and eosinophilic collagen fibers, and, sometimes, linear in appearance; and score three represented a fibrous ECM, typified by a marked deposition of markedly thick, compactly aligned eosinophilic collagen fibers, with mature fibroblasts and few vessels. A quantitative approach was performed for the variable neovascularization by calculating the average of blood vessels (tubular structures with endothelium and red blood cells in the lumen) in ten fields using a 40x objective, according to criteria adapted from Valacchi et al. (2011). The scores

zero (absent), one (discrete), two (moderate), and three (marked) were assigned for the other variables.

Histological slides were made and submitted picrosirius red staining for collagen fiber quantification. Photomicrographs of ten fields were captured under the 40x objective using a polarized light optical microscope and a camera (Sony® NEX-3) attached. The images were analyzed using the software Image J (NIH) version 1.3.1 through the color threshold tool to show the percentage of area occupied by type I and III collagen fibers (Brassolatti et al. 2016). This tool allows identify and differentiate the birefringence of collagen fibers, which is related to their morphological conformation. Thus, type I collagen fibers have strong birefringence, emitting a glow that varies from orange yellow to red, while type III emits a greenish glow. The total fibers were obtained by simultaneous disclosure in the Image J of areas occupied by type I and III collagen fibers.

Histological sections were distended on charged slides (Starfrost, Knittel Glass, Germany) for VEGF immunostaining (immunohistochemistry - IHC). The sections were immersed in blocking solutions of endogenous peroxidase and background proteins after the deparaffinization, hydration, and antigen recovery steps. Samples were incubated for 18 hours with primary anti-VEGF antibody (sc-53462 - Santa Cruz Biotechnology, Inc.) at a concentration of 1:100. The sections were then washed and incubated with secondary antibody and N-Histofine® Simple Stain Rat MAX PO (MULTI) reaction amplifier (Nichirei Biosciences Inc., Tokyo, Japan). The diaminobenzidine solution counterstained with Mayer's Hematoxylin was used for reaction visualization. The scores one (discrete), two (moderate), and three (marked) were assigned to immunostaining intensity.

Shapiro-Wilk test was used to verify residual abnormality. Due to non-normality, the Kruskal-Wallis nonparametric test at 5% significance was used to compare treatments at each moment of the macroscopic morphometric evaluation and during the analysis of microscopic parameters to histopathological and immunohistochemical examinations. The t-test with FDR adjustment was performed as a post-test. The results of the analyses of collagen quantification were subjected to analysis of variance and the means compared by the Duncan test. For this, the R software and the R easy ANOVA package (Arnhold 2013) were used.

RESULTS

The macroscopic morphometric evaluation showed that the wound of GA animals presented higher retraction in relation to that of animals of other groups (Table 1) at all evaluation moments, except on day 22, when all the wounds were closed. The wound in rats of GA and GCL groups had significantly higher retraction ($p<0.05$) compared to that of animals of groups GO and GAL on day 3. On day 8 (Fig.1), wound retraction of GA rats remained higher than that of groups GO and GAL, which statistically equaled the retraction observed in GCL animals.

Table 1. Means, medians and ranks sums, respectively, referring to the percentage of skin wound retraction in rats submitted to different treatments at 3, 8, 15, and 22 days after the surgical procedure

Group	Period			
	3 days	8 days	15 days	22 days*
GA	37.6/37.9/35.3[a]	84.9/84.8/27.2[a]	100/100/14.0[a]	100[a]
GCL	33.4/33.1/29.5[a]	78.1/80.2/20.4[ab]	99.5/100/12.3[a]	100[a]
GO	22.6/24.4/16.8[b]	69.8/72.7/14.0[b]	98.2/100/11.8[a]	100[a]
GAL	21.9/22.6/16.3[b]	62.4/66.7/12.3[b]	98.2/100/11.8[a]	100[a]

[a,b,ab] Within the same period, a value is only statistically different from another if there are no equal letters. A value identified as "a" is different from a value identified as "b", but not from a value identified as "ab", by the Kruskal-Wallis test ($p<0.05$); *At 22 days after the surgical procedure, all rats showed 100% wound retraction. Thus, the tests were not applied because all values are equal and therefore, there is no difference to be tested; GA = ozonized water group, GCL = sodium chloride group, GO = ozonized oil group, GAL = allantoin group.

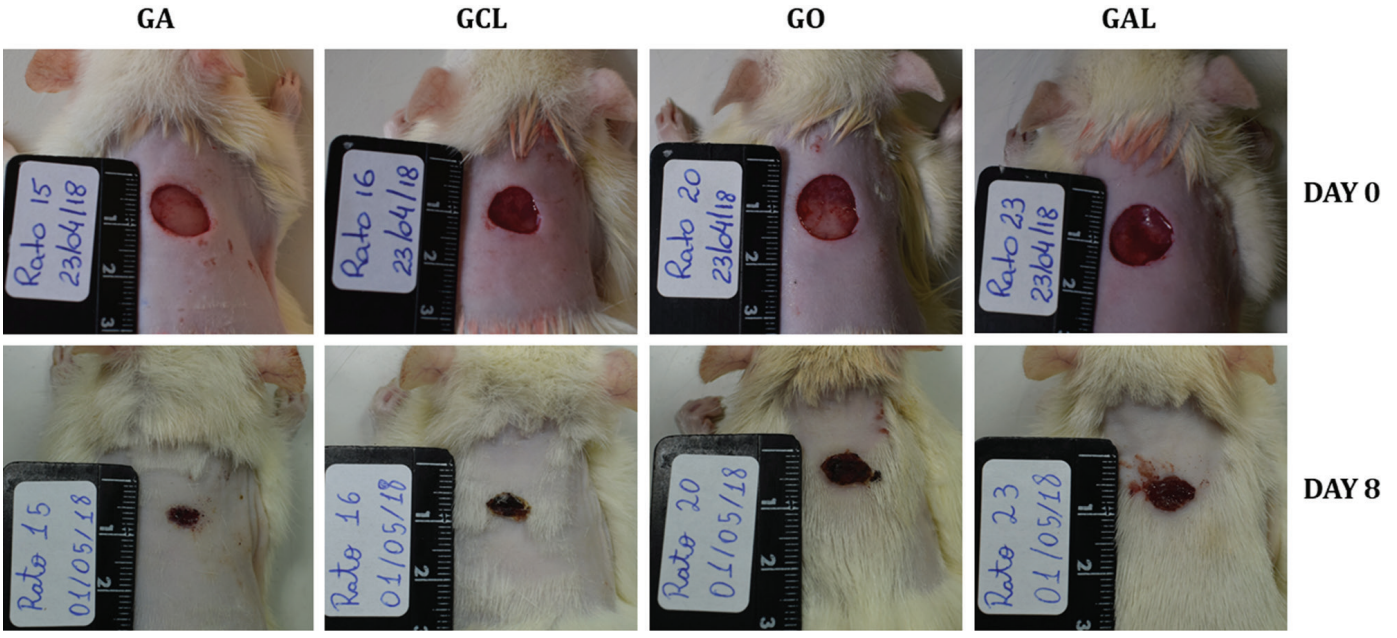


Fig.1. Wound on the back of rats of groups ozonized water (GA), 0.9% sodium chloride (GCL), ozonized oil (GO), and 0.2% allantoin cream (GAL) on days zero (upper) and eight (lower) after injury induction. Wounds of similar size to day zero in all groups; the GA rat wound exhibits greater retraction at 8 days after injury induction when compared to that of the other groups.

The variables polymorphonuclear infiltrate, mononuclear infiltrate, edema, type of ECM, reepithelization, and acanthosis, had no differences (p>0.05) between treatments of groups GA, GCL, GO, and GAL. Regarding neovascularization, the wound of GO rats presented the highest blood vessel count at all evaluated times, while GCL had the lowest count (Table 2). The wound of GO animals on day three was significantly more vascularized (p<0.05) than that of GCL animals. At 8 days, the wounds of groups GO and GAL stood out (p<0.05) in relation to those of GCL group, while at 15 days, the wounds of rats of GCL and GAL groups had the lowest vessel counts, with a difference between them and GO (Fig.2). At 22 days, the wounds of GA group animals presented the lowest neovascularization (p<0.05) when compared to those from the other groups.

The microscopic analysis with picrosirius red staining (Fig.3) at eight days showed that the wound of animals from groups GA and GCL had higher total collagen fibers than that observed for GAL rats (p<0.05) (Table 3). The percentage of type I and III collagen fibers (Table 4) in relation to total collagen showed that the wound of GA rats presented a higher amount (p<0.05) of more resistant collagen fibers (type I) at 15 days than that found for GAL animals. At 22 days, the wound of GO rats had a higher amount of resistant fibers when compared to groups GA and GAL.

Table 2. Means, medians and ranks sums, respectively, of neovascularization in the skin wound of rats at 3, 8, 15, and 22 days after induction and treatment with ozonized water (GA), sodium chloride (GCL), ozonized oil (GO), and allantoin (GAL)

Group	Period			
	3 days	8 days	15 days	22 days
GA	9.33/9/5.16^{ab}	18.66/19/6.50^{ab}	10.00/10/6.00^{ab}	6.33/6/2.00^{b}
GCL	8.33/8/3.50^{b}	14.66/14/2.33^{b}	9.33/9/4.50^{b}	8.66/9/7.66^{a}
GO	11.00/11/9.50^{a}	20.33/20/9.00^{a}	14.00/13/10.66^{a}	8.66/9/7.66^{a}
GAL	10.66/10/7.83^{ab}	20.00/20/8.16^{a}	9.33/9/4.50^{b}	8.50/8.5/7.00^{a}

^{a,b,ab} Within the same period, a value is only statistically different from another if there are no equal letters. Thus, a value identified as "a" is different from a value identified as "b", but not from a value identified as "ab", by the Kruskal-Wallis test (p<0.05).

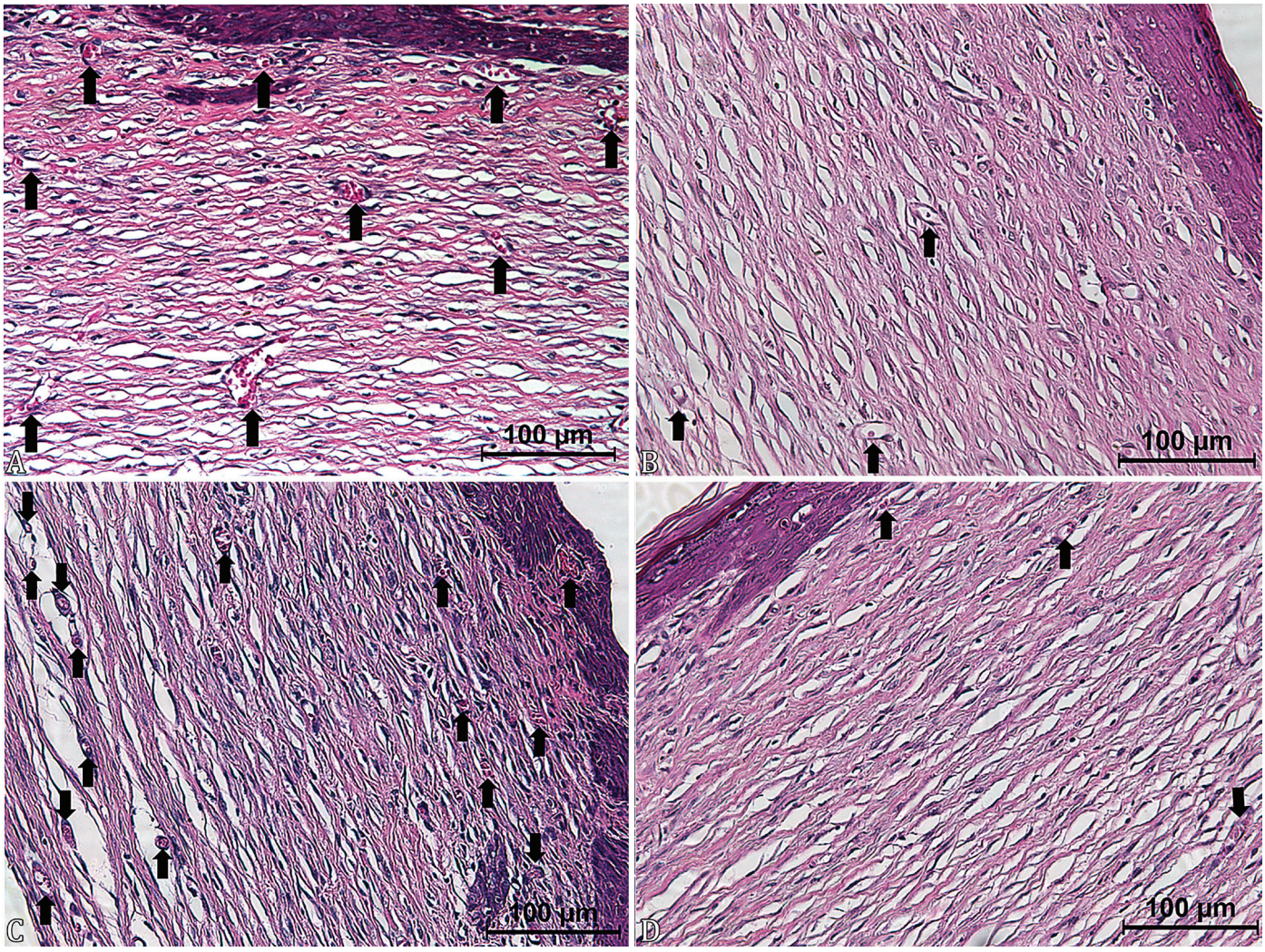


Fig.2. Photomicrographs of the skin of rats from different groups at 15 days after wound induction, showing newly formed vessels (arrows). (**A**) Group ozonized water (GA) showing moderate neovascularization. HE, obj.40x. (**B**) Group 0.9% sodium chloride (GCL) showing discreet neovascularization. HE, obj.40x. (**C**) Group ozonized oil (GO) showing marked neovascularization. HE, obj.40x. (**D**) Group 0.2% allantoin cream (GAL) showing discreet neovascularization. HE, obj.40x.

Immunostaining of vascular endothelium, inflammatory cells, fibroblasts, and, in some cases, epithelial cells were observed in the immunohistochemical evaluation of VEGF (Fig.4). The wound of GO rats presented higher VEGF immunostaining than that observed in animals of other groups (Table 5). Wounds of GO animals showed a higher VEGF immunostaining when compared to those of GCL rats at 8 and 22 days and those of GA animals at 15 and 22 days. Although the wound of GO rats showed higher medians than those observed in GAL animals, no difference was found between these groups at all evaluated times.

DISCUSSION

Ozonized water promoted the highest percentage of retraction of skin wounds at all evaluated times. Wounds of GA presented higher retraction when compared to those of groups GO and GAL 3 and 8 days after wound induction ($p<0.05$), and better numerical results compared to those of GCL. Similarly, Filippi (2001) treated wounds in the oral mucosa of humans and found that ozonized water at concentrations of 11 to 12mcg/mL induced higher rates of wound retraction in the first seven days of analysis. Another study showed that ozonized water at a concentration of 0.1mcg/mL could accelerate the regeneration process in zebrafish undergoing caudal fin amputation (Hao et al. 2015). These results demonstrate that ozonized water has a wide dose range, with positive effects on tissue repair. In this sense, the ozone concentration of 4.5mcg/mL used in this study and obtained from that described by Bocci (2005) is in the mentioned range of beneficial effects, being considered efficient in helping the repair of induced wounds in rats. It is in accordance with the information of this author when referring that concentrations of 5mcg/mL

Table 3. Means of collagen fibers in rat skin wounds at 8, 15, and 22 days after induction and treatment in groups GA, GCL, GO, and GAL

Group	Period		
	8 days	15 days	22 days
GA	4.71[a]	20.54[a]	22.87[a]
GCL	6.67[a]	15.51[a]	21.06[a]
GO	3.66[ab]	10.73[a]	25.12[a]
GAL	1.29[b]	10.17[a]	24.03[a]

[a,b,ab] Within the same period, a value is only statistically different from another if there are no equal letters. Thus, a value identified as "a" is different from a value identified as "b", but not from a value identified as "ab", by the Duncan test ($p<0.05$); GA = ozonized water group, GCL = sodium chloride group, GO = ozonized oil group, GAL = allantoin group.

Table 4. Mean percentage of type I and III collagen fibers in relation to total collagen in wounds at 8, 15, and 22 days after induction in groups GA, GCL, GO, and GAL

Period	Collagen fiber	Group			
		GA	GCL	GO	GAL
8 days	I	77.33[a]	75.93[a]	50.10[a]	54.83[a]
	III	22.67[A]	24.07[A]	49.90[A]	45.17[A]
15 days	I	93.46[a]	88.83[ab]	84.60[ab]	82.10[b]
	III	6.54[B]	11.17[AB]	15.40[AB]	17.90[A]
22 days	I	84.59[b]	86.49[ab]	90.20[a]	84.41[b]
	III	15.41[A]	13.51[AB]	9.80[B]	15.59[A]

[a,b,ab] Within the same period, groups followed by the same lowercase letter have statistically equal means (considering type I collagen fibers); [A,B,AB] within the same period, groups followed by the same uppercase letter have statistically equeal means (considering type III collagen fibers); For these comparisons, Duncan test was used ($p<0.05$); GA = ozonized water group, GCL = sodium chloride group, GO = ozonized oil group, GAL = allantoin group.

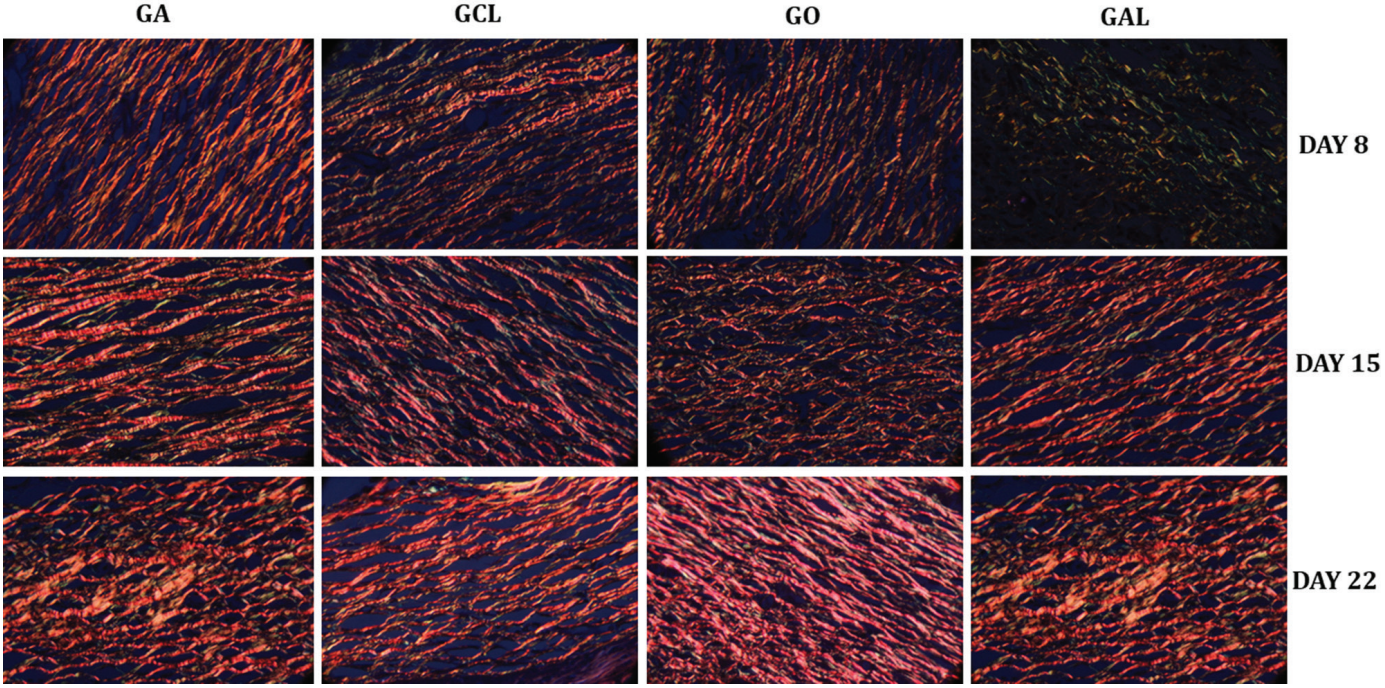


Fig.3. Photomicrographs of rat skin showing type I and III collagen fibers at 8, 15, and 22 days after induction in groups ozonized water (GA), 0.9% sodium chloride (GCL), ozonized oil (GO), and 0.2% allantoin cream (GAL). Picrosirius red, obj.40x.

of ozone in the water are ideal for helping the proliferation and remodeling phases of skin wounds.

Wounds that received ozonized oil with a peroxide index of 418.48mEq/kg showed an average wound retraction of 69.84% at 8 days of treatment compared to its original size, a value below that presented by GA. Other studies have shown that wounds treated with ozonized oil had a higher retraction in the first days of treatment compared to those receiving oil only, and the retraction process was complete from nine days after injury (Kim et al. 2009, Xiao et al. 2017). These findings have been confirmed by studies using ozonized oil with peroxide concentrations between 700 and 1631mEq/kg, which also reported higher wound retraction rates (Valacchi et al. 2011, Pai et al. 2014). In contrast, one of these studies (Pai et al. 2014) found that oil at 500mEq/kg of peroxide does not change the wound retraction rate compared to pure oil and commercial healing cream, while the other study (Valacchi et al. 2011) reports that a concentration of 3170mEq/kg of peroxides also does not promote positive effects on wound retraction. In this context, the oil peroxide concentration used in this research justifies the performance of the GO group below expectations regarding the aspect of wound retraction.

In addition, there is another reference for wounds treated with ozonized oil, showing no difference in retraction compared to control groups. However, peroxide concentration was not

Table 5. Means, medians and ranks sums, respectively, of VEGF immunostaining in the skin wound of rats at 3, 8, 15, and 22 days after induction in groups GA, GCL, GO, and GAL

Group	Period			
	3 days	8 days	15 days	22 days
GA	1.33/1/6.50[a]	2.66/3/6.67[ab]	1.00/1/3.00[b]	0.66/1/5.33[b]
GCL	1.00/1/4.50[a]	2.00/2/3.00[b]	1.66/2/6.66[ab]	0.33/0/3.66[b]
GO	1.66/2/8.50[a]	3.00/3/8.50[a]	2.33/2 /9.66[a]	1.66/2/10.0[a]
GAL	1.33/1/6.50[a]	3.00/3/7.83[ab]	1.66/2/6.66[ab]	1.00/1/7.00[ab]

[a,b,ab] Within the same period, a value is only statistically different from another if there are no equal letters. Thus, a value identified as "a" is different from a value identified as "b", but not from a value identified as "ab", by the Kruskal-Wallis test ($p<0.05$); GA = ozonized water group, GCL = sodium chloride group, GO = ozonized oil group, GAL = allantoin group.

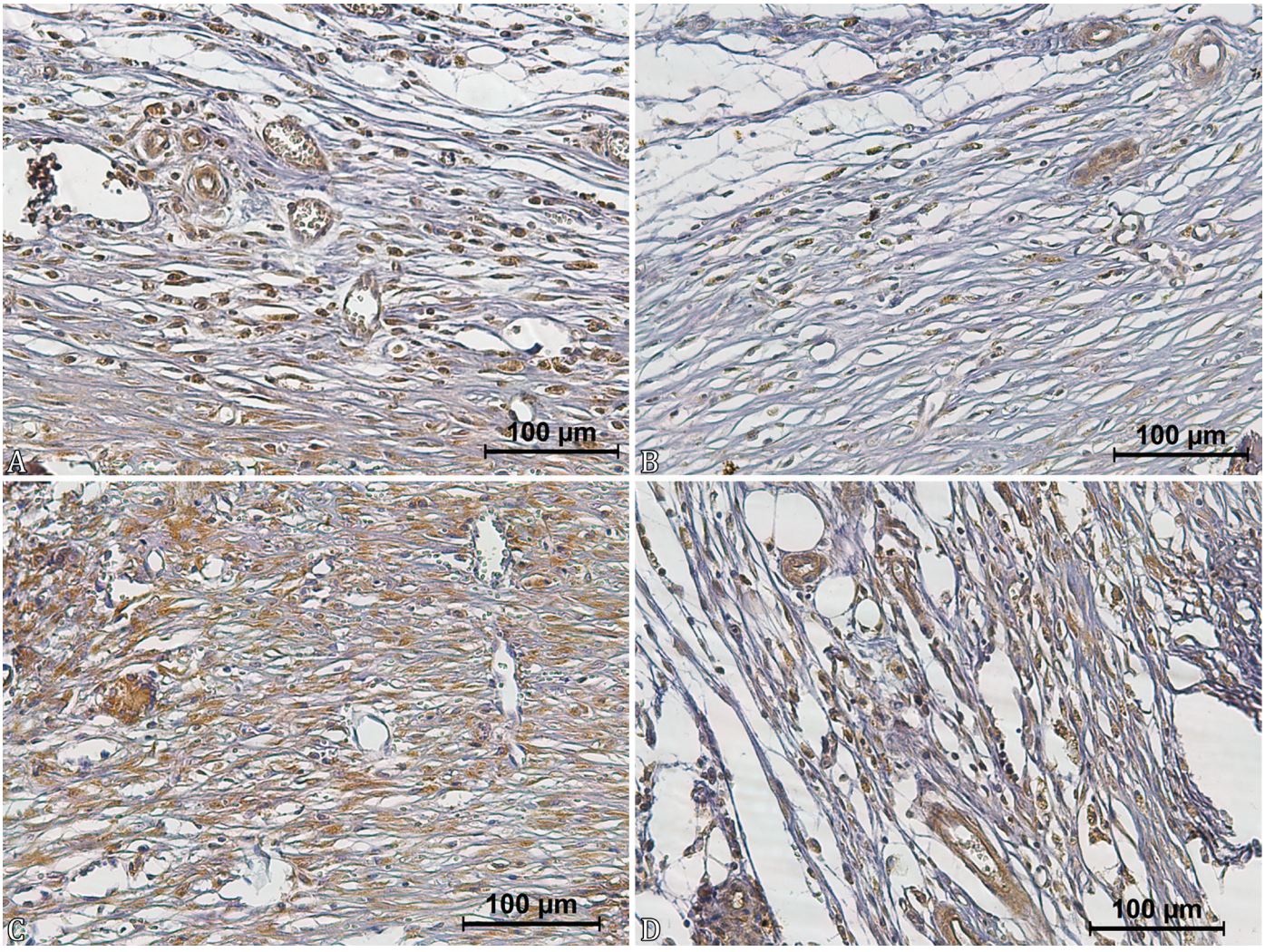


Fig.4. Photomicrographs of the skin of rats from different groups at 8 days after wound induction. (**A**) Group ozonized water (GA) showing moderate anti-VEGF immunostaining. IHC, obj.40x. (**B**) Group 0.9% sodium chloride (GCL) showing discreet anti-VEGF immunostaining. IHC, obj.40x. (**C**) Group ozonized oil (GO) showing marked anti-VEGF immunostaining. IHC, obj.40x. (**D**) Group 0.2% allantoin cream (GAL) showing moderate anti-VEGF immunostaining. IHC, obj.40x.

reported (Sakazaki et al. 2007). Thus, considering the results of this and other studies, knowing the peroxide concentration in the oil is of fundamental importance to justify possible beneficial effects or their absence. Considering the ozone action principle, an oil with low peroxide concentration may not produce sufficient oxidative reactions to activate organic biochemical pathways and trigger medicinal effects, and, on the contrary, high peroxide concentration may exceed the metabolic capacity of the organic antioxidant system, limiting the beneficial action of ozone. This hypothesis is based on a temporary situation of oxidative stress, with no deleterious effects, but enough to activate therapeutic responses in the body, which is considered the principle of ozone therapy (Bocci 2005). Therefore, considering the concentration of peroxides in the ozonized oil, there is a lack of standardization regarding the ideal concentration range to obtain the beneficial effects in each phase of wound healing.

Although no statistical differences were observed between groups for the analysis of the type of ECM and fibroblast population, treatments using ozonized solutions may help these variables. For example, an in vitro assay showed marked fibroblastic migration under the action of saline solution with ozone at a concentration of 8mcg/mL (Borges et al. 2017). On the other hand, Xiao et al. (2017) pointed out that ozonized oil increases the fibroblast migration process and increases its activation during the first five days after lesion, corroborating the results of Kim et al. (2009), who found more fibroblasts and collagen fibers on Masson's trichrome staining at 7 days after wound induction. Also, Pai et al. (2014) identified higher tensile strength in wounds treated with ozonized oil, which is attributed to the highest levels of collagenization observed in these wounds.

In this sense, the analysis of picrosirius red staining showed that wounds of groups GA and GO exhibited higher total collagen deposition at 15 and 22 days, respectively. When analyzing the type of collagen involved, wounds of these same groups presented more type I collagen (mature) on these days. The good performance regarding fibroblast population and ECM deposition in ozone studies is explained by an increase in growth factors in tissues exposed to this molecule (Kim et al. 2009), such as PDGF, which promotes the migration and proliferation of fibroblasts and their differentiation into myofibroblasts. In addition, there is the transforming growth factor-beta (TGF-β), also a promoter of fibroblast proliferation and extracellular matrix production (Abbas & Lichtmann 2013, DiPietro 2013, Kwan & Tredget 2017). The dose of ozone supplied, either by ozonized water or by oil, interferes with the number of biochemical components, which may generate positive, placebo, or even harmful effects.

Wounds of the GO group showed, at all evaluated times, a higher blood vessel count when compared to wounds of other groups. This finding was more pronounced at 3 and 8 days when wounds of GO group had a higher average of neovascularization ($p<0.05$) than that found for GCL, and at 15 days when GO wounds exhibited more neovessels than GCL and GAL. Groups GCL, GO, and GAL had a higher vascular count ($p<0.05$) when compared to GA at 22 days. Similarly, Valacchi et al. (2011) observed a higher angiogenesis index at 3 days after wound induction in groups treated with ozonized oil with a peroxide concentration from 949 to 3170mEq/kg. However, the highest degree of neovascularization was found in the group treated with oil at 1631mEq/kg of peroxide at 7 and 14 days after wound induction and in the group treated with 3170mEq/kg on day 14. Therefore, by inferring that the concentration of peroxide in the oil used in this study (418.48mEq/kg) had a positive impact on the increase of neovascularization in wounds compared to the other treatments, the therapeutic range of ozonized oil is still higher than that found in the literature is demonstrated, at least in relation to stimulating angiogenesis. In addition, the GA group was statistically similar to the treatment with ozonized oil at 3, 8, and 15 days, except on day 22, when it had a smaller amount ($p<0.05$) when compared to other groups. Considering that at this moment of tissue repair there is a remodeling of scar tissue, this group was more advanced in the maturation and remodeling phases when compared to the others.

As with other growth factors, ozone therapy induces higher release of factors acting on neovascularization (Kim et al. 2009), such as PDGF and TGF-β, stimulating VEGF production, which is responsible for promoting migration and proliferation of endothelial cells, as well as vasodilation (Abbas & Lichtmann 2013, DiPietro 2013, Kwan & Tredget 2017). The immunohistochemical analysis of VEGF performed in this study is in accordance with this information, as wounds of GO group presented a higher intensity of this growth factor on all analyzed days. An experimental model (Krkl et al. 2016) using ozonized oil showed that the application of this therapy in skin flaps improved wound neovascularization in relation to the group treated with oil and the control group also due to the higher VEGF immunostaining in animals exposed to ozonized oil.

Ozone therapy has great potential in the context of skin wound repair. However, the technique remains controversial regarding its reliability among health professionals. It may be the translation of inconsistent results into empirical ozone treatments, reflecting the need to establish scientific criteria for treating wounds with ozonized solutions. In this sense, researches involving ozone therapy should be strictly controlled regarding the route of administration, vehicle, and concentration of ozone, which generate data that allow establishing protocols.

CONCLUSION

Ozonized water and oil at the tested concentrations favor the tissue repair process in experimentally induced skin wounds in rats. Ozonized water at 4.5 mcg / mL potentiates wound retraction and accelerates the onset of healing in the maturation and remodeling phase, while ozonized oil at a concentration of 418.48 mEq / kg of peroxides promotes higher neovascularization during tissue repair and higher deposition of type I collagen from the third week of treatment.

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Isolation and characterization of the aerobic bacterial microbiota of the esophagus and its probable association with obstructive caseous lesions in green turtles (*Chelonia mydas*)[1]

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ABSTRACT.- Bindaco A.L.S., Calais Júnior A., Almeida I.C., Liesner C.O., Ferreira M.R., Donatele D.M., Carvalho G.D. & Nunes L.C. 2020. **Isolation and characterization of the aerobic bacterial microbiota of the esophagus and its probable association with obstructive caseous lesions in green turtles (*Chelonia mydas*).** *Pesquisa Veterinária Brasileira 40(11):922-932.* Centro de Ciências Agrárias e Engenharias, Universidade Federal do Espírito Santo, Alto Universitário, Alegre, ES 29500-000, Brazil. E-mail: louisianecn@gmail.com

Caseous lesions in the esophagus of green turtles (*Chelonia mydas*) from the coast of Brazil have been described as obstructive lesions and can lead to the death of these animals. However, their etiology remains unclear. The aim of this study was to isolate and characterize the aerobic bacterial microbiota of the esophagus of green turtles (*C. mydas*) from the Brazilian coast and to verify its possible participation in the etiology of caseous lesions. For this, 42 animals were used, 33 alive and healthy and 9 naturally dead that had esophageal lesions confirmed by necropsy, from Anchieta and Piúma beaches, Espírito Santo. Microbiological tests and morphological evaluation of the esophagus were performed. We isolated 14 different bacterial agents from healthy animal samples, with the prevalence of *Pseudomonas aeruginosa* being (36.36%), *Staphylococcus aureus* (33.33%), *Aeromonas hydrophila* (27.27%), and *Vibrio alginolyticus* (24.24%). In dead animals, only three distinct agents were isolated: *S. aureus* (50.00%), *A. hydrophila* (25.00%), and *V. alginolyticus* (25.00%). Morphological evaluation revealed a predominance of the lesions at the gastroesophageal junction, with multifocal-to-coalescent distribution, discrete intensity, and absence of obstruction. Ulcerations and caseous exudates, inflammatory infiltrates, parasitic eggs, and giant foreign body cells were also observed as well as bacterial lumps and glandular alterations, such as necrosis, adenitis, and fragments of adult parasites. There was a positive correlation between bacterial lumps and microbiological culture and a negative correlation between bacterial lumps and microbiological culture with parasites. Thus, it was noted that the esophageal aerobic microbiota of *C. mydas* was predominantly composed of Gram-negative bacteria such as *P. aeruginosa, A. hydrophila*, and *V. alginolyticus*, in addition to several enterobacteria and Gram-positive bacteria, such as *S. aureus*. These agents are opportunists and may be involved in the etiology of caseous esophagitis in association with other pathogens as co-factors working in association or, even in a secondary way.

INDEX TERMS: Aerobic bacteria, esophagus, obstructive caseous lesions, green turtles, *Chelonia mydas*, microorganism; esophageal microbiota, sea turtles, wildlife animals.

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RESUMO.- [Isolamento e caracterização da microbiota bacteriana aeróbia do esôfago e sua provável associação com lesões caseosas obstrutivas em tartarugas-verdes (*Chelonia mydas*)]. A ocorrência de lesão caseosa no esôfago de tartarugas-verdes (*Chelonia mydas*) da costa do Brasil tem sido descrita como de caráter obstrutivo e pode causar a morte dos animais. No entanto, sua etiologia permanece pouco esclarecida. Objetivou-se isolar e caracterizar a microbiota aeróbica esofágica das tartarugas-verdes (*C. mydas*) da costa

brasileira e verificar sua possível participação na etiologia das lesões caseosas. Foram utilizados 42 animais, 33 vivos e hígidos e nove mortos naturalmente que apresentavam lesão esofágica confirmada pela necropsia, provenientes de Anchieta e Piúma, Espírito Santo, nos quais foram feitos testes microbiológicos e avaliação morfológica do esôfago. Foram isolados 14 agentes bacterianos diferentes nas amostras de animais saudáveis, com prevalência de *Pseudomonas aeruginosa* (36,36%), *Staphylococcus aureus* (33,33%), *Aeromonas hydrophila* (27,27%) e *Vibrio alginolyticus* (24,24%). Nos animais mortos, foram isolados apenas três agentes distintos: *S. aureus* (50,00%), *A. hydrophila* (25,00%) e *V. alginolyticus* (25,00%). A avaliação morfológica revelou predominância da lesão em junção gastroesofágica, com distribuição multifocal a coalescente, intensidade discreta e ausência de obstrução. Observou-se ainda ulceração e exsudato caseoso, infiltrado inflamatório, ovos de parasitos e células gigantes do tipo corpo estranho, além de grumos bacterianos e de alterações glandulares, como necrose, adenite e fragmentos de parasitos adultos. Houve correlação positiva dos grumos bacterianos com cultivo microbiológico e negativa dos grumos bacterianos e cultivo microbiológico com parasitos. Assim, nota-se que a microbiota esofágica aeróbica de *C. mydas* é constituída predominantemente por bactérias Gram-negativas como *P. aeruginosa*, *A. hydrophila* e V. *alginolyticus*, além de diversas enterobatérias e por Gram-positivas, como *S. aureus*. Esses agentes são oportunistas e podem estar envolvidos na etiologia da esofagite caseosa em associação a outros patógenos como co-fatores agindo em associação, ou mesmo, por via de infecção secundária.

TERMOS DE INDEXAÇÃO: Bactéria aeróbica, esôfago, lesão caseosa obstrutiva, tartaruga-verde, *Chelonia mydas*, microrganismo, microbiota esofágica, tartarugas marinhas, animais silvestres.

INTRODUCTION

Currently, the green turtle (*Chelonia mydas*) is considered by the International Union for Conservation of Nature (IUCN) as a "Vulnerable" (VU) species in Brazil and an "Endangered" (EN) species on a global scale (Seminoff 2004). Although anthropogenic action is responsible for the high mortality rate of *C. mydas* from the Brazilian coast (Gagliardi et al. 2018), viral, parasitic, fungal, and bacterial diseases also represent important causes of death for these turtles (Glazebrook& Campbell 1990, Dominicano et al. 2017).

Several studies have suggested the involvement of the microbiota in the inflammatory lesions of different tissues in green turtles (Orós et al. 2004). In reptiles, bacterial infection can cause caseous inflammation, characterized macroscopically by a yellowish-white and friable material, and microscopically by the exudation of degranulated and degenerated heterophiles (Montali 1988).

In this context, the common occurrence of caseous esophagitis in green turtles is reported to be associated with trematode parasites and Gram-positive cocci (Santoro et al. 2007b, Calais Júnior 2015, Ribeiro et al. 2017, Jerdy et al. 2019). Santoro et al. (2007b) and Jerdy et al. (2019) proposed that bacteria have a probable origin from esophageal microbiota, while Calais Júnior (2015) suggested that they are more relevant in the development of the inflammatory response and formation of the caseous lesion; however, the genus and/or bacterial species found in these lesions were not informative for the authors.

Although the etiopathogenesis of caseous esophagitis has not been completely understood, Calais Júnior (2015) found a high prevalence (82.22%) of the disease in the animals evaluated on the Espírito Santo coast. The same author observed that caseous exudates generated by the inflammatory processes can sometimes completely obstruct the esophageal lumen. Histologically, ulceration of the mucosa is associated with the accumulation of heterophiles (caseous), parasitic adenitis, inflammation in the lamina propria, colonies of Gram-positive bacteria, and fibrous connective tissue (Ribeiro et al. 2017).

Given this scenario, it is observed that caseous esophagitis can become obstructive and cause a decrease in food intake, cachexia, and death of animals, which can contribute to the reduction of the species population. In addition, in the literature consulted, there are no data on the esophageal microbiota of *C. mydas*, as well as the types of bacterial agents found in the inflammatory processes of the esophagus. Thus, the aim of this study was to isolate and characterizate of the aerobic bacterial microbiota of the esophagus of *C. mydas* and to demonstrate its probable involvement in the etiology of caseous esophagitis. We also performed a macro and microscopic morphological evaluation of the affected esophagus, in order to contribute to a better understanding of the disease's etiology and pathophysiology.

MATERIALS AND METHODS

Free-living turtles, alive and dead were used to obtain microbiological samples. The live animals were used to compare the microbiota and initially had the body score assessment, including only healthy individuals. The dead animals were used for the microbiological tests (postmortem time of less than 6 hours) and morphological tests (postmortem time of a maximum of 12 hours). Live and dead animals were captured by members of the Instituto de Pesquisa e Conservação Marinha, situated in Anchieta, Espírito Santo. Some corpses came from the research project "Aspectos fisiológicos e patológicos de tartarugas marinhas no litoral sul capixaba" (protocol SISBIO No. 75593-1), coordinated by Professor and Doctor Gabriel Domingos Carvalho, from "Instituto Federal de Educação, Ciência e Tecnologia do Espírito Santo" (IFES), Piúma campus.

This research protocol has an authorization for activities with scientific purpose SISBIO nº 39329-4 and was approved by the "Comitê de Ética do Uso Animal" from the "Universidade Federal do Espírito Santo" (UFES), protocols 01/2018 and 16/2018.

Weight (kg), curved carapace length (CCL), curved carapace width (CCW), and the location of capture using the global positioning system (GPS) of each animal were obtained. Age was established by the parameters of CCL according to Hirth (1997). The *post mortem* time was assessed based on cadaveric phenomenons and was identified by the IPCMar team. Animals that presented significant cadaveric changes, such as exemple, skin and plaque detachment, enphisema, pseudomelanosis or signs of predation, were not included in the study.

Microbiological tests. In live animals, the collection of biological material was done by physical restraint of the animals, followed by the use of a mouth opener adapted from the model for small ruminants. Subsequently, a sterile swab (15cm) was introduced into the esophagus of the animals and the entire mucosa of the organ was exfoliated (Fig.1A). The adapted mouth opener was able to open the maximum of the rhamphoteca (turtle's beak) in order to guarantee the introduction of the swab as caudally as possible into

the esophagus. After removal, the swabs were individually packed in Stuart transport medium and transported under refrigeration (from 6 to 10°C) to the "Laboratório de Inspeção de Produtos de Origem Animal" of the "Hospital Veterinário", "Centro de Ciências Agrárias e Engenharias" (HOVET-CCAE) of UFES (Fig.1B). In dead animals, the collection of microbiological material was made by confirmation of the caseous lesion in the esophagus (suggestive of caseous esophagitis) evaluated during necroscopic exams. Then, a sterile swab was introduced into the injured area, which was immediately stored in Stuart transport medium and sent under refrigeration to the same laboratory previously mentioned. In these specimens, microbiological culture was recommended only for the caseous area in order to isolate the bacterial agent present in the lesions. All swabs were inoculated in selective culture media for Gram-negative bacteria, such as MacConckey Agar (Enterobacteria), *Pseudomonas aeromonas* Selective Agar (Glutamate Starch Phenol Red Agar - GSP Agar) *(Aeromonas* spp./*Pseudomonas* spp.), and thiosulfate citrate bile salt sucrose (TCBS) agar (*Vibrio* spp.). To identify Gram-positive bacteria, brain heart infusion (BHI) agar was used. All media were incubated in a bacteriological oven at 37°C for 24 to 48 hours. Colonies growing in the BHI medium and on the selective agar (GSP, MacConckey, and TCBS) were subjected to Gram staining for classification as Gram-positive or negative. For Gram-positive bacteria, biochemical tests were performed according to Anvisa (2013) to prove the genus and consequent species of the agents. For Gram-negative bacteria, colony color and texture were also evaluated and biochemical tests were performed according to Anvisa (2013), to identify *Aeromonas, Pseudomonas*, and Enterobacteriaceae species and according to the Ministério da Saúde (2010) for detection of *Vibrio* specimens.

Macro and microscopic morphological tests. The corpses were necropsied at the IFES-Piúma or at the Setor de Patologia Animal of the HOVET-CCAE-UFES. The necropsy was performed according to the technique established by Wolke & George (1981), with complete external examination and analysis of all organs. During the necroscopic examination, the esophagus that presented caseous exudates adhering to the mucosa was collected individually, fixed in 10% formalin, and sent to the "Laboratório de Patologia Animal" of the HOVET-CCAE-UFES for macro and microscopic evaluations. Initially, each organ was photo-documented, measured (cm), and in the injured areas, the location, distribution, intensity, and size of the case were described, in addition to the esophageal lumen obstruction status according to Calais Júnior (2015). Four esophageal regions were considered: proximal region (P), medial region (M), distal region (D), and gastroesophageal junction (GJ). The tissues were processed for routine histological examination, with impregnation and inclusion in paraffin, and finally cut with a manual rotating microtome into sections of 3-µm thickness. The resulting slides were stained with hematoxylin and eosin (HE), and GJ samples were also stained using the Periodic Acid-Schiff (PAS) technique. The samples were then analyzed using a light microscope. The variables evaluated in the microscopic description are shown in Figure 2. The data were evaluated by descriptive statistics, based on percentages (BioEstat 5.3) and for the variables of the caseous, bacterial lumps, parasites, and microbiological culture, the Spearman correlation test was used at the level of 5% probability.

RESULTS

In total, 42 specimens of *Chelonia mydas* were used, 33 of which were live, free-living; 84.85% (28/33) were obtained from Praia do Além (20°48'03.3"S 40°34'57.0"W) and 15.15% (5/33) from Praia Marvila (20°48'47.4" S, 40°39'58.8" W), both in the municipality of Anchieta/ES and nine dead animals, 77.78% (7/9) from Piúma (20°50'31.9" S, 40°43'46.0" W) and 22.22% (2/9) from Anchieta (20°48'03.3" S, 40°34'57.0" W).

The data on CCL, CCW, and weight of the 42 animals evaluated are shown in Table 1. Regarding the age of the

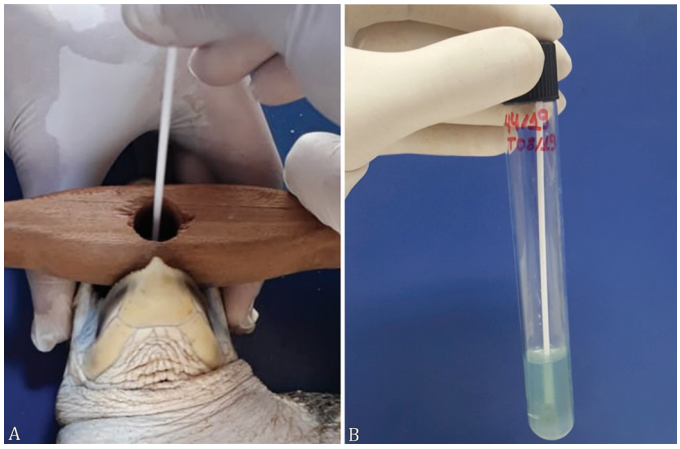


Fig.1. (**A**) Physical restraint and collection for microbiological samples in green turtles (*Chelonia mydas*) using a sterile swab and an adapted mouth opener. (**B**) Swab in the Stuart transport medium and identified.

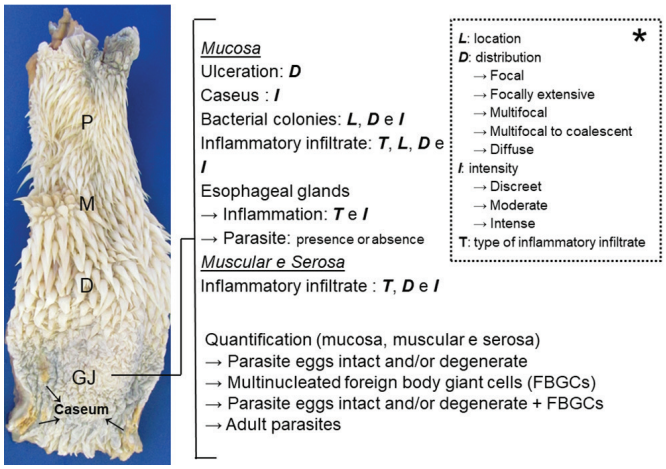


Fig.2. Variables used for the microscopic evaluation of the esophagus with caseous esophagitis of green turtles (*Chelonia mydas*) from the Brazilian coast from January to October 2019. The variables of location, distribution, intensity and type of inflammatory infiltrate were made according to Ackermann (2013) (asterisk).

Table 1. Average values and standard deviation of the curved carapace length (CCL), curved carapace width (CCW), and weight according to the age of the live and dead green turtles (*Chelonia mydas*) from the Brazilian coast from January to October 2019

C. mydas	N	Age	CCL (cm)	CCW (cm)	Weigth (kg)
Alive	26/42	Juvenile	34.89 (± 2.63)	32.37 (± 3.10)	4.76 (± 1.07)
	7/42	Sub-adult	43.04 (± 4.22)	39.97 (± 4.68)	9.19 (± 3.00)
Dead	9/42	Juvenile	34.39 (± 1.82)	31.73 (± 2.13)	4.59 (± 1.06)

animals, 83.33% (35/42) were juvenile, and 16.67% (7/42) were sub-adults. Among the dead animals in which it was possible to make sexual differentiation, 77.78% (7/9) male and 22.22% (2/9) females were analyzed.

In five corpses of *C. mydas*, no microbiological material was obtained from the lesion due to the postmortem time was greater than 6 hours, with only the morphological exams being performed on these specimens. The results microbiological culture showed the agents described in Table 2 and the biochemical tests used to identify the genus and species are shown in Table 3 and 4.

Of the nine dead animals with caseous esophagitis evaluated macroscopically, the mean esophagus length was 14.61cm (± 1.43). It was observed that 100% (9/9) of the lesions were located at the GJ, with 44.45% (4/9) being focal caseous exudates, 33.33% (3/9) being multifocal-to-coalescent, and 22.22% (2/9) being multifocal. The intensity was discrete in 77.78% (7/9) and moderate in 22.22% (2/9) of the cases. The size of the caseous varied from 0.1cm to 2.5cm, and 88.89% (8/9) of the animals were without esophageal obstruction, while 11.11% (1/7) had partial lumen obstruction (Fig.3).

Microscopically, focal ulceration was observed in 44.45% (4/9) of the epithelia, focally extensive in 33.33% (3/9) and multifocal-to-coalescent in 22.22% (2/9). There was also the presence of discrete caseous in 55.55% (5/9) and moderate caseous in 44.45% (4/9) of the ulcerated areas. In the lamina propria, mononuclear inflammatory infiltrates were observed with multifocal-to-coalescent distribution in 44.45% (4/9), multifocal in 33.33% (3/9), and focal in 22.22% (2/9) of the samples. The intensity of the inflammatory infiltrate was discrete at 55.55% (5/9) and moderate at 44.45% (4/9) (Fig.4).

The occurrence of bacterial lumps was found in 66.67% (6/9) of the lesions, concomitant with caseous and/or ulceration. The distribution was multifocal in 50.00% (3/6), multifocal-to-coalescent in 33.33% (2/6), and focal in 16.67% (1/6) of the samples, with 66.67% (4/6) and 33.33% (2/6) of the colonies having discrete and moderate intensities, respectively (Fig.4).

In all samples, golden brown parasite eggs (intact and degenerate) and/or multinucleated giant cells of the foreign body type were observed in the epithelium and/or lamina propria of the mucosa. There was a predominance of these structures in the lamina propria (Fig.4).

Microscopic analysis also revealed that 77.78% (7/9) of the esophageal glands had mononuclear inflammation and necrosis and thatin 57.14% (4/7) of the cases, the presence of adult parasite fragments was also found. In 22.22% (2/9) of the glands, there were no changes, while an adult parasite fragment was observed to be attached to the caseousin only 14.28% (1/9) of the samples (Fig.5).

Regarding the occurrence of changes in the other layers of the esophagus, only 22.22% (2/9) of the sampleshad an inflammatory infiltrate in the smooth musculature, discrete multifocal in both cases. No changes were observed in the serosa.

Spearman's correlation demonstrated that there was no correlation between the caseous, bacterial lumps, parasites, and microbiological culture. However, there was a positive correlation between bacterial lumps and microbiological culture (ρ= 0.16) and a negative correlation between bacterial lumps and parasites (ρ = -0.16) and between parasites and microbiological culture (ρ = -0.1).

DISCUSSION

The biometrics of the animals used in this study were similar to those of Forattini (2011), who also evaluated 31 animals from the Espírito Santo coast and found body scores from 2.6 to 20.2kg (6.8±2.9kg), a CCL value ranging from 27.6 to 53.4cm (38.4±5.09cm) and CCW from 26.1 to 49.7cm (34.6±4.6cm). This shows the high occurrence of juvenile *Chelonia mydas* on the coast of Espírito Santo. The predominance of individuals in the juvenile phase was also observed by Bezerra et al. (2012), as it is at this stage that the green turtle acquires herbivorous

Table 2. Aerobic bacteria isolated from the esophagus of the free-living green turtle's (*Chelonia mydas)* on the Brazilian coast, from June to October 2019

Bacteria isolated	Family and/or genus	Specie	*C. mydas* alive		*C. mydas* dead	
			Esophageal mucosa[a]	%[b]	Caseous lesion[c]	%[d]
Gram-negative	*Aeromonas*	*A. hydrophila*	9/33	27.27	1/4	25.00
	Enterobacteriaceae	*Citrobacter freundii*	2/33	6.06	0/4	00.00
		Enterobacter cloacae	4/33	12.12	0/4	00.00
		Escheria coli	6/33	18.18	0/4	00.00
		Proteus vulgaris	3/33	9.09	0/4	00.00
	Pseudomonas	*P. aeruginosa*	12/33	36.36	0/4	00.00
	Vibrio	*V. alginolyticus*	8/33	24.24	1/4	25.00
		V. fluvialis	4/33	12.12	0/4	00.00
		V. metschnikovii	3/33	9.09	0/4	00.00
		V. parahaemolyticus	5/33	15.15	0/4	00.00
Gram-positive	*Staphylococcus*	*S. aureus*	11/33	33.33	2/4	50.00
		S. epidermidis	3/33	9.09	0/4	00.00
		S. intermedius	2/33	6.06	0/4	00.00
	Bacillus	*Bacillus* spp.	2/33	6.06	0/4	00.00

[a] Number of bacteria isolated in relation to the total exfoliated esophageal mucosa in live animals, [b] percentage of bacteria isolated in relation to the total exfoliated esophageal mucosa in live animals, [c] number of isolated bacteria in relation to the total exfoliated caseous lesions in dead animals, [d] number of isolated bacteria in relation to the total exfoliated caseous lesions in dead animals.

Table 3. Isolation and characterization of the Gram-negative aerobic baterial microbiota from the esophagus of the free-living green turtle's (*Chelonia mydas*) on the Brazilian coast by biochemical tests, from June to October 2019

Tests	*Aeromonas*	*Pseudomonas*	*Vibrio*				Enterobacteria			
	A. hypdophila	*P. aueruginosa*	*V. alginolyticus*	*V. fluvialis*	*V. metschnikovii*	*V. parahaemolyticus*	*Citrobacter freundii*	*Enterobacter cloacae*	*Escheria coli*	*Proteus vulgaris*
Growth and color of the colonies in *Pseudomonas Aeromonas* Selective Agar Base (GSP Agar)	Excellent Yellow colonies	Excellent Red-violet colonies	X	X	X	X	X	X	X	X
Growth and color of the colonies in Thiosulfate-Citrate-Bile-Sucrose Agar (TCBS Agar)	X	X	Excellent Yellow colonies	Excellent Yellow colonies	Excellent Yellow colonies	Excellent Green colonies	X	X	X	X
Growth and color of the colonies in MacConkey Agar	X	X	X	X	X	X	Excellent Pink colonies	Excellent Pink colonies	Excellent Pink-red colonies	Excellent Colorless colonies
Form	Bacillus	Bacillus	Vibrion	Vibrion	Vibrion	Vibrion	Bacillus	Bacillus	Bacillus	Bacillus
Gram	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Enzymes										
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	-	+	-	-	-	-
DNase	+	-	+	+	+	+	-	-	-	+
Urease	-	-	-	+	-	+	-	+	-	+
Arginina dihidrolase	+	+	-	+	+	-	+	+	-	-
Lisina descarboxilase	+	-	+	-	-	+	-	-	+	-
Ornitina descarboxilase	-	-	+	-	-	+	-	+	+	-
Fenilalanina-desaminase	X	X	X	X	X	X	-	-	-	+
H_2S Production Test	-	-	-	-	-	-	+	-	-	+
Indole test	+	-	+	-	-	+	-	-	+	+
Motility	+	+	+	+	+	+	+	+	+	+
Citrate Utilization Test	-	+	X	X	X	X	+	+	-	-
Nitrate reductase test	+	+	+	+	-	+	+	+	+	+
Esculin hydrolysis	+	-	X	X	X	X	-	-	-	-
VP (Voges-Proskauer)	X	X	+	-	+	-	X	X	X	X
Gas production from glucose	+	+	+	-	-	+	+	+	+	+
Fermentation										
Glucose	+	+	+	-	-	+	+	+	+	+
Lactose	-	+	+	-	+	-	+	+	+	-
Sucrose	+	+	+	+	+	-	+	+	+	+
Mannitol	+	-	+	+	+	+	+	+	+	-
Arabinose	+	+	X	X	X	X	+	+	+	-
M*yo-inositol*	X	X	-	-	-	-	X	X	X	X
Growth in										
NaCl 3%	X	X	+	+	+	+	X	X	X	X
NaCL 6%	X	X	+	+	+	+	X	X	X	X

+ Positive result, - negative result, X = unrealized.

feeding habits and starts to inhabit coastal waters where marine vegetation is abundant. Thus, they are commonly observed in regions close to the coast (Bjorndal 1985).

The data from the present study revealed that the esophageal microbiota of *C. mydas* is constituted by a greater diversity of Gram-negative bacteria and by a lesser proportion of Gram-positive bacteria. The most prevalent bacteria in the esophagus of live animals were *Pseudomonas aeruginosa*, followed by *Staphylococcus aureus*, *Aeromonas hydrophila* and *Vibrio alginolyticus*. Similarly, Santoro et al. (2006) identified 21 species of Gram-negative and 8 Gram-positive agents in the nasal and cloacal regions of female green turtles in nidification, with the identification of *A. hydrophila*, *Escherichia coli*, *P. aeruginosa*, *S. aureus,* and *V. alginolyticus*. On the other hand, the same microorganisms were isolated by Orós et al. (2005) in cases of ulcerative and/or fibrinous esophagitis in *C. mydas* and free-living *Caretta caretta*, attributing the secondary infection of these agents to the trauma caused by fishing artifacts in the esophageal mucosa.

In this context, microbiological examinations of caseous esophagitis revealed bacteria that were also found in the esophagus of healthy *C. mydas*, with predominance of *S. aureus*, *A. hydrophila*, and *V. alginolyticus.* Thus, it is observed that the green turtle, like all reptiles, exhibits opportunistic aerobic bacterial species with pathogenic potential composing the esophageal microbiota, corroborating with Santoro et al. (2006) who revealed the opportunistic character of the isolated microorganisms in healthy *C. mydas* of their study. Also according to the same author, the presence of pathogenic bacteria in the tissues of the turtles is not synonymous with disease, and that in sea turtles the main entry points for microorganisms are injuries, mainly skin, and aspiration of sea water. However, diseases (viral and parasitic) and/or environmental conditions, for example, can affect the animal's immune system, making it more susceptible to infection by opportunistic agents that make up the healthy turtle microbiota. In this study, no traumatic injury by hooks or any other fishing device was found based on the examination of the oral cavity and no clinical signs of weakness or cachexia observed which are usually signs that may be associated with injuries caused by these artifacts, it is believed that bacterial infections in esophagitis can be triggered by several other factors, such as impairment of the immune system secondary to a concomitant underlying disease, for example.

It is worth mentioning that *A. hydrophila, Staphylococcus* spp., and *V. alginolyticus* are opportunistic pathogens commonly isolated from lesions in several organs of free and captive *C. mydas*. In captivity, these agents are commonly related to the development of ulcerative dermatitis and stomatitis, obstructive rhinitis, and bronchopneumonia, with stress and/or trauma as predisposing factors for infection (Glazebrook & Campbell 1990). However, Orós et al. (2005) isolated the same agents in free-living green turtles affected by ulcerative dermatitis, pleuritis and granulomatous pneumonia, necrotizing splenitis, ulcerative esophagitis, granulomatous/necrotizing hepatitis, and myositis. In addition, Orós et al. (2004) detected the *Staphylococcus* genus in 22.58% of bacterial diseases in free-living sea turtles. Thus, it can be suggested that free-living as well as captive green turtles have predisposing factors for the pathogenic action of the microbiota, such as trauma (of anthropic origin), pollution, viral diseases, and parasitism.

Table 4. Isolation and characterization of the Gram-positive aerobic baterial microbiota from the esophagus of the free-living green turtle's (*Chelonia mydas*) on the Brazilian coast by biochemical tests, from June to October 2019

Tests	*Staphylococcus*			*Bacillus*
	S. aureus	*S. epidermidis*	*S. intermedias*	*Bacillus* spp.*
Brain Heart Infusion Agar (BHI Agar)	Excellent	Excellent	Excellent	Excellent
Form	Coccus	Coccus	Coccus	Bacillus
Gram	+	+	+	+
Enzymes				
Catalase	+	+	+	+
Coagulase	+	-	-	-
Oxidase	-	-	-	-
DNAse	+	-	+	-
Urease	+	+	+	-
Arginina dihidrolase	-	+	-	X
Ornitina descarboxilase	-	-	-	X
H_2S Production Test	-	-	-	-
Motility	-	-	-	+
Indole test	-	-	-	-
Pyrrolidonil arilamidase (PYR)	-	-	+	X
Fermentation				
Glucose	+	-	+	X
Lactose	+	-	+	X
Sucrose	+	+	+	X
Mannitol	+	-	-	X
Novobiocin	Sensitive	Sensitive	Sensitive	X
Polymyxin	Resistant	Resistant	Sensitive	X

+ Positive result, - negative result, X = unrealized.

The occurrence of *Vibrio* species (*V. alginolyticus, V. fluvialis, V. metschnikovii,* and *V. parahaemolyticus*) can be explained by the fact that these microorganisms are considered autochthonous (native) from marine and estuary environments, which inhabit these places due to availability of sodium chloride (NaCl), salt necessary for its growth (Silveira et al. 2016). Reis et al. (2010) detected a positive result of 88.2% for *Vibrio* in the oral, nasal, and cloacal mucosa, and wounds of free-living juvenile *C. mydas*. The genus was also isolated in marine mammals, as observed by Pereira et al. (2007), who identified 17 species of *Vibrio* from lesions in whales, porpoises, dolphins and sea lions from the south and southeast coast of Brazil, with a higher prevalence of *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus* and *V. fluvialis*. Thus, it is believed that because the esophagus is in constant contact with saline water, through feeding and expelling excess water, these agents may favor the esophageal mucosa.

Aeromonas and *Pseudomonas* are present in fresh and salt water and are considered important opportunistic pathogens in reptiles (Troiano 2018), and their presence in the esophagus of *C. mydas* can be explained by the same factors as the occurrence of *Vibrio*. Reis et al. (2010) observed 59.2% of *Aeromonas* spp. in the oral, nasal, and cloacal mucosa, and wounds of juvenile green turtles. In the same context, Santoro et al. (2006) found 25.7% of *P. aeruginosa* and 22.8% of *A. hydrophila* in the nasal cavity and cloaca of adult female *C. mydas*.

Although it occurs naturally in fresh and salt water and estuarine environments, pollution can lead to an increase in *Aeromonas* and affect the distribution of bacteria in the environment (Marcel et al. 2002). *A. hydrophila*, for example, is not native to saline waters, but domestic sewage and tributaries of rivers to the sea lead to the isolation of the agent in the marine ecosystem (Hänninen et al. 1995). In the present study, *P. aeruginosa* was the microorganism with the highest frequency of isolation in the healthy esophagus of *C. mydas* and the explanation for this may be that the agent is a strong indicator of water contamination (Guilherme et al. 2000). The coastal region, where the animals were obtained, suffers from human pressure and occupation, which makes the space subject to anthropic action and the introduction of pollution sources, such as industries, agriculture, and domestic activities, which supply chemicals and organic matter to seas. Sewage drained directly into the sea, for example, contains several impurities, which can transmit diseases, cause intoxication, and even the death of marine flora and fauna (Fernandes 2016).

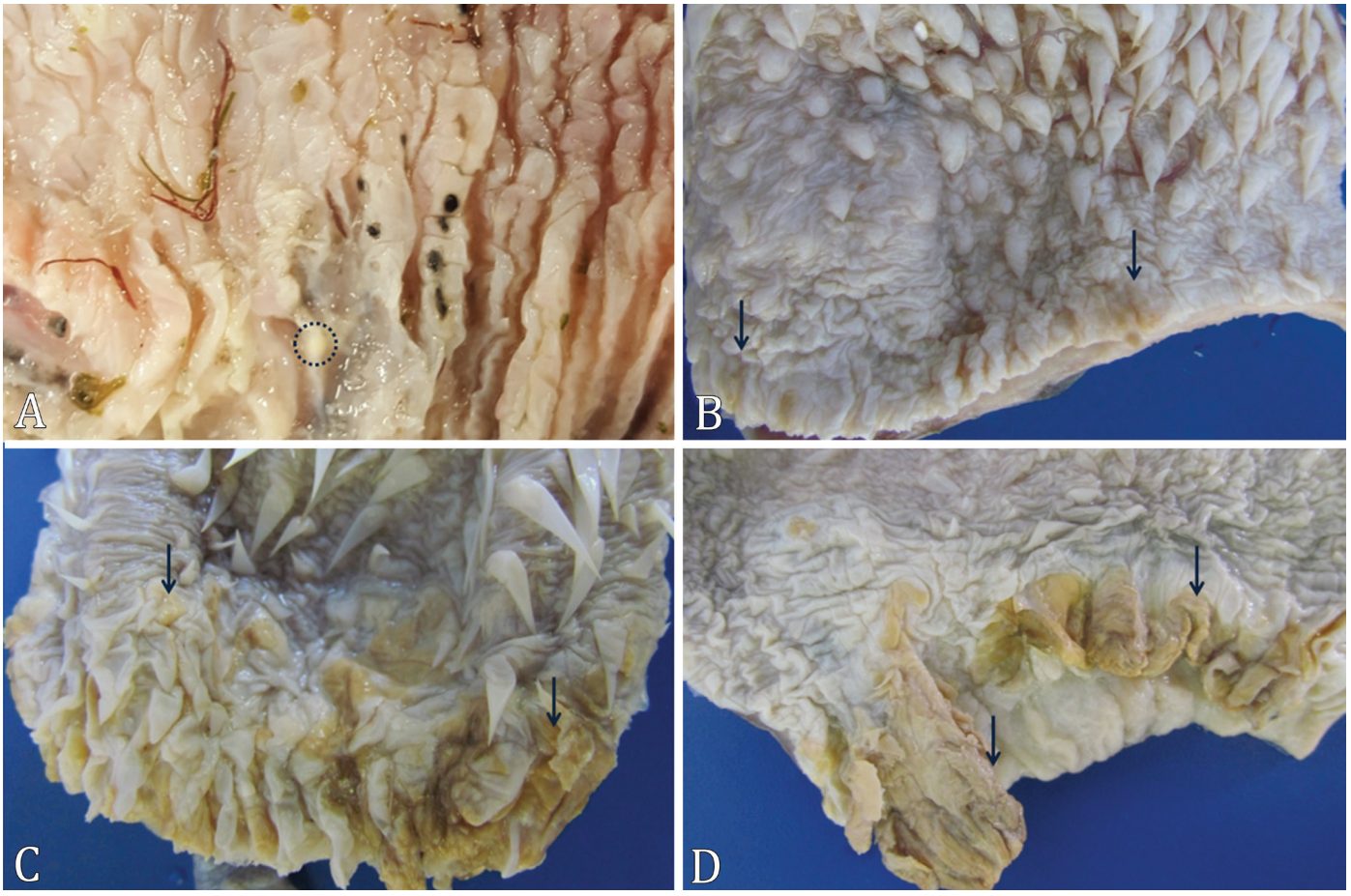


Fig.3. Caseous esophagitis in green turtles (*Chelonia mydas*) found naturally dead on the Brazilian coast from January to October 2019 with evidence of the caseous (dotted circle and arrows). (**A**) Focal distribution and discrete intensity (dotted circle). (**B**) Multifocal distribution and discrete intensity (arrows). (**C**) multifocal-to-coalescent distribution and moderate intensity (arrows). (**D**) multifocal-to-coalescent distribution with discrete partial lumen obstruction (arrows).

Four species of enterobacteria (*Citrobacter freundii, Enterobacter cloacae, Escherichia coli,* and *Proteus vulgaris*) were identified in the present study. The Enterobacteriaceae family includes 46 genera and 293 species and constitutes the largest and most heterogeneous family of Gram-negative bacteria (Moxley 2016), which justifies the diversity of agents of this family observed in the study. Santoro et al. (2006) found 53.1% of enterobacteria in the nasal and cloacal regions of green turtles, with isolation of *E. agglomerans, E. cloacae, E. coli, K. oxytoca, K. pneumoniae,* and *Serratia marcescens*.

Ribeiro et al. (2015) confirmed the predominance of these microorganisms in the gastrointestinal microbiota of jabuti-piranga (*Chelonoidis carbonaria*), with the highest occurrence of *Escherichia coli*, corroborating with the data of the present study. Enterobacteria in general, especially *E. coli*, are more commonly found in the digestive tract, because the conditions in the digestive tract such as nutrients and adequate temperature, are appropriate for their growth (Moxley 2016). In addition, microorganisms of the genus *Escherichia*, *Citrobacter*, *Enterobacter* and *Klebsiella* (coliforms) are indicators of water hygiene, especially *E. coli*, which is considered a strong indicator of faecal contamination (Fialho et al. 2014). With that, it can be reaffirmed that the coastal region where these animals live are subject to anthropic pollution and consequent water contamination.

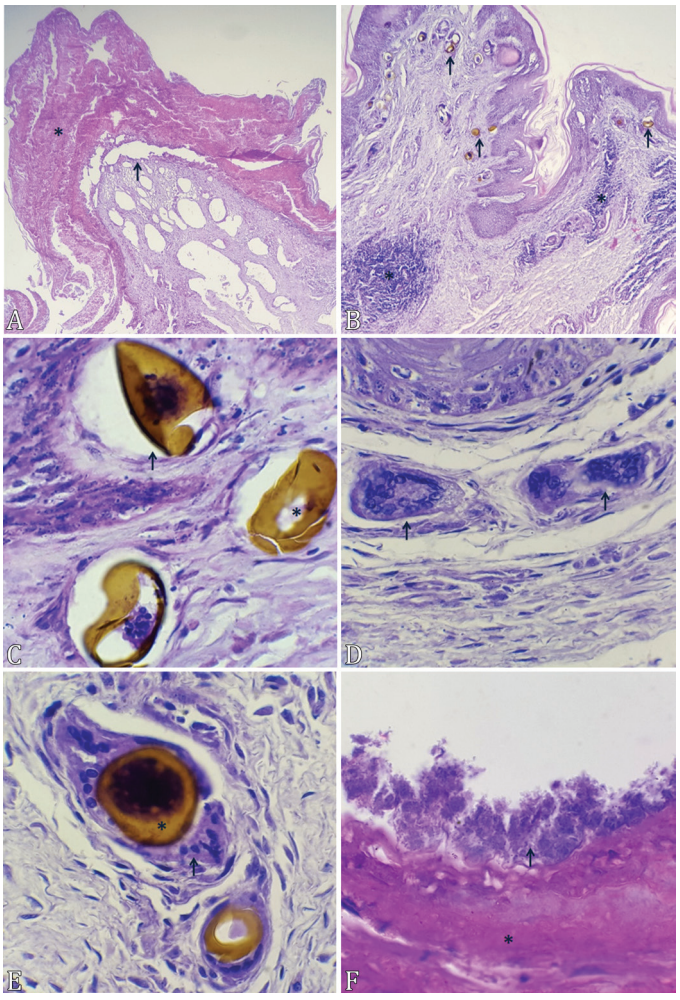


Fig.4. Caseous esophagitis in green turtles (*Chelonia mydas*) found naturally dead on the Brazilian coast from January to October 2019. (**A**) Caseous (asterisk) adhered to the ulcerated mucosa (arrow). HE, bar = 100µm. (**B**) Mononuclear inflammatory infiltrate (asterisk) and parasite eggs (arrows) in the lamina propria. HE, bar = 100µm. (**C**) Parasite eggs in the epithelium (arrow) and in the lamina propria (asterisk). HE, bar = 50µm. (**D**) Multinucleated giant cells of the foreign body type in the lamina propria (arrows). HE, bar = 50µm. (**E**) Parasite egg (asterisk) surrounded by multinucleated giant cells of the foreign body type in the lamina propria (arrow). HE, bar = 50µm. (**F**) Bacterial colonies (arrow) adhered to the caseous (asterisk). HE, bar = 50µm.

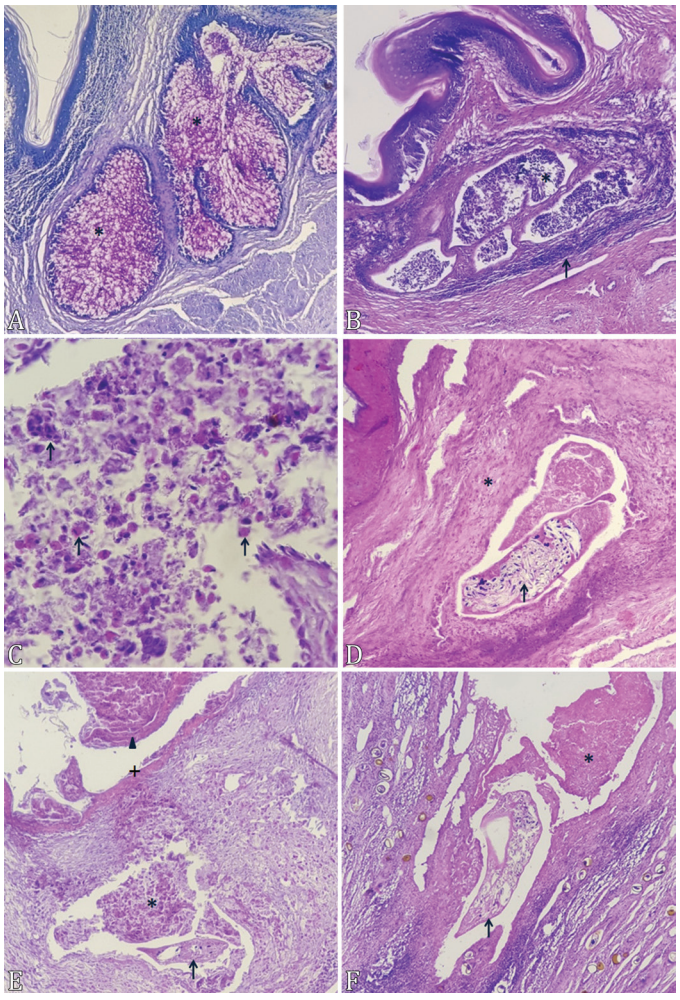


Fig.5. Esophageal glands of green turtles (*Chelonia mydas*) found naturally dead on the Brazilian coast from January to October 2019. (**A**) Gland without alterations with evidenced mucous content (asterisk). PAS, bar = 100µm. (**B**) Periglandular (arrow) and glandular lumen with mononuclear inflammatory infiltrate and necrosis (asterisk). HE, bar = 100µm). (**C**) Necrotic content in the glandular lumen (arrows). HE, bar = 50µm. (**D**) Fragment of adult parasite (arrow) associated with glandular necrosis (asterisk). HE, bar = 100µm. (**E**) Fragment of adult parasite (arrow) associated with glandular necrosis (asterisk), ulceration (cross), and caseous (triangle). HE, bar = 100µm). (**F**) Presence of adult parasite fragment in ulceration area (arrow) with caseous. HE, bar = 100µm).

Staphylococcus species are present on the skin and epithelial surfaces of all warm-blooded animals (Smeltzer & Beenken 2016), with reports in the cloaca and nasal cavity of green turtles (Santoro et al. 2006) and oral cavity of Geoffroy's side-necked turtles (*Phrynops geoffroanus*) (Ferronato et al. 2009). However, to date, this agent has not yet been reported in healthy *C. mydas* esophagus. The genus is commonly involved in the development of bacterial diseases in sea turtles, with the development of lesions in various tissues and organs of a secondary and opportunistic nature. Orós et al. (2004) detected the genus in 22.58% of sea turtle diseases, including ulcerative esophagitis, granulomatous splenitis, ulcerative stomatitis, granulomatous pneumonia, and pericarditis.

Considered to be omnipresent agents in nature, *Bacillus* spp. inhabits soil and water, occurring on different surfaces (Stewart & Thompson 2016). Similar to all microorganisms previously mentioned, they are part of the *C. mydas* microbiota, being isolated by Santoro et al. (2006) in the nasal cavity and cloaca of green turtles, similar to proportions isolated the present study.

In this scenario, the resident microbiota consists of the stable population of microorganisms that reside in the skin and mucous membranes of the host throughout its life. On the other hand, the transient microbiota is characterized by agents temporarily present in the tissue. In this study it was observed that the marine environment (sea water) influences the population of aerobic bacteria in the esophagus of *C. mydas*. In addition, the turtles used were free-living juveniles and are likely to travel along the Brazilian coast until they reach adulthood, when they move to the high seas (Hirth 1997). As a result, these animals inhabit different marine regions during their life cycle, and the esophagus may vary in the percentage of bacteria present, or even different genera and/or species. Therefore, it is suggested that there may be a variation in the population of bacterial agents in the esophagus of *C. mydas*, and the microbiota may be transient. However, it cannot be ruled out that the microorganisms found are part of the resident microbiota.

Regarding the morphological changes of caseous esophagitis, it was noted that all lesions were located at the GJ, similar to the findings by Calais Júnior (2015). The fact that GJ has mucus-producing glands (Calais Júnior et al. 2016), which can host trematode parasites (Calais Júnior 2015, Ribeiro et al. 2017, Santoro et al. 2007b) may justify the highest prevalence of injury in that portion. Parasites, such as *Rameshwarotrema uterocrescens,* are responsible for the inflammation and glandular necrosis in the esophagus of *C. mydas* (Santoro et al. 2007b).

The present study found the predominance of multifocal-to-coalescent caseous lesions macroscopically, diverging from Calais Júnior (2015), who observed the prevalence of multifocal distribution. In view of the fact that the caseous develops until it completely obstructs the esophageal lumen, it is possible that the lesion starts in several foci and joins until a larger caseous mass is formed.

Histologically, caseous and ulcerations were observed in all samples evaluated, in addition to mononuclear inflammatory infiltrates. Caseous inflammation is commonly observed in reptiles, and is characterized by the exudation of heterophiles (degranulated and/or degenerate) that accumulates and macroscopically forms a yellowish-white mass of friable consistency. The heterophile of reptiles corresponds to the neutrophils of mammals and plays an important role in combating the invasion of microorganisms such as bacteria, fungi, viruses, and protozoa (Montali 1988). Mucous ulceration was also observed by Santoro et al. (2007b) and Calais Júnior (2015), and may be related to the parasite, when it is located in the lamina propria, or even to the harmful enzymes released during the inflammatory process (Montali 1988).

The mononuclear inflammatory infiltrate in the lamina propria was also observed by Calais Júnior (2015). Thus, it can be inferred that the areas of inflammation indicate a chronic process in the esophageal mucosa, since according to Pereira (2011), chronic inflammation is characterized by exudation of mononuclear cells (such as macrophages and lymphocytes), necrosis and/or degeneration and regeneration and/or repair, and changes found in the esophageal tissues evaluated.

The study in question found bacterial colonies in caseous esophagitis, a fact also confirmed by other authors (Santoro et al. 2007b, Calais Júnior 2015, Ribeiro et al. 2017, Jerdy et al. 2019). The relevant bacterial association can be explained by the fact that the heterophile plays an important role in combating bacterial invasion (Montali 1988). Calais Júnior (2015) observed Gram-positive cocci in 56.75% of caseous lesions and concluded that bacteria are important for the formation of the caseous and obstruction of the esophagus.

In addition, the bacterial occurrence was concomitant with the presence of trematode eggs in the mucosa and/or fragments of adult parasites associated with necrosis and adenitis. Adult parasites are related to acute and chronic inflammatory processes in various tissues and organs of green turtles (Stacy et al. 2010). Santoro et al. (2007a) identified parasites of the Spirorchiidae family associated with chronic inflammation in cardiovascular, gastrointestinal, and pulmonary tissues, in addition to reporting cases of secondary bacterial infection associated with the parasitic lesions. Santoro et al. (2007b), Ribeiro et al. (2017) and Jerdy et al. (2019) described that esophagitis in green turtles is due to parasitism in esophageal glands by *Rameshwarotrema uterocrescens* with secondary bacterial infection by Gram-positive agents. However, Calais Júnior (2015), in a study carried out on the Espírito Santo coast, observed the occurrence of four different families of trematodes (Pronocephalidae, Microscaphidae, Cladorchiidae, and Rhytidodiae) in the injured esophagus, with the family of *Rameshwarotrema*, one of those described, in addition to Gram-positive bacteria. These data indicate that many other parasites, in addition to the genus *Rameshwarotrema*, may be involved in the etiology of obstructive esophageal lesions in *C. mydas*.

Thus, it is believed that these parasites act as primary harmful agents in esophageal inflammation, generating a gateway for infection by opportunistic and pathogenic bacteria from the microbiota itself, or even that the lesion is of multifactorial origin, in which the parasites and bacteria act as co-factors working in association and contribute to the formation of the case and consequent obstruction.

The parasites, mainly trematodes, are common in *C. mydas* and deposit their eggs in several organs, which do not lead to the death of the green turtle. However, they can contribute to the stranding and death of juvenile animals

(due to undeveloped immunity), or predispose to secondary diseases (Santoro et al 2007a).

In their research, Binoti et al. (2016) found intact or degenerate parasite eggs, with or without giant cells, in the spleen, lung, skeletal striated muscles, small and large intestine, kidney, salted glands, liver, stomach, heart muscle, pancreas, skin, esophagus, and adrenal glands without macroscopic lesions. The presence of the egg leads to the development of multinucleated giant cells of the foreign body type, which are formed by the fusion of macrophages, around non-immunogenic structures such as suture threads and parasite eggs (Pereira 2011). With this, it can be suggested that the observation of only multinucleated giant cells indicates that the egg was phagocyted and eliminated from the tissue. In addition, it is believed that the presence of eggs in the tissue does not necessarily cause injury.

In this study, adenitis and glandular necrosis were also observed in the esophageal mucosa and Santoro et al. (2007b), Ribeiro et al. (2017) and Jerdy et al. (2019), associated these to the *R. uterocrescens* agent; however, it was not possible to identify the species of the parasites, limiting themselves to classifying the agents as trematodes.

Thus, the location of intraglandular trematodes can justify the presence of eggs in the lamina propria. However, there are no data in the literature about the biological cycle of the parasites found in the esophagus of *C. mydas*; thus, it is not possible to exactly state the route taken by the parasitic agent to reachthe esophageal gland as well as the way in which they deposit their eggs.

On the other hand, the data obtained in this study by statistical tests also showed that when there is an increase in bacteria, either by observation of the bacteria by microscopy or by the number of isolated agents, there is a decrease in the parasitic load. However, the negative correlation observed was very weak and may be related to the chronicity of the lesion and the fact that the parasite is no longer observed in the tissue.

CONCLUSION

The esophageal microbiota of *Chelonia mydas* is predominantly composed of Gram-negative bacteria such as *Pseudomonas aeruginosa, Aeromonas hydrophila,* and *Vibrio alginolyticus*, in addition to several enterobacteria and Gram-positive agents, with the highest prevalence being *Staphylococcus aureus*. These agents are opportunists and may be involved in the etiology of caseous esophagitis in association with other pathogens as co-factors working in association or, even in a secondary way.

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

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Comparative retinal histomorphometry and visual acuity of three bat species of the genus *Artibeus* (Phyllostomidae: Stenodermatinae)[1]

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ABSTRACT.- Antonio E.A., de Sá F.B., Santos K.R.P., Lima Junior N.B., Silva F.R., Aguiar Júnior F.C.A. & Vieira J. R. C. 2020. **Comparative retinal histomorphometry and visual acuity of three bat species of the genus *Artibeus* (Phyllostomidae: Stenodermatinae)**. *Pesquisa Veterinária Brasileira 40(11):933-945*. Departamento de Morfologia e Fisiologia Animal, Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. E-mail: eribaldi17@hotmail.com

This study performed a histomorphometric analysis of the retina and estimated the visual acuity of three fruit-eating bats of the genus *Artibeus*, including *Artibeus lituratus*, *Artibeus planirostris* and *Artibeus obscurus*. In total, 13 animals were used for this study, in which the retinas were hemidisected, fixed, cut, and stained. The visual acuity was determined by the density of ganglion cells in the retina and the retinal layers were also measured from histological sections. The retinas of these bats are avascular, do not present *tapetum lucidum* in any quadrant, and have the 10 retinal layers common in mammals. Moreover, it was observed that the thickness of the retina in the central region was significantly higher in all measured parameters ($p<0.001$), except for the outer plexiform layer (OPL) which was significantly higher in the peripheral region ($p<0.001$). The retinas of the three species showed a horizontal visual streak with a higher concentration of retinal ganglion cells (RGCs) at the inferotemporal region. In addition, the species *A. lituratus* exhibited extras areas of high cell density in the retina. Thus, *A. lituratus* showed the highest visual acuity (1.92 cycles/degree), while *A. planirostris* showed a visual acuity of (1.77 cycles/degree) and *A. obscurus* exhibited the lowest visual acuity (1.50 cycles/degree). All these characteristics are related to the echolocation system and the eating habits of each species. Therefore, it can be concluded that bats of the genus *Artibeus* have a high visual acuity value compared to other echolocating bats and all these differences might be directly linked to the phylogeny of the genus.

INDEX TERMS: Fruit-eating bats, *Artibeus*, retinal histomorphometry, visual acuity, morpholgy.

RESUMO.- [**Histomorfometria comparada da retina e acuidade visual de três espécies de morcegos do gênero *Artibeus* (Phyllostomidae: Stenodermatinae).**]

Este estudo realizou uma análise histomorfométrica da retina e determinou os valores de acuidade visual de três espécies de morcegos do gênero *Artibeus*, incluindo *Artibeus lituratus*, *Artibeus planirostris* e *Artibeus obscurus*. Em total, 13 animais foram usados para esse estudo, no qual as retinas foram hemidissecadas, fixadas, cortadas e coradas. A acuidade visual foi determinada pela densidade de células ganglionares na retina e também foi realizado a mensuração das camadas retinianas a partir de cortes histológicos. As retinas dessas três espécies são avasculares, não apresentaram *tapetum lucidum* em nenhum quadrante e possuem as 10 camadas retinianas comum em mamíferos. Verificou-se também que a espessura da retina na região central foi significativamente maior em todos os parâmetros mensurados ($p<0,001$), com exceção da camada plexiforme externa (OPL) que foi

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significativamente maior na região periférica (p<0,001). As retinas das três espécies exibiram uma especialização retiniana em forma de faixa visual horizontal apresentando a maior concentração de células ganglionares na região inferotemporal. Além disso, a espécie *A. lituratus* exibiu áreas extras de alta densidade celular na retina. Assim, a espécie *A. lituratus* exibiu a maior acuidade visual (1,92 ciclos/grau), *A. planirostris* mostrou um valor intermediário entre essas duas espécies (1,77 ciclos/grau) e *A. obscurus* exibiu a menor acuidade visual (1,50 ciclos/grau). Todas estas características estão relacionadas aos padrões de ecolocalização e hábitos alimentares de cada espécie. Portanto, pode-se concluir que os morcegos do gênero *Artibeus* têm um alto valor de acuidade visual em comparação com outros morcegos ecolocalizadores e todas essas diferenças devem estar diretamente ligadas à filogenia do gênero.

TERMOS DE INDEXAÇÃO: Morcegos frugívoros, *Artibeus*, histomorfometria retiniana, acuidade visual, morfologia.

INTRODUCTION

The Order Chiroptera is the second largest group of mammals in number of species. These animals exhibit a unique morphology adapted to flight and a diverse food habits (Reis et al. 2007, Gardner 2008). Bats of the genus *Artibeus* Leach, 1821 (Phyllostomidae, Stenodermatinae) are *restricted to the Neotropical region and it currently* consists of 10 species *distributed from Mexico to the north of Argentina and southern Brazil (*Koopman 1982, Owen 1987, Marques-Aguiar 1994, Lim et al. 2004, Simmons 2005, Gardner 2008, Redondo et al. 2008, Solari et al. 2009, Reis et al. 2011, Ferreira et al. 2014). Besides, bats of this genus are mainly frugivorous and they play an important role in forest regeneration acting as seed dispersers, especially in early successional forests (Mikich 2002, Passos & Passamani 2003, Galetti & Morellato 2004, Passos & Graciolli 2004, Oprea et al. 2007, Oliveira & Lemes 2010, Martins et al. 2014, Bôlla et al. 2018, Pereira et al. 2019).

Recently, bats have been classified according to new molecular data and following the evolutionary process of the echolocation system (Teeling et al. 2000, Jones & Teeling 2006, Teeling 2009). Although most bats use echolocation for orientation and to forage in the dark, they also use the vision to perform these tasks (Rydell & Eklöf 2003, Altringham 2011, Gutierrez et al. 2018a, Warnecke et al. 2018). The vision is important for echolocating bats to find shelters (Ruczynski et al. 2011), migration and homing (Griffin 1970, Williams & Williams 1970), prey capture (Rydell & Eklöf 2003) avoidance-escape behavior of obstacles and predators (Gould 1977, Chase 1981, Koay et al. 2002, Eklöf & Jones 2003, Orbach & Fenton 2010) and visual recognition (Suthers et al. 1969, Gutierrez et al. 2014, Rodríguez-Herrera et al. 2019). Furthermore, frugivorous and nectarivorous bats can see the brightness and the spectral composition of different food resources (Neuweiler 2000, Winter et al. 2003, Müller et al. 2009). So far, researchers have found evidences that there is a relationship between nocturnal activity peaks and feeding strategies with the visual skills of echolocating bats (Chase 1981, Rydell & Eklöf 2003, Eklöf et al. 2014, Gutierrez et al. 2014, 2018a, 2018b, Kries et al. 2018).

The eyeballs of bats are constituted by three concentrically arranged tunics, one big lens, and a chambers system (Neuweiler 2000, Cunningham 2004). The retina covers internally the entire posterior region of the eyeball (Pedler & Tilley 1969, Leite et al. 2013). In echolocating bats, this nervous tunic is quite thin and it is formed by different types of neurons organized in 10 layers (Neuweiler 2000). Most bats have a dichromatic color vision due to the presence of two cone opsins, (S-) cone opsins and (L-) cone opsins related to sensitivity to short wavelengths and middle-long wavelengths, respectively. Furthermore, some species are able to see *into the ultraviolet spectrum* (Wang et al. 2004, Müller et al. 2007, Feller et al. 2009, Müller et al. 2009, Zhao et al. 2009, Boonman et al. 2013, Kries et al. 2018, Sadier et al. 2018, Simões et al. 2018, Gutierrez et al. 2018b). The eyes of these animals are adapted to operate in low light levels, resulting in poor visual acuity when compared to diurnal mammals (Suthers & Wallis 1970, Pettigrew et al. 1988, Heffner et al. 2001). In general, frugivorous bats have bigger eyes and better visual resolving power compared to other bat species (Blackwood et al. 2010). Moreover, it has been shown that bat eyes work better in dim light than in bright light, especially fruit-eating bats that are more efficient at detecting and capturing their food during luminosity similar to a full moon (Hope & Bhatnagar 1979, Gutierrez et al. 2014, Spoelstra et al. 2017, Gutierrez et al. 2018a).

The measurements of visual acuity are used to estimate the ability of an animal to perceive visual details. Thus, the visual acuity can be estimated anatomically by measuring the focal distance of the eyes and the peak density of retinal ganglion cells (RGCs) (Pedler & Tilley 1969, Collin & Pettigrew 1988, Pettigrew et al. 1988, Heffner et al. 2001, Müller et al. 2007, Ullmann et al. 2011, *Hauzman et al. 2014,* Veilleux & Kirk 2014). The high-density of RGCs might appear of two ways: Located in a central area or horizontally, forming a visual streak, usually found in species that needs a horizontal viewing in open fields which the distribution of cells may vary according to the habit of each species (Hughes 1977, Collin 2008).

Information about the vision or morphophysiology of the ocular bulb of bats is scarce, especially of those species found in Brazil. Therefore, this study aimed to carry out a histomorphometric analysis to evaluate the topographic distribution of neurons in the central and peripheral region of the retina of three fruit-eating bats of the genus *Artibeus* (*Artibeus planirostris*, *Artibeus lituratus* and *Artibeus obscurus*) from a fragment of the Atlantic Forest of Pernambuco, Brazil. Additionally, it was estimated their respective visual acuity and the relationship between the visual capability and the feeding habits of this genus.

MATERIALS AND METHODS

Bat captures. In total, 05 adult males of the species *Artibeus planirostris*, 05 adult males of the species *Artibeus lituratus* and, three adult males of the species *Artibeus obscurus* were used in this study. The bats were collected by 10 mist nets (12m long x 3m high) at an Atlantic Forest fragment located in Tamandaré, Pernambuco, Brazil (Latitude: 08°44'13" and 08°43'09 S and Longitude: 35°10'11" and 35°11'02" W). All procedures were carried out according to the international practices for animal use and care approved by the Ethics Committee on Animal Use of the "Universidade Federal de Pernambuco" (CEUA-UFPE), under protocol number 23076.040931/2014-76.

Eye enucleation. After each capture, the animals were anesthetized at the capture site with sodium pentobarbital at a concentration of 40mgkg-1 intraperitoneally, followed by euthanasia with a saturated solution of potassium chloride (Morais et al. 2013). Before enucleation, radial incisions were made on the dorsal pole of the cornea using a portable cautery. Briefly, both eyes were extracted from the orbit through lateral incisions in the periorbital fat and extrinsic muscles using a scalpel and microsurgical scissors, preserving a portion of the optic nerve as much as possible (Moshfeghi et al. 2000, Ullmann et al. 2011). Subsequently, the eye axial length (AL) was measured through digital calipers to an accuracy of 0.01mm. Finally, all right eyes were hemisected, the cornea and iris were cut and the lens and vitreous were removed, as well as a radial cut was made on the dorsal region of the eyecup for future orientation (Ullmann et al. 2011).

Tissue processing and preparation of retinal wholemounts. After enucleation, the tissue processing and the retinal wholemounts were prepared following the methods of Stone (1981) and Ullmann et al. (2011). The right eyecups were fixed in 4% paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.2-7.4) for 24 hours. After fixation, the eyecups were washed in PBS and the retinas were dissected by removing sclera and the choroid using surgical tweezers and a paintbrush. The bleaching process was not necessary because all retinas were easily detached from the retinal pigment epithelium. Subsequently, the dissected retinas were wholemounted on gelatinized slides with the optic fiber layer uppermost and other radial cuts were made to enable the retina to lie completely flat and adhere on the slide. The retinas were exposed to formalin vapor at 60°C for 2 hours and dried in the vessel for another 24 hours. Retinal wholemounts were immersed in 4% paraformaldehyde for 1 hour and then rehydrated in decreasing sequences of ethanol. After washing in distilled water, the tissues were Nissl stained with an aqueous solution of 0.05% cresyl violet for 10 minutes, dehydrated in an ethanol series, cleared in xylene, and coverslipped with Entellan (Stone 1981, Ullmann et al. 2011). The percentage of retinal tissue shrinkage may vary according to each species. Therefore, retinal pictures were taken and the retinal surface area was measured using the ImageJ Software version 1.44 (Research Services Branch, U.S. National Institutes of Health, Bethesda/MD, USA) before and after staining so that shrinkage could be estimated (Stone 1981, Lisney et al. 2012).

The left eyes were fixed following the same process of the right eyes. However, they were used to examine the microanatomy of the eye and the layered structure of the retina. Thus, the left eyes were processed according to routine histological techniques. They were embedded in paraffin and sectioned into 4μm thickness in serial sections, beginning from the temporal to nasal area in each eye. Then, the tissue preparations were stained with hematoxylin and eosin (HE) and analyzed by light microscopy (Tolosa et al. 2003).

Retinal ganglion cells topography and histomorphometric analysis of the retina. To count and mapping the topography of the retinal ganglion cells (RGCs), as well as to measure the retinal layers, several photomicrographs at 400x magnifications were taken at each quadrant in the central and peripheral retina of the three species. The photomicrographs were obtained by the computer program ScopePhoto using a digital capture camera (Moticam 2300), of 3.0 megapixels, coupled to an optical microscope (Nikon E-200). A systematic random sampling scheme was used to map the density distribution of the RGCs on each wholemount (Ullmann et al. 2011, Coimbra et al. 2012, Lisney et al. 2012). First of all, it was established that the central retina was a circular area with a radius of about 2,000μm from the optic nerve head, whereas the peripheral retina were those regions greater than 2,000μm from the optic nerve head (Schlamp et al. 2013). Counts of RGCs were performed using a counting frame of 100 x 100μm from the temporal region to the nasal region measured using the plugin Grid of the ImageJ software version 1.44. Counts were made at 100μm intervals in the periphery and 50μm intervals in the center of the retina. All RGCs within the counting frame and all RGCs intersected by the upper and right border of the counting frame were included, while all those intersected by the lower and left border of the counting frame were disregarded (Gundersen 1977). Moreover, only RGCs were considered for counting, and amacrine and glial cells were excluded. To distinguish the RGCs from other neurons was used the cytological criteria, which RGCs were those cells that always exhibited a large and irregular shape, a well-stained nucleus with a prominent nucleolus and Nissl granules in the cytoplasm (Hughes 1981, Collin 1988, Collin & Pettigrew 1988, Coimbra et al. 2012). Finally, to calculate the visual acuity and to map the topographic pattern distribution of RGCs the cell counts were converted to cells/mm^2. To create the topography maps, ganglion cell isodensity contour plots were created and smoothed by joining retinal regions of equal density (Ullmann et al. 2011, Moore et al. 2012, Garza-Gisholt et al. 2015). Topographic maps were created using Freehand (Adobe Systems Incorporated, San Jose/CA, USA). Subsequently, the average of RGCs density of each retinal contour plots was multiplied by its area and then the results of all contour plots were added up in order to estimate the total RGCs present in the retina of each species (Harahush et al. 2014).

Histomorphometric measurements were made using photomicrographs of cross-section of the retina at 400x magnifications. The photomicrographs were obtained using the same images capture devices for counting of RGCs. The total retinal thickness (excluding the retinal pigment epithelium) and seven retinal layers were measured in micrometer (μm) using the software ImageJ. The retinal layers measured were: photoreceptors outer and inner segment (PS), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL) and nerve fiber layer (NFL). Thus, 10 photomicrographs were considered for each quadrant in the central and peripheral regions of the retinas and the results were compared between regions and among the three species. Additionally, it was established the average number of layers of photoreceptors nuclei from the outer nuclear layer (ONL) in the central region of the retina.

Estimation of visual acuity. The visual acuity or anatomical spatial resolving power was calculated following the equations of Williams & Coletta (1987) and Pettigrew et al. (1988). First of all, the posterior nodal distance (PND) should be determined for each eye of the animals and it was calculated using a method established by Pettigrew et al. (1988). They proposed that to estimate the PND the axial length of the eyeballs should be multiplied by (0.52). This 0.52 value is a constant related to the mean ratio of PND regarding the axial length for nocturnal species, including microchiropteran bats. Second, to estimate the visual acuity from the retinal ganglion cells density, the retinal magnification factor (RMF) must be calculated. The RMF is the linear distance on the retina that subtends 1° of visual angle. Therefore, the RMF was estimated in micrometers per degree (μm/degree) and it was calculated according to the following formula (Pettigrew et al. 1988):

$$RMF = \frac{2\pi * PND}{360}$$

Finally, the RMF was used to figure out the visual acuity for the three species of *Artibeus*. Thus, the visual acuity was calculated using

the Nyquist limits of spatial resolution according to the following equation (Williams & Coletta 1987):

$$Fn = \frac{RMF}{2}\sqrt{\frac{2D}{\sqrt{3}}}$$

Where, Fn is the highest spatial frequency that can be detected under optimal light conditions by the eyes, and D represents the highest RGCs density located in the retina (cells/mm^2). Therefore, the visual acuity is the maximum number of retinal ganglion cells per linear degree and it was estimated in cycles per degree (cycles/degree).

Statistical analysis. The mean values obtained from each parameter analyzed for the three species were compared by Student's t-test and analysis of variance (ANOVA) using the software SPSS (Statistical Package for the Social Sciences, SPSS Inc. Chicago, EUA) version 15.0. The results were expressed as mean ± SD (standard deviation), with a significance level of 5% (P<0.05).

RESULTS

Axial length and retinal area

Anatomical measurement of the eyeball of these three bats showed that *Artibeus lituratus* have the biggest and *Artibeus obscurus* have the smallest eyes among these three species. The values of the eye axial length (averaged between the right and left eyes for each species) are shown in Table 1. The retina of the three bat species covers internally the posterior region of the eyes until the *ora serrata*. Thus, the area of the retina of the species *A. lituratus* is significantly larger (p<0.001) among these bats with approximately (18.96mm^2±0.12), while the retinal area of *Artibeus planirostris* is (14.23mm^2±0.10) and the retina of *A. obscurus* covers about (11.39mm^2±0.27) the inner region of the eye.

Retinal ganglion cells distribution

The ganglion cell density was significantly higher (p<0.001) in the center of the retina compared to the peripheral region. Besides, the highest concentration of RGCs was observed at the inferotemporal region of the retina, especially below the optic nerve head, ranging from 10,700 to 11,300 cell/mm^2 for the three bat species (Fig.1). No tissue correction was applied to cell density due to the shrinkage was minimal, located only in narrow zones in the borders of the retina and in the edges of some radial cuts. No apparent foveal pit or depressed part of the retina was identified. However, the highest concentration of RGCs is relatively a large circular area without any depression that was characterized as an *areae centrales*. Furthermore, a weakly pronounced horizontal visual streak was verified extended along the temporonasal axis in all three retinas. Thus, the peak density of RGCs is located within the visual streak close to the optic nerve head (Fig.2). The species *A. lituratus* exhibits another slightly horizontal visual streak beginning from the temporal quadrant until the nasal quadrant located just above the optic nerve head. Moreover, it shows a considerable circular area with a high density of RGCs at the superonasal quadrant (Fig.1A). Similarly, the retina of the species *A. planirostris* exhibits a great concentration of RGCs above the optic nerve head continuous within the horizontal visual streak (Fig.1B). The total number of RGCs for each species was estimated and the results are expressed in Table 1.

Visual acuity

Assuming the highest density of RGCs at a specific site in the retina, the species *A. lituratus* showed the highest visual acuity (1.92 cycles/degree) when compared with the other two. On the other hand, *A. obscurus* had the lowest visual acuity (1.50 cycles/degree), and the species *A. planirostris* exhibited an intermediate value of visual acuity (1.77 cycles/degree) (Table 1).

Table 1. Data base for visual acuity of *Artibeus lituratus*, *Artibeus planirostris* and *Artibeus obscurus*

Species	AL (mm)	Estimated PND (mm)	RMF (µm/degree)	Retinal area (mm^2)	Estimated total number of RGCs	Highest density of RGCs/mm^2	Estimated visual acuity (cycles/degree)
Artibeus lituratus							
No. 1	3.71	1.92	33.49	19.00	108,220	11,391	1.92
No. 2	3.74	1.94	33.84	18.99	107,005	11,322	1.93
No. 3	3.76	1.95	34.01	19.07	112,059	11,650	1.97
No. 4	3.68	1.91	33.31	18.75	100,371	10,950	1.87
No. 5	3.71	1.92	33.49	18.99	103,420	11,187	1.90
Mean ± SD	3.72 ± 0.03	1.93	33.66	18.96 ± 0.12	106,215 ± 4,493	11,300 ± 258	1.92 ± 0.04
Artibeus planirostris							
No. 1	3.52	1.83	31.92	14.24	80,938	10,776	1.78
No. 2	3.54	1.84	32.09	14.25	87,158	10,889	1.79
No. 3	3.51	1.82	31.74	14.20	82,447	10,935	1.77
No. 4	3.56	1.85	32.27	14.37	85,015	10,850	1.79
No. 5	3.51	1.82	31.74	14.09	78,442	10,550	1.75
Mean ± SD	3.52 ± 0.02	1.83	31.92	14.23 ± 0.10	82,800 ± 3,408	10,800 ± 151	1.77 ± 0.02
Artibeus obscurus							
No. 1	2.97	1.54	26.86	11.12	64,575	10,450	1.48
No. 2	3.03	1.57	27.38	11.66	66,950	10,700	1.52
No. 3	2.91	1.51	26.34	11.39	63,550	10,950	1.48
Mean ± SD	2.97 ± 0.06	1.54	26.86	11.39 ± 0.27	65,025 ± 1,744	10,700 ± 250	1.50 ± 0.02

AL = axial length, PND = posterior nodal distance, RMF = retinal magnification factor, RGCs = retinal ganglion cells. PND was calculated by multiplying the axial lengths by 0.52. AL = Axial length of the eye; PND = Posterior nodal distance; RMF = Retinal magnification factor; RGCs = Retinal ganglion cells; SD = Standard deviation.

Retinal morphometry

The retinas of these three species are avascular and did not present *tapetum lucidum* in any retinal quadrant. Furthermore, for all three species of *Artibeus* it is formed by 10 layers (Fig.3). It was observed that the thickness of the central region of all retinas is significantly higher (p<0.001) in all measured parameters for the three species, except for the outer plexiform layer (OPL) which is significantly higher in the peripheral region of the retina (p<0.001). However, the retinal thickness varies significantly in a great number of parameters when compared between the species (Table 2).

The species *A. lituratus* exhibited the highest total retinal thickness in the central region compared to the other two, whereas *A. obscurus* showed the highest total thickness in the peripheral region of the retina. The layer thickness of the outer and inner segments of photoreceptors (PS) was

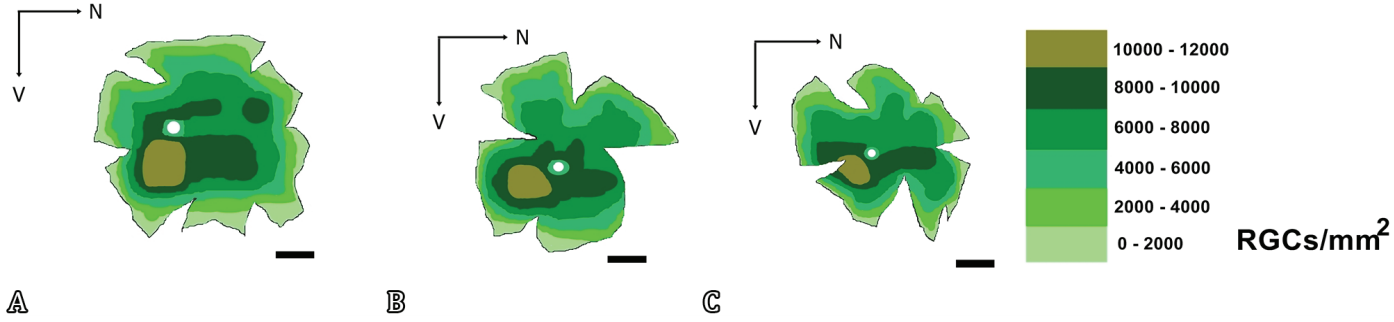


Fig.1. Isodensity topographic map representing the distribution of retinal ganglion cells (RGCs) in the retinas of (**A**) *Artibeus lituratus*, (**B**) *Artibeus planirostris* and (**C**) *Artibeus obscurus*. All three species show a horizontal visual streak and the highest concentration of RGCs located at the inferotemporal region of the retina (brown area) with a maximum density of approximately 11300 RGCs/mm^2, 10800 RGCs/mm^2 and 10700 RGCs/mm^2 to *Artibeus lituratus*, *Artibeus planirostris* and *Artibeus obscurus*, respectively. Ventral (V), nasal (N), optic nerve head (white dot). Bar = 1mm.

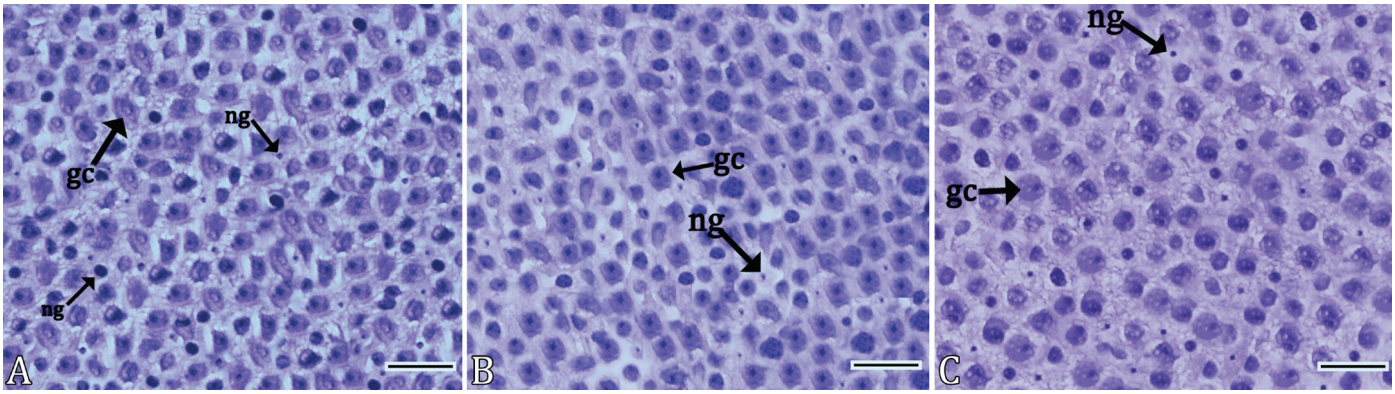


Fig.2. High-magnification digital photomicrographs of the Nissl-stained retinal ganglion cell layer of (**A**) *Artibeus lituratus*, (**B**) *Artibeus planirostris* and (**C**) *Artibeus obscurus* showing ganglion cells bodies (gc) and examples of nonganglion cells (ng), which were not included in the neuron counts. All three photomicrographs were taken from the inferotemporal region of the retinas (the highest concentration of RGCs). Nissl stain, obj.40x, bar = 20µm.

Table 2. Thickness of the central and peripheral layers of the retinas of *Artibeus lituratus, Artibeus planirostris* and *Artibeus obscurus*

Parameters	*Artibeus lituratus*		*Artibeus planirostris*		*Artibeus obscurus*	
	Center	Periphery	Center	Periphery	Center	Periphery
Total retinal thickness*	147.01 ± 1.46	131.13 ± 0.81	128.29 ± 0.66	124.79 ± 0.79	137.79 ± 2.15	132.18 ± 1.98
PS	36.07 ± 1.04	23.98 ± 1.31 (a)(b)	27.14 ± 0.73	23.69 ± 0.72 (a) (c)	26.51 ± 1.57	23.60 ± 1.05 (b)(c)
ONL	46.66 ± 0.75	40.32 ± 0.95	42.87 ± 2.14	36.09 ± 1.04	38.23 ± 2.2	33.62 ± 1.54
OPL	7.31 ± 0.41	8.39 ± 0.77	9.50 ± 0.17	15.95 ± 0.41	10.32 ± 1.08	13.59 ± 1.1
INL	20.95 ± 0.47	16.60 ± 0.93	22.81 ± 0.59	15.96 ± 0.41	22.25 ± 1.80	18.23 ± 1.51
IPL	24.07 ± 0.35 (d)	21.19 ± 0.96	24.12 ± 0.42 (d)	17.63 ± 0.75	25.57 ± 1.60	23.96 ± 1.30
GCL	8.99 ± 0.50 (e) (f)	7.28 ± 0.63(g)	9.17 ± 044 (e) (h)	7.73 ± 0.71	8.95 ± 0.58 (f) (h)	7.33 ± 0.64 (g)
NFL	10.09 ± 0.61	5.92 ± 0.36 (i) (j)	8.45 ± 0.66	5.82 ± 0.33 (i) (k)	7.63 ± 0.70	5.84 ± 0.39 (j) (k)

Thickness of the retinal layers (µm ± standard deviation); PS = photoreceptor outer and inner segment, ONL = outer nuclear layer, OPL = outer plexiform layer, INL = inner nuclear layer, IPL = inner plexiform layer, GCL = ganglion cell layer, NFL = nerve fiber layer, SD = standard deviation; * the total retinal thickness was measured without counting the retinal pigment epithelium; The same letters in the same line means that there was no significant difference between those pairs of parameters at 5% of significance level using Student's t test; P values: a = 0.195, b = 0.207, c = 0.678, d = 0.490, e = 0.062, f = 0.736, g = 0.758, h = 0.058, i = 0.139, j = 0.330, k = 0.809.

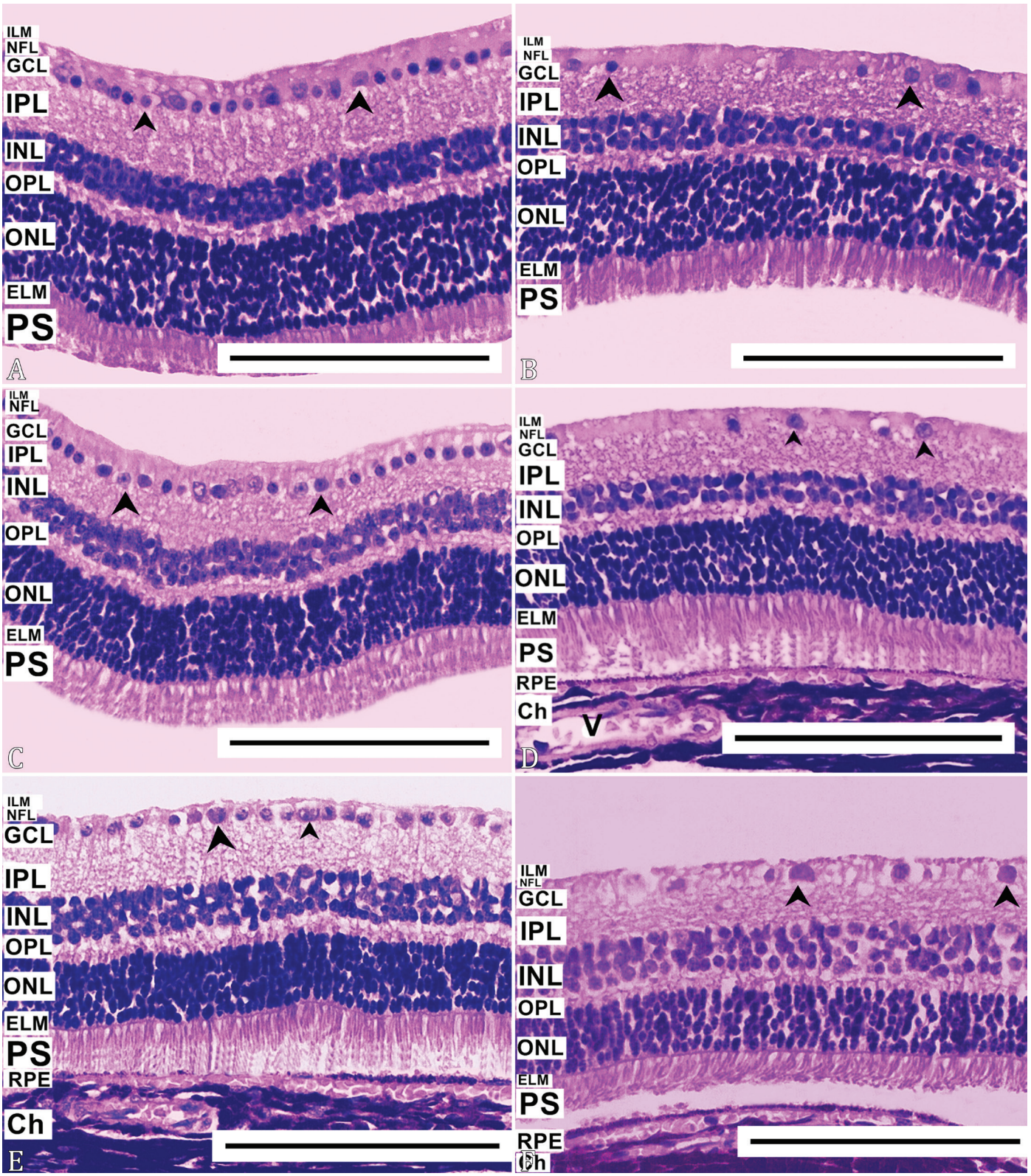


Fig.3. Photomicrographs of cross sections of the retinas of (**A**,**B**) *Artibeus lituratus*, (**C**,**D**) *Artibeus planirostris* and (**E**,**F**) *Artibeus obscurus*. (**A**,**C**,**E**) from the central retina and (**B**,**D**,**F**) from the peripheral retina. Inner limiting membrane (ILM), nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), external limiting membrane (ELM), photoreceptor outer and inner segments (PS), retinal pigment epithelium (RPE), choroid (Ch), venule (V), retinal ganglion cells (arrowhead). HE, obj.40x, bar = 100µm.

significantly higher for the species *A. lituratus* in the central region, while in the peripheral region there was no significant difference of this layer between any species (Table 2). On the photoreceptors layer was observed rods and cones cells. This layer has a rod-like dominance for all three species, mainly in the peripheral region of the retina, where its outer segments are thin and long. In contrast, cones were found in abundance within the central region presenting its outer segments wide and short, with a progressive decrease in amount over to the peripheral region of the retina. The external limiting membrane and the retinal pigment epithelium were easily identified in the retina of all three bats (Fig.3).

The outer nuclear layer (ONL) is thicker in the central area compared to the peripheral regions of the retina in the three species of *Artibeus* (Table 2). However, the ONL in the central region of the retina of *A. lituratus* is the thickest layer between the bats being formed by approximately 10 layers of photoreceptors nuclei, while the species *A. planirostris* showed an intermediate thickness with an average of nine layers of photoreceptors nuclei and the species *A. obscurus* presented the lowest thickness in this area with approximately seven layers of photoreceptors nuclei (Fig.3).

The outer plexiform layer (OPL) was the only retinal layer that showed thicker in the peripheral region for the three bats. Subsequently, the inner nuclear layer (INL) is formed by the nuclei of the horizontal cells, bipolar cells, amacrine cells, and nuclei of Müller cells (Park et al. 2017). Nevertheless, immunohistochemical analysis is required for the differentiation of these cells (Fig.3). The species *A. planirotris* showed the highest thickness of the (INL) and *A. lituratus* exhibited the lowest thickness of this retinal area. In contrast, the inner plexiform layer (IPL) was slightly thicker in the central and peripheral retina of the species *A. obscurus* compared to the other two (Table 2).

The ganglion cell layer in the three species of *Artibeus* is formed by only one layer of RGCs. These cells are irregular and have large sizes, containing granules in the cytoplasm, and have a well-stained nucleus with a prominent nucleolus (Fig.2). As observed in the topographic map of density and distribution of these cells, a high density of RGCs was verified in the central region of the retina, a moderate density is located at the transition between the central and peripheral regions of this tunic and a low density of RGCs was observed in the peripheral region of the retina of the three bat species (Fig.1).

The last parameter measured was the nerve fiber layer (NFL). This layer is composed of the axons from the RGCs that converge and unite to form the optic nerve at the posterior region of the eyes. This layer is significantly thicker in the central region of the retina for all three species. However, *A. lituratus* exhibited the highest thickness of the (NFL) in the central area of the retina compared among them, and there were no significant differences in the peripheral region of the retina between the three species (Table 2). Furthermore, the retinas of the three bats showed a well-defined inner limiting membrane (Fig.3).

DISCUSSION

Retinal ganglion cells distribution and visual acuity

According to the isodensity topographic map from the three species of *Artibeus* studied, it was possible to detect a horizontal visual streak with a high concentration of retinal ganglion cells (Fig.1). This characteristic is a type of retinal specialization that is responsible for increasing the spatial resolution across the horizon (Hughes 1977, Collin 1999). Species that have a horizontal visual streak in the retina generally live in open fields, have a great capacity to move over long distances, and tend to have their eyes located more laterally in relation to the head (Hughes 1977, Provis 1979, Oyster et al. 1981, Moore et al. 2012). The horizontal visual streak makes the animals able to scan the environment constantly using a monocular vision system with a very few head and eyes movements, which the field of view concentrate mainly on the horizon (Shinozaki et al. 2010, Coimbra et al. 2017). Furthermore, this is an especially useful feature for detecting predators (Hughes 1977).

This type of retinal specialization found in the retina is directly related to the behavior of these bats. Morrison (1980) studied the foraging behavior of *A. lituratus* and he observed that this species usually stays in the trees during the day and constantly changes the locations within an area ranging from 0.5 to 2.5 hectares. Moreover, this species changes the eating behavior according to the moon phases. During nights when full moons occur, feeding and agglomeration times decrease on the trees in order to avoid being perceived by predators such as owls. Gutierrez et al. (2014) performed behavioral tests with the same species referring to feeding ability in different luminosities. They have found that the species *A. lituratus* takes a long time to detect its targets with a high light intensity similar to twilight and this may be related to an exacerbated exposure to predators during this period of high luminosity. On the other hand, these bats were quite effective in detecting targets with an illumination similar to the full moon. In addition, the eyes of these three bats are located more laterally in relation to the head (Guerrero et al. 2003, Araújo & Langguth 2010) and these bats move both in open areas and dense forests during their foraging time (Bernard & Fenton 2003, Menezes Jr et al. 2008). All those behavioral characteristics are in agreement with our findings regarding the type of retinal specialization detected for these bats of the genus *Artibeus*, which is mainly related to the detection of either predators or food resources.

In animals that have a horizontal visual streak, the retina tends to present a central area of maximum density of RGCs in the temporal quadrant, especially in animals that have their eyes located more laterally (Hughes 1977). This central area was observed in the inferotemporal quadrant of the retina of the three bats and it has been characterized as *areae centrales*. This feature was also found in bats species of flying foxes, such as *Pteropus giganteus*, *Pteropus poliocephalus*, *Pteropus poliocephalus, Megaloglossus woermanni, Scotonyceteris zenkeri, Casynicteris argynnis, Rousettus aegyptiacus, Epomophorus wahlbergi, Epomops franqueti, Eidolon helvum, Eidolon helvum and Hypsignathus monstrosus* and some echolocating bats: *Rhinolophus rouxi, Rhinolophus ferrumequinum*, *Nyctophilus gouldi, Taphozous georgianus, Megaderma lyra, Dermanura cinerea, Macroderma gigas, Phyllostomus hastatus*, *Phyllostomus discolor and Artibeus jamaicensis* (Pedler & Tilley 1969, Pettigrew et al. 1988, Neuweiler 2000, Heffner et al. 2001, 2007, 2008, Müller et al. 2007, Kim et al. 2008, Hoffmann et al. 2016, Coimbra et al. 2017). The *areae centrales* provides a high-resolution vision in this point of the retina, frontal discrimination towards the horizon, and a type of binocular

fixation (Rapaport & Stone 1984). A high spatial resolution and frontal discrimination of the field of view are particularly important for fruit-eating bats, including those of the genus *Artibeus*, that need to detect their food resources located in fruit trees just in front of them.

The species *A. lituratus* showed an extra horizontal visual streak connected and a circular area with a high density of RGCs. According to Shinozaki et al. (2010) areas of high density of RGCs outside the horizontal visual streak might enhance the visual resolving power of the animal and increase the frontal field of view to discriminate objects. These characteristics in the retina of *A. lituratus* suggest that these bats have a wider field of view among these three species within the genus *Artibeus*.

The visual acuity of the three species of *Artibeus* was considered high when compared to other echolocating bats. Thus, *A. lituratus* exhibited the highest visual acuity among the three species. However, the other two species also showed a good spatial resolving power compared to other bats from different genera. The visual capability of bats is directly related to the food pattern of each species (Chase 1981, Rydell & Eklöf 2003). Bats that have a greater visual acuity tend to be frugivorous, such as flying foxes that present a high visual acuity ranging from 2.2 cycles/degree to 5.5 cycles/degree (Pedler & Tilley 1969, Neuweiler 2000, Heffner et al. 2008, Müller et al. 2007, Coimbra et al. 2017) and some echolocating fruit-eating bats, for instance, the three species of *Artibeus* studied, *A. lituratus* (1.92 cycles/degree), *A. planirostris* (1.77 cycles/degree) and *A. obscurus* (1.50 cycles/degree), *A. jamaicensis* (1.1 cycles/degree) (Heffner et al. 2001), *D. cinerea* (1.35 cycles/degree), *T. georgianus* (1.3 cycles/degree) and *P. hastatus* (1.2 cycles/degree) (Pettigrew et al. 1988). In addition, large carnivorous bats such as *M. gigas* (1.9 cycles/degree) and *M. lyra* (1.5 cycles/degree) show a high visual acuity among the echolocating bats as well (Pettigrew et al. 1988). Omnivorous species as *P. hastatus* (1.2 cycles/degree) (Heffner et al. 2007) and *P. discolor* (1.7 cycles/degree) (Hoffmann et al. 2016) also have a high visual acuity. In contrast, insectivorous species tend to have a lower visual acuity among the echolocating bats, for example, the species *R. rouxi* (0.35 cycles/degree) and *Eptesicus nilssonii* (0.6 cycles/degree) and in particular insectivorous bats of the family Vespertilionidae, including *Myotis brandtii, Myotis lucifugus, Myotis mystacinus* and *Myotis daubentonii* (0.3 cycles/degree) (Rydell & Eklöf 2003, Eklöf & Jones 2003, Feller et al. 2009, Eklöf et al. 2014). Furthermore, frugivorous bats exhibit a better morphological adaptation for low light vision compared to strictly insectivorous bats (Suthers 1966, Suthers et al. 1969, Sadier et al. 2018).

According to Veilleux & Kirk (2014), the evolution of visual acuity in certain groups of mammals is related to the high dependency of vision on basic ecological behaviors, including foraging techniques, migratory behavior, shelter locations and to avoid predators. However, for echolocating bats, the evolutionary pattern of vision differs from the general pattern observed in mammals. The visual system of bats is directly related to the evolutionary process of echolocation (Gutierrez et al. 2018a, 2018b). This sense has been acquired most recently among bats and the most ancestral bats on the evolutionary scale used to be more vision-dependent (Jones & Teeling 2006, Teeling 2009, Thiagavel et al. 2018). Boonman et al. (2013) stated that echolocating bats constantly integrate information acquired by echolocation and vision, and that, this sense of echolocation was refined to detect increasingly smaller targets in association with the use of vision, especially for insectivorous bats. Although it has been shown that echolocating bats use both vision and echolocation to locate objects, divert obstacles and to control their approach to the food site (Rother & Schmidt 1982, Joermann et al. 1988, Eklöf & Jones 2003, Orbach & Fenton 2010), gleaning (frugivorous) bats detects and find their food foraging in the passive mode using other sensory cues (olfactory and visual) rather than by echolocation (Kalko & Schnitzler 1998, Gutierrez et al. 2018a).

Bats that have a sophisticated echolocation system tend to exhibit a less developed visual capability, such as insectivorous bats which detect small prey. On the other hand, bats with a good visual acuity tend to have less dependence on echolocation, for example, frugivorous and nectarivorous bats that can distinguish brightness and spectral composition to find different food items (Pedler & Tilley 1969, Pettigrew et al. 1988, Neuweiler 2000, Heffner et al. 2001, Winter et al. 2003, Kim et al. 2008, Müller et al. 2009, Eklöf et al. 2014, Coimbra et al. 2017, Sadier et al. 2018, Simões et al. 2018, Gutierrez et al. 2018b, Kries et al. 2018). These statements corroborate our findings, in which the three species of the genus *Artibeus* showed a high visual acuity. Therefore, the visual capability is related to the echolocation system and also to the feeding guild that each bat belongs to. These characteristics of echolocation and vision apply to fruit-eating bats, and it was observed in a behavioral experiment performed by Gutierrez et al. (2014) showed that the species *A. lituratus* used visual clues to find food resources instead of echolocation.

Another important fact observed in this study is that even bats of different species belonging to the same genus and with similar eating habits, they exhibit different values of visual acuity (Table 1). This feature may be related to the size of the body of each bat and consequently to the size of their eyes that influences the value of retinal magnification factor (RMF) (Blackwood et al. 2010, Eklöf et al. 2014, Veilleux & Kirk 2014). This relation was observed at least among the three species of the genus *Artibeus* studied. The species *A. lituratus*, which has a total length ranging from 8.6-12cm and an average bodyweight of 75g (Guerrero et al. 2003, Araújo & Langguth 2010) showed the highest visual acuity and the highest RMF (33.66μm/degree), while the species *A. planirostris* that has a total length ranging from 7.5-11cm and body mass between 40-69g (Vizotto & Taddei 1973, Hollis 2005) exhibited an intermediate value of visual acuity and RMF (31.92μm/degree) between the three species and the species *A. obscurus* that exhibits an average length of approximately 7.9cm and body mass between 28-52.2g (Marques-Aguiar 1994, Haynes & Lee Jr 2004) showed the lowest visual acuity and the RMF (26.86μm/degree) among them. Thus, the visual acuity and the retinal magnification factor tends to be higher in those species that present larger body size and bigger eyes, at least among those bats which belong to the same genus. However, according to Eklöf et al. (2014), it is neither a rule nor a distinctive pattern.

According to Denzinger & Schnitzler (2013) species of bats with similar adaptations are attributed to the same guilds. Bats that exploit the same types of environmental resources tend to share morphological adaptations regardless of its

phylogeny. This can be demonstrated in the case of those bats of different genera and even of different families that share similar visual acuity values and explore the same food resources. Therefore, it was observed for the bats of the genus *Artibeus* of this study that present similar visual acuity to other frugivorous bats.

Retinal morphometry

The retinas of the three species of *Artibeus* are avascular and this characteristic is in agreement with Bojarski & Bernard (1988) and Neuweiler (2000) who claimed that echolocating bats usually exhibit avascular retinas and the nutrition of this tunic comes exclusively from the blood supply of the choroid and partly from the vitreous body. Moreover, the retinas of the three species studied did not show *tapetum lucidum*, corroborating Ollivier et al. (2004) and Moritz et al. (2013) who concluded that only flying foxes present this structure and microchiropteran bats do not have this reflective membrane and that it is not an indispensable feature for the eyes of nocturnal animals. Furthermore, the relationship between the size of the retinal area and eye axial length was directly proportional for the three species of bats, where the retinal area showed larger for *A. lituratus* and shorter for *A. obscurus*. These morphometric data follow the statements of Blackwood et al. (2010) who say that the retinal area is directly proportional to the size of the eye and its axial length, even to those bats that belong to the same genus.

Regarding to the total thickness of the retinas, it was observed that the retina of *A. lituratus* is thicker in comparison with the other two (Table 2). However, the thicknesses of the retinas for the three species are within the range of variances for echolocating bats, which range between 100 and 150μm (Wassle & Boycott 1991, Neuweiler 2000). In addition, all three species presented the central region of the retina thicker than the peripheral area, and it is due to this region be more important to vision, presenting the highest density of RGCs, photoreceptors and other neurons in general (Pettigrew et al. 1988, Collin 2008, Moore et al. 2012).

The prevalence of rods, mainly in the peripheral area of the retina, highlights the importance of these cells to the nocturnal activity of bats (scotopic vision). On the other hand, it was observed a greater abundance of cones in the central region of the retina indicating the capacity for color perception by these bats of the genus *Artibeus*. Müller et al. (2007) and Feller et al. (2009) observed the same pattern of distribution of photoreceptors in the retina in other echolocating bats. However, to determine the sensitivity of a cone to a specific wavelength, either immunohistochemical or electroretinographic tests are required. Echolocating bats and flying foxes are classified as dichromatic mammals having two types of visual pigments related to color perceptions, cone opsin (S-) and cone opsin (L-) which are associated with the sensitivity to short wavelength and medium-long wavelength, respectively (Hope & Bhatnagar 1979, Müller et al. 2007, Feller et al. 2009, Sadier et al. 2018, Simões et al. 2018, Gutierrez et al. 2018b, Kries et al. 2018). In addition, some species of nectarivorous bats show sensitivity to ultraviolet wavelength with sensitivity peaks at 365 nm and have cones that coexpress the opsins S and L (Winter et al. 2003, Müller et al. 2009).

The average number of photoreceptors nuclei layers might be directly related to the size of the outer nuclear layer (ONL). The species *A. lituratus* showed 10 layers of nuclei and the thickest ONL, while the species *A. obscurus* exhibited 07 layers of nuclei and the lowest thickness of the ONL. Ghassemi & Gholami (2015) observed that the species *Rhinopoma hardwickii* has 08 layers of photoreceptors nuclei in the retina, even it being an insectivorous species of large size. Therefore, the number of photoreceptors nuclei layers varies according to the species, but it tends to be higher for frugivorous bats (Blackwood et al. 2010).

The three species of bats exhibited a smaller thickness of the outer plexiform layer (OPL) in the central region of the retina by cause of the axons of the photoreceptors, essentially formed by rods, are shorter in this region due to this area be more sensitive to light (Masland 2001, Kolb et al. 2001). Although the inner nuclear layer (INL) was thicker in the central region of the retina of *A. planirostris* and *A. obscurus*, it is not a discrepant difference (Table 2). The inner plexiform layer (IPL) showed much thicker than the outer plexiform layer with little variations among the species of *Artibeus* studied. This layer is thicker than the OPL because it is the site of synapses between bipolar neurons and ganglion cells mediated by amacrine cells (Wassle & Boycott 1991, Rodrigues 2010).

The ganglion cell layer (GCL) exhibited a difference in the total number and density of RGCs among the three species (Table 1). This layer showed significantly thicker in the central region for all bats, remaining constant throughout the retina until decreasing its thickness in the peripheral area (Table 2). Furthermore, the quantity of RGCs was higher in the central area of the retina compared to the periphery as well (Fig.1). These data reinforce the idea of photoreceptor-RGCs proportions suggested by Collin (2008) and Hauzman et al. (2014) where the area of the retina that presents the highest density of RGCs will also have a higher density of cones and consequently a greater visual acuity and this characteristic was evidenced for the species of *Artibeus* in this study. The RGCs presented cell bodies of different sizes, which suggests that there are different types of RGCs in the retina of these bats (Fig.2). This differentiation in size and types of RGCs was also evidenced by Oyster et al. (1981), Pettigrew et al. (1988), Dacheux & Raviola (1994), and Brooks et al. (1999). Regarding the estimation of the total number of RGCs, which varied significantly between the three species even belonging to the same genus (Table 1), it may be directly related to the retinal area and eye size (Blackwood et al. 2010, Eklöf et al. 2014).

Finally, the thickness of the nerve fiber layer (NFL) for the three bats followed the pattern of vertebrate animals observed, where the retinal NFL thickness decreases from the optic disk (central region) toward the periphery of the retina following the decrease in the number of RGCs (Qijiu et al. 1981, Brooks et al. 1999). As a consequence of the greater number of RGCs in the central retina, the NFL of the species *A. lituratus* reveled thicker in this region, whereas *A. obscurus* showed the smallest thickness among these bats. In contrast, there was no significant difference in the thickness of this layer in the peripheral region between the three species of *Artibeus*, probably due to the low density of RGCs in this area (Table 2).

These histomorphometric data corroborate the information suggested by Jolicoeur & Baron (1980) who claimed that

frugivorous bats have larger eyes and a greater visual capability compared to insectivorous species. In addition, the visual capacity of echolocating bats is directly associated with the echolocation system and related to the eating habits of each species (Rydell & Eklöf 2003, Boonman et al. 2013, Denzinger & Schnitzler 2013). Regarding the fruit-eating bats that exhibit a good visual acuity and color perception, Veilleux & Kirk (2014) concluded that these visual features make those animals able to improve the success of foraging, feeding on specific fruits and increases the success in locating shelters and the chances to detect predators. Therefore, such evidence suggests that vision plays a very important role for these three fruit-eating bats of the genus *Artibeus*.

CONCLUSION

Bats of the genus *Artibeus* have a high visual acuity compared to other echolocating bats already described in the literature. The morphophysiological differences found between the retinas of the three bats might be related mainly due to the number of neurons, especially of retinal ganglion cells (RGCs), and the size of the eyes that consequently influenced in the retinal magnification factor (RMF) resulting in different values of visual acuity. The species *Artibeus lituratus* exhibited the greatest values of visual acuity and the highest number of RGCs distributed throughout the retina being arranged in a horizontal visual streak in the central area. The other two species showed a similar pattern of cell distribution, but the species *Artibeus obscurus* showed the lowest values of visual acuity and number of RGCs, whereas the species *Artibeus planirostris* demonstrated intermediate values of these parameters. In addition, we presuppose that all these differences among the three species may be also directly linked to the phylogeny of the genus.

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Em 15 de agosto de 2019

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

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

3. **Apresentação das Figuras:**

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

b) Enviar cada figura separadamente.

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

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

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

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

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

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

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

Exemplos de Referências

➢ Artigos publicados em periódicos:

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

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

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

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

- Livros:

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

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

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

- Capítulos de livros:

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

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

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

- Dissertações e Teses:

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

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

- Resumos publicados em eventos:

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

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

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

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GUIDE FOR AUTHORS

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

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

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

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

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

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

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

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

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

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

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

The author rights of the accepted papers are preserved.

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

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

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

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

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

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

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

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

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

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

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

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

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

m) Conflict of interest or none should be mentioned.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3. **Figure presentation:**

a) Save images at 300 dpi, TIF files.

b) Send each figure separately.

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

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

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

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

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

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

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

Exemples for References:

➢ Articles published in scientific journals:

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

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

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

➢ Books:

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

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

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

➢ Chapters of books:

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

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

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

➢ Dissertations and Theses:

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

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

➢ Abstracts published in Events:

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

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

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

(Note: Consult entire papers instead of only Abstracts)

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