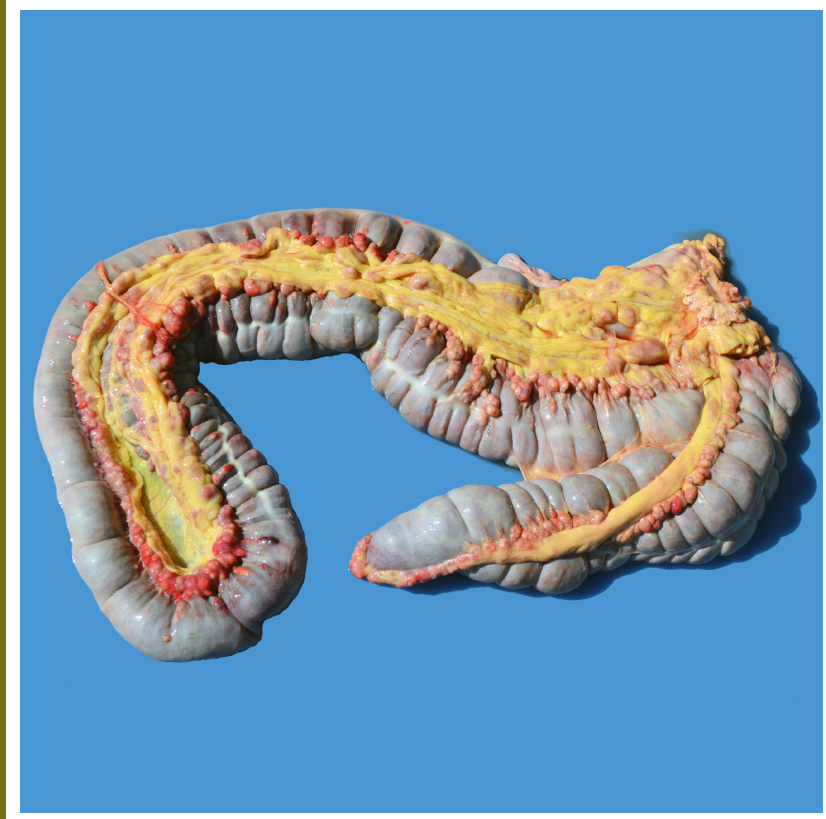
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Cover illustration: Lymphadenitis involving the colonic lymph nodes of a foal naturally infected with *Rhodococcus equi*. (Oliveira et al., p.852)

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Clinical and pathological findings of *Rhodococcus equi* infection in foals[1]

Luiz G.S. Oliveira[2], Tatiane T.N. Watanabe[3], Fabiana M. Boabaid[4], Flademir Wouters[5], Angelica T.B. Wouters[5], Paulo M. Bandarra[6], Priscila R. Guerra[7] and David Driemeier[8]*

ABSTRACT.- Oliveira L.G.S., Watanabe T.T.N., Boabaid F.M., Wouters F., Wouters A.T.B., Bandarra P.M., Guerra P.R. & Driemeier D. 2019. **Clinical and pathological findings of *Rhodococcus equi* infection in foals**. *Pesquisa Veterinária Brasileira 39(11):849-857.* Setor de Patologia Veterinária, Departamento de Patologia Clínica Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil. E-mail: davetpat@ufrgs.br

Infection by *Rhodococcus equi* is considered one of the major health concerns for foals worldwide. In order to better understand the disease's clinical and pathological features, we studied twenty cases of natural infection by *R. equi* in foals. These cases are characterized according to their clinical and pathological findings and immunohistochemical aspects. Necropsy, histologic examination, bacterial culture, *R. equi* and *Pneumocystis* spp. immunohistochemistry were performed. The foals had a mean age of 60 days and presented respiratory signs (11/20), hyperthermia (10/20), articular swelling (6/20), prostration (4/20), locomotor impairment (3/20) and diarrhea (3/20), among others. The main lesions were of pyogranulomatous pneumonia, seen in 19 foals, accompanied or not by pyogranulomatous lymphadenitis (10/20) and pyogranulomatous and ulcerative enterocolitis (5/20). Pyogranulomatous osteomyelitis was seen in 3 foals, one of which did not have pulmonary involvement. There was lymphoplasmacytic (4/20), lymphoplasmacytic and neutrophilic (1/20) or pyogranulomatous arthritis (1/20), affecting multiple or singular joints. Immunohistochemistry revealed to be a valuable tool for the detection of *R. equi*, confirming the diagnosis in all cases. Furthermore, pulmonary immunostaining for *Pneumocystis* spp. demonstrates that a coinfection with *R. equi* and this fungal agent is a common event in foals, seen in 13 cases.

INDEX TERMS: Clinic, patology, *Rhodococcus equi*, infection, foals, equine infectious diseases, pneumonias of foals, rhodococcosis, horses, equine.

RESUMO.- [**Achados clínicos e patológicos da infecção por *Rhodococcus equi* em potros.**] Infecção por *Rhodococcus equi* é considerado um dos maiores problemas sanitários para potros em todo o mundo. Para melhor compreender a apresentação clínica e patológica da enfermidade, foram avaliados vinte casos de infecção natural por *R. equi* em potros. Os casos são caracterizados de acordo com seus achados clínicos e patológicos e aspectos imuno-histoquímicos. Foram realizados exames de necropsia, histologia, bacteriologia e imuno-histoquímica para *R. equi* e *Pneumocystis* spp. Os potros tinham

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[2] Plataforma de Salud Animal, Instituto Nacional de Investigación Agropecuaria (INIA), Ruta 5 Km 386, Tacuarembó, TBO 45000, Uruguay.

[3] Louisiana Animal Disease Diagnostic Laboratory, Louisiana State University, School of Veterinary Medicine, River Road Baton Rouge, Louisiana, 70803, United States of America.

[4] Polo de Desarrollo Universitario del Instituto Superior de la Carne, Universidad de la República (UdelaR), Ruta 5 Km 386, Tacuarembó, TBO 45000, Uruguay.

[5] Setor de Patologia Veterinária, Departamento de Medicina Veterinária, Universidade Federal de Lavras (UFLA), Cx. Postal 3037, Lavras, MG 37200-000, Brazil.

[6] Núcleo de Reabilitação da Fauna Silvestre, Instituto de Biologia, Universidade Federal de Pelotas (UFPel), Campus Capão do Leão s/n, Pelotas, RS 96010-900, Brazil.

[7] Department of Veterinary and Animal Sciences, University of Copenhagen (UCPH), Grønnegårdsvej 15, Frederiksberg, C 1870, Denmark.

[8] Setor de Patologia Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9090, Porto Alegre, RS 95320-000, Brazil. *Corresponding author: davetpat@ufrgs.br

idade media de 60 dias e apresentaram sinais respiratórios (11/20), hipertermia (10/20), aumento de volume articular (6/20), prostração (4/20), distúrbios locomotores (3/20) e diarreia (3/20), entre outros. As lesões mais importantes eram pneumonia piogranulomatosa, vista em 19 potros, acompanhada ou não por linfadenite piogranulomatosa (10/20) e enterocolite ulcerativa (5/20). Osteomielite piogranulomatosa foi constatada em três potros, um dos quais não apresentava envolvimento pulmonar. Artrites afetando uma ou múltiplas articulações eram caracterizadas por infiltrado linfoplasmocítico (4/20), linfoplasmocítico e neutrofílico (1/20) e piogranulomatoso (1/20). A imuno-histoquímica demonstrou ser uma ferramenta valiosa na detecção de *R. equi*, permitindo confirmar o diagnóstico em todos os casos avaliados. Além disso, a imuno-histoquímica para *Pneumocystis* spp. demonstra que a coinfecção por *R. equi* e o agente fúngico é um evento frequente em potros, constatado em 13 casos.

TERMOS DE INDEXAÇÃO: Clinica, patologia, infecção, *Rhodococcus equi*, potros, doenças infecciosas de equinos, pneumonias em potros, rodococose, equinos.

INTRODUCTION

Rhodococcus equi infection is known worldwide as one of the major sanitary problems affecting foals up to six month old. In humans it is a concern, especially in immunosuppressed patients (Prescott 1987, 1991, Giguére & Prescott 1997). Variable concentrations of the rod-to-coccus gram-positive nocardioform actinomycete can be found in horse farms, but infections occur depending on foal population density, temperature, dust, soil pH, as well as, a high proportion of virulent *R. equi* strains in the soil (Prescott 1991, Takai 1997, Meijer & Prescott 2004).

The virulence of the strain is determined by a group of proteins called "Vap" (virulence-associated proteins), of which VapA is considered the most important for the development of the disease in foals (Takai et al. 1991, Tan et al. 1995, Monego et al. 2009). Secondary and immunosuppressive conditions can interact in the pathogenesis of the disease in foals. Pulmonary infection by the fungus *Pneumocystis* is sometimes seen in association to *R. equi* infection in foals (Ainsworth et al. 1993, Perron Lepage et al. 1999).

The main clinical and pathological feature in foals with *Rhodococcus equi* infection is severe pyogranulomatous pneumonia, frequently accompanied by ulcerative enterocolitis and mesenteric lymphadenitis (Zink et al. 1986, Caswell & Williams 2016). Furthermore, the infection can lead to arthritis, osteomyelitis, hepatic, splenic or skin abscesses, hypopyon, ulcerative lymphangitis, methritis, and abortion in horses (Zink et al. 1986, Szeredi et al. 2006, Caswell & Williams 2016). Histologically, there are extensive areas of necrosis with abundant pyogranulomatous infiltrate and macrophages containing numerous intracytoplasmic cocobacillary bacteria (Johnson et al. 1983, Caswell & Williams 2016). The aims of this study were (I) to describe the clinical and morphological findings of rhodococcosis in foals associated to the immunohistochemical detection of the organism; (II) determine the occurrence of pulmonary coinfection with *Pneumocystis* spp.

MATERIALS AND METHODS

Twenty foals naturally infected by *Rhodococcus equi* were submitted to postmortem examination from 1997 to 2012. Referring veterinarians provided the clinical background. Samples of multiple organs were collected, fixed in 10% formalin, routinely processed for histology and embedded in paraffin. Sections were cut at 3µm and stained with hematoxylin and eosin (HE), Brown-Hopps method, and Grocott's methenamine silver (GMS). Fresh samples of the tissues containing lesions were submitted to bacteriological testing according to standard laboratory protocols (Quinn et al. 2011).

Immunohistochemistry (IHC) for *R. equi* was performed with a polyclonal antibody produced by inoculation of an ATCC strain of *R. equi* in rabbits, at a 1:50 dilution. The antigen retrieval was made by a citrate buffer solution at pH 6.0 for 3 cycles of 5 minutes in a microwave. For the *Pneumocystis* IHC the commercial antibody anti-*Pneumocystis jirovecii* (*carinii*) (3F6): sc-57980 (Santa Cruz Biotecnology®) at a 1:1,000 dilution was used. The antigen retrieval was made by a 0.1% dilution of trypsin for over 15 minutes at 37°C, followed by a citrate buffer solution at pH 6.0 for 40 minutes at 96°C. Afterward, for both techniques, staining was obtained by the streptavidin-biotin immunoperoxidase technique, using 3.3-diaminobenzidine for *R. equi* IHC and 3-amino-9-ethylcarbazole for *P. jirovecii* (*carinii*) (3F6).

RESULTS

History and clinical features

The foals submitted to necropsy belonged to four different horse-breeding farms from Porto Alegre, Brazil. Seventeen out of 20 cases were from a single farm, with history of approximately eight deaths/year attributed to *Rhodococcus equi* infection. The affected foals were of different ages, varying from 30 to 95 days (average 60 days), belonging to different breeds, such as Brazilian Sport Horse (6/20), Holsteiner (6/20), American Quarter Horse (2/20), Brazilian Saddle Horse (1/20), unknown (5/20). No difference in sex was observed (10 females and 10 males). Clinical history was of respiratory signs, such as nasal discharge, cough and dyspnea (11/20); as well as hyperthermia (10/20); prostration (4/20); articular swelling (6/20); locomotor disorders, such as lameness, hindlimb paresis or paralysis (3/20), opacity of the anterior eye chamber (2/20); hyphema (2/20); diarrhea (3/20); and two foals had no clinical signs. The foal's clinical picture is described in Table 1.

Necropsy findings

At necropsy, the foals' body condition ranged from good or fair (17/20) to poor (3/20) (Table 2). Pulmonary lesions were seen in the majority of the cases (19/20) and consisted of multifocal to coalescing nodules ranging from a few millimeters up to 10cm in diameter (Fig.1A), containing a yellowish white creamy material (Fig.1B), accompanied by cranioventral consolidation in some foals. The lungs were also diffusely non-collapsed with rib impressions and elastic consistency in one foal (1/20). Other respiratory lesions included guttural pouch empyema (1/20), guttural pouch tympany (1/20), fibrinosuppurative pleuritis (1/20), pneumothorax associated with pleural bullous emphysema (1/20) and ulcerative tracheitis (1/20). One-half of the foals presented lymphadenitis (10/20), eight of them involving the mesenteric, colonic (Fig.2A) and/or gastric lymph nodes

Table 1. Signalment and clinical signs of the foals with *Rhodococcus equi* infection

Case	Age (days)	Sex	Breed	Main clinical signs
1	60	F	Quarter Horse	Prostration, anorexia, nasal purulent discharge, respiratory signs, hyperthermia
2	60	M	NA	Born weak, respiratory signs
3	90	M	NA	Respiratory signs, hyperthermia
4	45	F	NA	Respiratory signs, hyperthermia
5	30	F	Brazilian Sport Horse	Respiratory signs, nasal purulent discharge, hyperthermia
6	75	F	Brazilian Sport Horse	Respiratory signs
7	60	M	Quarter Horse	Underdeveloped, ocular opacity, blindness
8	45	F	NA	Found dead
9	95	M	Brazilian Saddle Horse	Underdeveloped, respiratory signs, prostration, staggering, walking in circles
10	45	F	Holsteiner	Not stated
11	60	M	Brazilian Sport Horse	Hindlimb paralysis, hyperthermia
12	30	M	Holsteiner	Dyspnea, cough, multiple articular swelling, hyphema, hyperthermia, prostration, hindlimb paresis
13	70	M	Holsteiner	Prostration, hyperthermia, hyphema, tarsal articular swelling, diarrhea, superficial lymphadenomegaly
14	75	F	Brazilian Sport Horse	Right hindlimb lameness with swelling and fistulation of the right tarsal joint; fluctuating nodule on the left retromandibular region
15	60	M	Brazilian Sport Horse	Respiratory signs, hyperthermia
16	52	F	Holsteiner	Respiratory signs, multiple articular swelling
17	81	M	Holsteiner	Chronic intermittent diarrhea accentuating at the final stage
18	60	F	Holsteiner	Cough, hyperthermia, carpal joint swelling
19	40	F	Brazilian Sport Horse	Found dead in a paddock presenting cases of acute diarrhea in foals
20	60	M	NA	Chronic intermitent diarrhea, multiple articular swelling, hyperthermia, ocular opacity

F = female, M = male, NA = not available.

Table 2. Macroscopical and histological lesions in the foals naturally infected with *Rhodococcus equi*

Case	BCS	Respiratory system and thoracic cavity	Non-respiratory
1	G	PGP, fibrinosuppurative pleuritis and guttural pouch empyema	Hydroperitoneum and jejunoileal intussusception
2	G	PGP	None
3	G	PGP, PU tracheitis	None
4	G	PGP, IP	None
5	G	PGP, IP, tracheobronchial PGL	Mesenteric PGL
6	P	PGP	Mesenteric PGL. PU typhlocolitis with colonic PG lymphangitis. FN glossitis, esophagitis, and gastritis.
7	G	PGP	Mesenteric PGL, hypopyon
8	G	PGP	None
9	G	PGP, tracheobronchial PGL	Inguinal hernia with segmental jejunal incarceration (15cm long)
10	G	PGP	PU typhlocolitis
11	P	PGP	PU typhlocolitis, mesenteric PGL. PG osteomyelitis of the second lumbar vertebra with spinal cord compression
12	G	PGP, IP	LP arthritis of the stifle and tarsal joints, hyphema
13	G	PGP, severe pulmonar hemorrhage, tracheobronchial PGL	Multifocal extensive hemorrhages in muscles, subcutaneous, lungs and urinary bladder, hyphema, hemarthrosis and LP arthritis of the tarsal joint. Hepatic and mesenteric PGL, peritoneal adhesions, ulcerative jejunitis and ileitis
14	G	None	PG osteomyelitis of the right side of the atlas, involving the adjacent soft tissues. PG and fibrosing arthritis of the right tarsal joint
15	G	PGP	None
16	G	PGP	Mesenteric PGL, colonic PG lymphangitis, LP and neutrophilic arthritis
17	G	PGP, IP, FN tracheitis, PG osteomyelitis of the third left rib	PU typhlocolitis, mesenteric PGL, FN glossitis, esophagitis and gastritis of the non-glandular portion
18	G	PGP, pneumothorax secondary to bullous emphysema, atelectasis, tracheobronchial PGL	PU typhlocolitis, LP arthritis of the carpal joint
19	G	PGP	FN enterocolitis, mesenteric PGL
20	P	PGP, IP, diffuse pulmonar mineralization. Guttural pouch timpany	Mesenteric PGL, hypopyon, LP arthritis, fibrinous peritonitis, extensive epicardial and endocardial mineralization.

BCS = Body condition score, FN = fibrinous-necrotizing, G = good or fair body condition, IP = interstitial pneumonia, LP = lymphoplasmacytic infiltration, P = poor body condition, PG = pyogranulomatous, PGL = pyogranulomatous lymphadenitis, PGP = pyogranulomatous pneumonia, PU = pyogranulomatous and ulcerative, UL = ulcerative.

and five implicating the tracheobronchial ones. Ulcerative typhlocolitis (Fig.2B) was observed in five cases. One case presented ulcerative enteritis of the distal segment of the jejunum and ileum and one had acute fibrinonecrotic enterocolitis. Two foals had thickened lymphatic vessels on the colonic serosa; one case had jejunoileal intussusception and one additional case had an inguinal hernia containing a 15cm long segment of the jejunum. Fibrinous peritonitis and ascites have been seen in one case each. Swollen joints containing abundant aqueous synovial fluid or hemorrhagic content were seen in the tibiotarsal, femorotibiopatellar or carpal joints (5/20). Alternatively, a fibrous thickening of the tarsal joint capsule, with a caseous exudate draining through a fistula was seen in one case (1/20, Fig.3A). Three foals (3/20) had bone lesions; one foal presented a purulent nodule with a complete destruction of the body of the second lumbar vertebra (Fig.3B), resulting in the compression of the spinal cord; one case consisted of an encapsulated nodule filled with a purulent exudate measuring 20cm on the left wing of the atlas; and the last one had a purulent mass on the costochondral junction of the third left rib. One foal presented a retromandibular fluctuating mass, measuring approximately 20cm in diameter, corresponding to an abscess involving the first cervical vertebra. The eyes were affected in four foals, with hypopyon (2/20) or hyphema (2/20). Additionally, there was one case with disseminated hemorrhages, involving different organs, including the subcutaneous tissue, skeletal muscles, lungs, urinary bladder and the already mentioned anterior chamber of the eye and a tarsal joint. There was also concurrent fibrinonecrotic esophagitis (2/20) with similar lesions in the nonglandular portion of the stomach in one of the foals, affecting the tongue and trachea in the other.

Histopathological findings

In the 19 foals with pulmonary gross lesions, there was multifocal inflammatory infiltrate of neutrophils, macrophages and multinucleated giant cells filling the alveolar spaces and

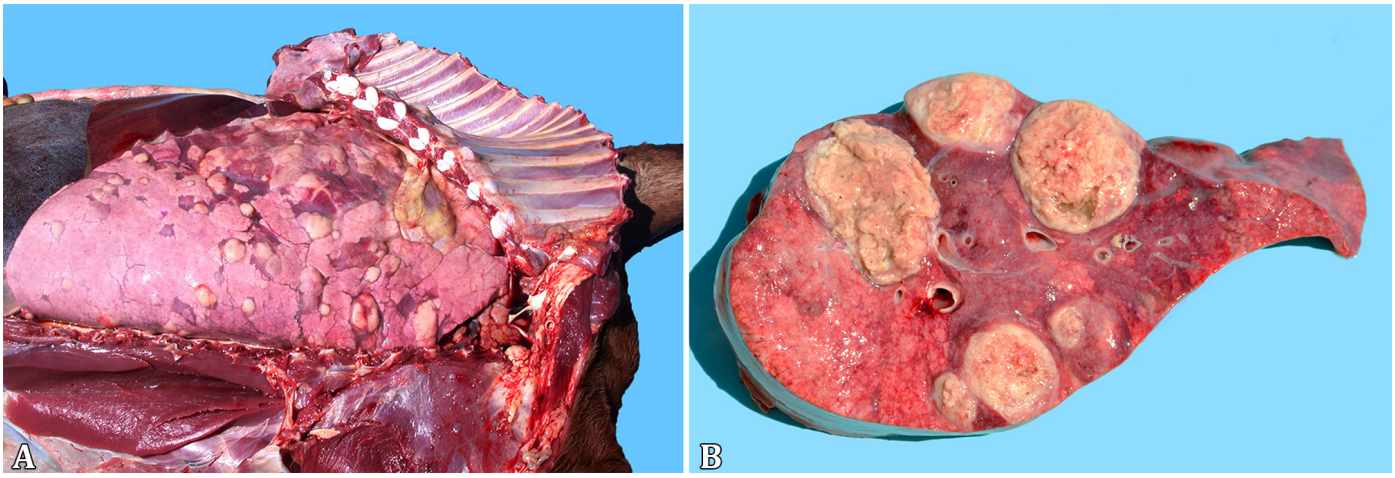


Fig.1. Lungs of *Rhodococcus equi* naturally infected foals. (**A**) Multifocal to coalescing lung nodules, and (**B**) containing a yellowish white creamy material, seen at the cut surface.

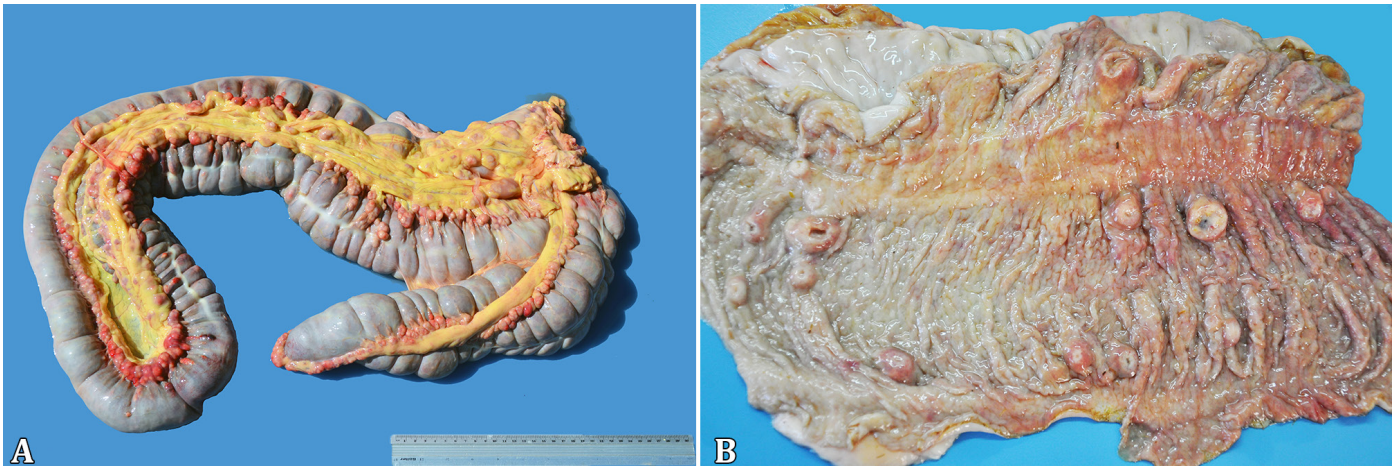


Fig.2. *Rhodococcus equi* naturally infected foals. (**A**) Lymphadenitis involving the colonic lymph nodes. (**B**) Ulcerative typhlocolitis with umbilicated nodules on the mucosa.

bronchioles (Fig.4A), associated with extensive necrosis of the pulmonary parenchyma. The macrophages occasionally contained myriads of cocobacillary basophilic bacteria (Fig.4B), which stained gram-positive with Brown-Hopps technique. The inflammatory infiltrate was sometimes delimitated by a fibrous capsule and also interspersed between the nodules. The alveolar capillaries had large amounts of neutrophils and multifocal thrombosis. There was also interstitial and alveolar edema, sometimes with abundant fibrin deposition. In five cases there was simultaneous interstitial pneumonia, characterized by alveolar walls thickening, due to either proliferation of type 2 pneumocytes as well as mononuclear cells infiltration. In one case, associated with the interstitial lesion, mineralization of the alveolar walls, arterial tunica media, as well as the endocardium was observed. In ten cases, marked pyogranulomatous infiltrate, sometimes containing bacterial laden macrophages was observed in the lymph nodes. Pyogranulomatous infiltrate in the lymphatic aggregates of the large intestine with superficial mucosal ulceration was seen in five cases, eventually accompanied by pyogranulomatous lymphangitis. Fibrinonecrotic enterocolitis with coccobacillary bacteria on the mucosal surface was seen in one case. Three foals had pyogranulomatous osteomyelitis with severe destruction of the bone matrix, involving a cervical and a lumbar vertebra, and a rib of each foal. One case presented pyogranulomatous and fibrosing arthritis of the tarsal joint, one had lymphoplasmacytic and neutrophilic articular infiltrate, and four other cases had lymphoplasmacytic arthritis, with severe hemarthrosis in one of them. There were two cases of hypopyon, with marked fibrinous exudate in the anterior eye chamber and two cases of hyphema. In the trachea of one foal, there

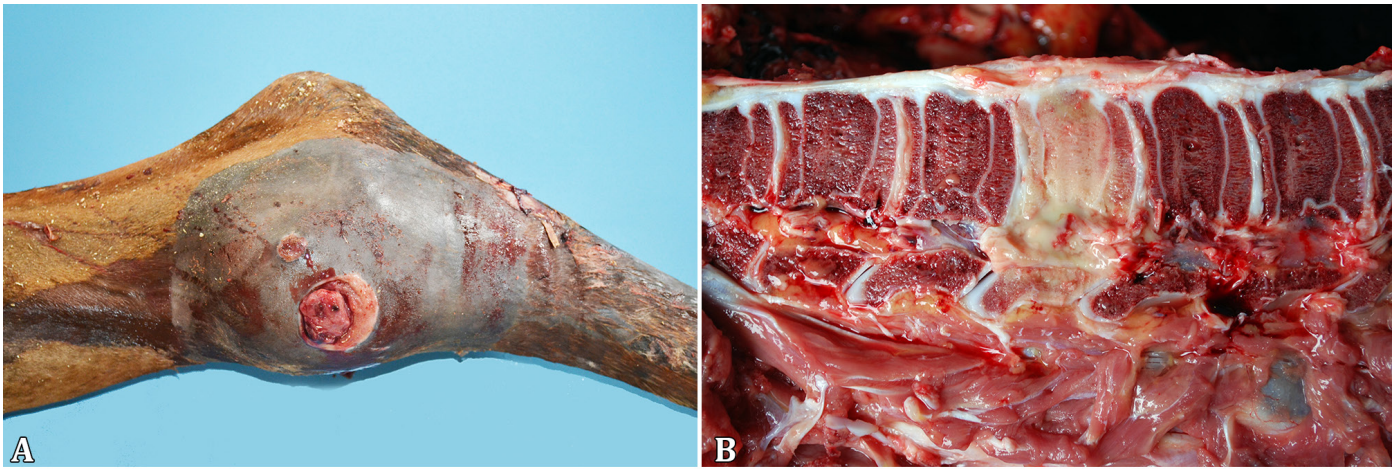


Fig.3. *Rhodococcus equi* naturally infected foals. (**A**) Swollen tibiotarsal joint with an inspissated exudate draining through a fistula. (**B**) Destruction of the body of the second lumbar vertebra.

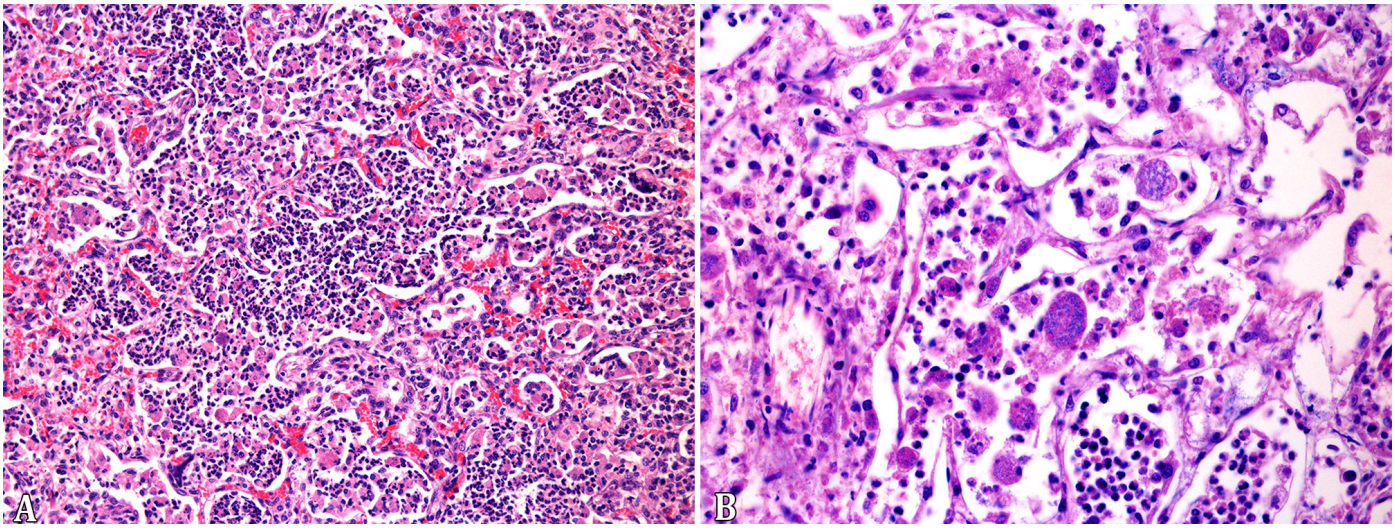


Fig.4. *Rhodococcus equi* naturally infected foals. (**A**) Neutrophils, macrophages, and eventual multinucleated giant cells filling the alveolar spaces and bronchioles. HE, obj.20x. (**B**) Macrophages expanded by myriads of cocobacillary basophilic bacteria. HE, obj.40x.

was a multifocal submucosal pyogranulomatous infiltrate with ulceration of the mucosa. The cases with ulcerative esophagitis, gastritis or tracheitis had multifocal extensive areas of superficial necrosis with neutrophilic infiltrate, interspersed with numerous Grocott positive yeasts and pseudohyphae, compatible with *Candida* spp.

Microbiological tests

Out of the 20 cases, 15 resulted in the isolation of pure cultures of *R. equi* of the lesions. In two foals there was also isolation of *Salmonella* sp. from the intestines. The two cases of ulcerative esophagitis resulted in *Candida albicans* isolation from the esophageal samples.

Immunohistochemistry

The 19 cases of pyogranulomatous pneumonia the intracytoplasmic bacteria seen in the intralesional macrophages were positive for *R. equi* in the IHC test. In addition, the bones of the 3 foals with osteomyelitis as well as the tarsal joint, tracheal and intestinal lesions of all horses with extra-pulmonary pyogranulomatous lesions were positive for *R. equi* (Fig.5A,B). The synovial membranes of four foals with lymphoplasmacytic arthritis and the foal with lymphoplasmacytic and neutrophilic arthritis were all negative. The IHC for *Pneumocystis* revealed 13 positive cases for the fungus, two with large amounts of the microorganism, either free in alveolar spaces or in the cytoplasm of alveolar macrophages (Table 3, Fig.6A,B).

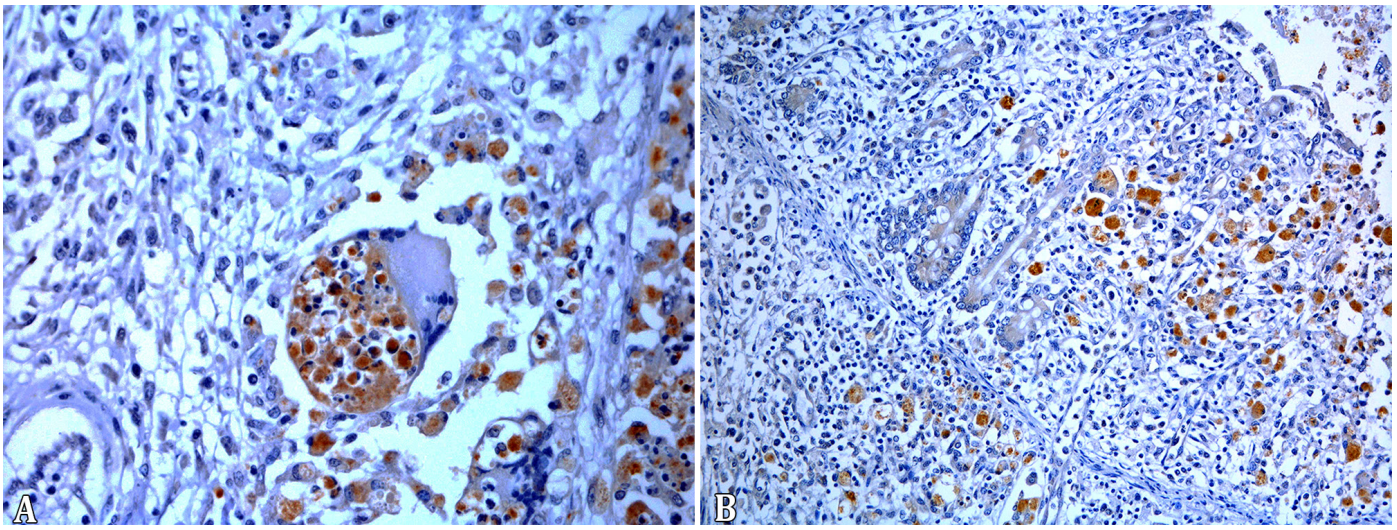


Fig.5. (**A**) Immunolabeled *Rhodococcus equi* in the cytoplasm of intralesional macrophages in pyogranulomatous pneumonia. Streptavidin-peroxidase, chromogen 3,3-diaminobenzidine (DAB), obj.40x. (**B**) Immunolabeled *Rhodococcus equi* in intestinal lesions of naturally infected foals. Streptavidin–peroxidase, chromogen 3,3-diaminobenzidine (DAB), obj.20x.

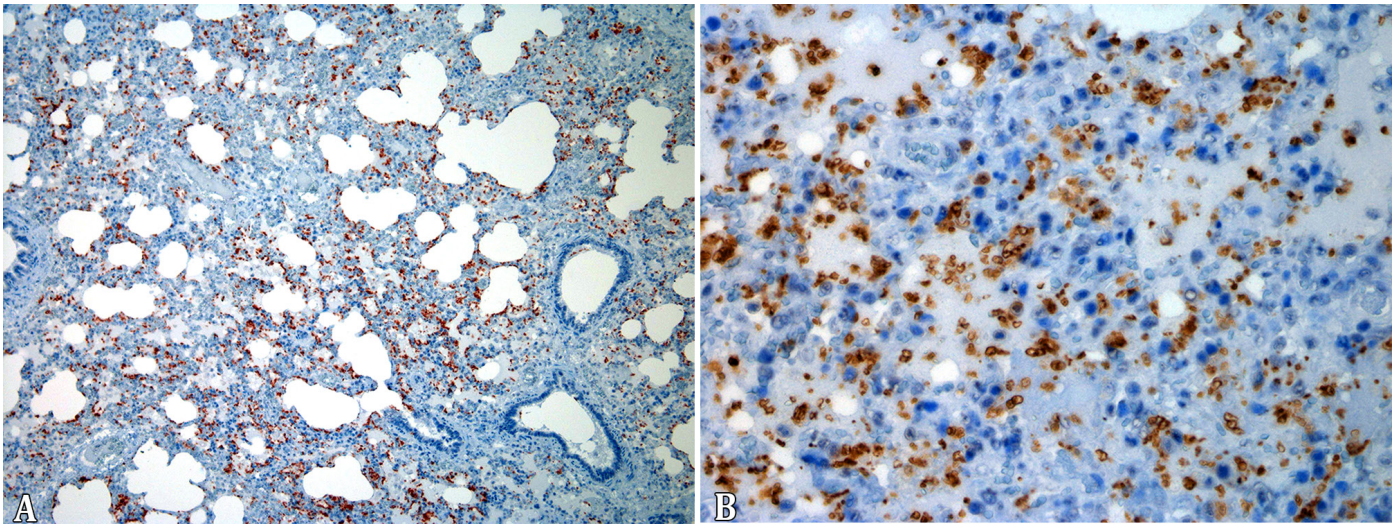


Fig.6. (**A**-**B**) Large amounts of the *Pneumocystis* sp., are seen free in alveolar spaces and in the cytoplasm of alveolar macrophages of foals naturally infected by *Rhodococcus equi*. Primary antibody *Pneumocystis jiroveci* (*carinii*) (3F6). Streptavidin-peroxidase, chromogen 3-amino-9-ethylcarbazole (AEC), obj.10x and obj.40x.

Table 3. Immunohistochemical findings for *Pneumocystis* spp. in lungs of foals naturally infected by *Rhodococcus equi*

Case	Immunostaining*	Distribution
1	-	NA
2	-	NA
3	++	Multifocal
4	+	Multifocal
5	+	Multifocal
6	-	NA
7	++	Multifocal
8	-	NA
9	+++	Diffuse
10	++	Multifocal
11	-	NA
12	-	NA
13	-	NA
14	++	Multifocal
15	+	Multifocal
16	++	Multifocal
17	+++	Multifocal to coalescing
18	+	Multifocal
19	+	Multifocal
20	++	Multifocal

NA = Not applicable; * Score: (-) no staining, (+) low or mild, (++) moderate, (+++) marked.

DISCUSSION

The clinical and pathological findings described herein reinforce *Rhodococcus equi* as an important horse pathogen, supporting studies of other countries (Zink et al. 1986, Giguère & Prescott 1997, Sönmez & Gürel 2013). In our cases, all foals came from horse-breeding farms; even though extensively raised workhorses are abundant in the State of Rio Grande do Sul (Costa et al. 2014), indicating that a high populational density can be, indeed, a determinant factor for the occurrence of the disease. An epidemiological study was however not performed to determine the disease prevalence in different types of production.

Pyogranulomatous pneumonia was the most common, single or combined finding, seen in 95% of the examined foals, highlighting the importance of the airborne way of infection. The only case with no respiratory lesions presented osteoarticular infection affecting the first cervical vertebra and the right tarsal joint. This singular case points to alternative routes of dissemination, such as hematogenic spreading (Giguère & Prescott 1997). The most common lesions, in addition to the pulmonary presentation, consist of pyogranulomatous lymphadenitis and pyogranulomatous and ulcerative enterocolitis, seen respectively in 50% and 25% of the cases, similar to other reports described in the literature (Zink et al. 1986, Caswell & Williams 2016). The articular infiltrate was lymphoplasmacytic (20%), lymphoplasmacytic and neutrophilic (5%) or pyogranulomatous (5%). Just in the case with pyogranulomatous infiltrate, microbiology and immunohistochemistry tests allowed the detection of *R. equi*. Lymphoplasmacytic arthritis is thought to be an immune-mediated reaction (Sweeney et al. 1987, Giguère & Prescott 1997), reinforced by the fact that none of the samples yielded bacterial growth nor immunohistochemical expression for *R. equi* at the lesion site. Vertebral bodies are other important sites of infection, resulting in compression of the spinal cord due to a compressive myelopathy, which led to locomotor impairment in one foal. The lack of evident clinical signs in spite of the severity of the lesions in many cases could be explained by the slow progression of the pulmonary lesions, combined to the remarkable capacity of the foal to compensate the progressive loss of viable lung (Giguère & Prescott 1997). The organism was detected in 100% of the cases by immunohistochemistry. This method is known to be a valuable tool in the routine detection of *R. equi* infection (Madarame et al. 1996, Mariotti et al. 2000, Sönmez & Gürel 2013).

Pulmonary infection with *Pneumocystis* revealed to be frequently associated with *Rhodococcus equi* infection in foals, with 65% of the lungs being positive in the immunohistochemical test, even in a case with no pulmonary pyogranulomatous lesion. Lung infection with *Pneumocystis* is thought to be an opportunistic condition in horses, similarly to what is observed in humans with Immunodeficiency Virus infection and in pigs with Porcine Circovirus type 2 infection (Cavallini Sanches et al. 2006, Ueno et al. 2014). However, it is important to emphasize that only four of the 13 foals with *Pneumocystis* infection had histologic evidence of interstitial pneumonia. Similarly, in pigs slaughtered in Brazil, pulmonary infection by *Pneumocystis* spp. is not associated with interstitial pneumonia in a large proportion of the cases (Cavallini Sanches et al. 2007). In our study immunohistochemistry was performed with a monoclonal antibody against *Pneumocystis jirovecii* from human lung; however a molecular investigation was not accomplished to determine the specific identity of the organism. In one case of *Pneumocystis* infection in a foal, the organism found revealed to be distantly related to the organism that infects humans, but close to the *Pneumocystis* spp. found in other hosts (Ueno et al. 2014).

Other concurrent infections observed in this study were, for example, candidiasis of the superior digestive tract (2/20) and enteric infection with *Salmonella* sp. (2/20). One of the cases with *Salmonella* sp. infection had a fibrinonecrotic enterocolitis, typical of salmonellosis in horses (Traub-Dargatz & Besser 2007, Juffo et al. 2016) and the other case had a pyogranulomatous and ulcerative colitis, similar to the enteric lesions due to *R. equi* infection (Traub-Dargatz & Besser 2007). Concomitant infections with microorganisms are considered opportunistic and could be attributed to an imbalance in the digestive tract microbiota secondary to antibiotic or anti-inflammatory therapy as well as environmental stress factors (Traub-Dargatz & Besser 2007, Uzal et al. 2016, Juffo et al. 2016).

Hemorrhagic diathesis (1/20) and soft tissue mineralization (1/20) were also present. The first condition presented as a systemic hemorrhage, involving the musculoskeletal system, eyes, as well as the lungs and urinary bladder. Although the macroscopic picture could suggest purpura, which is a possible complication of *Rhodococcus equi* infection in foals, the lack of microscopic evidence of vascular necrosis ruled out the diagnosis (Pusterla et al. 2003). Disseminated intravascular coagulation (DIC) is another hypothesis since it has been seen in foals intratracheally inoculated with *R. equi*. Albeit

the authors of this experiment considered unlikely that sepsis due to *R. equi* could lead to DIC (Johnson et al. 1983). The pathogenesis involving the pulmonary, epicardial, and endocardial mineralization seen in one foal remains speculative, but this finding could be attributed to hypercalcemia, a consequence of an upregulated production of activated vitamin D_3 by macrophages in granulomatous diseases, as demonstrated in bovine paratuberculosis (Driemeier et al. 1999). Diffuse pulmonary mineralization has also been seen in cases of salmonellosis in foals (Juffo et al. 2016).

CONCLUSIONS

Rhodococcus equi infection is an important sanitary problem of foals in our region, due to the severity of the lesions involving the respiratory and alimentary systems, as well as the locomotor apparatus.

Immuno-histochemistry was an important tool to reach the detection of *R. equi* infection and helped to elucidate the pathogenesis of different lesions, such as septic or immune-mediated arthritis.

Furthermore, infection with *Pneumocystis* shows to be frequent in foals infected with *R. equi* in the laboratory's range.

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

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Retrospective study of radiographic changes in athletic bulls with orthopedic disorders[1]

Bruno F. Cholfe[2], José R.B. Silva[3], Alfredo M. Filho[2], Caroline G. Souza[2], Antônio M.T. Filho[2], Silvia H.V. Perri[4], Luiz C. Vulcano[3] and Celso A. Rodrigues[3]*

ABSTRACT.- Cholfe F.B., Silva J.R.B., Filho A.M., Souza C.G., Filho A.M.T., Perri S.H.V., Vulcano L.C. & Rodrigues C.A. 2019. **Retrospective study of radiographic changes in athletic bulls with orthopedic disorders.** *Pesquisa Veterinária Brasileira 39(11):858-862.* Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rua Prof. Dr. Walter Maurício Correa s/n, Cx. Postal 560, Distrito de Rubião Júnior, Botucatu, SP 18 618-682, Brazil. E-mail: celso.a.rodrigues@unesp.br

The objectives of this study were to demonstrate the lesions that affect athlete bulls and to correlate the disorders with weight, age, affected limb and region of the limb. The present study was accomplished using radiographic images of athletic rodeo bulls collected from the medical and surgical records of the large animal service at the veterinary hospital. Radiographic images were evaluated for 136 bulls that were taken care of at the Veterinary Hospital, ranging in age from 4 to 13 years, with an average weight of 800kg and proven prior physical activity through participation in rodeos. The chi-square or Fisher's exact test was used to assess the association between the studied variables. It was observed that 71.6% of the bulls studied and suffering from lameness had radiographic lesions, predominantly in experienced animals. Enthesopathy in starter and experienced bulls, septic arthritis in starter bulls, and fractures and degenerative joint disease in experienced bulls were the most frequent radiographic lesions diagnosed. The region of limb where the majority of radiographic changes occurred was the digits. Risk factors and occurrence of diseases of the locomotor system in athletic bulls are similar to those in sport equines.

INDEX TERMS: Retrospective study, radiographic changes, athletic bulls, orthopedic disorders, bovine, osteoarthritis, bull, X-ray, joint diseases.

RESUMO.- [**Estudo retrospectivo das alterações radiográficas de touros atletas com doenças ortopédicas.**] O objetivo desse trabalho foi demostrar quais lesões acometem touros atletas, correlacionando as desordens com peso, idade, membro e região afetada. O presente estudo foi realizado utilizando radiografias de touros atletas de rodeio, após levantamento dos prontuários clínicos e cirúrgicos do serviço de grandes animais de um hospital veterinário. Foram avaliadas radiografias de 136 touros que haviam sido atendidos em tal hospital, com idade variando de quatro a 13 anos, com média de peso de 800kg e realizavam participação em rodeios. Para avaliar a associação entre as variáveis estudadas foi utilizado o teste exato de Fischer e o Qui-quadrado. Observou-se que 71,6% dos touros estudados que claudicavam, apresentavam lesões radiográficas, predominantemente em animais mais experientes. A entesiopatia em todos os animais, artrite séptica nos touros iniciantes, fraturas e doença articular degenerativa nos touros mais experientes, foram as alterações radiográficas mais frequentes. As regiões com maior ocorrência de lesões radiográficas foram os dígitos. Os fatores de risco para ocorrência de doenças no sistema locomotor de touros atletas são diversos, similares aos que ocorrem nos equinos atletas.

TERMOS DE INDEXAÇÃO: Estudo retrospectivo, alterações radiográficas, touros atletas, doenças ortopédicas, bovinos, osteoartrite, touro, raio-x, doenças articulares.

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[2] Departamento de Cirurgia de Grandes animais, Centro Universitário de Rio Preto (Unirp), Rodovia BR-153 Km 69, São José do Rio Preto, SP 15093-450, Brazil.

[3] Faculdade de Medicina Veterinária e Zootecnia (FMVZ), Universidade Estadual Paulista "Júlio de Mesquita Filho" (Unesp), Rua Prof. Dr. Walter Maurício Correa s/n, Cx. Postal 560, Distrito de Rubião Júnior, Botucatu, SP 18618-682, Brazil. *Corresponing author: celso.a.rodrigues@unesp.br

[4] Departamento de Apoio, Produção e Saúde Animal, Faculdade de Medicina Veterinária, Unesp, Campus de Araçatuba, Rua Clóvis Pestana 793, Dona Amélia, Araçatuba, SP 16050-680, Brazil.

INTRODUCTION

The appropriate diagnosis and monitoring of joint lesions should make use of diagnostic aid techniques, in addition to specific clinical examination of the locomotor system. Among these, the radiographic, sonographic, cytological and histopathological techniques can be highlighted (Stanek 1997, Parisi & Shakeri 2007, Ulian 2010). Radiographic and ultrasonographic examination remains the main tools for diagnosis of large animal locomotor system lesions (Parisi & Shakeri 2007, Kofler et al. 2014, Nichols & Lardè 2014).

Considering the importance of radiographic evaluation in cattle, Barnabé (2005) performed a radiographic study of lame cattle, finding foot illnesses according to the following prevalence: degenerative joint disease (28.72%), osteitis (24.22%), periostitis (11.42%), septic arthritis (7.96%) and osteomyelitis (7.26%). The main diseases in cattle in the "free stall" system were digital papillomatous dermatitis, flat sole and heel erosion, while cows in the "tie-stall" system presented sole bleeding, white line disease, heel erosion, double sole, interdigital dermatitis, vertical crack in corneum case, and overgrow toe/weak heels with a greater frequency (Silveira 2009). Borges et al. (2006), while evaluating 70 bovine forelimbs with foot illnesses, found 58% of limbs had osteitis; 32%, aseptic physitis; and 31%, periostitis.

Among the radiographic findings in cattle suffering from septic arthritis, osteolysis at the point of insertion of the deep flexor tendon and osteoperiostitis stand out, with a greater distancing between the articular surfaces of the middle and distal phalanges. In addition to these, irregularities in the articular bone surface are also frequent, with subchondral bone proliferation, periarticular osteophytes and enthesophytes, in the case of the distal portion of the limbs (Pharr & Bargai 1997).

Radiographic findings in degenerative joint disease are characterized by subchondral bone sclerosis, subchondral bone cysts, osteophyte formation, subluxation, articular and intra-articular space narrowing, and periarticular calcification (Bargai et al. 1989, Motta et al. 2017). Periosteal response in cattle is rapid and extensive (Fergusson 1997, Barnabé 2005). This reaction is the result of the spread of septic or traumatic injury to the periosteum. Soft tissue calcification occurs in a few weeks, and periosteal new bone formation results in increased bone diameter, with may lead to animal lameness in acute and chronic (Bargai et al. 1989).

Radiographic examination is an important and efficient tool to assess the changes caused by chronic enthesopathy. Among the many forms of presentation, the changes represented by bone erosions, soft tissue calcification, periosteal neoformation and changes in bone contour stand out (Stanek 1997). The high level of periosteum reactivity in the bovine is an aspect that influences the emergence of radiographic signs of many diseases commonly secondary to trauma and infections. This occurs by stimulating periosteal neoformation in young animals by increasing the radiopacity of the bone. However, these radiographic changes in older animals may be an incidental finding in an animal that has no lameness (Pharr & Bargai 1997). Digits fractures may be traumatic or pathological, open or closed. The pathological usually occur because of extensive bone destruction or in cases of chronic laminitis (Kofler et al. 2014).

Consequently, the objectives of this study were to conduct a retrospective study of radiographic findings of rodeo bulls suffering from lameness and treated at the Veterinary Hospital in order to characterize and quantify the major orthopedic injuries. In addition, the aim is to build a database for research in veterinary medicine for athletic cattle and to correlate the main types of radiographic lesions according to animal weight and age, location in relation to the limb and the region of the affected limb.

MATERIALS AND METHODS

This study was performed using the radiographic findings of 136 athlete bulls suffering from lameness and treated at the Veterinary Hospital "Dr. Halim Atique", of the "Centro Universitário de Rio Preto" (Unirp), located in São José do Rio Preto/SP, from 2005 to 2011.

Initially, the study was conducted as a survey of the records of all athlete bulls treated at the Veterinary Hospital from 2005 to 2011. Later, the study was narrowed to a screening of the records in which the animals were subjected to radiographic examination and had historical and clinical evidence of lameness. The screened records were from 136 athlete bulls from different breeds and owners, ranging in age from 4-13 years, with an average weight of 800kg and proven prior physical activity through participation in rodeos.

After review of the animals' medical records, a case selection was made and the radiographic images were gathered from the collection of the Veterinary Hospital. All analogic radiographs were obtained from the equipment Emik®, R 2000. Medical history was taken and physical examination conducted following hospital protocols. Animals were restrained in a chute with a mobile wall (Beckhauser®) and the radiographic examination was performed.

The radiographic diagnosis criteria used considered the normal anatomical relationship, changes caused by disease in the bone surfaces, joints, insertions of ligaments, tendons and joint capsule, following the method described by Bargai et al. (1989) and Steiner et al. (2010).

Radiographic examinations were performed with portable radiographic equipment and radiographic films of dimensions 18cm x 24cm. The projections used were dorsoplantar or palmar and lateromedial, in order to observe the condition of the distal interphalangeal joints, and proximal to observe the metacarpal/metatarsal phalangeal joint. The radiographic technique used for this region was 55kV exposure and duration of 0.5 seconds. In the joint region of the carpus and tarsus articulation, dorsoplantar or palmar and lateromedial projections were used, with radiographic technique of 65-75kV with a duration of 0.5 seconds. For the humeral-radioulnar and femorotibial-patellar joint a lateromedial projection was used, with the radiographic technique of 75-85kV and duration of 6 to 8 seconds.

For statistical analysis the Chi-square or Fisher's exact test was used to assess the association between the studied variables. Values were considered significant when $p<0.05$. The statistical analysis was performed using the computer program SAS® (Statistical Analysis System).

RESULTS

The physical containment of animals at the mobile wall stock allowed maintaining them in a standing position, without the need for sedation and proved to be suitable for carrying out radiographic examinations. This restraint provided a rapid

and efficient method of handling the bulls, providing safety to both the animal and the veterinarian.

The projections and radiographic techniques proved effective, allowing the best technical analysis because of the good quality of images.

When the variables of age and type of lesion were compared, a correlation was observed. Experienced animals were most affected by injuries, and the most common were, septic arthritis (Fig.1), osteoperiostitis (Fig.2), enthesopathy (Fig.3), degenerative joint disease and fracture. The beginner bulls were less affected by lesions, with the most common being enthesopathy, septic arthritis, fractures and osteoperiostitis (Table 1).



Fig.1. Dorso-plantar radiography evidenced septic arthritis in the proximal interphalangeal joint in the medial foot of right forelimb.

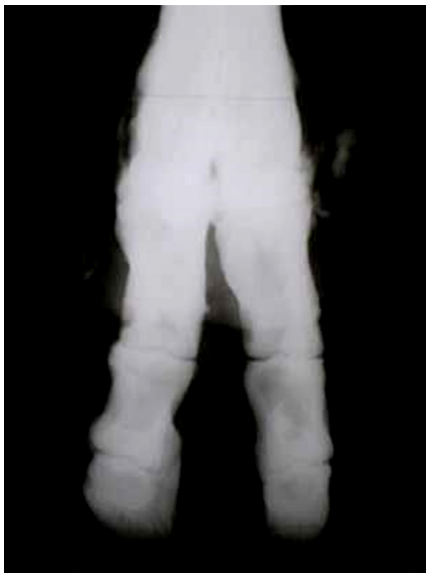


Fig.2. Plantar-dorsal radiography evidenced osteoperiostitis in the 3rd and 4th metatarsi of the left forelimb.

In the analysis of data on the relationship between the affected limb and weight, where we evaluated the categories of age and affected limb, there was a great disparity among the beginner animals in the percentage of animals injured in the forelimb (13.3%) and in the hindlimb (25.8%). In experienced and heavier bulls, the percentage of injuries corresponded to 27.7% in the forelimbs and 32.5% in the hindlimbs.

It was observed that when considering the variables of lesion and area of the affected limb, the most common type of injury in the proximal and distal interphalangeal joint was enthesopathy. While in the metacarpal/ metatarsal phalangeal and carpal joints, osteoperiostitis was the most frequent. In the tarsal joint the most frequent lesions were fractures, which are also more frequent in the humeral-radioulnar joint. Degenerative joint disease was most observed in the femorotibial-patellar joint, while a single case of osteoperiostitis was observed in the 3rd metatarsal joint (Table 2).

DISCUSSION

There are some reports in the literature of retrospective radiographic studies in cattle, especially in dairy and confined animals (Barnabé 2005, Lima et al. 2009) and in bulls (Motta et al. 2017). However, none of these studies evaluated lesions solely related to athlete bulls. Barnabé (2005), in his radiological study with dairy cattle with lameness, found the following prevalence of foot illnesses: degenerative joint disease (28.72%), osteitis (24.22%), periostitis (11.42%), septic arthritis (7.96%) and osteomyelitis (7.26%). In evaluating the limbs from confined animals for slaughter, there were abnormalities in 51 (25.5%) distal limbs evaluated (Lima et al. 2009). Remembering that dairy cattle were exposed to risk factors like as agglomeration, moisture and high ambient contamination. In the athlete bulls of the current study radiographic changes were noted in 71.5% of animals with historical and clinical evidence of lameness.



Fig.3. Plantar-dorsal radiography evidenced enthesophytes in the fetlock and distal interphalangeal joint of the medial and lateral foot, and in insertion of proximal interdigital cruciate ligament of medial foot.

Table 1. Distribution frequency of lesions related of age

		Lesion										
Age		NL	Osteoperiostitis	Enthesophytosis	Septic arthritis	DJD	Fracture	F.B.	C.L.	Ostei./ Osteo.	Osteoarthritis	Total
B	n	19	9	10	10	1	8	-	1	2	-	60
	%	12.6	6.0	6.6	6.6	0.7	5.3	-	0.7	1.3	-	40.3
E	n	24	11	25	5	8	16	1	-	-	1	91
	%	15.9	7.3	16.6	3.3	5.3	10.6	0.7	-	-	0.7	59.7

B = beginners, E = experienced, NL = no lesion, DJD = degenerative joint disease, F.B. = foreign body, C.L. = cysts lesions, Ostei./Osteo. = osteitis/ osteomyelitis. Fisher's exact test (P = 0.0407).

Table 2. Distribution frequency of radiographic images related to affected limb region

		Affected limb region							
		Claw	Fetlock	Carpus joins	Tarsus	Knee	Elbow	3rd metatarsus	Total
No lesion	n	31	2	-	6	4	-	-	43
	%	20.5	1.3	-	4.0	2.6	-	-	27.4
Osteoperiostitis	n	5	8	5	1	-	-	1	19
	%	3.3	5.3	3.3	0.7	-	-	0.7	13.3
Enthesophytosis	n	34	1	-	-	-	-	-	35
	%	22.5	0.7	-	-	-	-	-	23.2
Septic arthritis	n	12	2	-	1	-	-	-	15
	%	7.9	1.3	-	0.7	-	-	-	9.9
DJD	n	1	4	-	1	3	-	-	9
	%	0.7	2.6	-	0.7	2.0	-	-	6.0
Fracture	n	13	2	2	5	-	2	-	24
	%	8.6	1.3	1.3	3.3	-	1.3	-	14.8
Foreign body	n	-	1	-	-	-	-	-	1
	%	-	0.7	-	-	-	-	-	0.7
Cysts lesion	n	-	1	-	-	-	-	-	1
	%	-	0.7	-	-	-	-	-	0.7
Osteitis/Osteomyelitis	n	1	1	-	-	-	-	-	2
	%	0.7	0.7	-	-	-	-	-	1.4
Osteoarthritis	n	-	-	-	-	1	-	-	1
	%	-	-	-	-	0.7	-	-	0.7

DJD = degenerative joint disease. Chi-square test (P < 0.0001).

The frequency of the lesions observed by Lima et al. (2009) were: degenerative joint disease (27.5%), osteitis (19.6%), periostitis (15.7%), septic arthritis of the distal interphalangeal joint (11.8%), deep flexor tendon calcification (7.8%), extensor tendon calcification (5.9%), splinter fracture, arthritis, spondylitis and calcification of the proximal ligament (3.9%). Borges et al. (2006), evaluating 70 animals with foot illnesses, found 58% of limbs had osteitis; 32%, aseptic physitis; and 31%, periostitis. In this study, it is noteworthy that among the athlete bulls, the experienced animals were the most affected by injuries (with 60.2% affected), among which the most common were enthesopathy (16.6%), fractures (10.6%), osteoperiostitis (7.3%), degenerative joint disease (5.3%) and septic arthritis (3.3%). Beginner animals were least affected by injuries (with 39.2% affected); noteworthy injuries were enthesopathy (6.6%), septic arthritis (6.6%), periostitis (6.0%) and fractures (5.3%). These results are the same as in breeding bulls, where a positive correlation between age and degenerative join disease was observed (Motta et al. 2017).

The lesions most often found in dairy and beef cattle in confinement system are traditionally associated with environmental risk factors. These factors subject the claw to trauma or humid conditions and anaerobic bacteria Silveira et al. (2009). However, athlete bulls do high intensity physical activity for a short period of time (8 seconds). Thus, some of the factors differ from those previously described (Nicoletti 2004). Thus, the types of lesions, and their respective locations, better resemble those of performance horses (Ross 2003). Other aspects that explain the high frequency of lesions in the evaluated animals are: trauma related to transportation and rodeo, loading and unloading, fights and nutritional imbalance. To these are added risk factors commonly described for cattle (Ytrehus et al. 2007, Motta et al. 2017).

Possibly the high frequency of enthesophytosis and osteoperiostitis in young animals is due to the high periosteal reactivity associated with trauma and infections (Pharr & Bargai 1997, Nichols & Lardé 2014), which associates to the bovine's increased capacity of periosteal reaction. The radiographic characterization of observed enthesophytosis

and osteophytosis were consistent with those described by other studies (Bargai et al. 1989, Steiner et al. 2010), which report the presence of bone erosions, soft tissue calcification, periosteal neoformation and changes in bone contour. The high prevalence in young animals can still be explained by the greater susceptibility of this age group to detachment micro fractures at the insertion point of tendons and ligaments, predisposing the formation of enthesophytes. The stress on the ligaments, tendons and joint capsule is a major cause of the formation of enthesophytes in horses (Dyson 2003). However, one should carefully evaluate the clinical relevance of this finding, as it might not be the primary cause of lameness. Thus, the presence of enthesophytes and osteophytes in radiographic images of experienced animals can be an incidental finding in animals that do not have lameness, but it reflects the biomechanical stress that these structures had been suffering (Kofler et al. 2014). This consideration was also highlighted by Barnabé (2005), who did not correlate the enthesopathy with lameness in cattle.

In several studies of bovine animal production hindlimbs are most affected by lesions (Fergusson 1997, Nicoletti 2004, Barnabé 2005). The main explanation for this higher incidence of disease in the hindlimb would be due to weight distribution, where the pelvic limbs are responsible for supporting the major portion of the weight (Kofler et al. 2014). This distribution was confirmed in the beginner bulls of this study, which represented 67% of the animals. In this category, there was a wide disparity between the percentage of injuries in the forelimb (13.3%) and hindlimb (25.8%). In experienced and heavier bulls, the percentage of injuries corresponded to 27.7% in the forelimb and 32.5% in the hindlimb.

Another hypothesis for the balance between the percentage of lesions observed between hind- and forelimbs would be a change in weight distribution (Ross 2003). The adult animals, due to athletic activity, visually have higher muscular development in the forelimbs, changing forelimb weight bearing and directly increasing the number of injuries.

Ross (2003) reports that the weight distribution in horses is 60% on the forelimbs and 40 on hindlimbs, so lesions in the forelimbs are more frequent. In horses the rider's weight factor, also has a decisive role' in the occurrence of injuries. However, this factor probably exerts little influence on the incidence of injuries in athlete bulls because the competitor remains briefly mounted on the bull. However, it is observed that only the mainstream rodeo bulls are kept in athletic activity for long periods of time. The presence of extremely muscular forelimbs in athlete bulls and the act of jumping and landing on these limbs result in the distribution and types of injuries that most resemble equine athletes.

CONCLUSIONS

It was observed that 71.6% of the studied bulls suffering from lameness had radiographic lesion, these being predominant in experient animals. Enthesopathy in young and adult bulls, septic arthritis in young, fractures and degenerative joint disease in adults, were the most frequent radiographic lesions diagnosed. Radiographic lesions found in athlete bulls differ in part from other types of cattle production.

The occurrence of most locomotor system disorders in the rodeo bulls studied were related to intensity of exercise and athletic life.

Conflict of interest statement.- The authors have no conflict of interest.

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Original Article
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Experimental poisoning by *Crotalaria lanceolata* and *Crotalaria pallida* seeds in broilers[1]

Thaiza Savaris[2], Claudia P. Biffi[3], Daiane Ogliari[3], Nathalia Wicpolt[3], Franciéli Adriane Molossi[3], Elaine Melchioretto[3], Dale Gardner[4] and Aldo Gava[3]*

ABSTRACT.- Savaris T., Biffi C.P., Ogliari D., Wicpolt N., Molossi F.A., Melchioretto E., Gardner D. & Gava A. 2019. **Experimental poisoning by *Crotalaria lanceolata* and *Crotalaria pallida* seeds in broilers.** *Pesquisa Veterinária Brasileira 39(11):863-869.* Laboratório de Patologia Animal, Centro de Ciências Agroveterinárias, Universidade do Estado de Santa Catarina, Av. Luiz de Camões 2090, Conta Dinheiro, Lages, SC 88520-000, Brazil. E-mail: aldo.gava@udesc.br

Crotalaria lanceolata E. Mey. and *Crotalaria pallida* Aiton. are leguminous plants of family Fabaceae found in most of the Brazilian territory. They were initially used as green manure and due their easy spread they are currently considered weeds in crops. Soybean and corn contamination can occur through the mechanical harvesting of these grains along with seeds of the *Crotalaria* species, which end up in the formulation of feed for production animals. *Crotalaria* spp. genus has toxic pyrrolizidine alkaloids (PA). Most plant species belonging to this genus can cause acute or chronic liver injury. In a first stage, one-day old broilers were divided into three groups: Group A (*C. pallida* seeds), Group B (*C. lanceolata* seeds), and Group C (Control). Groups A and B were divided into five subgroups, each with eight broilers, which received the following doses of the respective seeds in feed as of the 7th day of age: daily doses of 0.4%, 0.8% and 2.5%, and single doses of 15% and 25%. Four broilers in each study group were euthanized at 28 days of age - completing 21 days of seed consumption, and the four remaining broilers were euthanized at 42 days of age - completing 35 days of seed consumption. In a second stage, experiments were conducted using seeds of both the aforementioned plants with 28-day old broilers. These were divided into three groups of four animals each: Group D (*C. pallida* seeds) and Group E (*C. lanceolata* seeds), which received the respective seeds at daily doses of 1% and 2% in feed for 20 days, and Group F (Control). These broilers were euthanized when they were 80 days old. *C. lanceolata* seeds showed higher toxicity to broilers than *C. pallida* seeds, both supplied as of the 7th day of life. Clinical signs included inappetence, ruffled feathers, and brown diarrhea. The following gross lesions were observed: subcutaneous edema, ascites, hydropericardium, yellowish liver with hypertrophy or atrophy and enhanced lobular pattern, and distended gallbladder. Histologic lesions present in all birds in varying degrees were characterized by tumefaction and vacuolar degeneration of hepatocytes. The following clinical conditions and gross lesions were observed in the broilers: hepatocyte megalocytosis and karyomegaly, slight biliary epithelial hyperplasia, eosinophilic spheroids, and nuclear invagination with loss of hepatocyte cord architecture.

INDEX TERMS: Experimental poisoning, *Crotalaria lanceolata* E. Mey., *Crotalaria pallida* Aiton., Fabaceae, hepatotoxic plants, pyrrolizidine alkaloids, *Crotalaria* sp., broilers, toxic plants, poisoning, toxicoses.

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[2] Universidade do Estado de Santa Catarina (UDESC), Av. Luiz de Camões 2090, Conta Dinheiro, Lages, SC 88520-000, Brazil.

[3] Laboratório de Patologia Animal, Centro de Ciências Agroveterinárias (CAV), UDESC, Av. Luiz de Camões 2090, Bairro Conta Dinheiro, Lages, SC 88520-000. *Corresponding author: aldo.gava@udesc.br

[4] Poisonous Plant Research Laboratory, Agricultural Research Service (ARS), United States Department of Agriculture (USDA), Logan, Utah, USA.

RESUMO.- [Intoxicação experimental pelas sementes de *Crotalaria lanceolata* E. Mey. e *Crotalaria pallida* Aiton. em frangos de corte.] *Crotalaria lanceolata* E. Mey. e *Crotalaria pallida* Aiton. são leguminosas da família Fabaceae presentes na maioria do território brasileiro. Inicialmente foram utilizadas como adubação verde e devido sua fácil disseminação são consideradas invasoras de culturas. Através da colheita mecanizada da soja e milho pode ocorrer a contaminação destes grãos com sementes dessas espécies e entrar na formulação de ração para animais de produção. O gênero *Crotalaria* spp. possui alcaloides pirrolizidínicos de ação tóxica. A maioria das espécies desse gênero causam lesões hepáticas, com evolução aguda, ou, crônica. Frangos de corte de um dia de vida foram divididos em três grupos: Grupo A (sementes de *C. pallida* - doses diárias de 0,4%, 0,8%, 2,5% e doses únicas de 15% e 25%), Grupo B (sementes de *C. lanceolata* - doses diárias de 0,4%, 0,8%, 2,5% e doses únicas de 15% e 25%) e Grupo C (Controle). Os Grupos A e B foram divididos em cinco subgrupos, com oito frangos cada, que a partir do sétimo dia de vida, receberam as doses estabelecidas. Quatro frangos de cada grupo foram sacrificados aos 28 dias, e os quatro restantes aos 42 dias de vida. Também foram conduzidos experimentos com as sementes dessas duas plantas com aves de 28 dias, as quais foram divididas em três grupos: Grupo D (*C. pallida* - doses de 1% e 2% diariamente, durante vinte dias), Grupo E (*C. lanceolata* - doses de 1% e 2% diariamente, durante vinte dias) e Grupo F (Controle). Cada grupo composto por quatro aves. Estas aves foram sacrificadas ao completarem 80 dias de vida. Sementes de *C. lanceolata* demonstraram maior toxicidade para frangos de corte do que sementes de *C. pallida.* Os sinais clínicos foram inapetência, penas arrepiadas e diarreia acastanhada. Na macroscopia observou-se edema subcutâneo, ascite, hidropericárdio, fígado de coloração amarelada com hipertrofia e, ou, atrofia, evidenciação do padrão lobular e vesícula biliar distendida. As lesões histológicas presentes em todas as aves, em diferentes graus caracterizaram-se por tumefação e degeneração vacuolar de hepatócitos. Nas aves que manifestaram alterações clínicas e lesões macroscópicas, havia megalocitose, cariomegalia, hiperplasia do epitélio biliar, leve, esferoides eosinofílicos e invaginação nuclear com perda da arquitetura dos cordões de hepatócitos.

TERMOS DE INDEXAÇÃO: Intoxicação experimental, *Crotalaria lanceolata* E. Mey., *Crotalaria pallida* Aiton., Fabaceae, plantas hepatotóxicas, alcaloides pirrolizidínicos, intoxicação por *Crotalaria* sp., frangos de corte, toxicoses.

INTRODUCTION

Genus *Crotalaria* spp., family Fabaceae is distributed throughout the Brazilian territory, with 42 known species (Flores 2015, Garcia et al. 2013). Plants of this genus have pyrrolizidine alkaloids (Williams & Molyneux 1987, Cheeke 1998) that are toxic not only to animals (Nobre et al. 2005), but also to humans (Wiedenfeld & Edgar 2011, Fu et al. 2004). Although most of them cause acute or chronic liver injuries, some species can affect the respiratory system (Tokarnia et al. 2012). Pyrrolizidine alkaloid (PA) poisoning is a significant cause of mortality in Brazilian livestock (Lucena et al. 2010). Spontaneous outbreaks of poisoning have been reported in cattle by the leaves and seeds of *Crotalaria pallida* (Lemos et al. 1997), *Crotalaria retusa* (Nobre et al. 2004b) and *Crotalaria incana* (Queiroz et al. 2013); in sheep by *C. retusa* leaves (Dantas et al. 2004, Riet-correa et al. 2011) and seeds (Anjos et al. 2010), and by *C. pallida* leaves (Borelli et al. 2016); in horses by seeds of *Crotalaria juncea* (Nobre et al. 1994) and *C. retusa* (Nobre et al. 2004a); in donkeys by seeds of *C. juncea* and *C. retusa* (Pessoa et al. 2013); in swine by *Crotalaria spectabilis* seeds (Ubiali et al. 2011). Experimental poisoning has been reproduced in cattle with leaves of *C. pallida* (Tokarnia & Döbereiner 1982) and *Crotalaria anagyroides* (Tokarnia & Döbereiner 1983) and with *C. pallida* seeds (Boghossian et al. 2007), and in swine with *C. spectabilis* seeds (Souza et al. 1997, Torres et al. 1997). Poisoning by *Crotalaria lanceotala* has not been reported in any animal species.

In Brazil, *C. lanceolata* and *C. pallida* are considered sub-spontaneous species (Garcia et al. 2013). The plants used in this study were found in fields cultivated in the municipality of São João do Sul, south coast of Santa Catarina state, and because these seeds are small, they can contaminate cereal grains during mechanized harvesting.

The objective of this study was to evaluate the toxicity of these two plant species, characterize the clinical and pathological conditions of the poisoning, and determine the toxic amounts for broilers.

MATERIALS AND METHODS

Crotalaria lanceolata E. Mey. *and Crotalaria pallida* Aiton. seeds were collected from January to June 2015 in the municipality of São João do Sul, southern coast of Santa Catarina state, Brazil. Plant samples were sent to the Department of Botany of the "Universidade do Estado de Santa Catarina" (UDESC) for identification. The seeds of both species were removed from the pods and dried in oven at 37°C. The samples were weighed daily for three days until they reached constant weight. Samples of the dried seeds were sent to the Poisonous Plant Research Laboratory, Agricultural Research Service (ARS), United States Department of Agriculture (USDA), in Logan, Utah, USA, for analysis and quantification of the specific dosages of pyrrolizidine alkaloids (PA) contained in the seeds used in the experimental study.

Throughout the study period, the birds were inspected daily, having their behavior and feed intake evaluated. At pre-established ages, they were euthanized using the cervical dislocation method, approved by the Federal Council of Veterinary Medicine (CFMV), normative resolution no. 1000, and by the Animal Research and Ethics Committee, Center of Agricultural Sciences (CAV), UDESC under protocol no. 6169200716. For histologic examination, samples were collected from all organs, fixed in 10% buffered formalin, and routinely processed.

First stage of the experimental study. Eighty-eight Cobb broiler chickens were housed at one day of age and fed water and feed *ad libitum* until the 7th day of life. As of this date, they were divided into three large groups: Group A, fed *C. pallida* seeds added to the ration at daily doses of 0.4% (A1), 0.8% (A2), 2.5% (A3) and single doses of 15% (A4) and 25% (A5), totaling five subgroups with eight broilers each (40 birds in total); Group B, fed *C. lanceolata* seeds added to the ration at daily doses of 0.4% (B1), 0.8% (B2), 2.5% (B3) and single doses of 15% (B4) and 25% (B5), totaling five subgroups with eight broilers each (40 birds in total); Group C (Control), composed of with eight broiler chickens that received seed-free ration throughout the experiment. Four birds randomly chosen from each study group were euthanized for necropsy and

sample collection for histopathologic examination at 28 and 42 days of age, respectively.

Second stage of the experimental study. Twenty Cobb broiler chickens were housed at 28 days of age and fed water and feed *ad libitum* throughout the experimental period, in addition to seeds of *C. pallida* and *C. lanceolata* until 50 days of age. The broilers were also separated into three groups as follows: Group D, fed *C. pallida* seeds added to the ration at daily doses of 1% (D1) and 2% (D2) for 20 days, totaling two subgroups of four broilers each (8 birds in total); Group E, fed *C. lanceolata* seeds added to the ration at daily doses of 1% (E1) and 2% (E2) for twenty days, totaling two subgroups of four broilers each (8 birds in total); Group F (Control), composed of with four broiler chickens that received free-seed ration throughout the experiment. At 80 days of age, 30 days after the end of seed supply in the feed, all birds were euthanized for necropsy and material collection for histopathologic examination.

RESULTS

First stage of the experimental study. *Crotalaria pallida*: Only birds from Group A3 showed signs of inappetence and brownish diarrhea from the second week of the seed supply. There was no mortality in the groups. Macroscopic and microscopic lesions are shown in Table 1 and 2.

Crotalaria lanceolata: Groups B2 and B5 showed signs of inappetence, ruffled feathers, and brownish diarrhea from the second week of the seed supply. There was no mortality in the groups. Macroscopic and microscopic lesions are described in Table 3 and 4, respectively.

Table 1. Gross lesions observed in broilers euthanized at 28 and 42 days of age. Experimental poisoning by seeds of *Crotalaria pallida*, Group A (1, 2, 3, 4 and 5)

Lesion	28 days					42 days				
	Group									
	A1 0.4%	A2 0.8%	A3 2.5%	A4 15%SD*	A5 25%SD*	A1 0.4%	A2 0.8%	A3 2.5%	A4 15%SD*	A5 25%SD*
Ascites	-	-	-	-	-	¼	-	-	-	-
Hydropericardium	¼	-	2/4	-	-	-	2/4	-	-	¼
Distended gallbladder	¼	-	¼	-	-	-	¼	¼	-	¼
Hepatic hypertrophy	¼	¼	¼	¼	¼	¼	2/4	¼	¼	2/4
Subcutaneous edema	-	-	-	¼	-	¼	-	-	¼	-
Enhanced lobular pattern liver	-	-	-	-	-	-	-	¼	-	-
Yellowish liver	-	-	¼	¼	-	¼	-	-	-	-

* SD = single dose.

Table 2. Microscopic lesions observed in broilers euthanized at 28 and 42 days of age. Experimental poisoning by seeds of *Crotalaria pallida*, Group A (1, 2, 3, 4 and 5)

Lesion	28 days					42 days				
	Group									
	A1 0.4%	A2 0.8%	A3 2.5%	A4 15%SD*	A5 25%SD*	A1 0.4%	A2 0.8%	A3 2.5%	A4 15%SD*	A5 25%SD*
Pulmonary edema	-	-	-	-	-	-	-	-	-	-
Vacuolar hepatocyte degeneration	-	-	-	-	-	-	+	+	-	-
Hepatocyte megalocytosis	-	-	-	-	-	-	-	-	-	-
Hepatocyte karyomegaly	-	-	-	-	-	-	+	++	-	+
Hepatocyte swelling	-	-	-	-	-	-	-	+	-	+
Nuclear hepatocyte invagination	-	-	-	-	-	-	-	-	-	-

* SD = single dose; + mild, ++ moderate, +++ severe.

Table 3. Gross lesions observed in broilers euthanized at 28 and 42 days of age. Experimental poisoning by *Crotalaria lanceolata* seeds, Group B (1, 2, 3, 4 and 5)

Lesions	28 days					42 days				
	Group									
	B1 0.4%	B2 0.8%	B3 2.5%	B4 15%SD*	B5 25%SD*	B1 0.4%	B2 0.8%	B3 2.5%	B4 15%SD*	B5 25%SD*
Ascites	-	-	-	-	-	-	-	-	¼	-
Hydropericardium	-	¼	¼	¼	-	-	¼	2/4	2/4	¾
Distended gallbladder	-	-	-	-	¼	-	¼	¼	¼	-
Hepatic hypertrophy	2/4	-	2/4	2/4	¼	2/4	-	¼	-	¾
Subcutaneous edema	-	-	2/4	-	¼	-	-	2/4	-	2/4
Enhanced lobular pattern liver	-	-	-	¼	-	-	-	-	-	-
Yellowish liver	-	¼	-	-	¼	2/4	-	-	2/4	-

* SD = single dose.

Table 4. Microscopic lesions observed in broilers euthanized at 28 and 42 days of age. Experimental poisoning by *Crotalaria lanceolata* seeds, Group B (1, 2, 3, 4 and 5)

Lesions	28 days					42 days				
	Group									
	B1 0.4%	B2 0.8%	B3 2.5%	B4 15%SD*	B5 25%SD*	B1 0.4%	B2 0.8%	B3 2.5%	B4 15%SD*	B5 25%SD*
Pulmonary edema	-	-	-	-	-	-	-	-	-	-
Vacuolar hepatocytes degeneration	+	-	+	+	+	-	-	+	-	+
Hepatocyte megalocytosis	+	+	-	-	++	-	-	+	-	-
Hepatocyte karyomegaly	+	+	+	+	++	+	+	+	-	+
Hepatocyte swelling	+	+	-	+	++	+	+	+	-	+
Nuclear hepatocyte invagination	-	-	+	+	+	-	+	-	-	-

* SD = single dose; + mild, ++ moderate, +++ severe.

Table 5. Gross lesions observed in broilers euthanized at 80 days. Experimental poisoning by *Crotalaria pallida* and *Crotalaria lanceolata* seeds, 1% and 2% in the diet, Group D (1 and 2) and Group E (1 and 2)

Lesions	Group			
	D1 1% *C. pallida*	D2 2% *C. pallida*	E1 1% *C. lanceolata*	E2 2% *C. lanceolata*
Ascites	-	-	¼	¼
Hydropericardium	-	-	2/4	2/4
Distended gallbladder	-	2/4	¼	-
Hepatic hypertrophy	-	-	¼	2/4
Hepatic atrophy	-	4/4	¼	¼
Pulmonary edema	-	-	¼	¼
Enhanced lobular pattern liver	-	¼	-	-
Yellowish liver	-	¼	-	¼

Second stage of the experimental study. The broilers that received 1% *Crotalaria lanceolata* (Group E1) in the diet presented apathy, ruffled feathers and inappetence nine days after the end of the seed supply, and one chicken had a bulging abdomen and died fifteen days after the end of the feed supply. The broilers that received 2% *C. lanceolata* (Group E2) presented reduced feed intake, and one chicken died twelve days after the end of the seed supply. The primary gross lesions observed were ascites, hypertrophy, and yellowish liver, whereas the following microscopic lesions were verified: mainly swelling and vacuolar hepatocyte degeneration accompanied by megalocytosis and loss of cord architecture in hepatocytes (Fig.1). The gross and microscopic changes are described in Tables 5 and 6, respectively. The birds in the Control Group showed no clinical signs or pathological conditions. Analysis of the seed samples of the two *Crotalaria* species used in this study revealed presence of toxic pyrrolic esters or dehydropyrrolizidine alkaloids (DHPA). Total alkaloids were quantified from the dry weight of the samples. *C. lanceolata* presented 910µg/g of toxic DHPA, i.e., 0.091% (dry weight), whereas *Crotalaria pallida* showed 737µg/g of toxic DHPA, i.e., 0.0737% (dry weight).

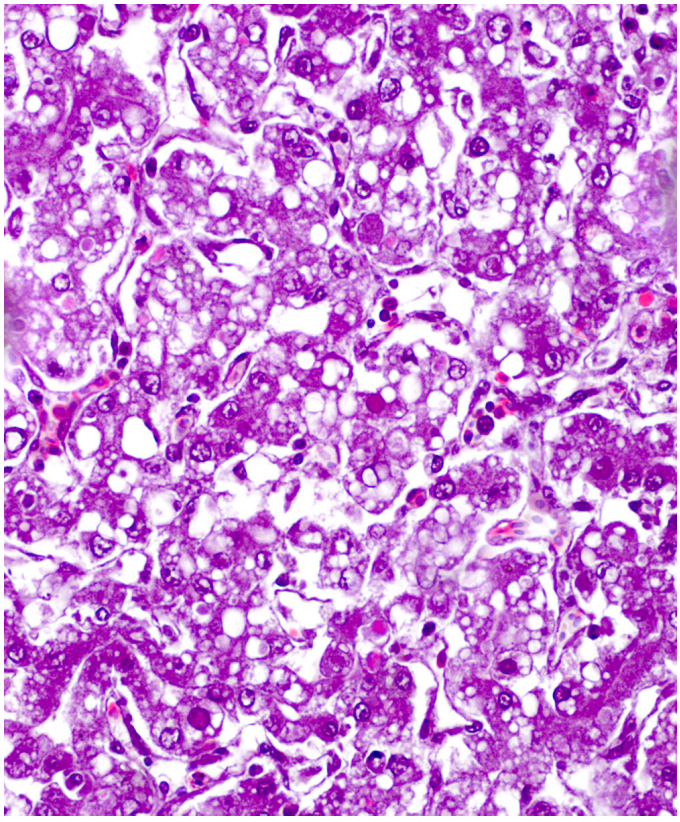


Fig.1. Experimental poisoning with 2% *Crotalaria lanceolata* seeds in the diet of broiler chickens (Group E2). Vacuolar degeneration, megalocytosis, cord architecture loss in hepatocytes, mild biliary proliferation, and hyaline spheres (arrows). HE, obj.40x.

DISCUSSION

This study verified that *Crotalaria lanceolata* seeds were toxic to broiler chickens that received, as of seven days of age, single doses of 15% and 25% and daily doses of 0.4%, 0.8% and 2.5% in feed for 21 and 35 days, as well as to those that received, as of the age of 28 days, daily doses of 1% and 2% in feed for 20 days.

Table 6. Microscopic lesions observed in broilers euthanized at 80 days. Experimental poisoning by *Crotalaria pallida* and *Crotalaria lanceolata* seeds, 1% and 2% in the diet, Group D (1 and 2) and Group E (1 and 2)

Lesions	Group			
	D1 1% *C. pallida*	D2 2% *C. pallida*	E1 1% *C. lanceolata*	E2 2% *C. lanceolata*
Pulmonary edema	-	-	++	-
Vacuolar hepatocyte degeneration	-	-	+	++
Hepatocyte megalocytosis	-	+	+	+++
Hepatocyte karyomegaly	-	-	+	+
Hepatocyte swelling	+	++	+	++
Biliary epithelium proliferation	-	-	+	+

+ Mild, ++ moderate, +++ severe.

Crotalaria pallida proved to be less toxic than *C. lanceolata*, and the doses that caused clinical changes and gross and microscopic lesions were as follows: single doses of 25% and daily doses of 0.8% and 2.5% in ration for 35 days. In broiler chickens aged 28 days, *Crotalaria pallida* seeds also showed toxicity at daily doses of 1% and 2% in feed for 20 days.

The following clinical changes were observed in some birds poisoned by both *C. lanceolata* and *C. pallida*: inappetence, ruffled feathers, and brownish diarrhea that arose from the second week of seed supply, as well as growth retardation. These changes, except for the diarrhea, have been previously observed in poisoning with *Crotalaria spectabilis* (Allen et al. 1960, Simpson et al. 1963, Hatayde et al. 1997a, 1997b, Pereira et al. 2011), with *C. spectabilis* and *Crotalaria striata* (*C. pallida*) (Bierer et al. 1960, Kelly et al. 1961), and with *Crotalaria retusa* (Hooper & Scanlan 1977).

Gross lesions included subcutaneous edema, ascites, hydropericardium, yellowish liver with hypertrophy, atrophy (Allen et al. 1960, Simpson et al. 1963, Hatayde et al. 1997b, Pereira et al. 2011) and enhanced lobular pattern, and distended gallbladder (Hooper & Scanlan 1977). These lesions were also observed in this study with both *C. lanceolata* and *C. pallida*; however, jaundice and bleeding in serosa and mucosa, as described by Figueredo et al. (1987) and Alfonso et al. (1993), were not verified.

The main microscopic lesions caused by *C. lanceolata* and *C. pallida* were similar but of varying intensity, and were more severe for *C. lanceolata*. Hepatocyte swelling, megalocytosis and vacuolar degeneration were also observed, and were similar to the lesions described for *C. striata* (*C. pallida*) (Bierer et al. 1960, Kelly et al. 1961). Loss of hepatocyte cord architecture was also observed in the present study. In the birds that presented gross liver lesions, eosinophilic spheroids or formation of Councilman hyaline bodies, marked megalocytosis and nuclear invagination were also observed, as described for *Crotalaria mucronata* (*C. pallida*) poisoning in cattle (Boghossian et al. 2007). Chromatin-thickening hepatocytes around the nuclear membrane have been frequently observed with enhanced and enlarged nucleoli, as described in bird poisoning by *C. spectabilis* seeds (Hatayde et al. 1997b, Pereira et al. 2011).

However, Pereira et al. (2011) also describe hepatic necrosis, bruising, connective tissue proliferation, and inflammatory infiltrate, which were not evidenced in this study. Fibrosis and proliferation of biliary epithelium in *Crotalaria* spp. seem to be more evident in the equine (Nobre et al. 2004a, 2004b), bovine (Nobre et al. 2004a) and ovine (Dantas et al. 2004, Riet-correa et al. 2011) species. In the birds of the present study, these changes were slight, which can be attributed to their slaughter time after the end of the feed intake. A common feature observed in poisoning by *C. lanceolata* and *C. pallida* at single doses of 15% and 25% drew attention: gross liver lesions seemed to be regressive, since birds euthanized at 28 days of age showed histologic lesions such as swelling, vacuolar hepatocyte degeneration, cariomegaly, and nuclear invagination. These lesions were not observed in the birds that received the same doses and were euthanized at 42 days of age. The daily dose of 2.5% caused progressive lesions for both *C. lanceolata* and *C. pallida* poisoning.

Poisoning by green leaves or seeds of *C. pallida* has caused pneumotoxicity in cattle (Tokarnia & Döbereiner 1982, Lemos et al. 1997, Boghossian et al. 2007). Acute clinical presentation of pulmonary edema was observed in sheep with gross and microscopic lesions restricted to the respiratory system. It was also found that non-lethal daily doses of *C. pallida* (0.63 and 1.25g/kg) given to sheep for 30 days did not have cumulative effect, as they did not become ill (Borelli et al. 2016). It is worth noting that sheep are more resistant to PA than other species (Lucena et al. 2010). According to what was suggested by Pessoa et al. (2013), in the reproduction of liver and lung disease in donkeys, it is hypothesized that prolonged exposure to low levels of DHPA results in mild hepatotoxicity, and that lung lesions do not occur until the dose is sufficiently high for DHPA to be metabolized in the lung, or that pneumotoxic compounds pass intact through metabolism and are activated only in the lung, causing injury. In the present study, pulmonary lesions were not significant.

Crotalaria lanceolata seeds were 1.23 times more toxic than *C. pallida* seeds to the poultry assessed in this study, resembling the toxicity of *C. retusa* described by Hooper & Scanlan (1977) and that of *C. spectabilis* reported by Pereira et al. (2011). Allen et al. (1960) reported that chickens poisoned with concentrations higher than 0.3% *C. spectabilis* seeds for 18 days reached 100% mortality. Chickens poisoned with 0.5% *C. retusa* seeds with monocrotaline concentration ranging from 4.0-4.4% began to die in the beginning of seed supply and, at 45 days of age, all birds were dead (Hooper & Scanlan 1977). Another previous study showed bird mortality by *C. spectabilis* at doses of 0.045% of seed fed to one-day chickens, 0.09% fed to four-week chickens, and 0.72% fed to turkeys, with the last resulting in 100% mortality (Simpson et al. 1963). Laying hens fed 0.02%, 0.04% and 0.06% seeds of *C. spectabilis* for

28 days showed 5%, 25% and 40% mortality, respectively (Pereira et al. 2011). In experiments performed with *C. pallida*, Kelly et al. (1961) verified bird mortality as of the 4th week of supplying 3% and 6% of seeds in the feed. Diaz et al. (2003) reported mortality of birds fed doses of 1%, 2% and 3% of seeds for 21 days. In this study, there were no deaths of birds poisoned by *C. pallida*, contrary to what was observed with birds poisoned by 1% and 2% *C. lanceolata* seeds. Difference in intensity of the lesions was observed between broiler chickens of the same group - a fact that may be associated with the difference in sensitivity between individuals and/or with feed selectivity regarding grain size.

Difference in toxicity between the aforementioned species may be associated with the type of DHPA and the amount present in its seeds, which may vary according to the time of year and even to desiccation (Hurst 1942) and storage time. The concentration of PA determines the plant toxicity and, consequently, the acute or chronic evolution of the poisoning condition. Monocrotaline, the most frequently PA isolated from plants of genus *Crotalaria* spp. causes pulmonary hypertension and right ventricular hypertrophy, which result from increased resistance to blood flow through the lungs caused by lung injury (Wilson et al. 1992, Pan et al. 1993). The main changes are pulmonary edema and congestion with consolidated areas in the parenchyma, causing interstitial and pulmonary arteriolar lesions, with inflammation, hemorrhage and fibrosis (Nobre et al. 1994). The PA isolated from *C. pallida* were millin, integerrimine, nilgirine, and crostastriatine (Smith & Culvenor 1981), but there was no evidence of monocrotaline. In the study by Diaz et al. (2014), *C. pallida* seed analysis for DHPA content detected usaramine and its N-oxide, in total alkaloid concentration of 0.18% (dry weight). In 1987, Williams & Molyneux isolated from a *C. mucronata* (*C. pallida*) sample a total amount of toxic alkaloids of 0.15%. From a sample of *C. lanceolata* from Brazil, they quantified 0.21% of total toxic alkaloids, but did not identify them. In this study, the total alkaloid quantification for *C. lanceolata* presented 910µg/g of toxic DHPA, i.e., 0.091% (dry weight), whereas *C. pallida* showed 737µg/g of toxic DHPA, i.e., 0.0737% (dry weight). This difference in DHPA concentration may explain the lower severity of *C. pallida* lesions when compared with those of *C. lanceolata.*

CONCLUSIONS

Crotalaria lanceolata seeds were toxic to broiler chickens as of the 7th day of life in single doses of 15% and 25% and daily doses of 0.4%, 0.8% and 2.5% in the feed for 21 and 35 days. In broilers aged 28 days, toxicity was also observed at the doses of 1% and 2% in the feed for 20 days.

Crotalaria pallida seeds were also toxic to broiler chickens, but to a lesser extent, at a single dose of 25% and at daily doses from 0.8% and 2.5% in the diet for 35 days.

The lesions caused to broilers by seeds of *C. lanceolata* and *C. pallida* are compatible with those caused by pyrrolizidine alkaloids, mainly affecting the liver.

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

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Serological response against bovine herpesvirus and bovine viral diarrhea virus induced by commercial vaccines in Holstein heifers[1]

Camila C. Baccili[2]*, Camila Cecilia Martin[2], Karen N. Silva[2], Marcílio Nichi[3], Eduardo F. Flores[4], Aníbal E. Vercesi Filho[5], Edviges Maristela Pituco[6] and Viviani Gomes[2]

ABSTRACT.- Baccili C.C., Martin C.C., Silva K.N., Nichi M., Flores E.F., Vercesi Filho A.E., Pituco E.M. & Gomes V. 2019. **Serological response against bovine herpesvirus and bovine viral diarrhea virus induced by commercial vaccines in Holstein heifers.** *Pesquisa Veterinária Brasileira 39(11):870-878.* Departamento de Clínica Médica, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Orlando Marques de Paiva 87, Cidade Universitária, Butantã, SP 05508-270, Brazil. E mail: camila.rcosta@usp.br

Vaccination is a strategy to the prevention and control of reproductive diseases caused by bovine viral diarrhea virus (BVDV) and bovine herpesvirus type 1 (BoHV-1), however the various compositions of commercial vaccines should be evaluated for their ability to induce protection mediated by antibodies. The objective of this research was to evaluate the production of specific neutralizing Abs against BVDV-1 and 2, and BoHV-1 induced by commercial vaccines composed by different adjuvants. Holstein heifers were vaccinated and distributed in three experimental groups: Group I (G1) was vaccinated with a commercial vaccine containing inactivated BVDV-1, BVDV-2 and BoHV-1 diluted in alum hydroxide as adjuvant (n=9); Group II (G2) was vaccinated with an product containing inactivated strains of BVDV-1, BVDV-2, BoHV-1 and BoHV-5 diluted in oil emulsion as adjuvant (n=10); Group III (G3) was vaccinated with a commercial vaccine containing inactivated BVDV-1 and BVDV-2, besides live modified thermosensitive BoHV-1, diluted in Quil A, amphigen and cholesterol (n=10); A control, non-vaccinated group (n=6) was mock vaccinated with saline. Heifers received two subcutaneous doses of 5mL of each commercial vaccine on the right side of the neck, with 21 days interval. Humoral immune response was assessed by the virus neutralization test (VN) against BVDV-1 (NADL and Singer strains), BVDV-2 (SV253 strain) and BoHV-1 (Los Angeles strain) in serum samples collected on vaccination days zero (D0), 21 (D21) and 42 (D42; 21 days after boosting). Neutralizing Abs against BVDV-1 NADL was detected only in D42, regardless of the vaccine used. Similar geometric mean titers (GMT) for BVDV-1 NADL were observed between G1 (log_2=5.1) and G3 (log_2=5.1). The seroconversion rate (%) was higher in G1 (78%) when compared to G2 (10%) and G3 (40%). For BVDV-1 Singer, it was also possible to detect Abs production in G1 (log_2=5.8, 100% seroconversion rate) and G3 (log_2=3.5, seroconversion rate = 60%), only after the booster dose (D42). Neutralizing Abs to BVDV-2 (SV253) were detected only in G3, observing 90% seroconversion associated with high titers of Abs (log_2=6.7) after the 2nd dose of vaccine (D42). Heifers from G1 and G3 responded to BoHV-1 after the first dose (D21): G1 (log_2=2.5,

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[2] Departamento de Clínica Médica, Universidade de São Paulo (USP), Av. Orlando Marques de Paiva 87, Cidade Universitária, Butantã, SP 05508-270, Brazil. *Corresponding author: camila.rcosta@usp.br

[3] Departamento de Reprodução Animal, USP, Av. Orlando Marques de Paiva 87, Cidade Universitária, Butantã, SP 05508-270.

[4] Graduate Program in Veterinary Medicine, Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria (UFSM), Av. Roraima 1000, Cidade Universitária, Camobi, Santa Maria, RS 97105-900, Brazil.

[5] Agência Paulista e Tecnologia do Agronegócio, APTA Gado de Leite, Rua Heitor Penteado 56, Nova Odessa, SP 13460-000, Brazil.

[6] Instituto Biológico, Laboratório de Viroses de Bovídeos, Av. Conselheiro Rodrigues Alves 1252, Vila Mariana, SP 04014-002.

seroconversion rate = 67%) and G3 (log_2=0.7, seroconversion rate = 80%). In D42, a higher magnitude response was observed in the heifers from G3 (log_2=6.1, 100%) compared with G1 (log_2=4.3, 100%) and G2 (log_2=2.7, 60%). Based on the data obtained, it can be concluded that the commercial vaccine contained aluminum hydroxide (G1) was most effective in the induction of antibodies against BVDV-1. On the other hand, this vaccine did not induce the production of neutralizing Abs against BVDV-2. Only the heifers from G3 (Quil A, amphigen and cholesterol) generated neutralizing Abs against BVDV-2. The animals that received commercial vaccine containing oil emulsion as adjuvant (G2) had a weak/undetectable response against BVDV-1 and BVDV-2. The best protective response against BoHV-1 was observed in heifers vaccinated with the live modified thermosensitive virus.

INDEX TERMS: Vaccine response, serology, bovine viral diarrhea virus, BVDV, bovine herpesvirus type 1, BoHV-1, vaccine, Holstein heifers, cattle.

RESUMO.- [**Resposta sorológica contra herpesvírus bovino e vírus da diarreia viral bovina induzida por vacinas comerciais em novilhas Holandesas.**] A vacinação é utilizada como estratégia para a prevenção e controle das doenças reprodutivas, causadas pelos vírus da diarreia viral bovina (BVDV) e herpesvírus bovino tipo 1 (BoHV-1), entretanto, as diversas composições de vacinas comerciais devem ser avaliadas quanto a sua eficiência protetiva mediada por anticorpos (Acs). O objetivo desta pesquisa foi avaliar a produção Acs neutralizantes específicos para cepas de BVDV-1 e 2, e BoHV-1 induzida por vacinas comerciais contendo diferentes tipos de adjuvantes. Para tal, novilhas Holandesas foram vacinadas e distribuídas em três grupos experimentais: Grupo I (G1) foi vacinado com uma vacina comercial composta por cepas inativadas de BVDV-1, BVDV-2 e BoHV-1 diluídas em hidróxido de alumínio como adjuvante (n=9); Grupo II (G2) foi vacinado com produto contendo as cepas inativadas de BVDV-1, BVDV-2, BoHV-1 e BoHV-5 em uma emulsão oleosa como adjuvante (n=10); O Grupo III (G3) foi vacinado com uma vacina comercial contendo BVDV-1 e BVDV-2 inativado, além do BoHV-1 vivo modificado e termosensivel, diluídos em adjuvante contendo Quil A, Amphigem e colesterol (n=10); O Grupo Controle não vacinado (n=6) foi inoculado com solução salina. As novilhas receberam duas doses das respectivas vacinas ou solução salina (5mL), com intervalo de 21 dias, por via subcutânea, na tábua do pescoço do lado direito. A resposta imune humoral foi avaliada pelo teste de vírus neutralização (VN) contra o BVDV-1 (cepas NADL e Singer), BVDV-2 (cepa SV253) e BoHV-1 (cepa Los Angeles) em amostras de soro coletadas nos dias (D) de vacinação zero (D0), 21 dias após 1ª dose (D21)e 42 (D42; 21 dias após A 2ª dose). Os anticorpos neutralizantes contra o BVDV-1 NADL foram detectados apenas em D42, independentemente da vacina utilizada. Os títulos médios geométricos (GMT) de anticorpos foram semelhantes entre G1 (log_2=5,1) e G3 (log_2=5,1). A taxa de soroconversão foi maior no G1 (78%) quando comparado ao G2 (10%) e G3 (40%). Para o BVDV-1 Singer, somente após D42 foi observada a produção de Acs no G1 (log_2=5,8; taxa de soroconversão de 100%) e G3 (log_2=3,5; taxa de soroconversão = 60%). Os anticorpos contra BVDV-2 (SV253) foram detectados apenas nas novilhas do G3, observando-se taxa de soroconversão de 90% com altos títulos de anticorpos neutralizantes (log_2=6,7) em D42. Novilhas G1 e G3 responderam ao BoHV-1 após a primeira dose (D21): G1 (log_2=2,5; taxa de seroconversão = 67%) e G3 (log_2=0,7; taxa de seroconversão = 80%). Em contrapartida, foi observada uma maior magnitude de resposta para as novilhas G3 (log_2=6,1; 100%) em D42, em relação aos animais G1 (log_2=4,3; 100%) e G2 (log_2=2,7; 60%). Com base nos dados obtidos, foi possível concluir que a vacina composta por hidróxido de alumínio (G1) foi mais eficaz na produção de anticorpos contra o BVDV-1, em contrapartida esse produto não induziu anticorpos contra o BVDV-2. Apenas as novilhas do G3 (Quil A, amphigen e colesterol) geraram Acs neutralizantes contra o BVDV-2. Os animais que receberam a vacina em emulsão oleosa (G2) como adjuvante apresentaram uma resposta fraca/indetectável contra o BVDV-1 e BVDV-2. A melhor resposta protetiva contra o BoHV-1 foi observada nas novilhas vacinadas com a vacina viva modificada termosensível.

TERMOS DE INDEXAÇÃO: Resposta vacinal, sorologia, vírus da diarreia viral bovina, BVDV, herpesvírus bovino tipo 1, BoHV-1, bovinos, novilha Holandesa, vacina comercial.

INTRODUCTION

Today the world population is growing exponentially and will reach 10 billion inhabitants by 2050 (FAO 2017), increasing the demand for food of animal origin. Brazil is the largest exporter of beef and holds the most significant commercial herd in the world represented by 218 million animals, effectively producing about 5.87 billion liters of milk and slaughtering around 7.37 million heads a year (IBGE 2017). Despite productive efficiency, world demand has put pressure on our country to achieve the best reproductive rates aiming at achieving maximum reproductive efficiency. In this scenario, herd health is a prerequisite for ensuring that investment in genetics and breeding is successful with the birth of healthy calves. Thus, the use of reproductive vaccines to control infectious diseases that act negatively in both the reproductive and productive scenario in the dairy and beef systems has intensified (Weber et al. 2013).

Among the main agents responsible for reproductive losses in cattle are bovine viral diarrhea virus (BVDV) and bovine herpesvirus type 1 (BoHV-1). Studies estimate that the financial losses associated with viral infections generate losses of $ 0.50 to 687.80/animal for BVDV in dairy herds (Richter et al. 2017) and $ 460 to 767 million per year in beef cattle (Givens & Marley 2013). For BoHV-1, it was estimated that an average cost is $ 379 per infected cow (Can et al. 2016).

Bovine Viral Diarrhea (BVD) is cited in the World Animal Health Organization's list of diseases as a worrying virus in cattle production worldwide (OIE 2018). BVDV has a wide antigenic variability, belongs to the family Flaviviridae, genus

Pestivirus, in this genus 11 species are included among them BVDV-1, BVDV-2 and Hobi-like *Pestivirus* (Ridpath et al. 1994, Schirrmeier et al. 2004, ICTV 2019). The impact generated by the BVDV virus is related to its ability to cross the placental barrier, and cause several problems in the embryo or fetus, including fetal resorption, immunosuppression, teratogeny, and mainly cause the birth of persistently infected (IP) calves (Martin et al. 2016, Walz et al. 2017, Jardim et al. 2018). BoHV-1 belongs to the family Herpesviridae, subfamily Alfaherpesviridae, genus *Varicellovirus*, and is associated with the Bovine Respiratory Disease Complex and reproductive disease in bovine females, including abortions (Becker et al. 2015, Costa et al. 2017).

Given this, reproductive vaccines represent an essential strategy for the control of infections caused by both BVDV and BoHV-1, in an attempt to limit losses related to lower conception rates, early embryonic death, abortions, stillbirths and birth of premature calves caused by viral infections (Walz et al. 2017). Vaccination of cattle against BVDV and BoHV-1 is not yet a widespread practice in our country (Silva et al. 2007b), but a large number of commercial vaccines licensed against these agents in Brazil shows a trend towards continuous use vaccination protocols associated with reproduction. In Brazil, there are around 20 commercial reproductive vaccines, most of them composed of inactivated strains of BVDV (Brasil 2019). To date, only one commercial live modified BVDV (MLV) vaccine has been licensed for use in Brazil by the "Ministério da Agricultura, Pecuária e Abastecimento" (MAPA). Besides, there are no commercial Hobi-like vaccines registered in our country.

Vaccine-induced protection is ensured by its ability to stimulate the production of neutralizing antibodies (Abs), which are capable of binding to epitopes on the surface of antigens. These Abs eliminate invading microorganisms by mechanisms of neutralization, agglutination, complement system activation with opsonization, and pathogen lysis (Chase 2007). The minimum Abs titers needed to protect animals from BVDV challenge suggested by Bolin & Ridpath (1995) are $\log_2$ 4 (1:16), and for BoHV-1, Pospísil et al. (1996) cited $\log_2$ neutralizing Abs titers ≥4 or 5 (≥1:16 or 1:32). However, it is estimated that higher neutralizing Abs titers will provide a greater protective capacity to the host.

Inactivated vaccines are composed of dead viral particles, unable to replicate in host cells, which require greater antigenic mass diluted in adjuvants, and play a role in improving the immunological and consequently protective efficiency of commercial formulations (Vartak & Sucheck 2016, Hogenesch et al. 2018). Adjuvants most commonly used in veterinary medicine are aluminum hydroxide, oily emulsions, saponins, and immune-stimulating complexes (ISCOM). Adjuvants generally act primarily at the site of application by stimulating innate immunity by enhancing antigen recognition and uptake by antigen-presenting cells (APCs) at the injection site, with subsequent migration to the regional lymph nodes for stimulation of a cellular and humoral immune response (Spickler & Roth 2003).

Given the context presented, considering the importance of using efficient vaccines that are capable of generating a protective and lasting immune response in the prevention of reproductive diseases, this research hypothesizes that commercial vaccines containing different types of adjuvants induce different degrees of the humoral response against BVDV and BoHV-1. Our hypothesis is based on the lack of information on the antibody production profile for each type of adjuvant present in commercial vaccines, making it necessary to evaluate the efficacy of these immunogens. Thus, the objective of this research was to evaluate the production of specific Abs for the main strains of BVDV-1 and 2, and BoHV-1 induced by commercial reproductive vaccines available in the Brazilian market containing different adjuvants.

MATERIALS AND METHODS

This research was approved by the Animal Use Ethics Committee of the "Faculdade de Medicina Veterinária e Zootecnia" of USP, protocol number 6229201216. The heifers from this research are part of the experimental farm herd of the "Agência Paulista e Tecnologia do Agronegócio (APTA) Gado de Leite", located in the city of Nova Odessa, São Paulo (22°75'S latitude and 47°27'W longitude). This property was selected for not performing BVDV and BoHV-1 vaccinations. The sanitary protocol adopted by the farm is composed only of FMD and Brucellosis vaccines, required by MAPA.

The field trial was conducted from December 2015 to April 2016. During this period, average temperatures ranged from 17°C to 33°C, according to the "Instituto Nacional de Meteorologia" (INMet). Prior selection of heifers was initiated by animals aged 15-24 months (n=35), seronegative for both BVDV-1 (NADL) and BoHV-1 (Los Angeles) viruses, according to the virus neutralization test (VN), (OIE 2015). Exclusion of persistently infected animals (PIs) was conferred by reverse transcriptase reaction followed by a polymerase chain reaction to BVDV (RT-PCR), according to Basqueira et al. (2017). All heifers remained during the experimental period under extensive management in *Brachiaria Brizantha* cv. *Marandu* with *ad libitum* water and mineral salt supply, the body score of the animals ranged from 2.75 to 4.

Heifers (n=35) were randomly assigned to four experimental groups: Group I (G1) was inoculated with a vaccine composed of inactivated strains of BVDV-1, BVDV-2 and BoHV-1 in aluminum hydroxide as adjuvant (n=9); Group II (G2) was inoculated with inactivated vaccine strains of BVDV-1, BVDV-2, BoHV-1 and BoHV-5 in an oily emulsion as adjuvant (n=10); and Group III (G3) was inoculated with a vaccine containing BVDV-1, inactivated BVDV-2 and thermosensitive modified live BoHV-1 in Quil A, amphigen and cholesterol adjuvant (n=10). The unvaccinated Control Group (n=6) was inoculated with saline, as shown in Table 1. Vaccination protocols followed the manufacturers' recommendations. Heifers received two doses of different commercial vaccines (5mL), 21 days apart, subcutaneously, in the right neck region. Animals in the unvaccinated Control Group received a saline injection (5mL) on the days of administration of the 1st and 2nd doses of vaccines.

The adaptive immune response to BVDV and BoHV-1 was determined at the time of vaccination (D0), the day of revaccination (D21) and 21 days (D42) after the second dose.

The research of neutralizing Abs against BVDV-1 (NADL) and BoHV-1 (Los Angeles) were performed according to the recommendations of the World Organization for Animal Health (OIE 2015) at the Bovine Virus Laboratory, Biological Institute. In 96-well flat-bottom polystyrene microtiter plates, column 1 of the test plate was intended for cell control, column two for toxicity control of each serum, and in columns three through 12, samples were diluted in logarithmic base 2 ($\log_2$) from dilutions 1:10 (BVDV-NADL) and 1:2 (BoHV-1-Los Angeles) in eight replications using the minimum essential medium cell culture medium as diluent (MEM) containing 1% antibiotics

Table 1. Distribution of experimental groups, the composition of commercial vaccines according to adjuvants and strains for BVDV-1, BVDV-2, and BoHV-1

Groups (G)	Strains
Group 1 (n=9)	BVDV-1 (Singer) and BVDV-2** - inactivated, strains provided by INTA and CEVAN BoHV-1 (Los Angeles) inactivated
Group 2 (n=10)	**BVDV-1 and BVDV 2 (inactivated) **BoHV-1 and BoHV-5 (inactivated)
Group 3 (n=10)	BVDV-1 (5960) and BVDV-2 (53637) - inactivated BoHV-1 (Cooper) - chemically altered thermosensitive
Control Group (n=6)	Saline inoculation

** Strains not provided by the manufacturer; CEVAN = Center for Animal Virology, Argentina, INTA = National Institute of Agricultural Technology, Argentina.

and 5% BVDV antibody-free fetal bovine serum. Duplicate serum 50μL was added to the wells of the plates, after which 50μL of the respective virus solution containing $TCID_{50}$/100μL (50% tissue culture infective doses) was added. The plates were incubated for 18-24h for BoHV-1 and one hour for BVDV in an oven at 37°C with 5% CO_2. After incubation an MDBK (Madin-Darby bovine kidney) cell suspension was added to each well of the plates. After this process, the plate was incubated in an oven at 37°C with 5% CO_2 for 4-5 days. The search for neutralizing antibodies against BVDV-1 Singer and BVDV-2 VS253 was performed in the Virology Sector of the "Universidade Federal de Santa Maria", following the protocol described by OIE (2015). Serum dilutions were 1:5, with 100 infective doses for 50% of the cell cultures (TCID50) of each virus used.

Plates were read after 96h of incubation by observing the visible cytopathic effect (CPE) on the cell monolayer under an inverted microscope. The neutralizing antibody titer was considered to be the reciprocal of the highest serum dilution capable of neutralizing viral replication. Samples that did not show neutralization at the lowest dilution were considered negative (Reed & Muench 1938).

The statistical analysis of neutralizing antibody titers of each animal and the frequency of seroconversion was performed using the SAS statistical program (version 9.4, SAS Institute Inc., Cary/NC). Data that did not present parametric distribution were transformed following two approaches. First, the data were ranked; after this step, the data were submitted to a log_{10}, square root, or inverse transformation, this methodology was described by Templeton (2011). In the case where the transformations occurred, the P presented refers to the tests related to the transformed values, while the described values are real.

All variables were evaluated for distribution concerning the gaussian curve by the guided data analysis function. The variables were tested for fixed effects of treatments (G1, G2, G3, and Control) and days (0.21 and 42), as well as the interaction of treatment and day effects by MIXED procedure (PROC-mixed, SAS), with post hoc test "least significant difference" (LSD). The models were tested according to covariance structures, using the "Akaike information criterion" (AIC).

The seroconversion rate was presented in frequency (%) because it is a quantitative data, and the comparison between groups was performed by Chi-square test and between times by Cochran's Q test. Analyses were considered significant when P≤0.05 (*).

RESULTS

The mean geometric titers (GMT log_2) of Abs and the seroconversion frequencies (%) for BVDV (type 1 and 2) and BoHV-1 strains produced by vaccinated heifers, as well as the differences between groups and times are presented in Table 2 and Figure 1.

It was possible to observe differences for the fixed effects detected by PROC-mixed in relation to BVDV-1 (NADL and Singer), BVDV-2 and BoHV-1 specific Abs, regarding groups (P=0.001), days (P=0.001) and Trt x day (P=0.001).

Regarding BVDV-1 NADL, none of the heifers belonging to vaccinated Groups 1, 2, and 3 seroconverted after the 1st dose of commercial vaccines (D21). In D42, the average of Abs (GMT-log_2) were similar between heifers G1 (GMT-log_2=5.1) and G3 (GMT-log_2=5.1), but the seroconversion rate was higher in G1 (78%, 7/9) compared to G3 (40%, 4/10). The G2 heifers had similar mean Abs titers to the unvaccinated Control Group, and only one animal (10%) responded to vaccination.

Regarding the BVDV-1 Singer strain, G1 heifers presented 22% (2/9) and GMT log_2=0.1 seroconversions after primo vaccination (D21), but this group did not differ statistically from G2 and G3. In D42, heifers belonging to G1 had higher mean titers of Abs (GMT-log_2=5.8) and 100% seroconversion when compared to G3 heifers (GMT-log_2=3.5; seroconversion rate 60%). The G2 animals did not respond to the BVDV-1 Singer strain.

The BVDV-2 VN test (VS253) showed that only Group 3 produced Abs after the first dose (GMT-log_2=1.0) and the 2nd vaccine dose (GMT-log_2=6.7), seroconversion presented was 10% (1/10) at D21 and 90% (9/10) at D42. The Groups 1 and 2 showed undetectable production of neutralizing Abs against BVDV type 2.

Regarding BoHV-1, specific Abs were detected in D21 only for G1 (GMT log_2=2.5) and G3 (GMT log_2=0.7), the observed seroconversion was 67% (6/9) and 80% (8/10). In D42 revaccination, higher response intensity was observed in G3 heifers (GMT-log_2=6.1; 100% seroconversion rate), followed by G1 (GMT-log_2=4.3; 100% seroconversion rate), G2 (GMT-log_2=2.7; seroconversion rate 70%) and negative control.

The intensity of antibody production in each group is shown in Table 3. The frequency of heifers with minimum protective titers (≥16) for BVDV-1 strains was detected only at D42. Regarding BVDV-1 (NADL), Groups 1 and 3 showed frequencies of 67% (6/9) and 20% (2/10), while for BVDV-1 (Singer) it was 89% (8/9) and 40% (4/10). Group 2 heifers had lower titers than 16. Regarding BVDV-2 (VS-253), only G3 presented Abs titers, being 80% (8/10) protective. Regarding BoHV-1 (Los Angeles) protective Abs (≥16) in D21 were detected only in 1/9 (11%) in G1. In D42, the vaccine used in G3 induced Abs in 90% (9/10) of animals, followed by G1 with 44% (6/9) and G2 with 20% (2/10).

Table 2. Geometric mean titers (GMT-log2) of neutralizing antibodies and seroconversion rates (%) for BVDV-1 (NADL), BVDV-1 (Singer), BVDV-2 (VS253) and BoHV-1 (Los Angeles) induced by commercial vaccines containing different types of adjuvants in Holstein heifers

Strains	Day	GMT (log_2)				Seroconversion (n/%)			
		Group 1 (n=9)	Group 2 (n=10)	Group 3 (n=10)	Control (n=6)	Group 1 (n=9)	Group 2 (n=10)	Group 3 (n=10)	Control (n=6)
BVDV-1 (NADL)	D0	0.0 ± 0^{b}	0.0 ± 0^{a}	0.0 ± 0^{b}	0.0 ± 0^{a}	0% (0/9)^{b}	0% (0/10)^{a}	0% (0/10)^{b}	0%(0/6)^{a}
	D21	0.0 ± 0^{b}	0.0 ± 0^{a}	0.0 ± 0^{b}	0.0 ± 0^{a}	0% (0/9)^{b}	0% (0/10)^{a}	0% (0/10)^{b}	0%(0/6)^{a}
	D42	5.1 ± 5.6^{Aa}	0.0 ± 0^{Ba}	5.1 ± 6.6^{Aa}	0.0 ± 0^{Ba}	78% (7/9)^{Aa}	10%(1/10)^{Ba}	40%(4/10)^{Aa}	0%(0/6)^{Ba}
BVDV-1 (Singer)	D0	0.0 ± 0^{b}	0.0 ± 0^{a}	0.0 ± 0^{b}	0.0±0	0% (0/9)	0% (0/10)	0% (0/10)	0%(0/6)
	D21	0.1 ± 1.1^{b}	0.0 ± 0^{a}	0.0 ± 0^{b}	0.0±0	22% (2/9)	0% (0/10)	0% (0/10)	0%(0/6)
	D42	5.8 ± 5.5^{Aa}	0.0 ± 0^{Ca}	3.5 ± 3.7^{Ba}	0.0±0	100% (9/9)	0% (0/10)	60%(6/10)	0%(0/6)
BVDV-2 (VS253)	D0	0.0 ± 0^{a}	0.0 ± 0^{a}	0.0 ± 0^{b}	0.0±0	0% (0/9)	0% (0/10)	0% (0/10)	0%(0/6)
	D21	0.0 ± 0^{a}	0.0 ± 0^{a}	1.0 ± 2.6^{b}	0.0±0	0% (0/9)	0% (0/10)	10%(1/10)	0%(0/6)
	D42	0.0 ± 0^{Ba}	0.0 ± 0^{Ba}	6.7 ± 6.9^{Aa}	0.0±0	0% (0/9)	0% (0/10)	90%(9/10)	0%(0/6)
BoHV-1 (Los Angeles)	D0	0.0 ± 0^{c}	0.0 ± 0^{b}	0.0 ± 0^{c}	0.0 ± 0^{a}	0% (0/9)^{c}	0% (0/10)^{b}	0% (0/10)^{c}	0%(0/6)^{a}
	D21	2.5 ± 3.3^{Ab}	0.0 ± 0^{Bb}	0.7 ± 0^{ABb}	0.0 ± 0^{Ba}	100% (9/9)^{Ab}	10%(1/10)^{Bb}	80%(8/10)^{ABb}	0%(0/6)^{Ba}
	D42	4.3 ± 4.0^{Ba}	2.7 ± 2.0^{Ca}	6.1 ± 5.6^{Aa}	0.0 ± 0^{Da}	100% (9/9)^{Ba}	70%(7/10)^{Ca}	100%(10/10)^{Aa}	0%(0/6)^{Da}

Group 1 = commercial vaccine containing aluminum hydroxide, Group 2 = commercial vaccine containing oily emulsion, Group 3 = commercial vaccine containing Quil A, amphigen and cholesterol; ^{A,B,C} uppercase letters in the same row demonstrate difference between groups, ^{a,b,c} lowercase letters in the same column demonstrate difference between times, data without letters showed no differences. For GMT differences were detected by the PROC MIXED test; seroconversion rate was tested between groups by Chi-square test and between times by Cochran's Q test; the analyzes were considered significant when P≤0.05.

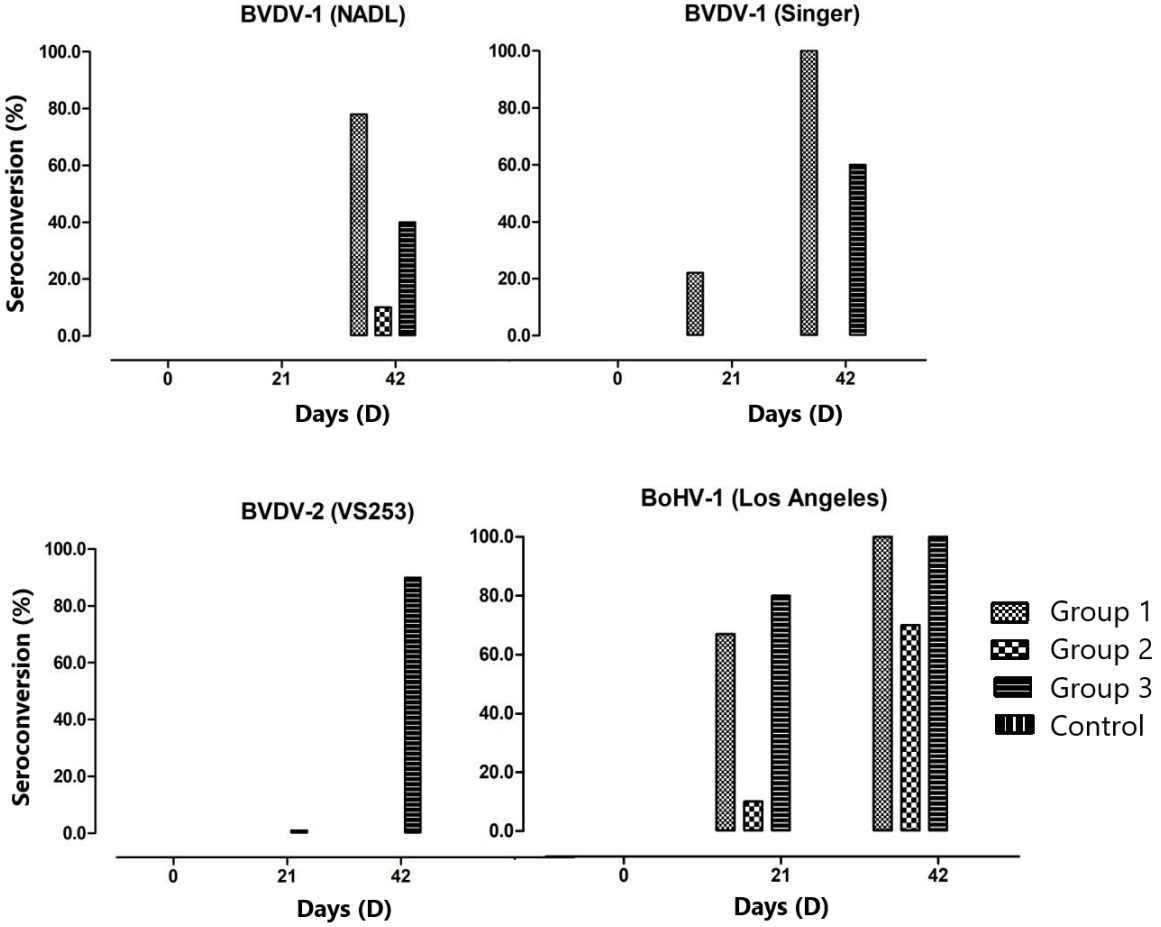


Fig.1. Seroconversion frequencies (%) of Group 1, 2 and 3 heifers inoculated with commercial vaccines containing different adjuvant types for BVDV-1 (NADL), BVDV-1 (Singer), BVDV-2 (VS253) and BoHV-1 (Los Angeles) viruses.

Table 3. Frequency distribution (%) according to antibody production intensity against BVDV-1 (NADL), BVDV-1 (Singer), BVDV-2 (VS253) and BoHV-1 (Los Angeles), induced by commercial vaccines containing different types of adjuvants in Holstein heifers

Virus (strains)	Neutralizing Ab titers	Group 1 (n=9)		Group 2 (n=10)		Group 3 (n=10)	
		D21	D42	D21	D42	D21	D42
BVDV-1 (NADL)	2	0% (0/9)	0% (0/9)	0% (0/10)	10% (1/10)	0% (0/10)	0% (0/10)
	10	0% (0/9)	11% (1/9)	0% (0/10)	0% (0/10)	0% (0/10)	20% (2/10)
	20	0% (0/9)	33% (3/9)	0% (0/10)	0% (0/10)	0% (0/10)	10% (1/10)
	40	0% (0/9)	22% (2/9)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)
	160	0% (0/9)	11% (1/9)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)
	316	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	0% (0/10)	10% (1/10)
	Titers ≥16	0% (0/9)	67% (6/9)	0% (0/10)	0% (0/10)	0% (0/10)	20% (2/10)
BVDV-1 (Singer)	5	22% (2/9)	11% (1/9)	0% (0/10)	0% (0/10)	0% (0/10)	10% (1/10)
	10	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	0% (0/10)	10% (1/10)
	20	0% (0/9)	11% (1/9)	0% (0/10)	0% (0/10)	0% (0/10)	30% (3/10)
	40	0% (0/9)	44% (4/9)	0% (0/10)	0% (0/10)	0% (0/10)	10% (1/10)
	80	0% (0/9)	22% (2/9)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)
	160	0% (0/9)	11% (1/9)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)
	Titers ≥16	0% (0/9)	89% (8/9)	0% (0/10)	0% (0/10)	0% (0/10)	40% (4/10)
BVDV-2 (VS-253)	10	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	0% (0/10)	10% (1/10)
	20	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	10% (1/10)	20% (2/10)
	40	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	0% (0/10)	10% (1/10)
	160	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	0% (0/10)	10% (1/10)
	≥320	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	0% (0/10)	20% (2/10)
	Titers ≥16	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	10% (1/10)	80% (8/10)
BoHV-1 (Los Angeles)	2	0% (0/9)	11% (1/9)	10% (1/10)	10% (1/10)	80% (8/10)	0% (0/10)
	4	55% (5/9)	0% (0/9)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)
	8	0% (0/9)	44% (4/9)	0% (0/10)	40% (4/10)	0% (0/10)	20% (2/10)
	16	0% (0/9)	11% (1/9)	0% (0/10)	20% (2/10)	0% (0/10)	0% (0/10)
	32	11% (1/9)	22% (2/9)	0% (0/10)	0% (0/10)	0% (0/10)	20% (2/10)
	64	0% (0/9)	11% (1/9)	0% (0/10)	0% (0/10)	0% (0/10)	20% (2/10)
	126	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	0% (0/10)	40% (4/10)
	Titers ≥16	11% (1/9	44% (4/9)	0% (0/10)	20% (2/10)	0% (0/10)	90% (9/10)

D = day, Group 1 = commercial vaccine containing aluminum hydroxide, Group 2 = commercial vaccine containing oily emulsion, Group 3 = commercial vaccine containing Quil A, amphigen and cholesterol.

DISCUSSIONS

The results obtained in this research vary in the magnitude of the humoral response to strains of BVDV-1, BVDV-2, and BoHV-1.

The mean neutralizing antibody titers against the BVDV-1 NADL strain were similar between vaccinated heifers G1 and G3. On the other hand, G2 vaccinated animals had similar antibody titers to the control. Seroconversion rates were observed only in the secondary vaccine response, with 78% reagents in G1, 40% G3, and 10% G2. Similar response pattern was observed for BVDV-1 Singer strain. The cutoff point for protective antibody titers was considered above 16 (Bolin & Ridpath 1995), so in this study, we found that the protective response profile for BVDV-1 (NADL and Singer) was 67% and 89% (G1), 20% and 40% for G3 in D42. Bolin & Ridpath (1995) found that animals with neutralizing antibody titers below 1:16 and challenged with the non-cytopathic BVDV virus (strain 890) showed acute disease with hyperthermia, leukopenia, thrombocytopenia, and diarrhea.

Vaccines used in cattle are generally multivalent, immunizing animals against several pathogens in a single administration. However, mixing bacterial and viral strains can present a significant immune challenge, as it requires a simultaneous response to immunodominant antigens and less immunogenic antigens, in addition to the possible inclusion of immunosuppressive agents and preparations containing both live virus and inactivated virus (Kreutz 2012).

The low immunogenicity of national BVDV vaccines has been reported in previous studies by Brazilian research teams. A study by Lima et al. (2005) verified effective humoral response in cattle after 30 days of vaccination with an experimental formulation containing attenuated BVDV-1 (GMT=1612.7) and BVDV-2 (GMT=151.0), while the other inactivated commercial vaccines showed weak response and partial. In the same research, two vaccines containing aluminum hydroxide induced BVDV-1 seroconversion in 32/36 (GMT=14.3) and 22/28 (GMT=25.1) animals. Regarding BVDV-2, seroconversion was observed in 27/36 (GMT=10.0) and 12/28 cattle (GMT=11.5) after vaccination with formulations containing aluminum hydroxide. The only oily vaccine in the study showed that 16/30 (GMT=40.0) and 10/30 (GMT=10.0) seroconverted to BVDV-1 and BVDV-2, respectively. Gomes et al.

(2014), reported low passive antibody transfer against BVDV after vaccination of commercially formulated prepartum cows containing BVDV-1 (NADL and Singer), BVDV-2 and BoHV-1 (Los Angeles) diluted in hydroxide aluminum as an adjuvant. The authors state that only 14.28% (1/7) of calves in the group of vaccinated mothers had neutralizing antibodies against BVDV in colostrum and blood serum from birth at 15 days of age. Baccili et al. (2018) evaluated the vaccine response of prepartum Holstein cows and their influence on passive immunity transfer, and it was found that only 33% (2/6) of vaccinated cows seroconverted after two doses of BVDV 1 polyvalent vaccine (strain 5960) and BVDV 2 (strain 53637) inactivated; live and heat-sensitive; BoHV-1 (strain RBL 106) and BBPI3-V (strain RLB 103); live attenuated; BRSV (strain 375), diluted in ISCOM adjuvant.

Inactivated vaccines contain inert viral particles, unable to replicate in the animal, abolishing the risk of fetal infection in pregnant cows; they are more stable under field conditions and are less costly to produce. The predominant response profile in this vaccine is the B-lymphocyte-mediated Th2 (humoral) type, which is critical for the production of antibodies that neutralize infectious viral particles before they infect host target cells. Despite these advantages, the search for vaccines that are also capable of stimulating cellular response (Th1) is essential, as this is the primary mechanism for host defense after the establishment of infection caused by viral agents (Kelling 2004, Silva et al. 2007b, Platt et al. 2008, Kreutz 2012). Given this context, this type of vaccine requires the addition of adjuvants in order to increase immunogenicity, improving antigen presentation, and thus amplifying both humoral and cellular immune response. The addition of adjuvants, on the other hand, imposes vaccine reactions on the animal, especially after multiple applications (Shams 2005, Newcomer et al. 2017).

In this research, it was found that vaccinated animals produced neutralizing antibody titers only at D42, regardless of the vaccine composition. Development of the humoral immune response after the first dose of a vaccine may take three to four weeks to occur, they should be driven by revaccination. At the time of the first vaccination, the antibody concentration does not increase until 10-14 days from the date of application of the first vaccine dose, and then the antibody concentration slowly increases. Therefore, the importance of booster should be reinforced with the application of the second dose of commercial formulations to boost the increase in antibody concentration. After the second dose, antibody concentration increases rapidly within 24 hours and peaks within a few days, with this response maintained for weeks or months (Chase et al. 2008).

A study by Anziliero et al. (2015) found different results from our research regarding seroconversion of animals vaccinated with a vaccine containing adjuvant ISCOM (G3). The authors analyzed eight commercial BVDV vaccines containing BVDV-1 and BVDV-2 strains. Animals immunized with the vaccine containing ISCOM adjuvant showed 100% seroconversion, with moderate to high titers for BVDV-types 1 and 2. In the present study, we observed that only 60% of animals seroconverted to BVDV-1 Singer. However, the work performed by Anziliero et al. (2015) used beef cattle of mixed breeds, aged 8 to 12 months, belonging to properties located in the central region of Rio Grande do Sul. These methodological differences may explain our divergent results since we use cattle with different aptitudes, breed, and age, as well as climatic variations between the south and southeast regions. Regarding the other seven vaccines researched by Anziliero et al. (2015), the authors detected partial seroconversion to BVDV-1 in animals receiving oily adjuvant, aluminum hydroxide, and Selenium Max adjuvant vaccine. Three other vaccines tested containing aluminum hydroxide as adjuvant did not induce the production of detectable antibodies against BVDV-1 in no vaccinated animals. Regarding BVDV-2, only two commercial vaccines induced partial responses against BVDV-2.

In general, the aluminum hydroxide-based adjuvant-containing vaccine stood out in this research in protecting against BVDV-1 NADL and BVDV-1 Singer, presenting higher antibody titers, seroconversion rate and neutralizing antibody production. Three main mechanisms are described in an attempt to explain the ability of aluminum hydroxide to stimulate the immune system. The first is its ability to adsorb antigenic particles, which retain the antigen at the injection site, increasing the recruitment time of antigen-presenting cells and the uptake of viral particles by the cells dendritic. Secondly, aluminum hydroxide generates cell necrosis at the injection site resulting in the release of molecular patterns, including DNA, uric acid, ATP, IL-1α, and IL-33, which induce APCs recruitment at the site injection (Hogenesch et al. 2018). Finally, aluminum hydroxide converts soluble antigen into particulate form (in association with adjuvant), facilitating its phagocytosis by macrophages and dendritic cells, which will migrate to local drainage lymph nodes, with the induction of a predominant immune response Th2 type (Lambrecht et al. 2009, Ghimire 2015).

Evaluation of response against BVDV-2 VS253 revealed that only G3 heifers showed a response against this viral strain. One hypothesis for this finding is that the vaccine used for G3 contains immunostimulating complexes (ISCOM) as an adjuvant, which is known to be a more potent adjuvant than the others. The ISCOMs form nanoparticles, similar to spherical capsules composed of cholesterol, amphigen (phospholipids and glycolipids) and saponin derivatives (Quil A). At nanoparticles formed by the mixture are approximately 30-40nm in diameter, which allows greater mobility for drainage to the lymph nodes, as well as local stability, thus increasing antigen absorption and presentation to dendritic cells, this set of factors increase the chances of cellular (Th1) and humoral (Th2) immune response induction (Sjölander et al. 1998, Saliba et al. 2017).

BVDV has high antigenic variability with low cross-serological reactivity between BVDV-1 and BVDV-2 species, which represents an obstacle to vaccination protocols (Ridpath et al. 1994). The frequencies of subgenotypes found in Brazilian herds according to Silveira et al. (2017) are approximately 35.9% for BVDV-type 1a, 31.4% BVDV-2b, 10.1% BVDV-1b, 6.7% BVDV-1d, 2.2% BVDV-2c and 1, 1% BVDV-1e. Flores et al. (2000) identified genotypes and subgenera through phylogenetic analysis of 17 BVDV isolates, with 23.5% of genotype 1a (BVDV-1a), 52.9% of genotype 1b (BVDV-1b) and 23.5% genotype 2 (BVDV-2). It is noteworthy that herds that use commercial formulations containing only BVDV-1 are more susceptible to BVDV-2 infections (Basqueira et al. 2017).

Regarding BoHV-1, a higher magnitude was observed for G3 of ($\log_2$=6.1), the mean titers in secondary response were $\log_2$=4.3 in G1 and $\log_2$=2.7 for G2. Probably this phenomenon

by the inclusion of thermosensitive modified live BoHV-1 in the commercial formulation used for vaccination of G3 heifers. Replicative vaccines generate a more potent immune response than those composed of inactivated viruses due to the ability of the vaccine virus to replicate in animal cells (Vartak & Sucheck 2016). Thermosensitive vaccines contain a chemically altered living virus that can replicate only at lower temperatures (30-33°C) than in the body (37°C), which precludes the development of systemic infections following vaccination with this type of antigen (Patterson et al. 2012).

Silva et al. (2007a) used calves aged 10 to 14 months to evaluate the immunogenicity of six commercial vaccines containing inactivated BoHV-1 antigens: one Brazilian (BR), one North American (US), two Uruguayan (UR1 and UR2) and two Argentine (ARG1 and ARG2). Only the US vaccine presented 87.5% of the animals with minimum titers (≥16 or 32), (Pospísil et al. 1996). The other vaccines had titers below 16 in 62.5% (5/8, BR), 33.3% (4/9, UR1), 75% (6/8, UR2) and 83.3% (5/6, ARG2) of calves. The ARG1 vaccine presented even lower performance, in which only three animals (37.5%) seroconverted. Considering the minimum protective antibody titer for BoHV-1 (≥16), we found in this research that 90% of G3 heifers had protective titer, while only 44% and 20% of G1 and G2 females were protected. Pospísil et al. (1996) found that vaccinated pregnant cows with neutralizing antibody titers above 1:16 and 1:32 challenged with BoHV-1 (TD strain) did not show respiratory disease and maintained their pregnancies without any abnormality.

Care must be taken when adjusting a vaccination protocol. In this research, the vaccine containing aluminum hydroxide (G1) presented better antibody production against BVDV-1, a result compatible with the higher capacity of the adjuvant aluminum hydroxide modular Th2 type immune response. The inoculum of G3 generated higher titers for BVDV-2 and BoHV-1. Importantly, this research did not evaluate the Th1 immune response mediated by cytotoxic lymphocytes. Theoretically, vaccines containing ISCOM complexes have a more exceptional ability to induce Th1 cellular immune response. Vaccination should induce both immune (humoral and cellular) responses to the protection and defense of cattle against viral agents, so further research is needed to associate the effect of adjuvants on both humoral and cellular responses.

CONCLUSION

Based on the data obtained, it was possible to conclude that the aluminum hydroxide (G1) vaccine was more effective in producing antibodies against BVDV-1, whereas this product did not induce antibodies against BVDV-2. Only G3 heifers (Quil A, amphigen and cholesterol) generated neutralizing Abs against BVDV-2. Animals receiving the oily emulsion (G2) vaccine as adjuvant showed a poor/undetectable response against BVDV-1 and BVDV-2. The best protective response against BoHV-1 was observed in heifers vaccinated with the modified thermosensitive live vaccine.

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Biocidal potential of *Eugenia pyriformis* essential oil in the control of *Rhipicephalus* (*Boophilus*) *microplus* in the free-living cycle[1]

Jaqueline P. Medeiros[2], Wanessa C. Bortollucci[2], Eloisa S. Silva[2], Herika L.M. Oliveira[2], Caio F.A.A. Campo[2], José Eduardo Gonçalves[2,3], Ranulfo P. Junior[4] and Zilda Cristiani Gazim[2]*

ABSTRACT.- Medeiros J.P., Bortollucci W.C., Silva E.S., Oliveira H.L.M., Campos C.F.A.A., Gonçalves J.E., Piau Junior R. & Gazim Z.C. 2019. **Biocidal potential of *Eugenia pyriformis* essential oil in the control of *Rhipicephalus* (*Boophilus*) *microplus* in the free-living cycle.** *Pesquisa Veterinária Brasileira 39(11):879-888.* Graduate Program in Biotechnology applied to Agriculture, Universidade Paranaense, Praça Mascarenhas de Moraes 4282, Umuarama, PR 87502-210, Brazil. E-mail: cristianigazim@prof.unipar.br

Brazil has one of the largest commercial cattle herds in the world, which naturally coexist with an enormous number of parasitic species. Southern cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*, is among these species, interfering with animal productivity and causing losses to the beef and dairy cattle sector. The use of chemical acaricides in the control of this mite has resulted in the emergence of resistant populations. In this sense, alternative control measures using plants as sources of botanical acaricides have shown to be effective. *Eugenia pyriformis* Cambess is a Brazilian plant with antioxidant and antimicrobial activity; however, there are no reports on its acaricidal activity in the literature. The present study aimed to evaluate the acaricidal and larvicidal potential of *E. pyriformis* leaf essential oil (EO) on southern cattle tick at different stages of the reproductive cycle. *E. pyriformis* leaves were collected and dried, and had their EO extracted by hydrodistillation (3h) using a modified Clevenger apparatus. Chemical analysis was performed by gas chromatography coupled with mass spectrometry (GC/MS), and 32 compounds belonging to the sesquiterpene class were identified: hydrocarbons (17.98%) and oxygenated forms (81.96%), with spathulenol (43.65%) and caryophyllene oxide (12.17%) as the most common. The EO was evaluated by the Adult Immersion Test at the concentrations (500.00 to 3.12mg/mL) in which the following parameters were measured: mortality of females (%), hatchability of eggs (%), and product efficiency (%). Larvae were assessed by the Larval Packet Test at concentrations ranging from 25.00 to 0.00004mg/mL. Lethal concentrations (LC) required for killing 50 and 99.9% of adult females and larvae were determined using Probit analysis. LC_{50} and $LC_{99.9}$ of EO were 0.06 and 24.60mg/mL and 1,208.80 and 2,538mg/mL for larvae and adult females, respectively. Action of the EO in the free-living cycle of *R.* (*B.*) *microplus* larvae was another parameter assessed. To this end, the larvae were deposited in pots containing *Brachiaria decumbens* and, after migration to the leaf apex, a solution containing $LC_{99.9}$ (24.60mg/mL) of the EO was sprayed. After 24h, 72.25% of the larvae had died, indicating stability of the EO when subjected to uncontrolled temperature and humidity conditions. The mechanism of action through which the EO killed the larvae and adult females was investigated by the Bioautographic Method, which showed

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[2] Graduate Program in Biotechnology applied to Agriculture, Universidade Paranaense (Unipar), Praça Mascarenhas de Moraes 4282, Umuarama, PR 87502-210, Brazil. E-mails: jaquepavel@hotmail.com, wanessa.bortolucci@edu.unipar.br, eloisa.s@edu.unipar.br, herika_line@hotmail.com; *Corresponding author: cristianigazim@prof.unipar.br

[3] Master of Science in Clean Technology, Instituto Cesumar de Ciência, Tecnologia e Inovação, Centro Universitário de Maringá, Avenida Guedner 1610, Jardim Aclimação, Maringá, PR 87050-900, Brazil. E-mails: jose.goncalves@unicesumar.edu.br, caiofaac@hotmail.com

[4] Graduate Program in Animal Science, Unipar, Praça Mascarenhas de Moraes 4282, Umuarama, PR 87502-210. E-mail: piau@prof.unipar.br

inhibition of 3.15mg/mL of the EO on the acetylcholinesterase (AChE) enzyme. The results found in the present experiment indicate that *E. pyriformis* essential oil is an alternative in the control of southern cattle tick in the larval (parasitic) and free-living cycle (non-parasitic) stages under field conditions.

INDEX TERMS: Biocidal, *Eugenia pyriformis*, essential oil, *Rhipicephalus (Boophilus) microplus*, free-living cycle, "uvaia", acaricide, larvicide, spathulenol, caryophyllene oxide, acetylcholinesterase, alternative acaricides, tick.

RESUMO.- [**Potencial biocida do óleo essencial de *Eugenia pyriformis* Cambess no controle de *Rhipicephalus* (*Boophilus*) *microplus* no ciclo de vida livre.**] O Brasil dispõe de um dos maiores rebanhos bovinos comerciais do mundo, sendo natural que junto a esse rebanho, coexista uma enorme quantidade de espécies parasitárias; dentre estes o carrapato *Rhipicephalus* (*Boophilus*) *microplus* que interfere na produtividade animal, causando prejuízos à pecuária de corte e leite. A utilização de acaricidas químicos no controle deste ácaro tem causado o surgimento de populações resistentes e neste sentido, controles alternativos utilizando plantas como fontes de acaricidas botânicos têm se mostrado eficazes. *Eugenia pyriformis* Cambess é uma planta brasileira com atividades antioxidante e antimicrobiana, entretanto não há relatos da atividade acaricida. O objetivo do presente estudo consistiu na avaliação do potencial acaricida e larvicida do óleo essencial das folhas de *E. pyriformis* sobre o carrapato bovino nos diferentes estágios do ciclo reprodutivo. As folhas foram coletadas, secas e o OE extraído por hidrodestilação (3 horas) em aparelho Clevenger modificado. A análise química foi realizada por cromatografia gasosa acoplada à espectrometria de massas (CG/EM) sendo identificados 32 compostos, pertencentes à classe sesquiterpenos: hidrocarbonetos (17.98%) e oxigenados (81.96%); tendo como majoritários o spathulenol (43,65%) e caryophylene oxide (12,17%). O OE foi avaliado pelo teste de imersão de adultos nas concentrações (500,00 a 3,12mg/mL) onde foram mensurados os parâmetros: mortalidade das fêmeas (%), eclodibilidade dos ovos (%) e eficiência do produto (%). As larvas foram avaliadas pelo teste de imersão larval (Larval Packet Test) nas concentrações que variaram de 25,00 a 0,00004mg/mL. Foram determinadas as concentrações letais (CLs) necessárias para matar 50 e 99,9% das fêmeas adultas e das larvas utilizando a análise de Probitos. As CL_{50} e $CL_{99,9}$ do OE foram (0,06 e 24,60mg/mL) para as larvas e (1.208,80 e 2.538mg/mL) para as fêmeas adultas, respectivamente. Outro parâmetro avaliado em nossa pesquisa foi mensurar a ação do OE no ciclo de vida livre das larvas de *R.* (*B.*) *microplus;* onde as larvas foram depositadas em vasos com *Brachiaria decumbens* e após migração destas para o ápice das folhas, foi aspergido solução contendo a $CL_{99,9}$ (24,60mg/mL) do OE. Após 24 horas, 72,25% das larvas morreram indicando que houve estabilidade do OE quando submetido a condições de temperatura e umidade não controladas. O mecanismo de ação pelo qual o OE matou as larvas e fêmeas adultas foi investigado pelo método bioautográfico, indicando uma inibição de 3,15mg/mL do óleo essencial sobre a enzima acetilcolinesterase (AChE). Os resultados encontrados no presente experimento indicaram que o óleo essencial de *E. pyriformis* é uma alternativa no controle do carrapato bovino no estágio larval (parasitário) e no ciclo de vida livre (estágio não parasitário) em condições de campo.

TERMOS DE INDEXAÇÃO: Biocida, óleo essencial, *Eugenia pyriformis*, *Rhipicephalus* (*Boophilus*) *microplus*, ciclo de vida livre, uvaia, acaricida, larvicida, spathulenol, caryophyllene oxide, acetilcolinesterase, alternative acaricida, carrapato.

INTRODUCTION

Livestock is one of the most important activities in Brazilian agribusiness, as the country has one of the largest commercial cattle herds and is the second largest producer and the largest exporter of beef in the world (De Carvalho & De Zen 2017). However, profitability of the livestock activity is significantly diminished by the effects of parasites affecting cattle. Southern cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*, is among these parasites (Ramírez et al. 2016). It is an ectoparasite with a parasitic life cycle of 21 days in live host, and a free-living cycle of up to 300 days in pasture without live host (Pereira et al. 2015). This tick causes losses in the order of billions of dollars per year in Brazil (Grisi et al. 2014) as a result of reduced beef and milk production, falling birth rates, increased mortality, devaluation of leather, transmission of important pathogens, and spending on an attempt to control it (Campos et al. 2012).

Control of this tick is based on the use of chemicals such as organophosphates, carbamates, amidines, synthetic pyrethroids, fluaruzon, and lactone macrolides (Mendes et al. 2013). One of the problems of the continuous use of chemicals in the control of ticks is the residues left in the body and milk of treated animals, presenting a long period of action. In humans, these residues can cause neurotoxic disorders, endocrine system disorders, allergies, cough, and abnormalities of lung function (Ferrarini et al. 2008). Thus, there is a demand for the implementation of research and technologies that promote sustainable development with the use of natural products (Zaman et al. 2012) that have acaricidal properties, which may be an alternative to control ticks with lesser environmental impact (Chagas et al. 2002).

There are several species of plants in the Brazilian flora, or cultivated in the country, that are used in southern cattle tick control programs in an attempt to reduce the use of conventional products. Citronella (*Cymbopogon nardus*), neem (*Azadirachta indica*), andiroba (*Carapa guianensis*), tobacco (*Nicotiana tabacum*), lemongrass (*Cymbopogon citratus*), eucalyptus (*Eucalyptus* spp. and *Corymbia*) and others comprise the group of herbal medicines with acaricidal efficacy (Alves et al. 2014).

Research in this area is promising, as evidenced by the several studies conducted on this theme. Clemente et al. (2010) observed the acaricidal activity of *Eucalyptus citriodora* and *Cymbopogon nardu* oils on ticks. De Souza et al. (2012) observed a tendency to low mammalian toxicity, rapid degradation and slow resistance development in plant-borne acaricides. Gomes et al. (2012) evaluated the effect of *Lippia sidoides* oil

on *R.* (*B.*) *microplus* larvae and females. De Monteiro et al. (2012) and Valente et al. (2014) confirmed the acaricidal activity of myrtaceae on larvae and females of *R. microplus*. De Assis et al. (2015) demonstrated the acaricidal activity of *Baccharis dracunculifolia* essential oil on non-engorged larvae and engorged females of *R. microplus*.

However, there are no reports of control studies addressing the southern cattle tick *R.* (*B.*) *microplus* using *Eugenia pyriformis* Cambess essential oil (EO).

E. pyriformis species belongs to family Myrtaceae, a plant commonly found in the states of São Paulo, Paraná, Santa Catarina, and Rio Grande do Sul, known by the popular names of "uvaia', 'uvaieira", or field bush (Armstrong et al. 2012). This plant is a 6-13m tall tree with brown, erect trunk and rounded crown. Flowering occurs from August to September with fruit ripening between November and December. The genus to which it belongs presents a large number of species in Brazil, which are known for the production of essential oils (Stieven et al. 2009). Research has shown that *E. pyriformis* EO has acaricidal (De Assis et al. 2011), antimicrobial, antioxidant (Stieven et al. 2009), and antifungal (Souza 2013) potential against *Tyrophagus putrescentiae* and *Suidasia pontifica* Oudemans. Studies conducted with the fruits of "uvaia" revealed the presence of total flavonoids and phenolic compounds, highlighting the antioxidant potential of this species (Silva et al. 2018, Da Silva et al. 2019). However, there are no reports in the literature on the application of *E. pyriformis* oil in the control of southern cattle tick, which led our research group to evaluate the chemical composition of *E. pyriformis* leaf EO and its effectiveness in the control of this pathogen *in vitro* and *ex situ* (free-living cycle), as well as to measure the acaricidal activity of this EO in application under semi-natural conditions for the control of *R.* (*B.*) *microplus* larvae.

MATERIALS AND METHODS

Data on plant material, soil analysis, temperature (°C), rainfall (mm) and relative humidity (%). *Eugenia pyriformis* Cambess leaves were collected in the municipality of Mangueirinha, southwestern Paraná state, Brazil (25°56'28" S; 52°10'32" W) 921m a.s.l. from January to August 2015. During this period, data on temperature (°C), rainfall (mm) and relative humidity (%) were obtained from the "Secretaria Estadual de Agricultura e Abastecimento" (SEAB) of the aforementioned municipality. The plant was identified and deposited at the Herbarium of the "Universidade Estadual de Maringá" (UEM) under no. 30.713. This species is registered in the National System of Genetic Heritage Management and Associated Traditional Knowledge (SisGen) under no. A06F4D8. The soil in the location where the *Eugenia pyriformis* leaves were collected at 0-20cm depth in different points was analyzed at the Santa Rita Soil Analysis Laboratory in the municipality of Mamborê, Paraná state.

Extraction of *Eugenia pyriformis* leaf essential oil (EO). The leaves were dried at room temperature, sectioned in a milling processor, and hydrodistilled using the modified Clevenger apparatus. The plant-to-water ratio was 300g fragmented leaves to 3000mL distilled water. Hydrodistillation was performed for 3h, and the oil was removed with *n*-hexane, filtered with anhydrous sodium sulfate (Na_2SO_4) (Simões & Spitzer 2002), stored in amber flasks, and kept under refrigeration at 4°C (Omolo et al. 2004) for complete solvent evaporation. EO yield was determined by the dry leaf mass to EO mass (%) ratio.

Physical and chemical indices of *Eugenia pyriformis* leaf EO. Relative density was obtained by the mass-to-volume ratio at 20°C, and determined according to the technique described by the Farmacopeia Brasileira (1988). The refractive index was determined using an RL-3 Abbe refractometer (Warsaw, Poland) (Farmacopeia Brasileira 1988).

Chemical characterization. Chemical identification of the EO was carried out by GC-MS using an Agilent 7890B Gas Chromatograph coupled with an Agilent 5977 A MSD Mass Spectrometer and an Agilent HP-5ms UI fused silica capillary (30×250µm × 0.25µm; Agilent Technologies), with initial oven temperature of 50°C (2min), followed by an increase to 60°C at 5°C/min for 1min, an increase to 250°C at 5°C/min for 15min and, finally, an increase to 300°C at 50°C/min for 1min. Helium was utilized as the carrier gas at the linear speed of 1mL/min up to 300°C and pressure release of 56kPa. The injector temperature was 250°C; injection volume was 1µL; injection occurred in split mode (20:1). The temperatures of the transfer line, ion source, and quadrupole were 240, 230 and 150°C, respectively. The MS detection system was used in scan mode at the mass-to-charge ratio (*m/z*) of 40-550 with solvent delay of 3min. The compounds were identified by comparing the mass spectra found in NIST Mass Spectral Library (NIST 11.0) and the retention indices (RI) obtained by a homologous series of *n*-alkane standard (C7-C28) (Adams 2012).

Essential oil activity on *Rhipicephalus* (*Boophilus*) *microplus*. Acaricidal activity of "uvaia" EO was determined by the adult immersion test (AIT) (Drummond et al. 1973) and the larval packet test (LPT), recommended by the Food and Agriculture Organization (FAO 2004), whereas larvicidal activity was verified in the free-living cycle (Araújo et al. 2015).

Adult immersion test (AIT). A total of 360 *Rhipicephalus* (*Boophilus*) *microplus* female ticks with maximum engorgement were collected from dairy cattle in a farm in the municipality of Umuarama, Paraná state, Brazil. Then *E. pyriformis* EO solutions were prepared at the following concentrations: 500.00, 400.00, 300.00, 200.00, 100.00, 50.00, 25.00, 12.50, 6.25 and 3.12mg/mL. A 2% (v/v) aqueous polysorbate-80) solution was used as EO emulsifier. The negative control was a 2% (v/v) aqueous polysorbate-80 solution, whereas the positive control was a 0.125% commercial solution containing 15% cypermethrin, 25% chlorpyrifos and 1% citronellal. Subsequently, the ticks cleaned with water and selected based on normal appearance and motility, body integrity, and maximum engorgement (Leite et al. 1995). The AIT was performed according to Drummond et al. (1973). From the data obtained (tick mass, egg mass, and egg hatching rate), reproductive efficiency (RE) and product efficiency (PE) were calculated according to Equations 1 and 2, respectively (Drummond et al. 1973).

$$RE = \frac{egg\ mass(g) \times hatching\ rate(\%) \times 20.000}{tick\ mass(g)} \quad (1)$$

$$RE = \frac{negative\ Control\ Group\ RE - Treated\ Group\ RE \times 100}{negative\ Control\ Group\ RE} \quad (2)$$

Larval packet test (LPT). Larvicidal activity of the EO on *R.* (*B.*) *microplus* larvae was determined by the Larval Packet Test (LPT). EO was used at concentrations of 25.00, 12.50, 6.25, 3.00, 1.50, 0.78, 0.39, 0.19, 0.09, 0.04, 0.02 0.01, 0.006, 0.003, 0.0015, 0.0007, 0.0003, 0.00019, 0.00009, and 0.00004mg/mL. Reading consisted in observing their motility, and those that did not show motility at touch after 24h were considered dead (Leite et al. 1995).

Larval mortality rate (%) and mean mortality (%) were calculated according to Equations 3 and 4.

$$Larval\ mortality(\%) = \frac{dead\ larvae \times 100}{total\ larvae} \quad (3)$$

$$Average\ mortality(\%) = \frac{1st\ repetition + 2nd\ repetition + 3rd\ repetition}{3} \quad (4)$$

Larvicidal activity of *Eugenia pyriformis* essential oil on the free-living cycle of *Rhipicephalus* (*Boophilus*) *microplus* larvae. Plastic pots (n=9), 25cm high and 25cm in diameter, were filled with 2.2kg of soil, previously autoclaved at 121°C for 2h. Each pot was fertilized with 1g of N, P, K fertilizer (08-20-10), and six *Brachiaria decumbens* seeds (Figueiredo et al. 2013) were planted at a depth of 2cm. Plants were allowed to grow in a greenhouse with daily watering for three months (Araújo et al. 2011). After development, each pot was again fertilized with 1g of N, P, K (20-05-20), and the *B. decumbens* leaves were pruned at the height of 40cm off the ground. At the edges of the pots, physical barriers of adhesive tape were placed to prevent the larvae from escaping. Then 30mg of larvae were deposited at the base of each *B. decumbens* seedling. After 24h, the larvae migrated to the apex of the grass leaves. After that, 5mL of a positive control triplicate (commercial solution at 0.000125mg/mL containing 0.015mg/mL cypermethrin, 0.025mg/mL chlorpyrifos and 0.001mg/mL citronellal) were sprayed on the grass leaves. Negative control (2% (v/v) aqueous polysorbate-80 solution) and *E. pyriformis* EO were prepared in a 2% (v/v) aqueous polysorbate-80 solution at the $CL_{99.9}$ previously established in *in vitro* tests. The application was concentrated at the apex of the plant, simulating the application of commercial acaricides on pastures. After 24h, the *Brachiaria* leaves were removed and the dead larvae were counted with the aid of an entomological magnifying glass. The mean number of live larvae in the negative control group was compared with the mean number of live larvae in the treatments. Extract efficacy (%) was obtained according to Equation 5 (Bittencourt et al. 2003), where: A = mean number of live larvae in the negative control group and B = mean number of live larvae in the treatment groups.

$$Efficiency(\%) = \frac{A - B \times 100}{A} \quad (5)$$

Anticholinesterase activity of the essential oil. Anticholinesterase activity was determined by the Bioautographic Method described by Marston et al. (2002), with modifications (Yang et al. 2009). *Eugenia pyriformis* leaf EO was tested at concentrations ranging from 500.00 to 0.18mg/mL, diluted in methanol. The samples were plotted on aluminum chromatoplates (10x10cm, 0.2mm thick, silica gel 60 F254). After plotting, the plates were dried and an acetylcholinesterase enzyme solution in TRIS buffer was sprayed on them, followed by spraying of an α-naphthyl acetate solution. The plates were kept at 37°C for 20min. After that, the chromatoplates were sprayed with Fast Blue B salt colorimetric reagent, resulting in a purple-colored surface. Anticholinesterase activity of the EO was determined by the appearance of white spots after 5min, demonstrating the inhibitory action of the concentrations evaluated on the enzyme activity, contrasting with the purple color of the colorimetric reagent (Collins et al. 1997). The positive pattern was Temephós® larvicide.

Statistical analysis. The experimental design was completely randomized. Data were processed and submitted to analysis of variance (ANOVA), and differences between arithmetic means and the standard deviation were determined by Tukey's test at 5% significance level. Lethal concentrations that killed 50% (LC_{50}) and 99.9% ($LC_{99.9}$) of adult teleogines and larvae and their CIs (5%) were calculated by Probit analysis (ED 50 Plus 1.0). All tests were performed in triplicate.

RESULTS AND DISCUSSION

Data on plant material, soil analysis, temperature (°C), rainfall (mm) and relative humidity (%)

Hydrodistillation of dried *Eugenia pyriformis* leaves yielded 0.023% ±0.06 colorless oil. These results are in accordance with those of Stefanello et al. (2009), who found yield of 0.02-0.04% for fresh leaves in the city of Curitiba, Paraná state, Brazil, collected from October to December 2005. However, Apel et al. (2004), using fresh leaves collected in the state of Rio Grande do Sul, Brazil, in June 1997, found a yield (0.4%) higher than that obtained in the present experiment. This difference may have been influenced by seasonality (Morais 2009), as this species is resistant to cold, light frost, and abundant rainfall (Sartori et al. 2010). This information coincides with the meteorological data for the region where the leaves were collected, whose data showed 19.65±3.62°C temperature, 70.86±44.53mm rainfall, and 77.56±4.87 (%) relative humidity.

Density and refractive index found in this study were 0.95 g/mL and 1.4835, respectively. These parameters confirm the identity and detection of essential oil impurities (Instituto Adolfo Lutz 1985). Care during the extraction and storage processes influences the quality of essential oils, as they are unstable in the presence of light, oxygen, heat, moisture, and metals. Thus, it is necessary to perform physicochemical analyses as a way of certifying and maintaining quality (Serafini et al. 2001).

The soil in which *E. pyriformis* was planted is classified as clayey, acidic (pH 4.13), of low fertility (V% 24.23), and normality for organic matter (23.19% C) (Ronquim 2010) (Table 1).

According to Nascimento et al. (1995), the ideal soils for "uvaia" cultivation are clayey neosols or latosols and perfect soil pH is 4.1-5.6. Sartori et al. (2010) consider the pH of 4.5-5.5 as ideal for the cultivation of sedimentary origin and average fertility. Thus, the location where the *E. pyriformis* of this study was cultivated corroborated the data in the literature regarding the appropriate soil characteristics.

Chemical characterization of the essential oil (EO)

Thirty-two compounds were identified by GC/MS in the EO extracted in this study. The predominant class was sesquiterpenes: oxygenates (82.02%) and hydrocarbons (17.98%), with spathulenol (43.65%) and caryophyllene oxide (12.17%) as the most commonly found (Table 2).

Comparison between the chemical composition of the *Eugenia pyriformis* leaf EO found in the present experiment and those of other studies showed that in Porto Alegre, Rio Grande do Sul state, Brazil, Apel et al. (2004) identified 29 compounds whose main class was sesquiterpenes: hydrocarbons (17.98%) and oxygenates, (81.96%) with predominance of α-cadinol (14.00%) and δ-cadinene (12.40%). In this same context, the study conducted by Stefanello et al. (2009) in Curitiba, Paraná state, Brazil, found 65 compounds whose predominant class was monoterpenes, with limonene (12.05%) and β-pinene

Table 1. Analysis of pH, base saturation, micro- and macro-nutrients of the soil for cultivation of *Eugenia pyriformis*

	pH and Macronutrients							
	cmol/dm^{-3}						mg dm^{-3}	
pH ($CaCl_2$)	Al^{3+}	$H^+ + Al^{3+}$	$Ca^{2+} + Mg^{2+}$	K^+	SB*	CEC*	P	C
4.13	1.90	10.85	3.07	0.4	3.47	14.32	4.36	23.19
Base saturation	Micronutrients (cmol/dm^{-3})							
V (%)	Fe	Cu	Mn	Zn	B	S	Ca/Mg	Ca/K	Mg/K
24.23	46.10	6.97	120	5.59	0.30	4.73	1.26	4.27	3.4

SB = sum of bases, CEC = cation exchange capacity, V = base saturation, pH = pH in $CaCl_2$, P = phosphorus, C = carbon, Al^{3+} = aluminum, H^+Al^{3+} = potential acidity, Ca^{2+} = calcium, Mg^{2+} = magnesium, K^+ = potassium.

Table 2. Chemical composition of *Eugenia pyriformis* leaf essential oil

Peak	Compounds[a]	Relative area[b] (%)	RI calculate[c]	Method identification
	Hydrocarbon sesquiterpenes			
1	α-copaene	1.11	1376	a,b,c
2	β-bourbonene	2.25	1388	a,b,c
3	β-elemene	t	1390	a,b,c
4	α-gurjunene	t	1409	a,b,c
5	β-caryophylene	1.49	1419	a,b,c
6	β-gurjunene	0.70	1433	a,b,c
7	Aromadendrene	1.40	1441	a, b,c
8	α-humulene	1.18	1454	a, b,c
9	*Allo*-aromadendrene	2.37	1460	a, b,c
10	α-amorphene	0.79	1484	a, b,c
11	Germacrene D	t	1485	a, b,c
12	Bicyclogermacrene	1.99	1500	a, b,c
13	α- muurolene	0.82	1500	a, b,c
14	γ-cadinene	1.06	1513	a, b,c
15	*Cis*-calamenene	0.79	1522	a, b,c
16	*Trans*-cadina 1,4 - diene	0.78	1534	a, b,c
17	α-cadinene	1.25	1538	a, b,c
	Oxygenated sesquiterpenes			
18	Valerenol	t		a, b,c
19	Spathulenol	43.65	1578	a, b,c
20	Caryophylene oxide	12.17	1583	a, b,c
21	Viridiflorol	5.00	1592	a, b,c
22	Globulol	1.86	1590	a, b,c
23	Guaiol	2.35	1560	a, b,c
24	Ledol	2.91	1602	a, b,c
25	N.i.	0.03		a, b,c
26	*Iso*-sphatulenol	1.55	1625	a, b,c
27	*Epi*-α-cadinol	4.29	1638	a, b,c
28	*Allo*-aromadendrene epoxide	1.54	1641	a, b,c
29	*Epi*-α-muurolol	4.18	1640	a, b,c
30	Cariofilla-3,8(13)-dien-5β-ol	0.89	1640	a, b,c
31	α-santalol	0.57	1674	a, b,c
32	β-santalol	1.00	1715	a, b,c
TOTAL identified		99.94		
Hydrocarbon sesquiterpenes		17.98		
Oxygenated sesquiterpenes		81.96		

[a] Compounds listed in order of elution in column HP-5MS, [b] Relative area (%) = percentage of the area occupied by the compounds in the chromatogram, [c] RI calculate = identification based on retention index (RI) using a homologous series of *n*-alkane C_7-C_{40} on Agilent HP-5MS column, [d] MS = identification based on comparison of mass spectra using Wiley 275 libraries, N.i = not identified, t = traces.

(15.60%) as the most common. The *Eugenia pyriformis* leaf EO composition in this experiment showed variability compared with the studies by Apel et al. (2004) and Stefanello et al. (2009). Interestingly, the experiments conducted with EO of this species were mainly conducted in the southern Brazil. Although this region presents similar climatic characteristics, differences in EO chemical composition were observed. This variability in EO chemical composition may be associated with the influence of environmental factors such as temperature and humidity, edaphic factors, and genetic variability (Borsato et al. 2007, Gobbo-Neto & Lopes 2007, Cerqueira et al. 2009). The EO analyzed in this study was obtained from a single species, in the same locality, and under the same conditions. Thus, it is considered that local climatic variations and soil composition (Table 1) contributed to its variability. Biotic and abiotic factors directly influence the chemical composition of essential oils. According to Morais (2009), information on the species used, the vegetative phase of the plant during collection, crop implantation location, soil information, temperature and altitude are fundamental for the correct knowledge of its chemical and biological characteristics.

EO activity on *Rhipicephalus* (*Boophilus*) *microplus in vitro*

The objective of the present experiment was to verify in which stage of the *R.* (*B.*) *microplus* reproductive cycle the EO showed the highest activity. The results of acaricidal activity indicated that *Eugenia pyriformis* EO, at the tested concentrations, did not significantly promote mortality of engorged females (Fig.1), with LC_{50} of 1,562.90mg/mL and $LC_{99.9}$ of 2,578.67mg/mL (Table 3), i.e., the EO did not act in the first stage of southern cattle tick reproductive cycle, which corresponds to the parasitic life cycle.

Another important step in this experiment was to evaluate whether *E. pyriformis* essential oil inhibited the hatchability of eggs (Fig.2). The results indicated that, at the concentration of 500.00mg/mL, the EO provided 54.36% inhibition, thus evidencing low reproductive efficiency of the tick (64.73%) (Fig.3).

However, the EO showed higher activity when tested in the free-living cycle, which corresponds to the larval stage (Fig.4), showing LC_{50} of 0.06mg/mL and $LC_{99.9}$ of 24.60mg/mL (Table 3).

Effect of the EO on the larvae were expressive because, according to Cheng et al. (2003), good larvicidal agents present LC_{50} values <0.10mg/mL, and the *E. pyriformis* EO LC_{50} found in the present experiment was 0.06mg/mL (Table 3). When comparing the LCs between engorged females and larvae, it was evident that the EO was more active on the larvae. The likely explanation for this difference may be associated with the cuticle composition of the larvae and adult females, as tick cuticle is formed by an outer layer, epicuticle (composed of waxes and proteins), and an inner layer, endocuticle (composed of chitin and protein). According to Odhiambo (1982), the lipid layer is observed in *R.* (*B.*) *microplus* only from nymph ecdysis and, in greater quantity in the adult tick, in which it is thicker. This difference in cuticle thickness is also explained by Furlong & Sales (2007), who stated that the increase in the cuticle thickness of engorged females occurs as a result of hormonal stimulation, so that there is no extravasation of their acquired proteins during the blood meal. According to Santos et al. (2015), cuticle thickening

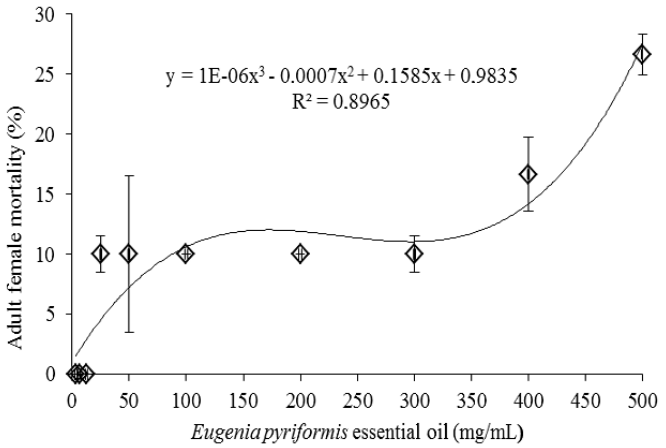


Fig.1. Mortality (%) of *Rhipicephalus* (*Boophilus*) *microplus* engorged adult females subjected to the adult immersion test (AIT) at different concentrations of *Eugenia pyriformis* essential oil. Positive control (commercial solution) = 100% adult female mortality and negative control (2% polysorbate-80) = 0% adult female mortality.

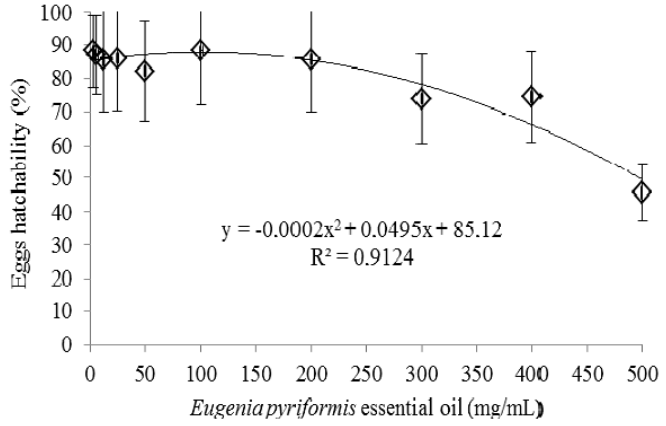


Fig.2. Hatchability of eggs (%) of *Rhipicephalus* (*Boophilus*) *microplus* subjected to adult immersion test (AIT) at different concentrations of *Eugenia pyriformis* essential oil. Positive control (commercial solution) = 0% hatchability of eggs and negative control (2% polysorbate-80) = 95.71±2.17% hatchability of eggs.

Table 3. Lethal concentrations (LC_{50} and $LC_{99.9}$ mg/mL) of *Eugenia pyriformis* leaf essential oil on adult females and larvae of *Rhipicephalus* (*Boophilus*) *microplus* by Probit analysis

	Rhipicephalus (Boophilus) microplus	
	CL_{50} (mg/mL)	$CL_{99.9}$ (mg/mL)
Positive control	0.019 ± 0.001[a] (*)	0.21 ± 0.01[a] (*)
Larvae CI	0.06 ± 0.003[a] (0.057 - 0.063)	24.6 ± 5.74[a] (18.86 - 30.34)
Adult females CI	1,208.80 ± 118.80[b] (1,111.90 - 1,305.70)	2,538 ± 238[b] (2,344 - 2,733)

Values presented as mean ±standard deviation. [a,b] Means followed by same letters in the same column do not statistically differ by Tukey's test at 5% significance level; LC_{50} = lethal concentration 50%, $LC_{99.9}$ = lethal concentration 99.9%, CI = confidence interval, Positive control = commercial organophosphorus (15% cypermethrin; 25% chlorpyrifos; 1% citronellal).

makes it difficult for EO to penetrate only through joints and natural orifices, leaving the adult tick less vulnerable to poisoning, which makes the lethal dose higher for teleogines compared with larvae.

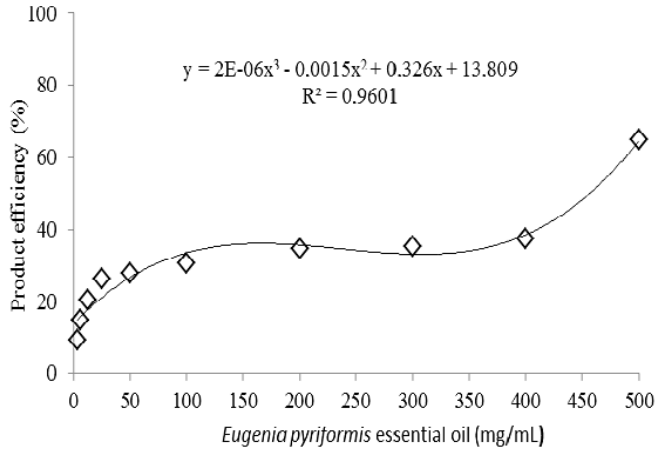


Fig.3. Product efficiency (%) of *Eugenia pyriformis* essential oil on *Rhipicephalus* (*Boophilus*) *microplus*. Positive control (commercial solution) = 100% product efficiency and negative control (2% polysorbate-80) = 0% product efficiency. Means obtained from the equation by Drummond et al. (1973).

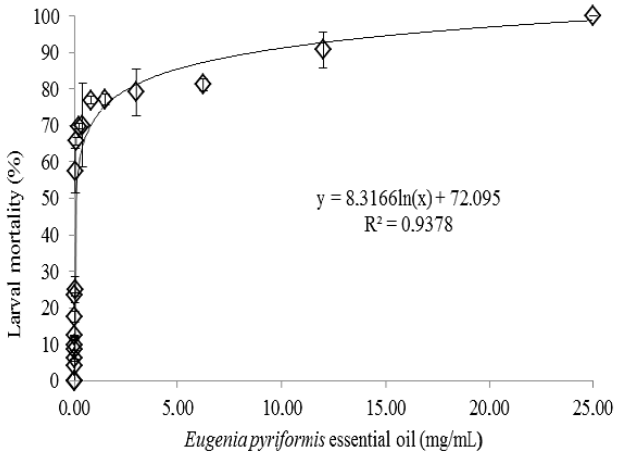


Fig.4. Mortality (%) of *Rhipicephalus* (*Boophilus*) *microplus* larvae subjected to the larval packet test (LPT) at different concentrations of *Eugenia pyriformis* essential oil. Positive control (commercial solution) = 100% larval mortality and negative control (2% polysorbate-80) = 0% larval mortality.

EO activity on *Rhipicephalus* (*Boophilus*) *microplus* under semi-natural conditions

Another stage of the reproductive cycle of the tick corresponds to the larval free-living cycle. It is an important stage because 95% of the larvae are distributed in pastures, and only 5% are parasitizing animals (Powell & Reid 1982). Thus the $LC_{99.9}$ of 24.60mg/mL (Table 3) found for larvicidal activity by the Larval Packet Test was used in the field tests for the tick free-living cycle (Table 4).

Twenty-four hours after EO spraying at 24.60mg/mL concentration on tick larvae positioned at the apex of the *Brachiaria decumbens* leaves, larva mortality of 72.25% was observed (Table 4).

Some essential oils have been shown to have proven acaricidal activity on *R.* (*B.*) *microplus* larvae in *in vitro* studies (Scoralik et al. 2012). However, there seem to be no reports on the application of *E. pyriformis* EO for tick control at any stage of the free-living cycle, which is the first to evaluate the activity of EO on *R.* (*B.*) *microplus* larvae under semi-natural conditions.

Regarding the application of EO and other substances of plant origin, many *in vitro* studies have been conducted, but there are still few investigations *ex sito*, simulating field conditions. Under semi-natural conditions, thymol found in the EO of Lamiaceae and Verbenaceae (Pengelly 2004) plants was tested at a concentration of 10mg/mL (LC90=9.25mg/mL) in *Brachiaria decumbens* pots containing of *R.* (*B.*) *microplus* larvae, reducing more than 95% the number of live larvae (Araújo et al. 2015), thus indicating the efficiency of the protocol established by this author.

From the results obtained in the present study, it is possible to conclude that *E. pyriformis* EO, when applied to *B. decumbens* pots under semi-natural conditions, showed a 72.25% reduction in tick larvae reproductive efficiency and stability when used in an uncontrolled environment (Table 4).

Anticholinesterase activity of the essential oil (EO)

Another important aspect within this research was to determine by which mechanism of action the EO acted on southern cattle tick. In conventional southern cattle tick control treatment, it is common to use organophosphate, a class of pesticides that promote the inhibition of the acetylcholinesterase (AchE) enzyme. Despite the high toxicity of these substances, they are widely used in livestock and agriculture, as well as domestically (Worek et al. 2005).

Thus the activity on AchE was evaluated by the Bioautographic Method (Table 5).

The results showed that the EO inhibited the enzyme at a concentration of 3.15mg/mL, which is superior to the results

Table 4. Larvicidal activity of *Eugenia pyriformis* leaf essential oil on larvae of *Rhipicephalus* (*Boophilus*) *microplus* by the *ex situ* test

Treatment	Mean number of live larvae	Treatment efficacy (%)
Positive control	0.00 ± 0.00[a]	100
EO (24.6 mg/mL)	66.00 ± 15.51[a]	72.25
Negative control	237.67 ± 55.19[b]	0.00

Values presented as mean ±standard deviation. [a,b] Means followed by same letters in the same column do not statistically differ by Tukey's Test at 5% significance level; Positive control = commercial organophosphorus (15% cypermethrin, 25% chlorpyrifos, 1% citronellal), Negative control = polysorbate-80 (2%).

Table 5. Inhibiting activity of acetylcholinesterase enzyme at different concentrations of *Eugenia pyriformis* leaf essential oil (EO) by the Bioautographic Method

Acetylcholinesterase inhibitor (AChEI)		
Concentration (mg/mL)	*Eugenia pyriformis* (EO)	Positive control
500.00	+	+
400.00	+	+
300.00	+	+
200.00	+	+
100.00	+	+
50.00	+	+
25.00	+	+
12.50	+	+
6.25	+	+
3.15	+	+
0.75	-	+
0.37	-	+
0.18	-	+

PC = Positive control; commercial organophosphorus (15% cypermethrin, 25% chlorpyrifos, 1% citronellal); +++ strong inhibition, ++ moderate inhibition, + weak inhibition of acetylcholinesterase enzyme; (-) absence of inhibition; EO = essential oil.

found in the *in vivo* larvae test ($LC_{99.9}$ 24.60mg/mL). This difference may be explained by the absence of physiological conditions that interfere with biochemical reactions *in vivo*, because the Bioautographic Method is performed in a controlled environment with all pre-established conditions, without interference of cell wall permeability, absorption characteristics of molecules, as well as solubility in hydrophilic and lipophilic media inherent in living beings (Benson 2005, Brain et al. 2007).

Molecules capable of inhibiting or reactivating the AChE enzyme are potential substitutes for organophosphates. Success in developing products of these classes depends on the efficiency and practicality of the tests employed to evaluate AchE activity (Araújo et al. 2016).

CONCLUSIONS

Eugenia pyriformis Cambess leaf essential oil (EO) presented sesquiterpenes as the predominant class, with spathulenol and caryophyllene oxide as major compounds.

In *in vitro* tests, the EO was active against *Rhipicephalus* (*Boophilus*) *microplus* southern cattle tick larvae at $LC_{99.9}$ of 24.6mg/mL. When tested under semi-natural conditions (*ex situ*), the EO showed a 72.25% reduction in tick larvae reproductive efficiency and stability when applied in an uncontrolled environment. The mechanism of action by which the EO killed the larvae was inhibition of the acetylcholinesterase enzyme at a concentration of 3.15mg/mL, thus indicating presence molecules with biocidal potential in the *E. pyriformis* leaf EO.

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Dystrophic mineralization in uremic dogs: an update[1]

Paula G.S. Cardoso[2], Marcela P.R. Pinto[2], Ludmila R. Moroz[3], Thanielle N. Fontes[2], Rosilane S. Santos[4], Júlia L. Freitas[2], Vivian A. Nogueira[5] and Tiago C. Peixoto[3]*

ABSTRACT.- Cardoso P.G.S., Pinto M.P.R., Moroz, L.R., Fontes T.N., Santos R.S., Freitas J.L., Nogueira V.A. & Peixoto T.C. 2019. **Dystrophic mineralization in uremic dogs: an update.** *Pesquisa Veterinária Brasileira 39(11):889-899.* Departamento de Anatomia, Patologia e Clínicas Veterinárias, Universidade Federal da Bahia, Avenida Adhemar de Barros 500, Ondina, Salvador, BA 40170-110, Brazil. E-mail: tcpeixoto@ufba.br

Pathological mineralization is the abnormal deposition of minerals in body tissues, previously injured or not. In these lesions, in addition to calcium, other minerals can be found at lower concentrations. Classically, mineralization is divided into two types: dystrophic and metastatic. However, currently, there is no consensus among researchers on the type of mineralization that occurs in uremic dogs. The objective of this study was to elucidate the type of pathological mineralization that occurs in dogs with uremic syndrome through the correlation of biochemical examinations with gross and histopathological changes, given the existence of controversial information on this theme in the specialized literature. The Shapiro-Wilk, D'Agostino and Pearson tests were used to evaluate data normality distribution, and analysis of variance (ANOVA) was applied to compare the data between more than two groups. Additionally, the Dunnett's multiple comparison test was used in the comparison between the Control Group (CG) and the Experimental Groups (G1, G2, and G3). Serum levels of urea, creatinine, total and ionized calcium, phosphorus, calcium-phosphorus product (CPP), parathyroid hormone (PTH), and albumin of 40 azotemic dogs with chronic kidney disease (CKD) were evaluated. Dogs were categorized by degree of azotemia (mild, moderate, and severe). Ionized hypocalcemia was observed in 97.5% (39/40) of the dogs, and no animals presented ionized hypercalcemia. Hyperphosphatemia was frequent (62.5%), especially in dogs with severe azotemia. PTH concentration increased with progression of azotemia, and high PTH levels were verified in 100% of the dogs with severe azotemia. CPP >60mg^2/dl^2 was observed in 75% (30/40) of the dogs. Of the 29 dogs that died during the study period, 16 were necropsied. Soft tissue mineralization was observed in 93.7% (15/16) of these dogs at gross and histopathological evaluation (HE and Von Kossa), regardless of the degree of azotemia, in nine organs/tissues: kidneys (75%), lungs (50%), stomach (31.2%), heart (25%), larynx (25%), intercostal muscles (25%), aorta (6.2%), intestines (6.2%), and tongue (6.2%). In one animal, the serosa of all segments of the small intestine showed whitish, rough, irregular, multifocal plaques of varying sizes, confirmed by histopathology as dystrophic mineralization of the longitudinal outer muscular layer, which presented necrosis of coagulation and of the intestinal serosa. This intestinal lesion has not been described in dogs with uremic syndrome to date. In conclusion, the laboratory and histopathologic data previously described, especially regarding tissue and vascular mineralization, which occur in association with previous degenerative/necrotic lesions in the absence of hypercalcemia in dogs with CKD, assist with clarifying inconsistencies found in the existing literature. Therefore, conceptually, mineralization that occurs in uremic dogs should be considered dystrophic.

INDEX TERMS: Dystrophy, mineralization, uremia, dogs, nephrology, pathological mineralization.

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[2] Graduate Studies Program in Animal Science in the Tropics (PPGCAT), Escola de Medicina Veterinária e Zootecnia (EMEVZ), Universidade Federal da Bahia (UFBA), Salvador, BA 40170-110, Brazil.

[3] Departamento de Anatomia, Patologia e Clínicas Veterinárias, EMEVZ, UFBA, Salvador, BA 40170-110. *Corresponding author: tcpeixoto@ufba.br

[4] Veterinarian, Salvador, BA, Brazil.

[5] Departamento de Epidemiologia e Saúde Pública, Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro (UFRRJ), Rodovia BR-465 Km 7, Seropédica, RJ 23890-000, Brazil.

RESUMO.- [**Mineralização distrófica em cães urêmicos: uma atualização.**] Mineralização patológica é a deposição anormal de minerais em tecidos previamente lesados ou não. Nessas lesões, além do cálcio, outros minerais podem ser encontrados em concentrações inferiores. Classicamente, as mineralizações são divididas em dois tipos: distrófica e metastática. Contudo, atualmente, ainda não há consenso entre os pesquisadores sobre o tipo de mineralização que ocorre em cães urêmicos. Objetivou-se com esse estudo elucidar o tipo de mineralização patológica que ocorre em cães com síndrome urêmica através da correlação de exames bioquímicos com alterações macroscópicas e histopatológicas, visto a existência de informações controversas na literatura especializada. Os dados obtidos foram submetidos ao teste de Shapiro-Wilk e teste de D'Agostino e Pearson para avaliação da normalidade da distribuição e para comparação de dados em mais de dois grupos foi utilizado o teste ANOVA. Adicionalmente, o teste de comparações múltiplas de Dunnett permitiu a comparação entre o grupo controle (GC) com os demais grupos (G1, G2 e G3). Foram avaliados os níveis séricos de ureia, creatinina, cálcio total e ionizado, fósforo, produto cálcio-fósforo (PCF), PTH e albumina de 40 cães azotêmicos com doença renal crônica (DRC). Os cães foram classificados quanto ao grau de azotemia (leve, moderada e severa). Verificou-se hipocalcemia ionizada em 97,5% (39/40) dos cães e, em nenhum animal houve hipercalcemia ionizada. Hiperfosfatemia foi frequente (62,5%), principalmente em cães com azotemia severa. A concentração do PTH aumentou conforme a progressão da azotemia, encontrando-se elevada em 100% dos cães com azotemia severa. Em 75% (30/40) dos cães o PCF foi superior a $60mg^2/dl^2$. Durante o estudo, 29 cães morreram, sendo 16 desses necropsiados. Em 93,7% (15/16) desses cães observou-se mineralização de tecidos moles, durante a avaliação macroscópica e histopatológica (HE e Von Kossa), independentemente do grau de azotemia, em nove órgãos/tecidos: rins (75%), pulmões (50%), estômago (31,2%), coração (25%), laringe (25%), músculos intercostais (25%), aorta (6,2%), intestino (6,2%) e língua (6,2%). Adicionalmente, em um animal verificou-se na serosa de todos os segmentos do intestino delgado placas multifocais brancacentas, rugosas, irregulares de tamanhos variados, cuja histopatologia confirmou tratar-se de mineralização distrófica da camada longitudinal muscular externa que apresentava necrose de coagulação e da serosa intestinal. Essa lesão intestinal nunca havia sido descrita em cães com síndrome urêmica. Em suma, os dados laboratoriais e histopatológicos aqui descritos, sobretudo, no que se refere à mineralização tecidual e vascular, que ocorrem relacionadas a lesões degenerativo-necróticas prévias, na ausência de hipercalcemia, em cães com DRC, ajudam a esclarecer as incongruências existentes na literatura. Por conseguinte, conceitualmente, as mineralizações que ocorrem em cães urêmicos devem ser consideradas distróficas.

TERMOS DE INDEXAÇÃO: Mineralização, distrofia, cães, uremia, caninos, nefrologia, mineralização patológica.

INTRODUCTION

Pathological mineralization is the abnormal deposition of minerals in body tissues, whether previously or injured not. In these lesions, in addition to calcium, other minerals such as iron, magnesium, and phosphorus can be found at lower concentrations. Classically, mineralization is divided into two types: dystrophic and metastatic. The dystrophic type occurs when there is deposition of minerals in tissues previously affected by lesions of necrotic, degenerative or inflammatory nature, regardless of serum calcium concentration (Peixoto et al. 2012, Doerr et al. 2013), whereas the metastatic type is characterized by precipitation of calcium salts in tissues due to persistently high serum calcium concentration with no previous tissue injury (Doerr et al. 2013, Foster 2016). However, Peixoto et al. (2012) have recently demonstrated that mineralization that occurs in soft tissues of animals intoxicated by vitamin D or calcinogenic plants should not be considered metastatic, as described in most textbooks, because in essence there is a tissue modification characteristic of metaplasia. Thus, Peixoto and collaborators proposed that this particular type of hormone-induced mineralization (vitamin D) should be more appropriately termed either mineralization over or by metaplasia, or simply metaplastic mineralization. Additionally, Peixoto et al. (2012) stated that there are still many gaps regarding the interaction that occurs between the hormones involved in physiological and pathological mineralization, and that descriptions are generic and inaccurate, which make the understanding of these phenomena even more difficult.

Currently, there is no consensus among researchers regarding the type of pathological mineralization that occurs in dogs with uremia. According to the national and international literature specialized in veterinary pathology, uremia-associated soft tissue mineralization represents both the metastatic and dystrophic types (Santos 1979, Confer & Panciera 1998, Cheville 2004, Newman 2013). In contrast, Jones et al. (2000) stated that metastatic mineralization occurs in uremia and Cheville (2004) recognized that hypercalcemia can rarely be directly associated with tissue calcification, but claimed that uremia is one of the most common causes of metastatic mineralization. However, according to Serakides (2011), soft tissue mineralization is dystrophic, because it is preceded by degenerative changes induced by toxins accumulated in the bloodstream, such as creatinine, ammonia, and parathyroid hormone (PTH), as well as by the absence of hypercalcemia (a condition necessary for metastatic mineralization to occur). Such textbooks, published until the present decade, have been adopted as primary literature in veterinary schools in Brazil.

Although there are several studies addressing necroscopic and histopathologic lesions in dogs with chronic kidney disease (CKD) (Cheville 1979, Dantas & Kommers 1997, Peters et al. 2005, Inkelmann et al. 2012, Silveira et al. 2015) in the literature, they do not correlate these lesions with serum calcium, phosphorus and PTH levels, which could better support discussion on the pathogenesis of pathological mineralization in the soft tissue of dogs with uremic syndrome. Thus, the objective of this study was to elucidate or provide subsidies to establish the type of pathological mineralization that occurs in uremic dogs.

MATERIALS AND METHODS

The present study was conducted at the "Hospital de Medicina Veterinária Renato de Medeiros Netto" (HOSPMEV), "Universidade Federal da Bahia" (UFBA), and at the Hospital of the Department of Veterinary Medicine (HOVET- DMV), "Universidade Federal Rural de Pernambuco" (FRPE). The study sample was composed of 40 dogs,

regardless of breed, age or gender, included in the routine assistance to small animals of HOSPMEV-UFBA and HOVET-UFRPE from December 2016 to August 2018, with clinical history and laboratory changes compatible with chronic kidney disease (CKD) (inclusion criteria: renal azotemia - creatinine ≥1.4mg/dL -, persistent for at least three months, or two measurements).

The selected dogs were divided into three experimental groups according to serum creatinine value: G1 (mild azotemia, 1.4-2.0mg/dl), G2 (moderate azotemia, 2.1-5.0mg/dl) and G3 (severe azotemia, >5.0mg/dl) using the same creatinine criteria of the IRIS staging system (IRIS 2017). Eight healthy dogs comprised the Control Group (CG). The term CKD was adopted to describe the nephropathy diagnosed in dogs of the three experimental groups, that is, it ranged from the initial and asymptomatic stages to the final stages of the disease (chronic renal failure and renal failure), according to the terminologies proposed by Romão Junior (2004) and Polzin (2008).

This study followed the norms for animal use and experimentation and was approved by the Animal Use Ethics Committee (CEUA) of the UFBA College of Veterinary Medicine and Zootechnics (EMEVZ) under protocol no. 75/2016, and only dogs with CKD whose guardians signed an Informed Consent Form (ICF) were included in the experimental groups. Dogs with urinary tract obstruction (post-renal azotemia), primary or nutritional hyperparathyroidism, hypoparathyroidism, hypercortisolinism, intoxication, neoplasms, malignancy (paraneoplastic) hypercalcemia or non-azotemic CKD were excluded from the study.

Blood samples were collected via puncture of the jugular, cephalic or femoral vein. Serum for biochemical analysis was obtained after clot retraction of the samples in tubes without anticoagulant. It was centrifuged at 3000rpm for 5min and then placed in 2.0ml polypropylene tubes. Serum urea, creatinine, and phosphorus measurements were performed at the Clinical Analysis Laboratory (LAC) at UFBA using the following methods: enzyme kinetic assay (urea), enzymatic colorimetric assay (creatinine), and colorimetric assay (phosphorus). Calcium and albumin measurements (colorimetric method) were performed at the Laboratory of Nutritional and Metabolic Diseases at UFRPE. When serum albumin values were <3.5mg/dl, total calcium (Cat) to corrected calcium (Cac) was corrected according to the formula [Cac = (Cat - serum albumin) +3.5], proposed by Chew & Meuten (1982). Ionized calcium (Cai) concentration was determined by the ion-selective electrode method using a gas analyzer (Cobas b 121 Analyzer). The calcium-phosphorus product (CPP) was calculated by multiplying the corrected calcium and phosphorus serum concentrations. CPP values<60mg^2/dl^2 were considered normal and ≥60mg^2/dl^2 were considered abnormal, according to Mcleland et al. (2014).

For determination of parathyroid hormone (PTH), whole blood samples packed in a dry tube were centrifuged at 1900rpm for 10min in a 4ºC refrigerated centrifuge to obtain at least 1.0ml of serum. After centrifugation, the serum was packed in light-protected 2ml polypropylene tubes and frozen at -80°C (Schenck et al. 2007) until examination. Intact PTH concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) (Canine Intact PTH, Immutopics, Inc., San Clemente, California, USA) previously validated in dogs (Kilpatrick et al. 2015). Reading was performed using a 450nm wavelength spectrophotometer.

After the death, or euthanasia *in extremis* of dogs with CKD, when authorized by the guardians, the animals were sent to the Veterinary Pathology Laboratory (LPV) at UFBA for necropsy. Euthanasia *in extremis* was performed in CKD terminal phase animals. The guardians opted for this procedure in case of unfavorable prognosis. During necropsy, fragments of organs of the abdominal and thoracic cavities, bones, and brain were collected and fixed in buffered formalin. Additionally, the bones were decalcified in formic acid. After fixation, the material was cleaved, routinely processed for histology, and stained with hematoxylin and eosin (HE) and special Von Kossa.

The data obtained were tabulated and collected in an orderly manner. A p value <0.05 was considered significant. The Shapiro-Wilk, D'Agostino and Pearson tests were used to evaluate data normality distribution, and analysis of variance (ANOVA) was applied to compare the data between more than two groups. The Dunnett's multiple comparison test was used in the comparison between the Control Group (CG) and the Experimental Groups (G1, G2, and G3).

RESULTS

The 40 dogs with chronic kidney disease (CKD) were divided into groups according to the following degrees of azotemia observed at the time of diagnosis: 15, mild (G1); 10, moderate (G2); 15, severe (G3). Table 1 shows the main biochemical changes found.

Hyperphosphatemia was common (62.5%, 25/40) in dogs with CKD, and serum phosphorus concentration severely increased as azotemia progressed, which was high in 26.6% in G1 (4/15 dogs, mean=5.1mg/dl), 70% in G2 (7/10 dogs, mean=8.1mg/dl), and 93.8% in G3 (14/15 dogs, mean=17.5mg/dl). No statistically significant differences in mineral concentration were verified when the G1 and G2 were compared with the CG, but dogs with severe azotemia (G3) presented higher phosphorus values than those of dogs in the other groups (p<0.0001).

No statistically significant differences were observed in total corrected calcium (Cac) concentrations in the comparison between the CG and the experimental groups (p=0.07), with means of 9.4, 11.2, 10.1 and 9.9mg/dl in dogs from the GC, G1, G2 and G3, respectively. Cai was below normal level in 97.5% (39/40) of the dogs (mean: CG=5.0mg/dl, G1=3.5mg/dl, G2=2.7mg/dl and G3=2.2mg/dl). All dogs in G1 and G3 had ionized hypocalcemia. In G2, nine (90%) dogs had reduced Cai, and only one animal (10%) presented normocalcemia. The serum Cai concentrations observed in dogs of the three experimental groups significantly differed from those of dogs in the CG (p<0.0001).

Among the 40 dogs with CKD, only 10 [25% (10/40)] had calcium-phosphorus product (CPP) values <60 mg^2/dl^2: nine in G1 and one in G2. The average CPP values were 161.8, 79.0, 57.8 and 36.74 mg^2/dl^2 in G3, G2, G1 and GC, respectively. Statistically significant differences in CPP values were observed in the comparison between CG and G3 (p<0.0001).

Parathyroid hormone (PTH) concentration increased as azotemia progressed, and was high in 73.3% of the dogs in G1 (11/15), 80% in G2 (8/10) and 100% in G3 (15/15). The following average values were found: 102.4pg/ml in G1, 145.4pg/ml in G2 and 551.3pg/ml in G3, and statistically significant differences were verified in the comparison of PTH values between G1 and G2 with G3 (p<0.0001). Statistically significant difference was also observed when the PTH reference value of healthy animals was compared with those of dogs in the three experimental groups (p<0.01).

Twenty-nine dogs (72.5%, 29/40) had a fatal outcome during the study, 15 (51.7%) due to natural death, and 14

(48.3%) undergoing euthanasia *in extremis*. Of the 16 necropsied dogs (40%), one belonged to G1, six to G2 and nine to G3. Extrarenal lesions of uremia are described ahead in order of frequency and presented in Table 2.

Both kidneys of each of the 16 necropsied dogs (32 kidneys) had severe gross (Fig.1A) and histologic lesions. In general, there was pallor, size reduction, uneven surface with occasional retention cysts, fibrosis, mineralization, and parenchyma retraction. Nephrosis was observed in 12 (75%) dogs, infarction areas was verified in 11 (68.7%) animals, retention cysts was found in seven (43.7%) dogs, and hydronephrosis was observed in only two (12.5%) animals.

The main renal lesions responsible for uremia and extrarenal lesions of the uremic syndrome were chronic interstitial nephritis (56.2%, 9/16), tubular nephrosis associated with chronic interstitial nephritis (18.7%, 3/16), glomerulosclerosis (6.2%, 1/16), fibrosis associated with chronic renal infarction (6.2%, 1/16), glomerulonephritis associated with interstitial nephritis (6.2%, 1/16), and progressive juvenile nephropathy (6.2%, 1/16). Histopathologic examination revealed changes such as interstitial nephritis (100%, 16/16), hyalinization and thickening of the Bowman's capsule (87.5%, 14/16), renal tubular ectasia (87.5%, 14/16), interstitial fibrosis (68.7%, 11/16), hyaline cylinder formation (68.7%, 11/16),

Table 1. Mean values, standard deviation and range of serum urea, creatinine, phosphorus, ionized calcium (Cai), calcium-phosphorus product (CPP) and PTH of the 40 dogs in the experimental and control groups, distributed according to degree of azotemia

Variables		Control Group (n = 10)	Degree of azotemia		
			Group 1 (n = 15)	Group 2 (n = 10)	Group 3 (n = 15)
Creatinine (mg/dL)	Mean ± SD[c]	0.8±0.3	1.7±0.2	3.5±0.8[a]	9.8±3.7[a]
	Interval	(0.4 - 1.1)	(1.4 - 2)	(2.5 - 4.7)	(5.5 -18.8)
Urea (mg/dL)	Mean ± SD	28.8±10.3	86.5±44.2	175±107.2[a]	378.9±133.3[a]
	Interval	(15.7 - 41.2)	(28.7 - 302.2)	(64 - 406)	(238 - 646)
Phosphorus (mg/dL)	Mean ± SD	3.9±1.4	5.1±1.3	8.1±2.9	17.5±7.6[a]
	Interval	(2.1 - 5.7)	(3.6 - 10)	(2.8 - 11.7)	(5.5 - 27.4)
Cai (mg/dL)	Mean ± SD	5.0±0.3	3.5±0.6[a]	2.7±1.4[a]	2.2±0.8[a]
	Interval	(4.6 - 5.4)	(2.5 - 4.5)	(1.2 - 5.3)	(1.2 - 3.9)
CPP (mg^2/dl^2)	Mean ± SD	36.7±13.0	57.8±14.8	79±26.1	161.8±58.5[a]
	Interval	(18.2 - 52.7)	(41.9 - 107)	(31.5 - 129.9)	(73 - 247.6)
PTH (pg/ml)	Mean ± SD	ND[d]	102.4±65.1[b]	145.4±103.8[b]	551.3±311.8[b]
	Interval		(20.2 - 213.2)	(15.4 - 356.2)	(121.4 - 1201)

[a] Statistically significant differences compared with healthy dogs, [b] statistically significant differences compared with the reference value for healthy dogs, [c] SD = standard deviation, [d] ND = not determined.

Table 2. Frequency of extrarenal lesions of uremia in 16 dogs with chronic kidney disease (CKD)

Lesion	Dogs	Frequency (%)
Uremic lung disease	6, 8, 10, 12, 13, 17, 18, 26, 29, 32, 33, 34, 35, 37, 39, and 40	100%(16/16)
Stained mucous membranes (anemia)	6, 8, 10, 12, 17, 18, 26, 29, 32, 33, 34, 35, 37, 39, and 40	93.7%(15/16)
Uremic gastropathy	6, 8, 10, 12, 13, 17, 18, 26, 29, 33, 34, 35, 37, 39, and 40	93.7%(15/16)
Soft tissue mineralization:		93.7%(15/16)
Kidneys[a]	6, 8, 10, 12, 18, 26, 29, 34, 35, 37, 39, and 40	75%(12/16)
Larynx	6, 10, 17, 18, 32, 33, 35, and 40	50%(8/16)
Lungs	12, 18, 26, 29, 32, 35, 37, and 40	50%(8/16)
Stomach	10, 18, 26, 34, 39, and 40	31.2%(5/16)
Heart	6, 17, 18, and 26	25%(4/16)
Intercostal muscles	12, 17, 32, and 35	25%(4/16)
Aorta	18	6.2%(1/16)
Intestines	18	6.2%(1/16)
Tongue	26	6.2%(1/16)
Arteriosclerosis	6, 12, 13, 17, 18, 26, 29, 32, 33, 34, 35, and 37	75%(12/16)
Necrotic and ulcerative stomatitis and/or glossitis	6, 12, 13, 18, 26, 29, 32, 33, 35, 39, and 40	68.7%(11/16)
Uremic enteropathy	6, 10, 18, 26, 33, 34, 35, 37, and 39	56.2%(9/16)
Parathyroid hyperplasia	6, 18, 26, 29, 33, 34, and 35	43.7%(7/16)
Fibrinonecrotical esophagitis	17, 26, and 35	18.7%(3/16)
Fibrous osteodystrophy	34	6.2%(1/16)
Fibrinous pericarditis	37	6.2%(1/16)

[a] Although not considered a true extrarenal lesion of uremia, as it occurs in the kidneys and secondary to CKD disorders, it has been described here.

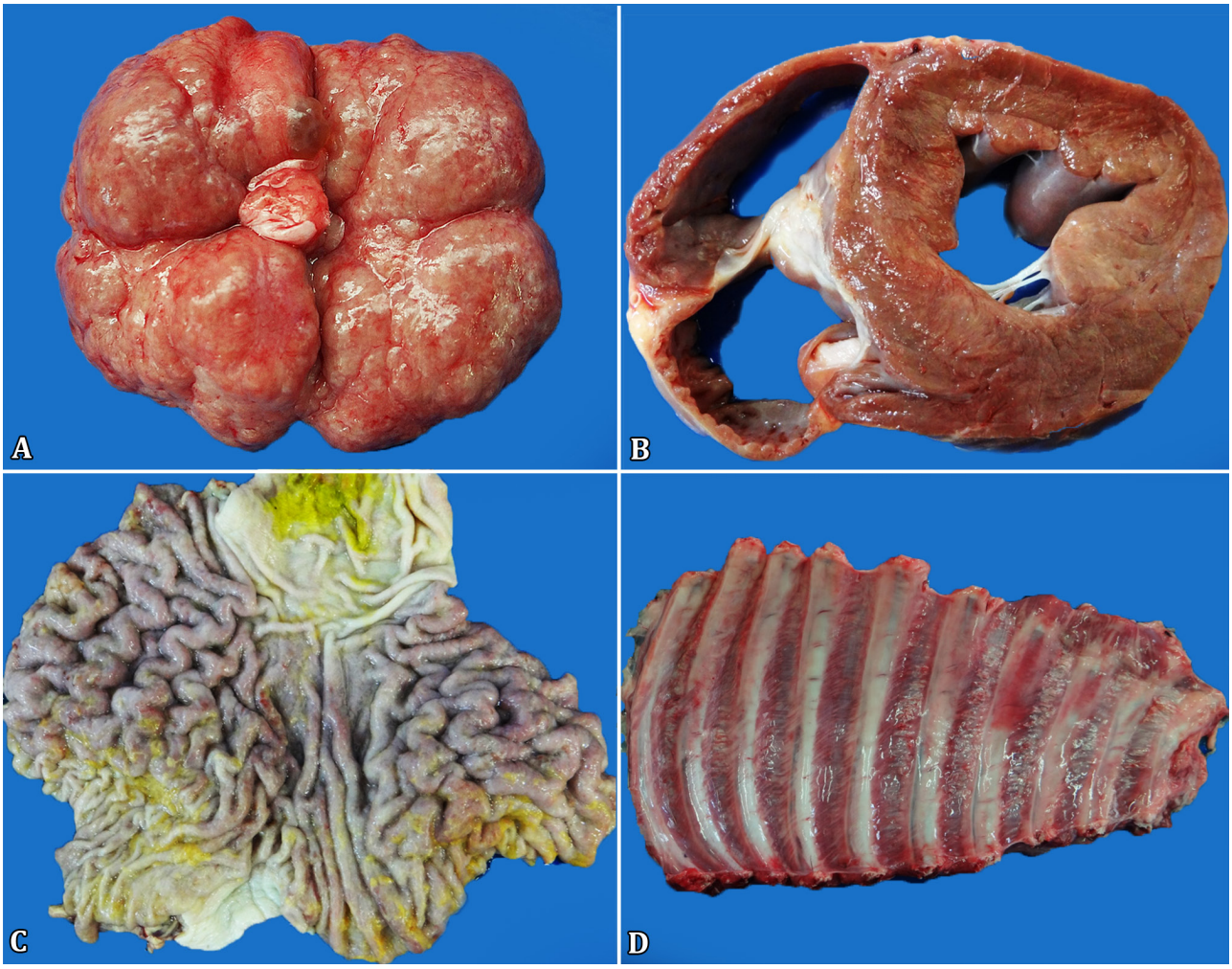


Fig.1. Chronic kidney disease (CKD) in dogs. (**A**) Kidney of Dog 37, G2 showing uneven natural surface and parenchyma retraction. (**B**) Small multifocal whitish foci in the myocardium (dystrophic mineralization). Heart of Dog 18, G3. (**C**) Uremic gastropathy. Marked edema and thickening of the gastric mucosal folds. Stomach of Dog 10, G3. (**D**) Grayish white granular pleural thickening with horizontal "scaffolding" arrangement (dystrophic mineralization). Intercostal muscle of Dog 35, G3.

glomerular atrophy (56.2%, 9/16), glomerular sclerosis (25%, 4/16), coagulation necrosis (12.5%, 2/16) and hydropic degeneration (12.5%, 2/16).

Anemia was the most frequent extrarenal lesion of uremia in this study. It was observed in all (100%, 16) necropsied dogs, and was characterized by mild to intensely stained mucosae, organ pallor, and reduced circulating blood volume. Uremic gastropathy was observed in 93.7% (15/16) of the dogs, which showed erosive and/or ulcerative gastritis, edema, and hyperemia of the gastric mucosa, in addition to ammoniac odor in most cases. The gastric mucosa was mild to intensely thickened (Fig.1C) and hyperemic in 62.5% (10/16) of the dogs in G2 and G3. Three dogs in the G3 presented gastric mucosa mineralization verified at necroscopic examination, which was confirmed by histopathology. Mineralization was observed only at histopathologic examination in only one dog in G1. Gastric content was hemorrhagic in two dogs in G3. The dogs in G2 showed loss of the gastric mucosa surface epithelium (erosion) focally, or of multifocal association with inflammatory infiltrate, predominantly lymphocytic and edematous. In the dogs in Group G3, in addition to these alterations, areas of gastric mucosa necrosis were observed, as well as mononuclear inflammatory infiltrate and multifocal hemorrhage.

Similarly, uremic pneumopathy was also observed in 15 (93.7%) dogs, with pulmonary edema as the most common finding (75%, 12/16). The lungs showed smooth, moist, shiny natural surface and varying amounts of free-flowing or translucent foamy fluid (edema) in the light of the bronchial and tracheal lumen. The gross and histologic lesions were compatible, and consisted of multifocal emphysema (56.2%, 9/16), pneumonia (37.5%, 6/16), congestion (37.5%, 6/16), focal or multifocal atelectasis (43.7%, 7/16) and hemorrhage (25%, 4/16), in addition to edema.

In this study, soft tissue mineralization was a frequently observed change (93.7%, 15/16), and involved multiple organs and tissues (Table 2). Mineralization was observed in nine different sites, namely in order of prevalence, kidneys (75%), lungs (50%), stomach (31.2%), heart (25%), larynx (25%), intercostal muscles (25%), aorta (6.2%), intestines (6.2%), and tongue (6.2%).

Twelve (75%, 12/16) animals, eight of them in G3, presented mineralization in the kidneys, in some cases macroscopically visible in the form of multiple millimeter points or multifocal whitish areas. On histopathologic examination, mineralization was most commonly observed in necrotic renal tubules (coagulation necrosis) in the cortical and medullary regions. Additionally, it was verified in the glomeruli, wall of small blood vessels of the cortical region and corticomedullary junction, as well as in the Bowman's capsule (Figs.2A,B).

The lung was the second organ most affected by mineralization (50%, 8/16) in animals in the three study groups. Lesion affected mainly the alveolar walls and, to a lesser extent, the blood vessel wall (Fig.2D). Three animals in G3 and one in G1 presented multifocal gastric mineralization, mainly in the mucosa and blood vessels, which was not macroscopically identifiable in the dog in G1.

In the heart of four (25%, 4/16) animals, three in G3 and only one in G2, dystrophic mineralization of necrotic cardiomyocytes (hyaline coagulation necrosis) was observed (Fig.1B and 3E). Four dogs also presented mineralization of the intercostal muscles (Fig.1D), characterized by whitish, uneven and firm sandy-looking protruding areas. In four cases, irregular, whitish, firm, sandy-looking multifocal areas were found in the larynx (mineralization).

Whitish, rough, irregular, firm multifocal plaques of varying sizes (from millimeters to approximately 3.0 cm)were observed in the serosa of all segments of the small and large intestine in one (6.2%, 1/16) dog (Fig.3). Histopathology confirmed that

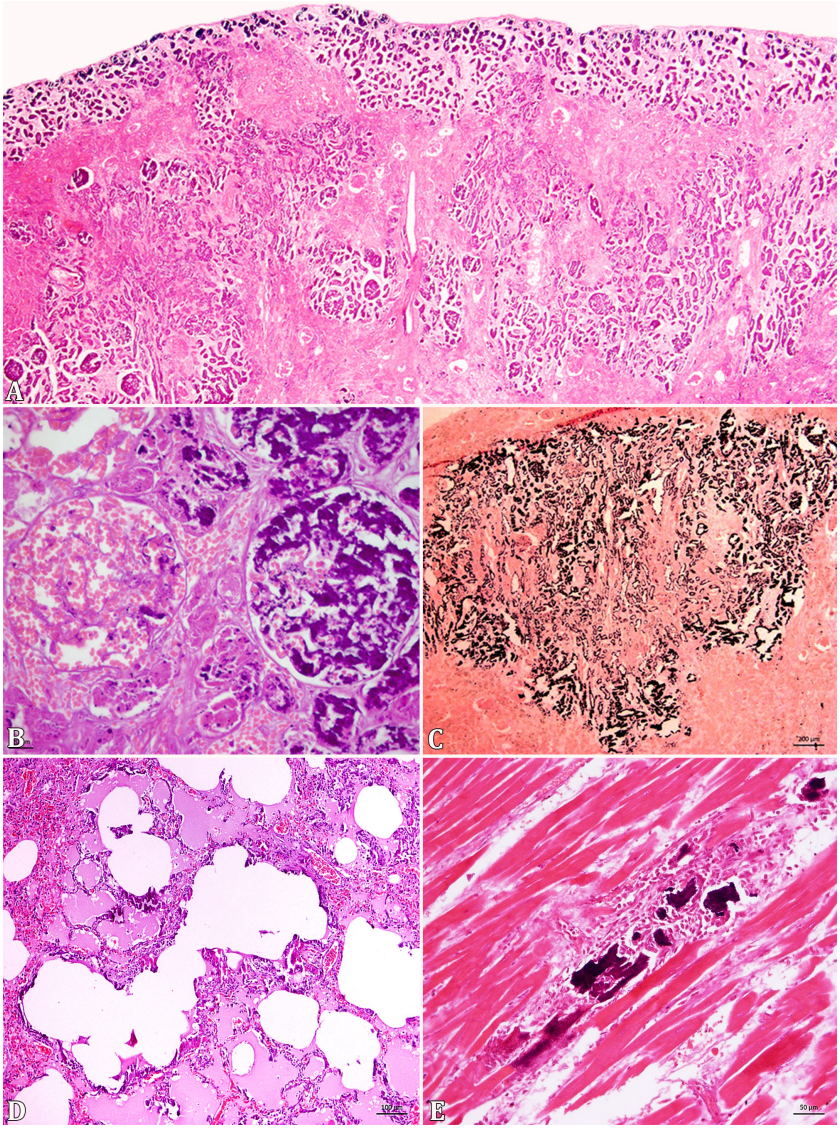


Fig.2. Dystrophic mineralization in dogs with chronic kidney disease (CKD). (**A**) Focal length area of dystrophic mineralization of renal glomeruli and tubules. HE, obj.5x. (**B**) Dystrophic mineralization of the early (left) and advanced (right) glomerular tuft associated with coagulation necrosis and renal tubule mineralization. Kidney of Dog 12, G3. HE, obj.40x. (**C**) Extensive areas of dystrophic mineralization of renal tubules and glomeruli (evidenced by the blackening of calcium deposits). Kidney of Dog 12, G3. Von Kossa, obj.5x. (**D**) Uremic lung disease. Multiple areas of dystrophic mineralization in the bronchioles, pulmonary alveolar wall, and small blood vessels associated with marked pulmonary edema. Lung of Dog 40, G2. HE, obj.10x. (**E**) Group of hypereosinophilic cardiomyocytes with loss of striations and foci of dystrophic mineralization over necrotic cardiomyocytes. Heart of Dog 18, G3. HE, obj.20x.

it was dystrophic mineralization of the external longitudinal layer of the muscle that presented necrosis of coagulation and of the intestinal serosa (Fig.4A,B). Mineralization of the aorta and necrotic tongue muscle fibers were also described in only one case each.

Aortic and/or coronary arteriosclerosis was found in 12 (75%, 12/16) dogs, five in G2 and seven in G3, and consisted of whitish, irregular, multifocal, plaque-shaped protruding areas in the intimal layer of the aorta that was sometimes diffusely irregular. Focal thickening was observed in the coronary arteries. Histopathologic examination revealed thickening of the tunica intima with smootvh muscle proliferation and internal elastic lamina fragmentation.

Eleven cases (68.7%, 11/16) presented necrotic stomatitis and/or ulcerative glossitis: seven dogs in G3, three in G2, and one in G1. The lesions were characterized by ulcerated areas, mainly on the lateral faces or apex of the tongue and on the mouth gum, sometimes covered by necrotic tissue with hemorrhagic edges.



Fig.3. Chronic kidney disease (CKD) in dogs. Uremic enteropathy. Multiple irregular whitish plaques of varying sizes in the small intestine serosa that deepen into the muscular layer (detail). Accentuated multifocal dystrophic mineralization. Dog 18, G3.

Uremic enteropathy was verified in nine (56.2%, 9/16) dogs, eight of them belonging to G3. In these animals, there was mild, moderate or severe thickening of the intestinal wall, diffuse or multifocal, and in the case of intestinal mineralization, the wall had irregular gelatinous and serous appearance. The intestines were often reddish or blackened, and the mucosa was irregular or hyperemic. Histopathology showed, predominantly, inflammatory infiltrate, lymphoplasmacyte, congestion, and activated endothelial cells.

Bilateral parathyroid enlargement (hyperplasia) was observed in seven (43.7%, 7/16) dogs, only one in G2 and six in G3. It was also verified that the seven dogs, six in G3 and one in G2, that presented parathyroid volume increase at necropsy showed high serum PTH concentrations, with values ranging from 124.2 to 1201pg/ml.

One dog in G1, two in G2 and three in G3 presented endocarditis or mural myocarditis. Atrioventricular valve endocardiosis was also found in varying degrees (25%, 4/16), as well as left ventricular hypertrophy (12.5%, 2/16). One animal had dilated cardiomyopathy (6.25%, 1/16). Histopathologic examination generally revealed predominantly multifocal mononuclear inflammatory infiltrate associated with multifocal interstitial edema. Areas of degeneration and hyaline necrosis were also observed in the groups with cardiomyocytes (43.7%, 7/16), and epicardial hemorrhage was verified in only one animal.

Fibrinonecrotic esophagitis occurred in two dogs in G3 and in one in G2 (18.7%, 3/16), and fibrinous pericarditis was observed in one (6.2%, 1/16) dog in G2.

Hyperosteotic fibrous osteodystrophy was found in only one (6.2%, 1/16) dog in G3. This animal presented a significant mineralization disorder caused by intense demineralization resulting from marked bone resorption and replacement with fibrous connective tissue, which resulted in severe fragility of the maxillary and mandible bones ("rubber jaw") with bilateral

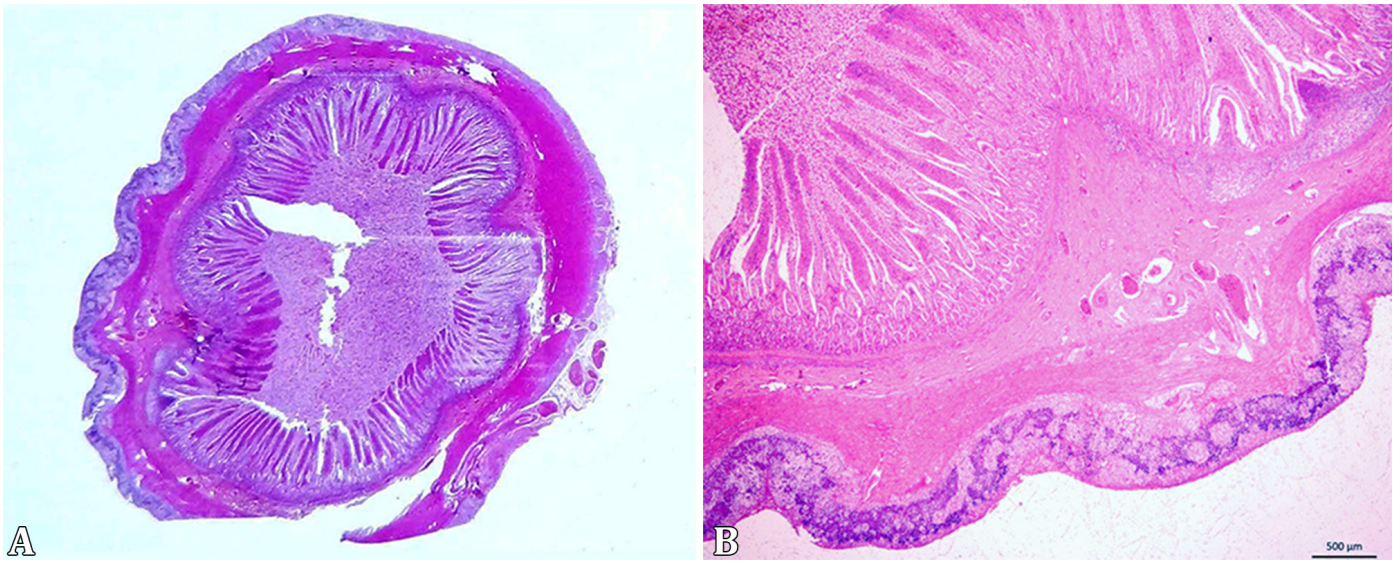


Fig.4. Chronic kidney disease (CKD) in dogs. Uremic enteropathy. Multifocal to coalescent dystrophic mineralization accentuated in the serosa and outer (longitudinal) muscular layer. (**A**) Submacroscopic imaging and (**B**) small bowel photomicrograph. Dog 18, G3. HE, obj.5x.

facial deformity, characterized by increased volume of these bones. In the oral cavity, there was misalignment and tooth mobility, as well as malocclusion, absence of dentin in the upper canines, exposure of the dental pulp, and wear of the incisor teeth. Histopathology revealed marked fibroplasia with replacement of bone trabeculae, osteopenia, thin, fragmented and disconnected bone trabeculae, numerous osteoclasts, and Howship's lacunae (bone resorption).

DISCUSSION

Changes in mineral metabolism are frequent in dogs with chronic kidney disease (CKD) (Brito Galvão et al. 2013). In fact, in this study, ionized hypocalcemia was the most frequently observed mineral biochemical change. According to Schenck et al. (2007), less than 10% of dogs with CKD have high serum ionized calcium (Cai) concentrations, which corroborates the findings of this study, since none of the 40 dogs assessed presented ionized hypercalcemia. Cortadellas et al. (2010) also reported reduction intotal calcium (Cat) and Cai in dogs with severe azotemia. It is worth noting that these authors did not observe significant difference in Cat concentrations in healthy and CKD dogs, and none of the dogs they evaluated presented ionized hypercalcemia. Regarding the decrease in Cai concentration, the same was observed in cats in the last stage of the disease, whereas the average concentrations remained within normality during all stages (Barber & Elliott 1998).

It is worth remembering that in CKD, Cat is useless in predicting the concentration of Cai, being fundamental to the measurement of the ionized fraction (Martinez & Carvalho 2010). Ionized calcium should be measured directly and, in cases of hyperparathyroidism, kidney disease and hypo- and hyperproteinemia, the measurement and interpretation of the ionized fraction have been superior to the measurement of serum corrected calcium (Cac) (Giovaninni 2015). The correction of serum concentrations concerning serum albumin or total protein concentration is relevant for an adequate diagnostic interpretation, because the degree of mineral-protein binding is significant (80-90% of serum calcium) (Schenck et al. 2007).

Hyperphosphatemia resulting from phosphorus retention due to progressive reduction in the glomerular filtration rate (GFR) (Geddes et al. 2013) was the second most frequent change in mineral metabolism observed in this study. Such abnormality is frequent since the early stages of CKD, and has a direct correlation with the worsening of azotemia, as it intensifies with the loss of nephrons, which was observed in this study, as well as in felines and humans (Elliott & Barber 1998, Lazaretti et al. 2006, Cortadellas et al. 2010, Martinez & Carvalho 2010, Giovaninni et al. 2013, Queiroz 2015, Valencia & Arango 2015).

Hyperphosphatemia can result in secondary renal hyperparathyroidism (SRHPT), a common complication of CKD, as well as in soft tissue mineralization and CKD progression (Giovaninni 2015, Foster 2016). In this study, the increase in PTH levels (73.3%) was considerably higher than the frequency of dogs with hyperphosphatemia (26.6%) in G1, which may be explained by the corrective effect of SRHPT in the early stages of CKD, which leads to high PTH concentrations and induces phosphaturia and maintenance of serum phosphorus levels. In dogs and cats with end-stage CKD (moderate to severe azotemia), such usual compensatory mechanisms often fail to prevent hyperphosphatemia (Alfrey 2004, Polzin 2011, Davis 2015). In the present study, all dogs with hyperphosphatemia already had high PTH concentrations. Cortadellas et al. (2010) demonstrated that, in dogs with CKD, SRHPT is frequent and may even be present at stage 1 of the disease, suggesting that reduced renal phosphorus excretion and, consequently, serum retention would start at the earliest stages of CKD. Giovaninni et al. (2013) also found increased phosphorus and PTH concentrations in cats with mild azotemia.

Most lesions resulting from uremic syndrome are of renal origin and are mainly attributed to tubulointerstitial nephritis, renal infarction, granulomatous nephritis, glomerulonephritis and acute tubular necrosis, or to associations between these lesions (Dantas & Kommers 1997, Inkelmann et al. 2012). The type and distribution of extrarenal uremia lesions found in the 16 autopsied dogs, including the frequent dystrophic mineralization observed in various organs and soft tissues, were similar to those described in the literature in cases of uremic syndrome (Cheville 1979, Dantas & Kommers 1997, Peters et al. 2005, Silveira et al. 2015, D'Ávila et al. 2016), except for the uremic enteropathy with severe multifocal dystrophic mineralization found in one dog. Macroscopically, this lesion drew attention because it is an atypical case of intestinal mineralization, mainly due to the severity and multifocal distribution in the small intestine - dozens of whitish, irregular and firm plaques measuring up to 4.0x1.6 cm, thus never described in dogs with uremic syndrome. Cianciolo & Mohr (2015) pointed out that intestinal lesions have lower intensity and frequency than those found in the stomach of dogs with CKD, and that intestinal mineralization has not been described in uremic dogs. However, this lesion has been reported in two dogs with CKD, being described in the mucosa and sub-mucosa and in the muscle vessels of one animal (Dantas & Kommers 1997), and as mild and located in the small intestine of the other (Silveira et al. 2015).

Some considerations should be made on the vascular mineralization found in the kidneys. At histopathologic examination, mineralization foci were more frequent in renal tubules, small vessel wall, glomeruli, and Bowman's capsule, in decreasing order of prevalence; however, such vascular mineralization has not been described in previous studies, although interstitial fibrosis, interstitial nephritis, tubular atrophy, tubular cell mineralization, and glomerulosclerosis were frequent (McLeland et al. 2014, D'Ávila et al. 2016); these lesions were attributed to prolonged uremia. In contrast, Newman (2013) reported that vessel calcification, tubular basement membranes, Bowman's capsule, and degenerate tubular epithelium are common in fibrotic kidneys because of changes in CKD-associated calcium and phosphorus metabolism.

In this study, coagulation necrosis and cardiac dystrophic mineralization were observed in 43.7% and 25% of dogs, respectively. Lesions in cardiomyocytes of this nature, as well as changes in cardiac vessels in chronic nephropathic dogs with uremia, have recently been reported to be much more frequent than those mentioned in the literature, and have been attributed to long-term uremia and/or hemodynamic and metabolic changes occurring in type IV cardiorenal syndrome (D'Ávila et al. 2016). In the present study, these vascular lesions were not observed probably due to the limited number of cardiac fragments evaluated. In contrast, atherosclerosis was observed in 75% of the necropsied dogs - a vascular lesion previously described in uremic dogs (Dantas & Kommers

1997). Observation of these degenerative, inflammatory or necrotic lesions in uremic dogs reinforces the hypothesis that a dystrophic lesion precedes mineralization.

Mineral metabolism-related injuries such as parathyroid hyperplasia and fibrous osteodystrophy are less frequent, and occur in cases of extreme CKD (Dantas & Kommers 1997, Silveira et al. 2015, Foster 2016); however, it is crucial to consider that bones are not often submitted to routine histologic examination, which makes the diagnosis of this change difficult in early stages of disease, snd can be underestimated in uremic dogs since clinical manifestations range from mild, focal or generalized bone loss to fibrous osteodystrophy (Davis 2015). In this study, fibrous osteodystrophy occurred in a 5-month-old pup, and was due to juvenile nephropathy. Interestingly, the primary renal disease with SRHPT (mineralization disorder) bone deformities due to the classic increase in surface volume was mistaken for the previous clinical examination of bone malformation. This error underscores the importance of knowledge about the interactions that occur between two or more systems in some diseases.

In the present study, the absence of ionized hypercalcemia shows that calcemia does not play a significant role in the tissue mineralization of uremic dogs, even in face of the possibility that ionized hypercalcemia may have been transient and, therefore, not detected in the exams performed. This is because tissue mineralization is a chronic lesion that occurs over the course of weeks or months. It is important to remember that transient hypercalcemia has previously been demonstrated in cases of experimental poisoning by calcinogenic plants (containing vitamin D analogs) in cattle (Dobereiner et al. 1971) and sheep (Riet-Correa et al. 1993). Only a slight increase in calcemia was observed within the first 24 and 48 hours after the administration of these plants (Dobereiner et al. 1971, Riet-Correa et al. 1993). Similarly, these findings demonstrated that the tissue mineralization observed in animals intoxicated by these plants also has no direct correlation with hypercalcemia (Peixoto et al. 2012), as they have been mistakenly disseminated in textbooks, which are based on the Virchow's classification, previously questioned by Seifert & von Hentig (1967). Nevertheless, Peixoto et al. (2012) recently proposed that hypervitaminosis D-induced mineralization should be more appropriately termed metaplastic mineralization.

It has previously been mentioned that animals with CPP >60-70mg^2/dl^2 present higher risk of developing soft tissue mineralization, although these high concentrations are likely to persist for weeks and months (McLeland et al. 2014, Foster 2016). Additionally, Peters et al. (2005) correlated the occurrence of gastric mineralization in dogs with CKD with high CPP values, and stated that dogs with CPP >60-70mg^2/dl^2 would present higher risk of developing tissue and vascular mineralization, and that these would be of the metastatic type. Importantly, in the present study, although CPP was elevated mainly in severely azotemic dogs, this increase was attributed to frequent hyperphosphatemia rather than to ionized hypercalcemia (absent all dogs). Other studies have also suggested or attributed soft tissue mineralization to changes in serum calcium and phosphorus values, but they did not evaluate serum mineral concentrations (McLeland et al. 2014, Silveira et al. 2015).

It should also be noted that the 15 dogs that developed tissue mineralization identified in the necroscopic and histopathologic examinations presented CPP values >70mg^2/dl^2 and ionic hypocalcemia, regardless of the degree of azotemia. McLeland et al. (2014) found that the administration of phosphorus chelators in cats with CKD - to correct hyperphosphatemia - did not interfere with the occurrence of mineralization, which once again reinforces the hypothesis that mineralization is dystrophic. Additionally, this hypothesis also corroborates the biochemical findings of the dog that developed fibrous osteodystrophy, which although having suffered extreme bone calcium resorption due to SRHPT in an attempt to correct calcemia (Polzin 2011, Brito Galvão et al. 2013), still presented ionized hypocalcemia with marked CPP increase caused by hyperphosphatemia.

Indeed, according to Serakides (2011), since nephropathy is invariably irreversible and fatal, in cases of SRHPT there is no recovery of bone injury and, consequently, the plasma condition of hyperphosphatemia and hypocalcemia is perpetuated. Thus, the data described herein show, in a substantiated manner, that hypercalcemia is not a requirement for soft tissue mineralization in dogs with CKD at different stages of azotemia.

We believe, however, that the association between CPP increase and tissue mineralization verified by other authors (Peters et al. 2005, McLeland et al. 2014, Foster 2016) is mistaken. What happens is that dogs in more advanced stages of CKD (renal failure) have higher hyperphosphatemia, which increases CPP. We believe that the most appropriate association would be between the occurrence of vascular and/or tissue lesions secondary to uremia and the development of tissue mineralization, as verified and discussed in this study. It is worth remembering that these vascular and/or tissue lesions are chronic, and more frequent and severe in more advanced stages of CKD (renal failure), which strengthens the idea that mineralization observed in dogs with CKD requires previous injury and is, therefore, considered dystrophic.

In conclusion, the laboratory (azotemia, hypocalcemia, hyperphosphatemia, and CPP) and histopathologic data described herein, especially concerning tissue and vascular mineralization, which occurs in association with previous degenerative-necrotic lesions (essential requirement for dystrophic mineralization) in the absence of hypercalcemia (essential requirement for metastatic mineralization) in dogs with CKD associated with information available in the principal studies on this subject (Dantas & Kommers 1997, Peters et al. 2005, Vlleet & Ferrans 2009, Silveira et al. 2015, D'Ávila et al. 2016), provide subsidies that assist with clarifying inconsistencies found in the literature. It is noteworthy that, although the mineralization observed in uremic dogs presents systemic distribution - a characteristic of metastatic mineralization - this is because uremia occurs with multisystemic lesions and, therefore, mineralization occurs on these multiple previous lesions.

CONCLUSION

Organ and tissue mineralization in dogs with chronic kidney disease is preceded by degenerative/necrotic vascular and tissue damage resulting from toxins accumulated in the bloodstream, and which occur in the absence of hypercalcemia. Therefore, conceptually, mineralization that occurs in uremic dogs should be considered dystrophic.

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

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Original Article
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Epidemiological, clinical, and laboratory aspects in a case series of canine hyperadrenocorticism: 115 cases (2010-2014)[1]

Francisco S.M. Martins[2]*, Guilherme Luiz C. Carvalho[2], Luciana Jesus[3], Álan Gomes Pöppl[2,4] and Félix H.D. González[2,5]

ABSTRACT.- Martins F.S.M., Carvalho G.L.C., Jesus L., Pöppl A.G. & González F.H.D. 2019. **Epidemiological, clinical and laboratory aspects in a case series of canine hyperadrenocorticism: 115 cases (2010-2014).** *Pesquisa Veterinária Brasileira 39(11):900-908.* Hospital de Clínicas Veterinárias, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Agronomia, Porto Alegre, RS 90540-000, Brazil. E-mail: savio_mmartins@hotmail.com

Diseases' clinical-epidemiological characterization assists in directing the diagnosis. The objective of this study was to describe epidemiological, clinical and laboratorial aspects of a case series of canine hyperadrenocorticism (HAC). One-hundred fifteen records of dogs diagnosed by the low dose dexamethasone suppression test and/or ACTH stimulation test were evaluated. Of the cases, 81.3% were HAC ACTH-dependent and 18.7% HAC ACTH-independent. Females were more affected, representing 69.3% of the cases. The mean age was 10.3±2.5 years and 64.9% were gonadectomized. Most of the patients were small size dogs, weighting less than 10kg (73.9%). The most frequent breeds were: Poodle (27%), Dachshund (17.4%), and Yorkshire Terrier (10.4%). The most frequent clinical manifestations were polyphagia (86%), polydipsia (82.6%), polyuria (80%), abdominal enlargement (82.6%), thin skin (79.1%), muscular weakness (78.3%) and panting (74.8%). However, eventually unusual HAC-associated signs would be present in some dogs in a more important way compared with the classic disease´s clinical signs. The CBC showed neutrophilia (66%), eosinopenia (58.3%) and lymphopenia (42.6%) as main hematological abnormalities. The most common findings in serum biochemistry were increased alkaline phosphatase activity (81.74%), increased ALT activity (62.6%), hypercholesterolemia (66%) and hypertriglyceridemia (54.7%). Urinalysis revealed hyposthenuria in 14.9% and isostenuria in 13.5%; besides proteinuria in 50% of the cases. Abdominal ultrasound showed bilateral adrenal hyperplasia (92.2%) with adrenal asymmetry in 20.8% of the cases, in addition to hepatomegaly (80.9%), biliary sludge (67.8%) and hepatic hyperechogenicity (47.8%). It was concluded that small size gonadectomized female dogs, mainly Poodles and Dachshunds, presented higher frequency in the population studied, and that the main changes observed in clinical and complementary tests were polyphagia, polyuria, polydipsia, abdominal enlargement, adrenal hyperplasia, increased phosphatase alkaline and hyperlipidemia. These results corroborated to a better disease characterization at Brazil. This work concluded that the population studied resembles the profile describe in European and North American epidemiologic studies, and that the HAC dog´s clinical picture looks similar worldwide.

INDEX TERMS: Epidemiology, clinics, laboratory, case series, canine hyperadrenocorticism, dogs, Cushing syndrome, hypercortisolism, glucocorticoids.

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[2] Graduate Program in Veterinary Science, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9090, Agronomia, Porto Alegre, RS 91540-000, Brazil. *Corresponding author: savio_mmartins@hotmail.com

[3] Faculdade de Veterinária, UFRGS, Av. Bento Gonçalves 9090, Agronomia, Porto Alegre, RS 91540-000.

[4] Departamento de Medicina Animal, Faculdade de Veterinária, UFRGS, Av. Bento Gonçalves 9090, Agronomia, Porto Alegre, RS 91540-000.

[5] Departamento de Patologia Clínica Animal, Faculdade de Veterinária, UFRGS, Av. Bento Gonçalves 9090, Agronomia, Porto Alegre, RS 91540-000.

RESUMO.- [**Aspectos epidemiológicos, clínicos e laboratoriais em uma série de cães com hiperadrenocorticismo: 115 casos (2010-2014).**] A caracterização clínica-epidemiológica de doenças auxilia no direcionamento do diagnóstico. O objetivo deste trabalho foi descrever aspectos epidemiológicos, clínicos e laboratoriais de uma série de casos de hiperadrenocorticismo (HAC) canino. Foram avaliados 115 prontuários de cães diagnosticados pelo teste de supressão por baixa dose de dexametasona e/ou teste de estimulação com ACTH. Os casos de HAC ACTH-dependentes representaram 81,3% da população, e 18,7% foram ACTH-independentes. As fêmeas foram mais acometidas, representando 69,3% dos casos. A média de idade foi 10,3 ± 2,5 anos e 64,9% eram gonadectomizados. A maioria dos cães foi de porte pequeno, de até 10 kg (73,9%). As raças mais frequentes foram Poodle (27%), Dachshund (17,4%) e Yorkshire (10,4%). As manifestações clínicas mais relatadas foram polifagia (86%), polidipsia (82,6%), poliúria (80%), abdome pendular (82,6%), atrofia cutânea (79,1%), fraqueza muscular (78,3%) e dispneia (74,8%). Entretanto, eventualmente sinais clínicos pouco associados ao HAC se manifestaram de forma mais importante que os sinais clássicos da doença. O hemograma revelou neutrofilia (66%), eosinopenia (58,3%) e linfopenia (42,6%) como principais alterações hematológicas. Na bioquímica sérica foi observado aumento de fosfatase alcalina (81,74% dos casos), aumento da atividade da ALT (62,6%), hipercolesterolemia (66%) e hipertrigliceridemia (54,7%). A urinálise revelou hipostenúria em 14,9% e isostenúria em 13,5%; além de proteinúria em 50% dos casos. A ecografia abdominal evidenciou hiperplasia bilateral de adrenal (92,2%) com assimetria de adrenais em 20,8% dos casos, além de hepatomegalia (80,9%), lama biliar (67,8%) e hiperecogenicidade hepática (47,8%). Concluiu-se que fêmeas castradas de pequeno porte, principalmente das raças Poodles e Dachshunds, apresentaram maior frequência na população estudada e que as principais alterações observadas clínicas e nos exames complementares foram polifagia, poliúria, polidipsia, aumento abdominal, hiperplasia da adrenal, aumento de fosfatase alcalina e hiperlipidemia. Estes resultados corroboram para melhor caracterização da doença no Brasil. Este estudo concluiu que a população estudada se assemelha ao perfil populacional de cães com HAC descrito em estudos Europeus e Norte Americanos de forma que o perfil dos casos ao redor do globo parece similar.

TERMOS DE INDEXAÇÃO: Epidemiologia, clínica, laboratório, série de casos, cães, hiperadrenocorticismo, síndrome de Cushing, hipercortisolismo, glicocorticoides.

INTRODUCTION

Cushing's syndrome or hyperadrenocorticism (HAC) is a multisystemic clinical condition caused by chronic higher exposure to glucocorticoids (Gilor & Graves 2011). In dogs, hypercortisolism may occur secondarily to adrenocorticotropic hormone (ACTH) increased secretion by the hypophysis, classified as ACTH-dependent HAC. Less often, HAC may result from an increase in cortisol production by an adrenal gland neoplasm, known as ACTH-independent HAC (Peterson 2007, Behrend 2015).

HAC is considered the most prevalent dog´s endocrine disease and a common disease in older dogs (Pöppl et al. 2016). Some breeds, such as Poodles and Dachshunds, present greater predisposition (Ling et al. 1979). Clinical manifestations begin, in most cases in a slow and progressive manner. Among such manifestations, polyuria, polydipsia, polyphagia and abdominal enlargement stand out. Regarding laboratorial aspects, stress leukogram, increase in alkaline phosphatase activity and hypertriglyceridemia are the main alterations (Behrend 2015).

Diagnosis is based on history and clinical evaluation, imaging exams and hormonal tests, such as the low-dose dexamethasone suppression test (LDDST) and the ACTH stimulation test (Behrend et al. 2013). Surgical and pharmacological approaches are among the therapeutic options. Therapeutic conduct is chosen based on HAC etiologic classification, since pituitary neoplasms are mostly treated with drugs whereas first-line treatment for adrenal gland neoplasms is adrenalectomy. Prognosis of canine HAC may vary according to case severity and the presence of comorbidities (Behrend 2015). There is a paucity of Brazilian data regarding HAC features. The aim of this study was to determine the frequency of clinical, laboratorial and ultrasonographic alterations in dogs with HAC, to foment recognition of suspicious cases in clinical routines and improve disease characterization at Brazil.

MATERIALS AND METHODS

Ethics Statement. This study was approved by the Research Ethics Committee from "Faculdade de Veterinária" of the "Universidade Federal do Rio Grande do Sul" (UFRGS) being also approved by the Ethics Committee on Animal Experimentation (CEUA) (Project 31755).

Study design. A retrospective study was conducted within a population with HAC diagnosis followed-up by an Endocrinology and Metabology Service from a Veterinary Teaching Hospital from 2010 to 2014. Clinical-epidemiological, laboratorial (CBC, serum biochemistry, and urinalysis), and ultrasonographic data of interest were obtained from standardized medical records.

Excluding criteria. Cases with diabetes mellitus, chronic kidney disease, and liver failure; or records that failed to contemplate information necessary for the study were not included. Also, dogs being treated with furosemide, glucocorticoids (iatrogenic HAC), and phenobarbital, were excluded from the study.

Hyperadrenocorticism classification. Etiological classification of HAC was characterized by the LDDST results profile and aspects of the abdominal ultrasonography, which classified HAC as either ACTH-dependent or ACTH-independent. Adrenal gland hyperplasia was considered in cases where the adrenal caudal pole thickness exceeded: 0.54cm in dogs with up to 10kg, 0.68cm in dogs from 10 to 30kg, and 0.8cm in dogs weighing over 30kg. Adrenal asymmetry was assumed as a difference between adrenal glands thickness greater than 0.4cm (Soulsby et al. 2015).

Statistical analysis. Quantitative and qualitative variables were analyzed, and means, medians and variances were obtained; regarding qualitative data, distribution, counting and proportion were obtained.

RESULTS

A total of 115 HAC cases out 143 HAC cases attended from 2010 to 2014 were included based on exclusion criteria.

Hyperadrenocorticism classification and population profile

From those cases, 81.3% had ACTH-dependent HAC and 18.7% ACTH-independent HAC. Mean cortisol concentrations during the LDDST (basal, four and eight hours post-dexamethasone) were 6.2µg/dL, 2.1µg/dL, and 4.7µg/dL in the ACTH-dependent

HAC group, while in the ACTH-independent HAC group the mean values were 5.3µg/dL, 3.6µg/dL, and 4.1µg/dL. There was a greater disease occurrence in females, accounting for 69.3% of cases. In the population assessed, 65% of the dogs were gonadectomized. Mean age was 10.3±2.5 years, ranging from four to 16 years. According to etiological classification, mean age of dogs with ACTH-dependent HAC was 9.3 years, and 11.4 years for dogs with ACTH-independent HAC. Fig.1 shows the distribution of number of cases by age and etiological classification. Regarding size, 78% were considered small dogs (up to 10kg), followed by 16% of medium size dogs (from 10 to 20kg), and large size dogs (over 20kg) corresponding to only 6% of cases. Table 1 shows breed distribution frequency.

Laboratorial abnormalities

The Figure 2 presents most frequent clinical manifestations in this case series. The mean CBC abnormalities were neutrophilia (66%), eosinopenia (58.3%), lymphopenia (42.6%) and monocytosis (30,5%). Mean values of red blood cells, hemoglobin and hematocrit were 6.4±1.0 x 10^6/µL, 15.8±3.9g/dL and 46±7.2%, respectively (Table 2). Normocytic normochromic anemia was present in 10.4% of the dogs with HAC, whereas 4.3% presented with macrocytic normochromic anemia. Among anemic dogs, 80% were ACTH-dependent. Thrombocytosis (platelets >500.000/µL) was documented in 33.9% of the cases, with a mean platelets value of 479.900/µL.

In 81.7% of the cases an increase in alkaline phosphatase activity was observed. Hypercholesterolemia was documented in 66% of the cases, while increase in ALT activity was present in 62.6%, hypertriglyceridemia in 54.8%, hyperglycemia in 30.5% and creatinine below 0.7mg/dL in 48.7% of cases. Urinalysis has shown proteinuria in 48.7% of the cases and urinary density lower than 1.015 in 26.1%. Table 2 shows mean values, standard deviation, minimum and maximum for each variable.

Abdominal ultrasound

The main alterations in the abdominal ultrasonography were bilateral adrenal gland hyperplasia (92.2%) and hepatomegaly (80.9%), followed by biliary sludge occurrence (67.8%), hepatic hyperechogenicity (47.8%) and renal parenchymal calcification (37.4%). In 26.1% of cases, irregularity was found in the adrenal gland surface, with caudal pole asymmetry detected in

Table 1. Breed distribution of 115 dogs diagnosed with hyperadrenocorticism

Breed	Number of cases	Percentage
Poodle	31	26.9
Dachshund	20	17.3
Mongrel	14	12.2
Yorkshire Terrier	12	10.4
Bichon Frisé	9	7.8
Maltese	5	4.3
Shi-tzu	4	3.4
Pinscher	3	2.6
Schnauzer	3	2.6
Cocker Spaniel	2	1.7
West Highland White Terrier	2	1.7
American Staffordshire Terrier	1	0.9
Beagle	1	0.9
Boxer	1	0.9
French Bulldog	1	0.9
Chihuaha	1	0.9
Chow Chow	1	0.9
Dogo Argentino	1	0.9
Labrador	1	0.9
Lhasa Apso	1	0.9
Scotish Terrier	1	0.9

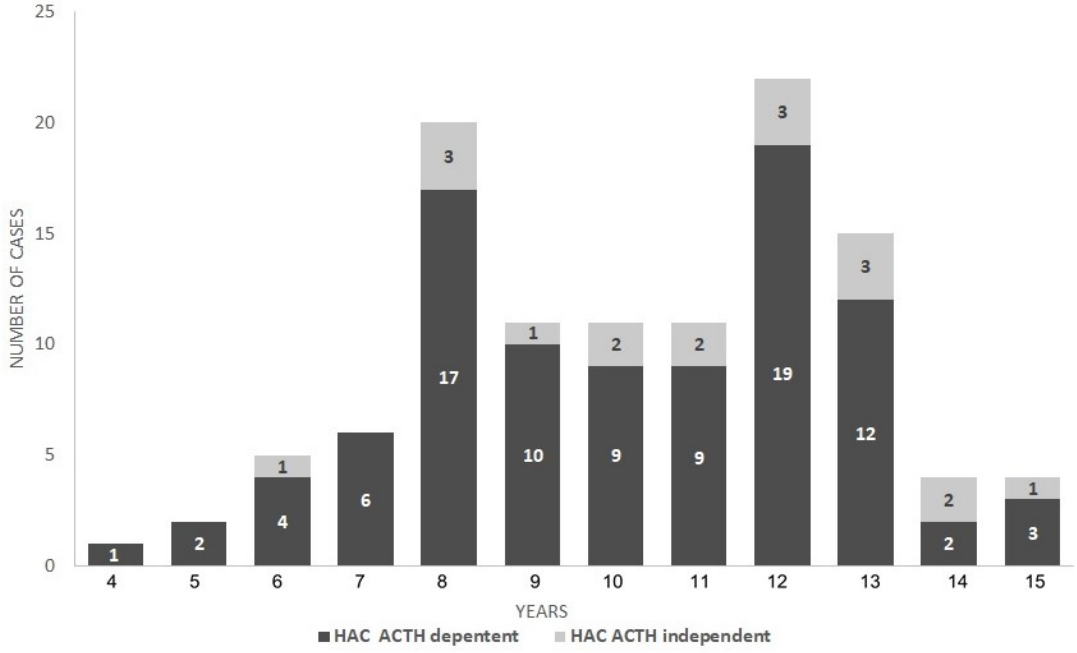


Fig.1. Dog´s number distribution according to age and hyperadrenocorticism etiological classification.

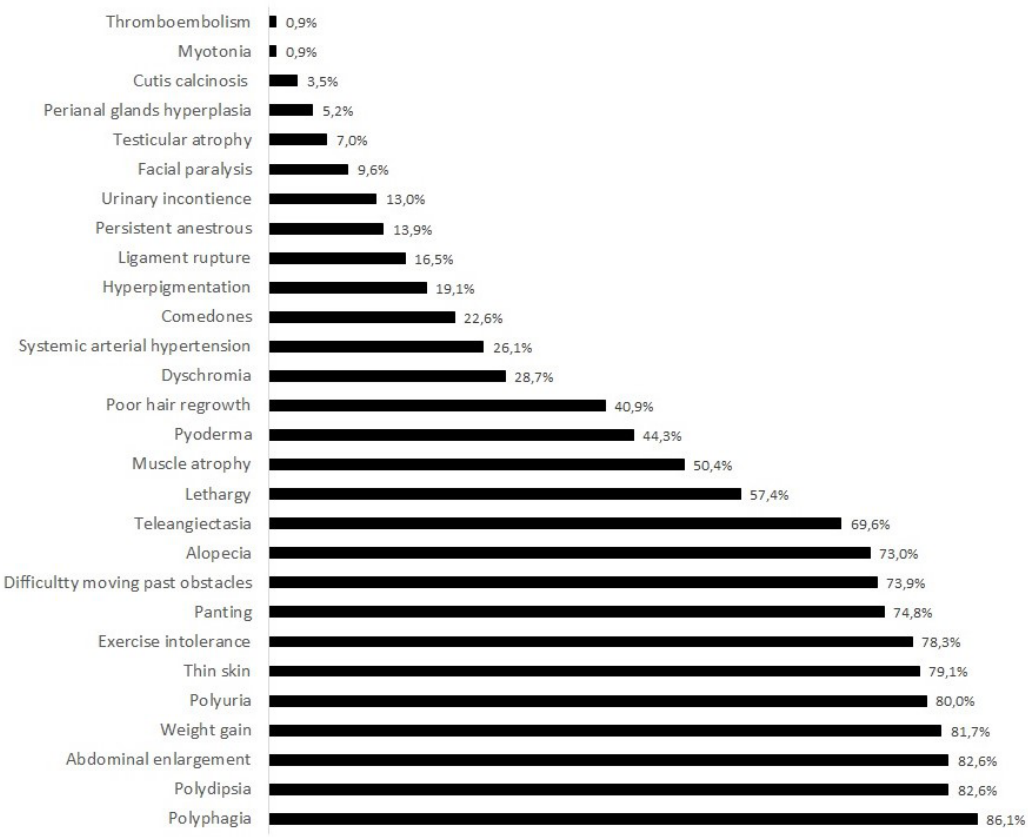


Fig.2. Clinical manifestation´s percentage distribution in dogs diagnosed with hyperadrenocorticism.

Table 2. Mean values, standard deviation, range, and reference interval for the hematologic, serum biochemistry and urinary specific density in 115 dogs with hyperadrenocorticism

Parameters	Mean (standard deviation)	Range	Reference range
Red blood cells	6.4 (± 1.06)	3.5 - 8.28	5.7 - 8.5 x10^6/µL
Hemoglobin	15.8 (± 3.9)	9.5 - 18.2	12 - 18g/dL
Hematocrit	46.0 (± 7.1)	29 - 63	37 - 55%
Platelets	479.9 (± 174.5)	342 - 1,000	200 - 500 x10^3/mm^3
Alkaline phosphatase	391 (± 1,102)	33 - 6,516	< 156 U/L
ALT	194.9 (± 177.9)	7.2 - 923	< 102 U/L
Cholesterol	330.8 (± 143.2)	100 - 1,013	135 - 270mg/dL
Triglycerides	310.04 (± 398)	23 - 2,956	32 - 138mg/dL
Creatinine	0.77 (± 0.28)	0.3 - 1.87	0.5 - 1.5mg/dL
Urea	40 (± 28.6)	10 - 171	21 - 60mg/dL
Glucose	105.3 (± 23.2)	59 - 163	65 - 118mg/dL
Urinary density	1.021 (± 0.011)	1.006 - 1.052	1.015 - 1.045

20.8% of cases, and parenchymal hyperechogenicity in 18.3%. Among adrenal gland alterations, in the ACTH-dependent HAC group, heterogeneous parenchyma was observed in 11.1% and irregular surface in 17.2%. In the ACTH-independent HAC group of dogs, asymmetry and heterogeneous parenchyma happened in 55% of the cases, and irregular shape in 60%.

Left adrenal gland caudal pole average measures in the ACTH-dependent HAC group were 0.9±0.4cm (range 0.4-3.3cm), while in right adrenal gland were 0.83±0.33cm (range 0.35-2.6cm). In the ACTH-independent HAC group, average of left adrenal glands was 1.31±0.76cm (range 0.69-3.65cm) and 1.46±1cm (range 0.5-4.5cm) for right adrenal glands. In small size dogs, average measures of adrenal glands caudal pole were 0.88±0.44cm for the left adrenal gland and 0.86±0.44cm for the right one. In medium size dogs, average measures of caudal pole were 1.17±0.6cm for the left adrenal gland and 1.12±0.78cm for the right one, whereas in large size dogs' average thickness of caudal pole of the left adrenal gland was 1.26±0.56cm and 1.43±0.48cm in the right one (Fig.3).

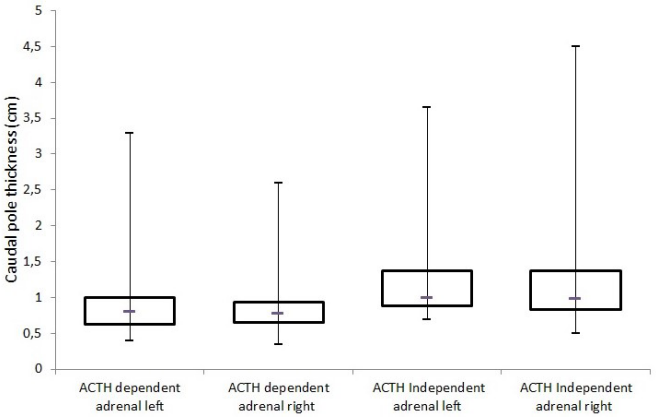


Fig.3. Left and right adrenal caudal pole thickness box plots of 115 dogs with hyperadrenocorticism according to etiological classification.

DISCUSSION

Characterization of the clinical-epidemiological profile collaborates to direct diagnosis process. Therefore, it is considered important to determine clinical, epidemiological and laboratorial aspects of diseases, especially ones that present a complex diagnostic, such as canine HAC. In the present study, classic disease´s presentations were observed, with well-characterized clinical findings; however, in some patients, clinical manifestations were more discreet. Those cases did not present alterations considered frequent such as polyuria, polyphagia, increase in alkaline phosphatase activity, and hyperplasia of adrenal glands. Despite all the improvement in techniques and methodologies concerning hormonal analysis, evaluation of clinical aspects and complementary exams composition are essential to determine diagnosis (Benchekroun et al. 2010, Behrend 2015).

ACTH-dependent HAC was identified as being more frequent, accounting for about 80% of the cases, which is a similar result to what can be found in other studies (Reusch & Feldman 1991, Theon & Feldman 1998). Determination of the etiological classification of HAC is important not only to establish therapeutic protocol and prognosis (Fracassi et al. 2014, Behrend 2015), but also as a basis for the development of studies on pathogenesis, identifying factors that influence on incidence and etiology of this endocrinopathy. In this aspect, it seems reasonably that Brazilian canine HAC´s population behaves in an analogous way that the patients worldwide.

Among possible factors, increased exposure to endocrine disruptors[1], such as food contaminants (Koestel et al. 2017), toys, or plastic utensils (Wooten & Smith 2013). Such substances interfere with several physiological mechanisms, from the inhibition of hormone synthesis (such as happens with lindane reducing StAR protein expression, decreasing steroidogenesis) to steroid receptors binding, altering the process of transduction, signaling, and modulating enzymatic pathways in the metabolism of sexual hormones (for example, analogues of dichlorodiphenyltrichloroethane) (Koestel et al. 2017, Wooten & Smith 2013).

Recent studies have evaluated the binding capacity of these pesticides to the glucocorticoid receptor (GR), demonstrating antagonistic effects from hormonal activity (Zhang et al. 2016). Such results, associated with knowledge of the cumulative potential and the possible effect over negative feedback on the pituitary gland, must be considered regarding the pathophysiological aspects of the disease. It is believed that a mutation in the structure of GR might reduce glucocorticoid sensitivity, causing an ACTH higher compensatory secretion. This hypothesis would justify the greater frequency of ACTH-dependent HAC. However, new evidence discredits this possibility (van Wijk et al. 2014). Despite significant improvement in the biomolecular techniques applied to endocrinology; etiopathogenesis and triggering factors of the mutations involved in HAC occurrence remain uncertain (Teshima et al. 2009, Kool et al. 2013). Those data therefore stress the clinical epidemiological studies importance and characterization.

HAC mainly affects middle-aged to older dogs, with a mean age of 10 years (Gallelli et al. 2010, Behrend 2015, Pöppl et al. 2016). It was previously found that dogs with ACTH-independent HAC tend to be more senile when compared to those with the ACTH-dependent form of the disease (Reusch & Feldman 1991), which was observed in the present study. According to Kennedy et al. (2012) this is likely to happen due to the accumulation of damage and detrimental mutations in DNA, leading to compromise of genetic information, and relating aging to senile diseases such as cancer and neurodegeneration.

Most research agreed that HAC happens more often in female dogs (Reusch & Feldman 1991, Gallelli et al. 2010, Pöppl et al. 2016). Nonetheless, there was no consensus regarding the existence of gender predisposition along time. In the present evaluation, ACTH-dependent HAC was more frequent in bitches, whereas ACTH-independent HAC was more frequent in male dogs. A study conducted in the seventies did not identify significantly differences in the sexual distribution between the groups assessed (Ling et al. 1979). In early nineties, Reusch & Feldman (1991) indicated females HAC percentage over 60%. Behrend (2015) stated a female gender predisposition.

Regarding reproductive condition, studies do not point out to greater HAC diagnosis incidence in gonadectomized dogs (O'Neill et al. 2016). Nonetheless, Belanger et al. (2017) have observed gonadectomy in males and females was significantly associated with a greater risk to develop HAC. In the study, spayed females were more frequent in the ACTH-dependent HAC group, which corroborates Peterson (2007) when stating that due to the interaction between female sexual hormones and the hypothalamic pituitary axis, females present greater predisposition to develop HAC, being the ACTH-dependent form more common. In fact, a recent study on canine HAC environmental risk-factors found gonadectomy as the only risk-factor identified within females' patients. HAC bitches in this study had an odds ratio greater than ten to be gonadectomized against control bitches paired by age and breed (Bock & Pöppl 2018).

[1] Note: Endocrine disruptors are substances exogenous to the organism that may interfere with synthesis, secretion, transport, metabolism or elimination of different hormones (Sonnenschtein & Soto 2010). Examples: polychlorinated biphenyls, dioxins (industrial solvents), bisphenol A (plastics), PBDEs (flame retardants), dichlorodiphenyltrichloroethane (pesticides), diethylstilbestrol (pharmaceutical products) and parabens (preservatives).

O'Neill et al. (2016) have also reported that large breed dogs present lower risk to develop ACTH-dependent HAC when compared to small breed dogs. This aspect may be due to the diversity among dog breeds, with significant size and muscle mass alteration resulting from selective pressure in the formation and maintenance of modern breeds. It is evident that medium and small sized dogs are more HAC affected, which could be explained by data from a study that compared gene expression among dog sizes, identifying variants regarding a lower expression of IRS-4, IGSF-1 and ACSL-4 in small and medium sized dogs (Plassais et al. 2017). Such substrates are important in pituitary development (Wang et al. 2017) and in steroidogenesis (Midzak & Papadopoulos 2016), which may explain the correlation between the occurrence of HAC and size. However, the breeds most reported in our study are also popular in our region, and the fact that they were overrepresented in this population does not absolutely means that they are more prone to HAC. Nevertheless, the breeds reported here are also commonly described in other South America and worldwide studies (Gallelli et al. 2010, Nozawa et al., 2014, Fracassi et al., 2015, Pöppl et al. 2016, Caragelasco et al. 2017, Miceli et al., 2017, Bennaim et al. 2019). Moreover, mongrel dogs may be less represented due to eventual owner´s economic or even social conditions.

Knowing that the cases studied severity was variable and there is no correlation between gravity and cortisol concentration (Behrend 2015), it is interesting to point out the role of the 11β-hydroxysteroid dehydrogenase type 1 enzyme. Morgan et al. (2014) have shown glucocorticoids activation by 11β-HSD1 in the peripheral tissues is a determining factor for the characterization of Cushing syndrome in mice. Data demonstrate that, even in high concentrations of glucocorticoids, in the absence of 11β-HSD1, mice are protected from clinical manifestations such as glucose intolerance, hyperinsulinemia, hepatic steatosis, adiposity, hypertension, myopathy and thin skin. This happens since glucocorticoids availability and action is also dependent on tissue-specific intracellular metabolism (Morgan et al. 2009), as well as on the level of expression of the enzyme, possibly affecting and changing clinical condition.

The results of the present study have resembled the data proposed by Behrend et al. (2013) that have classified the main HAC clinical manifestations as: frequent, less frequent, and infrequent. However, with the greater knowledge development on this endocrinopathy, identification of more subtle clinical signs becomes obvious. Besides the more classic manifestations seen in the study, less frequent ones such as lethargy/apathy, hyperpigmentation, comedones, pyoderma and poor hair regrowth were often observed. In the same way, infrequent ones, such as thromboembolism, ligament ruptures, facial nerve paralysis, cutis calcinosis, testicular atrophy, myopathy and persistent anestrous. Persistent anestrous low documentation may have occurred due to most females being already gonadectomized at diagnosis. The observation of a considerable number of patients with facial nerve paralysis in this case series calls attention to the necessity of broader studies to understand the origin of this little-reported alteration associated with HAC.

Regarding laboratory exams, complete blood count showed anemia in 17% of the cases. Normocytic normochromic anemia was the most frequent one, mainly affecting patients with ACTH-dependent HAC. Chervier et al. (2012) believes 14% of the anemia cases in dogs are associated with endocrinopathies, with 80% of them being considered mild anemia. Such data goes against the expectation of mild erythrocytosis due to the eventual role of adrenal androgens on erythropoiesis. In the present study, frequency of anemia was lower in ACTH-independent cases. Chervier et al. (2012) points out that cancer-related anemia happen more often than anemia from chronic inflammation. In some cases, anemia could be present as a result from an induced euthyroid sick syndrome, due to the suppressive action of chronic hypercortisolemia in the secretion of thyrotrophic hormone (TSH) and reduced thyroxine (T_4) deiodination to triiodothyronine (T_3) (Behrend 2015). It is important to note that anemia could be predictive of comorbidities and may help in patient's prognosis. Further investigation is warranted in those cases.

The most common findings in the leukogram were neutrophilia without left shift and eosinopenia. Behrend (2015) indicates that neutrophilia is due to neutrophils demargination from capillaries, monocytosis is secondary to a lower stimulus for circulation removal, lymphopenia is due to steroid-induced sequestration and cellular destruction, and eosinopenia results from bone marrow sequestration.

Thrombocytosis is considered a common finding in HAC. Studies indicate it may occur in 60% to 80% of dogs with HAC (Herrtage & Ramsey 2012, Pace et al. 2013, Rose et al. 2013). In the present study it was observed in 33.9% of the cases assuming 200.000-500.000/mm^3 platelets as reference range. Until the present moment, the role and relevance of thrombocytosis in HAC dogs is not known (Behrend 2015), since an association between hypercoagulability and canine HAC has not yet been proved. There is, however, indication to evaluate the coagulation profile of these patients, through monitoring of coagulation factors and counting of platelets, as well as identifying and treating possible factors associated, such as hypertension, hypercholesterolemia, and diabetes mellitus (Rose et al. 2013, Klose et al. 2011).

Concerning alterations in biochemical exams, the most frequent one was an increase in serum alkaline phosphatase (ALP) activity, due to the glucocorticoid-induced isoenzyme. However, hepatic vacuolation due to glycogen overload, favoring intrahepatic cholestasis also collaborate to ALP increases (Kooistra & Galac 2010). Higher ALP serum activity is one of the most common findings in HAC canine patients (Herrtage & Ramsey 2012, Behrend 2015). In the present study, however; about 20% of the cases did not show this abnormality. Therefore, it is of interest to emphasize that patients with hypercortisolemia may present values for ALP activity within reference range, and one should not discard HAC from differential diagnosis list based on a normal ALP.

In most cases, ALT shows a discrete elevation, caused by an increase in hepatocellular membrane permeability due to glycogen deposition in hepatocytes, with eventual cellular death low level (Behrend et al. 2013). Hence, greater hepatic compromise must be considered in HAC cases with higher ALT values. In the present study, increase in serum ALT activity was observed in more than half of the dogs, which may justify the adjunct use of drugs that act controlling oxidative stress and inflammation during HAC treatment (AU et al. 2013).

Urea values were elevated in almost 30% of the cases, possibly indicating glucocorticoid-induced protein catabolism. Nonetheless, both urea and creatinine may be at lower end

of reference range (creatinine <0.7mg/dL was observed in almost 50% of the cases in the study). This condition happens due to glucocorticoid-induced muscle catabolism along with polyuria, which promotes a greater urinary loss of nitrogen compounds, and an increased glomerular filtration rate caused by glucocorticoids (Behrend et al. 2013, Herrtage & Ramsey 2012).

Hyperlipidemia was present in about 60% of the dogs with HAC. According to Ramsey & Ristic (2007), hypercholesterolemia is observed in 90% of the dogs with HAC and many of these animals also present hypertriglyceridemia. It is likely that the condition happens due to chronic glucocorticoid-induced insulin resistance, with a lower lipoprotein lipase activation and greater hormone-sensitive lipase activation.

Miceli et al. (2017) have observed that dogs with fasting blood glucose levels above 105mg/dL, with dyslipidemia (triglycerides >221.2mg/dL and hypercholesterolemia >350mg/dL), and cortisol:creatinine ratio >100 x 10^{-6}, present greater risk of diabetes mellitus developing. The study highlights that 93.7% of the dogs with HAC and DM presented ACTH-dependent HAC, indicating a higher predisposition of this group to develop diabetes mellitus, and even suggests preventive insulin therapy. Other study has also shown an association between HAC and diabetes mellitus in 16% of cases (Pöppl et al. 2016). Biomolecular data has indicated a reduction in the expression of genes involved in insulin signaling mechanisms (IRS-1, IRS-2, PI3-K e Akt-2) of dogs with HAC, thus contributing to insulin resistance induction (Nozawa et al. 2014).

Urinalysis showed proteinuria in almost 50% of cases and hyposthenuria (urinary density <1,008) in 15% of them. These alterations are reported as being the main urinary findings in dogs with HAC (Behrend et al. 2013). It is important to note that even after treatment and decrease in glomerular filtration rate; proteinuria persists in some dogs (Smets et al. 2012a). The reason for this is related to glucocorticoid-induced dysfunction both at glomerular and tubular level in dogs with untreated HAC. This observation warrant monitoring of renal function and systemic hypertension control (Smets et al. 2012b). Moreover, other studies have shown that proteinuria is directly correlated with blood pressure (Vidal et al. 2018), and that HAC initial treatment with trilostane does not seem to be enough to normalize altered cardiovascular parameters in dogs (Soares et al. 2016). In this scenario, it is important to note that the overexpression of the 11β-hydroxysteroid desidrogenase-1 enzyme in visceral adipose tissue of HAC patients could play a role in the maintenance of hypercortisolism in dogs with HAC (Miceli et al. 2018).

In the abdominal ultrasounds, adrenal glands bilateral enlargement was observed in 92% of the cases. Benchekroun et al. (2010) state that the bilateral enlargement of the adrenals suggests that HAC is ACTH-dependent, whereas the unilateral enlargement of the gland points towards ACTH-independent HAC. Even though it was adrenal abnormalities in almost all the cases analyzed, studies indicate that the adrenal glands may present unusual sizes or alterations in any type of HAC (Benchekroun et al. 2010). Nevertheless, it was observed that the frequency of adrenal glands with irregular shape was of 60% in the group of dogs with ACTH-independent HAC, there is, 3.5-fold greater than in the ACTH-dependent HAC group. It is important to highlight that no alterations were found in the adrenal glands in 7% of the cases, which corroborates the concept that the possibility of HAC cannot be ruled out based on the identification of adrenal glands with normal size and aspect (Behrend et al. 2013). Pagani et al. (2017) have compared the alterations described in the ultrasonography against the histologic findings. Their results indicated bilateral lesions in the adrenals as predictive of benign lesions, such as cortical hyperplasia. On the other hand, large masses (>20mm) and surface irregularities, were predictive of malignant neoplasm. Such structural characteristics could help diagnosis and investigation. Even though, they are not pathognomonic, especially since some ACTH-dependent cases develop macronodular hyperplasia of the adrenal glands and since adrenal hyperplasia because of the hypothalamic pituitary adrenal axis (HPA) activation is a common finding.

Also, a high frequency of hepatomegaly was verified in abdominal ultrasounds, with liver hyperechogenicity being observed in almost 50% of the cases. This finding is compatible with a steatosis condition due to steroids-induced hepatic glycogen overload (Ramsey & Ristic 2007). Biliary sludge was observed in almost 70% of the cases, attesting to the high occurrence of cholestatic complications in dogs with HAC. It is believed that cholestatic disorders physiopathology in patients with ACTH-dependent HAC may be related to the cholesterol metabolism. Kim et al. (2017) have observed an association between ACTH-dependent HAC and cholestatic disease, and that patients with such comorbidity need higher doses of trilostane. A recent study has also shown it is possible that gallbladder mucoceles and biliary sludge have the same physiopathology, thus representing a continuous disease, with biliary sludge being considered the stage before gallbladder mucoceles appearance (Kutsunai et al. 2014, Mizutani et al. 2017).

CONCLUSIONS

This work concluded that the population studied resembles the profile describe in European and North American epidemiologic studies, and that the HAC dog´s clinical picture looks similar worldwide. This clinicopathological characterization of a South Brazilian cohort contributes for a better recognition of this important, challenging, and frequent disease at Brazil.

Special attention should be present in front of vague clinical signs or minor laboratory and ultrasound abnormalities compatible with HAC. Due to the greater dog´s life expectancy, and better early HAC recognition, to know how local HAC cases behave in contrast with international literature helps to support accurate diagnosis in clinic routine. Moreover, eventually unusual HAC-associated signs would be present in some dogs in a more important way compared with the classic disease´s clinical signs. To recognize the large spectrum of clinicopathological abnormalities secondary to this interestingly endocrine disease is a key factor to improve HAC suspicion and investigation.

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

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Canine *POMC* deletion (P187fs) allele frequency in Labrador Retrievers in Brazil[1]

Natalia O. Lourenço[2], Ana Luísa H. Albuquerque[2], Roberta M. Basso[2], Anelize S. Trecenti[2], Lukas G. Albertino[2], Alessandra Melchert[2], Alexandre S. Borges[2] and José P. Oliveira-Filho[2]*

ABSTRACT.- Lourenço N.O., Albuquerque A.L.H., Basso R.M., Trecenti A.S., Albertino L.G., Melchert A., Borges A.S. & Oliveira-Filho J.P. 2019. **Canine *POMC* deletion (P187fs) allele frequency in Labrador Retrievers in Brazil.** *Pesquisa Veterinária Brasileira 39(11):909-914*. Departamento de Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rua Prof. Doutor Walter Mauricio Correa s/n, Cx. Postal 560, Botucatu, SP 18618-681, Brazil. E-mail: jose.oliveira-filho@unesp.br

The Labrador Retriever is among the main breeds with the greatest predisposition to obesity. Several factors, especially the interrelationships between food management, exercise and social factors; influence the likelihood of a dog becoming obese. Furthermore, genetic factors are also responsible for obesity in dogs, and in Labrador Retriever, a frameshift mutation (P187fs) in *pro-opiomelanocortin* (*POMC*) gene is strongly associated with obesity. There is no knowledge of studies that have previously evaluated the prevalence of the canine *POMC* deletion (P187fs) in Brazilian Labrador Retriever. Therefore, the objective of this study was to investigate this mutation in Labrador Retriever dogs in Brazil. Of the 108 Labrador Retrievers that were assessed in this study, 59 were from a previous study, composed by animals assisted in a veterinary hospital with unknown lineage, and 49 were from a prospective study, composed of 19 pet and 30 assistance/rescue Labrador Retriever dogs. The obesity risk and appetite questionnaire were applied, with some modifications, to tutors of the animals used in the prospective study. Fragments of the DNA, containing the mutation, were amplified by PCR and submitted to direct gene sequencing. The allele frequency of the mutation was 21.3% and was out of Hardy-Weinberg equilibrium (P<0.05). Using only the data of animals with known lineage, the presence of the mutated allele was higher in the Assistance/rescue Group than Pet Group (P<0.01), furthermore, the allele frequencies observed in Assistance Group (31.7%) was out of Hardy-Weinberg equilibrium (P<0.05), while that in the Pet Group (18.4%) was in equilibrium (P>0.05). Although the mutation has increased the food-motivation in the assistance/rescue dogs, other variables, especially frequent exercising, favored that these animals maintained the ideal body weight (body condition score = 5). In summary, the Hardy-Weinberg disequilibrium observed in the allele distribution of the deletion *POMC*_P187fs in this study, independently of the Labrador Retriever group assessed, suggesting the possibility of positive selection of the mutated allele, which may lead to the maintenance of this deleterious allele in the studied population.

INDEX TERMS: Canine *POMC*, P187fs, allele frequency, Labrador Retriever, Brazil, gene, mutation, dogs, obesity, prevalence.

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[2] Departamento de Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia (FMVZ), Universidade Estadual Paulista "Júlio de Mesquita Filho" (Unesp), Rua Prof. Doutor Walter Mauricio Correa s/n, Cx. Postal 560, Botucatu, SP 18618-681, Brazil. *Corresponding author: jose.oliveira-filho@unesp.br

RESUMO.- [**Frequência de alelos mutados (P187fs) no *POMC* de cães Labrador Retriever no Brasil.**] O Labrador Retriever é uma das principais raças caninas com maior predisposição à obesidade. Vários fatores, especialmente as interrelações entre a alimentação, exercício e fatores sociais,

influenciam a probabilidade de um cão se tornar obeso. Além disso, fatores genéticos são também responsáveis pela obesidade em cães, e no Labrador Retriever a mutação "frameshift" P187fs no gene pró-opiomelanocortina (*POMC*) está fortemente associada à obesidade. Não existem estudos prévios de prevalência da deleção P187fs no gene *POMC* em cães Labrador Retriever no Brasil. Portanto, o objetivo deste estudo foi investigar esta mutação em cães da raça Labrador Retriever no Brasil. Dos 108 Labradores Retrievers avaliados neste estudo, 59 eram de um estudo retrospectivo (composto por animais atendido no hospital veterinário e sem linhagem conhecida) e 49 eram de um estudo prospectivo (composto por 19 cães pet e 30 cães de assistência/resgate). Um questionário de risco de obesidade modificado foi aplicado nos tutores dos animais usados no estudo prospectivo. Fragmentos de DNA, contendo a mutação, foram amplificados por PCR e submetidos ao sequenciamento gênico direto. A frequência alélica da mutação foi de 21,3% e estava fora do equilíbrio de Hardy-Weinberg (P<0,05). Usando somente os dados dos animais de linhagem conhecida, a presença do alelo mutado foi maior no Grupo de cães de Assistência/resgate que no Grupo de Pets (P<0,01), além disso, as frequências alélicas nos Grupos de Assistência/resgate (31,7%) e no de pets (18,4%) estavam fora e em equilíbrio de Hardy-Weinberg (P<0,05), respectivamente. Embora a mutação tenha aumentado a motivação pelo alimento em cães Labrador Retriever do Grupo de Assistência/resgate, outras variáveis, especialmente o frequente exercício, favoreceu a manutenção o peso corporal ideal (peso corporal = 5). Em resumo, o desequilíbrio de Hardy-Weinberg observado na distribuição do alelo *POMC*_P187fs observado neste estudo, independentemente do grupo de Labrador Retriever avaliado, sugere a possibilidade de uma seleção positiva para o alelo mutado, o qual poderá levar a manutenção desse alelo deletério nesta população.

TERMOS DE INDEXAÇÃO: Alelos mutados, *POMC*, caninos, P187fs, Labrador Retriever, Brasil, gene, mutação, cães, obesidade, prevalência.

INTRODUCTION

Obesity or excessive overweight is characterized by the accumulation of excessive amounts of adipose tissue in the body and is the most common nutritional disorder in companion animals (German 2006). As in humans, several disorders may be due to obesity in dogs, especially, metabolic and endocrine abnormalities, orthopedic disease, cardiorespiratory disease (Pöppl & González 2005, German 2006, Lund et al. 2006, Clark & Hoenig 2016, Frye et al. 2016). Obesity in dogs is affected by the interrelationships between food management, exercise and social factors (Bland et al. 2009, Aptekmann et al. 2014, Debastiani 2018). The higher prevalence of canine obesity seen in recent years may be also influenced by the increase in human obesity (Raffan et al. 2016).

Furthermore, genetic factors are also responsible for obesity in dogs (Skorczyk et al. 2007, Grzes et al. 2011, Skorczyk et al. 2011, Mankowska et al. 2015, Miyabe et al. 2015) and specifically in Labrador Retriever a frameshift mutation (P187fs) in *pro-opiomelanocortin* (*POMC*) gene is strongly associated with obesity (Raffan et al. 2016). This mutation provokes a 14bp deletion in the *POMC* gene, which causes loss of production of β-MSH (melanocyte-stimulating hormone) and β-endorphin (Raffan et al. 2016). Although, the mechanism by which reduced β-MSH and β-endorphin due to the mutation causes behavioral and weight phenotypes remains to be precisely elucidated, Raffan et al. (2016) observed that *POMC*_P187fs mutation is associated with increased body weight (per allele effect of 0.33 SD), adiposity, and greater food motivation. The *POMC*_P187fs mutation was found closely related to Flat-Coated Retriever, however, it was absent from dogs of 38 other diverse breeds (Raffan et al. 2016).

Obesity is the second major primary disorder observed in Labrador Retrievers in the United Kingdom (McGreevy et al. 2018). As in other countries (Payan-Carreira et al. 2015, McGreevy et al. 2018), the Labrador Retriever is among the main breeds with the greatest predisposition to obesity in Brazil (Braos et al. 2009, Aptekmann et al. 2014, Debastiani 2018). In addition, there is no knowledge of studies that have previously evaluated the prevalence of the canine *POMC* deletion (P187fs) in Brazilian Labrador Retriever. Therefore, the objective of this study was to investigate this mutation in Labrador Retriever dogs in Brazil.

MATERIALS AND METHODS

Ethics statement. All procedures were approved by the Board of Ethics and Animal Experimentation of the institution (Protocol no. 220/2016 - CEUA).

Experimental animals and samples. For the present molecular study, blood or buccal swab was randomly collected from 108 Labrador Retrievers mainly from states of São Paulo (89/108), followed by Santa Catarina (13/108), Minas Gerais (3/108), and Pernambuco (3/108), based on informed consent. Labrador Retriever cohort was composed of 69 females (64%) and 39 males (36%), with ages ranging from ten months to 15 years (average age of 6.6 years old). Of the 108 dogs assessed in this study, 59 were Labrador Retrievers that had been submitted to the Veterinary Hospital at FMVZ-Unesp, Botucatu, for medical examination, and blood samples from these dogs were used for genomic DNA purification. The prospective study used the remaining 49 Labrador Retrievers, where a buccal swab was collected from these animals and the Dog Obesity Risk and Appetite (DORA) questionnaire was applied, with some modifications, to tutors of these animals (Raffan et al. 2015).

According to age, the animals were divided into three groups: young animals (age under 5 years), adult animals (age ranging from 5 to 10 years), and old animals (age over 10 years). The body condition score (BCS) of all dogs was attributed by veterinary practitioners using previously validated standard chart of the WSAVA (World Small Animals Veterinary Association) (Laflamme 1997, Kealy et al. 2002, Mawby et al. 2004, German et al. 2010, Jeusette et al. 2010), who had representative images and accompanying descriptors. The dogs were classified on a scale of 1-9 (1 to 3-BCS under ideal; 4 to 5-BCS ideal; and 7 to 9-BCS over ideal), and the animals with BCS of 7 to 9 were considered obese.

DNA purification and genotyping analysis. Genomic DNA was purified from blood or buccal swab samples using the GenElute™ Genomic Blood DNA Kit (Sigma-Aldrich®) according to the manufacturer's instructions. The DNA obtained was used to genotype the deletion (P187fs) in the *POMC* gene previously associated with obesity in Labrador Retrievers (Raffan et al. 2016). Two specific primers (JPPOMC-F3 5'-AAGCCGGTGGGCAAGAA-3', and JPPOMC-R3 5'-GGAAGTGCTCCATCTTGTAGGG-3') were designed with Primer Express software (Applied Biosystems, Grand Island, NY) to genotype the *POMC*_P187fs (244-bp). The specificity of the PCR primers was evaluated in silico with the Basic Local Alignment

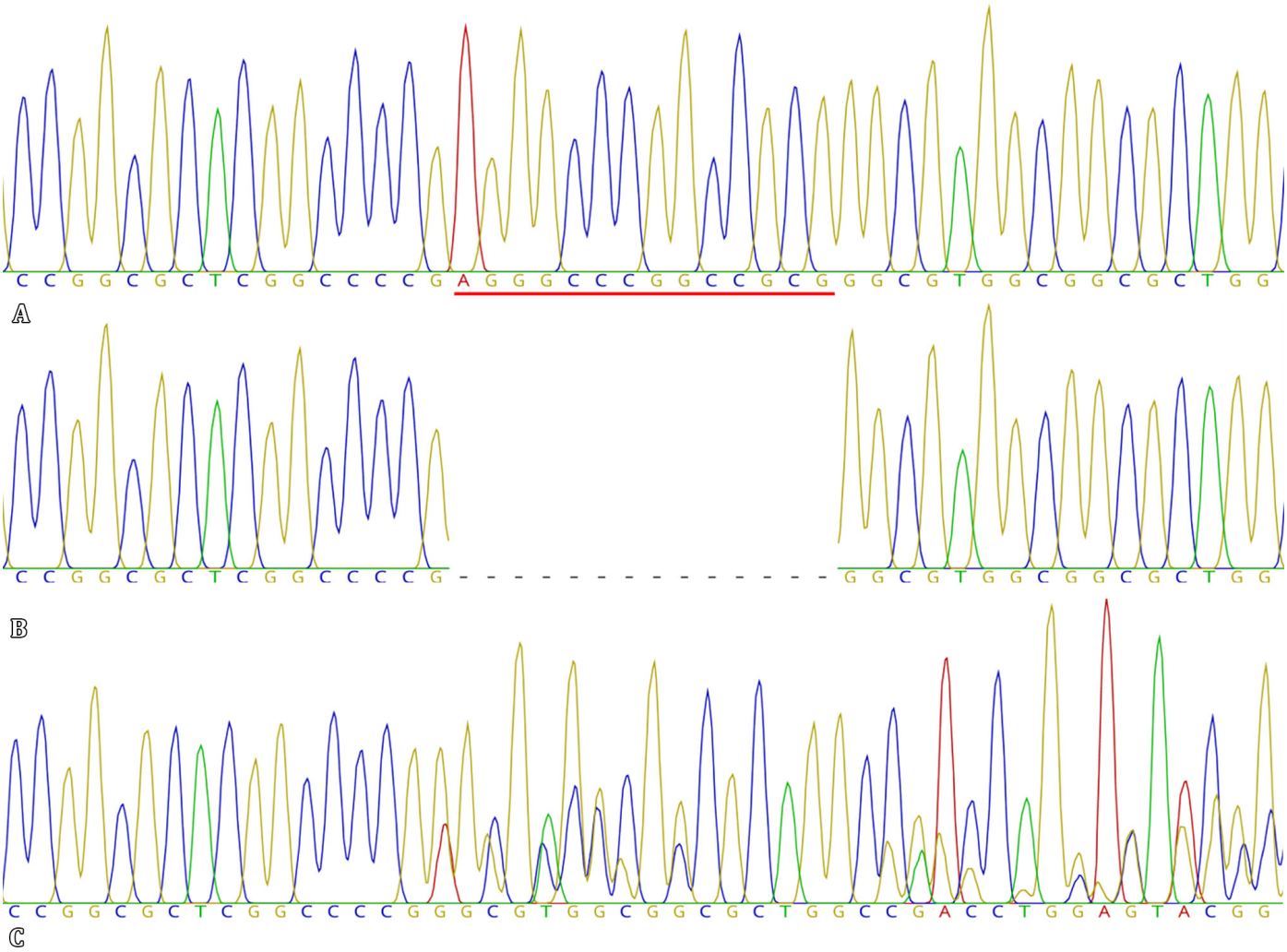


Fig.1 Partial chromatogram showing (**A**) capillary sequencing results from wild-type, (**B**) homozygous deletion, and (**C**) heterozygous Labrador Retriever dogs. (**A**) It is possible to observe the 14bp (AGGGCCCGGCCGCG) of the deletion *POMC*_P187fs (underlined in red), while at (**B**) these 14 bases are absent. (**C**) The double peaks may be observed from the beginning of the deletion *POMC*_P187fs, i.e., the 14bp are only deleted in one allele. Image obtained in the Geneious® 10.0.9 software (Biomatters Ltd, Auckland, New Zealand).

Search Tool (National Center for Biotechnology Information, USA). Polymerase chain reactions were performed in a total volume of 25μL, which contained 2.5μL of template DNA, 0.3μM each forward and reverse primer, 1.25μL of DMSO (5% v/v), 12.5μL of GoTaq® Green PCR Master Mix (Promega), and 7.5μL of nuclease-free water q.s.p. In addition, a no-template control reaction was performed to check for the possible presence of contamination in the PCR preparations. The amplification conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 63.5°C for 1 minute, and extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes. Amplicons were analysed by 1.5% agarose gel electrophoresis, purified, and subjected to direct sequencing. The obtained PCR products were analyzed via 1.5% agarose gel electrophoresis (Invitrogen™, Carlsbad, CA), stained with the Sybr® Safe DNA Gel Stain (Invitrogen™), purified using the QIAquick® PCR Purification Kit (Qiagen®), and then subjected to direct sequencing.

Sequencing analysis. To sequence the DNA, 10μL of each PCR product, 5μL of the purified forward primer and BigDye® Terminator Cycle Sequencing Kit were used (Life Technologies™ CA, USA). The sequences were determined using the ABI 3500 Genetic Analyzer (Life Technologies™ CA, USA). The obtained sequences and the electropherograms (Fig.1) were analyzed using Geneious® 10.0.9 (Biomatters Ltd, Auckland, New Zealand). The sequences were compared with the *Canis lupus familiaris POMC* sequence (XM_022404519.1), deposited in GenBank™, using BLAST[1].

Data analysis. Chi-square or Fisher tests were used to compare the proportion of obesity and genotype versus age, gender, and coat colour. The prospective of Assistance/rescue Group and Pets Group Labrador Retrievers were compared using Chi-square or Fisher tests, in order to analyse proportion of obesity and genotype versus exercise frequency, type of food (house feeding or commercial dog food), and behaviour features (excitement, unremitting interest, snack training, speed of intake, and selective behaviour). Statistical analyses were conducted using GraphPad Prism® version 7 for Windows and

[1] Basic Local Alignment Search Tool. Available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>

the level of statistical significance was set at 0.05. The genotypic frequency was estimated and the data were analyzed descriptively. The chi-square test was also used to test whether alleles were in Hardy-Weinberg equilibrium within different groups.

RESULTS AND DISCUSSION

The samples used in the previous study (n=59) belonged to the DNA bank of the Biomol Laboratory of the Department of Veterinary Clinical Science of the FMVZ-Unesp and were collected during 2015 and 2016, while the samples used in the prospective study (n=49) were collected during 2017 and 2018. Of the 108 Labrador Retrievers tested, 16 dogs (15%) were identified as homozygous for deletion *POMC*_P187fs, 14 dogs (13%) were identified as heterozygous, and 78 (72%) were homozygous for the wild-type (WT) allele (Table 1). Therewith, in the present study the allele frequency of the mutant allele (21.3%) was higher than that observed in UK and USA (12%; 2% homozygous for deletion, 20% heterozygous, and 78% WT) (Raffan et al. 2016). Differently from what was observed in these two countries (Raffan et al. 2016), the allelic distribution of the deletion *POMC*_P187fs was out of Hardy-Weinberg equilibrium in the population evaluated in the present study (P<0.01). Furthermore, when analysing separately the groups of animals of the previous study (composed of 59 Labrador Retrievers of unknown lineage.) and prospective (composed of 19 pet and 30 assistance/rescue Labrador Retriever dogs) were observed frequencies of 19.5% and 23.5%, respectively, and both frequencies were out of Hardy-Weinberg equilibrium (P<0.01). However, using only the data of animals with known lineage, the presence of the mutated allele was statistically higher (P=0.0423) in the assistance/rescue dogs than pets dogs, furthermore, the allele frequencies observed in this group (31.7%) was out of Hardy-Weinberg equilibrium (P<0.01), while the allele frequencies (18.4%) observed in the Pet Group was in equilibrium (P=0.122). Therefore, these results were similar to those of Raffan et al. (2016), which observed that the mutation was significantly more common in Labrador Retrievers selected to become assistance dogs than pets (allele frequencies, 45% vs. 12%), and the allelic distribution of the deletion *POMC*_P187fs in the assistance dogs was out of Hardy-Weinberg equilibrium, while in the Pet Group was in equilibrium. These authors believed that temperament and trainability are the main drivers for selection of assistance dogs, and positive reinforcement with food reward is a mainstay of puppy training, and with this, dogs carrying the *POMC* deletion may be more likely to be selected as assistance dogs.

Of the 69 females evaluated, nine (13%) were identified as homozygous for deletion *POMC*_P187fs, nine (13%) were identified as heterozygous for the deletion *POMC*_P187fs, and 51 (74%) were homozygous for the wild-type (WT) allele. While of the 39 males evaluated, seven (18%) were identified as homozygous for deletion *POMC*_P187fs, five (13%) were identified as heterozygous for the deletion *POMC*_P187fs, and 27 (69%) were homozygous for the WT allele. According to age, 29 (27%) Labrador Retrievers were young, 60 (55%) were adult, and 19 (18%) were old. The allele mutated distribution in this age group was as follows: young dogs were 4% homozygous for deletion, 17% heterozygous, and 79% WT; adult dogs were 23% homozygous for deletion, 12% heterozygous, and 65% WT; and old dogs were 5% homozygous for deletion, 11% heterozygous, and 84% WT. In the present study, there was no relationship between gender (P=0.7861) or age (P=0.0886) with the genotype of the animals. This was expected since the deletion *POMC*_P187fs is an autosomal mutation, not related to sex chromosomes and age of the animals (Raffan et al. 2016).

In the present study, more obese females (49%, 34/69) were observed than obese male (36%, 14/39), however, there was no statistical difference (P=0.2297) in the proportion of obese and lean Labrador Retrievers between males (14 obese and 25 leans) and females (34 obese and 35 leans). On the other hand, obesity was statistically more frequent in old animals than in young and adult animals (P=0.0408). Although, the correlation between age or gender with canine obesity is well established, being females and old dogs more susceptible to obesity (Braos et al. 2009, Aptekmann et al. 2014, Debastiani 2018), there are other variables that may increase the risk of a dog being obese.

Forty-seven percent of dogs assessed in the present study were yellow (51/108), 29% black (31/108), and 24% chocolate/liver (26/108). Although chocolate Labrador Retrievers had a significantly shorter lifespan than non-chocolate Labrador Retrievers (McGreevy et al. 2018), in the present study, no relationship was observed between the coat colour and obesity (P=0.8949) or genotype (P=0.0682).

In order to verify the relation between of the deletion *POMC*_P187fs and obesity versus other variables, the DORA questionnaire (Raffan et al. 2015) was applied, with some modifications, to tutors of the dogs of the prospective study. As previously stated in the present study, the presence of the mutated allele was statistically higher (P=0.0423) in the assistance/rescue dogs than pets Labrador Retrievers, nevertheless, the dogs of the assistance/rescue lineage (seven obese and 23 non-obese) were statistically lean (BCS≤5) than

Table 1. Genotype prevalence, allele frequency, and Hardy-Weinberg analysis of the deletion *POMC*_P187fs in Labrador Retrievers dogs

Group	Genotype						Allele frequency	Hardy-Weinberg equilibrium[a]	
	Wild-type		Heterozygous for deletion		Homozygous for deletion				
Total	72%	(78/108)	13%	(14/108)	15%	(16/108)	21.3%	Out	P = 0.0000007
Retrospective	76%	(45/59)	9%	(5/59)	15%	(9/59)	19.5%	Out	P = 0.00000004
Prospective	67%	(33/49)	19%	(9/49)	14%	(7/49)	23.5%	Out	P = 0.00002
Pet	69%	(13/19)	26%	(5/19)	5%	(1/19)	18.4%	In	P = 0.12230
Assistance/rescue	60%	(18/30)	17%	(5/30)	23%	(7/30)	31.7%	Out	P = 0.000000007

[a] Chi-square test, if P<0.05, not consistent with Hardy-Weinberg equilibrium.

the pets (ten obese and nine non-obese) (P=0.0062). Raffan et al. (2016), observed the positive influence of the *POMC* deletion with higher body weight (p<0.0001; mean effect size 1.9kg per deletion allele), and that assistance/rescue dogs were more affected by it. The controversial findings observed in the present study may be explained according to other aspects observed in the assistance/rescue dogs, i.e., these dogs were submitted to a greater frequency of exercise (three or more times a week) than the pets (P=0.0021), and the supply of human food only occurred in the Pet Group (P=0.0002). On the other hand, assistance/rescue dogs, influenced by the genotype (Raffan et al. 2016), were more excited (P=0.0183) and had an incessant interest in the food (P=0.0035); and received more snacks (P=0.0378) than pets. It is known that obesity in dogs is influenced by the interrelationship between food management, exercise and social factors (Bland et al. 2009, Aptekmann et al. 2014, Debastiani 2018). However, the higher frequency of exercise and the single supply of animal feed overrides the food-motivation observed in the Assistance/rescue Group, making them less obese.

CONCLUSIONS

In summary, the present study showed the existence of the deletion *POMC*_P187fs in Labrador Retriever in Brazil.

The Hardy-Weinberg disequilibrium observed in the allelic distribution of the deletion *POMC*_P187fs in this study, independently of the Labrador Retriever group assessed, suggesting the possibility of positive selection of the mutated allele, which may lead to the maintenance of this deleterious allele in this population.

The presence of the mutated allele was significantly more common in Labrador Retrievers selected to become assistance/rescue dogs. However, although the mutation has increased the food-motivation in these group of the Labrador Retrievers, other variables, especially the frequent exercise, favored that these animals maintained the ideal body weight (BCS=5).

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Original Article
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Genotyping of *Malassezia pachydermatis* disclosed genetic variation in isolates from dogs in Colombia[1]

Adriana Marcela Celis-Ramírez[2,3]*, Marcela Guevara-Suarez[2,3], Juan Camilo Galvis-Marín[4], María Ximena Rodríguez-Bocanegra[4], Rubiela Castañeda-Salazar[4], Melva Yomary Linares-Linares[4], Sergio Heli Triana-Sierra[2,3,5] and Adriana del Pilar Pulido-Villamarín[4]*

ABSTRACT.- Celis-Ramírez A.M., Guevara-Suarez M., Galvis-Marín J.C., Rodríguez-Bocanegra M.X., Castañeda-Salazar R., Linares-Linares M.Y., Triana-Sierra S.H. & Pulido-Villamarín A.P. 2019. **Genotyping of *Malassezia pachydermatis* disclosed genetic variation in isolates from dogs in Colombia**. *Pesquisa Veterinária Brasileira 39(11):915-922.* Unidad de Investigaciones Agropecuarias, Departamento de Microbiología, Facultad de Ciencias, Pontificia Universidad Javeriana, Carrera 7 43-82, Ed. 52, Of. 608, Bogotá, Colombia. Departamento de Ciencias Biológicas, Universidad de Los Andes, Carrera 1 18A-10, Bloque J, Of. J-214, Bogotá, Colombia. E-mails: adriana.pulido@javeriana.edu.co, acelis@uniandes.edu.co

Malassezia pachydermatis is a lipophilic and lipid-dependent yeast mostly isolated from animals' skin; hence, it is regarded as a zoophilic species causing otitis externa in dogs. Aspects associated with its epidemiology and pathogenicity is a matter of interest. This study aimed to conduct a molecular characterization of 43 isolates of *M. pachydermatis* obtained from dogs with otitis externa. For this purpose, the 5.8S internal transcribed spacer 2 (ITS2) and D1/D2 26S rRNA regions were amplified, sequenced and analyzed using restriction fragment length polymorphism (RFLP) with *Alu*I, *Cfo*I, and *BstF5*I endonucleases. Phylogenetic analyses revealed that these isolates grouped with the sequence types I, IV and V, previously proposed for *M. pachydermatis*. Interestingly, we found a new polymorphic RFLP pattern using *BstF5*I, these isolates were associated with the sequence types IV and V, nevertheless an association between polymorphic RFLP patterns, and fosfolipase activity or canine population data was not observed. These findings underline the genetic diversity of *M. pachydermatis* and provide new insights about the epidemiology of this species in the analyzed population.

INDEX TERMS: Genotype, *Malassezia pachydermatis*, genetic variation, isolates, dogs, Colombia, gene, otitis, PCR-RFLP, sequence types.

RESUMO.- [**Genotipagem de *Malassezia pachydermatis* revelou variação genética em isolados de cães na Colômbia**]. *Malassezia pachydermatis* é uma levedura lipofílica e dependente de lipídios, principalmente da pele de animais. Sendo, por essa razão, considerada uma espécie zoofílica e causadora de otite externa em cães. Neste sentido, aspectos associados à sua epidemiologia e patogenicidade constituem um tema de interesse científico. O objetivo deste estudo foi realizar a caracterização molecular de 43 isolados de *M. pachydermatis* obtidos a partir de cães com otite externa. Para esta propósito, foram amplificadas, sequenciadas e analisadas com enzimas de restrição as regiões do gene 5.8S, do espaçador interno transcrito 2 (ITS2) e D1/D2 do 26S do rRNA pelo método RFLP, com as endonucleases *Alu*I, *Cf*OI e *BstF5*I. Análises filogenéticas revelaram que os isolados se agruparam com as sequências tipo I, IV e V de *M. pachydermatis* como já descrito anteriormente. De maneira interessante, se observou um novo RFLP polimórfico utilizando *BstF5*I. Os isolados que mostraram esse padrão foram associados com os padrões IV e V. No entanto, não foi observada associação entre padrões

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[2] Grupo de Investigación Celular y Molecular de Microorganismos Patógenos (CeMoP), Departamento de Ciencias Biológicas, Universidad de Los Andes, Carrera 1 18A-10, Bloque M, Of. M-201, Bogotá, Colombia.

[3] Laboratorio de Micología y Fitopatología (LAMFU), Universidad de Los Andes, Carrera 1 18A-10, Bloque J, Of. J-214, Bogotá, Colombia. *Corresponding author: acelis@uniandes.edu.co

[4] Unidad de Investigaciones Agropecuarias (UNIDIA), Departamento de Microbiología, Facultad de Ciencias, Pontificia Universidad Javeriana, Carrera 7 43-82, Ed. 52, Of. 608, Bogotá, Colombia. *Corresponding author: adriana.pulido@javeriana.edu.co

[5] Structural and Computational Biology Unit, European Molecular Biology Laboratory, Meyerhofstraße 1, 69117, Heidelberg, Germany.

polimórficos de RFLP e atividade de fosfolipase ou dados da população canina. Estes resultados demonstram a diversidade genética de *M. pachydermatis* e fornecem novas perspectivas sobre a epidemiologia destas espécies na população analisada.

TERMOS DE INDEXAÇÃO: Genotipagem, *Malassezia pachydermatis*, variação genética, isolados, cães, Colômbia, genes, otite, PCR-RFLP, tipos de sequência.

INTRODUCTION

Malassezia genus comprises 18 species (Triana et al. 2015, Wu et al. 2015, Cabañes et al. 2016, Honnavar et al. 2016, Lorch et al. 2018), which are considered as the dominant species within animal and human skin mycobiota, characterized by being lipophilic and lipid dependent. Nevertheless, these commensal yeasts have also been related to several dermatological diseases (Sugita et al. 2010, Harada et al. 2015, Wu et al. 2015, Prohic et al. 2016).

Malassezia pachydermatis is one of the most common species within this genus, which is commonly isolated from mammals and birds. In dogs, it is frequently found in the external ear canal; moreover, this species may cause otitis externa and dermatitis (chronic, localized or generalized, external lesions) (Crespo et al. 2002, Bond et al. 2010, Pulido et al. 2010, Pulido-Villamarín et al. 2015). Furthermore, *M. pachydermatis* has been considered to be potentially zoonotic, mainly associated to fungaemia (Gueho et al. 1987, Morris et al. 2005, Bond et al. 2010, Seyedmousavi et al. 2015). Given the fact that a possible association of genotypes with specific hosts has been discerned, it can be suggested that *M. pachydermatis* has adapted to their ecological niche and could be related to the establishment of the disease (Puig et al. 2016). In addition, several studies have linked the pathogenicity of this species with phospholipase activity, biofilm production and immune response (Cafarchia et al. 2008, Buommino et al. 2016, Hurtado-Suárez et al. 2016).

Several studies have reported the isolation of *M. pachydermatis* from external ear canal samples of both ill and healthy canines (Bond et al. 1995, Duarte et al. 2001, Masuda et al. 2000, Castellá et al. 2005, Girão et al. 2006, Brito et al. 2007, Bond et al. 2010). In South America, only some reports regarding this matter has been conducted. In Brazil studies including canines and other domestic and wild animals reported the prevalence of *M. pachydermatis;* however, the identification of these isolates were mainly achieved using conventional methods (Duarte et al. 2001, Girão et al. 2006, Brito et al. 2007). Nevertheless, molecular biology techniques, such as restriction fragment length polymorphism (RFLP) and nucleic acid sequencing have become common place procedures. Molecular methods for species identification employ ribosomal DNA genes (rDNA) as diagnostic markers, including the 26S (D1D2-Fw/D1D2) and 5.8S (ITS3/ITS4) subunits (Mirhendi et al. 2005, Gaitanis et al. 2006, Ribeiro do Prado et al. 2007, Cafarchia et al. 2011a, Kim et al. 2015), this genotyping has allowed to identify high intra-species variability in *M. pachydermatis* with the presence of several sequence types (Guillot et al. 1997, Cafarchia et al. 2007, 2008, 2011b, Puig et al. 2016). These assessments have improved the characterization of these yeast populations to the better knowledge of their epidemiology.

PCR-RFLP is one of the most reliable techniques, usually trough the restriction enzyme digestion of LSU rDNA and ITS regions (Boekhout et al. 1998, Aizawa et al. 1999, Theelen et al. 2001, Gaitanis et al.2002, Celis & Cepero de García 2005, Mirhendi et al. 2005, González et al. 2009, Sugita et al. 2010, Amado et al. 2013). Nevertheless, other methods such as multi-locus phylogenetic analyses have evinced to be a useful tool to distinguish closely related species. Recently, these analyses showed that *M. pachydermatis* has a high intra-species variability due to the presence of several sequence types, at least five (I-V) for LSU and eleven (I-XI) for ITS (Puig et al. 2016). Moreover, the combination of LSU, ITS, CHS2 and β-tubulin gene sequences revealed the presence of fifteen genotypes clustered in two different clades; one clade mainly comprises isolates from dogs, while the other included isolates from other domestic animals such as cats (Puig et al. 2016).

To date, molecular characterization of *M. pachydermatis* isolates from animals in Colombia has not been reported. Therefore, the aim of the present study was to characterize at molecular level a set of isolates of this species obtained from dogs with otitis externa. This study can provide new insights on the genotypes of *M. pachydermatis* present in canine otitis.

MATERIALS AND METHODS

***Malassezia* isolates and growth conditions.** A total of 43 isolates of *M. pachydermatis* from dogs with clinical record of otitis externa from a previous study (Pulido et al. 2010) were included. The isolates were phenotypically characterized, including the evaluation of phospholipase activity (Pulido-Villamarín et al. 2015), preserved in 10% skin milk at -70 °C according to the methods for preservation and storage of *Malassezia* species (Crespo et al. 2000), and deposited at the microorganism collection from the "Unidad de Investigaciones Agropecuarias" (UNIDIA, Unit of Agricultural Research) of the "Pontificia Universidad Javeriana". In addition, *Malassezia* strains: *M. furfur* CBS 7019, *M. pachydermatis* CBS 1879, *M. sympodialis* CBS 7222 and *M. slooffiae* CBS 7956, obtained from the Fungal Biodiversity Center (Westerdijk Institute, Utrecht, The Netherlands), were used as phenotypic and genotypic controls. All strains were recovered using modified Dixon agar (mDixon agar; 36g L^{-1} Mycosel agar - BD, Franklin Lakes/NJ, USA -, 20g L^{-1} Ox bile, 36g L^{-1} malt extract - Oxoid, Basingstoke, UK -, 2mL L^{-1} glycerol, 2mL L^{-1} oleic acid, and 10 mL L^{-1} Tween 40), incubating for 4 to 5 days at 33°C (Mirhendi et al. 2005).

Molecular characterization. Total DNA was extracted using the fungal and yeast genomic DNA kit (Norgen Biotek, Thorold, ON, Canada), according to the manufacturer's instructions, and then treated with RNase (Sigma, St Louis/MO, USA) at a final concentration of 2mg/mL per sample at 37°C for 4 hours. The DNA samples were stored at -20°C until further use.

Two nuclear DNA targets were amplified by PCR and sequenced using primers previously reported (González et al. 2009): ITS3/ITS4 for the 5.8s rDNA-ITS2 region, and D1D2-Fw/D1D2 for 26S rDNA region (Table 1). Polymerase chain reaction amplification was carried out in a final volume of 50µL, where each reaction contained 45µL

Table 1. Forward and reverse primer sequences employed to amplify regions 5.8S rDNA-ITS2 and 26S rDNA

	Primer	Forward/reverse
5.8S rDNA-ITS2 region	ITS3	5'-GCATCGATGAAGAACGCAGC-3'
	ITS4	5'-TCCTCCGCTTATTGATATGC-3'
26S rDNA region (D1D2)	D1D2-Fw	5'-TAACAAGGATTCCCCTAGTA-3'
	D1D2-Rv	5'-ATTACGCCAGCATCCTAAG-3'

Reference: Mirhendi et al. (2005).

Table 2. Origin of *the Malassezia pachydermatis* isolates included in the present study

Sequence type	Isolate no.	Breed	GenBank accession numbers	
			D1/D2	ITS
I	11	Labrador	KU757185	LT962403
I	27	Labrador	KU757188	LT962406
I	35	Labrador	KU757189	LT962407
I	43	Labrador	KU757194	LT962411
I	58	Labrador	KU757199	LT962415
I	80	Labrador	KU757203	-
I	100	Labrador	KU757207	LT962422
I	103	Labrador	KU757209	LT962424
I	104	Labrador	KU757210	LT962425
I	109	Labrador	KU757212	LT962427
I	128	Labrador	KU757220	LT962432
I	129	Labrador	KU757221	LT962433
I	131	Labrador	KU757222	LT962434
I	134	Labrador	KU757223	LT962435
I	135	Labrador	KU757224	LT962436
I	141	Labrador	KU757226	LT962438
I	149	Labrador	KU757231	LT962440
I	153	Labrador	KU757232	LT962441
IV	18	Labrador	KU757187	LT962405
IV	40	Labrador	KU757193	LT962410
IV	65	Labrador	KU757201	LT962417
IV	70	Golden Retriever	KU757202	LT962418
IV	83	Labrador	KU757205	LT962420
IV	111	Golden Retriever	KU757213	LT962428
IV	115	Stray dog	KU757214	LT962429
V	15	Labrador	KU757186	LT962404
V	36	Beagle	KU757190	LT962408
V	38	Golden Retriever	KU757191	LT962409
V	39	Golden Retriever	KU757192	-
V	47	Stray dog	KU757195	LT962412
V	54	Golden Retriever	KU757197	-
V	55	Beagle	KU757198	LT962414
V	59	Golden Retriever	KU757200	LT962416
V	81	Labrador	KU757204	LT962419
V	88	Golden Retriever	KU757206	LT962421
V	101	Stray dog	KU757208	LT962423
V	107	French Poodle	KU757211	LT962426
V	116	Labrador	KU757215	LT962430
V	125	French Poodle	KU757219	LT962431
V	140	Labrador	KU757225	LT962437
V	144	Golden Retriever	KU757228	LT962439
V	156	Beagle	KU757233	LT962442
V	157	Beagle	KU757234	LT962443

PCR SuperMix (1.1X) (Thermo Scientific, St Peters/MO, USA), 1µL of each primer (10pmol/µL), and 3µL of genomic DNA (20ng/mL). A first analysis with 26S rDNA and ITS sequences using the nucleotide BLAST tool[6] was carried out to confirm the preliminary phenotypic identification. The amplification protocol consisted of an initial denaturation step for 5min at 95°C and 30 cycles consisting of 95°C for 1min, 55°C for 1 min, and 72°C for 1min, followed by a final 5min extension step at 72°C.

The restriction enzyme digestion for the amplicon of 5.8S region was done with *Alu*I (Thermo Scientific, St Peters/MO, USA) and for 26S region with *Cfo*I (Promega, Madison/WI, USA) and *BstF5*I (Thermo Scientific, St Peters/MO, USA), enzyme reactions were performed as previously reported (Mirhendi et al. 2005). The products were visualized in 1.8% (p/v) agarose gel electrophoresis using a Gel Doc System (Bio-Rad, Hercules/CA, USA).

Single-band PCR products were purified and sequenced using an ABI3730xl DNA analyzer (Applied Biosystems, Foster City/CA, USA). Consensus sequences were assembled using Geneious software v.4.8.5[7]. DNA sequences determined in this study have been deposited in GenBank, the accession numbers are reported in Table 2.

Phylogenetic reconstructions. In order to assess the intraspecific variability of our isolates, we performed phylogenetic analyses using

[6] Basic Local Alignment Search Tool. Available at <https://blast.ncbi.nlm.nih.gov/Blast.cgi>

[7] Geneious Bioinformatics Software for Sequence Data Analysis. Available at <http://www.geneious.com>

the sequences data of D1/D2 and ITS and complemented them with sequence types reported for *M. pachydermatis* retrieved from GenBank database (Puig et al. 2016). Additionally, sequences of type strains of *Malassezia* species were included. For phylogenetic analyses, sequences of the individual loci were aligned using ClustalW algorithm (Thompson et al. 1994) in MEGA v. 7.0 (Kumar et al. 2016), refined with MUSCLE (Edgar 2004), and manually adjusted using the same software platform. Phylogenetic reconstructions were made with the individual loci using maximum-likelihood (ML) in MEGA v. 7.0 and Bayesian inference (BI) under MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001). The best substitution model for all matrices was estimated using JModelTest v. 2.1 (Darriba et al. 2012). For ML analyses, nearest-neighbor interchange was used as the heuristic method for tree inference. Support for internal branches was assessed by 1,000 ML bootstrapped pseudoreplicates of data. The phylogenetic reconstruction by BI was performed using 1,000,000 Markov chain Monte Carlo (MCMC) generations, with two runs (one cold chain and three heated chains), and samples were stored every 1,000 generations. The 50% majority-rule consensus tree and posterior probability (pp) values were calculated after discarding the first 25% of the samples as burn-in. The resulting trees were plotted using FigTree v. 1.4.2[8], and edited in Adobe Illustrator CS6. Bootstrap support (bs) and pp values were labelled on nodes of the three. Values of ≥70bs and less than 0.95pp were considered significant.

***In silico* restriction.** To confirm the restriction pattern for all the sequences obtained from 26S rDNA region with the enzyme *BstF5*I, *in silico* digestions were performed using the platform Webcutter 2.0[9].

RESULTS

For all isolates amplification of the 5.8S rDNA-ITS2 region resulted in a band of ~500bp, and a slightly larger band, ~550bp was obtained for the 26S rDNA region. An analysis with 26S rDNA and ITS sequences using the nucleotide BLAST tool allows us to confirm that all canine isolates were *Malassezia pachydermatis.* The restriction analysis obtained by PCR-RFLP agreed with what is reported for the identification of *M. pachydermatis,* using the 5.8S rDNA-ITS2 region with *Alu*I, and for 26S rDNA region restriction analysis using *Cfo*I. However, the digestion of 26S rDNA region with *BstF5*I revealed two different restriction patterns. The first pattern was found in 18 out of 43 isolates (Fig.1, lanes 2-4) as well as in the reference strain *M. pachydermatis* CBS 1879, this pattern consisted of two bands of ~450 and 60bp; the second pattern was observed in the remaining isolates (n=25), this consisted of four bands ~450, 300, 180, and 60bp (Fig.1, lanes 5-11). To confirm the new restriction pattern for *BstF5*I, we performed an *in silico* restriction, where we observed the results matched with an additional cutting site within the alignment of the 26S rDNA region.

Phylogenetic analyses performed to identify sequence types of *M. pachydermatis* based on 26S rDNA are shown in Figure 2. The final alignment with this region was 496bp long, and the model selected was Kimura 2-parameter (K2) with gamma-distributed rates (G). This analysis showed that all canine isolates formed a major clade (97% bs/1pp) with ex-type strain of *M. pachydermatis*. Additionally, these isolates

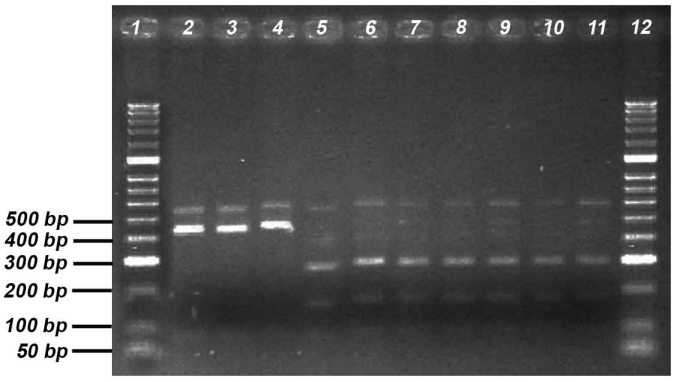


Fig.1. *Malassezia pachydermatis* 26S rDNA region amplicon digestion with *BstF5*I. Molecular-weight size marker (lane 1), *M. pachydermatis* CBS 1879 (lane 2), 26S rDNA region two pattern band (~450, 60bp) with *BstF5*I (lane 3-4), 26S rDNA region four pattern band (~450, 300, 180, 60bp) with *BstF5*I (lane 5-11), molecular-weight size marker (lane 12).

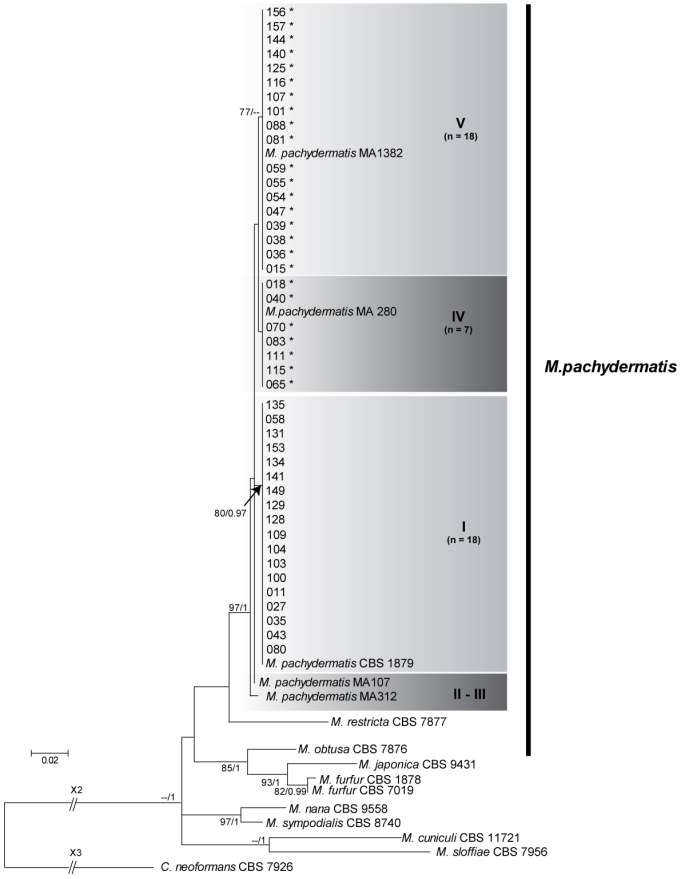


Fig.2. Phylogenetic diversity of isolates of *Malassezia pachydermatis* obtained from dogs in this study. Phylogenetic tree using maximum-likelihood (ML) and Bayesian inference (BI), tree inferred from 26S rDNA sequence data. Support values are above branches, and represent bootstrap values >70% for ML/posterior probabilities >0.95 for BI. The tree is rooted to *Cryptococcus neoformans* CBS 7926. Isolates with the four-banded pattern after digestion with *BstF5I* (*). The sequences types proposed by Puig et al. (2016) for 26S rDNA region are shown in dark boxes.

[8] Figtree Software, Molecular Evolution, Phylogenetics and Epidemiology. Available at <http://tree.bio.ed.ac.uk/software/figtree>

[9] WebCutter 2.0, A Restriction Mapping Tool. Available at <http://heimanlab.com/cut2.html>

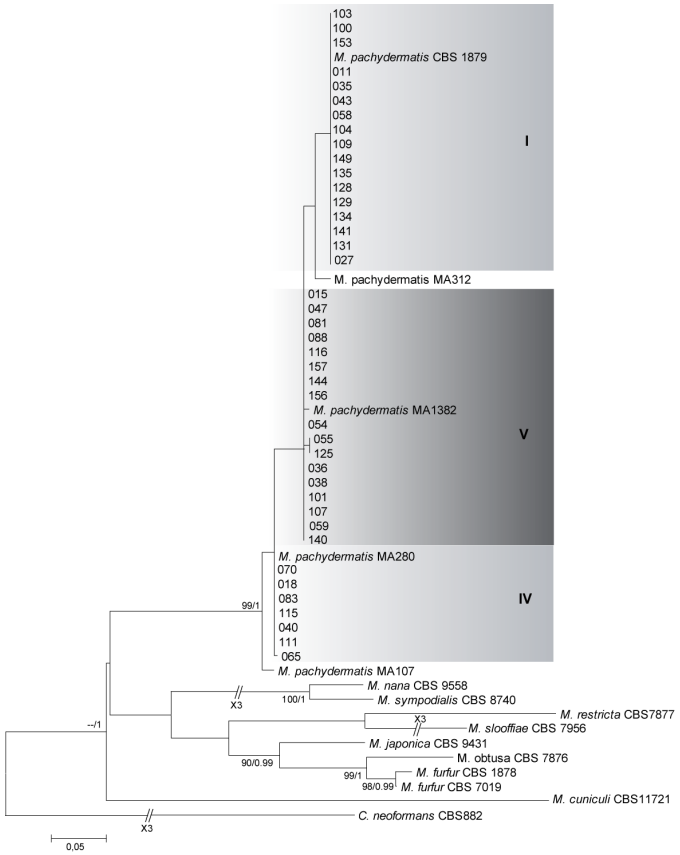


Fig.3. Phylogenetic diversity of isolates of *Malassezia pachydermatis* obtained from dogs in this study. Phylogenetic tree using maximum-likelihood (ML) and Bayesian inference (BI), tree inferred from 5.8S rDNA-ITS2 region. Support values are above branches, and represent bootstrap values >70% for ML/posterior probabilities >0.95 for BI. The tree is rooted to *Cryptococcus neoformans* CBS 882. The sequence types proposed by Puig et al. (2016) are shown in dark boxes.

were distributed among three sequence types, I (n=18), IV (n=7) and V (n=18), which have been previously reported. The analysis of the ITS sequences (Fig.3) showed similar topology to phylogenetic tree of 26S rDNA region. However, not all clades had a robust phylogenetic support.

Interestingly, we observed that two-banded *BstF5*I restriction pattern isolates grouped with the sequence type I, while the isolates with the four-banded pattern grouped with the sequence types IV and V (Fig.2). We did not find any association between the *BstF5*I restriction patterns for 26S rDNA and the phospholipase activity data (previously determined) nor with the breed, age, gender or ear type of the dogs (Pulido et al. 2010, Pulido-Villamarín et al. 2015, Hurtado-Suárez et al. 2016).

DISCUSSION

This is the first epidemiological study aimed to genotype *Malassezia pachydermatis* isolates causing canine otitis in Colombia. *M. pachydermatis* is an important colonizer of healthy canine skin and mucosae; however, it can cause otitis externa and dermatitis (Crespo et al. 2002, Ribeiro do Prado et al. 2007, Cafarchia et al. 2008, Pulido et al. 2010). In humans, this species has been reported in bloodstream infections, possibly caused by zoonotic transmission (Gueho et al. 1987, Morris et al. 2005, Seyedmousavi et al. 2015). The interest in the study of *M. pachydermatis* has increased in the last decades, given the better understanding of aspects regarding its epidemiology and ecology, as well as the unraveling aspects of the pathogenesis and its role in the developing of the diseases. Nevertheless, many aspects are still unknown.

Several molecular typing methods have been previously reported to resolve the species identification discordances between conventional methods, such as morphological and biochemical methods, commonly used for this purpose (Sugita et al. 2010). These advances have been crucial to progress in the diagnostic field. In our study, average size of the amplified 5.8S rDNA-ITS2 region fragment was similar to that reported by Gaitanis et al. (2002), who obtained an average amplicon size of 483bp for *M. pachydermatis* isolates from dogs lesions diagnosed with otitis. For the 26S rDNA region fragment, our findings agreed with those of Gupta et al. (2004) who reported an average amplicon size of approximately 550bp.

PCR-RFLP is a reliable tool to perform an undemanding identification of *Malassezia* species, having been widely reported in epidemiological studies in human skin diseases (Gupta 2004, Lee et al. 2006, Jang et al. 2009, Sugita et al. 2010, Sosa et al. 2013, Soares et al. 2015). Through the current study, we were able to identify *M. pachydermatis* obtained from otitis externa in dogs. The results obtained in our study for the analysis of the restriction patterns of 5.8S rDNA-ITS2 and 26S rDNA regions with *Alu*I and *Cfo*I, concurred with the previous reports for *M. pachydermatis* (Mirhendi et al. 2005, González et al. 2009).

The digestion of 26S rDNA fragment with *BstF5*I revealed a dimorphic banding pattern. In previous studies, the only reported banding pattern obtained by *BstF5*I restriction on *M. pachydermatis* isolates consisted of two bands of 500 and 70bp (Mirhendi et al. 2005, González et al. 2009). Remarkably, our results showed a new restriction pattern for the digestion of 26S rDNA region with *BstF5I,* this new pattern consisting of four bands was found in most of the isolates included in this study. The *in silico* restriction analysis corroborates this finding giving new insights on this species epidemiology in our country. This could be attributed to a genetic variability in the 26S rDNA region for this species and others in this genus, as it has been proposed by several researchers using diverse molecular tools (Aizawa et al. 1999, Cabañes et al. 2005, Cafarchia et al. 2007, 2011a, Duarte & Hamdan 2010, Wu et al. 2015, Puig et al. 2016). These differences highlight the importance of performing molecular typification for achieving a better identification, and broadening the epidemiological knowledge in animal health (Gupta 2004).

Additionally, different studies have revealed the presence of a high genetic variability into this species with the presence of different genotypes/subgenotypes that can be isolated from domestic and wild animals (Guillot et al. 1997, Cafarchia et al. 2007, 2008, 2011a, Gandra et al. 2008, Álvarez-Pérez et al. 2015, Puig et al. 2016). Here we were able to identify the presence of at least three sequence types (I, IV, V) for *M. pachydermatis* in D1/D2 and ITS regions. Particularly, the two restriction patterns found for 26S rDNA region with *BstF5*I were grouped with different sequence types, suggesting a high diversity of this species. These genotypes can be detected in other

animals such as cats and wild mammals, hence, is important to perform additional analysis including a wide number of isolates from this sources to understand this diversity.

Many other aspects should be explore to have better comprehension about the relation between the presence of this genotypes and a possible connection with the establishment of the disease, such as the relation with bacterial microbiota, pH and fatty acids composition, aspects that have been linked to the adaptation of this species to some anatomical sites of the host due to the differences found in the lipid metabolism of this yeast (Cafarchia et al. 2008, Buommino et al. 2016, Puig et al. 2016, Triana et al. 2017).

CONCLUSIONS

The results revealed the presence of different genotypic variants of *Malassezia pachydermatis* in Colombia.

The existence of different genotypes could be a tool to accurate the clinical management of the species, as well as to understand the ecology and epidemiology of *M. pachydermatis* and contribute with this data at local and global level.

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Ultrasonographic features of the normal thyroid gland in adult horses[1]

Gustavo F. Viana[2], Luciana Carandina[2], Alessandre Hataka[3], Monica Midon[4], Camila A. Sarkis[2], José N.P. Puoli Filho[5] and Vânia M.V. Machado[2]*

ABSTRACT.- Viana G.F., Carandina L., Hataka A., Midon M., Sarkis C.A., Puoli Filho J.N.P. & Machado V.M.V. 2019. **Ultrasonographic features of the normal thyroid gland in adult horses.** *Pesquisa Veterinária Brasileira 39(11):923-931.* Departamento de Reprodução Animal e Radiologia Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rua Prof. Dr. Walter Mauricio Correra s/n, Rubião Junior, Botucatu, SP 18618-970, Brazil. E-mail: vaniamvm@fmvz.unesp.br

Few reports have been published regarding the use of ultrasonography as a method of evaluating the normal thyroid gland in horses. For these reasons, this study aimed at determining reliably of the thyroid measurements from the comparison between the left and right thyroid lobes, as well as assessing the contour, format, echotexture and echogenicity of the healthy thyroid by mode-B ultrasonography. Additionally, the equine thyroid vascularization was quali-quantitatively characterized the by Doppler. The sample size initially was determined by the animal selection with advanced age and without volume increase in the neck proximal region. Finally, eleven horses were selected by laboratory test, search of thyroid neoformations by ultrasonography and cytology thyroid. Next, these animals were submitted to thyroid lobes ultrasonographic evaluation. Excellent reproducibility was observed for all measurements obtained. Among the comparisons made between the quantitative parameters of the left and right lobes, it was observed that there was only difference between their respective lengths. Qualitatively, there was a significant variation between the lobes elliptical format in the longitudinal plane of some glands, which oscillated between a rounded and flattened conformation. Thus, we can conclude that the difference between the lobes format of some thyroids can be explained by the significant difference observed between the length of the left and right lobes. Additionally, it was verified that there was no difference between the Doppler quantitative parameters. Therefore, we may suggest that unilateral analysis of the cranial thyroid artery by spectral Doppler can be used to evaluate equine thyroid diffuse disorders.

INDEX TERMS: Ultrasonography, thyroid gland, adult horses, thyroid lobes, ultrasound, equines, horses, morphology.

RESUMO.- [**Características ultrassonográficas da glândula tireoide hígida em equinos adultos.**] Poucas análises foram publicadas com relação ao uso da ultrassonografia como método de avaliação da glândula tireoide hígida em equinos. Alguns trabalhos incluem a determinação das dimensões e características do parênquima. Por essas razões, este estudo teve como objetivo determinar com segurança as medidas tireoidianas a partir da comparação entre o lobo tireoidiano esquerdo e direito, bem como avaliar os contornos, formato, ecotextura e ecogenicidade das glândulas tireoides hígidas pela ultrassonografia em modo B. Além disso, a vascularização da tireoide equina foi caracterizada quali-quantitativamente pela ferramenta Doppler. O tamanho da amostra inicialmente foi determinado pela seleção dos animais com idade avançada e sem aumento de volume na região proximal do pescoço. Por fim, onze equinos foram selecionados por meio de exame laboratorial, pesquisa de neoformações tireoidianas por ultrassonografia e citologia da tireoide. Em seguida, estes animais foram submetidos à avaliação de lobos tireoidianos

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[2] Departamento de Reprodução Animal e Radiologia Veterinária, Faculdade de Medicina Veterinária e Zootecnia (FMVZ), Universidade Estadual Paulista "Júlio de Mesquita Filho" (Unesp), Rua Prof. Dr. Walter Mauricio Correra s/n, Rubião Junior, Botucatu, SP 18618-970, Brazil. *Corresponding author: vaniamvm@fmvz.unesp.br

[3] Departamento de Clínica Veterinária, FMVZ, Unesp, Rua Prof. Dr. Walter Mauricio Correra s/n, Rubião Junior, Botucatu, SP 18618-970.

[4] Departamento de Cirurgia e Anestesiologia, FMVZ, Unesp, Rua Prof. Dr. Walter Mauricio Correra s/n, Rubião Junior, Botucatu, SP 18618-970.

[5] Departamento de Produção Animal, FMVZ, Unesp, Rua Prof. Dr. Walter Mauricio Correra s/n, Rubião Junior, Botucatu, SP 18618-970.

por ultrassonografia. Observou-se excelente reprodutibilidade em relação a todas as medidas deste estudo. Foram observadas diferenças qualitativas entre os lobos tireoidianos direito e esquerdo. Assim, podemos concluir que tais diferenças entre o formato dos lobos tireoidianos podem ser explicadas pela diferença significativa e/ou tendência observada no parâmetro comprimento entre os respectivos lobos. Além disso, não foi encontrada diferença significativa entre os valores da artéria tireoidiana cranial esquerda em relação aos obtidos na artéria tireoidiana cranial direita, sugerindo que a análise dos valores espectrais do Doppler de uma das artérias tireoidianas craniais possa ser utilizada como método de avaliação de distúrbios difusos da tireoide equina.

TERMOS DE INDEXAÇÃO: Ultrassonografia, glândula tireoide, equinos adultos, lobos tireoidianos, ultrassom, cavalos, morfologia.

INTRODUCTION

The horse thyroid gland (*Glandula thyreoidea*), located between the third and the sixth tracheal ring, consists of two lobes immediately caudal to the larynx, usually joined by a narrow fibrous isthmus. It is not uncommon to observe a developed isthmus, exerting glandular activity on the adult horse. The thyroid is highly vascularized, receiving blood from the cranial thyroid artery, the largest branch of the common carotid artery (Venzke 1975, Budras et al. 2008).

Neoplasia is the most common finding among thyroid gland changes in horses. It is usually unilateral, derived from adenomas of parafollicular cells (C cells) and occurs mainly in aged horses (Ueki et al. 2004). Malignant neoplasias of the thyroid are less frequent and, in some cases, can be responsible for triggering hypo- or hyperthyroidism (Hillidge et al. 1992, Held et al. 1985, Ramirez et al. 1998).

Few reports have been published regarding the use of ultrasonography as evaluation methodology of normal thyroid gland in horses. Some studies include the determination of the dimensions and parenchymal characteristics (Rantanen 1998, Reef 1998, Davies et al. 2010). Despite the lack of thyroid gland normality parameters by ultrasonography, some reports described the use of ultrasound in the characterization of thyroid neoformations in horses (Elce et al. 2003, Tan et al. 2008, Saulez et al. 2013). According to Troillet et al. (2016), the ultrasonographic exam was useful for evaluation of size, relation with adjacent anatomical structures and vascularization of neoplastic thyroids submitted to thyroidectomy. In human medicine, B-mode ultrasonography associated with Doppler method has been used in order to perform a more accurate evaluation of nodular and diffused thyroid diseases (Turgut et al. 2009). This technic can provide more than 90% of diagnosis accuracy in thyroid carcinoma (Fukunari 2002). However, in veterinary medicine, there are few studies regarding the use of Doppler tool as a thyroid evaluation method. These research are restricted to the use of power Doppler function for the purpose of mainly assess the vascularization in some case reports of equine (Tan et al. 2008, Saulez et al. 2013) and canine (Taeymans 2009) thyroid neoplasms.

Therefore, a broader knowledge of the normal thyroid gland ultrasonographic features becomes necessary. For these reasons, this study aimed at (1) determining reliably of the thyroid measurements from the comparison between the left and right thyroid lobes, as well as (2) assessing the contours, formats, echotexture and echogenicity of the healthy thyroid lobes by mode-B ultrasonography; and (3) quali-quantitatively characterizing the equine thyroid gland vascularization by Doppler tool.

MATERIALS AND METHODS

Ethics statement. This study was carried out from August 2014 to June 2015, and it was approved and performed in accordance with the Ethics Committee of the "Universidade Estadual Paulista" (protocol number: 165/2013-CEUA).

Animal selection. The sample size was initially determined by the selection of animals over the age of ten (risk factor for thyropathies) and clinically certified as without apparent volume increase in the ventrolateral proximal region adjacent to the larynx. Finally, 11 horses (seven males and four females) with healthy thyroid were selected by laboratory test for evaluating the thyroid function. These animals also were submitted to search for neoformation in the thyroid parenchyma by ultrasonography. The mean age of the horses used in this study was 16 (±2.49) years and the mean weight was 403.45 (±36.23) kg.

Laboratory exam and search of thyroid neoformations by ultrasonography. Blood samples were sent to a specialized laboratory for the diagnosis of veterinary endocrine disorders (B.E.T. Laboratories, Rio de Janeiro, Brazil) for the determination of the free and biologically active fractions of thyroxine (free T4) by the method of radioimmunoassay (equilibrium dialysis).

A portable ultrasound apparatus (MyLab™ 30VETGold; Esaote, Genova, Italy) with an 18MHz multi-frequency linear transducer was used to investigate the neoformations presence in the thyroid parenchyma.

Ultrasound-guided fine needle aspiration cytology (FNAC). The cytological exam was performed after the ultrasonography studies. This permitted a more reliable evaluation of the thyroid parenchyma without any influence from acute and/or chronic inflammatory alterations originated by the perforation of the needle in the evaluated glands.

The horses were sedated through the intramuscular and intravenous administration of acepromazine (0.05mg/kg) and xylazine hydrochloride (0.5mg/kg), respectively.

A portable ultrasound apparatus (the same one used for searching for thyroid nodular formations by ultrasound) was used. A transversal image of the examined lobe was generated. The following materials were used in this procedure: disposable hypodermic needles (50x7mm), 10-ml disposable syringes and a Valeri aspirator. The needle was introduced at a 45-degree angle on the skin and was directed toward the center of the thyroid lobe. During the trajectory of the needle, the cervical portion of the parotid salivary gland and the sternocephalic muscle were surpassed so the gland could be reached.

Ultrasonography. The ultrasound apparatus was the same as the one used for the search of thyroid neoformations. The examinations were performed by a master veterinary diagnostic imaging. These studies were supervised by a Veterinary Radiology Associate Professor at "Universidade Estadual Paulista", with 20 years of experience in equine ultrasonography.

B-Mode study. The B-mode analysis for thyroid was performed with the following settings: gain, 94-100%; frequency, 12-18MHz; depth, 3-4cm. Only one focus was utilized, and it was regulated up to the medial margin of the gland. Initially, the transducer was positioned perpendicularly to the larynx, slipped to the proximal portion of the trachea and then displaced laterally to the left and right until obtaining the transverse plane of the thyroid lobes. Starting

from the acquired prior image, the longitudinal plane was obtained by rotating the transducer 90°.

Mensuraments. The dimensions were acquired on the ultrasound apparatus itself. The width and height were determined in the transverse plane of the thyroid lobe with 18MHz of frequency. In the longitudinal plane, the length was obtained by total visualization of both poles (cranial and caudal), considering the greatest dimension of the lobe. The transducer was slipped 90° from the initial transverse position to get these images. Slight pressure and angulations were necessary for the total visualization of the cranial and caudal poles. For this evaluation, a frequency from 12 to 18MHz was utilized, varying according to the general dimensions of the thyroid lobe (Fig.1).

The determination of the volume of the left thyroid lobe (VLTL) and right TL (VRTL) was estimated by the equation: length × width × height × π/6. The total glandular volume corresponded to the sum of the two lobes in the same animal ($VLTLcm^3$ + $VRTLcm^3$). This formula, designed to obtain the volume of elliptical forms, has been employed in human and veterinary medicine to calculate thyroid volume (Brown & Spencer 1978, Wisner & Nyland 1998).

Format, contours echotexture and echogenicity. These parameters were evaluated qualitatively. For format, the approximate geometric form of the thyroid lobes was described in each image plane with its respective variations. In the longitudinal plane, the format was classified as flat and rounded. For the transverse plane, they were classified as spherical and elliptical.

The contours of the thyroid gland were evaluated according to the definition (clearly or poorly defined) and according to the regularity (regular or irregular) of their lateral and medial portions.

The images generated were used to evaluate parenchymal characteristics, such as the echogenicity and echotexture of the thyroid lobes.

Doppler study. The cranial thyroid artery (*Arteria thyreoidea cranialis*) was selected for the study of the thyroid lobe arterial vascularization due to its smaller anatomical variation, greater caliber and lower degree of tortuosity when compared with the branches near the thyroid lobe cranial pole and the caudal thyroid artery (*Arteria thyreoidea caudalis*). Additionally, the cranial thyroid artery was suitable for application of the angle of insonation (less than or equal to 60°).

The cranial thyroid artery was accessed in the longitudinal plane by displacing the transducer from the thyroid cranial pole to the medial aspect of the mandibular angle (*Angulus mandibulae*). Spectral Doppler was triggered after the precise identification of the cranial thyroid artery through the color Doppler in middle third of the vessel, at the thyroid lobe level.

For the color Doppler analysis, the apparatus was calibrated with a gain of 68-78% and a pulse repetition frequency (PRF) of 1.0-1.4KHz. For the assessment by spectral Doppler, the apparatus was calibrated with a PRF of 2.8-4.2KHz and with a wall filter of 50Hz. The size of the sample volume was regulated according to the diameter of the cranial thyroid artery. The frequencies for color and spectral Doppler analyses ranged from 6.6 to 8.0MHz.

From the Doppler protocols mentioned, quantitative and qualitative parameters were determined. By spectral Doppler, the means of the peak systolic velocity (PSV), pulsatility index (PI) and resistivity index (RI) of the left and right cranial thyroid artery were automatically calculated by the ultrasound apparatus starting from three pulses of arterial waves. The laminar flow velocity profile and the cranial thyroid artery waveforms were determined via spectral Doppler analysis.

The architecture of the blood flow of the thyroid parenchyma was characterized by a color Doppler study. For a better study of blood irrigation, each thyroid lobe was evaluated in the cranial, middle and caudal regions.

Statistical analysis. B-Mode and Doppler measurements were calculated by the analyses of three observers with at least five years of experience in veterinary ultrasonography.

All the statistical analyses were performed by using the Statistical Package for Social Sciences (IBM SPSS Statistics - version 24.0; SPSS Inc., Chicago/IL, EUA). The Kolmogorov-Smirnov statistical test was applied in order to verify a data normal distribution. Student's t test was utilized to compare the means of the right and left cranial thyroid arteries.

The interobserver reproducibility was used to evaluate the reliability of the measurements made by intraclass correlation coefficient. This coefficient was used for data analysis and classification, as proposed by Fleiss et al. (2003) (Table 1).

Table 1. Rating of interobserver agreement according to the intraclass correlation coefficient

Intraclass correlation coefficient	Interobserver agreement
< 40	Poor
0.40 - 0.75	Moderate
0.75 - 1.00	Excellent

Fleiss et al. (2003).

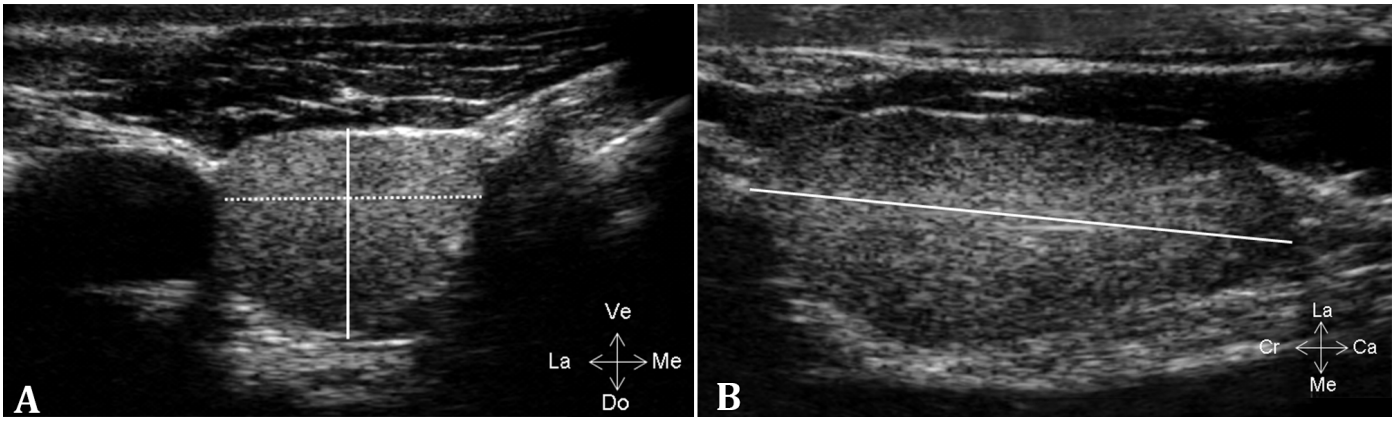


Fig.1. Thyroid lobe in the (**A**) transverse and (**B**) longitudinal planes by ultrasonography. (**A**) The height and width are represented by continuous and dotted lines, respectively. (**B**) The length is represented by a continuous line (largest axis). Dorsal (Do), lateral (La), medial (Me), ventral (Ve).

RESULTS

Animal selection

The free T4 levels showed a mean of 1.49 (±0.43) ng/dl (normal values range from 0.62 to 3.03ng/dl). One horse got levels above the limit (6.04ng/dl) and it was excluded from the experiment. In the screening for thyroid nodular formations by ultrasound, one horse presented a nodular formation of 0.5cm in height × 0.58cm in width × 0.77cm in length in the caudal portion of the right thyroid lobe. There were poorly defined contours, mixed echogenicity and discretely heterogeneous echotexture, compared to the adjacent thyroidal tissue. No significant vascularization was observed in the Doppler study. This animal was excluded from the study.

Finally, by ultrasound-guided FNAC, none of the slides presented alterations related to inflammatory, dysplastic, neoplastic or degenerative processes.

Ultrasonography

B-Mode study. The descriptive statistic values for width, height and volume did not differ significantly between the right and left thyroid lobes for all the observers. A significant difference was found related to the length parameter between the left and right thyroid lobes by Observers 1 and 2 (Table 2).

On the other hand, no significant difference was observed between the length of the left and right thyroid lobes by Observer 3. However, we can suggest that there was a tendency for this result to become statistically different, since this value was very close to 0.05. A fact that did not occur with the other parameters evaluated in this study (Table 2).

The reproducibilities were homogeneous and achieved an excellent reliability for the values obtained for the observers pairs (Table 3).

The means for the total volume of the thyroid gland calculated by ultrasonography were 13.03 (±4.91), 13.14 (±5.39) and 12.44 (±5.16) by Observers 1, 2 and 3, respectively.

The relation between the horses' weight and the total thyroid gland volume was evaluated. A moderate relation was observed between these variables: Observers 1 (33%), 2 (32%) and 3 (36%) (Fig.2).

In the transverse plane of the thyroid lobes presented a spherical form, and in some cases, it presented a discrete projection toward the thyroid lobe medial aspect (toward the margin of the trachea).

Table 2. The descriptive statistics and comparison between the left (Lt) and right (Rt) thyroid lobes according to the ultrasound parameters in a total of 11 horses with an average weight of 403.45 (±36.23) kilograms

	Site	Observer 1		Observer 2		Observer 3	
		Mean ± SD	P	Mean ± SD	P	Mean ± SD	P
W	Lt	1.97 ± 0.37	0.571	2.07 ± 0.44	0.238	1.97 ± 0.34	0.255
	Rt	1.90 ± 0.36		1.89 ± 0.41		1.84 ± 0.35	
H	Lt	1.66 ± 0.34	0.924	1.63 ± 0.34	0.644	1.61 ± 0.36	0.716
	Rf	1.67 ± 0.27		1.59 ± 0.25		1.58 ± 0.29	
L	Lt	3.85 ± 0.44	0.049*	3.90 ± 0.42	0.027*	3.85 ± 0.51	0.079
	Rt	3.59 ± 0.33		3.66 ± 0.34		3.64 ± 0.34	
V	Lt	6.96 ± 3.06	0.217	7.19 ± 3.38	0.110	6.69 ± 3.06	0.121
	Rt	6.11 ± 2.19		5.95 ± 2.43		5.75 ± 2.37	

SD = Standard deviation, W = width (cm), H = height (cm), L = length (cm), V = volume (cm^3); * statistical t test of paired samples t for P<0.05.

Table 3. The means of the intraclass correlation coefficients with 95% confidence intervals for ultrasound dimensions by interobserver agreement

		Interobserver agreement		
	Site	Observers 1 and 2	Observers 1 and 3	Observers 2 and 3
		ICC (CI at 95%)	ICC (CI at 95%)	ICC (CI at 95%)
W	Lt	0.879 (0.551 - 0.967)	0.973 (0.900 - 0.993)	0.913 (0.677 - 0.977)
	Rt	0.980 (0.924 - 0.995)	0,986 (0,950 - 0.996)	0.961 (0.854 - 0.989)
H	Lt	0.996 (0.986 - 0.999)	0.997 (0.987 - 0.999)	0.994 (0.976 - 0.998)
	Rf	0.983 (0.935 - 0.995)	0.971 (0.893 - 0.992)	0.970 (0.887 - 0.992)
L	Lt	0.971 (0.891 - 0.992)	0.971 (0.892 - 0.992)	0.925 (0.720 - 0.980)
	Rt	0.972 (0.894 - 0.992)	0.963 (0.864 - 0.990)	0.942 (0.784 - 0.984)
V	Lt	0.967 (0.877 - 0.991)	0.996 (0.986 - 0.999)	0.971 (0.892 - 0.992)
	Rt	0.988 (0.956 - 0.997)	0.992 (0.971 - 0.998)	0.988 (0.955 - 0.997)

ICC = Intraclass correlation coefficients, CI at 95% = 95% confidence intervals, W = width (cm), H = height (cm), L = length (cm), V = volume (cm^3).

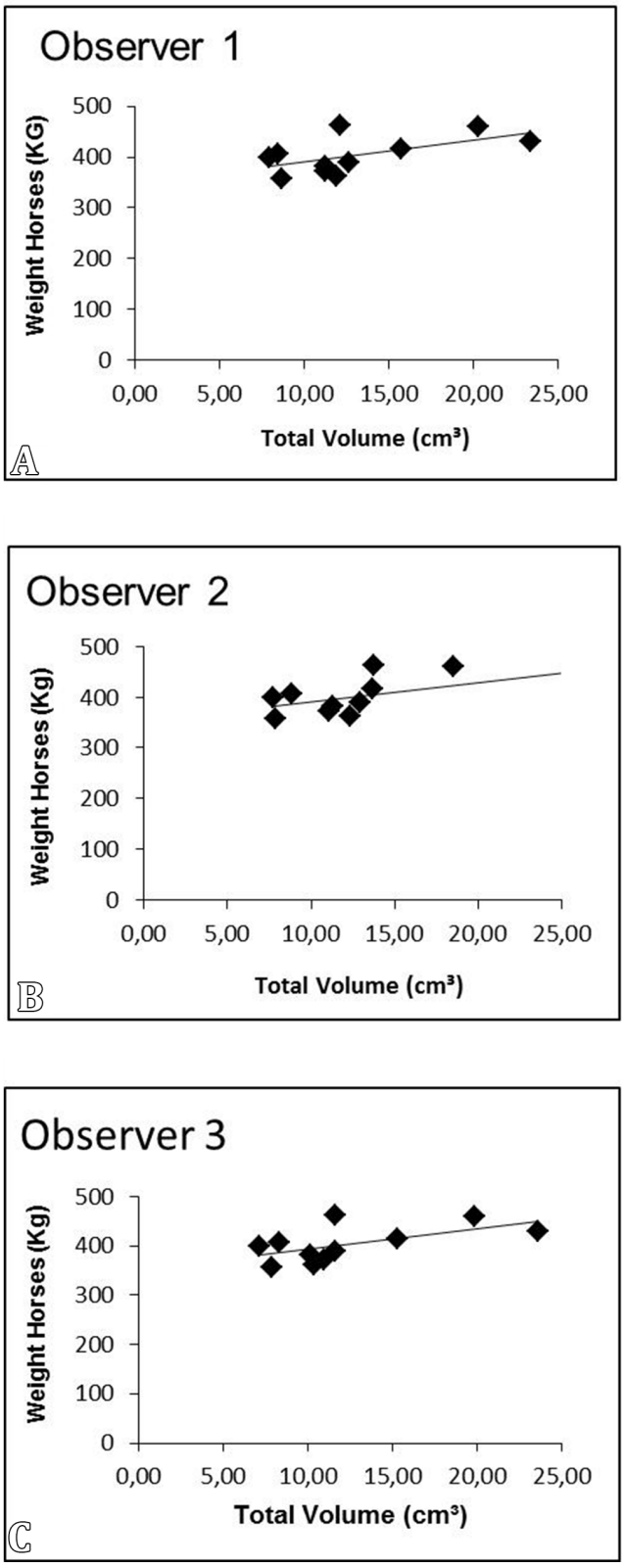


Fig.2. Relation between the thyroid gland total volume and horses' body weight by Observers 1 (R^2 = 0.33), 2 (R^2 = 0.32) and 3 (R^2 = 0.36). Lines represent linear regression line. Ultrasonography (USG), regression coefficient (R^2).

In the longitudinal plane, a high variation related to the elliptical format of the thyroid lobes was observed, oscillating between a rounded and flattened conformation (Fig.3). Six animals presented the lobes format of some thyroids with similar formats (right lobe similar to left lobe (54.54%) and five horses presented pair of thyroid lobes with different formats (45.46%).

Regarding the contours, 19 thyroid lobes had regular. One left and one right thyroid lobe were slightly irregular. Only one thyroid lobe (right) was shown to be moderately irregular.

All of the evaluated thyroid glands presented a slightly heterogeneous echotexture. Regarding the echogenicity, nineteen lobes (86.36%) displayed hyperechogenicity (Fig.3A,B,D) and two left lobes (9.09%) and one right lobe (4.55%) presented isoechogenicity in relation to the sternocephalic muscle (Fig.3C).

Doppler study. By spectral Doppler evaluation, the cranial thyroid arteries presented a parabolic flow velocity profile. The waveforms presented a flow pattern of low resistance, which is characterized by wide continuous systolic peaks and a high diastolic with a gradual reduction velocity. The beginning of the cranial thyroid artery waves tracing was characterized by the formation of a double systolic peak, with the second peak presenting higher amplitude (Fig.4).

No significant difference was observed between the spectral Doppler values of the left and right cranial thyroid arteries obtained by Observers 1, 2 and 3 (Table 4). In general, the Doppler parameters showed an excellent reproducibility among the three observers. However, these values were heterogeneous among pairs of evaluators (Table 5).

Color Doppler enabled a good characterization of the cranial, middle and caudal vascularization of the thyroid lobes in the longitudinal plane. The cranial thyroid artery and your branches were observed close to the cranial lobe of the thyroid lobes. The branches presented tortuous and discreetly calibrated (Fig.5A,B). The thyroid gland intralobar vessels presented a more evident vascularization in the medial portion of the gland than in the central and lateral regions (Fig.5D-F). These vessels were branched from the peripheral to the central regions and were a little tortuous through the thyroid parenchyma.

DISCUSSION

The parotid gland is the largest salivary gland of the horse. It occupies the space between the caudal border of the mandible, the wing of the atlas, and the base of the ear. Ventrally it extends to the linguofacial vein (Budras et al. 2008) place where the thyroid lobes are located. Thus, it becomes difficult to perform the ultrasound-guided FNAC of the thyroid without surpassing the parotid gland, in order to avoid the possibility of migration neoplastic cells from the thyroid to the parathyroid.

The observed echotexture and echogenicity of the equine thyroid gland were similar to those of previous descriptions (Rantanen 1998, Davies et al. 2010).

A significant difference regarding the length parameter between the left and right thyroid lobes was verified by Observers 1 and 2 and a tendency of these values to present a significant difference was noticed by Observers 3 (Table 2). The differences observed between the dimensions of the contralateral thyroid lobes could be reinforced by a previous

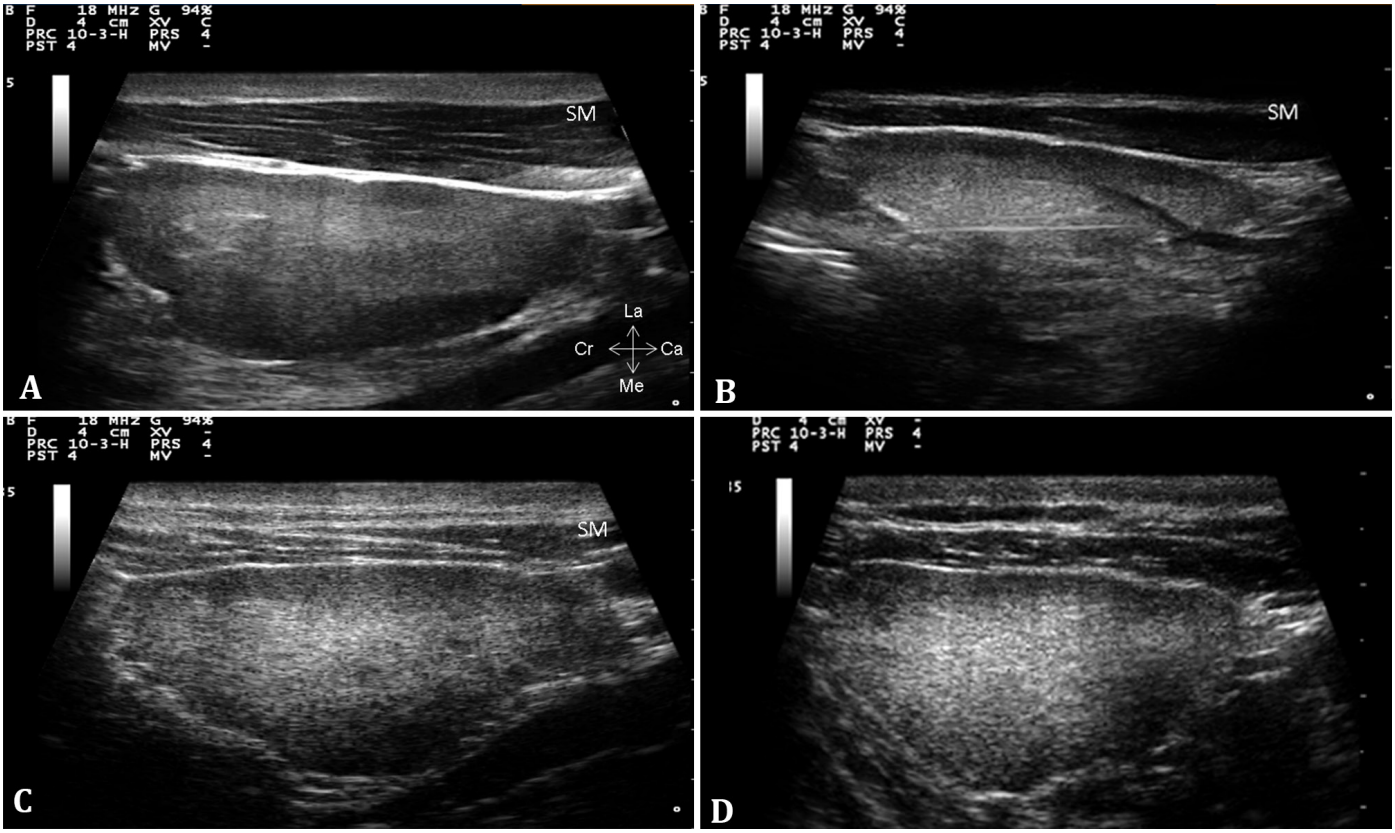


Fig.3. Presentation of the thyroid lobe formats, showing a pair of lobes with distinct aspects: (**A**) left side, (**B**) right side; and another pair of similar lobes (**C**) left side, (**D**) right side. (**A**,**B**,**D**) The gland is hyperechogenic in relation to the sternocephalic muscle (SM). (**C**) The lobe is isoecogenic in relation to the SM.

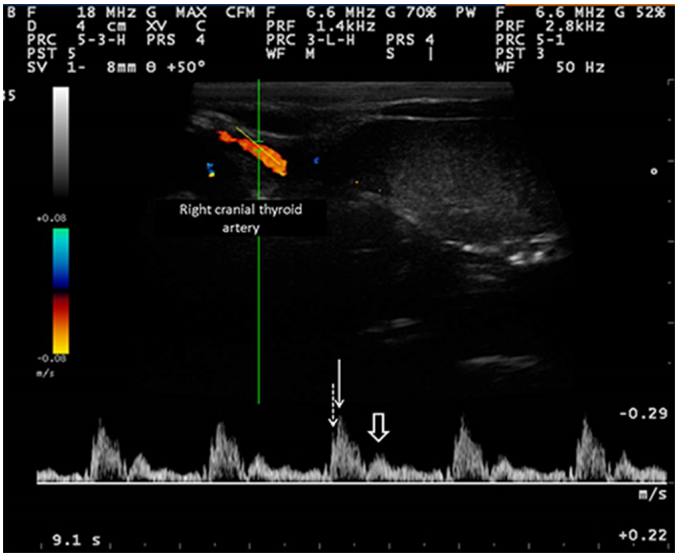


Fig.4. Spectral Doppler of cranial thyroid artery (PRF = 2.8KHz, angle 50°). The dotted and continuous arrows indicate a double systolic peak, with the second showing greater amplitude (early systolic peak, ESP). The velocity distribution is broad, without a spectral window, characterizing a parabolic flow velocity profile. The velocity decreases suddenly after the systolic peak but without diastolic reverse flow. Next, a high-velocity wave is observed (empty arrow), followed by gradual reduction of velocity, which characterizes the waveforms with a low-resistance flow pattern.

study involving ultrasonographic evaluation of Golden Retriever dogs with healthy thyroid glands and hypothyroidism. It was verified that there was a significant difference between the height of the healthy thyroid lobes on the left and right sides (Bromel et al. 2005).

In contrast to the reproducibility analyses performed in this study, an evaluation of the ultrasound measurement variability between different observers of canine thyroid found that the length presented the biggest variation (poor agreement) among them (Taeymans et al. 2005).

The discrete differences observed between thyroid lobes by transverse plane were not significant, as no differences were found between the width and height of the left and right thyroid lobes for the three observers. The difference of format observed between the left and right thyroid lobes could be explained by a significant tendency of the three observers' values to be different. Thus, the height, width and volume of a healthy thyroid lobe can be used more safely than length as reference parameters for evaluation an altered contralateral thyroid lobe in horses. Although it has been shown that radiological measurements performed in small animals cannot be used to evaluate organ changes (Lamb & Nelson 2015), based on the present results, quantitative parameters may serve as a basis for elucidating questions regarding the variation of the format of the unhealthy thyroid lobe.

The Doppler study of the thyroid vascularization confirmed anatomical information previously described by Venzke (1975),

Table 4. The descriptive statistics and comparison between the left (Lt) and right (Rt) cranial thyroid arteries according to the parameters of spectral Doppler ultrasonography in a total of 11 horses with an average weight of 403.45 (±36.23) kilograms

	Site	Observer 1		Observer 2		Observer 3	
		Mean ± SD	P	Mean ± SD	P	Mean ± SD	P
PSV	Lt	22.99 ± 5.74	0.981	24,04 ± 6.02	0.976	21.72 ± 5.46	0.529
	Rt	22.92 ± 8.85		23,95 ± 8.84		23.19 ± 9.41	
RI	Lt	0.71 ± 0.10	0.089	0.73 ± 0.10	0.081	0.76 ± 0.10	0.085
	Rt	0.76 ± 0.09		0.78 ± 0.05		0.82 ± 0.05	
PI	Lt	1.90 ± 0.57	0.549	1.81 ± 0.81	0.236	1.69 ± 0.62	0.142
	Rt	2.07 ± 0.57		2.16 ± 0.66		2.10 ± 0.65	

SD = Standard deviation, PSV = peak systolic velocity (cm/sec), PI = pulsatility index, RI = resistive index; statistical t test of paired samples t for p<0.05.

Table 5. The means of the intraclass correlation coefficients with 95% confidence intervals for spectral Doppler ultrasound parameters by interobserver agreement

		Interobserver agreement		
	Site	Observers 1 and 2	Observers 1 and 3	Observers 2 and 3
		ICC (CI at 95%)	ICC (CI at 95%)	ICC (CI at 95%)
PSV	Lt	0.846 (0.429 - 0.959)	0.805 (0.275 - 0.948)	0.790 (0.220 - 0.944)
	Rt	0.792 (0.226 - 0.944)	0.989 (0.958 - 0.997)	0.974 (0.903 - 0.993)
RI	Lt	0.990 (0.964 - 0.997)	0.929 (0.738 - 0.981)	0.954 (0.828 - 0.988)
	Rt	0.991 (0.967 - 0.998)	0.761 (0.110 - 0.936)	0.793 (0.232 - 0.944)
PI	Lt	0.952 (0.822 - 0.987)	0.855 (0.462 - 0.961)	0.914 (0.680 - 0.977)
	Rt	0.786 (0.205 - 0.942)	0.836 (0.391 - 0.956)	0.801 (0.261 - 0.947)

ICC = Intraclass correlation coefficients, CI at 95% = 95% confidence intervals, PSV = peak systolic velocity (cm/sec), PI = pulsatility index, RI = resistive index.

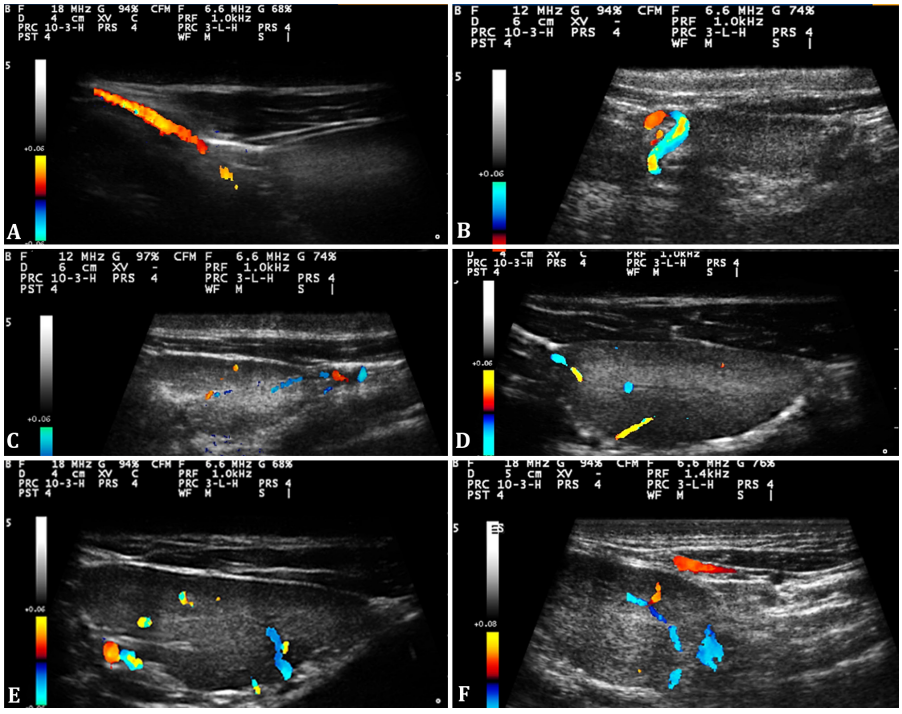


Fig.5. Vascularization assessment in the cranial, middle and caudal regions of the thyroid lobe by color Doppler. (**A**) Calibrated and non-tortuous cranial thyroid artery directed towards the cranial pole of the thyroid lobe. (**B**) The tortuous branch of the cranial thyroid artery is highlighted proximal to the cranial pole of the thyroid lobe. (**C**) The caudal thyroid artery supplying the thyroid lobe in its caudal portion. Intralobar vessel characteristics of the (**D**) cranial, (**E**) middle and (**F**) caudal regions of the thyroid lobe.

which emphasized that the equine cranial thyroid artery is a calibrous vessel that curves over the cranial pole of the thyroid lobe emitting several branches; and that the caudal thyroid artery is a small inconstant vessel that irrigates the caudal portion of the lobes.

In the spectral Doppler analysis of the cranial thyroid artery, the visualization of a low peak preceding a second peak of greater amplitude (early systolic peak, ESP) is a characteristic of low resistance arteries. These vessels supply organs that present a continuous demand for blood, and this demand can change according to the functional status of the organs, with consequent alterations of the Doppler flow pattern. In the case of thyroid hormonal disturbances, variations in the Doppler flow pattern of the cranial thyroid artery may be detected, indicating the presence of thyroid disorders with an abnormal release of thyroidal hormones (Szatmári et al. 2001, Chiou et al. 2006).

It is possible to observe several studies in human ultrasonography using reproducibility as methodology for the validation of their data from a single observer and then analyzed by different observers (Järvelä et al. 2003, Raine-fenning et al. 2003, Moron et al. 2010). This same methodology was applied in the present study, justifying the reliability of the measurements made by all of the observers.

The interobserver agreement for the Doppler parameters was concordant but with heterogeneous values. The discrete variations of the systolic peak and final diastole determined by the three observers may have contributed to this heterogeneity. However, a reliable reproducibility was achieved, suggesting that the measurement variations did not interfere with the Doppler tool application in the equine cranial thyroid artery. Considering that the diagnostic accuracy of a method is an essential resource for determining its usefulness, validation of the reproducibility of the Doppler parameters becomes useful for the identification of hemodynamic changes (Leão et al. 2008).

CONCLUSIONS

We can conclude that there was an excellent reproducibility for thyroid quantitative parameters obtained by ultrasonography in B-mode and Doppler.

Among the comparisons made between the quantitative parameters of the left and right lobes, it was observed that there was only difference between their respective lengths. Qualitatively, there was a significant variation between the lobes elliptical format in the longitudinal plane of some glands, which oscillated between a rounded and flattened conformation. Thus, we can infer that the difference between the lobes format of some thyroids can be explained by the significant difference observed between the length of the left and right lobes.

Additionally, it was verified that there was no difference between the Doppler quantitative parameters. Therefore, we may suggest that unilateral analysis of the cranial thyroid artery by spectral Doppler can be used to evaluate equine thyroid diffuse disorders.

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

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Se o artigo for submetido fora das normas de apresentação da PVB, a tramitação somente ocorrerá após as devidas correções feitas pelo autor.
A PVB publica Artigos Originais, Artigos de Revisão Crítica e Tópicos de Interesse Geral; não publica artigos com a denominação de *Short Communications*.
Relatos de Caso serão aceitos somente em artigos classificados como pertencentes à área de Animais Selvagens (*Wildlife Medicine*).
Os Artigos Originais devem conter resultados de pesquisa ainda não publicados ou submetidos para outros periódicos.
Artigos de Revisão de Literatura, submetidos a convite, devem constituir-se de análise crítica, de assuntos na área de experiência dos autores, isto é, quando os autores já tiverem publicado anteriormente artigos sobre o assunto.
Os raros Tópicos de Interesse Geral devem constituir-se de assuntos de grande importância atual baseado na vasta experiência dos autores.
As opiniões e conceitos emitidos nos artigos submetidos são de responsabilidade dos autores. O Conselho Editorial da PVB, com a assistência da Assessoria Científica, pode sugerir ou solicitar modificações. Os artigos submetidos são avaliados pelos pares (*peer review*) e, aceitos para publicação com dois pareceres favoráveis, ou rejeitados por dois pareceres desfavoráveis.
Os direitos autorais dos artigos aceitos para publicação permanecem com os autores.

1. **Os artigos devem ser organizados em TÍTULO, ABSTRACT, RESUMO, INTRODUÇÃO, MATERIAL E MÉTODOS, RESULTADOS, DISCUSSÃO, CONCLUSÕES (de preferência os últimos três separadamente), Agradecimentos, Declaração de conflito de interesse e REFERÊNCIAS:**
a) O **TÍTULO** deve ser conciso e indicar o conteúdo do artigo; pormenores de identificação científica devem ser colocados em MATERIAL E MÉTODOS.
b) **O(s) Autor(es) com numerosos primeiros nomes e sobrenomes, deve(m) padronizar o seu "nome para publicações científicas"**, como por exemplo: Cláudio Severo Lombardo de Barros, escreve Cláudio S.L. Barros ou Barros C.S.L.; Franklin Riet-Correa Amaral escreve Franklin Riet-Correa ou Riet-Correa F. **Os artigos devem ter no máximo 8 (oito) autores**. O autor para correspondência deve ser um autor que garanta o contato com o Conselho Editorial da PVB. Asteriscos de chamadas para o rodapé não devem ser sobrescritos.
c) O **Cabeçalho do ABSTRACT** deve conter além dos nomes dos autores abreviados invertido, o ano, o TÍTULO, o endereço postal do laboratório (inclusive o CEP) ou instituição principal onde foi desenvolvida a pesquisa. Endereços postais brasileiros

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3. **Apresentação das Figuras:**

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

b) Enviar cada figura separadamente.

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

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

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

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

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

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

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

Exemplos de Referências

➢ Artigos publicados em periódicos:

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

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

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

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

- Livros:

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

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

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

- Capítulos de livros:

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

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

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

- Dissertações e Teses:

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

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

- Resumos publicados em eventos:

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

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

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

Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

GUIDE FOR AUTHORS

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

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

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

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

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

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

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

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

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

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

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

The author rights of the accepted papers are preserved.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3. **Figure presentation:**

a) Save images at 300 dpi, TIF files.

b) Send each figure separately.

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

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

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

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

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

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

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

Exemples for References:

➢ Articles published in scientific journals:

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(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.

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

➢ Abstracts published in Events:

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