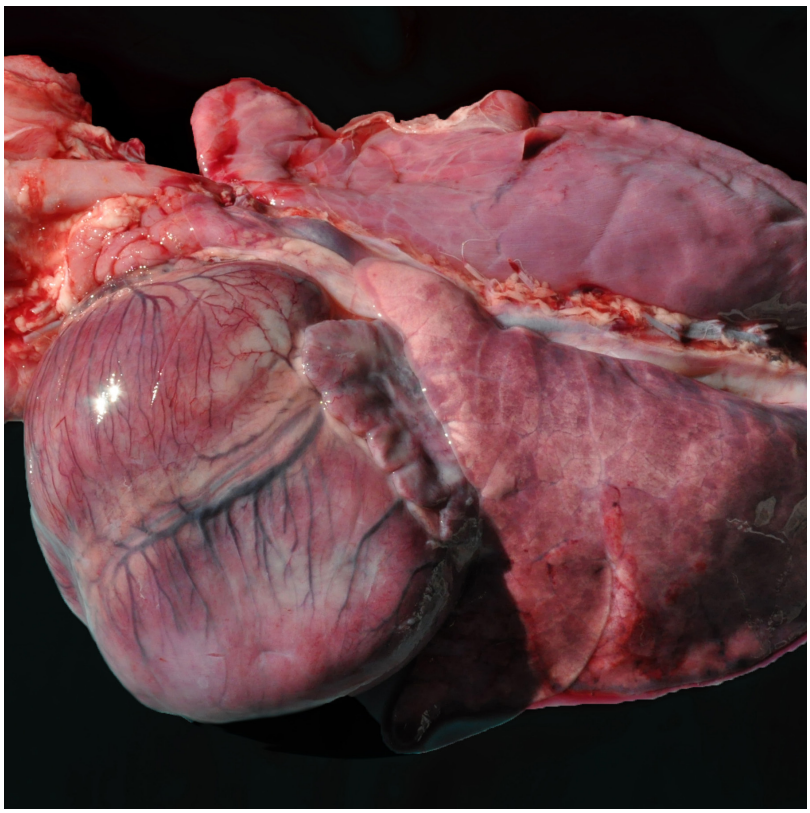
ISSN 0100-736X *(Print)*
ISSN 1678-5150 *(Online)*

Volume 39 Number 8
August 2019

PESQUISA VETERINÁRIA BRASILEIRA

Brazilian Journal of Veterinary Research



www.pvb.com.br

Colégio Brasileiro de Patologia Animal

PESQUISA VETERINÁRIA BRASILEIRA - Brazilian Journal of Veterinary Research (http://www.pvb.com.br/), edited by the scientific association Colégio Brasileiro de Patologia Animal (CBPA) in collaboration with institutions, such as Universidade de Brasília (UnB) and Universidade Federal Rural do Rio de Janeiro (UFRRJ). Pesq. Vet. Bras publishes original papers on animal diseases and related subjects divided by the following areas: **Livestock Diseases (LD), Small Animal Diseases (SA), Wildlife Medicine (WM) and Animal Morphophysiology (MF).**

Pesq. Vet. Bras. is edited from 1981, in continuation to the "Archives of the Institute of Animal Biology" (1959-1961) and the Veterinary Series (1968-1976) of the "Pesquisa Agropecuária Brasileira".

Pesq. Vet. Bras. publishes subjects about natural and experimental diseases. The journal details the diagnostic investigations of natural and emerging diseases of animals; especially herd diseases with occurrence in Brazil. The submission of topics on animal diseases related to public health is encouraged. Pathology in its broad sense is the flagship of the journal, including laboratory diagnostic, epidemiology, clinics and others. Each issue features Original Articles, Reviews or Topics of General Interest; Case Reports are only accepted in the area of Wildlife Medicine (WM). These articles aim a public consisting of veterinary practitioners, pathologists, veterinary diagnostic laboratory staff, toxicologic pathologists, comparative pathologists, medical pathology researchers, environmental scientists, and others involved in veterinary diagnostics and animal research across domestic species and wildlife.

For the preparation of the manuscript, authors must follow the format indicated by the journal, which can be found under "Instructions for Authors," "Submission of articles" in the journal webpage (http://www.pvb.com.br/instructions_to_authors.pdf). Submission of articles outside the presentation standards will be disregarded.

Critical review articles should only be submitted upon invitation by the Chief-Editor and should be supported by original publications of the author on the subject. The editors assume that papers submitted are not being considered for publication in other journals and do not contain material which has already been published. Submitted papers are peer reviewed.

Articles as of January, 2019 will be published in English, including a Portuguese Abstract when Portuguese is the native language of one of the authors.

The journal is indexed and/or abstracted by: SciELO-Scientific Electronic Library Online (www.scielo.br/pvb); ISI/Thomson Reuters, in its products Science Citation Index Expanded and BIOSIS Previews; CABI, in its key-databases CAB Abstracts and Global Health, and in several derived databases, such as: Animal Science Database and VetMedResources (for the internet), Index Veterinarius and Veterinary Science Database (abstract databases), and Veterinary Bulletin (printed), DOAJ-Directory of Open Access Journals (http://www.doaj.org).

Apoio:

Pesq. Vet. Bras. also has resources from the *Conselho Federal de Medicina Veterinária* (CFMV).

Pesquisa Veterinária Brasileira. - - vol.1, n.1 (1981) - . - - Rio de Janeiro: Colégio Brasileiro de Patologia Animal, 1981 -

il.; 28 cm

Mensal.

Resumos em inglês e português.

ISSN 0100-736X (*Print*).

ISSN 1678-5150 (*Online*).

Título traduzido: *Brazilian Journal of Veterinary Research*.

Continuação de: "Arquivos do Instituto de Biologia Animal" (1959-1961) e à Série Veterinária (1968-1976) da "Pesquisa Agropecuária Brasileira".

I. Colégio Brasileiro de Patologia Animal.

Impressão: Editora Cubo.
Tiragem: 100 exemplares.

ISSN 0100-736X (*Print*)
ISSN 1678-5150 (*Online*)

PESQUISA VETERINÁRIA BRASILEIRA

Brazilian Journal of Veterinary Research

The "Pesquisa Veterinária Brasileira" scientific journal is edited from 1981 in continuation to the "Arquivos do Instituto de Biologia Animal" (1959-1961) and the Veterinary Series (1966-1976) of "Pesquisa Agropecuária Brasileira".

Colégio Brasileiro de Patologia Animal

www.pvb.com.br

Cover illustration: Enlarged and globular heart in a pig with nutritional cardiomyopathy. (Cruz et al., p.576)

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(8):561-563, August 2019
DOI: 10.1590/1678-5150-PVB-5993

Original Article
Livestock Diseases

Seneciosis in cattle associated with ingestion of *Senecio brasiliensis* under different forms of consumption in Santa Catarina state, Brazil[1]

Claudia P. Biffi[2], Daiane Ogliari[2], Elaine Melchioretto[2], Sandra D. Traverso[2] and Aldo Gava[2]*

ABSTRACT.- Biffi C.P., Ogliari D., Melchioretto E., Traverso S.D. & Gava A. 2019. **Seneciosis in cattle associated with ingestion of *Senecio brasiliensis* under different forms of consumption in Santa Catarina state, Brazil.** *Pesquisa Veterinária Brasileira 39(8):561-563.* Laboratório de Patologia Animal, Centro de Ciências Agroveterinárias, Universidade do Estado de Santa Catarina, Av. Luiz de Camões 2090, Bairro Conta Dinheiro, Lages, SC 88520-000, Brazil. E-mail: aldo.gava@udesc.br

This study identified the different forms of ingestion of *Senecio brasiliensis* in cattle, diagnosed by the Animal Pathology Laboratory at CAV-UDESC, in the state of Santa Catarina, Brazil. A retrospective evaluation from 1987 to 2016 showed that ingestion has occurred voluntarily due to the presence of the adult plant in native field and/or involuntary, due to the presence of the plant in hay and silage, provided in the trough together with other pastures or by contamination in grain residues. These different forms of ingestion demonstrate the importance of epidemiologic investigation in the diagnosis of seneciosis.

INDEX TERMS: Seneciosis, cattle, *Senecio brasiliensis*, consumption, Santa Catarina, Brazil, diagnosis, epidemiology, forms of ingestion, investigation, poisoning by plants, toxicoses.

RESUMO.- [**Seneciose em bovinos por ingestão de *Senecio brasiliensis* sob diferentes formas de consumo, no estado de Santa Catarina.**]Esse estudo identificou as diferentes formas de ingestão de *Senecio brasiliensis* em bovinos, diagnosticados pelo laboratório de Patologia Animal do CAV-UDESC, no estado de Santa Catarina. Através de avaliação retrospectiva referente aos anos de 1987 a 2016, foi possível observar que as formas de ingestão da planta ocorreram de forma voluntária, pela presença da planta adulta em campo nativo, e/ou involuntária, pela presença da planta em fenos, silagens, fornecidas no cocho junto à outras pastagens ou por contaminação em resíduos de grãos. Essas diferentes formas de ingestão demonstram a importância da investigação epidemiológica no diagnóstico da seneciose.

TERMOS DE INDEXAÇÃO: Seneciose, bovinos, *Senecio brasiliensis*, consumo, Santa Catarina, diagnóstico, epidemiologia, formas de ingestão, investigação, intoxicação por plantas, toxicoses.

INTRODUCTION

Poisoning by *Senecio brasiliensis* (popular names: "maria mole" and flower of souls) frequently occurs in cattle in southern Brazil. It was first described in cattle by Tokarnia & Döbereiner (1984) and later by Méndez et al. (1987), Barros et al. (1987), and Driemeier et al. (1991). Other species of this gender were also described as poisoning for cattle: *Senecio cisplatinus, S. heterotrichius, S. selloi*, *S. leptolobus* (Barros et al. 1987, Méndez et al. 1987), *S. tweediei* (Méndez & Riet-Correa 1993), *S. oxyphyllus* (Karam et al. 2004), and *S. madagascariensis* (Cruz et al. 2010, Karam et al. 2011, Stigger et al. 2014).

Due to the low palatability of this plant, cattle poisoning is generally observed when they undergo extreme hunger and voluntarily ingest the plant in the field (Riet-Correa et al. 1993). Adult cattle, such as dairy cows, are the most affected, because they remain in the property for a longer time (Basile et al. 2005). In Santa Catarina state, the forms of ingesting this plant are quite variable and associated with cattle management in the field; it is more frequently observed in dairy cattle.

The objective of this study was to identify the main forms of ingestion of *Senecio brasiliensis* and evaluate the epidemiologic and clinical-pathological aspects of this plant in the cattle.

[1] Received on December 19, 2018.
Accepted for publication on February 16, 2019.
Part of the Doctoral Dissertation of the first author.

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

MATERIALS AND METHODS

The present study retrospectively evaluated epidemiologic and clinical-pathological data of the poisoning outbreaks by *Senecio brasiliensis* in cattle occurred in the coverage area of the Animal Pathology Laboratory (LAPA) of the "Universidade do Estado de Santa Catarina" (CAV-UDESC) from 1987 to 2016. This study includes the outbreaks diagnosed through clinical evaluation, necropsy, and histologic examination performed by the LAPA team as well as the samples of histologic evaluation sent to the laboratory by veterinarians. Fragments of the organs were collected, fixed in 10% buffered formalin, routinely processed, and stained with the hematoxylin and eosin (HE).

Data on epidemiology, clinical sign observations, and necropsy findings were collected during the visits to the property where the poisoning outbreaks by *S. brasiliensis* occurred, and they complemented by information obtained from the farm owners and veterinarians.

RESULTS

From 1987 to 2016, 177 necropsies were performed from 41 outbreaks, of which 17 were diagnosed through necropsy and histologic examination performed by the Animal Pathology Laboratory team and 24 were diagnosed through histologic examination of the samples sent to laboratory by veterinarians. The outbreaks occurred in following municipalities in Santa Catarina state: Lages, Otacílio Costa, Chapecó, Xanxerê, São José do Cedro, Treze Tílias, Água Doce, Campos Novos, Joaçaba, Itaiópolis, Benedito Novo, and Tubarão. The necropsy distribution of the 41 seneciosis outbreaks is illustrated in Figure 1.

The morbidity verified in the 17 outbreaks followed up by the LAPA team varied from 10 to 65%, with lethality of 100%. In the 24 outbreaks with diagnosis performed in the samples received by the LAPA, there was no information regarding the number of cattle affected and the form of ingestion of the plant. The data on the different forms of plant ingestion and the number of deaths in the outbreaks followed up at the LAPA are shown in Table 1. Figure 2 and 3 shows *Senecio brasiliensis* in alfalfa hay and in harvested pasture.

The clinical signs observed during the visits to the property include diarrhea, apathy, anorexia, progressive emaciation, and rectal tenesmus and prolapse. Aggressiveness, incoordination, walking in circles, and photosensitization were sometimes observed in animals with little pigmentation. In an outbreak in which only calves were affected, the animals also presented constipation.

At necropsy, the most frequently observed clinical signs included liver with reduced size, hardened, yellow or dark, whitish striations, distended gallbladder with thickened wall, and abdominal cavity edema at the abomasum and mesenteric submucose. At microscopy, the main lesions were found in the liver, and were characterized of proliferation of biliary ducts, megalocytosis, fibrous tissue proliferation in various degrees, fatty degeneration, and sometimes, at the nervous system, astrocyte degeneration and moderate spongiosis.

DISCUSSION

The largest number of cases of poisoning by *Senecio brasiliensis* diagnosed by the LAPA occurred from 2000 to 2008. Although by that time this plant had already been widely studied and its poisoning effect had been demonstrated (Tokarnia & Döbereiner 1984, Barros et al. 1987, Méndez et al. 1987, Driemeier et al. 1991), many producers continued to ignore its effects on cattle.

At that time, the state of Santa Catarina began to gain importance in the national milk production scenario, and today it is the fourth milk producer of the country (IBGE 2017). With the increase in productivity and the need for greater feeding availability, soil management for pasture ended up favoring the occurrence of *S. brasiliensis*. According to Pereira et al. (2011), this plant is mainly observed in native fields where there is no adequate management. In the present

Table 1. Forms of ingestion of *Senecio brasiliensis* observed in the outbreaks followed up by the Animal Pathology Laboratory, CAV-UDESC, Santa Catarina state, Brazil

S. brasiliensis ingestion ways	Outbreaks	Deaths
Adult plant in oat and/or ryegrass pasture	8	97
Young plant in native field	1	18
Green plant cut and supplied in the trough	2	9
Contamination of alfalfa and/or oat and/or tifton and/or ryegrass hay	4	54
Ryegrass pre-dried silage	1	8
Contamination in grain/corn residues	1	12
TOTAL	17	198

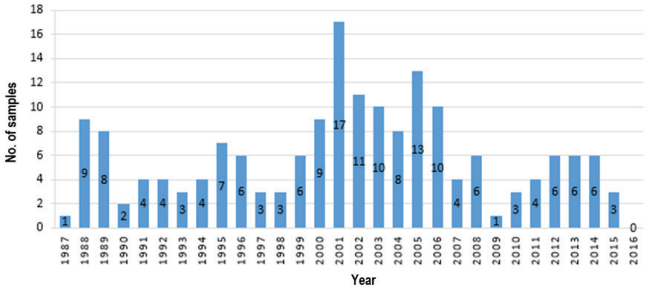


Fig.1. Number of necropsies in cattle affected by seneciosis performed from 1987 to 2016 within the coverage area of the Animal Pathology Laboratory, Santa Catarina state, Brazil.

Fig.2. Alfalfa hay contaminated with *Senecio brasiliensis* leaves.

Fig.3. Oat and ryegrass pasture being mechanically harvested for supply to cattle in the trough.

study, of the nine outbreaks resulting from voluntary plant ingestion, only two occurred in native fields, and one of them occurred through ingestion of the young plant. The octhers seven outbreaks occurred through ingestion of the adult plant in areas of oat and ryegrass pasture, differently from the poisoning situation observed by Barros et al. (1987) and Méndez et al. (1987).

In Brazil, there are few reports on the accidental consumption of this plant, (Riet-Correa et al. 1993). Due to the characteristic of being frequently present in the cropped pasture, In Santa Catarina state, because *S. brasiliensis* is frequently present in harvested pasture, it became a contamination source when it is cut together with the pasture and supplied to the cattle in the trough, or, when associated with hay and/or silage, and/or dry grain residues. Even after drying, the plant does not lose its toxicity (Méndez et al. 1987, Méndez et al. 1990, Méndez & Riet-Correa 2008). Poisoning by *S. brasiliensis* in cattle in Santa Catarina state, due to management diversity, can occur at any time of the year. This can be explained because, in Santa Catarina state, cattle farming generally occurs in small properties where harvested pasture is frequently used in winter and/or summer when the soil needs to be de-compacted, which favors the spread of *S. brasiliensis*. The seasonal spread of *S. brasiliensis* is also favored when the cattle are fed mechanically harvested pasture supplied in the trough, as well as hay, silage and dry grain residues, which can be used at different times of the year.

S. brasiliensis poisoning diagnosis was confirmed by the pathological clinical condition and observation of the plant consumed in the pasture and/or present in feed such as pre-dried alfalfa, oat and ryegrass, and residues of corn grain.

The seneciosis outbreaks followed up in the present study show that the cattle are exposed to different forms of ingestion of *S. brasiliensis*. This emphasizes that, in order to obtain the correct diagnosis and take prophylactic measures, accurate epidemiologic evaluation is essential.

CONCLUSION

Senecio brasiliensis poisoning in cattle in Santa Catarina state was observed due to voluntary ingestion, by hunger, of the adult plant, involuntary ingestion of the young plant in highly contaminated pastures, and/or ingestion of contaminated dry plant residues in hay, silage, grain, and pasture supplied to cattle in the trough.

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

REFERENCES

Barros C.S.L., Metzdorf L.L. & Peixoto P.V. 1987. Intoxicação por *Senecio* spp. em bovinos. Pesq. Vet. Bras. 7(4):101-107.

Basile J.R., Diniz J.M.F., Okano W., Cirio S.M. & Leite L.C. 2005. Intoxicação por *Senecio* spp. (Compositae) em bovinos no sul do Brasil. Acta Scient. Vet. 33(1):63-68. <http://dx.doi.org/10.22456/1679-9216.14565>

Cruz C.E.F., Karam F.C., Dalto A.C., Pavarini S.P., Bandarra P.M. & Driemeier D. 2010. Fireweed (*Senecio madagascariensis*) poisoning in cattle. Pesq. Vet. Bras. 30(1):10-12. <http://dx.doi.org/10.1590/S0100-736X2010000100002>

Driemeier D., Barros C.S.L. & Pilati C. 1991. Seneciose em bovinos. Hora Vet. 10(59):23-30.

IBGE 2017. Instituto Brasileiro de Geografia e Pesquisa. Available at <https://biblioteca.ibge.gov.br/visualizacao/periodicos/2380/epp_2017_4tri.pdf> Access on Mar. 7, 2018.

Karam F.C., Schild A.L. & Mello J.R.B. 2011. Intoxicação por *Senecio* spp. em bovinos no Rio Grande do Sul: condições ambientais favoráveis e medidas de controle. Pesq. Vet. Bras. 31(7):603-609. <http://dx.doi.org/10.1590/S0100-736X2011000700010>

Karam F.S.C., Soares M.P., Haraguchi M., Riet-Correa F., Méndez M.C. & Jarenkow J.A. 2004. Aspectos epidemiológicos da seneciose na região sul do Rio Grande do Sul. Pesq. Vet. Bras. 24(4):191-198. <http://dx.doi.org/10.1590/S0100-736X2004000400004>

Méndez M.C. & Riet-Correa F. 1993. Intoxication by *Senecio tweediei* in cattle in Southern Brazil. Vet. Human. Toxicol. 35(1):55. <PMid:8434457>

Méndez M.C. & Riet-Correa F. 2008. Plantas Tóxicas e Micotoxicoses. 2ª ed. Editora e Gráfica Universitária, Pelotas, p.25-32.

Méndez M.C., Riet-Correa F. & Schild A.L. 1987. Intoxicação por *Senecio* spp. (Compositae) em bovinos no Rio Grande do Sul. Pesq. Vet. Bras. 7(2):51-56.

Méndez M.C., Riet-Correa F., Schild A.L. & Martz W. 1990. Intoxicação experimental por cinco espécies de *Senecio* em bovinos e aves. Pesq. Vet. Bras. 10(3/4):63-69.

Pereira F.A.R., Verzignassi J.R., Arias E.R.A., Carvalho F.T. & Silva A.P. 2011. Controle de Plantas Daninhas em Pastagens. Embrapa Gado de Corte, Campo Grande, p.1-22.

Riet-Correa F., Méndez M.C. & Schild A.L. 1993. Intoxicações por Plantas e Micotoxicoses em Animais Domésticos. Editorial Hemisfério Sur, Montevideo. 340p.

Stigger A.L., Estima-Silva P., Fiss L., Coelho A.C.B., Santos B.L., Gardner D.R., Marcolongo-Pereira C. & Schild A.L. 2014. *Senecio madagascariensis* Poir. (Asteraceae): uma nova causa de seneciose em bovinos no Sul do Rio Grande do Sul. Pesq. Vet. Bras. 34(9):851-855. <http://dx.doi.org/10.1590/S0100-736X2014000900008>

Tokarnia C.H. & Döbereiner J. 1984. Intoxicação experimental por *Senecio brasiliensis* (Compositae) em bovinos. Pesq. Vet. Bras. 4:39-65.

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(8):564-572, August 2019
DOI: 10.1590/1678-5150-PVB-6215

Original Article
Livestock Diseases

Hypomagnesemia in dairy cattle in Uruguay[1]

Benjamín Doncel[2,3], Alsiane Capelesso[4], Federico Giannitti[3], Cecilia Cajarville[4], Melissa Macías-Rioseco[3], Caroline Silveira[3], Ricardo A. Costa[3] and Franklin Riet-Correa[3]*

ABSTRACT.- Doncel B., Capalesso A., Giannitti F., Cajarville C., Macías-Rioseco M., Silveira C., Costa R. A. & Riet-Correa F. 2019. **Hypomagnesemia in dairy cattle in Uruguay.** *Pesquisa Veterinária Brasileira 39(8):564-572*. Instituto Nacional de Investigación Agropecuaria, Plataforma de Investigación en Salud Animal, Estación Experimental INIA La Estanzuela, Ruta 50 km 11, 39173, Colonia, Uruguay. E-mail: frcorrea@inia.org.uy

An outbreak of hypomagnesemia is reported in Holstein dairy cattle grazing lush oat (*Avena sativa*) pasture in Uruguay. Nine of 270 (3.3%) cows died in May-July (autumn-winter) 2017. These nine cows were from 2 to 9-years-old (1st-6th lactation), with 22 to 194 days of lactation and 15.8 to 31.4L of daily milk production. Two cows with acute sialorrhea, muscle spasms, lateral recumbency, weakness, opisthotonos, and coma, were euthanized and necropsied. No significant macroscopic or histological lesions were found. One untreated clinically-affected cow and eight out of 14 clinically healthy cows of the same group under similar management and production conditions had low serum levels of Mg (lower than 0.7mmol/L). Secondarily, both clinically affected cows and six out of 14 healthy cows had low serum Ca levels. The K/(Ca+Mg) ratio of two oat forages, corn silage, and ration was 5.10, 7.73, 2.45, and 0.85, respectively. A K/(Ca+Mg) ratio lower than 2.2 represents a risk for hypomagnesemia. The difference between the contribution-requirement of minerals in the diet was established and a daily deficiency of Mg (-0.36g/day), Na (-25.2g/day) and Ca (-9.27g/day) was found, while K (184.42g/day) and P (12.81g/day) were in excess. The diet was reformulated to correct the deficiencies and the disease was controlled by the daily administration of 80g of magnesium oxide, 80g of calcium carbonate and 30g sodium chloride per cow. It is concluded that hypomagnesemia is a cause of mortality in dairy cattle in Uruguay, and that the condition can be prevented by appropriate diet formulation.

INDEX TERMS: Grass tetany, grazing dairy cows, hypocalcemia, hypomagnesemia, lush oat pasture, dairy cattle, Uruguay, cattle, pathology.

RESUMO.- [Hipomagnesemia em bovinos leiteiros no Uruguai.] Descreve-se um surto de hipomagnesemia em bovinos leiteiros da raça Holandês com alimentação a base de pastagens de aveia (*Avena sativa*) em crescimento no Uruguai, nos meses de maio a julho (outono-inverno) de 2017. De um rebanho de 270 vacas em ordenha, nove (3,3%) morreram. As nove vacas tinham entre dois e nove anos, estavam entre a primeira e a sexta lactação e a produção diária era de 15,8 a 31,4 litros de leite. Duas vacas que apresentaram sialorreia, espasmos musculares, decúbito lateral, debilidade, opistótono e posteriormente, estado comatoso foram eutanasiadas e necropsiadas. Não foram observadas lesões macroscópicas ou histológicas significantes. Uma vaca com sinais clínicos, não tratada, e oito de 14 vacas sem sinais clínicos, do mesmo rebanho, apresentaram baixos níveis séricos de Mg (menos de 0,7mmol/L). Adicionalmente, as duas vacas com sinais clínicos e seis das 14 vacas sem sinais clínicos apresentaram baixos níveis de Ca sérico. A relação do risco tetanizante K/(Ca+Mg) de duas pastagens de aveia, silagem de milho e ração concentrada foi estimada em 5; 10; 7,73; 2,15 e 0,85, respectivamente. Uma relação K/(Ca+Mg) maior de 2,2 é um indicador do potencial tetanizante da forragem, A diferença entre o aporte e os requerimentos dos minerais foi estabelecida constatando-se deficiência diária de

[1] Received on November 22, 2018.
Accepted for publication on March 22, 2019.

[2] Universidad Nacional de Colombia, Sede Bogotá, Facultad de Medicina Veterinaria y de Zootecnia, Laboratorio de Patología Veterinaria, Carrera 30 N° 45-03, Edificio 502, Código postal 11001, Bogotá, Colombia.

[3] Instituto Nacional de Investigación Agropecuaria (INIA), Plataforma de Salud Animal, Estación Experimental INIA La Estanzuela, Ruta 50 Km 11, 39173, Colonia, Uruguay. *Corresponding author: frcorrea@inia.org.uy

[4] Departamento de Nutrición, Instituto de Producción Animal (IPAV), Facultad de Veterinaria, Av. 18 de Julio 11200, 1824-1850 Montevideo, Uruguay.

Mg (-0,36g/dia), Na (-25,2g/dia) e Ca (-9,27g/dia). O potássio (K) (184,42g/dia) e o fósforo (P) (12,81g/dia) estavam em excesso. A dieta foi reformulada para corrigir as deficiências e a doença foi controlada mediante a administração diária de 80g de MgO, 80g de $CaCO_3$ e 30g de NaCl por vaca. Conclui-se que a hipomagnesemia é uma doença de importância crescente em gado leiteiro no Uruguai e que deve ser prevenida mediante a formulação correta da dieta.

TERMOS DE INDEXACAO: Vacas leiteiras em pastejo, pastagens de aveia, hipocalcemia, hipomagnesemia, Uruguai, patologia.

INTRODUCTION

Hypomagnesemia is a disorder of ruminants biochemically characterized by low serum magnesium (Mg) (Grunes et al. 1970, Crawford et al. 1998). It most commonly affects grazing cattle, which may be asymptomatic or develop clinical signs, or die abruptly sometimes leading to high mortality rates (Grunes et al. 1970, Meyer 1977, Smith & Edwards 1988, Constable et al. 2017, Zelal 2017). Mg is an essential cofactor in numerous enzymatic processes of the main metabolic pathways (Martín-Tereso & Martens 2014), an important intracellular second messenger (Li et al. 2011) and participates in osteogenic differentiation (Zheng et al. 2016), neurotransmission (Möykkynen et al. 2001) and muscle function (Goff 1999).

Mg is distributed mainly in bones (60-70%) (Rayssiguier & Larvor 1978), approximately 30% is intracellular and only 1% is in the extracellular space, including the blood (Storry & Rook 1962, Goff 1999). Blood levels of Mg depend on the balance between absorption and excretion rates (Martens et al. 2018) and are easily altered in cows because Mg in bones is not readily available (Storry & Rook 1962) and only 30% can be mobilized from this site (Alfrey & Miller 1973). In cattle, Mg is excreted in milk (4.1-4.9mmol/L) (Cerbulis & Farrell Junior 1976), urine (0.017-0.17mmol/day) (Kemp et al. 1961) and digestive secretions (0.00017mmol/kg body weight) (Schonewille et al. 2008). Additionally, in pregnant cattle, Mg requirements in developing fetuses during late gestation are 0.014 mmol/day (House & Bell 1993). Normal values of Mg in plasma for dairy cattle range between 0.7 and 1.2 according to Constable et al. (2017) or between 0.9-1.2 according to Martens et al. (2018). In the cerebrospinal fluid of cows, the Mg ranges from 0.62 to 0.81mmol/L (McCoy et al. 2001). The concentration of Mg in vitreous humor is 0.84 to 0.90mmol/L of Mg, and in the aqueous humor ranges from 0.73 to 0.79mmol/L (McCoy et al. 2001). Mg concentration in vitreous humor is stable for up to approximately 48h postmortem, which can be used in the postmortem diagnosis of hypomagnesemia (McCoy 2004). In the presence of mineral disorders, it is important to evaluate not only Mg but also Ca levels, because in general both minerals are low in blood (Reinhardt et al. 2011). In cases of Mg deficiency, low Ca values are attributed to hypomagnesemia (Van Mosel et al. 1991).

Hypomagnesemia affects cattle, sheep and goats, but cattle are more susceptible (Grunes et al. 1970). The annual morbidity and mortality registered in 120 dairy cattle farms in southwestern Victoria, Australia, was 2.1% and 0.53%, respectively (Harris et al. 1983). In England and Wales, a 7.8% mortality rate in in dairy cattle was recorded in 2004 (Watson et al. 2008), whereas in the province of Buenos Aires, Argentina, 3% and 4% mortality rates were registered in beef herds over periods of 15 and 20 years, respectively (Cseh & Crenovich 1996, Cantón et al. 2014). In Uruguay, outbreaks of hypomagnesemia have been recorded in beef cattle under stress caused by weaning, transportation and prolonged confinement without access to food or water (Dutra 2009, 2010).

Primary hypomagnesemia occurs when Mg deficit in the diet is less than 1.3g/kg dry matter (DM) (Ram et al. 1998), whereas secondary hypomagnesemia is due to low ruminal absorption of Mg despite adequate dietary concentration (Care et al. 1984). The rumen is the primary absorption site for Mg (Martens et al. 2018). The solubility and absorption of Mg decrease when the ruminal pH is less than 6.5 (Goff 2008). Potassium (K) ions interfere with ruminal absorption of Mg because it raises the apical membrane potential of the ruminal epithelial cells (Martens & Blume 1986, Martens & Schweigel 2000), increases the ruminal pH and causes ruminal and metabolic alkalosis (Fisher et al. 1994, Rérat et al. 2009). Therefore, high content of K in the diet affect the absorption of Mg in ruminants. Particularly when the K/(Ca+Mg) ratio is greater than 2.2, the diet is potentially tetanizing (Kemp & t'Hart 1957). Similar effects occur with phosphorous (P) and nitrogen (N) that also increase the ruminal pH and may form insoluble hydroxides with dietary Mg (Gabel & Martens 1986, Reinhardt et al. 1988). In contrast, fatty acids, such as linolenic, linoleic and palmitic, form insoluble salts with Mg decreasing its absorption in the rumen (Goff 2006). Transaconitate is a forage metabolite that chelates Mg and can contribute to its deficiency (Cook et al. 1994). Diets deficient in sodium (Na) and energy also negatively affect the active ruminal absorption of Mg (Martens 1985, Martens & Blume 1986).

Mg requirements increase during the end of gestation and the beginning of lactation because of the flow of Mg to the fetus and colostrum or milk (Cerbulis & Farrell Junior 1976, House & Bell 1993). Hypomagnesemia is more frequent in adult multiparous cows (Reinhardt et al. 2011), because Mg absorption and resorption in those animals are reduced (Van Mosel et al. 1991). Calves can be affected when consume only milk or milk replacers deficient in Mg (Naik et al. 2010, Constable et al. 2017).

Hypomagnesemia is also influenced by body condition. Cows with body condition greater than 3.75 (1 to 5 scale) increase lipolysis for energy supply (Rayssiguier 1977, Contreras et al. 2016, Alharthi et al. 2018). During lipolysis, catecholamines and adrenocorticotropic hormones stimulate Mg uptake by adipocytes (Elliott & Rizack 1974), since Mg is required by adenylate cyclase, ATPases, and lipases during this process (Rude 1998).

Hypomagnesemia is favored by environmental factors (Larvor 1976). In Argentina, winter and spring are seasons of higher risk for hypomagnesemia in grazing cattle (Cseh & Crenovich 1996). In general, the annual winter grasses (oat, wheat and ryegrass) are rich in K and deficient in Mg, therefore they pose a greater risk for grass tetany (Metson et al. 1966). The risk increases with fast growing forage, especially in the middle of autumn and early winter (Brizuela & Cseh 2003). Therefore, hypomagnesemia commonly occurs in cattle grazing perennial forages of autumn-winter growth (Metson et al. 1966) or lush annual forages such as ryegrass (*Lolium* sp.), wheat (Bohman et al. 1983), oat, and barley (Brizuela & Cseh

2003). These forages are rich in non-protein N that favor increased levels of ammonia and the ammonium ion, which precipitate Mg (Gabel & Martens 1986, Flores et al. 2014). The shortage of non-structural carbohydrates of these forages (Bohman et al. 1983, Chatterton et al. 1989, Chatterton et al. 2006, Cajarville et al. 2015) also contributes to the deficit of Mg since they facilitate Mg absorption (Giduck & Fontenot 1987).

Annual winter growing grasses also have high water content that increases the transit speed of the forage in the gastrointestinal tract and thus reduces the absorption of Mg in the rumen (Grunes et al. 1970, Maylan et al. 1976). Pasture management practices may affect the availability of Mg. For example, fumigation against broadleaf weeds reduces legumes with higher levels of Mg, such as clover and alfalfa (Jones 1963). In addition, the increase in animal density together with the excessive use of fertilizers containing N and K (Macdonald et al. 2017) reduces the availability of Mg to the plant and, consequently, the animal (Grunes et al. 1970, Fox & Piekielek 1984, Elliott 2008). Although hypomagnesemia is more common in winter and spring, it can also occur in autumn and summer (Allcroft & Burns 1968, Larvor 1976). The disease is exacerbated by stress conditions such as heat, overcrowding, transportation, changes in diet or starvation (Larvor 1976, Rayssiguier 1977).

Hypomagnesemia can be subclinical, with plasma Mg between 0.41-0.82 (Constable et al. 2017) or 0.7-0.8mmol/L (Martens at al. 2018), and clinical, with concentration on average of 0.21mmol/L (Constable et al. 2017). Clinical disease has three forms of presentation: acute, subacute and chronic (Smith & Edwards 1988). Acute illness is characterized by violent movements of the limbs, usually there is foam at the mouth and nostrils; sudden death can occur and is often the sole manifestation of the disease. Cows with subacute course show spasms in the face with continuous involuntary muscle movements. Some animals show nervousness, aggressiveness, teeth grinding, salivation or strong vocalization. Blindness, muscle spasms, excitability, rigid gait, dysmetria, and ataxia can be observed. Later they can remain in sternal or lateral recumbency, with rigid limbs, paddling movements or opisthotonos (Allcroft & Burns 1968, Martens & Schweigel 2000, McCoy et al. 2001, D'Angelo et al. 2015). Weight loss and decreased production are hallmarks of the chronic form of hypomagnesemia (Reinhardt et al. 1988, Smith & Edwards 1988).

Hypomagnesemia can be prevented by assessing the tetanizing potential of the forage before grazing (Kemp & t'Hart 1957, Brizuela & Cseh 2003). To prevent hypomagnesemia, it is necessary to supplement Mg in the diet by administering 0.35 to 0.40% Mg in the total dry ration from a source with adequate bioavailability (Goff 1999, Martens & Schweigel 2000, NRC 2001). Diets should be adjusted according to production requirements. Unnecessary management that imposes stress to the animals should be reduced, animals should not be transported in the last 6 weeks of gestation, and abrupt changes in diet should be avoided (Elliott 2009). Cattle consuming lush fast-growing grasses should be supplemented with Mg. Alternatively, farmers should implement the association of grasses with leguminous plants or add an appropriate amount of legume hay, that is rich in Mg, and provides fiber to decrease the transit speed of the forage through the digestive tract to improve ruminal Mg absorption (Smith & Edwards 1988, Muller 2003, Constable et al. 2017). It has been suggested that subclinical hypomagnesemia in the postpartum is a heritable trait (Tsiamadis et al. 2016).

Clinical cases of hypomagnesemia can be treated by administering Mg solutions intravenously or subcutaneously. Blood levels of Mg are recovered by administering 200ml of 50% Mg solution. Alternatively, 200 to 400ml of 25% solution of magnesium sulfate ($MgSO_4 7H_2O$) can be administered subcutaneously, a maximum of 50 to 100ml per site of application is recommended (Goff 1999). Clinical cases can be reduced by moving the herd or affected animals off the paddock and supplementing Mg in the diet (Sánchez 2000).

The objective of this work is to describe an outbreak of hypomagnesemia in dairy cattle in Uruguay and make recommendations for the prophylaxis and control of the disease.

MATERIALS AND METHODS

Nine lactating cows died acutely within 59 days (May 13-July 16, 2017) in a herd of 270 Holstein cows. Two cows with acute clinical signs that were in permanent recumbency and moribund were subjected to clinical examination, followed by euthanasia and necropsy. Tissue samples were collected and fixed in 10% buffered formalin, embedded in paraffin, cut into 4-5µm thick sections and stained with hematoxylin and eosin (HE) for microscopic examination.

Blood samples were collected from the two clinically affected cows and from 14 clinically healthy cows in the same herd under similar management and production conditions. The blood serum was separated and submitted to the clinical pathology section of the Direction of Veterinary Laboratories (Dirección de Laboratorios Veterinarios) (DILAVE) for determination of serum Ca by o-cresolphthalein complexone and 8-hydroxyquinoline procedures (Bazydlo et al. 2014). Total blood P was determined by the ammonium molybdate method measured at 340nm wave-length, and Mg concentration was assessed by the xylidyl blue reaction (Wiener lab®, Argentina) (Baginski et al. 1975, Bazydlo et al. 2014).

Tissue samples from lung, kidney, liver and cerebrospinal fluid (CSF) were subjected to microbiological culture on blood, chocolate and McConkey agars. The CSF was cultured in selective medium for *Listeria* spp. and incubated in a microaerophilic atmosphere for 48h at 37°C (Van Netten et al. 1989).

Oat grass samples (including 10 subsamples from each of two pastures A and B) were obtained for mineral evaluation. Pasture A was being grazed by the ill animals, and pasture B had previously been grazed by the same herd. Additionally, samples of the concentrate ration, corn silage and water were collected. These samples were assessed for P, Ca, Mg and K by atomic absorption, Mg and Ca by atomic emission, K by wet digestion and P by colorimetry at the Soil, Plant and Water Laboratories (INIA 2017) of the National Institute of Agricultural Research (INIA) La Estanzuela) (Jackson 1964). The P available in the water was determined by the digestion technique with ascorbic acid and colorimetry at the same laboratory.

Intake of DM was estimated based on the data obtained in the farm on diet and milk production, according to the NRC (2001). The mineral requirements (P, Ca, Mg, K and Na) of the cows and the water consumption were estimated from the NRC (2001). To calculate the mineral contributions, the ingested quantities of oat grass, corn silage, ration, water and the mineral supplement were considered. The difference contribution-requirements of the diet was calculated, and a correction of the deficiencies was formulated.

RESULTS

The outbreak occurred in a dairy farm located nearby Colonia Cosmopolita (latitude South 34°23'04", longitude West 57°24'01"), in the department of Colonia, Uruguay, in autumn-winter, from May 13 to July 16, 2017. The farm had 270 lactating Holstein cows managed in a single herd. The animals were consuming lush oat pastures in two daily grazing shifts, as well as corn silage and a ration consisting of ground corn kernels, ground barley grain, soybean meal, mineral supplement and monensin.

Mortality in the study period was 3.3%. The 9 dead cows were between 2 and 9-years of age (1st to 6th lactation), with 22 to 194 days of lactation and 15.8 to 31.4L of daily milk production. Two cows were found moribund. Cow #1131 was in right lateral recumbency and had opisthotonos, muscular spasms, profuse salivation and ruminal atony. The rectal temperature was normal (35.9°C), the respiratory rate was of 80 movements per minute (tachypnea) and the heart rate of 160 beats per minute (tachycardia). Cow #1175 was in sternal and later in lateral recumbency, with weakness, sialorrhea and comatose state.

The animals grazed growing oat pasture in two daily grazing shifts and consumed 15kg of corn silage administered in the paddock, and 4kg of ration/cow/day (administered in the dairy, 2kg per milking shift). The daily concentrate ration per cow was composed of 2.345kg of ground corn grain, 0.781kg of soybean meal, 0.781kg of milled grain barley, 0.09kg of commercial mineral supplement, and 0.0015kg of a commercial product containing 20% monensin.

Table 1 shows the data and the results of the evaluation of P, Ca and Mg in the serum of two clinically-affected cows and 14 healthy cows within 42 to 132 days of lactation and milk production between 17 and 32.2L per day. Cow #1131 with clinical signs, that had not received any medical treatment, had low serum levels of Mg and Ca. Cow #1715 with clinical signs, had been treated with a solution of Ca and Mg, and with methylene blue. In this cow, the serum Mg values were within normal parameters, but the values of Ca and P were low. The serum values of these minerals in the 14 clinically healthy untreated cows were distributed as follows: four cows had low values of Mg only, four cows had low values of Mg and Ca, two cows had low values of Ca and P, and four cows showed normal values of the three minerals.

The dietary values of minerals are shown in Table 2. The tetanizing potential was calculated by the K/(Ca+Mg) ratio of each component of the diet. The tetanizing potential of the oat forages (pastures A and B), corn silage and the ration were 5.10, 7.73, 2.45 and 0.85, respectively. The Ca:P ratio was 0.87 on average of components of the diet.

Table 3 shows the estimate of DM intake according to the components of the diet at the time of the visit to the farm. A daily consumption of 19.6 kg was estimated. Mineral concentrations in water were as follows: Ca=53.4mg/L, Mg=30.1mg/L, K=12.5mg/L and P=10.3mg/L.

The contributions of the total absorbed minerals of the diet, including Ca, P, Mg, K and Na are shown in Table 4. Daily deficiency was found in the contribution of Mg (-0.36g/day), Na (-25.2g/day) and Ca (-9.27g/day). K (184.42g/day) and P (12.81g/day) were in excess, and the Ca:P ratio was 0.71 (Table 4).

From the results of the difference of the contribution-requirement the amounts of magnesium oxide (MgO), calcium carbonate ($CaCO_3$) and sodium chloride (NaCl) that would be necessary to add in the diet as sources of Mg, Ca and Na, respectively, were calculated to balance the deficiencies. The daily addition per cow of 80g of MgO, 80g of $CaCO_3$ and 30g of NaCl in the diet resolved the deficits of the respective minerals (Table 5).

Table 6 shows the balance of Mg, Ca and Na minerals in the diet after making the respective corrections. The difference of

Table 1. Epidemiological data and blood serum levels of Ca, P and Mg of lactating cows with clinical signs or clinically healthy cows

Ear tag number	Age (years)	Number of lactations	Days in milk	Production average (L)	Calcium (mmol/L)	Phosphorus (mmol/L)	Magnesium (mmol/L)	Clinical signs
1131	9	6	48	26.8	**0.81**[a]	1.85	**0.5**	Yes
1715	6	5	194	29.2	**0.92**	**0.83**	0.94	Yes
1190	8	6	132	23.6	**1.51**	**1.21**	0.82	No
1314	5	4	69	31.2	**1.89**	1.84	**0.37**	No
1912	2	1	112	15	2.18	1.77	**0.42**	No
1590	4	3	42	32.2	2.16	1.72	**0.62**	No
1918	2	1	117	21.6	**1.91**	2.09	**0.25**	No
1950	4	2	107	30.2	2.22	1.82	0.94	No
1959	2	1	112	21.4	2.35	2.08	0.71	No
1972	2	1	112	15	**2.07**	**1.29**	0.88	No
1990	2	1	118	18.6	2.22	1.52	**0.68**	No
2000	2	1	123	17	2.37	1.57	0.88	No
1290	7	4	70	31	**2.09**	2.44	**0.41**	No
9919	2	1	120	22	2.12	1.70	0.75	No
1649	3	1	114	21	2.30	1.72	**0.41**	No
1446	5	3	90	24.2	**1.90**	1.52	**0.44**	No
Reference ranges (Constable et al. 2017)					2.1-2.6	1.4-2.6	0.7-1.2	

[a] The values in bold are below the reference range.

Table 2. Ca, P, Mg and K values, tetanizing potential, and Ca:P ratio in the diet

Diet component	Ca (%)	P (%)	Mg (%)	K (%)	K/(Ca+Mg) ratio	Ca:P ratio
Oat pasture A	0.28	0.32	0.13	2.07	5.10	0.86
Oat pasture B	0.30	0.40	0.14	3.41	7.73	0.75
Corn silage	0.24	0.26	0.15	0.95	2.45	0.91
Ration	0.63	0.68	0.17	0.69	0.85	0.94

Table 3. Dry matter intake considering all components of the diet

Diet component	% in the diet (DM basis)	kg DM/day
Lush oat pasture	64.51	12.62
Corn silage	19.82	3.88
Ground corn grain	8.00	1.57
Barley grain	3.63	0.71
Soybean meal	3.57	0.70
Mineral supplement[a]	0.46	0.09
Monensin[b]	0.01	0.0015

[a] Commercial formula reports: 9% of P, 18.8% of Ca, 1% of Mg, 12% of Na and 0% K, [b] commercial product with 20% monensin; DM = dry matter.

Table 4. Difference of the contribution-requirement of minerals of the original diet

	Ca	P	Mg	K	Na
Total absorbed mineral requirements (TAM g/day)	49.3	43.2	5.5	179.3	38.2
Gross diet contribution (g/day)	81.6	83.9	29.4	403.3	14.4
Diet contribution (TAM g/day)	38	56	4.7	362.9	13.0
Contribute water (TAM g/day)	2.03	0.01	0.44	0.82	
Difference Contribution-Requirement (g/day)	**-9.27**[a]	**12.81**	**-0.36**	**184.42**	**-25.2**
Ration density	0.42	0.43	0.15	2.06	0.07

[a] The values highlighted in bold are in excess or deficiency, the latter with the minus sign.

Table 5. Composition of the reformulated diet to correct Mg, Ca and Na deficiencies

Diet component	%DM	Kg DM/day
Lushreen oat pasture	64.51	12.62
Corn silage	19.82	3.88
Ground corn grain	8.00	1.57
Barley grain	3.63	0.71
Harina de soja	3.57	0.70
Mineral supplement	0.46	0.09
MgO	0.40	0.08
$CaCO_3$	0.40	0.08
NaCl	0.15	0.03

Table 6. Difference of the contribution-requirement of minerals in the reformulated diet

	Ca	P	Mg	K	Na
Total absorbed mineral requirements (TAM g/day)	49.3	43.2	5.5	179.3	38.2
Gross diet contribution (g/day)	95.7	83.9	74.4	403.3	45.9
Diet contribution (TAM g/day)	47.5	56	36.2	363	41.3
Contribute water (TAM g/day)	2.03	0.01	0.44	0.82	
Difference contribution-requirement (g/day)	**0.23**[a]	12.81	**31.14**	184.52	**3.10**
Ration density	0.48	0.42	0.38	2.04	0.23

[a] The values of Mg, Ca and Na minerals in bold were corrected.

the contribution-requirement is adequate for Ca (0.23g/day), Mg (0.31g/day) and Na (3.1g/day). The Ca:P ratio increased to 0.88.

At necropsy, both carcasses were in acceptable body condition score (3.5 on a scale of 1 to 5), with adequate fat reserves and moderate sinking of the eyes in the orbit (dehydration). In 80% of the endocardial surface, ecchymosis and suffusions were observed. In the epicardium as well as the subcutaneous tissue, there were multiple petechiae. The urinary bladder was moderately distended with dark amber urine. No remarkable lesions were observed in other organs.

Microscopically, the epicardial and endocardial hemorrhages observed at necropsy were confirmed and the cardiac muscle

revealed acute mild multifocal hydropic degeneration. There were no lesions of diagnostic importance in the other tissues examined. No pathogenic bacteria were isolated from lung, kidney, liver and CSF.

DISCUSSION

The diagnosis of hypomagnesemia in the outbreak described herein was based on epidemiological and clinical findings and confirmed by determination of low serum Mg values and by the analysis of the diet, which showed low concentrations of Mg and Na, high values of K and P, and a K/(Ca+Mg) ratio higher than 2.2. Serum Mg values within the normal range in one of the clinically ill cows can be explained by the treatment with a calcium and magnesium solution before death (Goff 1999, Martín-Tereso & Martens 2014).

Subclinical hypomagnesemia, with Mg values between 0.41-0.82 (Constable et al. 2017) or 0.7-0.8mmol/L (Martens et al. 2018) and clinical hypomagnesemia with values on average of 0.21mmol/L (Constable et al. 2017) have primary and secondary causes. The primary cause is the deficit in dietary Mg supply (Ram et al. 1998). Secondary hypomagnesemia might be triggered by high values of K, protein or non-protein N and low concentration of soluble carbohydrates (Bohman et al. 1983). In the current report, the estimated difference in dietary mineral contribution-requirement shows that the intake of Mg, Ca and Na were deficient according to the requirements (NRC 2001). The cows were fed lush oat pasture and corn silage, both deficient in Mg, which may have contributed to a primary Mg deficit. On the other hand, K values were high in both lush oat pastures and in corn silage, which probably contributed secondarily to the occurrence of the disease. The K/(Ca+Mg) ratio higher than 2.2 found in this work is an indicator of the tetanizing potential of forages (Kemp & t'Hart 1957). The diet that was consuming the herd had low concentrations of Mg and high values of K, what suggests that the absorption of the Mg may have been drastically diminished (Schonewille et al. 2008). P was elevated in the diet, which also interferes with Mg absorption (Reinhardt et al. 1988). In addition, the Na was below the recommended percentage for the species (NRC 2001). The low supply of Na in the diet reduces the absorption of Mg in the rumen (Goff 2014) by decreasing the active transport of Mg bound to Na (Martens et al. 1987). Other factors that contribute to secondary hypomagnesemia, which were not evaluated in this outbreak, are the high levels of protein and non-protein N that occur in the lush winter forages. The green oat forages have high values of protein (6.9-30.8%) (Pordomingo et al. 2007) and N (1.86-2.32%) (Flores et al. 2014) that favor the formation of ammonium and ammonia ions that interfere with Mg absorption (Care et al. 1984). Green oat forages also have low values of soluble carbohydrates (5.7% to 16.8%) (Pordomingo et al. 2007) which reduces the active transport of Mg (Martens 1985).

The epidemiological data of the outbreak indicate that several risk factors may have predisposed to hypomagnesemia. The herd consisted of high-producing lactating cows, which excrete around 116mg/L daily of Mg (Van Hulzen et al. 2009). Some of the deceased cows were within the oldest of the herd, from 6 to 9 years-old, age at which the absorption capacity of dietary Mg decreases (Van Mosel et al. 1991). In addition, the outbreak occurred in autumn and the beginning of winter, the time of greatest risk for elevated tetanizing potential of the fast growing winter forages, and probably by stressing climate changes such as intense cold, rain and cloudy days (Larvor 1976, Brizuela & Cseh 2003).

The blood Ca values in the clinically-affected and some of the healthy cows were below the normal range (2.1-2.6mmol/L) (Constable et al. 2017) but none had clinical hypocalcemia that occurs with values lower than 1.38mmol/L (Goff 2014). Sudden death, the acute neurological clinical manifestation in this outbreak, and the stage of lactation of the affected cows were not consistent with those of puerperal hypocalcemia. Most likely subclinical hypocalcemia in these cows was a consequence of hypomagnesemia, as previously reported (Van Mosel et al. 1991). Even though the Ca:P ratio was suboptimal, it has been shown that in the presence of low Mg values the production of parathormone and the sensitivity of bone cells to this hormone are reduced, leading to hypocalcemia (Fatemi et al. 1991, Kopic & Geibel 2013). In this outbreak, acute clinical signs and death occurred primarily due to hypomagnesemia, confirmed by serum Mg concentrations lower than 0.5mmol/L, as that found in experimental hypomagnesemia (McCoy et al. 2001).

Hypomagnesemia occurs frequently in New Zealand (Metson et al. 1966), Australia (Harris et al. 1983), Canada (Odette 2005) and Argentina (Cseh & Crenovich 1996, Cantón et al. 2014). In New Zealand, the intensification of grazing livestock improves milk production per cow, per hectare, and profitability, but requires greater forage production, which is achieved by applying fertilizers such as N and K (Macdonald et al. 2017). The increase of N and K in the soil is reflected in the forages and decreases the absorption and availability of Mg for the animals (Metson et al. 1966, Elliott 2008). Thus, supplementation with Mg is necessary to prevent hypomagnesemia (Goff 2006, Zelal 2017, Martens et al. 2018). In Uruguay, hypomagnesemia has been described in recently weaned beef cows locked in pens for long periods without access to water or food (Dutra 2009, 2010). We do not find publications on hypomagnesemia in dairy cattle in Uruguay, but it is possible that the disease is underdiagnosed. In recent years dairy farming in Uruguay has undergone a process of intensification (Inale 2014, Uruguay 2016), local dairy farmers and veterinary practitioners alike should be aware of the risk of hypomagnesemia in high-producing grazing dairy systems.

The recommendation to correct the dietary deficiencies of Mg, Ca and Na was based on the detected values of such minerals in the diet. The lush oat pastures used as the basis of the diet in the current outbreak were deficient in Mg, rich in K, the Ca:P ratio was lower than 1 and had also low levels of Na. The forages of autumn-winter lush growth contribute between 0.14% and 0.19% Mg in DM (Metson et al. 1966, Brizuela & Cseh 2003, Blackwood 2007), considering that K values were greater than 1%, it is recommended that 0.35% Mg be supplied in the total ration on DM basis (Schonewille et al. 2008, Martín-Tereso & Martens 2014). A cow that produces 20L of milk daily requires 70g of Mg daily (NRC 2001). The diet and water that the cows were consuming in this outbreak contributed approximately 30g of Mg per cow daily, so 40g of Mg were deficient and had to be supplemented in the reformulated diet. From 65 to 75g of MgO per cow daily as source of Mg was provided to correct the deficiency (Urdaz et al. 2003).

The occurrence of an outbreak of hypomagnesemia in a dairy farm that applies similar technology to many other dairy farms in Uruguay suggests that nutritional practices should be recommended for the prevention of this disease in the country. To administer the MgO preparing a ration that includes the amounts mentioned above is suggested. Another strategy is to supplement part of the MgO in the ration and the remaining in the mineral salt. In this case, 45g of MgO should be mixed in the ration and a mixture of 75% NaCl and 25% MgO should be offered *at libitum* concomitantly. This improves palatability and provides a source of Na. When Mg is added to the diet or salt, the DM and mineral salt intake should be monitored since MgO lacks palatability (Zelal 2017). The deficiency of Na can be corrected by adding 0.5% NaCl and the Ca:P ratio is improved by adding 1 to 1.5% $CaCO_3$ to the ration on DM basis, especially when commercial pellets with grains or by-products are used (corn, sorghum, bran of wheat or rice, soybean flour or expeller, corn lex) instead of balanced ration. Finally, it is recommended that each farm owner evaluates the situation of dietary contributions and requirements of cattle in production.

CONCLUSIONS

Hypomagnesemia is a cause of mortality in lactating dairy cattle in Uruguay and should be included in the differential diagnosis of acute neurological clinical signs and/or sudden death.

The risk for hypomagnesemia is increased when the base of the diet are lush grasses of fast autumn and winter growth, with low Mg and high K levels.

Hypomagnesemia can be prevented by an appropriate diet formulation.

Acknowledgements.- We thank Dr. Gonzalo Uriarte from the Division of Veterinary Laboratories (División de Laboratorios Veterinarios) (DILAVE) and Andrés Beretta from INIA´s Soil Plant and Water Laboratories (Laboratorio de Suelos, Plantas y Agua) for biochemical testing on various samples, and Dr. Martin Karlen for providing clinical and epidemiological data. We also thank Yisell Perdomo from INIA for assistance with histologic techniques.

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

REFERENCES

Alfrey A.C. & Miller N.L. 1973. Bone magnesium pools in uremia. J. Clin. Invest. 52(12):3019-3027. <http://dx.doi.org/10.1172/JCI107500> <PMid:4584344>

Allcroft R. & Burns K.N. 1968. Hypomagnesaemia in cattle. N.Z. Vet. J. 16(7):109-128. <http://dx.doi.org/10.1080/00480169.1968.33757> <PMid:4884842>

Alharthi A., Zhou Z., Lopreiato E., Trevisi E. & Loor J.L. 2018. Body condition score prior to parturition is associated with plasma and adipose tissue biomarkers of lipid metabolism and inflammation in Holstein cows. J. Anim. Sci. Biotechnol. 9(1):1-12. <http://dx.doi.org/10.1186/s40104-017-0221-1> <PMid:29387386>

Baginski E.S., Epstein E. & Zak B. 1975. Review of phosphate methodologies. Ann. Clin. Lab. Sci. 5(5):399-416. <PMid:1180482>

Bazydlo L.A.L., Needham M. & Harris N.S. 2014. Calcium, magnesium, and phosphate. Lab. Med. 45(1):e44-e50. <http://dx.doi.org/10.1309/LMGLMZ8CIYMFNOGX>

Blackwood I. 2007. Mineral content of common ruminant stockfeeds, crops and pastures. Primefact 522, NSW Department of Primary Industries. Orange 7p.

Bohman V.R., Horn F.P., Stewart B.A., Mathers A.C. & Grunes D.L. 1983. Wheat pasture poisoning. I. An evaluation of cereal pastures as related to tetany in beef cows. J. Anim. Sci. 57(6):1352-1363. <http://dx.doi.org/10.2527/jas1983.5761352x> <PMid:6674278>

Brizuela M.A. & Cseh S.B. 2003. Composición mineral y potencial tetanizante de verdeos de invierno sembrados en diferentes fechas. Revta Arg. Prod. Anim. 23(2):91-101.

Cajarville C., Britos A., Errandonea N., Gutiérrez L., Cozzolino D. & Repetto J.L. 2015. Diurnal changes in water-soluble carbohydrate concentration in lucerne and tall fescue in autumn and the effects on in vitro fermentation. N. Z. J. Agricult. Res. 58(3):281-291. <http://dx.doi.org/10.1080/00288233.2015.1018391>

Cantón G., Odriozola E. & Cseh S. 2014. Análisis de casos de hipomagnesemia en bovinos de producción de carne diagnosticados en INTA EEA Balcarce (1998-2013). XX Reunión Científica Técnica, Tucumán, Argentina. (Resumen)

Care A.D., Brown R.C., Farrar A.R. & Pickard D.W. 1984. Magnesium absorption from the digestive tract of sheep. Q. J. Exp. Physiol. 69(3):577-587. <http://dx.doi.org/10.1113/expphysiol.1984.sp002844> <PMid:6473696>

Cerbulis J. & Farrell Junior J. 1976. Composition of the milk of dairy cows. II. Ash, calcium, magnesium, and phosphorous. J. Dairy Sci. 59(4):589-593. <http://dx.doi.org/10.3168/jds.S0022-0302(76)84245-2> <PMid:1262573>

Chatterton N.J., Harrison P.A., Bennett J.H. & Asay K.H. 1989. Carbohydrate partitioning in 185 accessions of Gramineae grown under warm and cool temperatures. J. Plant Physiol. 134(2):169-179. <http://dx.doi.org/10.1016/S0176-1617(89)80051-3>

Chatterton N.J., Watts K.A., Jensen K.B., Harrison P.A. & Horton W.H. 2006. Nonstructural carbohydrates in oat forage. J. Nutr. 136(Suppl.7):2111S-2113S. <http://dx.doi.org/10.1093/jn/136.7.2111S> <PMid:16772513>

Constable P.D., Hinchcliff K.W., Done S.H. & Grünberg W. 2017. Veterinary Medicine. 11th ed. Saunders Elsevier, St Louis, Missouri, p.1662-1706.

Contreras G.A., Thelen K., Schmidt S.E., Strieder-Barboza C., Preseault C.L., Raphael W., Kiupel M., Caron J. & Lock A.L. 2016. Adipose tissue remodeling in late-lactation dairy cows during feed restriction-induced negative energy balance. J. Dairy Sci. 99(12):1-13. <http://dx.doi.org/10.3168/jds.2016-11552> <PMid:27720147>

Cook G.M., Wells J.E. & Russell J.B. 1994. Ability of *Acidaminococcus fermentans* to oxidize *trans*-aconitate and decrease the accumulation of tricarballylate, a toxic end product of ruminal fermentation. Appl. Environ. Microbiol. 60(7):2533-2537. <PMid:8074529>

Crawford R.J., Masie M.D., Sleper D.A. & Mayland H.F. 1998. Use of an experimental high-magnesium tall fescue to reduce grass tetany in cattle. J. Prod. Agricult. 11(4):491-496. <http://dx.doi.org/10.2134/jpa1998.0491>

Cseh S.B. & Crenovich H. 1996. Hipomagnesemia en el sudeste de la provincia de Buenos Aires, Argentina. Arch. Med. Vet. 28(2):111-116.

D'Angelo A., Bellino C., Bertone I., Cagnotti G., Iulini B., Miniscalco B., Casalone C., Gianella P. & Cagnasso A. 2015. Seizure disorders in 43 cattle. J. Vet. Intern. Med. 29(3):967-971. <http://dx.doi.org/10.1111/jvim.12592> <PMid:25857732>

Dutra F. 2009. Tetania del transporte. Arch. Vet. Este Ururguay 2:6-7.

Dutra F. 2010. Tetania del destete. Arch. Vet. Este Ururguay 2:10-11.

Elliott D.A. & Rizack M.A. 1974. Epinephrine and adrenocorticotropic hormone-stimulated magnesium accumulation in adipocytes and their plasma membranes. J. Biol. Chem. 249(12):3985-3990. <PMid:4365743>

Elliott M. 2008. Grass tetany in cattle: predicting its likelihood. Primefact 785. NSW Department of Primary Industries, Orange. 6p.

Elliott M. 2009. Grass tetany in cattle: treatment and prevention. Primefact 421. NSW Department of Primary Industries, Orange. 4p.

Fatemi S., Ryzen E., Flores J., Endres D.B. & Rude R.K. 1991. Effect of experimental human magnesium depletion on parathyroid hormone secretion and 1.25-dihydroxyvitamin D metabolism. J. Clin. Endocrinol. Metabol. 73(5):1067-1072. <http://dx.doi.org/10.1210/jcem-73-5-1067> <PMid:1939521>

Fisher L.J., Dinn N., Tait R.M. & Shelford J.A. 1994. Effect of level of dietary potassium on the absorption and excretion of calcium and magnesium by lactating cows. Can. J. Anim. Sci. 74(3):503-509. <http://dx.doi.org/10.4141/cjas94-071>

Flores F.E., Casillas H.M., Figueroa U.V. & Potisek M.C.T. 2014. Disponibilidad de nitrógeno y desarrollo de avena forrajera (*avena sativa* l.) con aplicación de biosólidos. Revta Terra Latinoamericana 32:99-105.

Fox R.H. & Piekielek W.P. 1984. Soil magnesium level, corn (*Zea mays* L.) yield, and magnesium uptake. Commun. Soil Sci. Plan. 15(2):109-123. <http://dx.doi.org/10.1080/00103628409367459>

Gabel G. & Martens H. 1986. The effect of ammonia on magnesium metabolism in sheep. J. Anim. Physiol. Anim. Nutr., Berlin 55(1/5):278-287. <http://dx.doi.org/10.1111/j.1439-0396.1986.tb00729.x>

Giduck S.A. & Fontenot J.P. 1987. Utilization of magnesium and other macrominerals in sheep supplemented with different readily-fermentable carbohydrates. J. Anim. Sci. 65(6):1667-1673. <http://dx.doi.org/10.2527/jas1987.6561667x> <PMid:3443586>

Goff J.P. 1999. Treatment of calcium, phosphorus, and magnesium balance disorders. Vet. Clin. N. Am., Food Anim. Pract. 15(3):619-639. <http://dx.doi.org/10.1016/S0749-0720(15)30167-5> <PMid:10573815>

Goff J.P. 2006. Macromineral physiology and application to the feeding of the dairy cow for prevention of milk fever and other periparturient mineral disorders. Anim. Feed Sci. Technol. 126(3/4):237-257. <http://dx.doi.org/10.1016/j.anifeedsci.2005.08.005>

Goff J.P. 2008. The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. Vet. J. 176(1):50-57. <http://dx.doi.org/10.1016/j.tvjl.2007.12.020> <PMid:18342555>

Goff J.P. 2014. Calcium and magnesium disorders. Vet. Clin. N. Am., Food Anim. Pract. 30(2):359-381, vi. <http://dx.doi.org/10.1016/j.cvfa.2014.04.003> <PMid:24980727>

Grunes D.L., Stout P.R. & Brownell J.R. 1970. Grass tetany of ruminants. Adv. Agron. 22:331-374. <http://dx.doi.org/10.1016/S0065-2113(08)60272-2>

Harris D.J., Lambell R.G. & Oliver C.J. 1983. Factors predisposing dairy and beef cows to grass tetany. Aust. Vet. J. 60(8):230-234. <http://dx.doi.org/10.1111/j.1751-0813.1983.tb05970.x> <PMid:6639526>

House W.A. & Bell A.W. 1993. Mineral accretion in the fetus and adnexa during late gestation in Holstein cows. J. Dairy Sci. 76(10):2999-3010. <http://dx.doi.org/10.3168/jds.S0022-0302(93)77639-0> <PMid:8227626>

Inale 2014. Encuesta lechera. Instituto Nacional de la Leche, Montevideo. Available at <https://www.inale.org/historico/wp-content/uploads/2018/08/Encuesta-lechera-2014.pdf> Accessed on Jun. 3, 2018.

INIA. Laboratorio de Suelos, Plantas y Aguas. 2017. Plantas y Aguas. Carpeta de técnicas oficiales. La Estanzuela, Uruguay.

Jackson M.L. 1964. Análisis Químico de Suelos. Ed. Omega, Barcelona. 662p.

Jones E. 1963. Studies on the magnesium content of mixed herbage and some individual grass and clover species. J. Brit. Grassl. Soc. 18(2):131-138.

Kemp A. & t'Hart M.L. 1957. Grass tetany in grazing milking cows. Neth. J. Agric. Sci. 5:4-17.

Kemp A., Deys W.B., Hemkes O.J. & Vann Es A.J.H. 1961. Hypomagnesaemia in milking cows: intake and utilization of magnesium from herbage by lactating cows. Neth. J. Agric. Sci. 9(2):134-149.

Kopic S. & Geibel J.P. 2013. Gastric acid, calcium absorption, and their impact on bone health. Physiol. Rev. 93(1):189-268. <http://dx.doi.org/10.1152/physrev.00015.2012> <PMid:23303909>

Larvor P. 1976. 28Mg kinetics in ewes fed normal or tetany prone grass. Cornell Vet. 66(3):413-429. <PMid:954445>

Li F.Y., Chaigne-Delalande B., Kanellopoulou C., Davis J.C., Matthews H.F., Douek D.C., Cohen J.I., Uzel G., Su H.C. & Lenardo M.J. 2011. Second messenger role for Mg2+ revealed by human T-cell immunodeficiency. Nature 475(7357):471-476. <http://dx.doi.org/10.1038/nature10246> <PMid:21796205>

MacDonald K.A., Penno J.W., Lancaster J.A.S., Bryant A.M., Kidd J.M. & Roche J.R. 2017. Production and economic responses to intensification of pasture-based dairy production systems. J. Dairy Sci. 100(8):6602-6619. <http://dx.doi.org/10.3168/jds.2016-12497> <PMid:28601460>

Martens H. 1985. The effect of dinitrophenol on magnesium transport across an isolated preparation of sheep rumen epithelium. Q. J. Exp. Physiol. 70(4):567-573. <http://dx.doi.org/10.1113/expphysiol.1985.sp002943> <PMid:3001811>

Martens H. & Blume I. 1986. Effect of intraruminal sodium and potassium concentrations and of the transmural potential difference on magnesium absorption from the temporarily isolated rumen of sheep. Q. J. Exp. Physiol. 71(3):409-415. <http://dx.doi.org/10.1113/expphysiol.1986.sp002999> <PMid:3763804>

Martens H. & Schweigel M. 2000. Pathophysiology of grass tetany and other hypomagnesemias. Implications for clinical management. Vet. Clin. N. Am., Food Anim. Pract. 16(2):339-368. <http://dx.doi.org/10.1016/S0749-0720(15)30109-2> <PMid:11022344>

Martens H., Kubel O.W., Gäbel G. & Honi H. 1987. Effects of low sodium intake on magnesium metabolism in sheep. J. Agric. Sci. 108(1):237-243. <http://dx.doi.org/10.1017/S0021859600064315>

Martens H., Leonhard-Marek S., Röntgen M. & Stumpff F. 2018. Magnesium homeostasis in cattle: absorption and excretion. Nutr. Res. Rev. 31(1):1-17. <http://dx.doi.org/10.1017/S0954422417000257> <PMid:29318981>

Martín-Tereso J. & Martens H. 2014. Calcium and magnesium physiology and nutrition in relation to the prevention of milk fever and tetany (dietary management of macrominerals in preventing disease). Vet. Clin. N. Am., Food Anim. Pract. 30(3):643-670. <http://dx.doi.org/10.1016/j.cvfa.2014.07.007> <PMid:25245611>

Maylan H.F., Grunes D.L. & Lazar V.A. 1976. Grass tetany hazard of cereal forages based upon chemical composition. Agron. J. 68(4):665-667. <http://dx.doi.org/10.2134/agronj1976.00021962006800040033x>

McCoy M.A., Hutchinson T., Davison G., Fitzpatrick D.A., Rice D.A. & Kennedy D.G. 2001. Postmortem biochemical markers of experimentally induced hypomagnesaemic tetany in cattle. Vet. Rec. 148(9):268-273. <http://dx.doi.org/10.1136/vr.148.9.268> <PMid:11292087>

McCoy M.A. 2004. Hypomagnesemia and new data on vitreous humor magnesium concentration as a post-mortem marker in ruminants. Magnes Res. 17(2):137-145. <PMid:15319147>

Metson A.J., Saunders W.M.H., Collie T.W. & Graham V.W. 1966. Chemical composition of pastures in relation to grass tetany in beef breeding cows. N.Z. J. Agric. Res. 9(2):410-436. <http://dx.doi.org/10.1080/00288233.1966.10420793>

Meyer H. 1977. Pathogenesis of the clinical symptoms of hypomagnesaemia in ruminants. Vet. Sci. Commun. 1(1):43-50. <http://dx.doi.org/10.1007/BF02267632>

Möykkynen T., Uusi-Oukari M., Heikkila J., Lovinger D.M., Lüddens H. & Korpi E.R. 2001. Magnesium potentiation of the function of native and recombinant GABA (A) receptors. Neuroreport 12(10):2175-2179. <http://dx.doi.org/10.1097/00001756-200107200-00026> <PMid:11447329>

Muller L.D. 2003. Supplementation of lactating cows on pastures. Available at <https://extension.psu.edu/supplementation-of-lactating-cows-on-pasture> Accessed on Jun. 3, 2018.

Naik S.G., Ananda K.J. & Rani B.K. 2010. Magnesium deficiency in young calves and its management. Vet. World 3(4):192-193.

NRC 2001. Nutrient Requirements of Dairy Cattle. 7th ed. National Academy Press, Washington, DC. 405p.

Odette O. 2005. Grass tetany in a herd of beef cows. Can. Vet. J. 46(8):732-734. <PMid:16187719>

Pordomingo A.J., Quiroga A., Jonas O., Santucho G., Otamendi H., Buffa H.G., Rolheiser D.O. & Albertario P. 2007. Producción y valor nutritivo de verdeos de invierno en siembra directa. Available at <https://www.engormix.com/ganaderia-carne/articulos/valor-nutritivo-verdeos-invierno-siembra-directa-t27006.htm> Accessed on Aug. 20, 2018.

Ram L., Schonewille J.T., Martens H., Van't Klooster A.T. & Beynen A.C. 1998. Magnesium absorption by wethers fed potassium bicarbonate in combination with different dietary magnesium concentrations. J. Dairy Sci. 81(9):2485-2492. <http://dx.doi.org/10.3168/jds.S0022-0302(98)70140-7> <PMid:9785240>

Rayssiguier Y. 1977. Hypomagnesemia resulting from adrenaline infusion in ewes: its relation to lipolysis. Horm. Metab. Res. 9(4):309-314. <http://dx.doi.org/10.1055/s-0028-1093519> <PMid:892696>

Rayssiguier Y. & Larvor P. 1978. Mineral bone composition and some elements of calcium metabolism in magnesium-deficient growing rats. Ann. Biol. Anim. Bioch. Biophys. 18(1):157-166. <http://dx.doi.org/10.1051/rnd:19780120>

Reinhardt T.A., Lippolis J.D., McCluskey B.J., Goff J.P. & Horst R.L. 2011. Prevalence of subclinical hypocalcemia in dairy herds. Vet. J. 188(1):122-124. <http://dx.doi.org/10.1016/j.tvjl.2010.03.025> <PMid:20434377>

Reinhardt T.A., Horst R.L. & Goff J.P. 1988. Calcium, phosphorus, and magnesium homeostasis in ruminants. Vet. Clin. N. Am., Food Anim. Pract. 4(2):331-350. <http://dx.doi.org/10.1016/S0749-0720(15)31052-5> <PMid:3061612>

Rérat M., Philipp A., Hess H.D. & Liesegang A. 2009. Effect of different potassium levels in hay on acid-base status and mineral balance in periparturient dairy cows. J. Dairy Sci. 92(12):6123-6133. <http://dx.doi.org/10.3168/jds.2009-2449> <PMid:19923615>

Rude R.K. 1998. Magnesium deficiency: a cause of heterogeneous disease in humans. J. Bone Miner. Res. 13(4):749-758. <http://dx.doi.org/10.1359/jbmr.1998.13.4.749> <PMid:9556074>

Sánchez J.M. 2000. Hipomagnesemia. Un desbalance metabólico subestimado en la producción de ganado lechero en Costa Rica. Nut. Anim. Trop. 6(1):75-95.

Schonewille J., Everts H., Jittakhot S. & Beynen A.C. 2008. Quantitative prediction of magnesium absorption in dairy cows. J. Dairy Sci. 91(1):271-278. <http://dx.doi.org/10.3168/jds.2007-0304> <PMid:18096949>

Smith R.A. & Edwards W.C. 1988. Hypomagnesemic tetany of ruminants. Vet. Clin. N. Am., Food Anim. Pract. 4(2):365-377. <http://dx.doi.org/10.1016/S0749-0720(15)31054-9> <PMid:3061613>

Storry J.E. & Rook J.A.F. 1962. The magnesium nutrition of the dairy cow in relation to the development of hypomagnesaemia in the grazing animal. J. Sci. Food Agric. 13(12):621-627. <http://dx.doi.org/10.1002/jsfa.2740131201>

Tsiamadis V., Banos G., Panousis N., Kritsepi-Konstantinou M., Arsenos G. & Valergakis G.E. 2016. Genetic parameters of subclinical macromineral disorders and major clinical diseases in postparturient Holstein cows. J. Dairy Sci. 99(11):1-14. <http://dx.doi.org/10.3168/jds.2015-10789> <PMid:27614830>

Urdaz J.H., Santos J.E., Jardon P. & Overton M.W. 2003. Importance of appropriate amounts of magnesium in rations for dairy cows. J. Am. Vet. Med. Assoc. 222(11):1518-1523. <http://dx.doi.org/10.2460/javma.2003.222.1518> <PMid:12784955>

Van Hulzen K.J., Sprong R.C., van der Meer R. & van Arendonk J.A. 2009. Genetic and nongenetic variation in concentration of selenium, calcium, potassium, zinc, magnesium, and phosphorus in milk of Dutch Holstein-Friesian cows. J. Dairy Sci. 92(11):5754-5759. <http://dx.doi.org/10.3168/jds.2009-2406> <PMid:19841235>

Van Mosel M., Van't Klooster A.T. & Wouterse H.S. 1991. Effects of a deficient magnesium supply during the dry period on bone turnover of dairy cows at parturition. Vet. Q. 13(4):199-208. <http://dx.doi.org/10.1080/01652176.1991.9694309> <PMid:1776234>

Van Netten P., Perales I., van de Moosdijk A., Curtis G.D. & Mossel D.A. 1989. Liquid and solid selective differential media for the detection and enumeration of *L. monocytogenes* and other *Listeria* spp. Int. J. Food Microbiol. 8(4):299-317. <http://dx.doi.org/10.1016/0168-1605(89)90001-9> <PMid:2518321>

Uruguay 2016. Estadística de la producción lechera 2016. Dirección de Estadísticas Agropecuarias, Ministerio de Ganadería, Agricultura y Pesca, Montevideo. Available at <http://www.mgap.gub.uy/unidad-organizativa/oficina-de-programacion-y-politicas-agropecuarias/publicaciones/anuarios-diea/anuario2016> Accessed on Jun. 3, 2018.

Watson E.N., David G.P. & Cook A.J.C. 2008. Review of diagnostic laboratory submissions of adult cattle 'found dead' in England and Wales in 2004. Vet. Rec. 163(18):531-535. <http://dx.doi.org/10.1136/vr.163.18.531> <PMid:18978365>

Zelal A. 2017. Hypomagnesemia tetany in cattle. J. Adv. Dairy Res. 5(2):1-9. <http://dx.doi.org/10.4172/2329-888X.1000178>

Zheng J., Mao X., Ling J., Chen C. & Zhang W. 2016. Role of magnesium transporter subtype 1 (MagT1) in the osteogenic differentiation of rat bone marrow stem cells. Biol. Trace Elem. Res. 171(1):131-137. <http://dx.doi.org/10.1007/s12011-015-0459-4> <PMid:26358767>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(8):573-579, August 2019
DOI: 10.1590/1678-5150-PVB-6248

Original Article
Livestock Diseases

Outbreaks of nutritional cardiomyopathy in pigs in Brazil[1]

Raquel A.S. Cruz[2], Daniele M. Bassuino[2], Matheus O. Reis[2], Cláudio J.M. Laisse[2], Saulo P. Pavarin[2], Luciana Sonne[2], Alexandre M. Kessler[3] and David Driemeier[2]*

ABSTRACT.- Cruz R.A.S., Bassuino D.M., Reis M.O., Laisse C.J.M., Pavarini S.P., Sonne L., Kessler A.M. & Driemeier D. 2019. **Outbreaks of nutritional cardiomyopathy in pigs in Brazil.** *Pesquisa Veterinária Brasileira 39(8):573-579*. Setor de Patologia 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

Dilated cardiomyopathy (DCM) is a condition that affects the myocardium, seldom reported in pigs. The DCM is characterized by ventricular dilation, which results in systolic and secondary diastolic dysfunction and can lead to arrhythmia and fatal congestive heart failure. This study described the clinical, pathological, chemical and toxicological findings of nutritional dilated cardiomyopathy (DCM) in nursery pigs through natural and experimental studies. Naturally occurring cases of DCM in three swine farms were investigated through necropsy (fourteen pigs), microscopic, virological, chemical and toxicological exams for the detection of the etiology. The experimental study was conducted with nine 40 days-old piglets, which were divided into three groups of three piglets each. Group 1 was fed with the suspected diet of the naturally occurring cases, Group 2 with half of the suspected diet and half of a control diet, and Group 3 received only the control diet. Clinical signs were recorded. All pigs were submitted of euthanized, necropsie and collection sample for laboratories exams, after 15 days of experiment onset. At the necropsy, all naturally occurring cases had bilateral cardiac dilatation associated to hepatic enhanced lobular pattern (nutmeg liver) and lungs edema. Microscopically, the heart revealed severe hypertrophy and vacuolization of cardiomyocytes, as well as myofiber disarray. Feed analysis revealed low-quality standard soybean meal. After the suspected feed was replaced, clinically ill pigs recovered, and mortality ceased. At the experimental study, two piglets from Group 1 had cough, dyspnea and diarrhea. At the necropsy, these animals had similar gross and microscopic lesions to the natural cases. The nutritional DCM in pigs may be associated to the diet with low-quality soybean meal, as it was further confirmed through an experimental study.

INDEX TERMS: Outbreak, nutritional cardiomyopathy, pigs, Brazil, swine, cardiomyopathy, heart, diet, soyben meal, heart failure.

RESUMO.- [**Surtos de cardiomiopatia nutricional em suínos no Brasil.**] Cardiomiopatia dilatada (CMD) é uma condição que afeta o miocárdio, raramente relatada em porcos. A DCM é caracterizada por dilatação ventricular, que resulta em disfunção sistólica e disfunção diastólica secundária e pode levar a arritmias e insuficiência cardíaca congestiva fatal. Este estudo descreve os achados clínicos, patológicos, químicos e toxicológicos da CMD em suínos de creche através de estudos naturais e experimentais. Investigaram-se três granjas com surtos de mortalidade por CMD através de exames de necropsia (catorze suínos), microscópicos, virológicos, químicos e toxicológicos para a detecção da etiologia. O estudo experimental foi conduzido com nove leitões de 40 dias de idade, divididos em três grupos de três leitões cada. O grupo 1 foi alimentado com a dieta suspeita dos casos naturais; o 2 com metade da dieta suspeita e metade de dieta controle; e o 3 recebeu apenas

[1] Received on March 12, 2019.
Accepted for publication on April 1, 2019.
[2] Setor de Patologia Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil. *Corresponding author: davetpat@ufrgs.br

[3] Laboratório de Ensino Zootécnico, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000.

a dieta controle. Sinais clínicos foram registrados. Todos os suínos foram submetidos a eutanásia, necropsia para a coleta de amostras para exames laboratoriais após 15 dias do início do experimento. Na necropsia, todos os leitões dos casos naturais apresentavam dilatação cardíaca bilateral associada a padrão lobular hepático aumentado (fígado de noz-moscada) e edema pulmonar. Microscopicamente, o coração revelou hipertrofia severa e vacuolização de cardiomiócitos, bem como desordem de miofibras. A análise da ração demonstrou que o farelo de soja apresentava baixa solubilidade o que indica baixa qualidade. Após a substituição da ração suspeita, os porcos clinicamente doentes recuperaram e a mortalidade cessou. No estudo experimental, dois leitões do grupo 1 apresentaram tosse, dispneia e diarreia. Na necropsia, esses animais apresentavam lesões macroscópicas e macroscópicas similar aos casos naturais. A CMD nutricional em suínos pode estar associado à dieta com farelo de soja de baixa qualidade, como foi confirmado através de um estudo experimental.

TERMOS DE INDEXAÇÃO: Surtos, cardiomiopatia nutricional, suínos, Brasil, coração, dieta, farelo de soja, insuficiência cardíaca.

INTRODUCTION

Cardiomyopathy (CM) refers to conditions that cause structural and/or functional abnormalities in the myocardium with concomitant dilatation or hypertrophy of one or both ventricles and possibly all four chambers of the heart (Richardson et al. 1996, Elliott et al. 2008, Harvey & Leinwand 2011). It affects humans and domestic animals and may be categorized into three morphologic forms: dilated (congestive), hypertrophic and restrictive (Richardson et al. 1996, Elliott et al. 2008).

Dilated cardiomyopathy (DCM) is a primary or secondary myocardial disorder of characterized by the reduced contractility and ventricular dilation involving the left or both ventricles (O'Grady & O'Sullivan 2004, Miller & Gal 2017). It is an important cause of congestive heart failure in animals (Czarnecki 1984, Edwards 1987, Lobo & Pereira 2002, O'Grady & O'Sullivan 2004, Miller & Gal 2017, Collins et al. 2015).

DCM is been studied in many species, but it has been extensively studied in dogs (Sisson et al. 1999, Lobo & Pereira 2002, O'Grady & O'Sullivan 2004). One form of DCM known as "round heart disease" has been discovered in turkeys, and this form of DCM does not have a well-defined etiopathogenesis (Czarnecki 1984, Stenzel et al. 2008). In cattle, DCM with a familial genetic origin has been determined to be an autosomal recessive inherited disease (Miller & Gal 2017). In swine, DCM has been associated with gossypol poisoning, fumonisin toxicosis and cardiomyopathy with an undetermined origin (Loynachan 2012, Sobestiansky 2012, Collins et al. 2015).

DCM causes ventricular dilation, systolic dysfunction, and secondary diastolic dysfunction, which may progress to congestive heart failure, arrhythmias, and eventually lead to death (Sisson et al. 1999, Sisson & Thomas 1999). Morphologically, myocardium is thinner, with dilated and flaccid cardiac chambers and, thus is weakened and unable to pump blood efficiently (Edwards 1987, Keene et al. 1994, O'Grady & O'Sullivan 2004).

The aim of this study was to describe the clinical, pathological, chemical and toxicological findings of nutritional DCM in nursery pigs through natural and experimental studies.

MATERIALS AND METHODS

Natural cases: clinical, epidemiological and histopathological features. From 2011 to 2014 eight porcine samples of multiple organs from three farms in Southern Brazil with high mortality rates of nursery-growing pigs Paraná (A), Rio Grande do Sul (B), and Mato Grosso do Sul (C)] had microscopical lesions consistent with congestive heart failure. On-site visits to the three farms were performed and data regarding the clinical signs and epidemiology were obtained with the clinician veterinarians. Fourteen piglets with clinical signs of cough and dyspnea from A (4 piglets), B (6) and farm C (4) were subjected to euthanasia followed by necropsy. Multiple tissue samples were collected, fixed in 10% neutral buffered formalin, processed for histopathology, and stained with hematoxylin and eosin (HE). Heart sections were also stained with periodic acid-Schiff (PAS) and Masson's trichrome (MT) to characterize morphologically the cardiac injury. Lung sections were also stained with Perl's Prussian blue stain to highlight the congestive heart failure lesions (heart failure cells).

Experimental study. The experimental study was conducted during 15 days with nine 40-day-old piglets (15kg of weight), which were further divided into three groups: 1 (suspected feed), 2 (50% suspected feed + 50% control feed) and 3 (100% control feed). The suspected feed was collected at the farm B, and was formulated with corn, soybean meal and commercial premix. The animals received their respective diet and water *ad libitum* for the experimental period, were daily monitored and weekly weighed.

All nine pigs were humanely euthanized as required by current legislation under the approval of the UFRGS Ethics and Animal Experimentation Committee (approval protocol number 29467). The carcasses were weighed and at necropsy multiple tissues, were collected and fixed in 10% buffered formalin. The whole heart from each pig was collected and weighed after the blood was drained. The heart weight/body weight (HW:BW) coefficient was then determined (Turk 1983, Richardson et al. 1996).

Chemical and toxicological analysis. Liver samples collected from the necropsied pigs of farm B and from the experimental study were frozen at -20°C to evaluate vitamin E and selenium levels. Liver and heart samples previously fixed in 10% formalin from piglets of farms B and C and from the experimental groups were used to determine the levels of other minerals. Feed samples from farms A and B were collected for chemical and toxicological analyses according to the substances and methodologies shown in Table 1.

A protein analysis (proteinogram) was performed on the feed sample from farm B, with the dosage of the following amino acids: alanine, arginine, aspartic acid, glycine, isoleucine, leucine, glutamic acid, lysine, cysteine, methionine, phenylalanine, tyrosine, threonine, tryptophan, proline, valine, histidine, and serine.

Molecular analysis. Fixed and paraffin-embedded myocardium samples of two pigs from each outbreak and from the experiment were subjected to molecular analysis for the detection of the major viral agents responsible for heart lesions in pigs: porcine circovirus, porcine parvovirus, influenza A, pestivirus and enterovirus, according to previously described protocols (Opriessnig et al. 2003, Vilcek et al. 2003, Vecchia et al. 2012).

RESULTS

Natural cases: clinical features and gross findings

Three outbreaks of acute respiratory failure, with dyspnea, cough, fatigue, and anorexia, followed by death within days in nursery-growing pigs (30-70 days-old) were observed

Table 1. Chemical and toxicological dosages in liver, heart, soybean meal and feed samples of the piglets with dilated cardiomyopathy

Elements	Samples	Technique
Selenium	Liver[b,e]	EAA
Gossypol	Soybean meal e feed[b,c]	HPLC
Ionophores	Feed[a,b]	HPLC
Monocrotaline	Soybean meal e feed[b]	UPLC-MS/MS
Minerals (Fe, Cu, Co, Zn, Ca, Mg, K e Na)	Liver and heart[b,c,e]	EAA
Toxic components	Soybean meal[b,c] and feed[b]	GC/MS
Mycotoxins (AFB)	Feed[a,b]	HPLC/FLD
Mycotoxins (FB e T-2)	Feed[a,b]	LC-MS/MS
Vitamin E	Liver[b,e]	HPLC

EAA = atomic absorption spectrometry, HPLC = high performance liquid chromatography, UPLC-MS/MS = ultra-high performance liquid chromatography-mass spectrometry, GC/MS = gas chromatography/mass spectroscopy, HPLC/FLD = high performance liquid chromatography with fluorescence detection, LC-MS/MS = liquid chromatography-mass spectrometry, AFB = aflatoxin B1, FB = fumonisin B, T-2 = toxin T-2; [a] farm A, [b] farm B, [c] farm C, [e] experimental study.

from March-June of 2011 and 2014. The farms were located in Paraná state (A), Rio Grande do Sul state (B) and Mato Grosso do Sul state (C), and during the outbreaks a total of 183 pigs died (60 at A, 53 at B and 80 at C). The pigs were treated unsuccessfully with antibiotics. Mortality levels ranged from 2 to 10%, which were considered above average within the farms.

Feed analysis from B and C revealed low-quality standard soybean meal, with a low urea activity index of 0.03 and 0.04 (reference values - RV: 0.05 to 0.2) and solubility analysis showed values between 50 and 60% (RV: 70-80%) (Berthol et al. 2001). In all three farms, after the suspected feed was replaced, clinically ill pigs recovered, and mortality ceased.

At necropsy, all 14 pigs had enlarged hearts which occupied almost the entire sternal surface (Fig.1A). The abdominal and thoracic cavity and the pericardial sac were filled with variable amounts of translucent fluid (ascites, hydrothorax and hydropericardium) (Fig.1B). In addition, there was severe hepatomegaly with an enhanced lobular pattern (nutmeg liver) (Fig.1C). The enlarged heart had a globular appearance (Fig.1D) with severe bilateral ventricular dilation and intraventricular clotted blood (Fig.1E). In some pigs from farm C, there was hepatic atrophy, with marked chronic lesions. The spleen was moderately enlarged and exuded blood at the cut surface. The lungs were not collapsed, and there was mild to moderate interlobular edema.

Experimental study: clinical features and gross findings

In the experimental study, one piglet from Group 1 had cough on the 8th day of feed consumption and severe dyspnea on the 13th day. Another piglet from the same group had diarrhea, with orange-stained feces on the 7th day of the experiment. The remaining pigs did not show any clinical abnormalities. Both piglets from Group 1 that presented clinical signs had similar gross lesions to those found in natural cases, with a mild amount of fluid in the abdominal and thoracic cavity, moderate bilateral ventricular dilatation (Fig.1F), and a nutmeg liver. The HW/BW evaluation of these two pigs (0.73% and 0.86%) revealed a significant difference when compared to the control pigs (average of 0.53%). Piglets from Group 2 and 3 did not present any gross lesions.

Natural cases and experimental study: histological findings

The histological examination of the myocardium in all the pigs of natural cases revealed marked cardiomyocyte hypertrophy and attenuated wavy fibers in the myocardium, shown in Figure 2A, and the heart from a control pig is shown in Figure 2B. Additionally, in two pigs (farms A and B), multifocal thrombosis in the myocardium, mild multifocal fibrinoid necrosis of blood vessel walls, and a mild multifocal infiltrate of lymphocytes in the epicardium and myocardium were observed. The liver had moderate to marked congestion, necrosis, and degeneration of the centrilobular hepatocytes (nutmeg liver) (Fig.2C,D). The lungs revealed moderate interlobular septal edema (Fig.2E) and a moderate number of hemosiderin-laden macrophages (heart failure cells) within the alveoli (Fig.2F), which were highlighted through Perl's Prussian blue stain. Fibrinoid necrosis of the vessel walls was highlighted through PAS stain, which also revealed lesions in the lymph nodes in one pig at farm A, and in the spleen and brain in two pigs at farm B.

Only two piglets from Group 1 that displayed clinical signs and gross lesions of DCM had microscopical abnormalities. These were similar to those observed in natural cases. Piglets from Group 2 and 3 did not present any microscopic lesions.

Chemical and toxicological analyses

Selenium values were below 2.16µg/kg (RV: 0.1 to 2.9µg/kg (Pallarés et al. 2002) in the analyzed samples of all the pigs for the natural and experimental cases. Vitamin E values in the liver of the pigs both from natural and experimental cases were below 8.55µg/kg in all pigs (RV: 3.8-10µg/kg), except for one pig in Group 1 (14.29µg/kg). Proteinogram analysis had values within the reference range.

All of the piglets with DCM in the natural and experimental cases had high magnesium levels (600-800µg/kg) in the myocardium compared to the control animals (300-400µg/kg). The iron levels in the liver were lower in affected animals from the Experimental Group (113-120µg/kg) than in animals in the Control Group (145-150µg/kg).

Gossypol, monocrotaline and ionophores were not detected in feed samples from B and C. Additionally, mycotoxin levels in the feed were below the toxic threshold (<10mg/kg for fumonisins and <50µg/kg for aflatoxin) (Sobestiansky 2012)

Fig.1. Gross features of nutritional dilated cardiomyopathy in pigs in Brazil. (**A**) The heart was markedly enlarged and occupied most of the thoracic cavity. There was also moderate ascites and a severe hepatomegaly. (**B**) The abdominal cavity was filled by abundant amount of translucent fluid (ascites). (**C**) The enlarged liver had an enhanced lobular pattern at the cut surface. (**D**) Natural cases had a severely enlarged heart with a globular appearance. (**E**) At the cut surface, there was severe bilateral ventricular dilation and intraventricular clotted blood. (**F**) Transversal sections of the heart of one piglet from the experimental cases displayed severe bilateral ventricular dilatation (above) and. the heart of a normal control pig is presented (below).

Molecular analysis

Porcine circovirus, porcine parvovirus, influenza A, pestivirus and enterovirus molecular analysis from heart samples of natural and experimental cases yielded negative results.

DISCUSSION

The diagnosis of congestive heart failure by nutritional DCM in pigs was obtained by the association of the pathological, epidemiological, and experimental findings. DCM in pigs has been linked to poisoning by gossypol, fumonisin toxicosis and unknown causes (Loynachan 2012, Sobestiansky 2012, Collins et al. 2015, Miller & Gal 2017). Which were ruled out in this study through the chemical and toxicological tests of the feeds from B and C. Magnesium levels in the heart were higher in piglets with DCM (both natural and experimental pigs) than in the Control Group, which may be related to the occurrence of cardiac alterations (Korpela 1991). However,

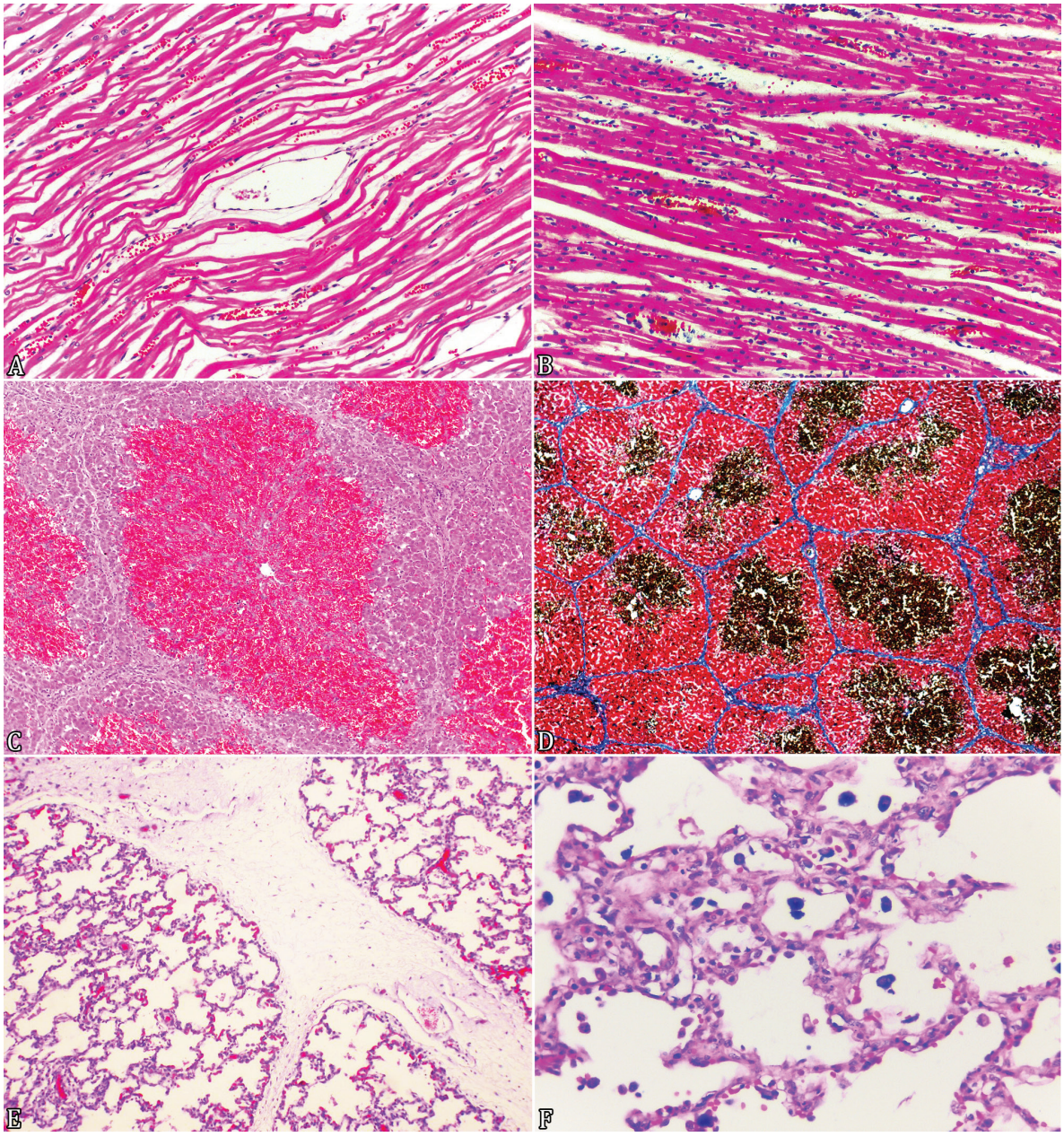


Fig.2. Histological features of nutritional dilated cardiomyopathy in pigs in Brazil. (**A**) Cardiomyocyte hypertrophy and attenuated wavy fibers in the myocardium. HE, obj.10x. (**B**) Cardiomyocytes of a control pig are arranged in homogenous parallel bundles. HE, obj.10x. (**C**) Moderate to marked hepatic congestion, degeneration and necrosis of the centrilobular hepatocytes (nutmeg liver). HE, obj.10x. (**D**) Moderate to marked centrilobular congestion (nutmeg liver). Masson's Trichrome, obj.10x. (**E**) Moderate interlobular pulmonary edema. HE, obj.10x. (**F**) Moderate numbers of macrophages with intracytoplasmic hemosiderin (heart failure cells) were evidenced within the alveoli. Perl's Prussian blue, obj.40x.

more studies are needed for clarification of this relationship in natural conditions. Additionally, high levels of iron were observed in the liver samples of the piglets with DCM, which likely occurred due to congestive heart failure and associated marked hepatic congestion.

In the outbreaks, the affected animals were mainly nursery piglets, which have a high energy requirement. External factors such as temperature, humidity, and facilities conditions, as well as factors related to diet, may interfere with the intake and utilization of food (Parsons et al. 1991, Berthol et al. 2001, Bunzen et al. 2008, Ramirez & Karriker 2012). In two of the farms (B and C), the soybean meal had poor quality, with low urea activity and low solubility. A low solubility indicates that the soybean meal did not have the recommended water content, and it has possibly undergone a process called "over toasting" (Parsons et al. 1991, Berthol et al. 2001). The consumption of low solubility soybean meal by the nursery pigs probably resulted in decreased digestibility rates and a decrease in

lysine availability, which most likely led to reduced weight gain (Parsons et al. 1991) However, the proteinogram of the low-solubility soybean meal, did not detect any abnormalities in the levels of amino acids. An hypothesis that these nutrients were absorbed in smaller amounts cannot be ruled out, since the nutritional value of protein in food does not solely depends on amino acids composition, but it also depends on digestibility and availability factors, which were not analyzed in this study (Bunzen et al. 2008).

HW:BW coefficient of the two piglets from the experimental study that had clinical signs and gross lesions were 0.86% and 0.73%, which are almost twice the estimated mean value of pigs (0.40%). In DCM, an increase of the heart chambers (mainly of the ventricles) is observed grossly, which acquire a rounded shape, with a flaccid appearance and a weight 20 to 50% above the normal range, as well as an increase in the HW:BW coefficient (O'Grady & O'Sullivan 2004, Robinson & Robinson 2016). In this study, dilation was observed in all heart chambers, which is similar to that described in dogs and humans (Edwards 1987, Lobo & Pereira 2002, O'Grady & O'Sullivan 2004). While it differs from the previously reported data in cats, mostly involves the left ventricle (Turk 1983).

The gross findings observed in piglets with nutritional DCM in this study are similar to the features of cardiomyopathy caused by taurine deficiency in cats, genetic cardiomyopathy in dogs and humans, gossypol toxicosis in pigs and "round heart disease" in turkeys (Edwards 1987, Sisson & Thomas 1999, O'Grady & O'Sullivan 2004, Stenzel et al. 2008, Sobestiansky 2012). The histological lesions observed both in the natural and experimental cases are characteristic of a congestive heart failure due to dilated cardiomyopathy, with wavy fibers in the myocardium. These histopathological features are consistent with the pattern of lesions observed in DCM in dogs, humans, turkeys, and pigs (Edwards 1987, Tidholm & Jönsson 2005, Stenzel et al. 2008, Collins et al. 2015).

There are few reports of DCM in pigs compared to humans and dogs, in which DCM is the main type of cardiomyopathy diagnosed (Sisson et al. 1999a, Lobo & Pereira 2002, O'Grady & O'Sullivan 2004, Elliott et al. 2008, Shen et al. 2011). Similary to the present study, the "round heart disease" in turkeys has an unknown etiology and pathogenesis (Stenzel et al. 2008); additionally, mulberry heart disease, which is an important heart disease that affects nursery pigs and does not have a defined etiology. However macroscopic and microscopic lesions are different from these cases (Shen et al. 2011).

CONCLUSION

Clinical history, pathological features, laboratory tests, and the results of the experimental study demonstrated the outbreaks of congestive heart failure by nutritional DCM in nursery pigs. This study suggests that the condition is related to the consumption of low-quality soybean meals. However, the specific cause is yet unknown, and further research on swine nutritional DCM is necessary since pigs may contribute as experimental models in human heart diseases.

Acknowledgements.- We thank Dr. Kent Schwartz, Dr. Scott Radke, Dr. Steve Ensley and Paulo Henrique Elias Arruda of Iowa State University for performing the GC/MS test; Professors Fernando Rosado Spilki and Andréia Henzel of Feevale University for performing the PCR test for Enterovirus. The authors thank so much all colleagues who have helped in some way.

Funding.- The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: "Coordenação de Aperfeiçoamente de Pessoal de Nível Superior" (CAPES), process number 406416/2016-8, "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq), process number 150386/2017-6 and "Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul" (FAPERGS), process number 17/255-000946-0.

Conflict of interest statement.- The authors declare no conflicts of interest with respect to publication of this manuscript. The authors received no specific grants from any funding agency in the public, commercial or not-for-profit sector for the preparation of this manuscript.

REFERENCES

Berthol T.M., Mores N., Ludke J.V. & Franke M.R. 2001. Proteínas da soja processadas de diferentes modos em dietas para desmame de leitões. Revta Bras. Zootec. 30(1):150-157. <http://dx.doi.org/10.1590/S1516-35982001000100022>

Bünzen S., Rostagno H.S., Lopes D.C., Hashimoto F.A.M., Gomes P.C. & Apolônio L.R. 2008. Digestibilidade do fósforo de alimentos de origem vegetal determinada com suínos em crescimento e terminação. Revta Bras. Zootec. 37(7):1236-1242. <http://dx.doi.org/10.1590/S1516-35982008000700014>

Collins D.E., Eaton K.A. & Hoenerhoff M.J. 2015. Spontaneous dilated cardiomyopathy and right-sided heart failure as a differential diagnosis for hepatosis dietetica in a production pig. Comp. Med. 65(4):327-332. <PMid:26310462>

Czarnecki C.M. 1984. Cardiomyopathy in turkeys. Comp. Biochem. Physiol. 77(4):591-598. <http://dx.doi.org/10.1016/0300-9629(84)90169-5> <PMid:6143637>

Edwards W.D. 1987. Cardiomyopathies. Hum. Pathol. 18(6):625-635. <http://dx.doi.org/10.1016/S0046-8177(87)80364-7> <PMid:3596582>

Elliott P., Andersson B., Arbustini E., Bilinska Z., Cecchi F., Charron P., Dubourg O., Kühl U., Maisch B., McKenna W.J., Monserrat L., Pankuweit S., Rapezzi C., Seferovic P., Tavazzi L. & Keren A. 2008. Classification of the cardiomyopathies: A position statement from the European society of cardiology working group on myocardial and pericardial diseases. Eur. Heart. J. 29(2):270-276. <http://dx.doi.org/10.1093/eurheartj/ehm342> <PMid:17916581>

Harvey P.A. & Leinwand L.A. 2011. Cellular mechanisms of cardiomyopathy. J. Cell. Biol. 194(3):355-365. <PMid:21825071>

Keene B.W., Panciera D.P., Atkins C.E., Regitz V., Schmidt M.J. & Shug A.L. 1994. Myocardial L-carnitine deficiency in a family of dogs with dilated cardiomyopathy. J. Am. Vet. Med. Assoc. 198(4):647-650. <PMid:2019534>

Korpela H. 1991. Hypothesis: increased calcium and decreased magnesium in heart muscle and liver of pigs dying suddenly of microangiopathy (mulberry heart disease): An animal model for the study of oxidative damage. J. Am. Coll. Nutr. 10(2):127-131. <http://dx.doi.org/10.1080/07315724.1991.10718136> <PMid:2030254>

Lobo L.L. & Pereira R. 2002. Cardiomiopatia dilatada canina. Port. Ciênc. Vet. 97:153-159.

Loynachan A.T. 2012. Cardiovascular and hematopoietic systems, p.189-193. In: Zimmerman J.J., Karriker L.A., Ramirez A., Schwartz K.J. & Stevenson G.W. (Eds), Diseases of Swine. 10th ed. Wiley-Blackwell, Iowa.

Miller L.M. & Gal A. 2017. Cardiovascular system and lymphatic vessels, p.561-616. In: Zachary J.F. (Ed.), Pathologic Basis of Veterinary Disease. 6th ed. Mosby Elsevier, St Louis. <http://dx.doi.org/10.1016/B978-0-323-35775-3.00010-2>

O'Grady M.R. & O'Sullivan M.L. 2004. Dilated cardiomyopathy: an update. Vet. Clin. N. Am., Small. Anim. Pract. 34:1187-1207.

Opriessnig T., Yu S., Gallup J.M., Evans R.B., Fenaux M., Pallares F., Thacker E.L., Brockus C.W., Ackermann M.R., Thomas P., Meng X.J. & Halbur P.G. 2003. Effect of vaccination with selective bacterins on conventional pigs infected with type 2 porcine circovirus. Vet. Pathol. 40(5):521-525. <http://dx.doi.org/10.1354/vp.40-5-521> <PMid:12949409>

Pallarés F.J., Yaeger M.J., Janke B.H., Fernández G. & Halbur P.G. 2002. Vitamin E and selenium concentrations in livers of pigs diagnosed with mulberry heart disease. J. Vet. Diagn. Invest. 14(5):412-414. <http://dx.doi.org/10.1177/104063870201400509> <PMid:12296394>

Parsons C.M., Hashimoto K., Wedekind K.J. & Baker D.H. 1991. Soybean protein solubility in potassium hydroxide: in vitro test of in vivo protein quality. J. Anim. Sci. 69(7):2918-2924. <http://dx.doi.org/10.2527/1991.6972918x> <PMid:1885400>

Ramirez A. & Karriker L.A. 2012. Herd evaluation, p.5-17. In: Zimmerman J.J., Karriker L.A., Ramirez A., Schwartz K.J., & Stevenson G.W. (Eds), Diseases of Swine. 10th ed. Wiley-Blackwell, Iowa.

Richardson P., McKenna W., Bristow M., Maisch B., Mautner B., O'Connell J., Olsen E., Thiene G., Goodwin J., Gyarfas I., Martin I. & Nordet P. 1996. Report of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force on the definition and classification of cardiomyopathies. Circulation 93(5):841-842. <http://dx.doi.org/10.1161/01.CIR.93.5.841> <PMid:8598070>

Robinson W.F. & Robinson N.A. 2016. Cardiovascular system, p.44-50. In: Maxie M.G. (Ed.), Jubb Kennedy and Palmer's Pathology of Domestic Animals. 6th ed. Saunders Elsevier, St Louis. <http://dx.doi.org/10.1016/B978-0-7020-5319-1.00012-8>

Shen H., Thomas P.R., Ensley S.M., Kim W.I., Loynachan A.T., Halbur P.G. & Opriessnig T. 2011. Vitamin E and selenium levelsare within normal range in pigs diagnosed with mulberry heart disease and evidence for viral involvement in the syndrome is lacking. Transbound. Emerg. Dis. 58(6):483-491. <http://dx.doi.org/10.1111/j.1865-1682.2011.01224.x> <PMid:21518323>

Stenzel T., Tykalowski B. & Koncicki A. 2008. Cardiovascular system diseases in turkeys. Pol. J. Vet. Sci. 11(3):245-250. <PMid:18942548>

Sisson D., O'Grady M.R. & Calvart C.A. 1999. Myocardial diseases of dog, p.581-620. In: Fox P.R., Sisson D. & Moise N.S. (Eds), Textbook of Canine and Feline Cardiology. 2nd ed. W.B. Saunders, Philadelphia.

Sisson D. & Thomas W.P. 1999. Myocardial disorders, p.1406-1410. In: Ettinger S.J. (Ed.), Textbook of Veterinary Internal Medicine Expert Consult. W.B. Saunders, São Paulo.

Sobestiansky J. 2012. Intoxicação por minerais, produtos químicos, plantas e gases, p.553-580. In: Sobestiansky J. & Barcellos D.E.S.N (Eds), Doenças dos Suínos. 2ª ed. Cânone Editorial, Goiânia.

Tidholm A. & Jönsson L. 2005. Histologic characterization of canine dilated cardiomyopathy. Vet. Pathol. 42(1):1-8. <http://dx.doi.org/10.1354/vp.42-1-1> <PMid:15657266>

Turk J.R. 1983. Necropsy canine heart: a simple technique for quantifying ventricular hypertrophy and valvular alterations. Compend. Contin. Educ. Vet. 5:905-910.

Vecchia A.D., Fleck J.D., Comerlato J., Kluge M., Bergamaschi B., Da Silva J.V., Da Luz R.B., Teixeira T.F., Garbinatto G.N., Oliveira D.V., Zanin J.G., Van der Sand S., Frazzon A.P., Franco A.C., Roehe P.M. & Spilki F.R. 2012. First description of Adenovirus, Enterovirus, Rotavirus and Torque teno virus in water samples collected from the Arroio Dilúvio, Porto Alegre, Brazil. Braz. J. Biol. 72(2):323-329. <http://dx.doi.org/10.1590/S1519-69842012000200013> <PMid:22735140>

Vilcek S., Nettleton P. & Paton D. 2003. Remarkable cross reaction of pan-Pestivirus PCR primers with poliovirus genome. J. Virol. Methods 114(2):167-170. <http://dx.doi.org/10.1016/j.jviromet.2003.08.003> <PMid:14625052>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(8):580-586, August 2019
DOI: 10.1590/1678-5150-PVB-6328

Original Article
Livestock Diseases

Salmonellosis in calves without intestinal lesions[1]

Carolina C. Guizelini[2], Rayane C. Pupin[2], Cássia R.B. Leal[3], Carlos A.N. Ramos[4], Saulo P. Pavarini[5], Danilo C. Gomes[2], Tessie B. Martins[2] and Ricardo A.A. Lemos[2]*

ABSTRACT.- Guizelini C.C., Pupin R.C., Leal C.R.B., Ramos C.A.N., Pavarini S.P., Gomes D.C., Martins T.B. & Lemos R.A.A. 2019. **Salmonellosis in calves without intestinal lesions**. *Pesquisa Veterinária Brasileira 39(8):580-586*. Laboratório de Anatomia Patológica, Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul, Av. Senador Filinto Muller 2443, Bairro Vila Ipiranga, Campo Grande, MS 79074-460, Brazil. E-mail: ricardo.lemos@ufms.br

Salmonellosis is a known cause of enteric disorders in calves. However, cases in the septicemic form may not present enteric lesions, which may lead the veterinary practitioner to not suspect salmonellosis, compromising the diagnosis. The current study describes the epidemiological, clinical, pathological and immunohistochemical aspects of septicemic salmonellosis in calves without enteric lesions. The protocols involving bovine material submitted to the Pathology Laboratory (LAP) of the "Faculdade de Medicina Veterinária e Zootecnia" (FAMEZ) of the "Universidade Federal do Mato Grosso do Sul" (UFMS) from January 1995 to July 2018 were studied. Cases confirmed or suggestive of septicemic salmonellosis in calves without enteric manifestations were selected. Fragments of the liver, lung, and spleen embedded in paraffin were submitted to immunohistochemistry (IHC). Only cases in which there was positive marking on the IHC or culture isolation of Salmonella were included in this study. Of a total of 5,550 cattle examined in the period, ten presented septicemic salmonellosis without enteric lesions. Clinical signs included mucosal pallor, apathy, hyperthermia, and dyspnea. Only three calves presented diarrhea, and two were found dead before clinical changes were observed. The most common necropsy findings were hepatosplenomegaly; yellow, orange or brown discolored livers; pale mucous membranes; inflated and sometimes red lungs; fibrin or fluid within body cavities; and gallbladder filled with inspissated bile. Jaundice was observed in three calves that had a concomitant infection with *Anaplasma* sp. Microscopically, paratyphoid hepatic nodules and interstitial pneumonia were the most frequent manifestations, followed by thrombosis and bacterial colonies in the spleen, lung, liver, and brain. A strong positive marking was observed in IHC, predominantly in the lung and to a lesser extent in the liver. Polymerase chain reaction (PCR) indicated the Dublin serotype as the causative agent in the samples of the four calves submitted to this procedure. In calves, the septicemic form was the major cause of death due to salmonellosis. Septicemic salmonellosis was usually not accompanied by diarrhea. The clinical signs of septicemia are nonspecific and of little assistance in the diagnosis. IHC has been shown to be efficient in the detection of the agent, mainly in the lung and especially in situations where it is not possible to perform bacterial culture.

INDEX TERMS: Salmonellosis, calves, diseases of cattle, *Salmonella* Dublin, septicemia, immunohistochemistry, cattle, bacterioses.

[1] Received on March 11, 2019.
Accepted for publication on March 20, 2019.
This work is part of the requirements for obtaining the Master's Degree by the first author.

[2] Laboratório de Anatomia Patológica, Faculdade de Medicina Veterinária e Zootecnia (FAMEZ), Universidade Federal de Mato Grosso do Sul (UFMS), Av. Senador Felinto Muller 2443, Bairro Vila Ipiranga, Campo Grande, MS 79074-460, Brazil. *Corresponding author: ricardo.lemos@ufms.br

[3] Laboratório de Bacteriologia, Faculdade de Medicina Veterinária e Zootecnia (FAMEZ), Universidade Federal de Mato Grosso do Sul (UFMS), Av. Senador Felinto Muller 2443, Bairro Vila Ipiranga, Campo Grande, MS 79074-460.

[4] Laboratório de Biologia Molecular, Faculdade de Medicina Veterinária e Zootecnia (FAMEZ), Universidade Federal de Mato Grosso do Sul (UFMS), Av. Senador Felinto Muller 2443, Bairro Vila Ipiranga, Campo Grande, MS 79074-460.

[5] Setor de Patologia Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil.

RESUMO.- [**Salmonelose em bezerros sem manifestações intestinais.**] A salmonelose é uma causa conhecida de distúrbios entéricos em bezerros. Porém, casos na forma septicêmica podem não apresentar manifestação entérica, o que leva o médico veterinário a não suspeitar de salmonelose, comprometendo o diagnóstico. Este estudo descreve os aspectos epidemiológicos, clínicos, patológicos e imuno-histoquímicos da salmonelose septicêmica em bezerros sem lesões entéricas. O estudo foi realizado a partir dos protocolos referentes a materiais de bovinos enviados para diagnóstico ao Laboratório de Anatomia Patológica (LAP) da Faculdade de Medicina Veterinária e Zootecnia (FAMEZ) da Universidade Federal de Mato Grosso do Sul (UFMS) de janeiro de 1995 a julho de 2018. Foram selecionados os casos de bezerros confirmados ou sugestivos de salmonelose septicêmica sem lesões entéricas. Fragmentos de fígado, pulmão e baço embebidos em parafina foram submetidos ao exame de imuno-histoquímica (IHQ). Somente foram incluídos neste estudo casos em que houve marcação positiva na IHQ ou isolamento da bactéria em cultura. De um total de 5.550 bovinos examinados no período, dez apresentaram salmonelose septicêmica sem lesão entérica. Os sinais clínicos incluíram palidez de mucosas, apatia, hipertermia e dispneia. Apenas três bezerros apresentaram diarreia e dois foram encontrados mortos sem terem sido observadas alterações clínicas. Os achados mais frequentes de necropsia foram hepatoesplenomegalia, fígado amarelado, alaranjado ou acastanhado, palidez de mucosas, pulmões inflados e, por vezes, vermelhos, fibrina ou líquido nas cavidades do organismo e vesícula biliar repleta de bile grumosa. Icterícia foi observada em três bezerros que apresentavam infecção concomitante por *Anaplasma* sp. Microscopicamente, os nódulos paratifoides hepáticos e pneumonia intersticial foram as manifestações mais encontradas, seguidas por trombose e colônias bacterianas no baço, pulmão, fígado e encéfalo. Na IHQ, marcação fortemente positiva foi observada, predominantemente, no pulmão e, em menor intensidade, no fígado. A técnica de reação em cadeia de polimerase (PCR) tipificou o sorotipo Dublin como agente etiológico nas amostras dos quatro bezerros submetidos a este procedimento. Em bezerros, a forma septicêmica foi a principal responsável pelas mortes por salmonelose. Na maioria das vezes essa forma não estava acompanhada por diarreia. Os sinais clínicos da forma septicêmica são inespecíficos e de pouco auxílio no direcionamento do diagnóstico. A IHQ mostrou-se eficiente na detecção do agente principalmente no pulmão e especialmente nas situações em que não é possível a realização da cultura bacteriana.

TERMOS DE INDEXAÇÃO: Salmonelose, bezerros, doenças de bovinos, *Salmonella* Dublin, septicemia, imuno-histoquímica, bovinos, bacterioses.

INTRODUCTION

Salmonellosis is a worldwide-distributed disease affecting several animal species (Radostits et al. 2007). It is a significant cause of economic loss in the cattle industry (Marques et al. 2013) due to reduced performance of affected animals, losses related to deaths, and costs involved in diagnostic methods, treatment and implementation of preventive and control measures (Radostits et al. 2007). There are two species: *Salmonella enterica* (comprising six subspecies) and *Salmonella bongori*. Most *Salmonella* of veterinary importance belong to the subspecies *S. enterica* subsp. *enterica* (Brenner et al. 2000). Within this subspecies, the Typhimurium and Dublin serotypes are the most frequent in cattle (Wray 1991). The former is responsible for causing enteric disease, especially in calves up to six months of age (Hughes et al. 1971). The second can affect cattle of all ages and is principally associated with severe septicemia (Baumler et al. 1998).

Reports describing the epidemiology, clinical and pathological aspect and laboratory diagnosis of septicemic salmonellosis in calves are frequent (Hall & Jones 1977, Loeb et al. 2006, Marques et al. 2013, Costa et al. 2018). However, we were unable to find published documentation of the historical series of this disease addressing all of the previously mentioned aspects simultaneously.

Salmonellosis is considered one of the most important causes of diarrhea in calves up to 30 days old (El-Seedy et al. 2016). Accordingly, fecal samples are traditionally sent for bacteriological culture. However, septicemic animals may not present diarrhea (Wray 1991), which often makes diagnosis difficult. Recent studies show that although the pathogenesis of salmonellosis in calvevs is well characterized, the histological lesions of naturally acquired disease are still poorly detailed (Pecoraro et al. 2017). The present retrospective study describes the epidemiological, clinical, pathological and immunohistochemical aspects of septicemic salmonellosis in ten calves without enteric lesions and strongly positive immunostaining, especially in lung lesions.

MATERIALS AND METHODS

The protocols of 5,550 bovine necropsies were reviewed. These protocols were filed at the Anatomic Pathology Laboratory (LAP) of the "Faculdade de Medicina Veterinária e Zootecnia" (FAMEZ) of the "Universidade Federal de Mato Grosso do Sul" (UFMS). The necropsies studied were performed from January 1995 to July 2018 either by the faculty of LAP-FAMEZ or by practicing veterinarians. Materials from research projects and necropsied cattle from other states were not included in this survey. Only cases with calves aged 0 to 12 months with a confirmed or suggestive diagnosis of septicemic salmonellosis without enteric lesions were selected from the surveyed files.

Diagnostic criteria for previous selection of the cases were the presence of gross and microscopic findings considered to be characteristic of salmonellosis. These cases were confirmed by culture isolation of the bacterium and its identification in tissues by immunohistochemistry (IHC). In cases in which bacterial culture was not performed, diagnosis was confirmed solely by IHC.

Information on the year of occurrence, age and breed of calves, total number of exposed cattle, number of sick and dead animals, and results of complementary tests were also obtained from the protocols. The calves (Cases 1-10) were divided into dairy (Holstein-Friesian, Gir, Jersey, and Girolando) and beef (Angus, Brangus, and Senepol) cattle. Calves of unknown breeds were classified as mixed. The cases were organized chronologically by using Arabic numbers. An outbreak was considered when several cattle in the same farm were affected over a short period. The morbidity and lethality rates were calculated considering the number of cattle at risk (Thrusfield 2004), which was defined as the total cattle that remained in the same environment as the affected calves.

Liver, lung, and spleen sections (Cases 3-10) were also subjected to IHC with a polyclonal anti-Salmonella antibody, as previously described (Juffo et al. 2017).

RESULTS

In the 23-year period of the study, samples from 5,550 bovine necropsies were analyzed, and 14 cases of salmonellosis were identified. Of these 14 cases, eleven were diagnosed with septicemic salmonellosis, ten of which had no enteric lesions. Eight (Cases 1-6, 8 and 9) of these ten cases were confirmed by isolating the bacterium in culture. Polymerase chain reaction (PCR) identified the agent as *Salmonella* Dublin in four cases (Cases 2, 3, 5 and 6). Spleen smears confirmed coinfection with *Anaplasma* sp. in three calves (Cases 1, 6 and 9) that also presented icterus. Diarrhea was reported in only three calves, but in these calves, there were no gross or microscopic changes consistent with diarrhea. Epidemiological data and information on the examinations performed on affected calves are presented in Table 1. Clinical signs are listed in Table 2. Necropsy findings refer to ten necropsied calves. There was pallor of the mucosae in five of the cases and icterus in three cases. Feces were adhered to the perineum in one case. In four cases, there was excess fluid in the thoracic and abdominal cavities, and fibrin could be observed covering the serosal surfaces of the body cavities in three cases. In nine of the cases, there was hepatomegaly, and the liver parenchyma had a yellow, orange, or tan hue (Fig.1A). In three cases, the gallbladder was distended by inspissated bile. Splenomegaly was observed in seven of the cases (Fig.1B). In half of the cases, the lungs were heavy, and impressions of the ribs in the pleural surface did not collapse when the thoracic cavity was opened (Fig.1C). At the cut surface, there was marked interstitial edema characterized by distension of the interlobular septa by gelatinous translucent exudate (Fig.1D). The results of the histopathological findings are summarized in Table 2 and illustrated in Figure 2A-D.

By using IHC, immunoreactivity was found in the liver (7/8 cases), lung (7/8 cases), and spleen (2/8 cases). The strongest marking was found in the lung (Fig.3). The morbidity and lethality rates varied from 2.5 to 85.71% and from 20 to 100%, respectively.

Table 1. Epidemiological data and diagnostic tests performed to confirm the diagnosis of the cases of salmonellosis in calves without enteric lesions

Outbreak no.	Year	Age (months)	Breed	At risk	Sick	Dead	Tests		
							Culture	PCR[d]	IHC[f]
1[b]	2009	7	Brangus	NI[a]	10	2	Positive	NP[e]	NP[e]
2[c]	2014	NI[a]	Holstein-Friesian	NI[a]	4	4	Positive	*S. dublin*	NP[e]
3[c]	2014	3	Senepol	NI[a]	2	2	Positive	*S. dublin*	Positive
4[c]	2015	1	Senepol	NI[a]	1	1	Positive	NP	Positive
5[c]	2016	2	Jersey	80	20	20	Positive	*S. dublin*	Positive
6[c]	2016	2	Mixed	122	3	3	Positive	*S. dublin*	Positive
7[c]	2016	3	Girolando	NI[a]	1	1	NP[e]	NP[e]	Positive
8[c]	2017	2	Mixed	NI[a]	2	2	Positive	NP[e]	Positive
9[c]	2017	1	Girolando	7	6	2	Positive	NP[e]	Positive
10[b]	2018	2	Girolando	NI[a]	2	2	NP[e]	NP[e]	Positive

[a] Not informed, [b] mailed-in samples, [c] necropsied by the authors, [d] polymerase chain reaction, [e] not performed, [f] immunohistochemistry.

Table 2. Microscopic findings on cases of salmonellosis in calves (1995-2018)

Organ	Microscopic lesion	Number of cases	Total (%)
Liver	Paratyphoid nodules (Fig.2A,B)	10/10	100
	Thrombosis	4/10	40
	Cholestasis	4/10	40
	Bacterial clusters	3/10	30
Lung	Interstitial pneumonia (Fig. 2C)	9/10	90
	Hyaline membrane (Fig.2C,D)	6/10	60
	Bacterial clusters	4/10	40
	Proliferation of type II pneumocyte	2/10	20
	Thrombosis	1/10	10
Spleen	Follicular necrosis	3/10	30
	Paratyphoid nodules	3/10	30
	Thrombosis	2/10	20
	Bacterial clusters	1/10	10
Rete mirabile	Thrombosis	1/10	10
	Bacterial clusters	1/10	10
Leptomeninges	Mononuclear/neutrophilic infiltrate	3/10	30
Brain	Neutrophilic perivascular cuffings	1/10	10
	Thrombosis	1/10	10

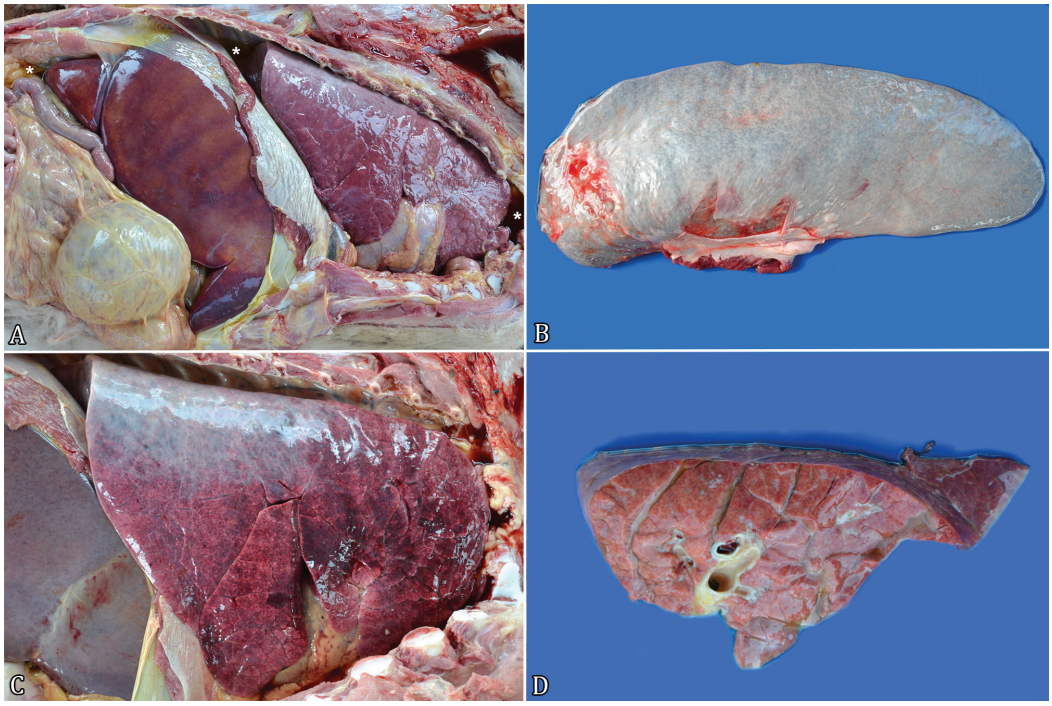


Fig.1 Gross findings in calves with septicemic salmonellosis. (**A**) There is an excess of clear yellow fluid in the thoracic (hydrothorax) and abdominal (ascites) cavities (asterisks). The liver is enlarged and displays the impression of the ribs on its capsular surface. Compressed liver areas have an orange hue. The lung is poorly collapsed and has a smooth, bright and reddened surface, which is marked by impression of the ribs. (**B**) Enlargement of the spleen is evident from the rounded borders and slight capsular tension. (**C**) Lung not collapsed. The pleural surface is smooth, reddened and bright. (**D**) Lung, cut surface, marked distension of interlobular septa by translucent gelatinous exudate (edema).

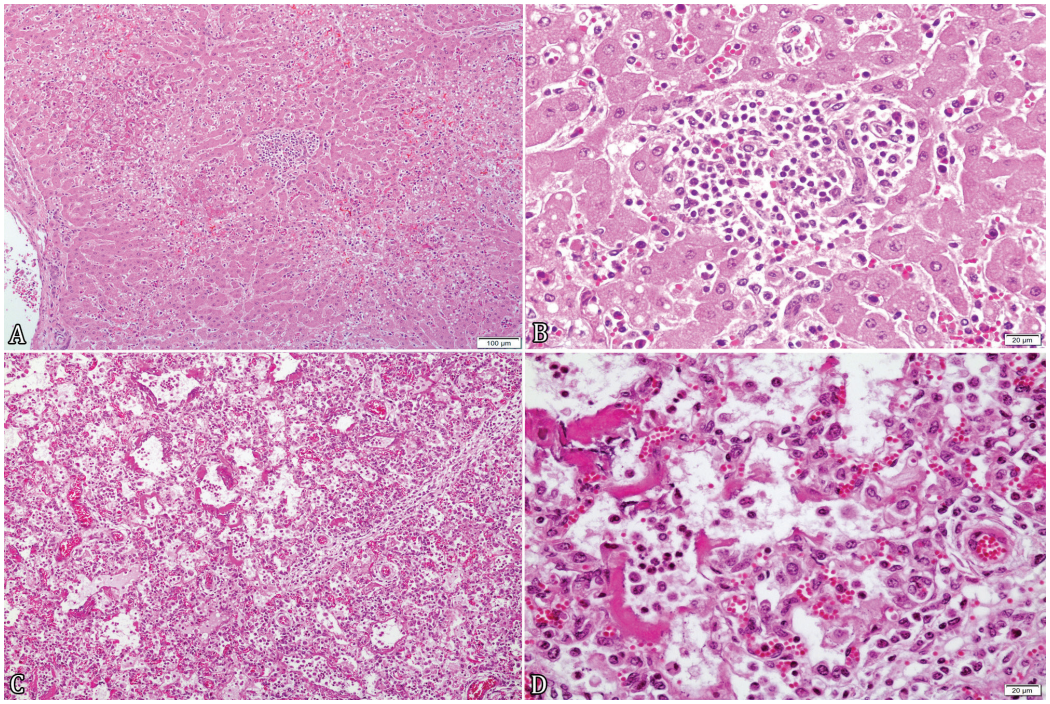


Fig.2 Histological lesions in calves with septicemic salmonellosis. (**A**) Paratyphoid nodules. Multiple granulomas are distributed throughout the liver parenchyma. HE, obj.20x. (**B**) One granulomatous nodule was composed of mononuclear cells (macrophages and lymphocytes). Red blood cell precursors can be seen with sinusoids. HE, obj.40x. (**C**) Lung. Bird's eye view of the pulmonary lesion. Interstitial pneumonia. The alveolar septa are thickened by a mononuclear infiltrate, and the alveolar line is covered by a hyaline membrane. Pink transudate (edema) is observed in the alveoli lumen, and the interlobular septa are distended and infiltrated by inflammatory mononuclear cells. HE, obj.20x. (**D**) Lung. A higher magnification of Figure 2C shows the proliferation of type II pneumocytes. Note also the thickened alveolar septa and hyaline membrane. HE, obj.40x.

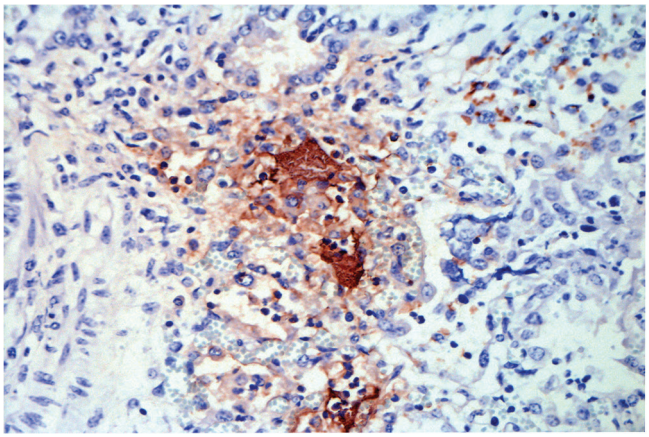


Fig.3. Polyclonal anti-*Salmonella* antibody. Lung. The strongest marking was found in the lung. IHC, obj.40x.

DISCUSSION

The number of cases of septicemic salmonellosis was low (0.2%) compared to the total number of bovine samples analyzed during the studied period. The prevalence of the disease was also low in surveys conducted by other diagnostic laboratories in Brazil, ranging from 0.6% (Lucena et al. 2010) to 0.9% (Assis-Brasil et al. 2013).

Although bovine salmonellosis is a frequent disease in other countries (Hughes et al. 1971, Carrique-Mas et al. 2010), surveys describing the clinical, epidemiological and pathological aspects of salmonellosis are not universal. Although salmonellosis can affect cattle of all ages (Hughes et al. 1971), only calves between seven and 40 days of age were affected in the present survey. Cattle from one to three months of age were the most frequently affected, accounting for 80% of all cases. Similar data were observed in cattle from other regions of Brazil (Marques et al. 2013), Uruguay (Costa et al. 2018) and the United States (Pecoraro et al. 2017).

Documentation of salmonellosis is more common in dairy cattle (Radostits et al. 2007); however, in the present study, it was observed in both dairy and beef calves. Regardless of cattle breed, several risk factors may contribute to the manifestation of this disease: 1) keeping calves of different ages in the same environment, resulting in exposure of the very young that still do not have enough colostral immunity; 2) providing drinking water that may contain feces contaminated with *Salmonella* spp.; 3) moving calf carriers, which reactivates the bacteria and releases them into the environment after a period of stress; 4) keeping calves close to rodents and wild birds, which are common sources of the bacteria (Wray 1991, Vaessen et al. 1998, Warnick et al. 2001, Fenwick & Collett 2004, Radostits et al. 2007, Blanchard 2012).

Clinical signs included diarrhea, mucosa pallor, apathy, hyperthermia, and respiratory distress. Two calves were found dead, and no previous clinical signs were observed. Considering that salmonellosis frequently causes diarrhea (Radostits et al. 2007) and interstitial pneumonia (Pecoraro et al. 2017), intestinal and respiratory clinical signs may be considered essential in the differentials list for this disease. As some of these clinical signs may also occur in several other diseases, their diagnostic value is less significant than the others. The three calves with jaundice in this study had concomitant infection with *Anaplasma* sp. Thus, jaundice cannot be attributed to salmonellosis.

Hepatosplenomegaly; yellowish, orange or brownish liver; pallor of mucous membranes, noncollapsed red and wet lungs; serosal body cavities containing serous fluid or fibrin-covered serous membranes; and gallbladder containing inspissated bile were common gross findings observed in 20-90% of the affected calves. As reported by others (Marques et al. 2013, Pecoraro et al. 2017), paratyphoid nodules (found in all calves) and interstitial pneumonia were the most common histopathological findings. Although fibrinous cholecystitis is considered a pathognomonic finding of salmonellosis (Mohler et al. 2009), it was not found in the calves of this study. Thus, the mere absence of this characteristic lesion cannot be the grounds for ruling out salmonellosis, and systematic sampling of tissues for complementary tests is advised in cases presenting other characteristic signs and lesions.

PCR detected *Salmonella* Dublin as the cause in four calves. Although *Salmonella* Typhimurium may also cause septicemia (Baumler et al. 1998, La Ragione et al. 2013), *Salmonella* Dublin is the main serotype associated with this form of clinical presentation (Wray 1991, Santos et al. 2001). This form of salmonellosis was described in an outbreak in Northern Brazil that involved the serovar Dublin (Marques et al. 2013).

Surveys describing the main types of manifestation of salmonellosis involving periods of several years have not been performed in Brazil, while in Europe (Carrique-Mas et al. 2010), the Dublin serovar has been reported to have a high prevalence and cause clinical signs suggestive of septicemia. However, in that account, the diagnosis was based mainly on the database of the UK diagnostic service, no details of necropsy or histopathological findings were reported, and diarrhea was noted as the most common manifestation (Carrique-Mas et al. 2010).

In the ten cases of septicemic salmonellosis in this study, diarrhea was observed only in three cases, which is in agreement with previous studies (Hughes et al. 1971, Wray 1991, Holschbach & Peek 2018) and demonstrates that diarrhea is not always associated with salmonellosis. This may be a reason for the underreporting of the disease because, in the absence of diarrhea, many veterinarians do not seek further testing for bacterial isolation from fresh or refrigerated samples of feces, liver, and bile nor do they order histopathological examination of organs such as intestine, liver, gallbladder, and lung.

It is noteworthy that most of the described clinical and pathological data described in this study were observed in cases where the LAP-FAMEZ staff performed the necropsy. Most of the mailed-in cases were not correctly sampled and were accompanied by limited clinical information. This, of course, compromises the diagnosis, as incorrect sampling may not provide fragments from all required organs for microscopic evaluation and no samples for bacterial isolation. When dead calves are submitted, usually advanced autolysis and putrefaction hinder the possibility of bacterial isolation (Strafuss 1988). This nonproper submission of material for diagnosis is not restricted to cases of salmonellosis and is frequently observed in many veterinary diagnostic laboratories (Lucena et al. 2010, Souza et al. 2015).

The use of IHC for the diagnosis of salmonellosis is described in sheep (Dagleish et al. 2010), horses (Juffo et al. 2017), swine (Watanabe et al. 2011), turkeys (Beyaz et al. 2010) and cattle (Pecoraro et al. 2017, Costa et al. 2018). In all analyzed materials in the present study, there was positive immunoreactivity in the liver and lung. In two calves, the spleen also tested positive under IHC. In contrast to a case described previously (Pecoraro et al. 2017), in which the liver was the principal organ of detection of the bacterium, the lung was the organ with the strongest positive marking by IHC in this study. Accordingly, lung fragments should always be included in the list of organs sent for IHC analysis in suspected cases of salmonellosis.

Similar to previous studies (Beyaz et al. 2010, Juffo et al. 2017), in two cases in this study, confirmation of salmonellosis was only possible with IHC because sampling-related problems precluded bacterial culture. In such cases, IHC becomes an essential tool for verifying the diagnosis of bovine salmonellosis. IHC should be used even in cases of negative bacterial culture results and for which epidemiological, clinical and pathological data suggest salmonellosis.

CONCLUSIONS

Septicemia was the leading cause of death by salmonellosis in calves.

In most cases, diarrhea was not a clinical sign.

The clinical signs were mostly nonspecific and of little assistance in directing the diagnosis.

IHC proved to be efficient in detecting the agent, which was detected mainly in the lung, and it was especially valuable when it was not possible to perform bacterial culture.

In cases of death of calves without the manifestation of diarrhea, *Salmonella* should be included in the list of differential diagnoses, thus requiring samples of liver, spleen, lung, and intestine for complementary laboratory diagnostic tests.

Acknowledgements.- One of the authors (RAAL) has a research fellowship from the Brazilian "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq). This study was partially funded by the "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES), Brasil, Finance Code 001, and by "Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul" (Fundect).

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

REFERENCES

Assis-Brasil N.D., Marcolongo-Pereira C., Hinnah F.L., Ladeira S.R.L., Sallis E.S.V., Grecco F.B. & Schild A.L. 2013. Enfermidades diagnosticadas em bezerros na região sul do Rio Grande do Sul. Pesq. Vet. Bras. 33(4):423-430. <http://dx.doi.org/10.1590/S0100-736X2013000400002>

Bäumler A.J., Tsolis R.M., Ficht T.A. & Adams L.G. 1998. Evolution of host adaptation in *Salmonella enterica.* Infect. Immun. 66(10):4579-4587. <PMid:9746553>

Beyaz L., Atasever A., Aydin F., Gümüşsoy K.S. & Abay S. 2010. Pathological and clinical findings and tissue distribution of *Salmonella gallinarum* infection in Turkey poults. Turk. J. Vet. Anim. Sci. 34(2):101-110.

Blanchard P.C. 2012. Diagnostics of dairy and beef cattle diarrhea. Vet. Clin. N. Am., Food Anim. Pract. 28(3):443-464. <http://dx.doi.org/10.1016/j.cvfa.2012.07.002> <PMid:23101670>

Brenner F.W., Villar R.G., Angulo F.J., Tauxe R. & Swaminathan B. 2000. *Salmonella* nomenclature. J. Clin. Microbiol. 38(7):2465-2467. <PMid:10878026>

Carrique-Mas J.J., Willmington J.A., Papadopoulou C., Watson E.N. & Davies R.H. 2010. *Salmonella* infection in cattle in Great Britain, 2003 to 2008. Vet. Rec. 167(15):560-565. <http://dx.doi.org/10.1136/vr.c4943> <PMid:21257417>

Costa R.A., Casaux M.L., Caffarena R.D., Macias-Rioseco M., Schild C.O., Fraga M., Riet-Correa F. & Giannitti F. 2018. Urocystitis and ureteritis in holstein calves with septicaemia caused by *Salmonella enterica* serotype Dublin. J. Comp. Path. 164:32-36. <http://dx.doi.org/10.1016/j.jcpa.2018.08.005> <PMid:30360910>

Dagleish M.P., Benavides J. & Chianini F. 2010. Immunohistochemical diagnosis of infectious diseases of sheep. Small Rum. Res. 92(1):19-35. <http://dx.doi.org/10.1016/j.smallrumres.2010.04.003>

El-Seedy F.R., Abed A.H., Yanni H.A. & Abd El-Rahman S.A.A. 2016. Prevalence of *Salmonella* and *E. coli* in neonatal diarrheic calves. Beni-Seuf Univ. J. Appl. Sci. 5(1):45-51.

Fenwick S.G. & Collett M.G. 2004. Bovine salmonellosis, p.1582-1594. In: Coetzer J.A.W. & Tustin R.C. (Eds), Infectious Diseases of Livestock. Oxford University Press, Southern Africa.

Hall G.A. & Jones P.W. 1977. A study of the pathogenesis of experimental *Salmonella* Dublin abortion in cattle. J. Comp. Pathol. 87(1):53-65. <http://dx.doi.org/10.1016/0021-9975(77)90079-2> <PMid:557060>

Holschbach C.L. & Peek S.F. 2018. *Salmonella* in dairy cattle. Vet. Clin. N. Am., Food Anim. Pract. 34(1):133-154. <http://dx.doi.org/10.1016/j.cvfa.2017.10.005> <PMid:29224803>

Hughes L.E., Gibson E.A., Roberts H.E., Davies E.T., Davies G. & Sojka W.J. 1971. Bovine salmonellosis in England and Wales. Brit. Vet. J. 127(5):225-238. <http://dx.doi.org/10.1016/S0007-1935(17)37588-7> <PMid:4931663>

Juffo G.D., Bassuino D.M., Gomes D.C., Wurster F., Pissetti C., Pavarini S.P. & Driemeier D. 2017. Equine salmonellosis in southern Brazil. Trop. Anim. Health Prod. 49(3):475-482. <http://dx.doi.org/10.1007/s11250-016-1216-1> <PMid:28013440>

La Ragione R., Metcalfe H.J., Villarreal-Ramos B. & Werling D. 2013. *Salmonella* infections in cattle, p.233-262. In: Barrow P.A. & Methner U. (Eds), *Salmonella* in Domestic Animals. CABI, Oxfordshire. <http://dx.doi.org/10.1079/9781845939021.0233>.

Loeb E., Toussaint M.J., Rutten V.P. & Koeman J.P. 2006. Dry gangrene of the extremities in calves associated with *Salmonella* Dublin infection; a possible immune-mediated reaction. J. Comp. Pathol. 134(4):366-369. <http://dx.doi.org/10.1016/j.jcpa.2006.01.005> <PMid:16707135>

Lucena R.B., Pierezan F., Kommers G.D., Irigoyen L.F., Fighera R.A. & Barros C.S.L. 2010. Doenças de bovinos no Sul do Brasil: 6.706 casos. Pesq. Vet. Bras. 30(5):428-434. <http://dx.doi.org/10.1590/S0100-736X2010000500010>

Marques A.L.A., Simões S.V.D., Garino Junior F., Maia L.A., Silva T.R.D., Riet-Correa B., Lima E.F. & Riet-Correa F. 2013. Surto de salmonelose pelo sorovar Dublin em bezerros no Maranhão. Pesq. Vet. Bras. 33(8):983-988. <http://dx.doi.org/10.1590/S0100-736X2013000800006>

Mohler V.L., Izzo M.M. & House J.K. 2009. *Salmonella* in calves. Vet. Clin. N. Am. Food Anim. Pract. 25(1):37-54. <http://dx.doi.org/10.1016/j.cvfa.2008.10.009>

Pecoraro H.L., Thompson B. & Duhamel G.E. 2017. Histopathology case definition of naturally acquired *Salmonella enterica* serovar Dublin infection in young Holstein cattle in the northeastern United States. J. Vet. Diagn. Invest. 29(6):860-864. <http://dx.doi.org/10.1177/1040638717712757> <PMid:28599615>

Radostits O.M., Gay C.C., Hinchcliff K.W. & Constable P.D. 2007. Diseases associated with *Salmonella* species, p.896-920. In: Constable P.D.,

Hinchcliff K.W., Done S.H. & Grünberg W. (Eds), Veterinary Medicine: a textbook of the diseases of cattle, sheep, goats, pigs and horses. W.B. Saunders, Philadelphia.

Santos R.L., Zhang S., Tsolis R.M., Kingsley R.A., Adams L.G. & Baumler A.J. 2001. Animal models of *Salmonella* infections: enteritis versus typhoid fever. Microbes Infect. 3(14/15):1335-1344. <http://dx.doi.org/10.1016/S1286-4579(01)01495-2> <PMid:11755423>

Souza R.I.C., Santos A.C., Ribas N.L.K.S., Colodel E.M., Leal P.V., Pupin R.C., Carvalho N.M. & Lemos R.A.A. 2015. Doenças tóxicas de bovinos em Mato Grosso do Sul. Semina, Ciênc. Agrárias 36(3):1355-1368. <http://dx.doi.org/10.5433/1679-0359.2015v36n3p1355>

Strafuss A.C. 1988. Specimen collection and submission, p.71-86. In: Thomas C.C. (Eds), Necropsy: simplified procedures and basic diagnostic methods for praticing veterinarians. Thomas Books, Springfield.

Thrusfield M.V. 2004. Descrevendo ocorrência de doenças, p.47-76. In: Thomas C.C. (Eds), Epidemiologia Veterinária. Roca, São Paulo.

Vaessen M.A., Veling J., Frankena K., Graat E.A. & Klunder T. 1998. Risk factors for *Salmonella* Dublin infection on dairy farms. Vet. Q. 20(3):97-99. <http://dx.doi.org/10.1080/01652176.1998.9694848> <PMid:9684297>

Warnick L.D., Crofton L.M., Pelzer K.D. & Hawkins M.J. 2001. Risk factors for clinical salmonellosis in Virginia, USA cattle herds. Prev. Vet. Med. 49(3/4):259-275. <http://dx.doi.org/10.1016/S0167-5877(01)00172-6> <PMid:11311958>

Watanabe T.T.N., Zlotowski P., Oliveira L.G.S., Rolim V.M., Gomes M.J.P., Snel G. & Driemeier D. 2011. Rectal stenosis in pigs associated with *Salmonella typhimurium* and porcine circovirus type 2 (PCV2) infection. Pesq. Vet. Bras. 31(6):511-515. <http://dx.doi.org/10.1590/S0100-736X2011000600009>

Wray C. 1991. Salmonellosis in cattle. In Practice 13(1):13-15. <http://dx.doi.org/10.1136/inpract.13.1.13>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(8):587-591, August 2019
DOI: 10.1590/1678-5150-PVB-6011

Original Article
Livestock Diseases

Enterotoxin-encoding genes in *Staphylococcus aureus* from buffalo milk[1]

Emmanuella O. Moura[2], Adriano H.N. Rangel[2], Cláudia S. Macêdo[2], Stela A. Urbano[2], Luciano P. Novaes[2] and Dorgival M. Lima Júnior[3]*

ABSTRACT.- Moura E.O., Rangel A.H.N., Macêdo C.S., Urbano S.A., Novaes L.P. & Lima Júnior D.M. 2019. **Enterotoxin-encoding genes in *Staphylococcus aureus* from buffalo milk**. *Pesquisa Veterinária Brasileira 39(8):587-591*. Universidade Federal de Alagoas, Campus Arapiraca, Av. Manoel Severino Barbosa s/n, Bom Sucesso, Arapiraca, AL 57309-005, Brazil. E-mail: juniorzootec@yahoo.com.br

This paper investigated the occurrence of *Staphylococcus aureus* and the detection of enterotoxin-encoding genes of these strains in milk collected from 30 Murrah buffaloes used to produce dairy products in Brazil. A total of 68 strains of *Staphylococcus aureus* were found as identified by conventional laboratory tests, and thus screened for *sea, seb*, *sec, sed, see, seg, seh* and *sei* enterotoxin-encoding genes by polymerase chain reaction (PCR). Twelve strains containing enterotoxin-amplified genes were found, with higher expression for the *sei* and *seh* genes. These results can be attributed to animal health and inadequate cleaning of the equipment, indicating the need for better quality control in animal production and health lines. The results of this study with the presence of pathogens and their enterotoxigenic potential indicate a source of food poisoning, as well as being a pioneering study in the detection of new enterotoxins for buffalo milk.

INDEX TERMS: Enterotoxin, encoding genes, *Staphylococcus aureus,* buffalo, animal origin food, food microbiology, mastitis, milk quality, bovine.

RESUMO.- [Genes codificadores de enterotoxinas em *Staphylococcus aureus* no leite de búfalas.] Este estudo investigou a ocorrência de isolados de *Staphylococcus aureus* e a detecção de genes que codificam a enterotoxigenicidade dessas cepas em leite de búfala utilizado na produção de laticínios no Brasil. As amostras foram coletados em 30 búfalos da raça Murrah, identificado por testes laboratoriais convencionais, foram identificados um total de 68 cepas de *S. aureus* e rastreados para os genes que codificam a enterotoxina *sea, seb, sec, sed, see, seg, seh* and *sei* por reação em cadeia da polimerase (PCR). Doze cepas contendo genes da enterotoxina foram amplificadas, com maior expressão para os genes *sei* e *seh*. Esses resultados podem ser atribuídos à saúde animal e à higiene inadequada do equipamento, indicando a necessidade de melhor controle de qualidade nas linhas de produção e saúde animal. Os resultados desta pesquisa, com a presença de patógenos e seu potencial enterotoxigênico, indicam uma fonte de intoxicação alimentar, além de ser uma pesquisa pioneira na detecção de novas enterotoxinas para o leite de búfala.

TERMOS DE IDEXAÇÃO: Genes codificadores, enterotoxinas, *Staphylococcus aureus*, búfalos, produtos de origem animal, microbiologia de alimentos, mastite, qualidade de leite, leite de búfala, bovinos.

INTRODUCTION

Staphylococcus aureus is among the most prevalent etiological agents of subclinical mastitis in different animal species, and it stands out for having high pathogenic potential and virulence. The *S. aureus* microorganism is equipped with pathogenic mechanisms that damage the host's tissue and facilitate colonization; moreover, *S. aureus* is one of the major causative agents of foodborne diseases worldwide and contributes to high food poisoning incidence (Silva et al. 2013). As it is frequently isolated in raw milk, it is considered responsible for an increase in milk somatic cell count

[1] Received on November 19, 2018.
Accepeted for publication on March 31, 2019.
[2] Graduate Studies Program in Animal Production, Unidade Acadêmica Especializada em Ciências Agrárias, Universidade Federal do Rio Grande do Norte (UFRN), RN-160 Km 3, Cx. Postal 07, Distrito de Jundiaí, Macaíba, RN 59280-000, Brazil.

[3] Universidade Federal de Alagoas (UFAL), Campus Arapiraca, Av. Manoel Severino Barbosa s/n, Bom Sucesso, Arapiraca, AL 57309-005, Brazil. *Corresponding author: juniorzootec@yahoo.com.br

(SCC), and consequent reduction in productivity while also compromising the milk's nutritional composition (Silva et al. 2000). The enterotoxins produced by *S. aureus* (known as staphylococcal enterotoxins - SEs) are heat-resistant, which preserves their biological activity after milk pasteurization or ultra-high-temperature processing. This fact explains disease outbreaks where milk and milk products have been directly implicated via SEs (Rosa et al. 2015).

Therefore, the objective of this study was to investigate the occurrence of *Staphylococcus aureus* in raw buffalo milk and also assess the presence of staphylococcal enterotoxin-encoding genes.

MATERIALS AND METHODS

Ethics statement. The project for this study was submitted to the Ethics Committee in Animal Use (CEUA), receiving opinion number 007/2015, being free and approved for implementation from the legal point of view, according to Law No. 11,794, 2008.

Sample collection and Staphylococcus identification. Raw milk samples for *Staphylococcus* spp. isolation which had been collected from 30 Murrah buffaloes in a commercial herd operation located in Taipu (Rio Grande do Norte - RN, Brazil) were put into a refrigerator at 4°C for 8 hours. Group 1 consisted of 15 randomly selected animals in early lactation stage with production ≥15kg milk/day, and Group 2 consisted of 15 animals in late lactation stage with production ≤8kg milk/day. Buffalo cows were kept in pasture with access to concentrate supplementation according to their productivity, and were milked twice daily at 5:00 a.m. and 3:00 p.m. by a mechanized system (milking parlor). Milk was collected from all mammary quarters directly from the animals' teats. The samples were collected in sterile bottles (20mL) after application of hygienic procedures consisting of the first jets of milk, asepsis of the ceilings by a chlorinated solution, drying of the ceilings with a disposable towel, followed by disinfection of the posterior end of the ceiling and sphincter with cotton soaked in 70% alcohol (v/v), with the handler using disposable gloves at all times.The samples were then transported under refrigeration to the Quality Control Laboratory and the Microbiology Laboratory of Food Engineering at the "Universidade Federal do Rio Grande do Norte", Natal/RN, Brazil.

For *Staphylococcus aureus* isolation, 25mL of milk was taken from each sample and diluted in 225mL of sterile peptone water (0.1% wt./vol.). Decimal serial dilutions (10^{-1} and 10^{-2}) of each sample were then made from this stock solution, and 1-mL aliquots were plated in duplicate into Baird-Parker agar enriched with egg yolk emulsion and 50% potassium tellurite at 1.0% and were then incubated at 36°C for 48h for colony growth (Silva et al. 2001). Next, 5 typical and 5 atypical colonies were randomly selected and inoculated into brain heart infusion broth at 36°C for 24h. Isolated colonies were used for additional identification tests such as Gram staining (Koneman et al. 2001), as well as assays for catalase and coagulase production (Silva et al. 2001). Tests for acetoin production (Voges Proskauer test), 0.04IU bacitracin resistance, and the fermentation of mannitol, maltose, and trehalose sugars were performed according to Koneman et al. (2001). In addition, an acriflavine resistance test was performed according to Brito et al. (2002).

Antimicrobial susceptibility testing. An antibiotic sensitivity test was also performed by the VidaVet laboratory through the disk diffusion method in agar for the identified samples, according to the methodology recommended by the National Committee for Clinical Laboratory Standards (NCCLS 2003), using the bases of commercial products available for clinical treatments of samples from cows with mastitis and different antimicrobials of samples from each buffalo. For this, 17 active ingredients were used in these sensitivity tests, as these drugs are the most used by breeders and all commercial antibiotics that the veterinarian can choose as the one which best applies to the inflammatory situation of each animal. The antibiotics applied for the antibiogram were: PMN = Novobiocin (40µg) + Penicillin G procaine (40µg), CEQ = Cefquinome (30µg), AMO = Amoxicillin (10µg), DUL = Danofloxacin (5µg), CTF = Ceftiofur (30µg), CL = Cephalexin (30µg), CN = Gentamicin (10µg), TE = Tetracycline (30µg), AM = Ampicillin (10µg), ENO = Enrofloxacin (5µg), NEO = Neomycin (30µg), SUT Sulfatrim (25µg), AMC = Ampicillin + Colistin (30µg), CNM = Cefalonium (30µg), ACA = Amoxicillin (20µg) + clavulanic acid (10µg), OXA = Oxacillin (1µg), and CIP = Ciprofloxacin (5µg).

Detection of enterotoxin-encoding genes. Genomic DNA of isolates was extracted by thermal lysis according to the protocol recommended by Pacheco et al. (1997). After extraction, the DNA was kept at -20°C and quantified in a spectrophotometer (Nanodrop 2000 Termo Cientific®, Wilmington/DE). Polymerase chain reactions for the detection of *sea, seb, sec, sed, see, seg, seh*, and *sei* genes (Johnson et al. 1991, Mehrotra et al. 2000, Jarraud et al. 2002) consisted of a mixture of 1µL of primer (Invitrogen, Carlsbad, California) (Table 1), 12.5µL of 1X Master Mix (dNTP, $MgCl_2$, and Taq DNA polymerase; Promega, Madison/WI), 5.5µL of nuclease-free water (Promega), and 5µL of total DNA for a final volume of 25µL. Amplification for all genes was performed in a thermocycler (BIO-RAD, Hercules/CA) with the following cycles: initial denaturation for 5 minutes at 94°C and then 30 cycles at 94°C for 2 minutes (denaturation) and 72°C for 1 minute (extension). The various temperatures used in the annealing step are shown in Figure 1. Final extension was performed at 72°C for 5 minutes (Rall et al. 2010).

The PCR-amplified samples were analyzed by electrophoresis for 50 minutes at 110V through a 1.5% agarose gel (Invitrogen, USA) in 0.5X TBE (0.09 M Tris-HCl, 0.09M boric acid, 2mM EDTA,

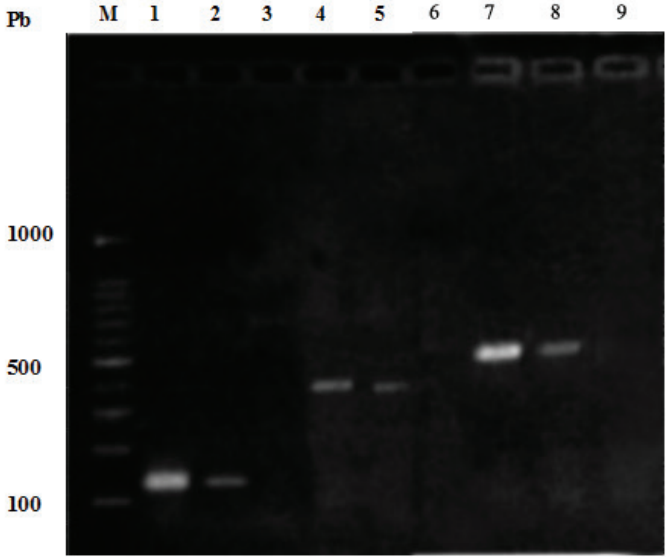


Fig.1. Agarose gel containing the amplified PCR products from enterotoxin-encoding genes of *Staphylococcus aureus*. M = 1,000bp DNA ladder, 1 = positive control for *sea* (120 bp), 2 = positive sample for *sea* (120 bp), 3 = negative control for *sea*, 4 = positive control for *seh* (376 bp), 5 = positive samples for *seh* (376 bp), 6 = negative control for *seh*, 7 = positive control for *sei* (577bp), 8 = positive samples for *sei*, and 9 = negative control for *sei*.

Table 1. Primers and temperature used for the detection of staphylococcal enterotoxin genes

Gene	Primer	Sequence	Base pair	Annealing temperature	Reference
sea	SEA-F	ttggaaacggttaaaacgaa	120	52.3°C	Johnson et al. (1991)
	SEA-R	gaaccttcccatcaaaaaca			
seb	SEB-F	tcgcatcaaactgacaaacg	478	52.3°C	Johnson et al. (1991)
	SEB-R	gcaggtactctataagtgcc			
sec	SEC-F	gacataaaagctaggaattt	257	52.3°C	Johnson et al. (1991)
	SEC-R	aaatcggattaacattatcc			
sed	SED-F	ctagtttggtaatatctcct	317	51.1°C	Johnson et al. (1991)
	SED-R	taatgctatatcttataggg			
see	SEE-F	aggtttttttcacaggtcatcc	209	50°C	Mehrotra et al. (2000)
	SEE-R	ctttttttttcttcggtcaatc			
seg	SEG-F	aattatgtgaatgctcaacccgatc	642	55°C	Jarraud et al. (2002)
	SEG-R	aaacttatatggaacaaaaggtactagttc			
seh	SEH-F	caatcaactcatatgcgaaagcag	376	61.8°C	Jarraud et al. (2002)
	SEH-R	catctacccaaacattagcacc			
sei	SEI-F	ctcaaggtgatattggtgtagg	577	61.8°C	Jarraud et al. (2002)
	SEI-R	aaaaaacttacaggcagtccatctc			

pH 8.3; Bio-Rad, USA). A 100bp ladder (New England Biolabs, USA) was used for reference. The gel was stained in a solution of Gel Red 3X (Biotium, USA) for 30 minutes and visualized by a Gel Doc™ EZ System (Bio-Rad, USA). Positive controls used for comparison included *Staphylococcus aureus* FRI 996 (*sea, seb,* and *sed*), *S. aureus* ATCC 19095 (*sec, seh,* and *sei*), *S. aureus* ATCC 27664 (*see*), and *S. aureus* ATCC 23235 (*seg*).

Descriptive statistics were used (M S. Exel©).

RESULTS

Staphylococcus identification

One hundred fifty (150) isolates of *Staphylococcus* spp. were found in the 30 analyzed samples of buffalo milk. Using the coagulase test, 93 isolates (62%) were identified as producers of the coagulase enzyme *Staphylococcus* (CPS), and of these, 38 (40.9%) were isolated from the milk samples from Group 1 and 55 (59.1%) from Group 2. Of the total CPS, 68 (73.1%) were identified as *S. aureus*. In addition, 57 (38%) isolates were identified as coagulase-negative *Staphylococcus*.

Antimicrobial susceptibility testing

The antibiogram results indicated that 13 (76.47%) of the 17 antibiotics tested were sensitive to all isolates with 100% efficiency, being: cefquinome, danofloxacin, tetracycline, ampicillin, enrofloxacin, ceftiofur, sulfatrim, amoxicillin + clavulanic acid, cefalonium, ciprofloxacin, oxacillin, amoxicillin, novobiocin + penicillin G procaine. The other tested antibiotics (cephalexin, gentamicin, neomicin and ampicilin + colistin) showed 22.22% resistance for the first three and 11.11% for the last one.

Detection of enterotoxin-encoding genes

Thirty randomly selected *Staphylococcus aureus* isolates were submitted to encoding gene detection for classic staphylococcal toxins (*sea, seb, sec, sed* and *see*) and new enterotoxins (*seg, seh* and *sei*). Of these 30 isolates, enterotoxigenic genes were found in 12 isolates (40%), including 8 (66.7%) from animals in Group 1, and 4 (33.3%) from animals in Group 2. Genes involved in *sea* synthesis were amplified in 1 sample (3.33%, Group 2), the *seh* gene was amplified in 5 samples (16.6%, Group 1), and the *sei* gene was amplified in 9 samples (30.0%), being 2 from Group 1 and 7 from Group 2. Moreover, 3 samples (25%) were amplified for 2 genes in association (1 sample for *sea* plus *sei* and 2 samples for *seh* plus *sei*) as shown in Table 2 and Figure 1. Regarding the other *seb, sec, sed, see* and *seg* genes, 18 (60%) were not amplified for any isolates in this study.

Table 2. Enterotoxin-encoding genes found in buffaloes belonging to Group 1 (early lactation animals) and Group 2 (late lactation animals)

Enterotoxins[a]	n (%)	Group 1	Group 2
A	1 (8.3)	0	1
H	5 (41.7)	0	5
I	9 (75)	3	6
Total	12	6	6

[a] The presence of genes in association were observed in 3 samples among the enterotoxins found, specifically A+I in 1 sample, and H+I in 2 samples.

DISCUSSION

A lack of strict hygienization of equipment and animal health for food production of animal origin is considered as the main source of food contamination, since these deficiencies can lead to the cultivation of enterotoxin-producing bacteria. *Staphylococcus aureus* isolated from raw buffalo milk presented most of the *sei* and *seh* genes for staphylococcal enterotoxins; this is important because these genes are capable of causing food poisoning outbreaks and only a few of these genes have been studied for this species.

Antibiotic resistance found in this study was also the same as those found by all opportunists as etiologic agents of mastitis, and similar to the results found by Barros et al. (2013). The antibiotic widely used in the treatment of mastitis is tetracycline, which showed 100% efficiency in this study, where all isolated samples of *Staphylococcus* spp. were sensitive to the same active principle. Gentamicin and neomycin, which

has shown high efficacy in vitro against mastitis agents in different studies, can be ineffective, especially when its use is frequent and inappropriate (Brito et al. 2001), and which may have occurred in animals of the present study which showed resistance to these active principles. Antimicrobial therapy is one of the main tools for controlling mastitis in herds, enabling treatment to be performed with greater efficiency and safety, but it is important to emphasize the importance of assessing antimicrobial susceptibility in vitro for each case prior to indication of treatment, since each property has a different reality and differences in milking hygiene.

Studies of enterotoxin-encoding gene expression in *Staphylococcus aureus* strains obtained from raw milk samples of different animal species have demonstrated a large variability among isolates. Such variation included differences in the presence or absence of genes and the diversity of the identified types, as well as differences in the number of strains producing staphylococcal enterotoxins and the types of enterotoxins produced (Paulin et al. 2012). For the *S. aureus* strains isolated in the present study, the *sei* gene was the most commonly found, followed by the *seh* gene and *sea.* A low occurrence (3.33%) of classic enterotoxigenic *S. aureus* isolates was observed in this study, with the *sea* gene being the only classic staphylococcal enterotoxin gene detected. Silva et al. (2013) found that the genes involved in the synthesis of *seb, sec, sed* and *see* were not amplified in any of the isolates of mastitic milk from dairy cows in Brazil.

Oliveira et al. (2011) and Rahimi & Alian (2013) reported the presence of the *sea* and *sea* and *sed* classic staphylococcal enterotoxin genes, respectively, when characterizing *Staphylococcus* spp. isolated from buffalo milk, which is corroborated by the current results. A pioneering study of toxigenic *S. aureus* in milk from buffaloes with mastitis was described by Bonna et al. (2004), who showed that only enterotoxin A was detected among the classic staphylococcal enterotoxins, and which were observed in 51.6% of the studied herds.

Similar to the present study, Luz (2009) also did not find the presence of the *seb*, *sec*, *sed* and *see* genes; however, at least 1 of the enterotoxin-encoding H and I genes was detected in 93.6% of the *Staphylococcus aureus* strains, and the presence of *seh* plus *sei* (1.1%) genotype association was found among the isolates which were positive for the toxigenic *seg*, *seh*, *sei*, and *sej* genes. The absence of genes for classic toxins and the presence of genes for other toxins may be associated with the duration and geographical distribution of the toxigenic isolates. In fact, according to Luz (2009), the gene profiles for staphylococcal enterotoxins seem to be variable among different years and geographical origins.

The occurrence of staphylococcal enterotoxins found in 40% of the isolated *Staphylococcus aureus* in the present study is high compared to Ferreira et al. (2014), who reported an occurrence rate of 19.5%. Therefore, our results indicate that the presence of *S. aureus* which have enterotoxinogenic potential in raw buffalo milk may be a source of food poisoning.

CONCLUSIONS

Several studies have investigated enterotoxins in cattle and goats, but there are few such studies on buffaloes. Additionally there is a lack of information on the occurrence of staphylococcal non-classical enterotoxin-encoding genes from milk. We highlight the need for further research and greater attention to this species to estimate its impact on buffalo milk.

Although this study does not assess the expression of enterotoxin-encoding genes, its detection is directly indicated by the enterotoxigenic potential of these strains, highlighting the need for better quality control in animal production and health lines, as well as future studies to ensure the health of the population, as buffalo milk is exclusively intended for manufacturing dairy products and such transmissions of enterotoxins will also be present in the products.

Acknowledgements.- The authors would like to acknowledge the financial support received from "Fundação de apoio à Pesquisa do Estado Rio Grande do Norte" (FAPERN)/"Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq) and Dr. Haíssa Roberta Cardarelli of the "Universidade Federal da Paraíba" (UFPB) for collaborating on the review and correction of this work.

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

REFERENCES

Barros J.P.N., Lopes L.V., Lima D.M., Oliveira A. & Botteon R.C.C.M. 2013. Limitações ao uso do antibiograma no tratamento e controle das mastites na rotina das propriedades leiteiras. Revta Bras. Med. Vet. 35(3):212-216.

Bonna I.C.F., Ferreira G.S., Carmo L.S. & Vieira-da-Mota O. 2004. Estudo da microbiota da glândula mamária de búfalas, com ênfase para *Staphylococcus aureus* produtores de toxinas. Revta Ciênc. Vida, UFRRJ 24(1):485-486.

Brito M.A.V.P., Brito J.R.F., Silva M.A.S. & Carmo R.A. 2001. Concentração mínima inibitória de dez antimicrobianos para amostras de *Staphylococcus aureus* isoladas de infecção intramamária bovina. Arq. Bras. Med. Vet. Zootec. 53(Suppl.5):531-537. <http://dx.doi.org/10.1590/S0102-09352001000500003>

Brito M.A.V.P., Campos G.M.D.M. & Brito J.R.F. 2002. Esquema simplificado para identificação de estafilococos coagulase-positivos isolados de mastite bovina. Ciência Rural 32(1):79-82. <http://dx.doi.org/10.1590/S0103-84782002000100014>

Ferreira D.H., Carvalho M.D.G.X., Nardelli M.J., Sousa F.G.C. & Oliveira C.J.B. 2014. Occurrence of enterotoxin-encoding genes in *Staphylococcus aureus* causing mastitis in lactating goats. Pesq. Vet. Bras. 34(7):633-636. <http://dx.doi.org/10.1590/S0100-736X2014000700004>

Jarraud S., Mougel C., Thioulouse J., Lina G., Meugnier H., Forey F., Nesme X., Etienne J. & Vandenesch L. 2002. Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. Infect. Immunity J. 70(2):631-641. <http://dx.doi.org/10.1128/IAI.70.2.631-641.2002> <PMid:11796592>

Johnson W.M., Tyler S.D., Ewan E.P., Ashton F.E., Pollard D.R. & Rozee K.R. 1991. Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. J. Clin. Microbiol. 29(3):426-430. <PMid:2037659>

Koneman E.W., Allen S.D., Janda W.M., Schreckenberger P.C. & Winn Junior W.C. 2001. Diagnóstico Microbiológico: texto e atlas colorido. 5th ed. Editora Médica e Científica, Rio de Janeiro. 1465p.

Luz I.S. 2009. Molecular characterization of toxins in *Staphylococcus aureus* isolated from milk and 'coalho' cheese in cities from the Agreste region of Pernambuco. Revta Inst. Med. Trop. 51(3):177. <http://dx.doi.org/10.1590/S0036-46652009000300010>

Mehrotra M., Wang G. & Johnson W.M. 2000. Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. J. Clin. Microbiol. 38(3):1032-1035. <PMid:10698991>

NCCLS 2003. Performance Standards for Antimicrobial Disk Susceptibility Tests. National Committee for Clinical Laboratory Standards, Wayne. 31p.

Oliveira A.A.F., Pinheiro Junior J.J.W., Mota R.A., Cunha M.L.R.S., Lopes C.A.M. & Rocha N.S. 2011. Phenotype characterization of *Staphylococcus* species strains isolated from buffalo (*Bubalus bubalis*) milk. J. Vet. Diagn. Invest. 23(6):1208-1211. <http://dx.doi.org/10.1177/1040638711428946> <PMid:22362803>

Pacheco A.B., Guth B.E., Soares K.C., Nishimura L., Almeida D.F. & Ferreira L.C. 1997. Random amplification of polymorphic DNA reveals serotype-specific clonal clusters among enterotoxigenic Escherichia coli strains isolated from humans. J. Clin. Microbio. 35(6):1521-1525. <PMid:9163473>

Paulin S., Horn B. & Hudson J.A. 2012. Factors influencing staphylococcal enterotoxin production in dairy products. Paper No.2012/07, MPI Technical, Ministry for Primary Industries, New Zealand Government. 78p.

Rahimi E. & Alian F. 2013. Presence of enterotoxigenic *Staphylococcus aureus* in cow, camel, sheep, goat, and buffalo bulk tank milk. Veterinarski Arch. 83:23-30.

Rall V.L.M., Sforcin J.M., Augustini V.C.M., Watanabe M.T., Fernandes Junior A., Rall R., Silva M.G. & Araújo Junior J.P. 2010. Detection of enterotoxin genes of *Staphylococcus* sp. Isolated from nasal cavities and hands of food handlers. Braz. J. Microbiol. 41(1):59-65. <http://dx.doi.org/10.1590/S1517-83822010000100011> <PMid:24031464>

Rosa D.L.S.O., Acúrcio L.B., Sant'Anna F.M., Castro R.D., Rosa B.O., Sandes S.H.C., Silva A.M., Souza M.R. & Cerqueira M.M.O.P. 2015. Detecção de genes toxigênicos, susceptibilidade antimicrobiana e antagonismo in vitro de *Staphylococcus* spp. isolados de queijos artesanais. Vigil. Sanit. Debate, Fundação Oswaldo Cruz 3:37-42.

Silva N., Junqueira V.C.A., Silveira N.F.A., Taniwaki M.H., Santos R.F.S. & Gomes R.A.R. 2001. Manual de Métodos de Análise Microbiológica de Alimentos e Água. Editora Varela, São Paulo. 317p.

Silva N.C., Guimaraes F.F., Manzi M.P., Budri P.E., Gomez-Sanz E., Benito D., Langoni H., Rall V.L. & Torres C. 2013. Molecular characterization and clonal diversity of methicillin-susceptible *Staphylococcus aureus* in milk of cows with mastitis in Brazil. J. Dairy Sci. 96(11):6856-6862. <http://dx.doi.org/10.3168/jds.2013-6719> <PMid:24054305>

Silva W.P.D., Destro M.T., Landgraf M. & Franco B.D.G.M. 2000. Biochemical characteristics of typical and atypical *Staphylococcus aureus* in mastitic milk and environmental samples of Brazilian dairy farms. Braz. J. Microbiol. 31(2):103-106. <http://dx.doi.org/10.1590/S1517-83822000000200008>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(8):592-599, August 2019
DOI: 10.1590/1678-5150-PVB-6031

Original Article
Livestock Diseases

Campylobacter jejuni and *Campylobacter coli* originated from chicken carcasses modulate their transcriptome to translate virulence genes in human cells[1]

Roberta T. Melo[2]*, Eliane P. Mendonça[2], Edson C.Valadares Júnior[2], Guilherme P. Monteiro[2], Phelipe A.B.M. Peres[2] and Daise A. Rossi[2]

ABSTRACT.- Melo R.T., Mendonça E.P., Valadares Júnior E.C., Monteiro G.P., Peres P.A.B.M. & Rossi D.A. 2019. ***Campylobacter jejuni* and *Campylobacter coli* originated from chicken carcasses modulate their transcriptome to translate virulence genes in human cells.** *Pesquisa Veterinária Brasileira* 39(8):592-599. Laboratório de Epidemiologia Molecular, Faculdade de Medicina Veterinária, Universidade Federal de Uberlândia, Rua Ceará s/n, Bloco 2D, Sala 43, Bairro Umuarama, Uberlândia, MG 38402-018, Brazil. E-mail: roberta-melo@hotmail.com

The aim was to determine the spread of genetically similar profiles of *Campylobacter* in chicken carcasses and evaluate their ability to produce transcripts for *ciaB*, *dnaJ*, *p19* and *sodB* genes, before and after cultivation in Caco-2 cells. The strains used were isolated from 420 samples of chicken carcasses chilled and frozen ready for marketing. The species were identified by PCR-multiplex, the phylogeny was determined by RAPD-PCR and the presence of transcripts was performed by RT-PCR. We identified 74 (17.6%) of *Campylobacter* strains, being 55 (74.3%) *C. jejuni* and 19 (25.7%) *C. coli*. The phylogenetic relationship demonstrated heterogeneity between isolates of the same species, with absence of clones, indicating the high level of diversity of circulating genotypes. The gene transcription showed conflicting results before and after the culture in Caco-2 cell, so that before cultivation isolates showed greater capacity to transcribe genes related to survival and after the interaction with human cells, the strains showed higher potential to transcribe genes associated with virulence. The result of this study contributes to the understanding of how these seemingly fragile microorganisms are the most prevalent bacterial agents in human gastroenteritis.

INDEX-TERMS: *Campylobacter jejuni*, *Campylobacter coli*, chicken, carcasses, transcriptome, virulence genes, human cells, campylobacteriosis, RT-PCR, gene, cells, virulence.

RESUMO.- [***Campylobacter jejuni* e *Campylobacter coli* originadas de carcaças de frango modulam seu transcriptoma para traduzir genes de virulência em células humanas.**] O objetivo foi determinar a disseminação de perfis geneticamente semelhantes de *Campylobacter* em carcaças de frango e avaliar sua capacidade de produzir transcritos para os genes *ciaB*, *dnaJ*, *p19* e *sodB*, antes e após o cultivo em células Caco-2. As cepas utilizadas foram isoladas de 420 amostras de carcaças de frango resfriadas e congeladas prontas para comercialização. As espécies foram identificadas por PCR-multiplex, a filogenia foi determinada por RAPD-PCR e a presença de transcritos foi realizada por RT-PCR. Identificamos 74 (17,6%) das cepas de *Campylobacter*, sendo 55 (74,3%) *C. jejuni* e 19 (25,7%) *C. coli*. A relação filogenética demonstrou heterogeneidade entre isolados da mesma espécie, com ausência de clones, indicando o alto nível de diversidade dos genótipos circulantes. A transcrição gênica mostrou resultados conflitantes antes e após a cultura em células Caco-2, de modo que, antes do cultivo, os isolados apresentaram maior capacidade de transcrever genes relacionados à sobrevivência e após a interação com células humanas, as linhagens apresentaram maior potencial para transcrever genes associados à virulência. O resultado deste estudo contribui para a compreensão de como esses microrganismos aparentemente frágeis são os agentes bacterianos mais prevalentes na gastroenterite humana.

TERMOS DE INDEXAÇÃO: *Campylobacter jejuni*, *Campylobacter coli*, carcaças de frango, transcriptoma, genes de virulência, células humanas, campilobacteriose, RT-PCR, virulência.

[1] Received on February 13, 2019.
Accepted for publication on March 31, 2019.
[2] Laboratório de Epidemiologia Molecular, Faculdade de Medicina Veterinária, Universidade Federal de Uberlândia (UFU), Rua Ceará s/n, Bloco 2D, Sala 43, Bairro Umuarama, Uberlândia, MG 38402-018, Brazil. Fone: +55 34 3213-2319. E-mails: daise.rossi@ufu.br, eliane_vet@yahoo.com.br, edson2campos@hotmail.com, guil.paz@hotmail.com, lipe-peres1@hotmail.com; *Corresponding author: roberta-melo@hotmail.com

INTRODUCTION

Campylobacter spp. is the main zoonotic agent that causes gastroenteritis in developed countries (CDC 2013, WHO 2013, EFSA 2014). Campylobacteriosis has serious repercussions for public health and a significant socio-economic impact (EFSA 2013). The species most involved in diarrheal cases are *Campylobacter jejuni* and *Campylobacter coli*, being *C. jejuni* the most prevalent and more associated with autoimmune complications such as Guillain-Barré Syndrome (EFSA 2014).

Among the foods involved in *Campylobacter* transmission to humans, chicken meat and its derivatives are considered the main source (EFSA 2014). Data from the European Union countries and USA show contamination rate in chicken carcasses at high rates, ranging from 25 to 100% (CDC 2013, EFSA 2014).

Unlike other enterobacteria such as *Salmonella* and *Shigella*, this microorganism presents a series of signal transduction systems which may contribute to its adaptation to various stress conditions. These systems also help in gene expression related to virulence (Mourik 2011), as *ciaB* genes (intracellular invasion), *dnaJ* (thermotolerance), *p19* (iron transport during stress) and *sodB* (defense to oxidative stress).

This study aimed to identify the positivity of *Campylobacter sp*. in chicken carcasses to determine the genetic proximity profiles of isolated and changes in the transcriptome of these agents when isolated from food and after passage in human intestinal cells (in vitro).

MATERIALS AND METHODS

We performed 21 collections of chilled and frozen chicken carcasses in the period of June 2011 to February 2012. In each collection were sampled 20 carcasses produced and slaughtered in three Brazilian states (Distrito Federal, Goiás and Minas Gerais), totaling 420 samples. The chicken carcasses were from refrigerators under federal inspection whose products are marketed across the country and also exported to Asia, Africa and the Middle East.

The processing of the samples was conducted at the Laboratory of Molecular Epidemiology at the Faculty of Veterinary Medicine and at the Center of Electron Microscopy of the Institute of Biomedical Sciences of "Universidade Federal de Uberlândia" (UFU). To perform all analyzes, it was used as positive control strains of *Campylobacter jejuni* ATCC 33291, *C. coli* ATCC 43478 and *C. jejuni* NCTC 11351, and milli-Q water in place of the sample as a negative control.

For isolation of *Campylobacter* spp., 420 chicken carcasses were subjected to rinsing process in 400mL of 0.1% of casein peptone (Difco®), as described by Zhao et al. (2010). A 30 mL portion of the product washings were pre-enriched in Bolton broth (Oxoid®) in double concentration and supplemented with antibiotics (Oxoid®) and 5% of defibrinated sheep blood (Laborclin®). The samples were incubated at 37°C for 44±4 hours in microaerophilic atmosphere. After this time, 100mL of each sample was inoculated on agar plates of m-CCDA (*Campylobacter* Blood-Free Selective Medium - Modified CCDA Preston) (Oxoid®) containing their antibiotic supplement (Oxoid®) and a membrane filter with porosity of 0,65µm (Millipore®) to reduce contamination. Then the plates were incubated at 37°C for 44±4 hours in microaerophilic atmosphere according to the instructions of ISO 10272-1 (ISO 2006).

The identification of *C. jejuni* and *C. coli* were verified by PCR-multiplex, with use of primers *pg3* (5'GAACTTGAACCGATTTG3')/*pg50* (5'ATGGGATTTCGTATTAAC3') and *C1* (5'CAAATAAAGTTAGAGGTAGAATGT3')/*C4* (5'GGATAAGCACTAGCTAGCTGAT3'). DNA extraction was performed with DuPont™ PCR Reagent kit, according to the manufacturer's instructions. The primers, the PCR reagents concentrations and the amplification protocol were performed as Harmon et al. (1997).

After identifying, the strains were evaluated for the ability to produce transcripts. For this, RNA extraction was performed using the Trizol method as Li et al. (2008) with modifications. The isolates from each sample obtained from the four cropping agar plates of m-CCDA, were transferred to microcentrifuge tubes containing 2mL of NaCl solution 0.85% (Synth®). The mixture was centrifuged at 12,000G for ten minutes at 4°C. To the obtained pellets was added 1mL of Trizol (Invitrogen®) and homogenized by vortexing (Phoenix®). Thereafter, 200µL of chloroform was added (Isofar®) and repeated the procedure of vortex homogenization followed by centrifugation at 12,000G for 15 minutes at 4°C. The formed aqueous portion was transferred to a new microtube, to which 500µL of isopropanol was added (Sigma Aldrich®), homogenized and centrifuged again at 12,000G for 10 minutes at 4°C. At the pellet formed was added 1mL of ethanol 75% (Sigma Aldrich®) and after, homogenization and centrifugation at 7.500g at 4°C for 5 minutes, the obtained supernatant was discarded. The RNA pellets were dried at room temperature to be diluted in 20µL of DEPC water (Invitrogen®). The concentration of RNA used was 200ng/µL, quantified in a spectrophotometer device NanoDrop (Thermo Scientific®).

Reverse transcription was performed with 10U of RNase inhibitor, 40U of MMLV-RT (Amersham Biosciences®), 1X of MMLV-RT buffer (Amersham Biosciences®), 200µM of dNTPs (dGTP, dATP, dTTP and dCTP), 126pmol of random hexamer oligonucleotides as primers (Invitrogen), 20µL of DEPC water (Invitrogen®) and 1µL of RNA, all kept at 37°C for one hour to obtain complementary DNA (cDNA). Subsequently, 3µL of the cDNA was used for amplification in a reaction volume of 25µL, comprising: 0,625U of Taq DNA polymerase, 5mM $MgCl_2$, 200µM of dNTPs and 4 pmoles of each primer (Table 1) (Invitrogen). The amplification and electrophoresis were performed as Birk et al. (2012).

The evaluation of gene transcription was also performed after culture in Caco-2 cells (human colon adenocarcinoma cells of the cell bank of Rio de Janeiro, Brazil - BCRJ: CR069), to compare the change in behavior of the strains. Polarized Caco-2 cells grown in the bottles (BD Falcon™) were used to evaluation with 24 hours after inoculation (Pinto et al. 1983). For infection, an aliquot of 100mL of NaCl 0.85% solution containing 10^6 CFU of each strain was placed in a bottle (BD Falcon™) with approximately 80% of confluency, containing 5mL of DMEM (Invitrogen) pure. After incubation, the cells inside of the bottle (BD Falcon™) were scraped with a cell scraper aid (BD Falcon™) and the contents were transferred to microcentrifuge tubes of 2mL containing DMEM medium (Invitrogen). The samples were centrifuged at 12,000G (Cientec®) for ten minutes at 4°C. To the pellet was added 1mL of Trizol (Invitrogen®) and the remaining steps for the realization of the RNA extraction and RT-PCR, were performed as described previously.

The genetic diversity of strains was determined by RAPD-PCR (Random Amplification of Polymorphic DNA), according to Akopyanz et al. (1992), using the primers HLWL (5'ACGTATCTGC3') and 1290 (5'GTGGATGCGA3'). Images of the gels were captured by transluminator (Loccus Biotechnology®) for further computational analysis in the program GelCompar II®. All formed bands were considered in the analysis and the similarity matrix was obtained using the Dice similarity coefficient. The final analysis was based on the average of the results obtained in both primers (average

Table 1. Primers used to verify the production of transcripts for *ciaB, dnaJ, p19* and *sodB* genes by *Campylobacter jejuni* and *C. coli* before and after cultivation in Caco-2 cells

Genes	Sequence 5'→3'	Molecular weight (mw)	Reference
ciaB	ATATTTGCTAGCAGCGAAGAG	157	Li et al. (2008)
	GATGTCCCACTTGTAAAGGTG		
dnaJ	AGTGTCGAGCTTAATATCCC	117	Li et al. (2008)
	GGCGATGATCTTAACATACA		
p19	GATGATGGTCCTCACTATGG	206	Birk et al. (2012)
	CATTTTGGCGTGCCTGTGTA		
sodB	TATCAAAACTTCAAATGGGG	170	Birk et al. (2012)
	TTTTCTAAAGATCCAAATTCT		

from experiments). It was used UPGMA (unweighted pair group method with arithmetic mean) for the construction of dendrograms (Madden et al. 2007).

The tests were performed in triplicate and the results were tabulated and submitted to descriptive statistics to calculate the percentages for *Campylobacter* occurrence and the species *C. jejuni* and *C. coli*. To analyze the production of transcripts in each species, at the time of isolation in food and after cultivation in Caco-2 cells was used the Fisher one tailed exact test, by GraphPad Software Inc.

RESULTS AND DISCUSSION

Of 420 chickens' carcasses investigated, 74 (17.6%) were positive for *Campylobacter*. Of these, 55/74 (74.3%) were identify by multiplex PCR as *Campylobacter jejuni* and 19/74 (25.7%) as *C. coli*.

Positivity was lower than that found in other studies, but the higher incidence of *C. jejuni* in this type of food agrees with the global data of the WHO (2013) and EFSA (2014). According to EFSA report (2014), overall, 23.6% of individual samples or in flocks were positive for *Campylobacter* in 15 member states of the European Union in 2012. A study carried out in Ireland in 2008 showed that from 394 chicken cooled carcasses that were studied, 98% were contaminated with *C. jejuni* (EFSA 2010). In Canada, the percentage was 33.7% (2146/6367) of positive samples in cooled chicken meat from 2003 to 2010 (Agunos et al. 2013). In Iran, was 63% and in Japan 45.8% (FAO 2009).

The analysis by RT-PCR showed the presence of transcripts in 62.0% (49/74) of the evaluated strains after isolation from carcasses, and before cultivation in Caco-2 cells. Of this total, 69.4% (34/49) treated up as genes transcribed by *C. jejuni,* and 30.6% (15/49) by *C. coli*. The studied genes showed different importance for virulence and adaptation processes of the strains to stress related to cold.

The potential pathogenic of *Campylobacter* can be identified by the presence of *ciaB* and *dnaJ* genes. The gene *ciaB* is essential to encode CiaB protein, which is important in the process of epithelial cell invasion (Poly & Guerry 2008) and also the invasion of intestinal mucosa (Ziprin et al. 2001). The *dnaJ* gene encodes a heat shock protein, which guarantees thermotolerance, and thus the micro-organism is able to overcome to the abrupt temperature variations (Stintzi 2003), facilitating their adaptation to the conditions of human intestine.

The *p19* and *sodB* genes are associated with the cold adaptation mechanisms. The *p19* gene encodes a periplasmic iron-dependent protein whose function is to transport iron (Palyada et al. 2004). The regulation of this protein indicates a way to control the level of intracellular iron during stress, a key element in the metabolic processes (Birk et al. 2012). The translation of SodB protein triggers a defense mechanism of the cell against oxidative stress resulting from the cold shock. In addition, this gene specifically protects the cellular components, including various cytoplasmic enzymes, DNA and membrane factors against damage caused by oxygen free radicals (Stintzi & Whitworth 2003).

In this study, in *C. jejuni*, the transcription of *ciaB* gene occurred in 54.5% (30/55) of the strains, *dnaJ* gene in 40.0% (22/55), *p19* gene in 30.9% (17/55) and *sodB* gene in 25.4% (14/55). For *C. coli* species, the values found were 31.6% (6/19), 26.3% (5/19), 57.9% (11/19) and 68.4% (13/19), respectively, for the same genes (Table 2).

These data shows that, although strains isolated from samples kept under the same conditions (low temperatures), the species showed different behavior. Transcription of genes associated with virulence (*ciaB* and *dnaJ*) was most evident in *C. jejuni*, with significant difference in *ciaB* (P=0.04). In other hand, in *C. coli* was observed that, statistically, had a greater production of transcripts for genes related to cold shock stress (*p19* and *sodB*) (P<0.01).

Therefore, it can be inferred that even when subjected to injury conditions, *C. jejuni* showed pathogenic potential associated with the invasion process and expressed in lower percentages the adaptive mechanisms to survive. According to Mourik (2011) the gene transcription in *Campylobacter* is regulated to prevent the unnecessary production.

The virulent profile of *C. jejuni* was quoted by Thakur et al. (2010) and Melo et al. (2013), showed that the greatest potential of this species over the other in causing clinical cases in humans (81.1%) (EFSA 2014) is due to the properties of invasion, colonization and toxin production which are essential to elicit its pathogenesis.

In contrast, *C. coli* showed that its priority is to ensure the survival, through the consolidation of the P19 and SodB proteins. The expression of mechanisms of adaptation to low temperatures for *C. coli* allows the acquisition or the biosynthesis of cryoprotectant molecules, leading to changes in the lipid composition of the membrane ensuring the survival under these conditions (Stintzi & Whitworth 2003).

After cultivation in Caco-2 cells was observed that there was a change in the transcriptome of the strains. The transcription was observed in 90.5% (67/74) of the strains, whereas 82.1% (55/67) were produced by *C. jejuni* and 17.9% (12/67) by *C. coli*.

Table 2. Production of transcripts of the *ciaB, dnaJ, p19* and *sodB* genes by 74 *Campylobacter* strains isolated from chicken carcasses before and after culturing in Caco-2 cells

Gene transcription	Beforeculture in Caco-2 cells		Afterculture in Caco-2 cells	
	C. jejuni(N=55) n (%)	*C. coli* (N=19) n (%)	*C. jejuni*(N=55) n (%)	*C. coli* (N=19) n (%)
ciaB	30 (54,5)^{aA}	6 (31,6)^{bC}	36 (65,4)^{cA}	8 (42,1)^{dC}
dnaJ	22 (40,0)^{aA}	5 (26,3)^{aC}	42 (76,4)^{cB}	10 (52,6)^{dD}
p19	17 (30,9)^{aA}	11(57,9)^{bC}	7 (12,7)^{cB}	2 (10,5)^{cD}
sodB	14 (25,4)^{aA}	13 (68,4)^{bC}	0 (0)^{cB}	3 (15,8)^{dD}

N = number of evaluated strains, n = number of strains that transcribed, % = percentage of strains that transcribed, a,b = difference between species before passing through Caco-2 cells, c,d = difference between species after cultivation, A,B = difference in transcription by *C. jejuni* before and after cultivation in cells, C,D = difference in transcription by *C. coli* before and after the culture (one tailed Fisher Test - P<0.05).

The transcripts' production were found in all 55 studied strains of *C. jejuni*, to *ciaB* gene the occurrence was of 65.4% (36/55) of the strains, to *dnaJ* was 76.4% (42/55) and to *p19* was 12.7% (7/55). There were no transcripts for *sodB*. To *C. coli*, the percentages were 42.1% (8/19), 52.6% (10/19), 10.5% (2/19) and 15.8% (3/19) for the same genes, respectively (Table 2).

The modulation of gene transcription in this moment (after inoculation in Caco-2 cells) proves the fact that these microorganisms have highly sensitive and specific mechanisms for rapid adaptation and change in their behavior (Mourik 2011).

Ma et al. (2009) evaluated the virulence expression associated with the genes *cdtB*, *cadF* and *ciaB*, when *C. jejuni* was submitted to nutritional stress. The *cdtB* transcription was observed only in the periods from 2 to 6 hours, while the expression of *cadF* and *ciaB* had been significantly reduced between 24 to 48 hours under nutritional stress. Distinctively, exposure to zinc oxide (ZnO), a food additive, did not significantly affect the transcription of *cdtB*, *cadF* and *ciaB* (Xie et al. 2011).

It was observed a tendency to increase transcription of virulence genes (*ciaB* and *dnaJ*) and decreased of cold adaptation genes (*p19* and *sodB*) when both species were cultured in Caco-2 cells. However, the change was not significant for *ciaB* gene. By species' comparison, it was observed that this behavior was more evident (P<0.05), with the exception of the *p19* gene, which showed no significant difference. This confirms the higher pathogenic potential of *C. jejuni* and a lower necessity for expressing characteristics of thermal adaption when compared to *C. coli*. These study verifies further evidence that *C. jejuni* has greater advantage over *C. coli* in causing clinical cases in humans.

This was expected, since according to Konkel et al. (2004) a greater transcription of genes associated with invasion occurs when the strains are in contact with the host intestinal epithelial cells. The presence of CiaB proteins increases the disease's severity with development of diarrhea symptoms 24 hours after contact, differently of strains with a lack of translation of these proteins that can carry this symptom only after three days of incubation (Konkel et al. 2001).

Moreover, the cultivation of the strains in higher temperature (37°C) and appropriate environment (5% of CO_2) in the presence of nutrients and host cells that enable the development of pathogenesis may have contributed to the expression of *ciaB* and *dnaJ* genes.

It is important to emphasize that, although most studies give greater importance to *C. jejuni* pathogenic potential (Ma et al. 2009, Mourik 2011, Xie et al. 2011), it is essential to highlight merit also to *C. coli* (Di Giannatale et al. 2014) in the transcription of genes that enhance virulence-associated to the pathogenic potential when presented in a human host. The submission to the different conditions promoted variations in the transcriptome of these agents as a way to overcome the environment and adaptation to allow their survival and colonization.

The homology among isolates of both species not identified the presence of clones, but were detected clusters with genetic similarity greater than 80% and were classified as belonging to the same genotype (Fig.1 and 2).

Were identified 15 clusters in *C. jejuni* corresponding to A to O profiles and 18 distinct genotypes (Fig.1), which proves the high genetic diversity among 55 isolates. As for the place of isolation, it was found that the profiles D, E, J, K, L and O had strains originated from the same place, Minas Gerais. However, the most prevalent was the presence of strains' clusters from chickens of different places, in groups A, B, C, F, G, H, I, M and N. This indicates that there is dissemination of different genotypes between three regions studied, and probably for other states in the country, besides the different nations that import these products.

The comparison on the day of collection of the samples indicates that the profiles B, C, G, J, K, M and O represents clusters of strains on the same day, and the groups J, K and O have isolated strains of the same flock. These three clusters infer the possibility of cross contamination in some stage of the production process, which promoted the presence of strains with high similarity in different chickens from the same flock. The other groups, A, D, E, F, H, I, L and N are composed of strains from different flocks, which suggest a possible neglection of biosecurity standards, which may have contributed to the persistence of the micro-organism along the production chain. This hypothesis can be sustained in the observation of cluster E, which has 84.8% of similarity, consisting of four strains from Minas Gerais chickens identified in September and November 2011 and in January and February 2012, confirming the persistence of genotype for at least six months.

As for genotypic characteristics related to gene transcription before and after cultivation in Caco-2 cells was observed homology to clusters B, D, E, G, I, L and O for the *dnaJ* gene and in clusters F, H and J to *ciaB* gene. Profiles A, C and N had in common the transcription of *ciaB* and *dnaJ*. The strains

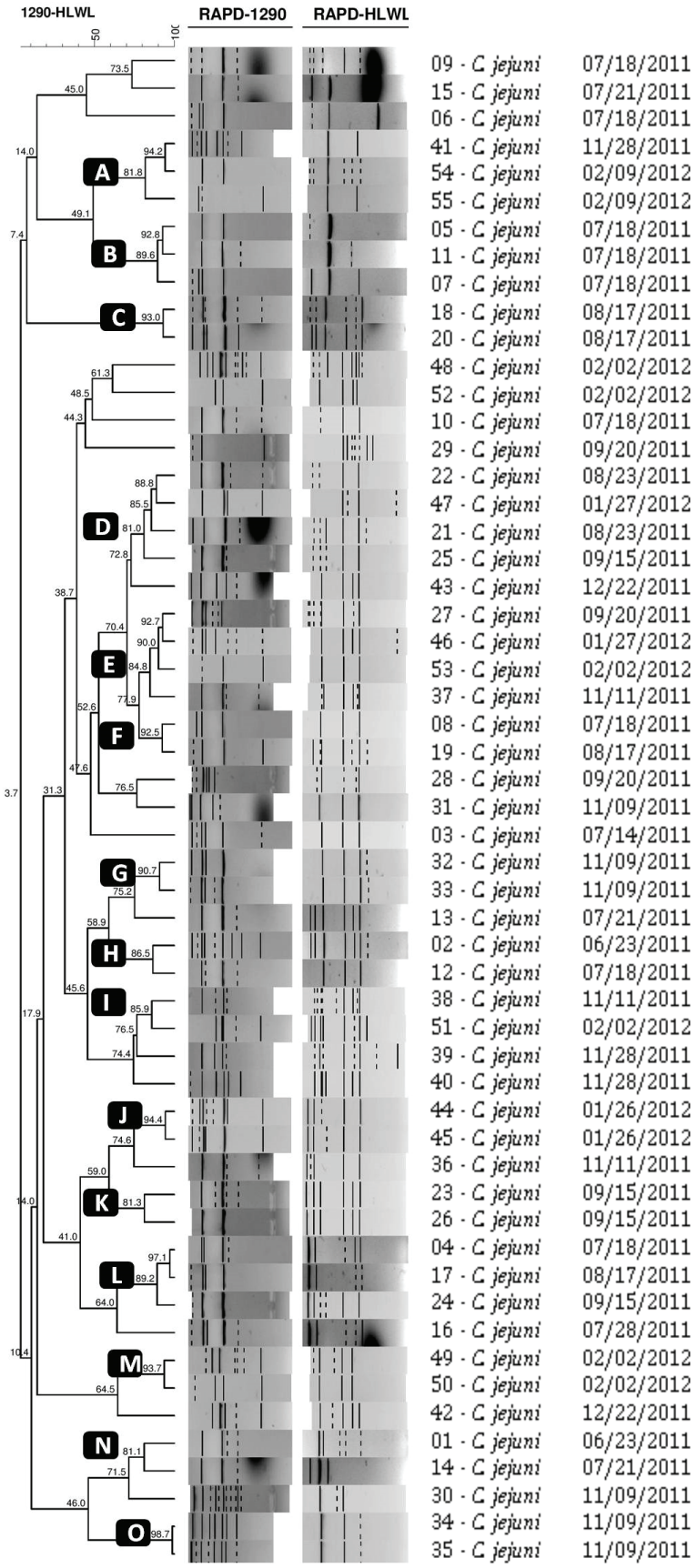


Fig.1. Dendrogram of 55 *Campylobacter jejuni* isolates from cold and frozen chickens using the RAPD-PCR technique with primers 1290 and HLWL, using the average of experiments with a tolerance of 1.5% and UPGMA method with optimization of 80%, by the program GelCompar.

that form the K group obtained as standard the presence of transcripts for *ciaB*, *dnaJ* and *p19*.

The dendrogram analysis of 19 strains of *C. coli* allowed the detection of three clusters identified by the profiles A, B and C and 12 distinct genotypes (Fig.2). These results also show the high level of genetic diversity among the isolates of this species.

The profile A was composed of strains from different states (Distrito Federal and Minas Gerais) and different flocks. The presence of a subgroup with 95.4% of homology was detected in the cluster B, which is composed of strains originating from the same flock provided from state of Goiás, suggesting that there is horizontal transmission of this genotype strain of chickens during primary processing or production. The other strain differs from the others, since it is provided from Minas Gerais and was collected in different date. A different pattern was found in the cluster C, where both strains were isolated from Minas Gerais, but in separate flocks. It is possible that the permanence of this genotype between consecutive flocks is a result of not meeting the standards of biosecurity from one flock to another.

In genotypic level, the clusters A and C presented transcripts for *sodB* in common. And the B subgroup showed no profile for transcripts of all the genes studied.

The genetic diversity observed by RAPD-PCR in *C. jejuni* and *C. coli* was also reported by Aquino et al. (2010), Madden et al. (2007), Ridley et al. (2008) and Workman et al. (2008) in *Campylobacter* isolates from chickens, pigs and medical patients in Brazil, United Kingdom and Barbados. This genotypic variation may be due to over exposure to a source of contamination during the production process of chickens, or genetic changes in bacterial population after colonization (Workman et al. 2008). Furthermore, *Campylobacter* spp. has a natural ability to change and undergo genomic rearrangements, which may also explain the higher genetic diversity (Ridley et al. 2008). Other reasons include the contamination of samples with multiple strains and cross-contamination (Workman et al. 2008).

The persistence of strains with high percentage of phylogenetic similarity in different flocks was also reported by Petersen & Wedderkopp (2001) in chicken samples. The authors associated the problem of the lack of hygienic conditions and the presence of insects and rodents as indicative of the microorganism permanence in the production chain of chickens.

In this study, similar genealogical groups were found in the three regions studied, indicating a probable association of genotypes in different parts of the country. Evidence of highly similar strains circulating in the country and possibly in other countries may indicate that *Campylobacter* have the capacity to act in a widespread form, as a global transmission network in humans. This fact was also observed by Sheppard et al. (2010) who observed high similarity among isolates of the Netherlands, USA and Senegal, revealing the international diffusion of highly phylogenetically close lines.

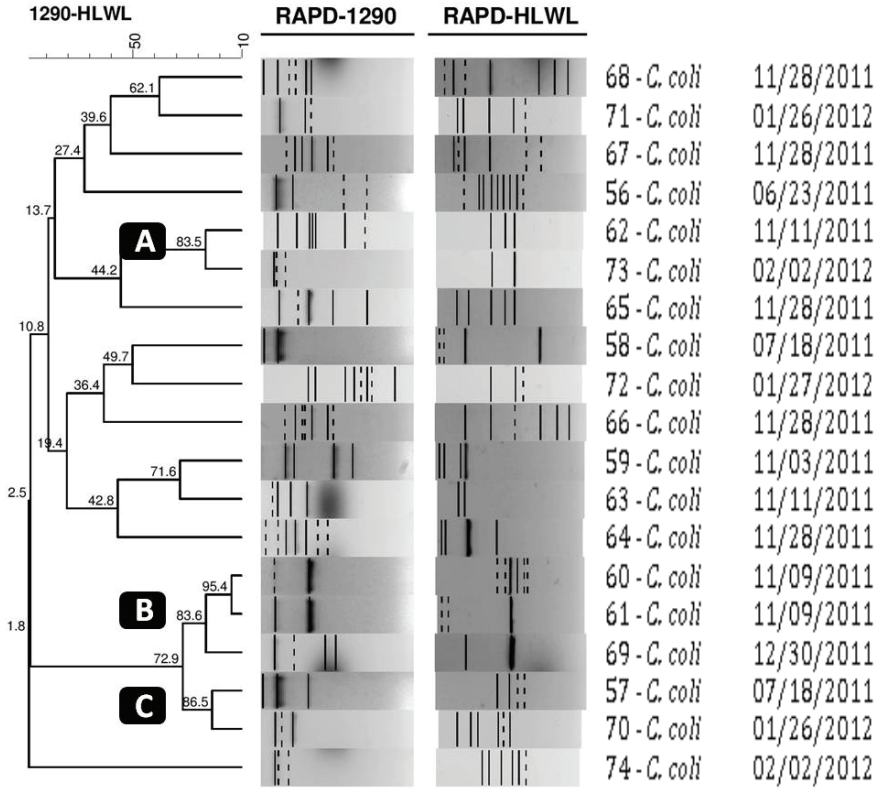


Fig.2. Dendrogram of the 19 isolates of *Campylobacter coli* coming from chilled and frozen chickens, the RAPD-PCR with the primers 1290 and HLWL, using the average from experiments with a tolerance of 1.5% and UPGMA method with optimization of 80% by GelCompar program.

CONCLUSIONS

The percentages of isolation found in chicken carcasses indicate the potential risks to the consumer, since these are strains that have withstood the processing of slaughter, in addition to cooling or subsequent freezing. This reinforces the need to implement more stringent control programs.

Changes in gene transcription observed as the growing conditions of the microorganism demonstrated the capacity of *Campylobacter* to modulate their virulence, by changing its transcriptome. This variation shows that the agent is able to adapt to a condition imposed on it, sometimes to ensure their survival as in stressful situations, and sometimes to express their pathogenic potential as observed in cell cultures of human origin, directly related to symptoms and the development of clinical disease.

The genotypic diversity observed revealed that there are probably several sources of of poultry infection and contamination of carcasses during the production process.

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

REFERENCES

Agunos A., Léger D., Avery B.P., Parmley E.J., Deckert A., Carson C.A. & Dutil L. 2013. Ciprofloxacin resistent *Campylobacter* spp. in retail chicken, Western Canada. Emerg. Infect. Dis. 19(7):1121-1124. <http://dx.doi.org/10.3201/eid1907.111417> <PMid:23764141>

Akopyanz N., Bukanov N.O., Westblom T.U., Kresovich S. & Berg D.E. 1992. DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. Nucleic Acids Res. 20(19):5137-5142. <http://dx.doi.org/10.1093/nar/20.19.5137> <PMid:1408828>

Aquino M.H.C., Filgueiras A.L.L., Matos R., Santos K.R.N., Ferreira T., Ferreira M.C.S., Teixeira L.M. & Tibana A. 2010. Diversity of *Campylobacter jejuni* and *Campylobacter coli* genotypes from human and animal sources from Rio de Janeiro, Brazil. Res. Vet. Sci. 88(2):214-217. <http://dx.doi.org/10.1016/j.rvsc.2009.08.005> <PMid:19765787>

Birk T., Wik M.T., Lametsch R. & Knochel S. 2012. Acid stress response and protein induction in *Campylobacter jejuni* isolates with different acid tolerance. BMC Microbiol. 12(1):174. <http://dx.doi.org/10.1186/1471-2180-12-174> <PMid:22889088>

CDC 2013. *Campylobacter*. Division of Foodborne, Waterborne, and Environmental Diseases (DFWED), National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), Center for Disease Control and Prevention (CDC). Available at <http://www.cdc.gov/nczved/divisions/dfbmd/diseases/campylobacter/technical.html> Accessed on Apr. 2, 2014.

Di Giannatale E., Di Serafino G., Zilli K., Alessiani A., Sacchini L., Garofolo G., Aprea G. & Marotta F. 2014. Characterization of antimicrobial resistance patterns and detection of virulence genes in *Campylobacter* isolates in Italy. Sensors, Basel 14(2):3308-3322. <http://dx.doi.org/10.3390/s140203308> <PMid:24556669>

EFSA 2010. Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU, 2010. EFSA J. 8(8):1-132.

EFSA 2013. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011, 2013. EFSA J. 11(4):1-250.

EFSA 2014. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2012. EFSA J. 12(2):1-312. <http://dx.doi.org/10.2903/j.efsa.2014.3547>

FAO 2009. Risk assessment of *Campylobacter* spp. in broiler chickens: technical report. Microbiological Risk Assessment Series. Vol. 12. Food and Agriculture Organization of the United Nations, World Health Organization, Geneva, p.132.

Harmon K.M., Ransom G.M. & Wesley I.V. 1997. Differentiation of *Campylobacter jejuni* and *Campylobacter coli* by polymerase chain reaction. Mol. Cell. Probes 11(3):195-200. <http://dx.doi.org/10.1006/mcpr.1997.0104> <PMid:9232618>

ISO 2006. ISO 10272-1: Microbiology of food and animal feeding stuffs: horizontal method for detection and enumeration of *Campylobacter* spp. Part 1: Detection method. 2nd ed. International Standards Organization, Geneva.

Konkel M.E., Monteville M.R., Rivera-Amill V. & Joens L.A. 2001. The pathogenesis of *Campylobacter jejuni*: mediated enteritis. Curr. Issues. Intest. Microbiol. 2(2):55-71. <PMid:11721281>

Konkel M.E., Klena J.D., Rivera-Amill V., Monteville M.R., Biswas D., Raphael B. & Mickelson J. 2004. Secretion of virulence proteins from *Campylobacter jejuni* is dependent on a functional flagellar export apparatus. J. Bacteriol. 186(11):3296-3303. <http://dx.doi.org/10.1128/JB.186.11.3296-3303.2004> <PMid:15150214>

Li Y.P., Ingmer H., Madsen M. & Bang D.D. 2008. Cytokine responses in primary chicken embryo intestinal cells infected with *Campylobacter jejuni* strains of human and chicken origin and the expression of bacterial virulence-associated genes. BMC Microbiol. 8(1):107. <http://dx.doi.org/10.1186/1471-2180-8-107> <PMid:18588667>

Ma Y., Hanning I. & Slavik M. 2009. Stress-induced adaptive tolerance response and virulence gene expression in *Campylobacter jejuni.* J. Food Safety 29(1):126-143. <http://dx.doi.org/10.1111/j.1745-4565.2008.00147.x>

Madden R.H., Moran L. & Scates P. 2007. Diversity of *Campylobacter coli* genotypes in the lower porcine gastrointestinal tract at time of slaughter. Lett. Appl. Microbiol. 45(6):575-580. <http://dx.doi.org/10.1111/j.1472-765X.2007.02246.x> <PMid:17922816>

Melo R.T., Nalevaiko P.C., Mendonça E.P., Borges L.W., Fonseca B.B., Beletti M.E. & Rossi D.A. 2013. *Campylobacter jejuni* strains isolated from chicken meat harbour several virulence factors and represent a potential risk to humans. Food Control 33(1):227-231. <http://dx.doi.org/10.1016/j.foodcont.2013.02.032>

Mourik A.V. 2011. Host adaptation mechanisms and transcriptional regulation in *Campylobacter jejuni*. Doctoral Dissertation, Infection and Immunity Center Utrecht, Universiteit Utrecht. 152p.

Palyada K., Threadgill D. & Stintzi A. 2004. Iron acquisition and regulation in *Campylobacter jejuni.* J. Bacteriol. 186(14):4714-4729. <http://dx.doi.org/10.1128/JB.186.14.4714-4729.2004> <PMid:15231804>

Petersen L. & Wedderkopp A. 2001. Evidence that certain clones of *Campylobacter jejuni* persist during successive broiler flock rotations. Appl. Environ. Microbiol. 67(6):2739-2745. <http://dx.doi.org/10.1128/AEM.67.6.2739-2745.2001> <PMid:11375189>

Pinto M., Robine S. & Appay M.D. 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol. Cell 47:323-330.

Poly F. & Guerry P. 2008. Pathogenesis of *Campylobacter.* Curr. Opin. Gastroenterol. 24(1):27-31. <http://dx.doi.org/10.1097/MOG.0b013e3282f1dcb1> <PMid:18043229>

Ridley A.M., Toszeghy M.J., Cawthraw S.A., Wassenaar T.M. & Newell D.G. 2008. Genetic instability is associated with changes in the colonization potential of *Campylobacter jejuni* in the avian intestine. J. Appl. Microbiol. 105(1):95-104. <http://dx.doi.org/10.1111/j.1365-2672.2008.03759.x> <PMid:18298527>

Sheppard S.K., Colles F., Richardson J., Cody A.J., Elson R., Lawson A., Brick G., Meldrum R., Little C.L., Owen R.J., Maiden M.C.J. & McCarthy N.D. 2010. Host association of *Campylobacter* genotypes transcends geographic

variation. Appl. Environ. Microbiol. 76(15):5269-5277. <http://dx.doi.org/10.1128/AEM.00124-10> <PMid:20525862>

Stintzi A. 2003. Expression profile of *Campylobacter jejuni* in response to growth temperature variation. J. Bacteriol. 185(6):2009-2016. <http://dx.doi.org/10.1128/JB.185.6.2009-2016.2003> <PMid:12618466>

Stintzi A. & Whitworth L. 2003. Investigation of the *Campylobacter jejuni* cold-shock response by global transcript. Genome Lett. 2(1/2):18-27.

Thakur S., Zhao S., McDermott P.F., Harbottle H., Abbott J., English L., Gebreyes W.A. & White D.G. 2010. Antimicrobial resistance, virulence, and genotypic profile comparison of *Campylobacter jejuni* and *Campylobacter coli* isolated from humans and retail meats. Foodborne Pathog. Dis. 7(7):835-844. <http://dx.doi.org/10.1089/fpd.2009.0487> <PMid:20367499>

Workman S.N., Mathison G.E. & Lavoie M.C. 2008. An investigation of sources of *Campylobacter* in a poultry production and packing operation in Barbados. Int. J. Food. Microbiol. 121(1):106-111. <http://dx.doi.org/10.1016/j.ijfoodmicro.2007.10.014> <PMid:18061296>

WHO 2013. The global view of campylobacteriosis. World Health Organization, Geneva. Available at <https://extranet.who.int/iris/restricted/bitstream/10665/80751/1/9789241564601_eng.pdf> Accessed on Mar. 27, 2014.

Xie Y.P., He Y.P., Irwin P.L., Jin T. & Shi X.M. 2011. Antibacterial activity and mechanism of action of zinc oxide nanoparticles against *Campylobacter jejuni.* Appl. Environ. Microbiol. 77(7):2325-2331. <http://dx.doi.org/10.1128/AEM.02149-10> <PMid:21296935>

Zhao S., Young S.R., Tong E., Abbott J.W., Womack N., Friedman S.L. & McDermott P.F. 2010. Antimicrobial resistance of *Campylobacter* isolates from retail meat in the United States between 2002 and 2007. Appl. Environ. Microbiol. 76(24):7949-7956. <http://dx.doi.org/10.1128/AEM.01297-10> <PMid:20971875>

Ziprin R.L., Young C.R., Byrd J.A., Stanker L.H., Hume M.E., Gray S.A., Kim B.J. & Konkel M.E. 2001. Role of *Campylobacter jejuni* potential virulence genes in cecal colonization. Avian Dis. 45(3):549-557. <http://dx.doi.org/10.2307/1592894> <PMid:11569726>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(8):600-605, August 2019
DOI: 10.1590/1678-5150-PVB-5891

Original Article
Livestock Diseases

Evaluation of pulmonary maturity in bovine neonates: analysis of amniotic fluid[1]

Gabriela N. Dantas[2]*, Bianca P. Santarosa[2], Vitor H. Santos[3], Fernando J. Benesi[4] and Roberto C. Gonçalves[2]

ABSTRACT.- Dantas G.N., Santarosa B.P., Santos V.H., Benesi F.J. & Gonçalves R.C. 2019. **Evaluation of pulmonary maturity in bovine neonates: analysis of amniotic fluid**. *Pesquisa Veterinária Brasileira 39(8):600-605.* Departamento de Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Distrito de Rubião Júnior s/n, Botucatu, SP 18618-970, Brazil. E-mail: gabrielan.dantas@gmail.com

Considering the representativeness of dairy cattle in our country, the concern about the mortality rates of the animals increases each time. Regarding to calf mortality, the Respiratory Distress Syndrome (RDS) has an important relevance during the neonatal period, and it is present in immature lungs. The amniotic fluid is in direct contact with the fetus, and it is able to offer evidence about his maturity. The aim of this study was to standardize the characteristics of the amniotic fluid, color, aspect, viscosity, quantification of lamellar body and surfactant evaluation by the Clements test and cytology, of term-born, mature and healthy calves. There were used 50 Black and White Holstein calves, which mothers were observed at calving in order to collect the amniotic fluid by puncture in the moment of exposure of the fetal membrane through the vaginal canal. Most amniotic fluid had a clear and hazy appearance due to varying degrees of viscosity and the presence or absence of clots. The Clements test could be adapted to the bovine species by the modification consisting in the addition of 3mL of amniotic fluid and 1mL of 95% ethanol. The methodology of the lamellar body count by the automated particle counter is not applicable for the bovine because of the small size of their lamellar body. The Nile Blue staining is unsatisfactory on predicting fetal maturity on the bovine species, different from cytology using Hematoxylin-Shorr stain. The presence of orange cells, increase in large amounts at the end of pregnancy. The cell stained orange counting, cells which are found in great amounts at the end of pregnancy. The present study stablished new parameters for evaluation of fetal and pulmonary maturity in the bovine species.

INDEX TERMS: Evaluation, pulmonary maturity, bovine neonates, analysis, calf, fetal maturity, lamellar bodies, Clements test, amniotic fluid, cytology.

RESUMO.- [**Avaliação maturidade pulmonar em neonatos bovinos: análise do líquido amniótico.**] O objetivo desse estudo foi reunir novos dados práticos sobre a avaliação da maturidade pulmonar em neonatos bovinos, padronizando as características do líquido amniótico de bezerros maduros e hígidos, o que proporcionará a oportunidade de tratamento precoce dos animais prematuros, evitando prejuízos econômicos, principalmente quando consideramos os animais de alto valor genético. Amostras de líquido amniótico foram coletadas de 50 vacas da Raça Holandesa Preta e Branca. Corpos lamelares foram identificados por microscopia eletrônica de transmissão como estruturas de tamanho aproximado de 130nm, o que impede sua contagem em analisadores automáticos. O teste de Clements sofreu adaptações de técnica e se mostrou viável com a diluição de 3mL de líquido amniótico em

[1] Received on February 22, 2019.
Accepted for publication on March 31, 2019.
Master's Thesis of the first author. Financed by "Fundação de Amparo à Pesquisa do Estado de São Paulo" (FAPESP) through scholarship (Proc. 2012/24836-8).

[2] Departamento de Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia (FMVZ), Universidade Estadual Paulista (Unesp), Distrito de Rubião Júnior s/n, Botucatu, SP 18618-970, Brazil. E-mails: biancasantarosavet@gmail.com, calderonmedvet@gmail.com; *Corresponding author: gabrielan.dantas@gmail.com

[3] Departamento de Cirurgia e Anestesiologia Veterinária, Faculdade de Medicina Veterinária e Zootecnia (FMVZ), Universidade Estadual Paulista (Unesp), Campus de Botucatu, Distrito de Rubião Júnior s/n, Botucatu, SP 18618-970, Brazil. E-mail: vitor.santos@grupointegrado.br

[4] Departamento de Clínica Médica, Faculdade de Medicina Veterinária e Zootecnia (FMVZ), Universidade de São Paulo (USP), Av. Dr. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP 05508-270, Brazil. E-mail: febencli@usp.br

1mL de etanol a 95%. A citologia utilizando o método de Hematoxilina-Shorr, diferentemente do teste de Azul de Nilo, foi eficaz na identificação das células orangiofílicas, indicativas de maturidade fetal. Esses métodos mostraram-se originais e úteis ferramentas para a avaliação de maturidade pulmonar na espécie bovina, porém estudos com bezerros prematuros ainda são necessários.

TERMOS DE INDEXAÇÃO: Avaliação, maturidade pulmonar, neonatos bovinos, análise, bezerro, maturidade fetal, corpos lamelares, teste de Clements, citologia, líquido amniótico.

INTRODUCTION

Lung maturation at birth coincides with fetal maturity and is directly related to surfactant production (Eigenmann et al. 1984). The function of surfactant is to reduce surface tension at the air-liquid interface of the alveoli, preventing their collapse and facilitating the newborn's respiratory work (Rebello et al. 1996). The consequence of pulmonary immaturity is respiratory distress syndrome (RDS), or neonatal asphyxia, which causes economic and genetic damage to breeding due to animal death (Benesi 1993).

In the last decade, studies have been conducted to clarify the effectiveness of tests to determine the stage of pulmonary development, being most of them with application to the human species (Gil et al. 2010). Research of this scope is scarce in veterinary medicine, and most of these data do not exist for the bovine species.

The tests used to assess fetal and pulmonary maturation from amniotic fluid samples can be summarized as follows: Nile Blue Sulfate or Hematoxylin-Shorr cell staining, which indicates immaturity (Martins & Prestes 2003); test of Clements, a qualitative method that indicates the formation of a complete or incomplete bubble ring due to the saponification reaction present, whether or not there is, respectively, adequate surfactant in the amniotic fluid (Barreto et al. 2011); lamellar body count, the phospholipid reservoirs, components of the pulmonary surfactant, present in the amniotic fluid, which is performed in an automatic hematological counter in the platelet count channel, due to the equivalent size of these structures (Gil et al. 2010). For the lamellar body count to be applied to the bovine species, they must necessarily have the same size as the platelets, so that the hematology analyzer can perform the exam. As the size and appearance of these structures in bovine are not known, it is necessary to perform electron microscopy in amniotic fluid of the bovine species in order to initially recognize the lamellar bodies before testing the applicability of the test.

Amniotic fluid is an important component of the intrauterine environment. It provides a wealth of information on fetal health and maturation as it is in direct contact with its respiratory tract and skin, thus reflecting the amount of surfactant present and the predominant cell type in its epithelium (Campana et al. 2003).

Thus, this work aimed at contributing to the improvement of techniques for measuring the degree of pulmonary maturity in bovine by analyzing the characteristics of the amniotic fluid as to coloration, appearance, viscosity, presence of surfactant by Clements test, evaluation of lamellar bodies and cytology of Black and White Holstein calves born mature, term and healthy, prioritizing tests of practical application in the clinical routine. Thus, a study that standardizes the characteristics of the amniotic fluid of mature calves will provide the opportunity for early treatment of premature animals, avoiding economic losses to the breeder, especially regarding to animals of high genetic value, *in vitro* fertilization products (IVF) and cloning. In addition, understanding and standardizing indicators of physiological pulmonary maturation through the abovementioned diagnostic tests will contribute to the understanding of the pathogenesis of RDS in bovine species, a necessary prerequisite for the development of therapeutic tools that mitigate or prevent lung lesions and the death of animals as a result of the syndrome.

MATERIALS AND METHODS

Fifty healthy Black and White Holstein newborn calves (30 females and 20 males) from a farm located in Descalvado/SP born from artificial insemination were used. Females of advanced gestational age were kept in semi-intensive regime in the farm's Maternity Sector. The collections were performed from February to June 2014. All deliveries were accompanied. Twice a day the paddocks were inspected for animals with signs of imminent calving, and cows with deliver scheduled to that week were separated from the flock. The selected animals were kept in farrowing pens where the support staff monitored them 24 hours a day.

In order for newborns to be classified as healthy and mature, they were evaluated at birth considering the following aspects: gestation duration and mother's health; size of the newborn; screening by Apgar score (Born 1981) in the first minute of life with selection of only 7 or 8 newborns; general physical examination (Feitosa & Benesi 2014); and blood pH measurement by jugular vein puncture in a portable blood gas meter (I-STAT®, Abbott Laboratories, Illinois, USA). Any evidence of prematurity, immaturity, disease, low vitality or contamination of amniotic fluid by meconium, were exclusion factors in this study.

Amniotic fluid samples (20mL) were collected during labor with the aid of a 20mL syringe and 40×12 needle by puncture and aspiration of the amniotic sac at the time it was exposed in the birth canal. The collected volume was transferred to a dry 30mL polyethylene tube and immediate tests were performed (analysis of color and appearance, and Clements test). The remaining material was transferred to identified *Eppendorf* tubes, kept at 20°C until further testing (lamellar body analysis and cytology).

For color and appearance analysis, the liquid was rated for color (light, yellowish or reddish), appearance (cloudy or not) and viscosity (low viscous/+, viscous/++ or very viscous/+++). In addition, the presence or absence of lumps was recorded. The original Clements test (Clements et al. 1972), modified by Barreto (Barreto et al. 2011) and technique adapted by the authors of this work were performed, which consisted of mixing 3mL of the amniotic fluid sample with 1mL of ethanol at 95% and performing the subsequent steps similarly to the original Clements test. The technique used for lamellar body counting was standardized for automatic counters (Neerhof et al. 2001). For the transmission electron microscopy processing, the collected material was fixed in Karnovsky solution (8% paraformaldehyde, 25% glutaraldehyde and 0.2M phosphate buffer at pH 7.3 in a 5:1:4 ratio) and kept at room temperature for 4 hours. After the fixation time, the material was refrigerated at 4°C until processing, performed on a 150-200 mesh carbon film copper grid and stained with 4% uranyl acetate. The material examination was performed under a transmission electron microscope Tecnai®. For cytology using Nile Blue, one drop (50µl) of amniotic fluid and one

drop of 0.1% Nile Blue dye were added to the slide, then mixed. After covering with a slide, the optical microscope reading was performed. Five fields were counted and the absolute and relative frequency of each cell type (orangiophilic and cyanophilic) was recorded. For staining using the Hematoxylin-Shorr technique, the methodology proposed by Moya (2005) was used, and after slide preparation, a reading similar to the Nile Blue method was performed.

Regarding the statistical analysis, all variables had their distribution analyzed for normality. Continuous variables were initially submitted to descriptive statistics. Frequency distributions were produced to present the results of nominal and ordinal variables. To compare the mean blood pH between animals from dystocia and eutocic births, the unpaired t-test was used. To estimate the correlation between the proportion of orange and blue cells in the two types of stains used in this work, we used the "Pearson coefficient". Analyses were performed at 5% statistical significance level.

This study was approved by the Animal Experimentation Ethics Committee of the School of Veterinary Medicine and Zootechnics of Unesp, Botucatu, (Protocol 241/2012).

RESULTS AND DISCUSSION

Of the 50 animals studied, 20 were born eutocia (40%) and the other 30 calves (60%) through mild forced traction (only one person) with the aid of obstetric currents if the amniotic pouch was ruptured for a period longer than 2 hours. All the dystocia observed were of fetal origin: relatively large fetuses in relation to the mother (70%) or with abnormal static (30%), represented by posterior presentation, flexion of the thoracic limbs or neck. One hypothesis that would explain this fact would be that there were, in the experimental period, many calving (primiparous) calves, which are known to have smaller pelvic sizes and higher incidence of dystocia calves (Jainudeen & Hafez 2004).

During labor, disturbances in the uteroplacental blood circulation arise as a result of the contractions of the myometrium, resulting in lower oxygen delivery to the fetus (Walser & Maurer-Schweiser 1979). Thus, in the case of delayed deliveries, this deficiency in fetal oxygenation is greater, predisposing the neonate to more severe degrees of acidosis with consequent decrease in vitality. The logical relationship between dystocia and the impairment of maternal-fetal circulation was the subject of discussion in the study by Massip & Mocsy (1965). The authors concluded that in the case of dystocia calves presented more pronounced acidosis than animals from eutocia calving; yet the greatest impairment came from neonates obtained by emergency cesarean section. However, other authors (Maurer-Schweizer et al. 1977) have shown that calves from spontaneous births and calves with mild traction-assisted births exhibit similar vitality and blood pH characteristics. Due to constant observation of pregnant cows, cases of dystocia were detected early and resolved. Consequently, the experimental animals did not remain in dystocia for a long time and did not develop loss of vitality due to possible acidosis (Table 1). Interestingly, 100% of calves had high vitality, i.e., Apgar score 7 or 8, as this was one of the inclusion criteria in the experiment.

Surfactant synthesis is known to be influenced by pH, body temperature and perfusion and is compromised in acidosis (Bittar 2000). It was already commented in the exclusion criteria that the newborns used in this research presented normal rectal temperature and CPT for the species, according to Feitosa & Benesi (2014), showing good capillary perfusion. There was no statistical difference in the pH of eutocia and dystocia animals (p=0.44), which allows them to be considered as a homogeneous group.

Table 1. Number of samples (N), means, confidence interval (95% CI), standard error (SE), standard deviation (SD), minimum (Min.) and maximum (Max.) values for blood pH of Black and White Holstein calves and mature, term and healthy at birth: general pH, pH of eutocia animals and pH of animals born by dystocia

Variable	N	Average	IC 95%	EP	DP	Min.	Max.
pH	50	7.22	7.20-7.24	0.01	0.06	6.99	7.34
pH eutocia	20	7.21	7.19-7.23	0.01	0.05	7.1	7.32
pH dystocia	30	7.22	7.20-7.25	0.01	0.07	6.99	7.34

Of the 50 samples of amniotic fluid analyzed, 43 (86%) were light colored, five (10%) yellowish and two (4%) reddish. Light color is considered normal in amniotic fluid (Zogno et al. 2004). The yellow color originated from the contamination of the amniotic fluid with the allantoid at the time of collection because a large amount was released moments before the exposure of amnion, leaving part of this fluid in the vaginal canal, although for the types of analysis performed there was no influence on the results. This hypothesis strengthens the fact that no animal was born meconium-dyed by discarding the contamination of amniotic fluid by this substance as a cause of yellowish color. The reddish color occurred due to blood contamination at the time of puncture, which sometimes reached small vessels of the amniotic membrane.

Regarding appearance, all samples were turbid, and 13 of them (26%) also had white lumps. The results of the viscosity classification were distributed as follows: 7 low viscous samples (+/14%), 30 intermediate viscosity samples (++/60%) and 13 very viscous samples (+++/26%). According to the protocol adopted at the School of Medicine of Unesp, Botucatu, where the present study is based, when the amniotic fluid is transparent and without lumps, it indicates pulmonary immaturity, and the cloudy and viscous fluid with lumps should be from mature fetus. However, in calves this relationship was not observed, since all the animals were mature and yet some presented little viscous amniotic fluid or without lumps.

There were no positive samples in the Clements test as originally proposed (Clements et al. 1972), and the same result was obtained after applying the modified Cleto test by Barreto (Barreto et al. 2011), i.e., all amniotic fluid samples were negative. Due to the failure of these two tests, the methodology was adapted again and, from successive attempts with increasing amounts of amniotic fluid, it was found that when mixed 3 mL of amniotic fluid with 1mL of 95% ethanol exclusively, without the addition of physiological solution, the samples formed the complete ring of bubbles, indicating test positivity. After this modification, 100% of the tested liquids were positive, showing parameters of fetal lung maturity in bovine species, since mature animals will have positive test under these dilution conditions. Amniotic fluid samples from premature animals with clinical signs of RDS need to be further tested to confirm that the adapted technique (3mL dilution) will be negative in animals deficient in the amount or quality of surfactant produced. For this, it

would be necessary to adopt the technique of amniocentesis during the final period of pregnancy, since the collection of LA from the slaughterhouse is not effective. It is probably because during the slaughter process, the fetus suffers and contaminates the amniotic fluid with meconium, making the Clements test always false positive, regardless of dilution. This test is particularly interesting because it is a simple technique and applicable to clinical routine, but more studies are needed to establish more precisely the cutoff point for dilution between mature and premature animals.

It was not possible to count the lamellar bodies in the automatic particle counter. Despite successive tests with refrigerated and/or frozen samples, all resulted in close to zero reading. In addition, the device often clogged due to the high viscosity and presence of mucus in the samples. Thus, the transmission electron microscopy was chosen to verify the existence of lamellar bodies in the amniotic fluid of bovine, since there were no studies describing the structure in this species. Castagnetti et al. (2007), evaluating the lung maturity of newborn foals by amniotic fluid analysis by electron microscopy, reported that lamellar bodies were structures composed of several concentric lamellae with a diameter ranging from 1.6 to 3.3μm, the particle counter methodology being applicable to equine species. After electron microscopy of the amniotic fluid in order to characterize the lamellar bodies of bovine, it was found that these structures are approximately 130nm in diameter (Fig.1). Thus, the morphology of the lamellar bodies of bovine species, still unpublished in the literature, was characterized. As these structures are small, it has been confirmed that their observation by optical microscopy is impossible, proving that the lamellar bodies of the amniotic fluid of cows are much smaller than those of humans and horses. Therefore, this fact explains the unsuccessful attempts to quantify lamellar bodies by automated particle counters. As the lamellar bodies disintegrate when invoked to cover the surfactant socket, the finding of concentric lamella structures, as described in the literature, was not possible. Thus, for further research the study of these structures still within the pneumocyte is indicated.

Cellularity results with Nile Blue and Hematoxylin-Shorr staining and their proportions and morphologies (Fig.2 and 3) showed no correlation between orangiophilic cell proportions among the different stains studied (Pearson coefficient = –0.14), indicating that the two methodologies do not reflect their results to each other (Tables 2-4). In women, the proportion of orange cells in amniotic fluid during pregnancy is already standardized, representing 50% to 95% of the cell population (Souza et al. 2000). Thus, the high proportion of orangiophilic cells is indicative of fetal maturity. Thereby, the cytology using Nile Blue staining was not satisfactory, since even mature calves presented low percentage of these cells, with an average of 8%. This finding agrees with the results of Moya (2005), who worked with cattle, and Martins & Prestes (2003) in their study with female canine amniotic fluid. In this study, a percentage ten times higher than the one obtained using the Nile Blue staining technique was found, that is, on average 80% of the amniotic fluid cell population of healthy Black and White Holstein calves and the term was composed by orangiophilic cells, which shows that the Hematoxylin-Shorr technique, although more laborious and time consuming, is the most appropriate to predict fetal maturity in the bovine species.

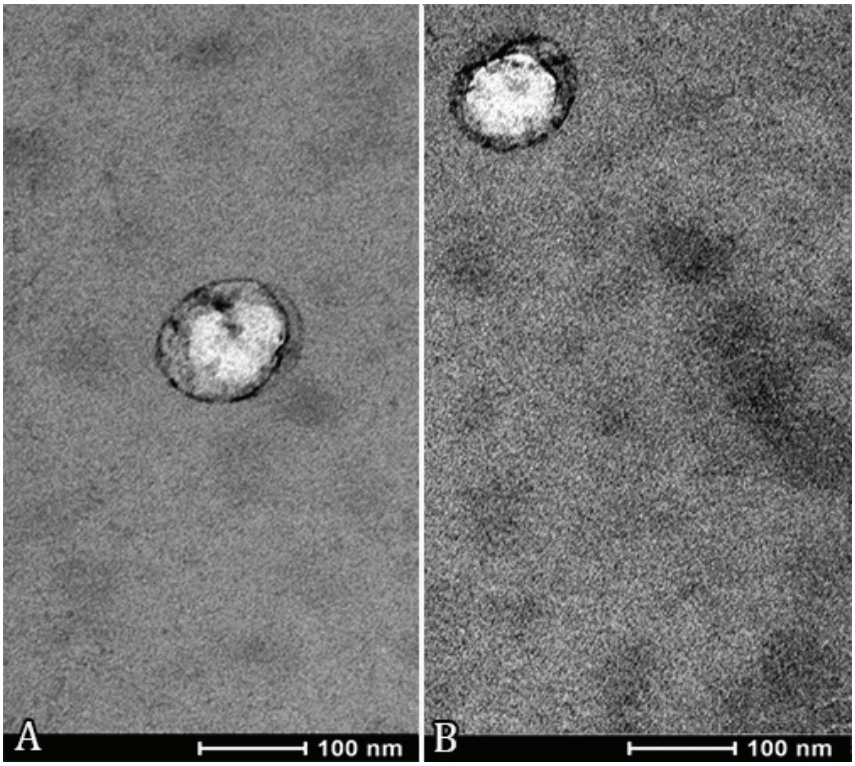


Fig.1. (**A**-**B**) Lamellar bodies present in amniotic fluid of the bovine species obtained by the transmission electron microscopy technique. Bar = 100nm.

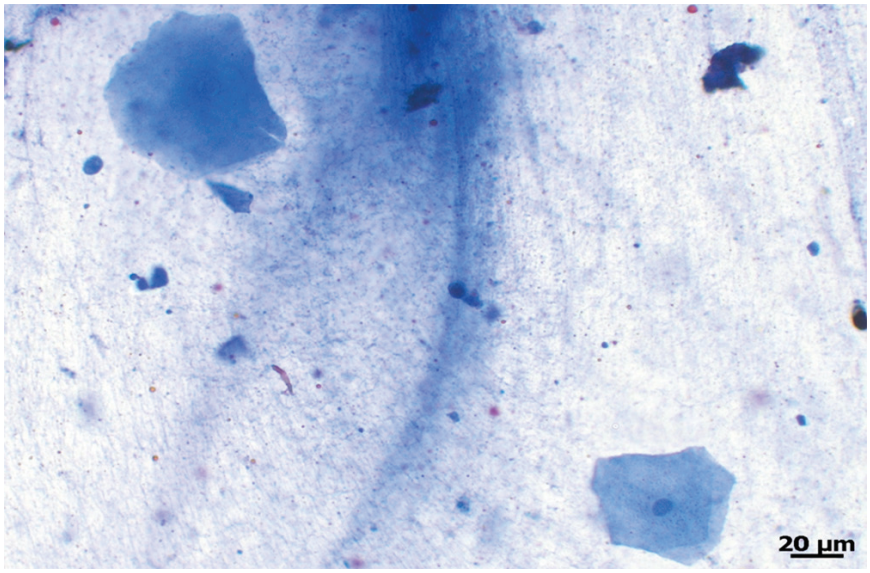


Fig.2. Acidophilic cell morphology (blue cell) obtained by optical microscopy of the cytology of amniotic fluid. Nile Blue, bar = 20µm.

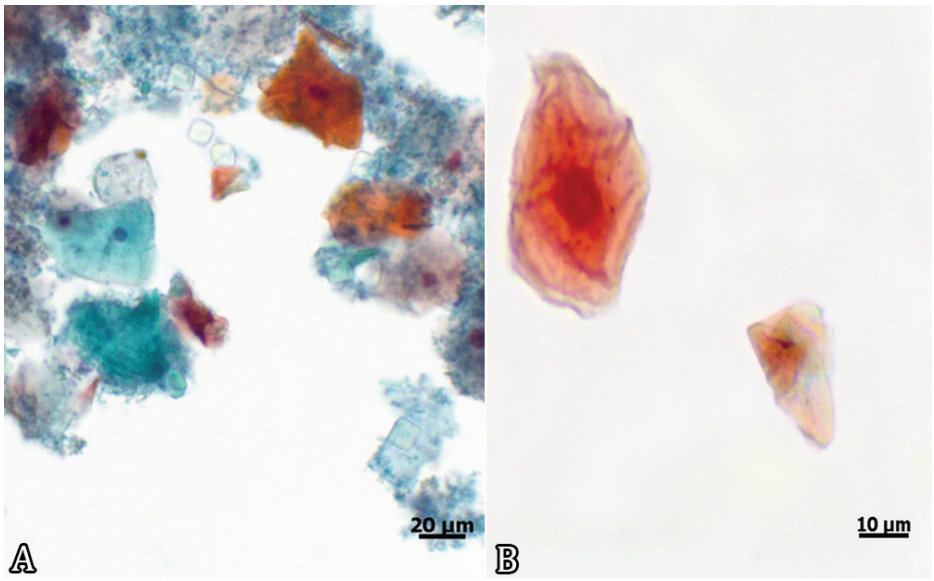


Fig.3. (**A**-**B**) Orangiophilic cell morphology (orange cell) obtained by optical microscopy of the cytology of the amniotic fluid. Hematoxylin-Shorr, bar = 20µm, bar = 10µm.

Table 2. Number of samples (N), means, confidence intervals (95% CI), standard errors (SE), standard deviations (SD), minimum values (Min.) and maximum values (Max.) for blue and orange cells obtained from amniotic fluid cytology of mature Black and White Holstein calves at term and healthy at calving by Nile Blue staining

Variable	N	Average	IC 95%	EP	DP	Min.	Max.
Blue cells	50	21	16-27	2,7	19	5	103
Orange cells	50	1.7	1.4-2.1	0,18	1.2	1	5

Table 3. Number of samples (N), means, confidence intervals (95% CI), standard errors (SE), standard deviations (SD), minimum values (Min.) and maximum values (Max.) for blue and orange cells obtained from amniotic fluid cytology of mature Black and White Holstein calves at term and healthy at calving using Hematoxylin-Shorr staining

Variable	N	Average	IC 95%	EP	DP	Min.	Max.
Blue cells	50	19	18-21	0.8	5.7	4	28
Orange cells	50	81	79-82	0.8	5.7	28	96

Table 4. Number of samples (N), means, standard deviations (SD), minimum values (Min.) and maximum values (Max.) for proportions between orange and blue cells (O:B) obtained by amniotic fluid cytology of Black and White Holstein calves mature at term and healthy at birth by staining with Nile Blue and Hematoxylin-Shorr

Variable	N	Average	DP	Min.	Max.
O:B Proportion (Nile Blue)	50	8%	2%	4%	16%
O:B Proportion (Hematoxilina-Shorr)	50	80%	5%	72%	96%

CONCLUSIONS

This work found results that characterize the fetal and pulmonary maturity of bovine neonates in the proposed tests, mainly regarding the bovine lamellar body size (approximately 130nm) and the ratio of LA to complete bubble ring formation in the Clements test, 3mL. However, future studies with immature lungs are still needed.

In addition, amniotic fluid cellularity data and surfactant carrier structure morphology, which were previously poorly established for the species, are important for the formation of basic knowledge about fetal maturation. Further studies in this area are needed in different animal species, so that Veterinary Neonatology can match evolution and knowledge with that established in Medicine.

Acknowledgments.- To FMVZ-Unesp/Botucatu, for the physical structure and equipment used to carry out the experiment. To FAPESP (São Paulo State Research Support Foundation) for the Master's Scholarship granted (Process 2012/24836-8).

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

REFERENCES

Barreto C.S., Prestes N.C., Souza F.F., Santos R.V., De Vita B., Matayoshi P.M. & Leal L.S. 2011. Uso do teste de clements modificado e densidade óptica do líquido amniótico e alantoideano para avaliação da maturidade pulmonar em cães. Vet. Zootec. 18(1):63-69.

Benesi F.J. 1993. Síndrome da asfixia neonatal dos bezerros: importância e avaliação crítica. Arq. Escola Med. Vet. UFBA 16(1):38-48.

Bittar R. 2000. Doença das membranas hialinas, p.103-106. In: Rugolo L.M.S.S. (Ed), Manual de Neonatologia. 2ª ed. Revinter, Rio de Janeiro.

Born E. 1981. Untersuchungen über den Einfluss der Schnittentbindung auf die Vitalität neugeborener Kälber. Dissertation, Tierärztliche Hochschule, Hannover. 47p.

Campana S.G., Chávez J.H. & Haas P. 2003. Diagnóstico laboratorial do líquido amniótico. J. Bras. Patol. Med. Lab. 39(3):215-218.

Castagnetti C., Mariella J., Serrazanetti G.P., Grandis A., Merlo B., Fabbri M. & Mari G. 2007. Evaluation of lung maturity by amniotic fluid analysis in equine neonate. Theriogenology 67(9):1455-1462. <http://dx.doi.org/10.1016/j.theriogenology.2007.02.013> <PMid:17448529>

Clements J.A., Platzker A.C.G., Tierney D.F., Hobel C.J., Creasy R.K., Margolis A.J., Thibeault D.W., Tooley W.H. & Oh W. 1972. Assessment of the risk of the respiratory distress syndrome by a rapid test for surfactant in amniotic fluid. New England. J. Med. 286(20):1077-1081. <http://dx.doi.org/10.1056/NEJM197205182862004> <PMid:5067186>

Eigenmann U.E.J., Schoon H.A., Jahn D. & Grunert E. 1984. Neonatal respiratory distress syndrome in the calf. Vet. Rec. 114(6):141-144. <http://dx.doi.org/10.1136/vr.114.6.141> <PMid:6546630>

Feitosa F.L.F. & Benesi F.J. 2014. Semiologia de recém-nascidos ruminantes e equídeos, p.69-97. In: Ibid. (Eds), Semiologia Veterinária. 3ª ed. Roca, São Paulo.

Gil B.M.K., Souza E., Silva C.A.J. & Figueiredo C.P. 2010. Avaliação da maturidade pulmonar fetal pela contagem dos corpos lamelares no líquido amniótico. Revta Bras. Ginecol. Obstetr. 32(3):112-117. <http://dx.doi.org/10.1590/S0100-72032010000300003>

Jainudeen M.R. & Hafez E.S.E. 2004. Gestação, fisiologia pré-natal e parto, p.141-155. In: Hafez B. & Hafez E.S.E. (Eds), Reprodução Animal. 7ª ed. Manole, Barueri.

Martins L.R. & Prestes N.C. 2003. Ensaio sobre exame citológico do líquido amniótico de cadelas colhido no momento da cesariana. Ars Vet. 19(3):294-299.

Massip J. & Mocsy J. 1965. Tratado de Diagnóstico Clínico de Las Enfermedades Internas de Los Animales Domésticos. 3ª ed. Labor, Barcelona. 675p.

Maurer-Schweizer H., Wilhelm U. & Walser K. 1977. Blutgase und Saure-Basenhaushalt bei lebensfrischen Kalbern in den ersten 24 Lebensstunden. Berliner und Munchener Tierarztliche Wochenschrift 90(10):192-196. <PMid:883945>

Moya C.F. 2005. Tipificação citológica do líquido amniótico de bezerros oriundos de produção in vitro, transferência de embrião e inseminação artificial no momento do parto. Master's Thesis, Faculdade de Medicina de Botucatu, Universidade Estadual Paulista, Botucatu, SP. 64p.

Neerhof M.G., Haney E.I., Silver R.K., Ashwood E.R., Lee I. & Piazze J.J. 2001. Lamellar body counts compared with traditional phospholipids analysis as an assay for evaluating fetal lung maturity. Obstet. Gynecol. 97(2):305-309. <PMid:11165600>

Rebello C.M., Ikegami M., Polk D.H. & Jobe A.H. 1996. Postnatal lung responses and surfactant function after fetal or maternal corticosteroid treatment of preterm lambs. J. Applied Physiol. 80(5):1674-1680. <http://dx.doi.org/10.1152/jappl.1996.80.5.1674> <PMid:8727554>

Souza C., Prestes N.C. & Lopes R.S. 2000. Pesquisa de células orangiofílicas e aplicação do teste de Clements no fluido amniótico de ovinos (*Ovis aries*), colhidos em matadouro, em vários estágios da gestação. Anais XVII Congresso Brasileiro de Medicina Veterinária, São Paulo, SP, p.29.

Walser K. & Maurer-Schweiser H. 1979. Acidosis and clinical state in depressed calves, p.551-563. In: Hoffman B., Mason I.L. & Schmidt J. (Eds), Calving Problems and Early Viability of the Calf. Current Topics in Veterinary Medicine and Animal Science. Vol.4. Springer Nature, Switzerland.

Zogno M.A., Miglino M.A. & Oliveira M.F. 2004. Análise bioquímica dos líquidos fetais e citologia do fluido amniótico da fêmea de mocó (*Kerodon rupestris*). Braz. J. Vet. Res. Anim. Sci. 41(4):226-235. <http://dx.doi.org/10.1590/S1413-95962004000400002>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(8):606-613, August 2019
DOI: 10.1590/1678-5150-PVB-5826

Original Article
Livestock Diseases

Risk factors for high bulk milk somatic cell counts in dairy herds from Campos das Vertentes region, Minas Gerais State, Brazil: a case-control study[1]

Geraldo M. Costa[2]*, Alan A. Mesquita[3], Christiane M.B.M. Rocha[2], Fabio R.P. Bruhn[4], Rafaella S. Andrade[2], Dircéia A.C. Custódio[2], Mirian S. Braz[2] and Sandra M. Pinto[5]

ABSTRACT.- Costa G.M., Mesquita A.A., Rocha C.M.B.M., Bruhn F.R.P., Andrade R.S., Custódio D.A.C., Braz M.S. & Pinto S.M. 2019. **Risk factors for high bulk milk somatic cell counts in dairy herds from Campos das Vertentes region, Minas Gerais State, Brazil: a case-control study.** *Pesquisa Veterinária Brasileira 39(8):606-613*. Departamento de Medicina Veterinária, Universidade Federal de Lavras, Campus Universitário, Cx. Postal 3037, Lavras, MG 37200-000, Brazil. E-mail: gmcosta@ufla.br
High bulk milk somatic cell counts (BMSCC) are indicative of failures related to the control of mastitis in the herd, which compromises the quality of the milk and generates great losses for the producers and for the industry. A case-control study was carried out in dairy herds in the Campos das Vertentes region, Minas Gerais State, Brazil, in order to contribute to the knowledge of the risk factors involved with elevated BMSCC. The study involved 46 dairy herds, of which 30 were considered cases (BMSCC ≥700,000 cells/mL of milk) and 16 control farms (BMSCC ≤200,000 cells/mL of milk). Sixteen qualitative variables and four quantitative variables were analyzed. The results showed that the risk factors for BMSCC ≥700,000 cells/mL were the presence of *Staphylococcus aureus* and *Streptococcus agalactiae* pathogens in bulk milk, non-use of pre and post-dipping, non-use of disposable paper towel for drying of mammary glands, non-monitoring of mastitis in the herd by means of California Mastitis Test (CMT) or individual somatic cell counts (SCC), non-implementation of the milking line and therapy of dry cows and failures in hygiene of teats and udders before milking. Moderate correlations were also observed between the elevation of BMSCC and counts of *S. aureus* and BMSCC and counts *S. agalactiae* in bulk milk, and a moderate correlation between *S. aureus* and *S. agalactiae* counts in bulk milk. Failures with regard to the maintenance and use of milking equipment, including manual pressure application in milking assemblies, unregulated milking vacuum pressure, and vacuum loss during milking, and maintenance failures of the milking machine and bulk milk tank were also pointed out as important risk factors of BMSCC elevation. The results of this study provided subsidies for the elaboration of more effective programs for mastitis control and improvement of raw milk quality, reducing the losses caused by the disease to producers and industry.

INDEX TERMS: Risk factors, milk, somatic cell, dairy herds, Minas Gerais, Brazil, mastitis, case-control study, cattle.

[1] Received on December 9, 2018.
Accepted for publication on February 21, 2019.
[2] Departamento de Medicina Veterinária, Universidade Federal de Lavras (UFLA), Campus Universitário, Cx. Postal 3037, Lavras, MG 37200-000, Brazil. *Corresponding author: gmcosta@ufla.br

[3] Departamento de Zootecnia, Universidade Federal de Lavras (UFLA), Campus Universitário, Cx. Postal 3037, Lavras, MG 37200-000.

[4] Faculdade de Veterinária, Universidade Federal de Pelotas (UFPel), Cx. Postal 354, Campus Universitário s/n, Capão do Leão, RS 96010-900, Brazil.

[5] Departamento de Ciências dos Alimentos, Universidade Federal de Lavras (UFLA), Campus Universitário, Cx. Postal 3037, Lavras, MG 37200-000.

RESUMO.- [**Fatores de risco para contagens elevadas de células somáticas no leite de tanque em rebanhos de bovinos da região de Campos das Vertentes, Minas Gerais, Brasil: um estudo de caso-controle.**] Altas contagens de células somáticas no leite do tanque (CCSt) são indicativas de falhas relacionadas com o controle da mastite no rebanho, o que compromete a qualidade do leite e gera grandes perdas para os produtores e para a indústria. Visando identificar os fatores de risco envolvidos com a CCSt elevada, foi realizado

um estudo de caso-controle em rebanhos bovinos leiteiros da região de Campos das Vertentes, em Minas Gerais. O estudo envolveu 46 propriedades, das quais 30 foram consideradas casos (CCSt ≥700.000 cels/mL de leite) e 16 propriedades controles (CCSt ≤200.000 cels/mL de leite). Foram analisadas 16 variáveis qualitativas e quatro variáveis quantitativas. Os resultados demonstraram que os fatores de risco para valores de CCSt ≥700.000 cels/mL de leite foram a presença dos patógenos *Staphylococcus aureus* e *Streptococcus agalactiae*, não utilização do pré e de pós-*dipping,* não utilização de papel toalha descartável para a secagem dos tetos, não monitoramento da mastite por meio do California Mastitis Test (CMT) ou CCS individual, não implementação da linha de ordenha e da terapia de vacas secas e falhas na higiene de tetos e de úbere antes da ordenha. Também se observaram correlações moderadas entre a CCSt e as contagens de *S. aureus* e entre CCSt e as contagens de *S. agalactiae*, e correlação moderada entre as contagens de *S. aureus* e de *S. agalactiae* no leite do tanque. Falhas com relação à manutenção e utilização dos equipamentos de ordenha, aplicação de pressão manual nos conjuntos da ordenha, pressão de vácuo da ordenha desregulada, perda de vácuo durante a ordenha e falhas de manutenção da ordenhadeira e do tanque de expansão foram também apontadas como fatores de risco para elevação da CCSt. Os resultados deste estudo possibilitaram identificar fatores de risco importantes para contagens elevadas de CCSt que poderão fornecer subsídios para a elaboração de programas de controle mais efetivos para a mastite e para a melhoria da qualidade do leite, mitigando o impacto que a doença causa para os produtores e para a indústria.

TERMOS DE INDEXAÇÃO: Fatores de risco, contagem de células, células somáticas, leite de tanque, bovinos, Minas Gerais, Brasil, mastite, estudo caso-controle.

INTRODUCTION

In Brazil, the consumption of milk and dairy products has showed a tendency of growing, as seen by the 42.6% increase in the annual per capita acquisition of these products from 2000 to 2015, totaling 174 liters of dairy products/year per inhabitant (IBGE 2017).The elevated nutritional value of milk, due to its proteins, fats, carbohydrates, vitamins and mineral salts has contributed to this increased of dairy consumption (Ribeiro 2008). Although, the consumers are increasingly worried about the quality, functionality and risks associated to food products (Carvalho 2010).

This increased concern with quality led to changes in legislation, aiming to adequate the milk production to the consumer market requirements. Thus, the Normative Instructions IN51 (Brasil 2002), IN62 (Brasil 2011) and IN7 (Brasil 2016) were created to regulate the production and quality parameters of the milk produced in Brazil. The adequation to legislation led to changes in the payment of milk acquired by dairy producers, introducing bonus for products with higher percentage of nutritional constituents and higher quality indexes, indicated mainly by reduction in the total bacterial count (TBC) and bulk-milk somatic cell count (BMSCC) (Guerreiro et al. 2005).

The milk from herds with high values of TBC and BMSCC results in worse quality dairy products (Politis & Ng-Kwai-Hang 1988). Changes in milk composition due to breast infections reduce the products nutritional values and raise processing problems, which can lead to products out of the desired quality standards. High values of BMSCC are also directly related to lower yield of the dairy production (Santos & Fonseca 2007, Lopes et al. 2012). In addition, increased BMSCC due to intramammary infections (IMI) caused by certain mastitis pathogens, such as *Streptococcus agalactiae,* reduces the shelf life of milk and its derivates (Barbano et al. 2006).

Among the milk quality parameters, BMSCC is the main index of mammary gland health of the cows of the herd. In the presence of bacterial infection and inflammatory process, the somatic cell count (SCC) can reach high values due to the increase in leukocytes that migrate to the mammary glands to struggle the infectious agents (Dong et al. 2012). Thus, periodic BMSCC evaluations can determine the average frequency of mastitis in the herd (Machado et al. 2000). According to the National Mastitis Council (NMC 1996), the prevalence of mastitis in the herd is directly related to the BMSCC. Counts of 200,000 cells/mL of milk indicate that approximately 6% of the herd's mammary quarters are infected and, when these counts reach 1,500,000 cells/mL of milk, it indicates that approximately 48% of the herd's mammary quarters may have mastitis.

Mastitis is a multifactorial disease, thus characteristics specific to the animals and also of the environment where the milk is produced and handled are considered important risk factors to IMI and milk quality (Omore et al. 1996). Among the first we can cite the number of parities, stage of lactation and individual production (Souza 2005). Risk factors related to the milking handling, such as manual or mechanical milking and lack of antisepsis of the teats before and after milking are associated with the occurrence of IMI and high values of BMSCC (Brito et al. 1998).

According to Cavazos (2003), inadequate functioning of milking equipment, lack of training and low motivation of the milkers are also associated with increased BMSCC. Periodic microbiological tests are also important to control and prevent mastitis and consequently reduce BMSCC; they allow to evaluate the present pathogens and monitor the index of infections in the herd, helping the adoption of control measures more adequate according to the agents present in the herd (NMC 2000).

Even though several contagious and environmental pathogens can be involved in causing mastitis, *Staphylococcus aureus, Streptococcus uberis, S. agalactiae, S. dysgalactiae* and *Escherichia coli* are the most common (Ranjan et al. 2006) and are usually associated with more severe mastitis (Reyher et al. 2012). According to Keefe (2012), *S. agalactiae* and *S. aureus* are considered bovine mastitis primary pathogens due to its impact in the production and quality of milk, especially in BMSCC. The main propagation method of these pathogenic agents within the herd is through infected cows, which serve as reservoirs. According to Brito et al. (1999), among these bacteria, named primary pathogens, *S. aureus* e *S. agalactiae* are the most commonly isolated microorganisms in cases of mastitis, being responsible for approximately 26% of the IMI in herds of Minas Gerais state.

Due to mastitis multifactorial aspect, the study of its risk factors is of great importance, considering that the intervening factors of this disease are dynamic and vary among herds. One of the alternatives for this kind of study is the case control

study, which includes epidemiological evaluations that are observational, longitudinal, retrospective and analytical. This type of study aims to create hypotheses for future investigations of diseases with risk factors that are not completely known, or for confirmatory studies to test pre-established hypotheses through the investigation of suspected factors (Rêgo 2010).

Despite several studies focused on the BMSCC aspects and its impact in the milk's quality, and studies related to mastitis risk factors in Brazilian bovine herds (Vilela & Nogueira 2010, Costa et al. 2012, Mendonça et al. 2016), there is still a lack of recent studies on the risk factors for the BMSCC increase. Such studies can help in the adoption of public policies for mastitis control and improvement of milk's quality, resulting in higher productivity for the producers, higher yield for the dairy industry e higher security for the consumer. Thus, the objective of this case control was to identify the main risk factors related to increase of the BMSCC, aiming to contribute to the knowledge of mastitis risk factors and the increase of milk quality in bovine dairy herds from the region of Campos das Vertentes, Minas Gerais, state.

MATERIALS AND METHODS

We performed a case control study to test the association of biological variables, sanitary handling and milking with the herds BMSCC. The BMSCC was considered the dependent variable. Thus, to perform the study, we randomly selected 46 milk producing properties located in the dairy farming region of Campos das Vertentes, in Minas Gerais state. The properties analyzed had approximately 1600 total animals (FAEMG 2006), 30 of them were considered cases and the remaining properties (16) were considered controls. The farms selected as cases presented BMSCC ≥700,000 cells/mL of milk, values that indicate a high prevalence of mastitis in the herd. The properties considered controls presented BMSCC ≤200,000 cells/mL of milk, indicating low prevalence of mastitis and superior milk quality.

We gathered information on the risk factors related to high scores of BMSCC, i.e., the biological variables, sanitary handling and milking. We also considered as possible risk factors the presence or absence of the pathogens *Staphylococcus aureus* and *Streptococcus agalactiae* in the milk samples obtained from the bulk milk tanks of these properties.

We used the results of the three last BMSCC analyses of each property and the milk quality data provided by the dairy, obtained with the laboratories of the Brazilian Network of Milk Quality.

The independent variables were obtained by using a questionnaire and *in loco* observation through technical visits to the properties. Data were obtained by a trained team composed of assistant technicians of the dairy that receive the milk from the studied properties. The criteria for deciding the tested variables included those that offered biological explanation for the increased BMSCC.

The following independent variables were raised in the interview: use of Tamis test, ; pre and post dipping; drying of the teats with disposable paper towel after the pre-dipping; monthly California mastitis test (CMT) or SCC evaluations; use of milking line for animals identified with clinical and subclinical mastitis; dry cow therapy and treatment of clinical cases. In addition, we evaluated whether the properties had cows with hyperkeratosis; whether the body score of all animals indicated good nutrition of the herd; and whether there were animals with thermal stress in any moment of the milking.

Related to the milking, we evaluated: the teats and udder hygiene before milking; the use of manual pressure during milking; entry of air in the milk sets during milking; the vacuum pressure of the milking machine according to the manufacturer's indication; periodic maintenance and adjustments of the milking machines and bulk milk tanks; and the automated hygiene of the milking machine and bulk milk tanks. We also verified if the milking period was superior to 2 hours and 30 minutes after the animals' arrival at the waiting room, and if the milking routine was calm, without the presence of dogs or other stress factors to the animals.

The presence of *S. aureus* and/or *S. agalactiae* was evaluated in samples of approximately 40 mL of milk, collected directly from the bulk milk tank in the studied properties; the samples were submitted to microbiological analysis, using selective media for the identification and quantification of these pathogens. For that, we performed serial dilutions of milk in sterile saline, which were plated in the selective culture media. The count of *S. aureus* was performed with Agar *Baird-Parker* (Oxoid®), according to the manufacturer's instructions. The count of *S. agalactiae* was performed with modified Agar *Edwards* (Oxoid®) enriched with 5% bovine blood, according to the manufacturer's instructions. In the plates showing growth of colonies suggestive of the agents of interest, we randomly selected five colonies that were submitted to confirmatory tests, according to Oliver et al. (2004a).

We performed descriptive analyses of all variables raised. For quantitative variables, we performed the data *Kolmogorov-Smirnov normality test.* We used non-parametric tests, including the *Spearman* coefficient, to test the correlations, and the *Mann-Whitney* test for comparison of rated means, considering 5% significance level.

The associations were tested with univariate analyses using the Pearson's chi-square test with 5% significance level. In a following step, we calculated the Odds Ratio with a 95% confidence interval.

The correlation tests between the BMSCC quantitative variables, daily milk production, *S. aureus* count and *S. agalactiae* count were performed using the non-parametric Spearman's test, with 1% and 5% significance levels.

The statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, Chicago, USA) version 20.0 for Windows.

RESULTS AND DISCUSSION

Among the case properties (BMSCC ≥700,000 cells/mL), 80% presented the pathogen *Streptococcus agalactiae* and 76.6% presented *Staphylococcus aureus*, while in the control properties (BMSCC ≤200,000 cells/mL) only 18.75% were positive for *S. aureus* and 50% for *S. agalactiae*. The results show that the presence of these pathogens in the herd is an important risk factor for increased BMSCC (Table 1). The presence of *S. aureus* increased the risk for BMSCC above 700,000 cells/mL of milk 14 times ($p<0.001$), while the presence of *S. agalactiae* increased this risk four times ($p=0.035$).

The presence of *S. aureus* and/or *S. agalactiae* is strictly associated with the increased BMSCC in the herd, due to its contagious nature and for predominantly causing subclinical mastitis, which negatively impacts milk quality (Jayarao et al. 2004, Keefe 2012). According to Philpot & Nickerson (2002), increased BMSCC is due to the increment of IMI, especially the subclinical mastitis that occurs when these pathogens are present in the herd.

The inflammatory process of mastitis triggers the release of proinflammatory cytokines that induce the passage of leucocytes from the blood to the interior of the gland, leading to increased individual SCC and consequent increase of BMSCC. The lesions in the mammary tissue decrease the efficiency

Table 1. Sanitary handling variables associated with the values of image result for bulk milksomatic cell counts (BMSCC) ≥700,000 cells/mL

Variables	Categories	Herds		P-values*	Odds Ratio (95% CI)
		Cases	Controls		
Staphylococcus aureus	Absent	7	13	0.000	14.23 (3.13-64.70)
	Present	23	3		
Streptococcus agalactiae	Absent	6	8	0.035	4.00 (1.06-15.07)
	Present	24	8		
Tamis test	Perform	16	4	0.117	-
	Does not perform	14	12		
Pre-dipping	Perform	16	14	0.026	0.163 (0.03-0.084)
	Does not perform	14	2		
Teats dried with paper towel	Perform	9	14	0.001	0.061 (0.01-0.32)
	Does not perform	21	2		
Post-dipping	Perform	10	14	0.001	0.071 (0.01-0.37)
	Does not perform	20	2		
CMT or individual SCC	Perform	5	17	0.000	0.01 (0.00-0.10)
	Does not perform	27	1		
Milking line	Perform	2	6	0.015	0.119 (0.02 – 0.68)
	Does not perform	28	10		
Dry Cow Therapy	Perform	20	17	0.012	0.10 (0.01-0.83)
	Does not perform	12	1		
Treatment of clinical cases	Perform	27	17	0.290	-
	Does not perform	5	1		
Teat hygiene before milking	Good	14	17	0.000	0.05 (0.01-0.39)
	Poor	18	1		

*p-values ≤ 0.05 were statistically significant using the Pearson $\chi 2$ (95%).

of the secretory cells, i.e., they produce and secret less milk (Brito et al. 2001).

Our results showed that the most of the studied variables were confirmed as risk factors of BMSCC above 700,000 cells/mL of milk (Table 1). This can be observed for the variables pre-dipping (OR=0.163, p=0.026), teats dried with disposable paper towel (OR=0.061, p=0.001) and post-dipping (OR=0.071, p=0.001). The use of these practices reduced the risk for increased BMSCC, indicating that they are protective factors. The lack of pre-dipping, drying of teats with disposable paper towel and post-dipping increased the risk for BMSCC in 6.1, 16.4 and 14.08 respectively.

The variables treatment of cows with clinical mastitis, Tamis test and automated hygiene of milk machine were not correlated with the BMSCC scores, suggesting that they did not impact this parameter(Table 2). Indeed the equipment hygiene and sanitizing procedures have a more significant impact in the total bacterial count (TBC) compared to BMSCC; however, they may lead to new IMI and consequently increased BMSCC when these requisites are not fully met.

Souza et al. (2005) observed herd characteristics and handling practices associated to the increase of BMSCC in 175 milk herds located in the Zona da Mata in the Minas Gerais state, verifying that performing the Tamis test did not correlate with BMSCC, a result that is corroborated in our study. The same author also observed that the nonuse of milking line and lack of pre and post dipping were associated to increase of BMSCC, again corroborating with our results.

The mastitis risk factors were studied by Mendonça et al. (2016) in 186 herds with BMSCC above 400,000 cells/mL, also located Zona da Mata region in the Minas Gerais state. The results showed that the main risk factors for increased BMSCC in the herds were related to inadequate handling practices: nonuse of Tamis test; feed the cows while milking and lack of post dipping.

Our study showed that the variable use of Tamis test was not associated with BMSCC. This was an unexpected result. This test allows to identifying animals affected with clinical mastitis for immediate treatment and disposal of their milk, which usually present high SCC. Thus, we expected that the use of this test to be a protective factor against BMSCC. However, we observed that this test is usually performed inappropriately. Many times, positive animals do not receive any treatment and their milk, affected by clinical mastitis, is not always discarded which may justify the results found in our study.

In this study, in agreement to results of Allore et al. (1998) and Berry & Hillerton (2002), the use of post dipping, treatment of dry cows and implantation of milking line were observed as protection factors for the increment of BMSCC. However, differently from the results obtained by these authors, the early treatment of mastitis clinical cases was not observed as a risk factor for high BMSCC. This may be due to the fact that almost all studied properties applied this procedure, both those with low and high BMSCC, but the frequency and method of use of this control measure, wich usually varies among herds, were not evaluated.

In the properties with mastitis monitoring through CMT, treatment of dry cow and milking line use, we observed

Table 2. Sanitary handling variables associated with bulk milk somatic cell counts (BMSCC) ≥700,000 cells/mL

Variables	Categories	Herds		P-values*	Odds Ratio (95% CI)
		Cases	Controls		
Automated milking cleaning	Yes	18	6	0.217	-
	No	12	10		
Pressure in the milking collectors	Perform	11	1	0.021	8.90 (1.05-76.04)
	Does not perform	21	17		
Milking vacuum pressure	Normal	22	17	0.036	0.13 (0.02-1.11)
	Unregulated	10	1		
Loss of vacuum during milking	Yes	9	1	0.055	6.65 (0.77-57.63)
	No	23	17		
Maintenance of milking machine and bulk milk tank	Good	0	16	0.000	Undefined
	Poor	30	0		

*p-values ≤ 0.05 were statistically significant using the Pearson $\chi 2$ (95%).

protective effect ($p < 0.05$), and the nonuse of these practices increased the risk of BMSCC above 700,000 cells/mL in 100, 10 and 8.4 times, respectively (Table 1).

Oliveira et al. (2012) studied the risk factors associated with bovine mastitis (SCC > 200,000 cells/mL) in 21 properties of the microregion of Garanhuns, Pernambuco state. The authors observed that the risk factors associated to the disease were the non-treatment of dry cows, non-drying of the teats with disposable paper towel and the nonuse of pre dipping; these results corroborate with ours.

Our results showed that, with exception of the Tamis test and the treatment of clinical cases, the deficiency or absence of mastitis control and prevention measures lead to increse of the BMSCC in the studied herds.

According to Lopes et al. (2011), increased BMSCC can lead to direct economic loss to the producer, since a large portion of the dairies adopts payment according to the milk quality, a fact that directly affects the mastitis economic impact. In another study, Lopes et al. (2012) verified that expenses with mastitis prevention represent a much lower cost compared to corrective measures and the production loss caused by increased BMSCC, which shows the advantages of investing in good milking handling practices that significantly contribute to the reduction of BMSCC, and consequently to reduction of the mastitis economic impact.

All the variables related to milking equipments were confirmed as risk factors for BMSCC above 700,000 (Table 2). The variable maintenance of the milking machine and bulk milk tank revealed complete association, i.e., all properties with any kind of failure in the maintenance or adjustments of the milking machine and/or milk bulk tank had BMSCC ≥700,000. Those properties without these problems had BMSCC ≤200,000 ($p \leq 0.001$), showing that these variables are highly associated to BMSCC. The variables inadequate pressure in the milking cluster and loss of vacuum during milking were also considered as risk factors. The presence of these variables in the properties led to increased risk of BMSCC ≥700,000 cells/mL in 8.90 and 6.65 respectively (Table 2).

Coentrão et al. (2008) studied subclinical mastitis risk factors (SCC >200,000 cells/mL of milk) in 2,657 cows from 24 herds from Minas Gerais state from November 2005 to June 2006. They observed that the main subclinical mastitis risk factors were related to cracks and fissures in the rubber parts of the milking equipment, inadequate teat cups, lack of maintenance of the pulsers, lack of milkers' training and the nonuse of mastitis microbiological diagnosis. Many of the risk factors related to inadequate functioning and lack of maintenance of the milking machine pointed out by these researchers also were observed in our study.

Dysregulated or defective milking equipment, lack of staff training and not adopting the recommended procedures for control and prevention of mastitis were the risk factors detected by Brito et al. (2002) and Cavazos (2003) for increased SCC. These variables interact and affect the frequency of increase in the herd's BMSCC and, consequently, of mastitis presence.

As for the quantitative variables, we observed a positive correlation between BMSCC and the counts of *S. aureus* and *S. agalactiae* in the milk (Table 3), suggesting that the increase of these agents in the bulk milk tank would be associated to the their higher prevalence in the herd. However, according to Oliver et al. (2004b), the counts of these pathogens in the bulk milk tank are not directly associated to their prevalence in the herd.

Souza et al. (2009) evaluated the effect of mastitis pathogens on SCC in 3,987 samples of milk from 2,657 animals from 24 milking herds located in the states of Rio de Janeiro and Minas Gerais. In this study they verified that SCC presented an average of 264,000 cells/mL of milk samples without

Table 3. Quantitative variables associated with bulk milk somatic cell counts (BMSCC) ≥700,000 cells/mL

Variables	Controls[a]			Cases[b]		
	Mean	Q_1-Q_3	Min.-Max.	Mean	Q_1-Q_3	Min.-Max.
BMSCC (cells/mL × 10^3)	162.25	130.25-190,79	51-209	934.00	786.96-1,118.21	707-1,573
Dialy milk production (L)	224.50	139.50-323.00	52-3673	290.58	168.98-566.75	59-1,736
Count of *Staphylococcus aureus* (UFC/mL)	0	0.00-0.00	0-1400	110.00	9.00-1,325.00	0-83,000
Count of *Streptococcus agalactiae* (UFC/mL)	525	0.00-2,380.00	0-3,800	3,650	52.75-25,250.00	0-16,6000

[a] Properties with BMSCC bellow 200, 000 cells/mL, [b] properties with BMSCC above 700,000 cells/mL.

Table 4. Correlations between bulk milk somatic cell counts (BMSCC), dialy milk production, count of *Staphylococcus aureus* and count of *Streptococcus agalactiae*

Varibles	BMSCC	Dialy milk production	Count of *S. aureus*	Count of *S. agalactiae*
BMSCC	1.000	0.194	0.400[a]	0.320[b]
Dialy milk production	0.194	1.000	0.110	-0.075
Count of *S. aureus*	0.400[b]	0.110	1.000	0.406[a]
Count of *S. agalactiae*	0.320[a]	-0.075	0.406[a]	1.000

[a] Spearman correlation with significant level of 0.01, [b] correlation with significant level of 0.05.

bacterial growth. When *S. agalactiae* was isolated, the SCC average was of 1,520,000 cells/mL of milk, with 50% of the samples presenting SCC ≥923,000 cells/mL. The same authors also observed that the presence of *S. aureus* was responsible for the second highest increase of SCC, with an average of 966,000 cells/mL. These results corroborate with ours, showing that these pathogens have great impact on BMSCC when present in the herd.

The results for the correlation between daily milk production and count of *S. aureus* (p=0.467), and between daily milk production and count of *S. agalactiae* (p=0.627) were not significant, showing that the farms daily production does not affect the numbers of these pathogens in the milk (Table 4). However, we observed moderate correlations between BMSCC and counts of *S. aureus* (p=0.06) and a positive correlation between BMSCC and counts of *S. agalactiae* (p=0.032). In concordance of our resultas, Cortinhas (2013) studied the correlation between SCC and the count of mastitis pathogens in herds from the state of São Paulo. This author found low correlation between the count of de *S. aureus* and BMSCC, and moderate positive correlation between the count of *S. agalactiae* and BMSCC (r=0.49).

Botaro et al. (2013) evaluated the correlation between the numbers of CFU/mL of *S. aureus* and SCC in samples of milk from individual mammary quarts. The authors observed no dependent correlation between the variables (p=0.1948), which is related to the intermittent elimination of the agent (Oliver et al. 2004b). This discordance with our results may be due to differences in the samples analyzed, since our study was performed with milk from bulk tanks. However, Sears et al. (1990) e Shoshani et al. (2000), even though using individual samples, pointed out the increased rate of elimination of *S. aureus* in the milk of animals chronically affected by *S. aureus*, concomitantly with increased SCC.

Djabri et al. (2002) observed that one single mammary quart infected with *S. aureus* and *S. agalactiae* resulted in 357,000 cells/mL and 857,000 cells/mL of milk, respectively, proving that the presence of these two pathogens is directly correlated with SCC and BMSCC. The presence of these microorganisms in milk, considered primary pathogens, was also related to the incremented SCC by Reis et al. (2011). The correlation between the counts of *S. aureus* and *S. agalactiae* with BMSCC suggests that this parameter may indicate the prevalence of these pathogens and the level of subclinical mastitis in the herd.

We also found a positive correlation between the counts of these pathogens (Table 4), showing that the increased count of one pathogen is associated to increased count of the other. This fact can be related to their contagious nature and similar epidemiological determinants.

Our results show that practices of prevention and control of mastitis related to the herd handling, maintenance of milking equipment and monitoring of mastitis are strictly correlated to the levels of BMSCC in the herd, justifying investments in these practices implementation, as shown by Lopes et al. (2011). These researchers reported that expenses with mastitis prevention, including culture and antibiogram, monitoring of BMSCC and individual SCC, pre and post dipping, vaccination against mastitis pathogens, treatment of dry cows and milking machine maintenance represent at most 10.8% of the economic impact associated with increased BMSCC in the herd. This highlights the economic return that will occur due to the implantation of these measures, which will significantly contribute to the reduction of BMSCC and improved milk quality.

Our results offer subsides for the elaboration of strategies to solve sanitary problems that influence BMSCC, improving the herds productivity, and not less important, the quality of the raw material, leading to higher profitability to producers and industry.

CONCLUSIONS

The presence and concentration of the pathogens *Staphylococcus aureus* and *Streptococcus agalactiae* are relevant risk factors for BMSCC ≥700,000 cells/mL.

There are moderate correlations between BMSCC and *S. aureus* and *S. agalactiae* counts, and moderate correlation between the *S. aureus* and *S. agalactiae* counts.

The nonuse of classical measures of control and prevention of mastitis related to the handling and milking hygiene, pre and post dipping, use of disposable paper towel to dry the teats, monitoring of the herd through CMT, implementation of milking line, dry cow therapy and lack of hygiene of the teats and udders were indicated as relevant risk factors for BMSCC ≥700,000 cells/mL.

The variables related to milking equipments, pressure applied to the milking sets, dysregulated milking vacuum pressure during milking and maintenance failures of the milking machine and bulk milk tank were identified as risk factors for BMSCC ≥700,000 cells/mL.

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

REFERENCES

Allore H.G., Erb H.N., Schruben L.W. & Oltenacu P.A. 1998. A simulation of strategies to lower bulk tank somatic cell count below 500,000 per milliliter. J. Dairy Sci. 81(3):694-702. <http://dx.doi.org/10.3168/jds.S0022-0302(98)75625-5> <PMid:9565872>

Barbano D.M., Ma Y. & Santos M.V. 2006. Influence of raw milk quality on fluid milk shelf life. J. Dairy Sci. 89(Suppl.1):15-19. <http://dx.doi.org/10.3168/jds.S0022-0302(06)72360-8> <PMid:16527874>

Berry E.A. & Hillerton J.E. 2002. The effect of selective dry cow treatment on new intramammary infections. J. Dairy Sci. 85(1):112-121. <http://dx.doi.org/10.3168/jds.S0022-0302(02)74059-9> <PMid:11860103>

Botaro B.G., Cortinhas C.S., Março L.V., Moreno J.F.G., Silva L.F.P., Benites N.R. & Santos M.V. 2013. Detection & enumeration of *Staphylococcus aureus* from bovine milk samples by real-time polymerase chain reaction. J. Dairy Sci. 96(11):6955-6964. <http://dx.doi.org/10.3168/jds.2013-6559> <PMid:24054287>

Brasil 2002. Instrução Normativa 51, de 18 de setembro de 2002. Regulamento Técnico de Produção, Identidade e Qualidade do Leite Tipo A, tipo B, Tipo C e Cru Refrigerado, Diário Oficial da União, Seção 1, Ministério da Agricultura, Pecuária e Abastecimento, Brasília, DF, p.13.

Brasil 2011. Instrução Normativa 62, de 29 de dezembro de 2011. Dispõe sobre Regulamentos Técnicos de Produção, Identidade, Qualidade, Coleta e Transporte do Leite, Diário Oficial da União, Seção 1, Ministério da Agricultura, Pecuária e Abastecimento, Brasília, DF.

Brasil 2016. Instrução Normativa 07, de 3 de maio de 2016. Dispõe sobre Alterações nos Regulamentos Técnicos de Produção, Identidade, Qualidade, Coleta e Transporte do Leite, Diário Oficial da União, Seção 1, Ministério da Agricultura, Pecuária e Abastecimento, Brasília, DF.

Brito M.A.V.P., Brito J.R.F., Souza H.M. & Vargas O.L. 1998. Avaliação da sensibilidade da cultura de leite do tanque para isolamento de agentes contagiosos da mastite bovina. Pesq. Vet. Bras. 18(1):39-44. <http://dx.doi.org/10.1590/S0100-736X1998000100007>

Brito M.A.V.P., Brito J.R.F., Ribeiro M.T. & Veiga V.M.O. 1999. Padrão de infecção intramamária em rebanhos leiteiros: exame de todos os quartos mamários das vacas em lactação. Arq. Bras. Med. Vet. 51(2):129-135. <http://dx.doi.org/10.1590/S0102-09351999000200001>

Brito M.A.V.P., Brito J.R.F., Silva M.A.S. & Carmo R.A. 2001. Concentração mínima inibitória de dez antibióticos para amostras de *Staphylococcus aureus* isoladas de infecção intramamária bovina. Arq. Bras. Med. Vet. 53(5):531-537. <http://dx.doi.org/10.1590/S0102-09352001000500003>

Brito J.R.F., Brito M.A.V.P. & Arcuri E.F. 2002. Como (Re)conhecer e Controlar a Mastite em Rebanhos Bovinos. Circular Técnica 70, Embrapa Gado de Leite, Juiz de Fora, MG, 8p.

Carvalho G.R. 2010. A Indústria de Laticínios no Brasil: passado, presente e futuro. Circular Técnica 102, Embrapa Gado de Leite, Juiz de Fora, 12p.

Cavazos G.F. 2003. Useful ideas and principles for the implementation of reinforcement programs to keep milkers motivated. Proceedings of the Annual Meeting of National Mastitis Council, Fort Worth, TX, p.23-37.

Coentrão C.M., Souza G.N., Brito J.R.F., Paiva e Brito M.A.V. & Lilenbaum W. 2008. Fatores de risco para mastite subclínica em vacas leiteiras. Arq. Bras. Med. Vet. 60(2):283-288. <http://dx.doi.org/10.1590/S0102-09352008000200001>

Cortinhas C.S. 2013. Qualidade do leite cru e práticas de manejo em fazendas leiteiras. Doctoral Dissertation in Veterinary Medicine, Universidade de São Paulo, Pirassununga, SP. 127p. <http://dx.doi.org/10.11606/T.10.2013.tde-21082014-111709>.

Costa G.M., Paiva L.V., Figueiredo H.C.P., Figueira A.R., Pereira U.P. & Silva N. 2012. Population diversity of *Staphylococcus aureus* isolated from bovine mastitis in brazilian dairy herds. Res. Vet. Sci. 93(2):733-735. <http://dx.doi.org/10.1016/j.rvsc.2011.09.014> <PMid:22035658>

Djabri B., Bareille N., Beaudeau F. & Seegers H. 2002. Quarter milk somatic cell count in infected dairy cows: a meta-analysis. Vet. Res. 33(4):335-357. <http://dx.doi.org/10.1051/vetres:2002021> <PMid:12199362>

Dong F., Hennessy D.A. & Jensen H.H. 2012. Factors determining milk quality and implications for production structure under somatic cell count standard modification. J. Dairy Sci. 95(11):6421-6435. <http://dx.doi.org/10.3168/jds.2012-5522> <PMid:22981577>

FAEMG 2006. Diagnóstico da pecuária leiteira do estado de Minas Gerais em 2005: relatório de pesquisa. Federação de Agricultura do Estado de Minas Gerais, Belo Horizonte, MG. 156p.

Guerreiro P.K., Machado M.R.F., Braga G.C., Gasparino E. & Franzener A.S.M. 2005. Qualidade microbiológica de leite em função de técnicas profiláticas no manejo de produção. Ciênc. Agrotec. 29(1):216-222. <http://dx.doi.org/10.1590/S1413-70542005000100027>

IBGE 2017. Estatística da Produção Pecuária 2017. Instituto Brasileiro de Geografia e Estatística, Brasília, DF, p.1-78.

Jayarao B.M., Pillai S.R., Sawant A.A., Wolfgang D.R. & Hegde N.V. 2004. Guidelines for monitoring bulk tank milk somatic cell and bacterial counts. J. Dairy Sci. 87(10):3561-3573. <http://dx.doi.org/10.3168/jds.S0022-0302(04)73493-1> <PMid:15377636>

Keefe G. 2012. Update on control of *Staphylococcus aureus* and *Streptococcus agalactiae* for management of mastitis. Vet. Clin. N. Am., Food Anim. Pract. 28(2):203-216. <PMid:22664203>

Lopes M.A., Demeu F.A., Abreu L.R. & Franco Neto A. 2011. Influência da contagem de células somáticas sobre o impacto econômico da mastite em rebanhos bovinos leiteiros. Arqs Inst. Biológico, São Paulo, 78(4):493-499.

Lopes M.A., Demeu F.A. & Franco Neto A. 2012. Avaliação do impacto econômico da mastite. Arqs Inst. Biológico, São Paulo. 79:477-483.

Machado P.F., Pereira A.R. & Sarríes G.A. 2000. Composição do leite de tanques de rebanhos brasileiros distribuídos segundo sua contagem de células somáticas. Revta Bras. Zootec. 29(6):1883-1886. <http://dx.doi.org/10.1590/S1516-35982000000600038>

Mendonça J.F.M., Brito M.A.V.P., Mendonça L.C., Souza G.N., Lange C.C., Brito J.R.F. & Monteiro D.L. 2016. Fatores de risco para mastite subclínica em rebanhos leiteiros localizados na região de Santos Dumont, estado de Minas Gerais, Brasil. Revta Educ. Cont. Med. Vet. Zootec. CRMV-SP. 14(3):71.

NMC 1996. Current concepts of bovine mastitis. National Mastitis Council, Madison, WI. 64p.

NMC 2000. Recommended mastitis control program. National Mastitis Council, Madison, WI. 78p.

Oliver S.P., González R.N., Hogan J.S., Jayarao B.M. & Owens W.E. 2004a. Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality. 4th ed. National Mastitis Council, Verona, WI, 46p.

Oliver S.P., Gillespie B.E., Headrick S.J., Moorehead H., Lunn P., Dowlen H.H., Johnson D.L., Lamar K.C., Chester S.T. & Moseley W.M. 2004b. Efficacy of extended ceftiofur intramammary therapy for treatment of subclinical mastitis in lactating dairy cows. J. Dairy Sci. 87(8):2393-2400. <http://dx.doi.org/10.3168/jds.S0022-0302(04)73361-5> <PMid:15328260>.

Oliveira J.M.B., Vanderlei D.R., Moraes W.S., Brandespim D.F., Mota R.A., Oliveira A.A.F., Medeiros E.S. & Pinheiro Júnior J.W. 2012. Fatores de risco associados à mastite bovina na microrregião Garanhuns, Pernambuco. Pesq. Vet. Bras. 32(5):391-395. <http://dx.doi.org/10.1590/S0100-736X2012000500005>

Omore A.O., McDermott J.J., Arimi S.M., Kyule M.N. & Ouma D. 1996. A longitudinal study of milk somatic cell counts and bacterial culture from cows on smallholder dairy farms in Kiambu District, Kenya. Prev. Vet. Med. 29(1):77-89. <http://dx.doi.org/10.1016/S0167-5877(96)01054-9>

Philpot W.N. & Nickerson S. 2002. Vencendo a Luta Contra a Mastite. Westfalia Landtechnik do Brasil Ltda., São Paulo. 189p.

Politis I. & Ng-Kwai-Hang K.F. 1988. Effects of somatic cell count & milk composition on cheese composition and cheese making efficiency. J. Dairy Sci. 71(7):1711-1719. <http://dx.doi.org/10.3168/jds.S0022-0302(88)79737-4>

Ranjan R., Swarup D., Patra & Nandi R.C.D. 2006. Bovine protothecal mastitis: a review. CAB Revs. Perspectives Agricult. Vet. Sci. Nutr. Nat. Resources 1(17):1-7.

Rêgo M.A.V. 2010. Case-control studies: a brief review. Gaz. Méd. 80(1):101-110.

Reis C.B.M., Barreiro J.R., Moreno J.F.G., Porcionato M.A.F. & Santos M.V. 2011. Evaluation of somatic cell count thresholds to detect subclinical mastitis in gyr cows. J. Dairy Sci. 94(9):4406-4412. <http://dx.doi.org/10.3168/jds.2010-3776> <PMid:21854914>

Reyher K.K., Haine D., Dohoo I.R. & Revie C.W. 2012. Examining the effect of intramammary infections with minor mastitis pathogens on the acquisition of new intramammary infections with major mastitis pathogens: a systematic review and meta-analysis. J. Dairy Sci. 95(11):6483-6502. <http://dx.doi.org/10.3168/jds.2012-5594> <PMid:22981582>

Ribeiro M.G. 2008. Princípios terapêuticos na mastite em animais de produção e de companhia, p.759-771. In: Andrade S.F. (Ed), Manual de Terapêutica Veterinária. 3ª ed. Roca, São Paulo.

Sears P.M., Smith B.S., English P.B., Herer P.S. & Gonzalez R.N. 1990. Shedding pattern of *Staphylococcus aureus* from bovine intramammary infections. J. Dairy Sci. 73(10):2785-2789. <http://dx.doi.org/10.3168/jds.S0022-0302(90)78964-3> <PMid:2283409>

Santos M.V. & Fonseca L.F.L. 2007. Estratégias para Controle de Mastite e Melhoria da Qualidade do Leite. Manole, Barueri. 150p.

Shoshani E., Leitner G., Hanochi B., Saran A., Shpigel N.Y. & Berman A. 2000. Mammary infection with staphylococcus aureus in cows: progress from inoculation to chronic infection and its detection. J. Dairy Res. 67(2):155-169. <http://dx.doi.org/10.1017/S002202990000412X> <PMid:10840670>

Souza G.N. 2005. Fatores de risco para mastite bovina. Doctoral Dissertation in Veterinary Medicine, Universidade Federal de Minas Gerais, Belo Horizonte, MG. 87p.

Souza G.N., Brito J.R.F., Moreira E.C., Brito M.A.V.P. & Bastos R.R. 2005. Fatores de risco associados à alta contagem de células somáticas do leite do tanque em rebanhos leiteiros da Zona Da Mata de Minas Gerais. Arq. Bras. Med. Vet. 57(Suppl.2):251-260. <http://dx.doi.org/10.1590/S0102-09352005000800018>

Souza G.N., Brito J.R.F., Moreira E.C., Brito M.A.V.P. & Silva M.V.G.B. 2009. Variação da contagem de células somáticas em vacas leiteiras de acordo com patógenos da mastite. Arq. Bras. Med. Vet. 61(5):1015-1020. <http://dx.doi.org/10.1590/S0102-09352009000500001>

Vilela P.S. & Nogueira A.E.C. 2010. Perfil do Produtor de Leite nas Mesorregiões da Zona da Mata e Campo das Vertentes de Minas Gerais. Pólo de Excelência do Leite e Derivados, Secretaria de Ciência, Tecnologia e Ensino Superior, Governo do Estado de Minas Gerais, Belo Horizonte, MG. 119p.

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(8):614-621, August 2019
DOI: 10.1590/1678-5150-PVB-5883

Original Article
Livestock Diseases

Protocols for preparation of platelet rich plasma (PRP) in Quarter Horses[1]

Stephania Miranda[2], Maria Fernanda Mello Costa[3,4]*, Natália Rebouças[2,5], Márcia T. Ramos[4,6], Daniel A.B. Lessa[2] and Nayro X. Alencar[2]

ABSTRACT.- Miranda S., Mello Costa M.F., Rebouças N., Ramos M.T., Lessa D.A.B. & Alencar N.X. 2019. **Protocols for preparation of platelet rich plasma (PRP) in Quarter Horses.** *Pesquisa Veterinária Brasileira 39(8):614-621.* Departamento de Patologia e Clínica Veterinária, Faculdade de Veterinária, Universidade Federal Fluminense, Rua Vital Brazil Filho 64, Niterói, RJ 24230-340, Brazil. E-mail: mfveterinaria@hotmail.com

This study compared two protocols for preparation of platelet rich plasma (PRP) and evaluated the association between manual and automated methods for platelet count using a prospective study design. Eight clinically healthy Quarter Horses had venous blood samples collected at rest. After collection, blood samples were centrifuged twice, using two different protocols including a period of sample resting, either at the start or at the end of the protocol. Platelet counting at the start of the protocol, during, and after obtaining PRP was conducted manually or with an automated counter, followed by comparison of the two methods. In order to investigate platelet degranulation during the protocol, vascular endothelial growth factor (VEGF) was measured at each preparation stage. The protocol with sample resting before centrifugation yielded a more concentrated PRP, and the study verified that both manual and automated methods are comparable and can be used interchangeably for platelet counting. VEGF concentration did not differ significantly between protocols, or among protocol stages. The results indicate that choice of protocol for PRP preparation will affect the quantity of platelets in the final product, although platelet degranulation was not observed as evidenced by the stable VEGF concentrations measured. A larger yield of non-degranulated platelets in PRP is desirable since more α-granules will be present, therefore Protocol II is recommended. Both manual and automated counts reliably allow clinicians to obtain platelet counts and the choice of utilizing a manual or automated method is unlikely to interfere with evaluation of the final PRP product.

INDEX TERMS: Platelet rich plasma, PRP, Quarter Horses, biotechnology, blood, equidae, investigative techniques, biological factors, horses.

RESUMO.- [**Protocolos para o preparo de plasma rico em plaquetas (PRP) em cavalos Quarto de Milha.**] Este estudo comparou dois protocolos de preparo de plasma rico em plaquetas (PRP) e avaliou a associação entre dois métodos de contagem plaquetária – um manual e o outro automático através de um estudo prospectivo. Sangue venoso de oito equinos da raça Quarto de Milha foi coletado e em seguida foi centrifugado duas vezes utilizando-se dois protocolos distintos: um com descanso antes da primeira centrifugação e outro após a segunda centrifugação. A contagem plaquetária ao início, no meio e ao final dos protocolos foi realizada manualmente e pelo método automatizado, seguida de comparação entre os dois métodos. Para investigar a degranulação plaquetária ocorrida durante o preparo do PRP, o fator de crescimento vascular endotelial (VEGF) foi mensurado em cada estágio dos protocolos. O método utilizando o descanso da amostra antes da primeira centrifugação proporcionou a obtenção de um PRP mais concentrado, além de o estudo verificar que ambos os métodos de contagem plaquetária (manual e automatizado)

[1] Received on April 3, 2019.
Accepted for publication on April 16, 2019.

[2] Departamento de Patologia e Clínica Veterinária, Faculdade de Veterinária, Universidade Federal Fluminense (UFF), Rua Vital Brazil Filho 64, Niterói, RJ 24230-340, Brazil.

[3] Waikato Institute of Technology (Wintec), Tristram Street, Whitiora, Hamilton 3200, New Zealand.

[4] Faculdade de Medicina Veterinária, Universidade Severino Sombra, Av. Expedicionário Oswaldo de Almeida Ramos 280, Centro, Vassouras, RJ 27700-000, Brazil. *Corresponding author: mfveterinaria@hotmail.com

[5] Faculdade de Medicina Veterinária, Universidade Castelo Branco, Avenida Brasil 9727, Penha, Rio de Janeiro, RJ 21030-000, Brazil.

[6] Universidade Federal Rural do Rio de Janeiro (UFRRJ), Rodovia BR-465 Km 7, Zona Rural, Seropédica, RJ 23890-000, Brazil.

são comparáveis e podem ser usados indiferentemente. A concentração de VEGF não foi significativamente diferente entre os estágios de preparo do PRP. Os resultados indicam que o método de preparo afeta a quantidade de plaquetas obtidas no PRP, apesar da degranulação plaquetária não ter sido observada, como evidenciado pela concentração estável de VEGF. Uma maior concentração de plaquetas no PRP é desejável, pois indica que um maior número de α-grânulos estará presente na amostra, portanto, conclui-se que o Protocolo II é mais recomendável. Tanto o método manual, quanto o automatizado, pode ser usado de maneira confiável para a contagem plaquetária, não interferindo com a avaliação do produto final (PRP).

TERMOS DE INDEXAÇÃO: plasma rico em plaquetas, PRP, cavalos Quarto de Milha, biotecnologia, equídeos, fatores biológicos, sangue, técnicas de investigação.

INTRODUCTION

Platelets are produced in the bone marrow from cytoplasmic fragments from megakaryocytes and have essential functions in healing, reepithelization, and conservation of vascular integrity through interactions with endothelial cells (Comar et al. 2009). Numerous growth factors (GFs) are stored inside α-granules in the platelets, and once released will act on regulatory processes, including tissue regeneration, chemotaxis, cell proliferation, angiogenesis, immunological regulation and modulation of inflammatory processes (Anitua et al. 2004).

Platelet rich plasma (PRP) is an autologous biological preparation with regenerative properties conferred by the large concentration of platelets and GFs it contains (Andrade et al. 2016), including vascular endothelial growth factor (VEGF), which is the most important angiogenic growth factor, but also promotes vascular permeability and chemotaxis of cells during inflammation (Ng et al. 2006). PRP was first described in the 1970s, although the biological functions of GFs contained in PRP were only investigated later (Ross et al. 1986). More recently, interest in PRP as a therapeutic agent for the treatment of orthopedic (Carmona Ramírez & Prades 2006, Bosch et al. 2010, Brossi et al. 2015) and skin conditions has increased (DeRossi et al. 2009, Maciel et al. 2012).

Recent reviews on the effects of PRP application in different fields of veterinary (Marcazzan et al. 2018, Tambella et al. 2018) and human medicine (Sheth et al. 2012, Laudy et al. 2015, Bos-Mikich et al. 2018) and although opinions vary as far as the possible applications of PRP in regenerative medicine most critics agree that lack of standardization of protocols for preparation of PRP is negatively impacting on the advance of the use of this bioproduct (Chahla et al. 2017).

Various techniques for equine PRP preparation have been suggested (DeRossi et al. 2009, Vendruscolo et al. 2012) but there is no consensus regarding the gold standard protocol. Differences in centrifugation time, speed, and resting of the sample could potentially influence the concentration of platelets and GFs in the final product, which likely influences the effects of PRP itself (Da Fontoura Pereira et al. 2013). Table 1 summarizes information available in the current literature concerning GFs, while Table 2 summarizes published protocols for harvesting PRP.

The goal of PRP preparation protocol should be to obtain a small volume of plasma with extremely high concentrations of platelets and GFs and minimal concentrations of erythrocytes and leukocytes when compared to the original blood sample (Vendramin et al. 2006, Da Fontoura Pereira et al. 2013).

Table 1. Summary of the current literature regarding source and function of growth factors

Growth factor	Source	Function	References
TGF-b	Platelets, neutrophils, macrophages, monocytes, natural cell killers, Th1 cells, bone extracellular matrix and cartilaginous matrix	Regulates the mitogenic effect of other growth factors, stimulates the proliferation of undifferentiated mesenchymal cells, fibroblast and osteoblast mitogen, endothelial regulator and regulator of the collagen synthesis and secretion of collagenase, stimulates angiogenesis and endothelial chemotaxis, inhibits the proliferation of macrophages and lymphocytes.	Raines & Ross (1982), Martelossi Cebinelli et al. (2016), Gatica et al. (2018)
FGF	Platelets, macrophages, chondrocytes, osteoblasts and mesenchymal cells	Mitogen for mesenchymal cells, chondrocytes and osteoblasts, stimulates the growth and differentiation of chondrocytes and osteoblasts.	Raines & Ross (1982), Kharitonenkov & Dimarchi (2017), Ornitz & Itoh (2015)
PDGF a-b	Platelets, macrophages/ monocytes, endothelial cells, osteoblasts and smooth muscle cells	Stimulates the chemotaxis and mitosis of fibroblasts, smooth and glia muscle cells, regulates the secretion of collagenase and collagen synthesis, mitogen for mesenchymal cells and osteoblasts, stimulates the chemotaxy of macrophages and neutrophils.	Raines & Ross (1982), Hye Kim et al. (2015), Heldin et al. (2018)
Epidermic growth factor	Platelets, macrophages/ monocytes	Stimulates mitosis of mesenchymal cells, regulates the secretion of collagenase, stimulates chemotaxis and angiogenesis of endothelial cells.	Raines & Ross (1982), Wee & Wang (2017), Brown et al. (2016)
VEGF	Platelets, endothelial cells	Stimulates mitosis of endothelial cells, increases angiogenesis and permeability of the vessel.	Raines & Ross (1982), Simons et al. (2016), Dehghani et al. (2018)
IGF	Platelets, macrophages, osteoblasts, bone matrix and mesenchymal cells	Stimulates the differentiation and mitogenesis of mesenchymal cells and of lining cells, stimulates osteoblasts and the production of type I collagen, osteocalcin and alkaline phosphatase.	Raines & Ross (1982), Frater et al. (2018)

Table 2. Summary of the current literature regarding protocols for harvesting platelet rich plasma

Reference	Number of centrifugations	Gravitational acceleration/ minutes	Initial platelet concentration (10^3/µL)	Final platelet concentration (PRP) (10^3/µL)	Resting time	Result achieved expressed as % of concentration
Lee at al. (2018)	2	900g for 15 min 200g for 15 min	101.83	542.50	None	5.3%
Giraldo et al. (2015)	3	120g for 5 min 240g for 5 min 3500g for 8 min	143.8	390.6	None	2.7%
Zuffova et al. (2013)	1	1500rpm for 5 min	87	466.5	1 hour	5.3%
Frye et al. (2016)	2	1200g for 4 min 1,050g for 9 min	214.70	1383.96	30-120 min	6.4%
Kwirant et al. (2019)	2	224g for 10 min 440g for 10 min	180.12	840.82	None	4.6%
Miranda et al. (2018a)	1	133g for 8 min	133.36	189.21	30 min	1.4%
Miranda et al. (2018b)	2	120g for 10 min 240g for 10 min	159.6	567.7	2 hours	3.5%
Bonilla-Gutiérrez et al. (2018)	2	120g for 5 min 240g for 5 min	130	370	None	2.8%
Tian et al. (2019)	2	900g for 5 min 1500g for 15 min	219	1218	None	5.5%
Xiong et al. (2018)	1	180g for 10 min	216.4	525.7	1 hour	2.4%

Platelet degranulation usually starts within one hour of blood collection and the clinical effects of this degranulation are controversial (Prado Vendruscolo et al. 2014). One way of assessing platelet degranulation is the measurement of P-selectin (Vestweber & Blanks 1999) but other substances present in the α-granules, such as VEGF, are likely candidates (Engels et al. 2015). The literature suggests that procedures with either single (Messora et al. 2009) or double (Carmona Ramírez & Prades 2006, Vendramin et al. 2006) centrifugations, or consecutive centrifugations with increasing speeds can produce satisfactory PRP, bearing in mind that to qualify as "PRP" the end product must have at least a three-fold increase in platelets in relation to the original blood sample (Marx et al. 1998).

Platelet counts can be done via the direct manual method or using an automated protocol, although the International Council for Standardization of Haematology (ICSH) considers the manual method utilizing dilution, lysis and direct count with a hemocytometer as the gold standard (Tasker et al. 2001). Other authors argue that hemocytometer counting of platelets is heavily influenced by operator experience and the quality of the microscope (Olsen et al. 2004), and that in high throughput situations the manual count is unrealistic and should be replaced by the automated method, which provides a coefficient of variation below 10% in platelet counts between 40,000 and 500,000/µL (Veloso et al. 2011).

This study evaluated two different methods of PRP preparation to investigate if there was a significant difference in the concentration of platelets between the two, and compared platelet counts via the manual and the automated methods to investigate their association. In order to evaluate platelet degranulation during PRP preparation, VEGF was measured. The working hypotheses, based on the current status of knowledge, were that protocol preparation would influence platelet concentration in PRP, that manual and automated methods for platelet counting would provide similar results at different stages of the preparation protocols, and that one protocol might be more efficacious in preventing platelet degranulation.

MATERIALS AND METHODS

Ethics statement. This project was approved by the "Universidade Federal Fluminense" Ethics Committee (CEUA-UFF) under number 767.

Animals. Eight clinically healthy Quarter Horses, between five and seven years of age, including four males and four females, stabled at the same property under the same management conditions were recruited to participate in this research. Animals were kept in individual stalls during the night with daily access to paddocks. Feeding regimes included *Pennisetum purpureum* Schumach grass, alfalfa, commercial hard food twice daily, mineral salt, and fresh water *ad libitum*. Animals were not fasted prior to collection, although blood samples were taken early in the morning, prior to any food being provided. Inclusion criteria were: absence of hematological alterations, a normal clinical examination, no lameness or signs of active inflammation (localized or general), and not having been vaccinated against infectious diseases in the previous 60 days. After collection of blood samples, initial platelet count via the manual method had to be between 100.000 and 260.000/µL for the horse to remain in the study.

Study design. Blood collection was conducted by venipuncture of the external jugular vein utilizing vacuum tubes containing 3.2% sodium citrate[a].

Twelve tubes per animal were collected to obtain an initial volume of blood of 60mL; tubes were separated in two groups of six tubes, one group per PRP protocol. The blood in the tubes was mixed by gentle inversion (10x) after which a 20µL aliquot was separated and diluted in 1.980µL of ammonium oxalate 2% for cell lysis. This aliquot was then submitted to the initial platelet count in the hemocytometer according to the method described in the

a Vacutainer, Becton Dickinson®, Brazil.

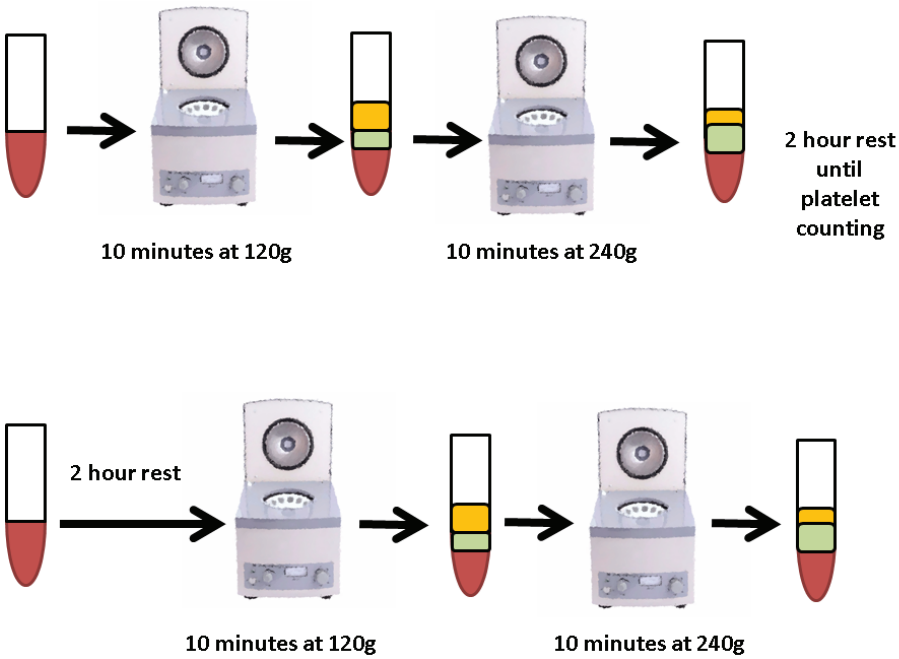


Fig.1. Diagrammatic view of the two protocols for PRP preparation with sample resting points highlighted, and supernatant aliquoting and aspiration indicated. The samples got separated into three layers by the first centrifugation. The first layer, comprising platelet poor plasma (represented as a yellow rectangle) got aliquoted for VEGF measurements. The second layer, including the buffy coat and platelet rich plasma (PRP, depicted as a green rectangle) was transferred to clean glass tubes and submitted to a second centrifugation. The third and final layer (red blood cells) was discarded. After the second centrifugation the process was repeated. The major difference between the protocols was the time point where samples were rested at room temperature for two hours. In Protocol I, shown at the top of the diagram, the sample was only rested as the very last step before platelet counting in PRP, while in the second protocol, shown at the bottom of the diagram, the sample was rested before the first centrifugation and that yielded better results.

literature (Jain 1993). Simultaneously another 100µL aliquot of the sample was processed in the automated equipment[b] for automated cell (including leukocytes) and platelet counts. Packed cell volume was done in a microtube centrifuge[c] and calculated hematocrit in the automated equipment calibrated for horses.

Protocols. PRP Protocol I consisted of immediate centrifugation of the six vacuum tubes in a bench top centrifuge[d] for 10 minutes at 120g for initial plasma separation, after which the top 2/3 of the supernatant in each of the vacuum tubes was aspirated with a single channel pipette, aliquoted into microtubes, and frozen in liquid nitrogen for VEGF measurement. The final third of the supernatant and the buffy coat were aspirated as a pool into glass tubes and submitted to a second centrifugation for 10 minutes at 240g, after which the separation of the top 2/3 and the final third of the supernatant was repeated as described above. The final third, considered to be the PRP, was subjected to a period of rest at room temperature for two hours. Average PRP yield volume was 2.5ml. At the end of the rest period a platelet count on the PRP product was conducted manually and with the automated equipment, as described for the initial sample. Aliquots for VEGF measurement were also separated from the PRP final product.

PRP Protocol II consisted of an initial rest period of two hours at room temperature followed by the same centrifugations and supernatant separations as described above for Protocol I. The final PRP product, (average PRP yield volume obtained was 2.5ml), was then submitted to platelet counts, both manually and by the automated method, as described above. Figure 1 details the methods, sample rest moments and aliquot separation times.

Concentration of VEGF. For VEGF concentration, Equine VEGF-A "Do-it-yourself" ELISA kit (Kingfisher, DIY0705E-003) was used according to manufacturer´s instructions. Buffer dilution and concentrations were optimized in our lab and details are published elsewhere (Mello Costa 2017). Aliquots from three distinct points during PRP preparation were analyzed (after the first centrifugation, after the second centrifugation and at the completion of PRP preparation) and compared, as described in the PRP protocol description.

Statistics. ANOVA was utilized for investigating the influence of gender and age on the platelet counts obtained, to investigate the effect of protocol on VEGF concentration and to compare mean VEGF concentrations at the three distinct points. Significance was set at 95% ($p \leq 0.05$).

Statistical analysis was conducted with specific software[e] and consisted of paired T-test analysis of initial and final counts for each of the protocols. Significance was set at 95% ($p \leq 0.05$).

Statistical evaluation of the automated method in relation to the manual method was conducted through dispersion and regression graphs. R values above 0.80 were considered to show a significant

b Coulter T890®, Beckman Coulter, USA.
c Hematocrit, model.
d Centribio.

e Minitab® 17.3.1, © 2013, 2016 Minitab Inc., USA.

correlation between the two methods. The association between the two methods was confirmed by plotting Bland Altman dispersion graphs.

Paired T-tests comparing the means of platelet counts from manual and automated processing methods (i.e. initial, intermediate and final platelet counts) was also conducted. Significance was set at 95% (p≤0.05).

RESULTS

Results are presented as mean ± standard deviation (SD) and test statistic results are followed by the 95% confidence interval, when pertinent.

There were no significant differences for any of the platelet counts, regardless of protocol or method, regarding animal gender or age.

Average initial platelet count by the manual method was 244.2x103/μL ± 21.3x103/μL and 256.5x103/μL ± 37.8x103/μL by the automated one. Final platelet counts for Protocol I with manual and automated methods were 463.0x103/μL ± 71.0x103/μL, and 513. 2x103/μL ± 69.6x103/μL, respectively, while counts for Protocol II with manual and automated methods were 761.8x103/μL ± 41.9x103/μL and 867x103/μL ± 219.3x103/μL respectively. Protocol I offered a 202.3% increase in platelet count, while Protocol II offered a 307.5% increase.

Final platelet counts were significantly higher than the initial counts for Protocols I and II, when the manual count was considered (p<0.01; 166.9 to 343.1 and p<0.01; 478.9 to 555.4 respectively).

Similarly, final platelet counts were significantly higher for Protocols I and II, when the automated count was considered (p<0.01; 203.0 to 346.5 and p<0.01; 387.2 to 765.6 respectively).

Protocol II offered a significantly higher final platelet count than Protocol I (manual count p<0.01; 179.0 to 345.3; automated count p=0.02; 73.8 to 525.3).

No significant differences in platelet counts were found between automated and manual methods for either protocol: p-values were 0.12 (initial manual compared to initial automated), 0.10 (final manual Protocol I versus final manual Protocol II), and 0.34 (final automated Protocol I versus final automated Protocol II).

Analysis of the association between the two methods (manual and automated) was conducted utilizing dispersion graphs according to Altman & Bland (1983), as can be seen in Figure 2, which corroborates the good correlation between the two methods.

Visual inspection of platelets in blood smears did not show platelet activation as evidenced by the absence of pseudopods. As far as VEGF concentration, mean values and SD can be seen in Table 3. Mean VEGF concentration for Protocol I was 0.82ng/mL ± 0.04ng/mL; 0.79 to 0.85 and 0.84ng/mL ± 0.04ng/mL; 0.80 to 0.87. Pooled SD was 0.04.

There was no significant difference in VEGF concentrations among the three points for Protocol I (p=0.57) nor Protocol II (p=0.06), and there was no difference in VEGF concentrations between Protocols I and II at any time (p=0.42; f=0.69).

Packed cell volume obtained from centrifugation and manual reading (30.1% ± 1.3) was not significantly different (p=0.26; -3.9 to 1.6) from the hematocrit by the automated method (31.2% ± 1.3).

The differences in final erythrocyte count (p=494; -0.56 to 0.28) and final leukocyte count (p=0.084; -3.02 to 40.02) between Protocol I and II were not significantly different. Both protocols offered an increase in leukocytes (345.3% for Protocol I and 525.6% for Protocol II) and a decrease in erythrocyte numbers (8.1% of initial erythrocytes left in Protocol I and 10.2% left in Protocol II).

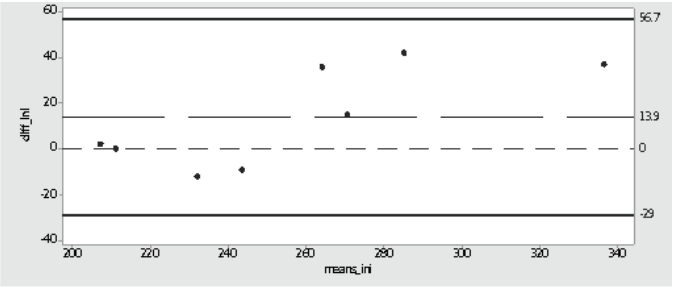


Fig.2. Altman & Bland (1983) dispersion graph showing good association between the manual and automated platelet counts. diff_ini = difference between average platelet counts from manual and automated methods, means_ini = averages from reference method (manual).

Table 3. Mean VEGF concentrations and SD

Measurement point	Protocol	Mean VEGF (ng/mL)	SD (ng/mL)
After first centrifugation	I	0.82	0.04
	II	0.84	0.04
After second centrifugation	I	0.82	0.05
	II	0.82	0.05
PRP	I	0.92	0.27
	II	0.88	0.04

VEGF = vascular endothelial growth factor, SD = standard deviation, PRP = platelet rich plasma.

DISCUSSION

Both protocols used in this study allowed concentration of platelets but only Protocol II (3.18x increase in platelet concentration) allowed for true production of PRP since final concentrations should be at least three times the initial ones (Marx 2004). Final platelet count with Protocol II was 761.8x103/μL ± 41.9x103/μL, which is higher than previous values reported in the literature by others (Carmona Ramírez & Prades 2006, Vendruscolo et al. 2012). Similar research describing differences between protocols for PRP preparation present results in terms of fold-concentration obtained. In the case of this study a 3.18-fold increase was observed with Protocol II, while others report a 4-fold increase with their own protocols (DeRossi et al. 2009, Da Fontoura Pereira et al. 2013).

In this study, both protocols utilized two centrifugation cycles with increasing centrifugal forces, according to what has been described in the literature (DeRossi et al. 2009, Vendruscolo et al. 2012), but differing in relation to the moment of sample rest. It appears that allowing the sample to rest for two hours at room temperature at the start of the protocol, before centrifugation, is beneficial to obtaining a higher platelet count in the PRP harvested from the blood of Quarter Horses. The results corroborate the hypothesis that the protocol used influences the final PRP product. That,

in return, might influence outcomes of possible therapeutic uses for the product.

One of the key aspects of the clinical outcome of PRP application is likely to rest in the concentration of active substances, rather than simply in the platelet counts. This work is part of a larger study which includes evaluation of active substances in the PRP and a review of its clinical application in a controlled trial.

In both protocols, a small amount of red blood cells was present in the PRP, with a smaller amount in Protocol I (0.51/μL ± 0.79) in relation to Protocol II (0.65/μL ± 0.44) with a similar outcome for leukocyte counts. It is unknown whether a higher concentration of erythrocytes and leukocytes would affect the usage and outcomes of therapeutic PRP (Marx 2004, Carmona Ramírez & Prades 2006, Vendramin et al. 2006), but previous investigations suggested that a small concentration of leukocytes would be desirable in order to maximize benefits arising from the increased platelet and GF concentrations in PRP (Pereira et al. 2013).

Both manual and automated methods provided similar counts for the parameters measured, in contrast with information provided previously stating that automated methods underestimate platelet values due to formation of aggregates (Tasker et al. 2001). This is likely because in both instances, manual and automated counts will tally clumped platelets as a single unit. In the present study, manual platelet count was conducted by a trained professional, reducing the possibility of operator error, as suggested by the literature (Olsen et al. 2004).

Therapeutic use of PRP in equine medicine, especially in dermatology, orthopaedics and inflammatory respiratory disorders has increased over the last few decades. Before studies investigating the effects of PRP use are properly conducted, an excellent working knowledge of the protocols for PRP preparation is required, including the use of a consistent method for aspiration and separation of PRP. This will ensure that the PRP is at optimal concentration and can provide the best possible outcome as far as platelet and GFs are concerned. Therapeutic effects can only be properly evaluated once the PRP protocols and techniques are standardized.

One issue that may arise during PRP preparation is the premature release of granules due to platelet activation. In the current study, PRP obtained was immediately used in a therapeutic protocol as part of ongoing research. VEGF concentration results indicate that platelet degranulation was avoided as evidenced by the lack of concentration differences among the points after each centrifugation and the PRP. Lack of platelet degranulation is reinforced by the observation of similar VEGF concentrations in Protocols I and II in the face of a significant larger platelet yield in Protocol II. It is important to emphasize that platelet activation and degranulation leading to release of α-granule content might be desirable prior to therapeutic application in patients with inflammatory conditions, as is the case with PRP antimicrobial properties (Drago et al. 2013). It is also documented that PRP can be used with or without platelet activation (Amable et al. 2013).

The current research provides evidence of variation in the PRP final product depending on the protocol used for its preparation. The effects of the presence of erythrocytes and leukocytes in the PRP final product is yet to be clarified, and from this perspective, Protocol II in this research provided more blood cell contamination, although this difference was not statistically significant.

CONCLUSIONS

The results of this study indicate that the protocol for PRP preparation which includes resting the sample at room temperature for two hours, followed by two consecutive centrifugations at centrifugal forces of 120g and 240g for 10 minutes each, should be used.

The variation in VEGF was not significant, indicating preservation of α-granules and lack of platelet degranulation during PRP preparation.

REFERENCES

Altman D.G. & Bland J.M. 1983. Measurement in medicine: the analysis of method comparison studies. Statistician 32(3):307-317. <http://dx.doi.org/10.2307/2987937>

Amable P.R., Carias R.B., Teixeira M.V., Cruz Pacheco I., Corrêa do Amaral R.J., Granjeiro J.M. & Borojevic R. 2013. Platelet-rich plasma preparation for regenerative medicine: optimization and quantification of cytokines and growth factors. Stem Cell Res. Ther. 4(3):67. <http://dx.doi.org/10.1186/scrt218> <PMid:23759113>

Andrade M., Rodrigues G., Lima D., Faria L., Silva L., Souza R. & Eurides D. 2016. Utilização de plasma rico em plaquetas de coelhos com poucos leucócitos e hemácias para a consolidação de ossos. Arq. Bras. Med. Vet. Zootec. 68(2):276-282. <http://dx.doi.org/10.1590/1678-4162-8304>

Anitua E., Andia I., Ardanza B., Nurden P. & Nurden A.T. 2004. Autologous platelets as a source of proteins for healing and tissue regeneration. Thromb. Haemost. 91(1):4-15. <http://dx.doi.org/10.1160/TH03-07-0440> <PMid:14691563>

Bonilla-Gutiérrez A.F., Castillo-Franz C., López C., Álvarez M.E., Giraldo C.E. & Carmona J.U. 2018. Equine suspensory ligament and tendon explants cultured with platelet-rich gel supernatants release different anti-inflammatory and anabolic mediators. Biomed. Pharmacother. 108:476-485. <http://dx.doi.org/10.1016/j.biopha.2018.09.065> <PMid:30241051>

Bosch G., Van Schie H.T.M., De Groot M.W., Cadby J.A., Van De Lest C.H.A., Barneveld A. & Van Weeren P.R. 2010. Effects of platelet-rich plasma on the quality of repair of mechanically induced core lesions in equine superficial digital flexor tendons: a placebo-controlled experimental study. J. Orthop. Res. 28(2):211-217. <PMid:19714688>

Bos-Mikich A., De Oliveira R. & Frantz N. 2018. Platelet-rich plasma therapy and reproductive medicine. J. Assist. Reprod. Genet. 35(5):753-756. <http://dx.doi.org/10.1007/s10815-018-1159-8> <PMid:29564738>

Brossi P.M., Moreira J.J., Machado T.S. & Baccarin R.Y. 2015. Platelet-rich plasma in orthopedic therapy: a comparative systematic review of clinical and experimental data in equine and human musculoskeletal lesions. BMC Vet. Res. 11(1):98. <http://dx.doi.org/10.1186/s12917-015-0403-z> <PMid:25896610>

Brown J., Su Y., Nellesen D., Shankar P. & Mayo C. 2016. Management of epidermal growth factor receptor inhibitor-associated rash: a systematic review. J. Commun. Support. Oncol. 14(1):21-28. <http://dx.doi.org/10.12788/jcso.0193> <PMid:26870839>

Carmona Ramírez J.U. & Prades M. 2006. Use of autologous platelet concentrates for the treatment of musculoskeletal injuries in the horse. Doctoral Dissertation, Universitat Autònoma de Barcelona, Bellaterra. 100p.

Chahla J., Cinque M.E., Piuzzi N.S., Mannava S., Geeslin A.G., Murray I.R., Dornan G.J., Muschler G.F. & LaPrade R.F. 2017. A call for standardization in platelet-rich plasma preparation protocols and composition reporting: a systematic review of the clinical orthopaedic literature. J. Bone Joint Surg. 99(20):1769-1779. <http://dx.doi.org/10.2106/JBJS.16.01374> <PMid:29040132>

Comar S.R., Danchura H.S. & Silva P.H. 2009. Contagem de plaquetas: avaliação de metodologias manuais e aplicação na rotina laboratorial. Revta Bras. Hematol Hemoter. 31(6):431-436. <http://dx.doi.org/10.1590/S1516-84842009005000087>

Dehghani S., Nosrati R., Yousefi M., Nezami A., Soltani F., Taghdisi S.M., Abnous K., Alibolandi M. & Ramezani M. 2018. Aptamer-based biosensors and nanosensors for the detection of vascular endothelial growth factor (VEGF): a review. Biosens. Bioelectron. 110:23-37. <http://dx.doi.org/10.1016/j.bios.2018.03.037> <PMid:29579646>

DeRossi R., Coelho A.C.A.D.O., Mello G.S.D., Frazílio F.O., Leal C.R.B., Facco G.G. & Brum K.B. 2009. Effects of platelet-rich plasma gel on skin healing in surgical wound in horses. Acta Cir. Bras. 24(4):276-281. <http://dx.doi.org/10.1590/S0102-86502009000400006> <PMid:19705027>

Drago L., Bortolin M., Vassena C., Taschieri S. & Del Fabbro M. 2013. Antimicrobial activity of pure platelet-rich plasma against microorganisms isolated from oral cavity. BMC Microbiol. 13(1):47. <http://dx.doi.org/10.1186/1471-2180-13-47> <PMid:23442413>

Engels E.A., Jennings L., Kemp T.J., Chaturvedi A.K., Pinto L.A., Pfeiffer R.M., Trotter J.F., Acker M., Onaca N. & Klintmalm G.B. 2015. Circulating TGF-B1 and VEGF and risk of cancer among liver transplant recipients. Cancer Med. 4(8):1252-1257. <http://dx.doi.org/10.1002/cam4.455> <PMid:25919050>

Frater J., Lie D., Bartlett P. & McGrath J.J. 2018. Insulin-like growth factor 1 (IGF-1) as a marker of cognitive decline in normal ageing: a review. Ageing Res. Rev. 42:14-27. <http://dx.doi.org/10.1016/j.arr.2017.12.002> <PMid:29233786>

Frye C.W., Enders A., Brooks M.B., Struble A.M. & Wakshlag J.J. 2016. Assessment of canine autologous platelet-rich plasma produced with a commercial centrifugation and platelet recovery kit. Vet. Comp. Orthop. Traumatol. 29(1):14-19. <http://dx.doi.org/10.3415/VCOT-15-03-0046> <PMid:26603823>

Gatica S., Cabello-Verrugio C. & Simon F. 2018. Transforming growth factor-beta family: advances in vascular function and signaling. Curr. Protein Pept. Sci. 19(12):1164-1171. <http://dx.doi.org/10.2174/1389203719666171128114730> <PMid:29189145>

Giraldo C.E., Álvarez M.E. & Carmona J.U. 2015. Effects of sodium citrate and acid citrate dextrose solutions on cell counts and growth factor release from equine pure-platelet rich plasma and pure-platelet rich gel. BMC Vet. Res. 11(1):60. <http://dx.doi.org/10.1186/s12917-015-0370-4> <PMid:25889052>

Heldin C.H., Lennartsson J. & Westermark B. 2018. Involvement of platelet-derived growth factor ligands and receptors in tumorigenesis. J. Intern. Med. 283(1):16-44. <http://dx.doi.org/10.1111/joim.12690> <PMid:28940884>

Hye Kim J., Gyu Park S., Kim W.K., Song S.U. & Sung J.H. 2015. Functional regulation of adipose-derived stem cells by PDGF-D. Stem Cells 33(2):542-556. <http://dx.doi.org/10.1002/stem.1865> <PMid:25332166>

Jain N.C. 1993. Essentials of Veterinary Hematology. Lea and Febiger, Philadelphia. 417p.

Kharitonenkov A. & DiMarchi R. 2017. Fibroblast growth factor 21 night watch: advances and uncertainties in the field. J. Intern. Med. 281(3):233-246. <http://dx.doi.org/10.1111/joim.12580> <PMid:27878865>

Kwirant L.A.D.A., De La Corte F.D., Cantarelli C., Cargnelutti J.F., Martins M., Cabral M.W., Maciel N. & Rubin M.I.B 2019. Cooling and cryopreservation of equine platelet-rich plasma with dimethyl sulfoxide and trehalose. J. Equine Vet. Sci. 72:112-116. <http://dx.doi.org/10.1016/j.jevs.2018.10.009> <PMid:30929774>

Laudy A.B., Bakker E.W., Rekers M. & Moen M.H. 2015. Efficacy of platelet-rich plasma injections in osteoarthritis of the knee: a systematic review and meta-analysis. Brit. J. Sports Med. 49(10):657-672. <http://dx.doi.org/10.1136/bjsports-2014-094036> <PMid:25416198>

Lee E.B., Kim J.W. & Seo J.P. 2018. Comparison of the methods for platelet rich plasma preparation in horses. J. Anim. Sci. Technol. 60(1):20. <http://dx.doi.org/10.1186/s40781-018-0178-4> <PMid:30147942>

Maciel F.B., DeRossi R., Módolo T.J., Pagliosa R.C., Leal C.R. & Delben A.A. 2012. Scanning electron microscopy and microbiological evaluation of equine burn wound repair after platelet-rich plasma gel treatment. Burns 38(7):1058-1065. <http://dx.doi.org/10.1016/j.burns.2012.02.029> <PMid:22683140>

Marcazzan S., Weinstein R.L. & Del Fabbro M. 2018. Efficacy of platelets in bone healing: a systematic review on animal studies. Platelets 29(4):326-337. <http://dx.doi.org/10.1080/09537104.2017.1327652> <PMid:28643535>

Martelossi Cebinelli G.C., Paiva Trugilo K., Badaró Garcia S. & Brajão de Oliveira K 2016. TGF-β1 functional polymorphisms: a review. Eur. Cytokine Netw. 27(4):81-89. <http://dx.doi.org/10.1684/ecn.2016.0382> <PMid:28396298>

Marx R.E. 2004. Platelet-rich plasma: evidence to support its use. J. Oral Maxillofac. Surg. 62(4):489-496. <http://dx.doi.org/10.1016/j.joms.2003.12.003> <PMid:15085519>

Marx R.E., Carlson E.R., Eichstaedt R.M., Schimmele S.R., Strauss J.E. & Georgeff K.R. 1998. Platelet-rich plasma: growth factor enhancement for bone grafts. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 85(6):638-646. <http://dx.doi.org/10.1016/S1079-2104(98)90029-4> <PMid:9638695>

Mello Costa M.F. 2017. Investigação de substâncias endógenas e expressão gênica de biomarcadores de enfermidades respiratórias em equinos. Relatório de Pesquisa, Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Rio de Janeiro, p.20.

Messora M.R., Nagata M.J.H., Melo L.G.N.D., Furlaneto F.A.C., Deliberador T.M., Garcia V.G. & Bosco A.F. 2009. Análise de um protocolo de única centrifugação para o preparo do plasma rico em plaquetas (PRP): estudo em coelhos. RSBO 6(2):135-141.

Miranda A.L.S., Soto-Blanco B., Lopes P.R., Victor R.M. & Palhares M.S. 2018a. Influence of anticoagulants on platelet and leukocyte concentration from platelet-rich plasma derived from blood of horses and mules. J. Equine Vet. Sci. 63:46-50. <http://dx.doi.org/10.1016/j.jevs.2018.01.003>

Miranda S., Costa M.F.D.M., Senna J.J., Frapoint J.C., De Alencar N.X. & Lessa D.A.B. 2018b. Effects of breed/species and gender on platelet concentration in autologous platelet rich plasma. Acta Vet. Brno 68(4):474-483. <http://dx.doi.org/10.2478/acve-2018-0038>

Ng Y.-S., Krilleke D. & Shima D.T. 2006. VEGF function in vascular pathogenesis. Experim. Cell Res. 312(5):527-537. <http://dx.doi.org/10.1016/j.yexcr.2005.11.008> <PMid:16330026>

Olsen L.H., Kristensen A.T., Qvortrup K. & Pedersen H.D. 2004. Comparison of manual and automated methods for determining platelet counts in dogs with macrothrombocytopenia. J. Vet. Diagn. Invest. 16(2):167-170. <http://dx.doi.org/10.1177/104063870401600215> <PMid:15053372>

Ornitz D.M. & Itoh N. 2015. The fibroblast growth factor signaling pathway. WIREs Develop. Biol. 4(3):215-266. <PMid:25772309>

Pereira R.C.F., Zacarias G.V.F., Cantarelli C., Corrêa M.M.B., Silva G.B., Barbosa A.L.T., Brass K.E. & Côrte F.D.D.L. 2013. Avaliação de sete protocolos para obtenção de plasma rico em plaquetas na espécie equina. Ciência Rural 43(6):1122-1127. <http://dx.doi.org/10.1590/S0103-84782013005000052>

Prado Vendruscolo C., Garcia Alves A.L., Monaco Brossi P. & Arantes Baccarin R.Y. 2014. Uso do soro autólogo condicionado e do plasma rico em plaquetas na terapia ortopédica de equinos. Semina, Ciênc. Agrárias 35(5):2607-2624.

Ross R., Raines E.W. & Bowen-Pope D.F. 1986. The biology of platelet-derived growth factor. Cell 46(2):155-169. <http://dx.doi.org/10.1016/0092-8674(86)90733-6> <PMid:3013421>

Raines E.W. & Ross R. 1982. Platelet-derived growth factor. I. High yield purification and evidence for multiple forms. J. Biol. Chem. 257(9):5154-5160. <PMid:7068680>

Sheth U., Simunovic N., Klein G., Fu F., Einhorn T.A., Schemitsch E., Ayeni O.R. & Bhandari M. 2012. Efficacy of autologous platelet-rich plasma use for orthopaedic indications: a meta-analysis. J. Bone Joint Surg. 94(4):298-307. <http://dx.doi.org/10.2106/JBJS.K.00154> <PMid:22241606>

Simons M., Gordon E. & Claesson-Welsh L. 2016. Mechanisms and regulation of endothelial VEGF receptor signalling. Nat. Rev. Mol. Cell Biol. 17(10):611-625. <http://dx.doi.org/10.1038/nrm.2016.87> <PMid:27461391>

Tambella A.M., Attili A.R., Dupré G., Cantalamessa A., Martin S., Cuteri V., Marcazzan S. & Del Fabbro M. 2018. Platelet-rich plasma to treat experimentally-induced skin wounds in animals: a systematic review and meta-analysis. PloS One 13(1):e0191093. <http://dx.doi.org/10.1371/journal.pone.0191093> <PMid:29324848>

Tasker S., Cripps P. & Mackin A. 2001. Evaluation of methods of platelet counting in the cat. J. Small Anim. Pract. 42(7):326-332. <http://dx.doi.org/10.1111/j.1748-5827.2001.tb02467.x> <PMid:11480897>

Tian J., Cheng L.H.H., Cui X., Lei X.X., Tang J.B. & Cheng B. 2019. Application of standardized platelet-rich plasma in elderly patients with complex wounds. Wound Repair Regen. 27(3):268-276. <http://dx.doi.org/10.1111/wrr.12702> <PMid:30693614>

Veloso W.A., Alencar S.M.F. & Cardozo S.V. 2011. Avaliação dos critérios adotados no interfaceamento dos resultados dos hemogramas automatizados. Revta Acad. Saúde Amb. 6(1):4-10.

Vendramin F.S., Franco D., Nogueira C.M., Pereira M.S. & Franco T.R. 2006. Plasma rico em plaquetas e fatores de crescimento: técnica de preparo e utilização em cirurgia plástica. Revta Col. Bras. Cir. 33(1):24-28. <http://dx.doi.org/10.1590/S0100-69912006000100007>

Vendruscolo C.P., Carvalho A.M., Moraes L.F., Maia L., Queiroz D.L., Watanabe M.J., Yamada A.L.M. & Alves A.L.G. 2012. Avaliação da eficácia de diferentes protocolos de preparo do plasma rico em plaquetas para uso em medicina equina. Pesq. Vet. Bras. 32(2):106-110. <http://dx.doi.org/10.1590/S0100-736X2012000200002>

Vestweber D. & Blanks J.E. 1999. Mechanisms that regulate the function of the selectins and their ligands. Physiol. Rev. 79(1):181-213. <http://dx.doi.org/10.1152/physrev.1999.79.1.181> <PMid:9922371>

Wee P. & Wang Z. 2017. Epidermal growth factor receptor cell proliferation signaling pathways. Cancers 9(5):52. <PMid:28513565>

Xiong G., Lingampalli N., Koltsov J.C., Leung L.L., Bhutani N., Robinson W.H. & Chu C.R. 2018. Men and women differ in the biochemical composition of platelet-rich plasma. Am. J. Sports Med. 46(2):409-419. <http://dx.doi.org/10.1177/0363546517740845> <PMid:29211968>

Zuffova K., Krisova S. & Zert Z. 2013. Platelet rich plasma treatment of superficial digital flexor tendon lesions in racing Thoroughbreds. Vet. Med. 58(4):230-239. <http://dx.doi.org/10.17221/6761-VETMED>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(8):622-629, August 2019
DOI: 10.1590/1678-5150-PVB-6266

Original Article
Small Animal Diseases

Lesions in 224 spleens of splenectomized dogs and evalution of alternative techniques for previous microscopic diagnosis[1]

Rafael S. Figueiredo[2], Caterina Muramoto[3], Thanielle N. Fontes[2], Iris D.S. Meneses[4], Paula G.S. Cardoso[2], Carlos H.C. Vieira Filho[2], Alessandra Estrela-Lima[3] and Tiago C. Peixoto[3*]

ABSTRACT.- Figueiredo R.S., Muramoto C., Fontes T.N., Meneses I.D.S., Cardoso P.G.S., Vieira Filho C.H.C., Estrela-Lima A. & Peixoto T.C. 2019. **Lesions in 224 spleens of splenectomized dogs and evalution of alternative techniques for previous microscopic diagnosis.** *Pesquisa Veterinária Brasileira 39(8):622-629.* Departamento de Anatomia, Patologia e Clínicas Veterinárias, Universidade Federal da Bahia, Av. Adhemar de Barros 500, Ondina, Salvador, BA 40170-110, Brazil. E-mail: tcpeixoto@ufba.br

When detecting a proliferative splenic lesion, veterinarians usually choose splenectomy before a conclusive diagnosis, which can provide a deleterious effect to the dog. The most appropriate would be to perform splenectomy as a therapeutic procedure only in cases with real surgical indication, which can be established after defining microscopic diagnosis and prognosis. The objectives of this study were: to determine the frequency of different types of lesions in spleens of splenectomized dogs in the period of 12 years (2006-2017); determine the representativity of neoplastic lesions (benign and malignant) and non-neoplastic lesions; to evaluate and compare the safety and efficiency of fine needle aspiration biopsy (FNA) and ultrasound-guided Tru-cut needle biopsy for cytological and histopathological diagnosis, respectively, of splenic nodular lesions. In the studied period 224 cases of lesions were found in splenectomized spleens. The frequency of non-neoplastic lesions (50,45%, 113/224) and neoplastic lesions (49,55%, 111/224) was very similar. Among the neoplastic lesions, the malignant ones were more frequent (79,27%, 88/111), and the hemangiosarcoma was the most common (52,25%, 58/111). The possibility of malignant neoplasm was about 74% greater than a benign one. In summary, it was verified that 60.71% (136/224) of the cases corresponded to benign lesions without indication to splenectomy. FNA techniques and Tru-cut biopsy showed a low risk of complications. Regarding the diagnostic efficacy, the FNA obtained 71.43% (15/21) of conclusive diagnoses, 60% (9/15) of which were compatible with the final result of the histopathological evaluation, after splenectomy (gold standard). The Tru-cut biopsy obtained 71.43% (5/7) of conclusive diagnoses and 28.57% (2/7) of inconclusive diagnoses. Among the conclusive one, in 100% of the cases the diagnosis was compatible with the gold standard. Thus, since the possibility of benign splenic lesions in dogs is 1.52 times greater than malignancies, splenectomy should be recommended as a therapeutic procedure only in cases with proven surgical indication, which can be established after definition of microscopic diagnosis and prognosis. The use of FNA and Tru-cut biopsy should be recommended, especially for small and focal splenic lesions, since such techniques are good alternatives for establishing diagnosis previously to

[1] Received on April 22, 2019.
Accepted for publication on April 30, 2019.
Part of Master's Thesis of the first author.

[2] Graduate Studies Program in Animal Science in Tropics (PPGCAT), Escola de Medicina Veterinária e Zootecnia (EMEVZ), Universidade Federal da Bahia (UFBA), Av. Adhemar de Barros 500, Ondina, Salvador, BA 40170-110, Brazil.

[3] Departamento de Anatomia, Patologia e Clínicas Veterinárias, Escola de Medicina Veterinária e Zootecnia (EMEVZ), Universidade Federal da Bahia (UFBA), Av. Adhemar de Barros 500, Ondina, Salvador, BA 40170-110. *Corresponding author: tcpeixoto@ufba.br

[4] Laboratório de Análises Clínicas, Hospital de Medicina Veterinária Prof. Renato Rodenburg de Medeiros Netto, Universidade Federal da Bahia (UFBA), Av. Adhemar de Barros 500, Ondina, Salvador, BA 40170-110.

splenectomy, which may reduce the number of unnecessary splenectomies. The importance of recommending such techniques is emphasized, especially for dogs with focal splenic lesions smaller than three centimeters.

INDEX TERMS: Lesions, dogs, evalution, alternative techniques, diagnosis, splenectomy, splenic biopsy, Tru-cut, neoplasms, cytology.

RESUMO.- [**Lesões em 224 baços de cães esplenectomizados e avaliação de técnicas alternativas para diagnóstico microscópico prévio.**] Rotineiramente, frente à detecção de uma lesão proliferativa esplênica, médicos veterinários costumam optar pela esplenectomia antes de se obter o diagnóstico definitivo da lesão esplênica, o que pode trazer consequências deletérias ao cão. O mais apropriado seria, contudo, a sua adoção, como medida terapêutica, apenas nos casos com comprovada indicação cirúrgica, o que pode ser estabelecido após o diagnóstico microscópico e prognóstico definidos. Os objetivos desse estudo foram: determinar a frequência dos diferentes tipos de lesão em baços de cães esplenectomizados no período de 12 anos (2006-2017); determinar a representatividade das lesões neoplásicas (benignas e malignas) e não neoplásicas; avaliar e comparar a segurança e a eficiência da punção aspirativa por agulha fina (PAAF) e da biópsia por agulha *Tru-cut* guiadas por ultrassom para o diagnóstico citológico e histopatológico, respectivamente, de lesões nodulares esplênicas. No período estudado foram encontrados 224 casos de lesões em baços esplenectomizados. A frequência de lesões não neoplásicas (50,45%, 113/224) e neoplásicas (49,55%, 111/224) esplênicas foi muito semelhante. Entre as neoplásicas, as de caráter maligno foram mais frequentes (79,27%, 88/111) e o hemangiossarcoma o mais comum (52,25%, 58/111). A possibilidade de ocorrência de uma neoplasia maligna foi cerca de 74% maior do que uma benigna. Em suma, verificou-se que em 60,71% (136/224) dos casos estudados, tratava-se de lesões benignas que não teriam indicação de esplenectomia. As técnicas de PAAF e a biópsia por agulha *Tru-cut* demonstraram ter baixo risco de complicações. Quanto à eficácia diagnóstica, pela PAAF obteve-se 71,43% (15/21) de diagnósticos conclusivos, sendo 60% (9/15) desses compatíveis com o resultado final realizado pela avaliação histopatológica, após a esplenectomia (padrão ouro). Já a biópsia por *Tru-cut* obteve 71,43% (5/7) de diagnósticos conclusivos e 28,57% (2/7) de inconclusivos. Dentre os conclusivos, em 100% dos casos o diagnóstico foi compatível com o padrão ouro. Desta maneira, visto a possibilidade de ocorrência de lesões esplênicas benignas em cães serem 1,52 vezes maior do que as malignas deve ser recomentado a adoção de esplenectomia, como medida terapêutica, apenas nos casos com comprovada indicação cirúrgica, o que pode ser estabelecido após o diagnóstico microscópico e prognóstico definidos. O uso da PAAF e da biópsia por *Tru-cut* deve ser recomentado, sobretudo, para lesões esplênicas pequenas e focais, uma vez que tais técnicas representam boas alternativas para o estabelecimento do diagnóstico prévio a esplenectomia, o que pode reduzir o número de esplenectomias desnecessárias. Enfatiza-se a importância da recomendação de tais técnicas, sobretudo para cães com lesões esplênicas focais menores do que três centímetros.

TERMOS DE INDEXAÇÃO: Lesões, caninos, avaliação, diagnóstico, esplenectomia, biópsia esplênica, *Tru-cut*, neoplasias, citologia.

INTRODUCTION

The spleen is the largest secondary lymphoid organ in the body and stands out for its role as a defense against microorganisms and antigenic particles, in hematopoietic lymphopoiesis and erythropoiesis, hemocateresis, red blood cell and platelet reserve, in addition to acting on iron metabolism. By virtue of its anatomical and physiological characteristics, the spleen may be involved in many disorders related to inflammatory disease, hyperplasias, as well as benign, malignant and metastatic neoplasms (Tilson 2003).

Routinely, after identifying focal, multifocal or generalized proliferative splenic changes in dogs, veterinarians opt for surgical treatment (total or partial splenectomy) without a prior definitive diagnosis of the lesion. Nevertheless, surgical excision of the spleen eliminates an important source of blood reserve. It may also cause less tolerance to exercise, inadequate response to hypoxia and shock, besides increasing susceptibility to microbial infections, erythrocyte parasitism, sepsis and cardiovascular complications (Nyland et al. 2005). Thus, ideally, this procedure would be performed only when beneficial for the patient (Eberle et al. 2012).

Many retrospective studies show that non-neoplastic spleen lesions in splenectomized dogs are more frequent than neoplastic lesions (Day et al. 1995, Christensen et al. 2009, Campos 2010, Bandinelli et al. 2011, Eberle et al. 2012, Dionísio 2016). There are more international epidemiological studies on splenic lesions (Hosgood 1987, Day et al. 1995, Christensen et al. 2009, Eberle et al. 2012, Dionísio 2016) than Brazilian ones (Campos 2010, Bandinelli et al. 2011, Rodigheri et al. 2015, Martins 2017).

Abdominal ultrasound imaging is a valuable diagnostics modality in the clinical routine of small animal care; it is non-invasive, fast, and low-cost compared to more complex techniques, and allows for evaluation of the internal architecture and identification of focal or diffuse lesions in abdominal organs, as well as detection of free abdominal fluid (Watson & Bunch 2010). Nevertheless, a definitive diagnosis of proliferative lesions (hyperplastic or neoplastic) requires a cytological or histopathological evaluation (Thrall 2004).

To avoid unnecessary invasive surgical procedures (laparotomy with incisional/excisional biopsy or partial/total splenectomy) or to confirm diagnoses of splenic lesions identified by ultrasound scan, one alternative would be collecting lesion samples for microscopic evaluation by fine-needle aspiration (FNA) or ultrasound-guided Tru-Cut biopsy of the spleen. A study recently evaluated the use of ultrasound-guided FNA to diagnose splenic lesions in 24 dogs. The study obtained 70.83% diagnostic precision, which indicates that FNA followed by cytological analysis is a satisfactory complementary technique associated with ultrasound imaging (Martins 2017). Some medical studies have shown that ultrasound-guided fragment biopsies are safe and allow for conclusive diagnosis of splenic lesions

(Lindgren et al. 1985, Lucey et al. 2002, Lieberman et al. 2003, Tam et al. 2008).

It is worth noting that, in order to prevent complications related to the collection of splenic samples by means of these techniques, particularly abdominal bleeding, the coagulogram must be previously evaluated and sample collection from hypervascular or cystic lesions must be avoided (Lucey et al. 2002).

This study aimed to determine the frequency of the different types of lesion occurring in splenectomized dogs' spleens over a period of 12 years (2006-2017) and the representativeness of neoplastic (benign and malignant) and non-neoplastic lesions, to evaluate and compare the safety and efficiency of fine-needle aspiration (FNA) and ultrasound-guided Tru-Cut needle biopsy for cytological and histopathological diagnosis, respectively, of splenic nodular lesions.

MATERIALS AND METHODS

This study was conducted in accordance with the animal use and experimentation regulation with the approval of the Animal Research Ethics Committee (CEUA) of the "Escola de Medicina Veterinária e Zootecnia" (EMEVZ) of "Universidade Federal da Bahia" (UFBA) under protocol number 38/2017. This study analyzed the biopsy protocols (test orders and histopathological reports) and record books from the Veterinary Pathology Laboratory (LPV) of UFBA's "Hospital de Medicina Veterinária Professor Renato Rodenburg de Medeiros Netto" (HOSPMEV-UFBA) from 2006 to 2017, and from a private veterinary pathology laboratory (LPPV) in the city of Salvador, state of Bahia, referring to the period from 2012 to 2017.

The study included all cases of splenic histopathological evaluation of splenectomized dogs performed by the laboratories, regardless of breed, age or gender. The spleens (fragments or whole) sent to LPV-UFBA belonged mostly to dogs routinely seen at Small Animals Surgical Clinic of HOSPMEV-UFBA and, to a lesser extent, referred by other veterinarians from Salvador and the city's metropolitan region. The samples evaluated by the LPPV came from several veterinary clinics in the city of Salvador and its metropolitan region.

The information on breed, gender, and age described in the biopsy protocols was collected and analyzed, as well as the data on the macroscopic aspect of the splenic lesions and the histopathological diagnosis described in the reports. The histopathological reports evaluated had been written by experienced veterinarian pathologists. The dogs were individually classified according to gender (male and female), breed, and age - puppies (<1 year old), adults (1-8 years old), and elderly (>8 years old). The macroscopic splenic changes were grouped according to their distribution (focal, multifocal, and multifocal to coalescent) and size – nodule (<3.0cm) or mass (≥3.0cm) (Carvalho 2016).

The histopathological diagnoses of the neoplastic lesions were established according to the terminology proposed by Meuten (2002). The diagnoses were grouped into non-neoplastic and neoplastic lesions, and the latter classified as benign or malignant. The non-neoplastic splenic lesions were classified into atrophy, congestion, splenitis, hyperplasia, hematoma, infarction, or rarefaction. All data obtained from the retrospective study were tabulated and systematically aggregated, and the frequency of each lesion was calculated.

In addition to the retrospective study, before the experimental study the veterinarian responsible for the dogs' spleen sample collection received specialized training by an experienced professional. In the pilot experiment, three different semi-automatic disposable Tru-Cut needle gauges (G) - 20, 18, and 16G - were assessed before choosing the one used in this study. All needles were Gallini® 160mm long. At this stage, the size and quality of six spleen samples were assessed from the slides made for histopathological analysis by a pathologist. The tissues obtained from the 16G Tru-Cut needle were considered the best-quality tissues that better represented the lesion; therefore, this gauge was chosen for sample collection from the patients in the experimental study.

Seven dogs with splenic focal lesions (nodules or masses, focal or multifocal, larger than 1.5cm in diameter) identified by abdominal ultrasound scan were selected regardless of breed, gender or age. All of them had been referred for surgical treatment with splenectomy. These dogs were identified from the routine outpatient care of a private veterinary hospital in Feira de Santana, state of Bahia (BA), and the procedure was performed with the guardians' permission and upon their signature of the free and informed consent form. The ultrasound scan evaluated the following characteristics of the splenic lesion: size, shape, echogenicity, echotexture, vascularization and location in the organ (head, body or tail of the spleen, proximity to parietal or visceral surface, distance to the main splenic vein). The following exclusion criteria were used: 1) splenic lesions that were hypervascularized under evaluation on Doppler mode, due to increased risk of hemorrhage; 2) abdominal effusion, as it hinders ultrasound evaluation of hemorrhage following the procedure; and 3) splenic lesions without solid areas or areas predominantly formed by extensive cavitations with a risk of rupture. The selected dogs underwent biochemical tests – alanine aminotransferase (ALT), alkaline phosphatase (AF), urea and creatinine, hematology (blood count, platelet count and coagulogram), prothrombin time (PT), and activated partial thromboplastin time (APTT). The tests were ran by a private laboratory in Feira de Santana-BA before the procedure for sample collection. The dogs presenting laboratory changes hindering anesthesia or increasing the risk of bleeding/hemorrhage were excluded from the study. The coagulogram tests were performed on the same day or one day before the fine-needle aspiration (FNA) and biopsy.

Twelve-hour food fasting and four-hour water fasting were required prior to anesthesia. Left ventrolateral abdominal trichotomy was performed followed by an ultrasound scan to identify the lesion(s). The exam was conducted using an ultrasound system Mindray Z6, Logic-e GE or Logic F8 GE (ranging from 7 to 12MHz and 5 to 12MHz frequency linear transducers). The dogs were anesthetized with IV propofol at 6 to 10mg/kg and later intubated and monitored during the entire procedure, until complete recovery. Following anesthesia, the skin was sterilized with chlorhexidine while the animals lied in supine or right lateral position, depending on the location of the lesion to be accessed. The dogs initially underwent FNA followed by the Tru-Cut biopsy with a 16G needle. In lesions classified as nodules, at least three samples were collected by FNA and one sample by Tru-Cut biopsy. On the other hand, for lesions classified as masses, at least three samples were collected by FNA and two by Tru-Cut biopsy.

The FNA was carried out according to the ultrasound-guided technique described by Menard & Papageorges (1995) with hypodermic needles of length and gauge chosen as per the depth of the target-lesion. In cases where no contents were perceived by expelling, we attempted aspiration by traction of the 3ml syringe plunger as a means of pressing material into the needle by the vacuum. After collection, the material obtained was expelled onto a glass slide with the aid of a 10 ml syringe, followed by Squash smear, air drying, and Romanosvsky staining (rapid panoptic).

For the fragment biopsies, after localization of the lesion with the ultrasound, a small cutaneous incision was made at the entry point of the Tru-Cut needle. The prepared needle was introduced until its tip was positioned at approximately 1cm from the region of interest. It was then triggered and the cutting edge advanced to the nodule/mass and quickly returned to the inside of the needle with the collected material. The needle was then removed and the collected sample was placed in a glass vial with 10% formalin solution for fixation.

Immediately after removing the FNA or biopsy needle from the animal, external pressure was manually applied on the skin with the aid of a chlorhexidine-soaked gauze for approximately 2 minutes with the intention of helping hemostasis. In cases where more than one splenic lesion was found, it was possible to collect samples of more than one nodule, as they could constitute different lesions.

Following the FNA collection and Tru-Cut biopsy, the abdominal ultrasound evaluation was performed in order to subjectively investigate the possibility of hemorrhage by means of identifying free abdominal fluid adjacent to the lesion. After evaluation, each patient was classified into absent, mild, moderate or severe, according to the amount of fluid present. The patients were followed-up for 30 minutes after the procedure; if no fluid was found around the lesion, they were discharged. If fluid was found, the animals were evaluated every 30 minutes for comparison of the quantity of fluid at different times.

All dogs in the experimental group were splenectomized, normally up to 48 hours after collection of ultrasound-guided samples, having the spleen or its nodule fragments referred for gold standard diagnostics histopathological evaluation.

The spleen fragments collected by biopsy and the spleen or fragments referred after splenectomy fixed in 10% formalin were referred to LPV-UFBA for histopathological analysis, where they were cleaved, dehydrated in absolute ethyl alcohol, clarified in xylol, embedded in paraffin and cut in microtomes at a thickness of 5µm. The histological sections obtained were stained with hematoxylin-eosin (HE) and the slides were examined under an optical microscope by two experienced pathologists (1 and 2). The diagnosis followed the same classification proposed in the retrospective study.

The cytological slides were blindly evaluated by three different clinical pathologists (Pathologists A, B, and C). The ultrasound evaluations and all collections of splenic material samples of the animals in the experiment, both for cytology and histopathology, were performed by the same examiner.

RESULTS AND DISCUSSION

Lesions in 224 spleens of splenectomized dogs

During the retrospective study period (2006-2017), histopathological exams were performed in 7,733 samples from several domestic animal species. Among them, 3,994 samples came from the UFBA LPV diagnostics service (from May 2006 to December 2017) and 3,739 from the LPPV (from April 2012 to December 2017). Of the total, 74.47% (5,759/7,733) corresponded to samples from dogs, from which 45.98% (2,648/5,759) and 54.02% (3,111/5,759) belonged to the LPV-UFBA and LPPV caseloads, respectively. Of the 5,759 dog biopsy protocols reviewed, 3.89% (224) cases of lesions in splenectomized spleens were found, from which 28.12% (63/224) came from LPV-UFBA and 71.88% (161/224), from LPPV.

Of the 224 samples of splenic lesions evaluated, 156 (69.65%) came from total splenectomy and 68 (30.35%), from partial. All 224 cases presented conclusive histopathological diagnoses. Table 1 shows the categories of splenic lesions identified with their respective frequencies. Of these 224 cases, 49.55% (111) were diagnosed as neoplastic lesions and 50.45% (113) as non-neoplastic. Of the animals with splenic neoplasia diagnosis, 79.27% (88/111) presented malignant neoplasias and 20.73% (23/111), benign (hemangioma).

Among the non-neoplastic lesions, the hyperplastic changes were the most frequent (59.29%, 67/113), followed by splenic hematoma (18.58%, 21/113), splenic infarction (10.62%, 12/113), splenitis (5.31%, 6/113), splenic atrophy (4.42%, 5/113), and splenic congestion (1.77%, 2/113). With regard to neoplastic lesions, the most frequent was hemangiosarcoma (52.25%, 58/111), followed by hemangioma (20.72%, 23/111), lymphoma (17.11%, 19/111), poorly differentiated sarcoma and fibrosarcoma (2.71%, 3/111 each), and mammary carcinoma, spindle cell sarcoma, histiocytic sarcoma, leiomyosarcoma, and round cell tumors (0.90%, 1/111 each).

With regard to the total of diagnoses found in this study, 20 different diagnoses of splenic lesions were found, divided into two categories (neoplastic and non-neoplastic lesions). Among them, the hyperplastic were the most frequent, corresponding to 29.91% (67/224) of the total of diagnoses. The second most common change among the total of diagnoses was hemangiosarcoma (HSA), which was observed in 25.89% (58/224) of cases. Upon separate analysis of the five subtypes among the 67 hyperplastic lesions found, in the group of non-neoplastic changes (113 cases), nodular lymphoid

Table 1. Frequency of neoplastic and non-neoplastic lesions in 224 spleens of splenectomized dogs (2006-2017)

Histopathological diagnosis	Number of diagnoses	% of category	% of total
Neoplastic (n = 111)			
Hemangiosarcoma	58	52,25%	25,89%
Hemangioma	23	20,73%	10,26%
Lymphoma	19	17,12%	8,48%
Splenic fibrosarcoma	3	2,70%	1,34%
Poorly differentiated sarcoma	3	2,70%	1,34%
Leiomyosarcoma	1	0,90%	0,45%
Metastasis Mammary carcinoma	1	0,90%	0,45%
Round cell sarcoma	1	0,90%	0,45%
Spindle cell sarcoma	1	0,90%	0,45%
Histiocytic sarcoma	1	0,90%	0,45%
Non-neoplastic (n = 113)			
Splenic hyperplasia			
Nodular lymphoid	56	49,55%	25,00%
Nodular age-related (senile)	6	5,31%	2,68%
White pulp	3	2,65%	1,34%
Follicular	1	0,89%	0,45%
Intense reactive	1	0,89%	0,45%
Hematoma	21	18,58%	9,37%
Splenic infarction	12	10,62%	5,35%
Splenitis	6	5,31%	2,68%
Atrophy	5	4,42%	2,22%
Splenic congestion	2	1,77%	0,89%
Total of diagnoses	224		100%

hyperplasia was the most frequent (83.58%, 56/67), followed by age-related (senile) nodular hyperplasia (8.95%, 6/67), white pulp hyperplasia (4.47%, 3/67), follicular hyperplasia, and intense reactive hyperplasia (1.5%, 1/67 each).

Considering the total of cases, the possibility of benign splenic lesions occurring (60.71%, 136/224 corresponding to 23 benign neoplasias and 113 non-neoplastic lesions) was higher than the malignant (39.29%, 88/224), with a ratio of 1.52 between them. This result is corroborated by international studies, in which non-neoplastic lesions in dog spleens were more frequent than neoplastic lesions (Day et al. 1995, Christensen et al. 2009, Dionísio 2016), as was also verified in a study conducted at "Universidade de São Paulo" (Campos 2010). In spite of the small differences in percentage of the neoplastic and non-neoplastic diseases in the abovementioned studies - 4% in 109 cases (Campos 2010), 12% in 87 cases (Day et al. 1995), 14% in 120 cases (Christensen et al. 2009), and 20% in 91 cases (Dionísio 2016) -, these differences were considerably higher than that found in this study (0.9%).

Conversely, in other works, neoplastic lesions were more frequent than non-neoplastic ones, with a difference between them of 4% in 31 cases (Hosgood 1987), 18.6% in 32 cases (Rodigheri et al. 2015), and 38.6% in 179 cases (Bandinelli et al. 2011). In this study, by analyzing neoplasias individually (111), the biologically malignant ones (79.27%, 88/111) were found to be considerably more frequent than the benign (20.73%, 23/111). The ratio between them is 3.83, i.e. the possibility of a malignant neoplasia occurring was 74% higher than that of a benign neoplasia. It is worth noting that the number of cases evaluated in our study (224) was much higher, which may justify the difference found. It is reasonable to consider that the greater the caseload evaluated, the higher the representability of the samples. Therefore, there may in fact be a similar prevalence between neoplastic and non-neoplastic lesions in dogs.

Eberle et al. (2012) verified, in a retrospective study conducted in Hannover with 249 dogs, 132 (53%) cases of neoplastic splenic lesions. Among them, 97 (73.5) consisted in HSA, which corroborates our findings, since HSA was the most frequent neoplasia representing 52.25% (58/111) of neoplasias. Differing malignant and benign splenic lesions before splenectomy is important to allow for more appropriate therapeutic conduct and, consequently, to keep the organ in cases of uncomplicated benign lesions, which would avoid greater-risk situations caused by the absence of the spleen due to lower tolerance to exercise, inadequate response to hypoxia and shock, besides increasing susceptibility to microbial infections, erythrocyte parasitism, sepsis and cardiovascular complications. Upon prior diagnosis, it is suggested that splenectomy be conducted only in cases where surgical excision can be beneficial for the patient (Eberle et al.2012).

Regarding gender, of the 224 biopsy protocols analyzed, only one (0.45%, 1/224) did not indicate the gender. Of the 223 (99.55%) dogs whose gender was informed, the female (52.47%, 117/223) were a little more affected than the male dogs (47.53%, 106/223). In females, there was a higher occurrence of both neoplastic (52.25%, 58/111) and non-neoplastic lesions (52.68%, 59/112) in relation to the males. However, this difference was not significant. Other works also report insignificant differences between the genders of the affected animals (Hammond & Pesillo-Crosby 2008, Christensen et al. 2009, Campos 2010, Bandinelli et al. 2011, Rodigheri et al. 2015, Dionísio 2016).

The age was informed in 94.64% (212/224) of the cases. Elderly animals were most frequently affected and represented more than three quarters of cases (77.83%, 165/212) followed by adults, with representativity below one quarter of cases (22.17%, 47/212). No puppies were affected. The 12 dogs whose ages were not specified corresponded to 5.36% (12/224) of the total of cases. Among the neoplastic lesions, the mean age of the affected dogs was 10.3 years and, for non-neoplastic lesions, 10.1 years. Hyperplastic splenic lesions were the most frequent among the non-neoplastic lesions (59.29%), with a mean age of 11.2 years; the least frequent lesion found in this study was HSA (52.25%), occurring in dogs with an average age of 10 years. This confirms that the probability of splenic lesions in aged dogs is considerably higher than in adults, as the ratio between them is 3.51. Similar findings have been described in the literature (Hosgood 1987, Prymak et al. 1988, Johnson et al. 1989, Day et al. 1995, Hammond & Pesillo-Crosby 2008, Bandinelli et al. 2011, Rodigheri et al. 2015), which reiterates the importance of routinely investigating lesions of this nature in these patients, especially due to their predisposition to develop HSA and splenic hematomas (Aronsohn et al. 2009).

As to the breeds of the dogs affected by splenic lesions, a great variety was found in this study (31 different breeds). Of the 212 dogs with identified breed pattern, 47 (22.17%) were mixed-breed (MBD) and 165 (77.83%) were purebred. Among them, the most frequent were Poodle (13.68%, 29/212), Shih Tzu and Yorkshire (7.55%, 16/212 each), Cocker Spaniel (6.13%, 13/212), and Pit Bull (5.19%, 11/212). The breed was not informed on the biopsy protocol in 12 cases (5.36%, 12/224).

In the present study, mixed-breed dogs (MBD) were the most frequently affected, which may reflect the fact that mixed-breed animals represent the majority of the canine population in Brazil (Fonseca 1999, Hataka 2004) and the patient population seen at HOSPMEV-UFBA itself, which comes mostly from low-income neighborhoods – as per the IBGE (Brazilian Institute of Geography and Statistics) classification – according to a recent georeferencing study conducted by Machado (2017). Bandinelli et al. (2011) suggested higher rates of development of HSA in MBD, which represented 27.9% (50/179) in their study, followed by the breeds Rottweiler 8.9% (16/179) and Cocker Spaniel 8.4% (15/179).

With regard to the size of splenic lesions, of the 113 (100%) non-neoplastic changes, 112 (99.1%) had their size specified in the biopsy protocols and, of these, 67% (75/112) were classified as nodules (smaller than 3.0 cm) and 33% (37/112), as a mass (larger than 3.0 cm) (Table 2). Upon evaluation of the size of the 111 (100%) neoplastic lesions, 75% (83/111) were found to be masses and 25% (28/111), nodules. Among

Table 2. Size of lesions found in the 224 spleens of splenectomized dogs

Nature of lesion	Nodule (<3.0cm)	Mass (>3.0cm)	NI[a]	Total
Non-neoplastic				
Size	75	37	1	113
Neoplastic				
Size	28	83	0	111

[a] NI = not informed.

Table 3. Distribution of lesions found in the 224 spleens of splenectomized dogs

Nature of lesion	Focal	Multifocal	Multifocal to coalescent	NI[a]	Total
Non-neoplastic					
Distribution	73	39	0	1	113
Neoplastic					
Distribution	55	49	7	0	111

[a] NI = not informed.

the 56 cases of nodular lymphoid hyperplasia diagnosed in this study, 83.92% (47/56) were nodules and 16.08% (9/56), masses; among the 58 HSA, 67.24% (39/58) were nodules and 32.76% (19/58), masses.

As to the distribution of non-neoplastic lesions described in 99.11% (112/113) of the cases, the focal lesions were more frequent (65.17%, 73/112) than the multifocal (34.83%, 39/112). With regard to the neoplastic lesions, 49.55% (55/111) were focal, 44.15% (49/111) multifocal and, less frequently, 6.30% (7/111) multifocal to coalescent (Table 3). In the cases of nodular lymphoid hyperplasia and HSA, the focal distribution was more frequent and corresponded to 71.42% (40/56) and 58.62% (34/58) of cases, respectively. As to the size and distribution of non-neoplastic splenic lesions, 67% were smaller than 3.0 cm, where 65.17% were focal. This finding indicates that small focal lesions are more likely to constitute non-neoplastic changes. In the experimental study, it was verified that, despite the low frequency (n=7), 100% of the multifocal to coalescent lesions were neoplastic. In the reviewed literature, no works evaluating these parameters in canine splenic nodules were found.

Evaluation of alternative techniques for prior microscopic diagnosis

In the experimental study, it was verified that both the FNA and the Tru-Cut biopsy with a 16G needle presented low risk of complications; therefore, they may be considered safe. No complications were observed in the dogs submitted to FNA and only 14.28% (1/7) of the Tru-Cut biopsies resulted in a mild fluid collection adjacent to the lesion, identified by means of an ultrasound scan performed after the procedure. In this isolated case, the lesion appeared to be multifocal heterogeneous with cavitations, which may explain the increased risk of bleeding or even rupture of one of the cavity areas. However, the patient did not present clinical changes and, during the ultrasound monitoring period, the amount of fluid gradually decreased and the animal was subsequently discharged. No correlation was found between the coagulogram lab findings and the occurrence of hemorrhagic complications after collection of samples by ultrasound-guided FNA and fragment biopsy using 16G Tru-Cut needles. It is worth highlighting that, in all collections carried out by both techniques, none of the dogs presented changes in the clinical signs indicating significant blood loss, and no surgical intervention was required to control bleeding.

It is noteworthy that the Tru-Cut biopsy has been more widely performed in the medicine and has proven to be an accurate and safe procedure with high diagnostic value for splenic lesions, with over 91% of reportedly conclusive diagnoses and reduced complication rate (Lucey et al. 2002, Lieberman et al. 2003). Cunha (2009) compared the FNA and 14G Tru-Cut biopsy techniques for canine testicles and concluded that both methods presented low risk for the patient, are easily employed, and allow for collection of enough material for diagnosis. However, in humans, similar studies presented more expressive complications, especially in the Tru-Cut biopsies compared to FNA, which required, in some cases, blood transfusions and even splenectomy due to hemorrhage, in up to 10.3% of cases (Lindgren et al. 1985, Lucey et al. 2002).

The splenic ultrasound evaluation identified nodular lesions bigger than 1.5cm or splenic masses in all seven dogs in this study. However, the ultrasound characteristics of the splenic lesions varied and, due to the small size of the sample and the variation of final diagnoses, it was not possible to establish a correlation of the image patterns of the lesions with the histopathological diagnoses.

In the five cases of malignant lesions confirmed by the histopathological exam following splenectomy, a tendency for multiple lesions identified as masses was observed. The ultrasound scan was useful in guiding the needle to the lesion of interest, as they were easily recognizable during the procedure. Most of the time, it was necessary to apply superficial pressure for the Tru-Cut needle to pass through the subcutaneous tissue, musculature and peritoneum, even with prior skin incision. This is attributed to the gauge of the needle and the cutting capacity of the needle brand chosen.

The list of microscopic diagnoses of the splenic nodular lesions obtained by the different techniques pre-splenectomy (FNA and Tru-Cut) and post-splenectomy (histopathology) are found in Table 4 for comparison. The cytological samples were normally hypercellular; however, some slides were fixed with formalin steam, which hindered proper staining and, consequently, microscopic visualization. At times, accentuated blood contamination was also observed, which led to inconclusive diagnosis of the samples.

Of the 21 possible cytological diagnoses for the seven animals evaluated by the three clinical pathologists, 28.57% (6/21) were inconclusive and 71.43% (15/21), conclusive. Of the conclusive diagnoses, 73.33% (11/15) were classified as neoplastic and 26.67% (4/15), non-neoplastic lesions. Of the conclusive diagnosis, 60% (9/15) were compatible with the histopathological splenectomy diagnosis. These cytological results obtained by the FNA showed that this technique, simple and low-cost, may help investigate splenic nodular lesions in veterinary clinical routine, since the rate of conclusive diagnoses was high (71.43%), with 60% accuracy in relation to the post-splenectomy histopathological results (gold standard).

A similar result was obtained in a recent survey, which performed ultrasound-guided FNA in 24 dogs with splenic lesions and produced diagnoses with 62.5% sensitivity, 87.5% specificity, and 70.83% accuracy (Martins 2017). Therefore, FNA may be considered a complementary technique providing satisfactory results when associated with ultrasound scan and histopathology.

Medical studies of this nature have also presented good results. About three samples obtained by FNA per splenic lesion with needles gauge 20, 22, and 23G achieved a definitive diagnosis in 95% of the cases (Lucey et al. 2002). In a retrospective study conducted in the United States, of 156 cases of material

Table 4. Comparison of the microscopic diagnoses of nodular splenic lesions obtained by alternative techniques pre- and post-splenectomy

Dog	Diagnosis prior to splenectomy				Histopathological diagnosis post-splenectomy
	FNA cytology			Biopsy by Tru-Cut	
	Pathologist A	Pathologist B	Pathologist C	Pathologist 1 and 2	Pathologist 1 and 2
1	Inconclusive	Hemangiosarcoma	Inconclusive	Hemangiosarcoma	Hemangiosarcoma
2	Inconclusive	Extramedullary hematopoiesis	Extramedullary hematopoiesis	Inconclusive	Lymphoma
3	Mastocytoma metastasis	Mastocytoma metastasis	Mastocytoma metastasis	Inconclusive	Hemangiosarcoma
4	Lymphoma	Lymphoma	Lymphoma	Lymphoma	Lymphoma
5	Sarcoma	Hemangiosarcoma	Sarcoma	Hemangiosarcoma	Hemangiosarcoma
6	Inconclusive	Inconclusive	Inconclusive	Hematoma	Hematoma
7	Reactive spleen	Lymphoma	Reactive spleen	Nodular hyperplasia	Nodular hyperplasia

collected with imaging guidance, a conclusive diagnosis with cytological exam was obtained in 131 cases (83.9%), most of them with 22G needles and collection of 2.8 samples per lesion, on average, with a variation of 1 to 6 samples. Thus, in only 25 cases (16.1%), the samples collected by FNA were not enough to close the diagnosis and required fragment collection by Tru-Cut needles (Tam et al. 2008).

Some factors may explain the fact that the results obtained by the FNA, in our study, were not more satisfactory, such as faults in smearing, blood contamination of samples and even the number of samples collected per animal. According to Christopher (2003), poor exfoliation of mesenchymal tumor cells during cytological collection hinders conclusive diagnosis. Controlling these factors can significantly reduce these faults and, consequently, increase the chances of success in cytological diagnosis (FNA) of splenic nodular lesions prior to splenectomy. In this study, most of the lesions not diagnosed by the cytological exam were hemangiosarcoma, a highly vascularized neoplasia that is difficult to diagnose by cytology due to low exfoliation of neoplastic cells and high blood contamination.

As to Tru-Cut biopsy, of the seven samples evaluated, 71.43% (5/7) presented conclusive diagnoses, while 28.57% (2/7) were inconclusive (Table 4). Among the conclusive diagnoses, 60% (3/5) of the lesions were neoplastic and 40% (2/5), non-neoplastic. All of the samples collected by Tru-Cut that resented conclusive histopathological diagnoses were compatible with the histopathological diagnoses obtained after splenectomy (gold standard) and, therefore, may be considered a good alternative technique for prior microscopic diagnosis of nodular splenic lesions. Studies conducted with dogs evaluating Tru-Cut biopsy techniques in splenic lesions were not found.

Laufer-Amorim et al. (2002) evaluated, the kidney biopsy technique with 14G Tru-Cut needles in dogs, having concluded that it was possible to determine the morphological diagnosis of diffuse nephropathies. Melchert et al. (2010) stated that the use of 16G Tru-Cut needles in dogs and also for kidney biopsies allowed for diagnosis without major complications or death, which points to a high diagnostic value. In medicine, it was also possible to diagnose primary or recurrent lymphoma by ultrasound-guided splenic biopsy using a 20 or 22G needle in 18 patients (90%) (Lieberman et al. 2003).

In this study, the results of the histopathological exams of the fragments collected by the 16G Tru-Cut needle were considered satisfactory, although in two cases the diagnosis were inconclusive, which was attributed to the limited number of samples (one per lesion), as well as to the choice of place for collection – which is expected, in some cases, in this type of exam. It may be said that a higher amount of samples is required to evaluate the real frequency of errors and successes of the different diagnostic modalities and to propose an agreement rate between them.

Post-splenectomy histopathological evaluation of splenic lesion samples allowed for the comparison of Tru-Cut cytological and histopathological results of the seven dogs. The samples generated by the splenectomies presented conclusive diagnoses in 100% (7/7) of the cases (Table 4), 71.43% (5/7) of which were neoplastic and 28.57% (2/7), non-neoplastic. It is worth noting that the splenic lesion samples obtained post-splenectomy allowed for evaluation of large extents of nodules, which allowed for the establishment of the definitive diagnosis and the conclusion and diagnostic agreement between both pathologists.

Preliminarily, although the three clinical pathologists (A, B, and C) presented the same diagnostic agreement rate with the post-splenectomy histopathological results (3/7, 42.8%), the different results obtained among evaluators shows that the pathologist's experience in cytological evaluation may influence the results of reading the slides. Therefore, it is clear that the alternative techniques for pre-splenectomy microscopic diagnosis of splenic nodular lesions evaluated here present some technical limitations and require particular care. This fact was detected by the occurrence of technical difficulties during collection (individual ability and choice of place), as well as by the reduced number of samples collected from each lesion.

These conclusions reiterate the importance and need for a training both for material collection (FNA and Tru-Cut) and reading and interpretation of cytological slides. Professional qualification in sample collection by means of training in animal corpses with the aim to perfect individual abilities may be an alternative.

CONCLUSIONS

Splenic lesions in dogs represented about 4% of the caseloads of the Veterinary Pathology Laboratories. Among them, the most frequent are hemangiosarcoma, splenic hyperplasia, hemangioma, hematoma, lymphoma, and

infarction. The frequency of neoplastic and non-neoplastic splenic lesions was similar.

The possibility of benign splenic lesions in dogs is 1.52 times higher than malignant; however, splenic neoplasias are four times more likely to be malignant than benign. Splenectomy could have been avoided in 61% of the dogs if conclusive microscopic exams had been performed before surgery to diagnose the nature of the lesion and prevent unnecessary excision of the spleen in animals affected by benign lesions.

Both techniques evaluated, FNA and Tru-Cut needle biopsy, present low risk of complication after sample collection. They were considered safe and a good alternative to microscopic diagnosis of nodular splenic lesions. These techniques are recommended mostly for dogs with focal splenic lesions under three centimeters.

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

REFERENCES

Aronsohn M.G., Dubiel B., Roberts B. & Powers B.E. 2009. Prognosis for acute nontraumatic hemoperitoneum in the dog: a retrospective analysis of 60 cases (2003-2006). J. Am. Anim. Hosp. Assoc. 45(2):72-77. <http://dx.doi.org/10.5326/0450072> <PMid:19258418>

Bandinelli M.B., Pavarini S.P., Oliveira E.C., Gomes D.C., Cruz C.E.F. & Driemeier D. 2011. Estudo retrospectivo de lesões em baços de cães esplenectomizados: 179 casos. Pesq. Vet. Bras. 31(8):697-701. <http://dx.doi.org/10.1590/S0100-736X2011000800011>

Campos A.G. 2010. Esplenomegalias em cães: estudo retrospectivo e análise imunohistoquímica do Fator de Crescimento Endotelial Vascular (VEGF). Master's Thesis, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP. 74p.

Carvalho F.C. 2016. Ultrassonografia intervencionista, p.229-308. In: Carvalho F.C. (Ed.), Ultrassonografia em Pequenos Animais. 2ª ed. Roca, São Paulo.

Christensen N.I., Canfield P.J., Martin P.A., Krockenberger M.B., Spielman D.S. & Bosward K.L. 2009. Cytopathological and histopathological diagnosis of canine splenic disorders. Aust. Vet. J. 87(5):175-181. <http://dx.doi.org/10.1111/j.1751-0813.2009.00421.x> <PMid:19382924>

Christopher M.M. 2003. Cytology of the spleen. Vet. Clin. N. Am., Small Anim. Pract. 33(1):135-152. <http://dx.doi.org/10.1016/S0195-5616(02)00082-7> <PMid:12512380>

Cunha G.N. 2009. Estudo da viabilidade do uso da punção biópsia aspirativa por agulhas fina comparada ao da "tru-cut", em testículos de cães. Doctoral Dissertation, Faculdade de Ciências Agrárias, Universidade Estadual Paulista, Jaboticabal, SP. 45p.

Day M.J., Lucke V.M. & Pearson H. 1995. A review of pathological diagnoses made from 87 canine splenic biopsies. J. Small Anim. Pract. 36(10):426-433. <http://dx.doi.org/10.1111/j.1748-5827.1995.tb02769.x> <PMid:8583757>

Dionísio M.I.M. 2016. Prevalência da doença esplénica em cães e sobrevivência após esplenectomia - estudo retrospetivo. 2016. Doctoral Dissertation, Universidade de Lisboa, Portugal. 94p.

Eberle N., von Babo V., Nolte I., Baumgärtner W. & Betz D. 2012. Splenic masses in dogs. Part 1: epidemiologic, clinical characteristics as well as histopathologic diagnosis in 249 cases (2000-2011). Tierärztliche Praxis Kleintiere 40(4):250-260. <PMid:22911256>

Fonseca C.S. 1999. Avaliação dos níveis séricos do estradiol e progesterona em cadelas portadoras de neoplasias mamárias. Master's Thesis, Faculdade de Ciências Agrárias, Universidade Estadual Paulista, Jaboticabal, SP. 87p.

Hammond T.N. & Pesillo-Crosby S.A. 2008. Prevalence of hemangiosarcoma in anemic dogs with a splenic mass and hemoperitoneum requiring a transfusion: 71 cases (2003-2005). J. Am. Vet. Med. Assoc. 232(4):553-558. <http://dx.doi.org/10.2460/javma.232.4.553> <PMid:18279091>

Hataka A. 2004. Citologia aspirativa com agulha fina e histopatologia: valor e significado para diagnóstico e prognóstico do câncer de mama em cadelas. Doctoral Dissertation, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Botucatu, SP. 98p.

Hosgood G. 1987. Splenectomy in the dog: a retrospective study of 31 cases. J. Am. Anim. Hosp. Assoc. 32(3):275-283.

Johnson K.A., Powers B.E., Withrow S.J., Sheetz M.J., Curtis C.R. & Wrigley R.H. 1989. Splenomegaly in dogs - predictors of neoplasia and survival after splenectomy. J. Vet. Intern. Med. 3(3):160-166. <http://dx.doi.org/10.1111/j.1939-1676.1989.tb03092.x> <PMid:2778749>

Laufer-Amorim R., Bandarra E.P., Monteiro L.A. & Moura V.M.B.D. 2002. Avaliação quantitativa e qualitativa da técnica de biopsia renal "de janela" em cães. Ciênc. Anim. Bras. 3(2):41-45.

Lieberman S., Libson E., Maly B., Lebensart P., Ben-Yehuda D. & Bloom A.I. 2003. Imaging-guided percutaneous splenic biopsy using a 20- or 22-Gauge cutting-edge core biopsy needle for the diagnosis of malignant lymphoma. Am. J. Roentgenol. 181(4):1025-1027. <http://dx.doi.org/10.2214/ajr.181.4.1811025> <PMid:14500223>

Lindgren P.G., Hagberg H., Eriksson B., Glimelius B., Magnusson A. & Sundstrom C. 1985. Excision biopsy of the spleen by ultrasound guidance. Brit. J. Radiol. 58(693):853-857. <http://dx.doi.org/10.1259/0007-1285-58-693-853> <PMid:3916062>

Lucey B.C., Boland G.W., Maher M.M., Hahn P.F., Gervais D.A. & Mueller P.R. 2002. Percutaneous nonvascular splenic intervention: a 10-year review. Am. J. Roentgenol. 179(6):1591-1596. <http://dx.doi.org/10.2214/ajr.179.6.1791591> <PMid:12438060>

Machado G.A.C. 2017. Dermatopatias diagnosticadas em cães no hospital de medicina veterinária da Universidade Federal da Bahia por avaliações histopatológicas (2007-2016) e clínico-laboratoriais (2015-2017), UFBA. Master's Thesis, Universidade Federal da Bahia, Salvador, BA. 117p.

Martins K.P.F. 2017. Caracterização ultrassonográfica e citológica de lesões esplênicas de cães comparadas ao diagnóstico histopatológico. Master's Thesis, Universidade de Cuiabá, Cuiabá, MT. 68p.

Melchert A., Moutinho F.Q., Mamprim M.J. & Santos F.A.M. 2010. Biopsia renal percutânea monitorizada por ultrassonografia em cães. Ciênc. Anim. Bras. 11(2):447-453. <http://dx.doi.org/10.5216/cab.v11i2.4915>

Menard M. & Papageorges M. 1995. Ultrasound corner technique for ultrasound-guided fine needle biopsies. Vet. Radiol. Ultrasound 36(2):137-138. <http://dx.doi.org/10.1111/j.1740-8261.1995.tb00233.x>

Meuten D.J. 2002. Tumors in Domestic Animals. 4th ed. Iowa State Press, Ames, Iowa. 800p. <http://dx.doi.org/10.1002/9780470376928>>.

Nyland T.G., Mattoon J.S., Herrgesell E.J. & Wisner E.R. 2005. Baço, p.131-145. In: Nyland T.G. & Mattoon J.S. (Eds), Ultra-som Diagnóstico em Pequenos Animais. 2ª ed. Roca, São Paulo.

Prymak C., Mckee L.J., Goldschmidt M.H. & Glickman L.T. 1988. Epidemiologic, clinical, pathologic, and prognostic characteristics of splenic hemangiosarcoma and splenic hematoma in dogs: 217 cases. J. Am. Vet. Med. Assoc. 193(6):706-712. <PMid:3192450>

Rodigheri S.M., Campos G.F. & Romani M.S. 2015. Avaliação clínica, hematológica e histopatológica de tumores esplênicos em cães. 42º Congresso Brasileiro de Medicina Veterinária e 1º Congresso Sul-Brasileiro da ANCLIVEPA, Curitiba, PR, p.2305-2309. (Resumo)

Tam A., Krishnamurthy S., Pillsbury E.P., Ensor J.E., Gupta S., Murthy R., Ahrar K., Wallace M.J., Hicks M.E. & Madoff D.C. 2008. Percutaneous image-guided splenic biopsy in the oncology patient: an audit of 156 consecutive cases. J. Vasc. Interv. Radiol. 19(1):80-87. <http://dx.doi.org/10.1016/j.jvir.2007.08.025> <PMid:18192471>

Thrall D.E. 2004. Oncologic imaging: focus on form or function? Proceedings World Small Animal Veterinary Association World Congress. (Resumo)

Tilson D.M. 2003. Spleen, p.1046-1062. In: Slatter D.H. (Ed.), Textbook of Small Animal Surgery. W.B. Saunders, Philadelphia.

Watson P.J. & Bunch S.E. 2010. Biopsia hepática, p.512. In: Nelson R.W. & Couto C.G. (Eds), Medicina Interna de Pequenos Animais. 4ª ed. Elsevier, Rio de Janeiro.

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(8):630-634, August 2019
DOI: 10.1590/1678-5150-PVB-5987

Original Article
Small Animal Diseases

Detection of enteric agents into a cats' shelter with cases of chronic diarrhea in Southern Brazil[1]

Ana Cristina S. Mósena[2], Dafne L. Cruz[3], Cláudio W. Canal[2], Sandra M.T. Marques[4], Stella F. Valle[4]*, João Fábio Soares[4], Mary Jane T. Mattos[4] and Fernanda V.A. Costa[5]

ABSTRACT.- Mósena A.C.S., Cruz D.L., Canal C.W., Marques S.M.T., Valle S.F., Soares J.F., Mattos M.J.T. & Costa F.V.A. 2019. **Detection of enteric agents into a cats' shelter with cases of chronic diarrhea in Southern Brazil.** *Pesquisa Veterinária Brasileira 39(8):630-634*. Serviço de Medicina Felina, Hospital de Clínicas Veterinárias, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil. E-mail: stella.valle@ufrgs.br

This study carried out a survey about enteropathogenic agents in domestic cats' shelter as a stage of investigation for the intermittent chronic diarrhea. Individual fecal samples from 39 cats with free access to the external environment were submitted to parasitological examination, parvovirus, and coronavirus by PCR, and *Cryptosporidium* spp., *Giardia* spp. and *Tritrichomonas foetus* by real-time PCR. From the cats evaluated, 30 (76.9%) were positive for one or more enteric agents, and coinfections were observed in 11 cats samples (28.2%). Helminth eggs were observed in 48.7% of cats (19/30), 16 (41%) were positive for parvovirus or coronavirus and 25.6% (10/30) were infected by protozoa. From the positives for protozoa, five cats were positive to *T. foetus* (12.82%). The first finding of this protozoan through PCR was in the southern Brazil, and the second was in the whole country. Chronic diarrhea in cats may be multifactorial in shelter animals where the population density is high and the control of parasitic, and viral infections are deficient. Moreover, it is due to poor hygiene conditions in these shelters. The factors associated with the proliferation of infectious diseases in shelters are correlated with new pathogens infections such as *T. foetus*.

INDEX TERMS: Enteric agents, cat shelter, chronic diarrhea, Southern Brazil, coinfection, *Tritrichomonas foetus*, intestinal parasitosis, gastrointestinal disorders, parasitoses.

RESUMO.- [Detecção de agentes enteropatogênicos associados à diarreia crônica em um gatil no Sul do Brasil.] Uma pesquisa de agentes enteropatogênicos em gatos domésticos de um abrigo foi realizado como etapa da investigação das causas de diarreias crônicas intermitentes. Amostras fecais individuais de 39 gatos, com livre acesso ao ambiente externo, foram obtidas para pesquisa de helmintos através do exame parasitológico, investigação de parvovírus e coronavírus e de *Cryptosporidium* spp., *Giardia* spp. e *Tritrichomonas foetus* através de PCR em tempo real. Dos gatos avaliados, 30 (76,9%) foram positivos para algum ou mais de um destes agentes entéricos. Desses, 11 (28,2%) apresentaram co-infecções parasitárias. Ovos de helmintos foram observados em 48,7% dos gatos (19/30), 16 felinos (41%) foram positivos para parvovírus ou coronavírus e 25,6% (10/30) estavam infectados por protozoários. Dos positivos para protozoários, cinco apresentaram *Tritrichomonas foetus* (12,82%), um organismo pouco relatado no Brasil, sendo este o primeiro relato de detecção deste protozoário através de PCR em fezes de gatos no Sul do Brasil e o segundo no país. A diarreia crônica em gatos pode ser multifatorial em animais de abrigo onde a densidade populacional é elevada e os meios de controle parasitário e viral são deficitários, além das condições de

[1] Received on February 20, 2019
Accepted for publication on March 8, 2019.

[2] Laboratório de Virologia, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil. E-mails: ana.mosena@ufrgs.br, claudio.canal@ufrgs.br

[3] Laboratório IBASA Ltda., Rua Almirante Tamandaré 530, Porto Alegre, RS 90220-030. E-mail: dafne@ibasa.com.br

[4] Departamento de Patologia Clínica Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000. E-mails: sandra.marques@ufrgs.br, mary.gomes@ufrgs.br, joao.soares@ufrgs.br; *Corresponding author: stella.valle@ufrgs.br

[5] Professor Adjunto IV, Departamento de Medicina Animal, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000. E-mail: fernanda.amorim@ufrgs.br

higiene precárias. Os fatores associados à proliferação de doenças infecciosas em abrigos promovem o surgimento de infecções por novos patógenos como o *Tritrichomonas foetus*, até então pouco relatado no Brasil.

TERMOS DE INDEXAÇÃO: Agentes enteropatogênicos, diarreia crônica, gatil, Sul do Brasil, co-infecção, *Tritrichomonas foetus*, parasitoses gastrointestinais, viroses gastrointestinais, parasitoses.

INTRODUCTION

Diarrhea is a common clinical sign that affects dogs and cats living in shelters and densely populated places. Factors such as overcrowding, stress, inappropriate diets and hygiene conditions associated with precarious vaccination predispose to the emergence of diseases caused by multiple agents and some emerging diseases (Pesavento & Murphy 2014). Infectious diarrhea in cats is a common problem and is associated with viral causes (feline parvovirus, Panleucopenia, feline enteric coronavirus), bacterial causes (*Campylobacter, Clostridium perfringens*), those caused by protozoan (*Giardia duodenalis, Tritrichomonas foetus, Cryptosporidium* spp., and *Cystoisospora* spp.) and those caused by helminths such as *Toxocara cati*, (Pedersen 1991, Cook 2008) *Toxascaris leonina, Ancylostoma* spp., *Strongyloides* spp. Young cats with access to the street are commonly affected, which makes definitive diagnosis difficult (Cook 2008).

There are few studies relating the agents that cause chronic diarrhea in dogs and cats kept in shelters. In a previous study in cats, the enteric coronavirus infection was more common than parvovirus (Sabshin et al. 2012) in cats in shelters and accumulators (Polak et al. 2014). Parvovirus is the viral agent most commonly found in cases of enteritis in young felines and is an important infectious cause of diarrhea and leukopenia in this age group, accounting for up to 25% of cub deaths (Cave et al. 2002). In the case of feline enteric coronavirus infection, this is an asymptomatic infection and may be accompanied by mild and self-limiting enteritis; however aggravations are possible, especially in the presence of other concomitant enteropathogenic agents.

Protozoal infection can also occur with acute or chronic diarrhea, which is already evident in cats from shelters or accumulators (Polak et al. 2014). The most common agents are *Giardia* spp., *Cryptosporidium* spp., and *Tritrichomonas foetus*, already described as causing chronic and debilitating intestinal infection in cats (Gookin et al. 2004). This last agent was observed in cats living in accumulators (Polak et al. 2014), in young animals from catteries and shelters with many animals (Tysnes et al. 2011). Recently, its first molecular detection in Brazil has been found (Hora et al. 2017) in a cat with persistent diarrhea. Infections with *Giardia* spp. have diarrhea, nutrient absorption deficits, steatorreic stools and consequent weight loss (De Santis-Kerr et al. 2006). Infection with *Cryptosporidium* spp. can cause persistent watery diarrhea and weight loss, and maybe severe in immunosuppressed animals (Ballweber et al. 2009). On the other hand, nematode parasitism, such as *Toxocara* spp., *Ancylostoma* spp. and *Trichuris* spp. is often unattractive, but immune-compromised cubs and adults may suffer from underdevelopment and anemia (Cook 2008).

This study aimed to identify enteropathogenic agents in cats from a shelter with a high frequency of diarrhea, through a parasitological examination to detect helminths and PCRs to detect viruses and protozoal, and to reporting the first PCR detection of *Tritrichomonas foetus* in cats in the south of Brazil, as well as the co-infection of T. *foetus* with other enteropathogenic agents.

MATERIALS AND METHODS

Fecal samples from 39 cats were collected from a cat shelter, which had free access to the external environment, located in the Municipality of Viamão, RS. The animals had a history of intermittent diarrhea, no defined breed. Among them, there were 16 males and 24 females, one cat less than one-year-old, and the others were adults.

For this study, fecal samples were collected individually for two weeks. Thus, each cat was separated and kept isolated until it was possible to obtain sample collection. The samples were conditioned in an isothermal box and sent under refrigeration for parasitological examination by Dennis et al. (1954) and Willis (1921) techniques in the Laboratory of Helminthology, and conventional PCR for the detection of parvovirus and coronavirus in the Laboratory of Virology, both at the Faculty of Veterinary Medicine of UFRGS. An aliquot of each sample was also sent under refrigeration for detection of protozoa by real-time PCR (Laboratory LABSAN, Curitiba/PR).

For viral detection, each sample was stored in phosphate-buffered saline (PBS, pH 7.4) at -20ºC for further processing. The DNA was extracted from the supernatant using a commercial silica-based kit (Simbios Biotecnologia, Canoas/RS, Brazil), with a previously described protocol (Boom et al. 1990) and stored at -20ºC for subsequent PCR use for parvovirus detection. Total sample RNA was extracted from the supernatant using TRIzol® LS (Invitrogen, USA) according to the manufacturer's instructions and stored at -80°C for further detection of coronavirus. For coronavirus RT-PCR, reverse transcription was performed using Superscript® III Reverse Transcriptase (Invitrogen, USA) with a final volume of 20µL and incubated for 5 minutes at 65°C with a subsequent cycle of 1 hour at 50°C and 15 minutes at 70°C. In PCR for coronavirus, a 409bp M gene fragment was amplified using primers CCoV1 and CCoV2 (Herrewegh et al. 1998), with an annealing temperature of 55°C. PCR for parvovirus detection amplified a 583bp fragment of the VP2 gene using primers 555for and 555rev with an annealing temperature of 50°C (Buonavoglia et al. 2001). PCRs were performed using Platinum Taq DNA Polymerase (Invitrogen, USA), according to the manufacturer's instructions. The PCR products were subjected to 2% agarose gel electrophoresis and visualized in U.V.

For the detection of protozoa by real-time PCR (qPCR), the extraction of DNA from the samples was performed using the standard commercial laboratory kit (Corbett XTractor-Gene, Qiagen, Valencia/CA, USA). Before the analyses, a housekeeping gene (18 S rRNA) was used to quantify genomic DNA and confirm DNA integrity. All samples were tested by qPCR for a panel of intestinal protozoan cats composed by *Cryptosporidium* spp., *Giardia* spp., and *Tritrichomonas foetus*. For the detection of these agents, the samples were analyzed based on the platform by real-time PCR (qPCR) amplifying a fragment of the 18S rRNA gene (IDEXX Laboratories, Inc., Westbrook/ME, USA) (Gizzi et al. 2014).

Stool samples were tested by qPCR based on the IDEXX RealPCR ™ platform. To perform the technique, commercial master mix (LC480 ProbesMaster, Roche Applied Science, Indianapolis/IN, USA) was used in a qCCR cycler (Roche LightCycler 480). "IDEXX Laboratories" does not provide information on primers and probes used for commercial reasons. The samples were tested by qPCR for a panel of intestinal

protozoa of cats, which comprises detection of *Cryptosporidium* spp., *Giardia* spp., and *Tritrichomonas foetus*.

RESULTS AND DISCUSSION

Thirty (76.9%) of the 39 cat feces samples were positive in one or more enteral tests (Table 1). In the shelter, the guardian of the animals often sought for veterinary care due to the presence of diarrhea in the cats, which motivated the epidemiological investigation. The feline population of this study had highlighted features such as high population density, free access to outside and stress factors, and chronic diarrhea followed by ineffective therapeutic measures. Some of these characteristics are considered risk factors, because of the chronic infectious diarrhea symptoms in the cats (Pesavento & Murphy 2014). The number of animals with diarrhea at the time of collection was not identified, mainly because this symptom had the characteristic of being intermittent in most of the animals.

The number of positive animals in the coproparasitological test (19 cats 48.7%) was similar to previous studies with cats in houses and in catteries in Brazil. In a mixed sample, the study carried out with cats in houses and ranches, 47% of the cats were parasitized (Pivoto et al. 2013). In cats in houses, a proportion of 34% was positive (Gavioli et al. 2011). In street cats, a higher occurrence of parasitized animals (87.9%) was observed (Serra et al. 2003). Periods greater than one year without worming can be considered a risk factor for the presence of parasites (Pivoto et al. 2013, Andersen et al. 2018). The animals of this study were considered dewormed for over a year and this factor may have contributed to the rate of parasitism observed.

In the PCR detection for parvovirus and coronavirus, 16 cats (41%) were positive for one or more viruses. Parvovirus was detected in only three cats (7.7%), a much lower occurrence than in two shelters in the USA, where the authors observed an occurrence of 33.9% and 26% of the population (Clegg et al. 2012). This smaller number possibly occurred due to vaccination of some cats' shelter when they were cubs, reducing environmental contamination and exposure of the animals to the agent. Additionally, a higher prevalence of parvovirus infection can be verified in cats less than 12 months old (Dupont et al. 2013).

Coronavirus RNA was detected in 13 animals (33.33%), similar to an animal shelter in the USA (Pedersen et al. 2004). This occurrence can be attributed to the exception of the virus in high amounts and for long periods in the feces of healthy cats (Herrewegh et al. 1997). Some of these cats remain chronically infected as healthy carriers, excreting the virus for prolonged periods, possibly for a lifetime that can infect other cats via the fecal-oral route (Foley et al. 1997).

Table 1. Enteropathogenic agents according to test type using fecal samples from 39 cats' shelter with chronic diarrhea

Test	Found agents	N	%
Parasitological	*Ancylostoma* spp.	16	41
	Toxocara spp.	3	7,7
	Dypilidium caninum	1	2,5
PCR	Coronavirus	13	33,3
	Parvovirus	3	7,7
PCR real-time	*Tritrichomonas foetus*	5	12,8
	Cryptosporidium spp.	3	7,7
	Giardia spp.	2	5,1

A total of 10 cats (25.6%) were positive in the panel for the detection of protozoa by qPCR. Also, three cats (7.7%) were positive for *Cryptosporidium* spp., a higher number than that previously verified in Rio Grande do Sul by co-analysis (Pivoto et al. 2013) demonstrating the importance of molecular tests for the detection of enteropathogenic agents in animals and in the investigation of cases of intermittent diarrhea.

Giardia spp. infection has a worldwide occurrence with prevalence varying from 1.2% to 14% (De Santis-Kerr et al. 2006). In this study, only two samples were positive for *Giardia* spp. (5%), similar to the values verified in previous studies using coproparasitological examination (Coelho et al. 2009, Pivoto et al. 2013), and inferior to other studies in cats' shelter (Gennari et al. 1999, Ferreira et al. 2013). Cubs appear to be more susceptible to parasitism by *Cryptosporidium* spp. and *Giardia* spp. (Mtambo et al. 1991), probably because of the immaturity of their immune system. The fact that the studied population is composed by adult animals may explain the lower occurrence (14.44%) than in other studies, such as Gennari et al. (1999), who evaluated animals of different ages.

The protozoan *Tritrichomonas foetus* is a recognized cause of abortion in cattle, and in the last decade, it has been an important cause of chronic large intestinal diarrhea in cats, especially young animals (Holliday et al. 2009) in densely populated areas (Tysnes et al. 2011). Studies that investigate *Tritrichomonas* as the etiology of diarrhea in cats in Brazil are scarce. The identification of *Tritrichomonas* as a cause of diarrhea in cats is limited to case reports (Snel et al. 2006, Hora et al. 2017), in which in the first report, the diagnosis was through the culture of the agent. Recently, through qPCR, *T. foetus* was identified in a cat with chronic diarrhea, previously identified in the direct feces examination. In this study, the first test that evaluated a population of cats with intermittent diarrhea observed five (12.8%) samples positive for *T. foetus* (12.8%), besides associating the infection of this organism with other enteropathogenic agents, reporting in a new way the co-infection of this protozoan with viruses and helminths.

Co-infection by two or more enteropathogenic agents may have distinct clinical consequences and interfere with the diagnostic and therapeutic approach. Eleven animals (28.2%) were positive for more than one type of infectious agent (Table 2). Three animals (7.5%) were positive for coronavirus, *T. foetus,* and *Ancylostoma* spp., one of them was also positive for *Dypilidium caninum*. Four animals (10.2%) were positive for a protozoan and a virus, and two samples were positive for both *Cryptosporidium* spp. coronavirus in co-infection. In a sample, the coronavirus was associated with *T. foetus* and in another, co-infection between *Giardia* spp., coronavirus and parvovirus was verified. One animal was positive for both *Ancylostoma* spp. as for *Cryptosporidium* spp. Three animals were positive in coproparasitological examination and viral detection, and two of them were positive for *Ancylostoma* spp. and parvovirus and another to *Ancylostoma* spp. and coronavirus. Multiple infections have already been described (Paris et al. 2014) and should be considered for the diagnosis of chronic diarrhea in cats, mainly due to the clinical consequences and the treatment decision.

Table 2. Enteropathogenic co-infections in 39 cats' shelter with chronic diarrhea

Agent Groups	Agents	N
Helminths + protozoa	*Ancylostoma* spp. + *Cryptosporidium* spp.	1
Virus + protozoa	Coronavirus + *Cryptosporidium* spp.	2
	Coronavirus + *Tritrichomonas foetus*	1
	Coronavirus + parvovirus+ *Giardia* spp.	1
Virus + helminths	Coronavirus + *Ancylostoma* spp.	1
	Parvovirus + *Ancylostoma* spp.	2
Viruses + protozoa + helminths	Coronavirus + *Tritrichomonas foetus* + *Ancylostoma* spp.	2
	Coronavirus + *Tritrichomonas foetus* + *Dypilidium caninum*	1

CONCLUSIONS

The use of medications to make a therapeutic diagnosis for cases of chronic diarrhea can be expensive and the time spent may end up contributing to the worsening of the patient's clinical picture.

The knowledge of the main causal agents of circulating diarrhea in certain geographic regions, which in this study were numerous and causing concomitant infections, improves the selection of diagnostic tests and the use of more targeted therapy.

In addition to commonly known agents, the parasite *Tritrichomonas foetus* should be included in the list of infectious agents that may cause diarrhea (especially chronic) in felines, especially in animals housed in denser shelters than recommended.

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

REFERÊNCIAS

Andersen A.A., Levy J.K., McManus C.M., McGorray S.P., Leutenegger C.M., Piccione J., Blackwelder L.K. & Tucker S.J. 2018. Prevalence of enteropathogens in cats with and without diarrhea in four different management models for unowned cats in the southeast United States. Vet. J. 236:49-55. <http://dx.doi.org/10.1016/j.tvjl.2018.04.008> <PMid:29871750>

Ballweber L.R., Panuska C., Huston C.L., Vasilopulos R., Pharr G.T. & Mackin A. 2009. Prevalence of and risk factors associated with shedding of *Cryptosporidium felis* in domestic cats of Mississippi and Alabama. Vet. Parasitol. 160(3/4):306-310. <http://dx.doi.org/10.1016/j.vetpar.2008.11.018> <PMid:19117680>

Boom R., Sol C.J., Salimans M.M., Jansen C.L., Wertheim-van Dillen P.M. & van der Noordaa J. 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28(3):495-503. <PMid:1691208>

Buonavoglia C., Martella V., Pratelli A., Tempesta M., Cavalli A., Buonavoglia D., Bozzo G., Elia G., Decaro N. & Carmichael L. 2001. Evidence for evolution of canine parvovirus type 2 in Italy. J. Gen. Virol. 82(12):3021-3025. <http://dx.doi.org/10.1099/0022-1317-82-12-3021> <PMid:11714979>

Cave T.A., Thompson H., Reid S.W., Hodgson D.R. & Addie D.D. 2002. Kitten mortality in the United Kingdom: a retrospective analysis of 274 histopathological examinations (1986 to 2000). Vet. Rec. 151(17):497-501. <http://dx.doi.org/10.1136/vr.151.17.497> <PMid:12430997>

Clegg S.R., Coyne K.P., Dawson S., Spibey N., Gaskell R.M. & Radford A.D. 2012. Canine parvovirus in asymptomatic feline carriers. Vet. Microbiol. 157(1/2):78-85. <http://dx.doi.org/10.1016/j.vetmic.2011.12.024> <PMid:22257775>

Coelho W.M.D., Amarante A.F.T., Soutello R.V.G., Meireles M.V. & Bresciane K.D.S. 2009. Ocorrência de parasitos gastrintestinais em amostras fecais de felinos no município de Andradina, São Paulo. Revta Bras. Parasitol. Vet. 18(2):46-49. <http://dx.doi.org/10.4322/rbpv.01802010>

Cook A. 2008. Feline infectious diarrhea. Top. Companion Anim. Med. 23(4):169-176. <http://dx.doi.org/10.1053/j.tcam.2008.07.001> <PMid:19081550>

De Santis-Kerr A.C., Raghavan M., Glickman N.W., Caldanaro R.J., Moore G.E., Lewis H.B., Schantz P.M. & Glickman L.T. 2006. Prevalence and risk factors for *Giardia* and *Coccidia* species of pet cats in 2003-2004. J. Feline Med. Surg. 8(5):292-301. <http://dx.doi.org/10.1016/j.jfms.2006.02.005> <PMid:16678461>

Dennis W.R., Stone W.M. & Swanson L.E. 1954. A new laboratory and field diagnostic test for fluke ova, in feces. J. Am. Vet. Med. Assoc. 124(922):47-50. <PMid:13117747>

Dupont S., Butaye P., Claerebout E., Theuns S., Duchateau L., Van de Maele I. & Daminet S. 2013. Enteropathogens in pups from pet shops and breeding facilities. J. Small Anim. Pract. 54(9):475-480. <http://dx.doi.org/10.1111/jsap.12119> <PMid:23915246>

Ferreira F.P., Dias R.C.F., Martins T.A., Constantino C., Pasquali A.K.S., Vidotto O., Freire R.L. & Navarro I.T. 2013. Frequência de parasitas gastrointestinais em cães e gatos do município de Londrina, PR, com enfoque em saúde pública. Semina, Ciênc. Agrárias 34(6):3851-3858.

Foley J.E., Poland A., Carlson J. & Pedersen N.C. 1997. Patterns of feline coronavirus infection and fecal shedding from cats in multiple-cat environments. J. Am. Vet. Med. Assoc. 210(9):1307-1312. <PMid:9143535>

Gavioli F.A., Borsa A., Diogo J.E., De Lara Pinto A.Z., Azevedo L.S. & Sousa V.R.F. 2011. Ocorrência de endoparasitos em gatos de Cuiabá, Mato Grosso, Brasil. Arch. Vet. Sci. 16(3):25-30. <http://dx.doi.org/10.5380/avs.v16i3.19498>

Gennari S.M., Kasai N., Pena H.F.J. & Cortez A. 1999. Ocorrência de protozoários e helmintos em amostras de fezes de cães e gatos da cidade de São Paulo. Braz. J. Vet. Res. Anim. Sci. 36(2):87-91. <http://dx.doi.org/10.1590/S1413-95961999000200006>

Gizzi A.B., Oliveira S.T., Leutenegger C.M., Estrada M., Kozemjakin D.A., Stedile R., Marcondes M. & Biondo A.W. 2014. Presence of infectious agents and co-infections in diarrheic dogs determined with a real-time polymerase chain reaction-based panel. BMC Vet. Res. 16(1):10-23. <http://dx.doi.org/10.1186/1746-6148-10-23> <PMid:24433321>

Gookin J.L., Stebbins M.E., Hunt E., Burlone K., Fulton M., Hochel R., Talaat M., Poore M. & Levy M.G. 2004. Prevalence of and risk factors for feline *Tritrichomonas foetus* and *Giardia* infection. J. Clin Microbiol. 42(6):2707-2710. <http://dx.doi.org/10.1128/JCM.42.6.2707-2710.2004> <PMid:15184456>

Herrewegh A.A.P.M., Mähler M., Hedrich H.J., Haagmans B.L., Egberink H.F., Horzinek M.C., Rottier P.J.M. & De Groot R.J. 1997. Persistence and evolution of feline coronavirus in a closed cat-breeding colony. Virology 234(2):349-363. <http://dx.doi.org/10.1006/viro.1997.8663> <PMid:9268167>

Herrewegh A.A., Smeenk I., Horzinek M.C., Rottier P.J. & De Groot R.J. 1998. Feline coronavirus type II strains 79-1683 and 79-1146 originate from a double recombination between feline coronavirus type I and canine coronavirus. J. Virol. 72(5):4508-4514. <PMid:9557750>

Holliday M., Deni D. & Gunn-Moore D.A. 2009. *Tritrichomonas foetus* infection in cats with diarrhoea in a rescue colony in Italy. J. Feline Med. Surg. 11(2):131-134. <http://dx.doi.org/10.1016/j.jfms.2008.06.004> <PMid:18774326>

Hora A.S., Miyashiro S.I., Cassiano F.C., Brandão P.E., Reche-Junior A. & Pena H.F.J. 2017. Report of the first clinical case of intestinal trichomoniasis caused by *Tritrichomonas foetus* in a cat with chronic diarrhea in Brazil. BMC Vet. Res. 13(1):109. <http://dx.doi.org/10.1186/s12917-017-1026-3> <PMid:28412947>

Mtambo M.M., Nash A.S., Blewett D.A., Smith H.V. & Wright S. 1991. Cryptosporidium infection in cats: prevalence of infection in domestic and feral cats in the Glasgow area. Vet. Rec. 129(23):502-504. <PMid:1664551>

Paris J.K., Wills S., Balzer H.J., Shaw D.J. & Gunn-Moore D.A. 2014. Enteropathogen co-infection in UK cats with diarrhoea. BMC Vet. Res. 10(1):13. <http://dx.doi.org/10.1186/1746-6148-10-13> <PMid:24410914>

Pedersen N.C. 1991. Feline Husbandry: diseases and management in the multiple-cat environment. American Veterinary Publications, Goleta, CA. 453p.

Pedersen N.C., Sato R., Foley J.E. & Poland A.M. 2004. Common virus infections in cats, before and after being placed in shelters, with emphasis on feline enteric coronavirus. J. Feline Med. Surg. 6(2):83-88. <http://dx.doi.org/10.1016/j.jfms.2003.08.008> <PMid:15123152>

Pesavento P.A. & Murphy B.G. 2014. Common and emerging infectious diseases in the animal shelter. Vet. Pathol. 51(2):478-491. <http://dx.doi.org/10.1177/0300985813511129> <PMid:24265288>

Pivoto F.L., Lopes L.F.D., Vogel F.S.F., Botton S.D.A. & Sangioni L.A. 2013. Ocorrência de parasitos gastrointestinais e fatores de rico de parasitismo em gatos domésticos urbanos de Santa Maria, RS, Brasil. Ciência Rural 43(8):1453-1458. <http://dx.doi.org/10.1590/S0103-84782013000800018>

Polak K.C., Levy J.K., Crawford P.C., Leutenegger C.M. & Moriello K.A. 2014. Infectious diseases in large-scale cat hoarding investigations. Vet. J. 201(2):189-195. <http://dx.doi.org/10.1016/j.tvjl.2014.05.020> <PMid:24934262>

Sabshin S.J., Levy J.K., Tupler T., Tucker S.J., Greiner E.C. & Leutenegger C.M. 2012. Enteropathogens identified in cats entering a Florida animal shelter with normal feces or diarrhea. J. Am. Vet. Med. Assoc. 241(3):331-337. <http://dx.doi.org/10.2460/javma.241.3.331> <PMid:22812469>

Serra C.M.B., Uchôa C.M.A. & Coimbra R.A. 2003. Exame parasitológico de fezes de gatos (*Felis catus domesticus*) domiciliados e errantes da Região Metropolitana do Rio de Janeiro, Brasil. Revta Soc. Bras. Med. Trop. 36(3):331-334. <http://dx.doi.org/10.1590/S0037-86822003000300003>

Snel G.G.M., Bercht B.S. & Gomes M.J.P. 2006. Tricomonose em gatos no Rio Grande do Sul. Anais do XVII Congresso Estadual de Medicina Veterinária, Gramado, RS, p.25. (Resumo expandido)

Tysnes K., Gjerde B., Nødtvedt A. & Skancke E. 2011. A cross-sectional study of *Tritrichomonas foetus* infection among healthy cats at shows in Norway. Acta Vet. Scand. 53(1):39-45. <http://dx.doi.org/10.1186/1751-0147-53-39> <PMid:21689400>

Willis H.H. 1921. A simple levitation method for the detection of hookworm ova. Med. J. Aust. 8:375-376.

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(8):635-642, August 2019
DOI: 10.1590/1678-5150-PVB-6264

Original Article
Small Animal Diseases

Prognostic value of coupling interval, prematurity index and heart rate variability in Boxer dogs[1]

Elizabeth Regina Carvalho[2]*, Evandro Zacché[2], Michelli Fenerich[2], Aparecido Antônio Camacho[2] and Marlos G. Sousa[3]

ABSTRACT.- Carvalho E.R., Zacché E., Fenerich M., Camacho A.A. & Sousa M.G. 2019. **Prognostic value of coupling interval, prematurity index and heart rate variability in Boxer dogs.** *Pesquisa Veterinária Brasileira 39(8):635-642.* Faculdade de Medicina Veterinária e Ciências Agrárias, Universidade Estadual Paulista, Via de Acesso Prof. Paulo Donato Castellane s/n, Jaboticabal, SP 14884-900, Brazil. E-mail: beth_rcarvalho@hotmail.com

Boxer dogs with arrhythmogenic right ventricular cardiomyopathy (ARVC) can experience sudden cardiac death regardless of presence/absence of clinical signs. The aims of this retrospective study were two-fold: 1) to investigate the coupling interval (CI) and prematurity index (PI) of ventricular arrhythmias (VA), and the heart rate variability (HRV) in Boxers, and 2) to evaluate their impact on overall survival time. The first 24-hour Holter 36 client-owned Boxer dogs meeting inclusion/exclusion criteria were evaluated for the number, morphology, site of origin, complexity, CI and PI, of ventricular premature complexes (VPCs), and time domain HRV. The effect on survival was assessed, considering the presence/absence of ventricular tachycardia (VT), and syncope. All-cause mortality was considered as the end-point, with median survival times being obtained by Kaplan-Meier analyses and compared by log-rank test. Polymorphic VPCs were more common in symptomatic dogs than asymptomatic. VPCs in dogs with VT were less premature, due to the influence of heart rate on PI despite comparable CI. The PI and mean heart rate (HRme) were significantly different between VT and non-VT dogs but did not discriminate adequately between groups as standalone tests. Median survival time was shorter in Boxer dogs with VT (463 vs 1645 days, HR: 4.31, P=0.03). The HRV parameters, SDNN and SDANN, were both associated with prognosis. The CI and PI were not demonstrated to be prognostic surrogates in Boxer dogs with VA. HRme≥112bpm is 100% sensitive but only 46% specific for detecting VT in Boxers on the 24-hour Holter. Presence of VT, SDNN≤245ms, or SDANN≤134ms at the time of the first 24-hour Holter was associated with a shorter survival.

INDEX TERMS: Prognostic, coupling interval, prematurity index, heart rate, Boxer dogs, arrhythmias, sympathetic nervous system, sudden death, dogs.

RESUMO.- [Valor prognóstico do intervalo de acoplamento, índice de prematuridade e variabilidade da frequência cardíaca em cães da raça Boxer.] Cães da raça Boxer com cardiomiopatia arritmogênica do ventrículo direito (CAVD) podem apresentar morte súbita independentemente da presença/ausência de sinais clínicos. Os objetivos desse estudo retrospectivo foram: 1) investigar o intervalo de acoplamento (IA) e o índice de prematuridade (IP) das arritmias ventriculares e a variabilidade da frequência cardíaca (VFC) em Boxers, e 2) avaliar o impacto de tais características sob o tempo de sobrevida global. O primeiro Holter de 24 horas de 36 Boxers selecionados para os critérios de inclusão/exclusão foram avaliados para o número, morfologia, local de origem, complexidade, IA e IP dos complexos ventriculares prematuros (CVPs) e da VFC no domínio do tempo. O efeito na sobrevida foi avaliado, considerado a presença/ausência de taquicardia ventricular (TV), e síncope. O desfecho final foi a mortalidade global, com o tempo de sobrevida mediano sendo obtido pela análise de Kaplan-Meier e comparado pelo teste de log-rank. CVPs polimórficos foram mais comuns em cães sintomáticos. Os CVPs em Boxers com TV foram menos prematuros, devido

[1] Received on January 22, 2019.
Accepted for publication on April 1, 2019.

[2] Faculdade de Medicina Veterinária e Ciências Agrárias, Universidade Estadual Paulista (Unesp), Via de Acesso Prof. Paulo Donato Castellane s/n, Jaboticabal, SP 14884-900, Brazil. *Corresponding author: beth_rcarvalho@hotmail.com

[3] Laboratório de Cardiologia Comparativa, Universidade Federal do Paraná (UFPR), Rua dos Funcionários 1540, Curitiba, PR 80035-050, Brazil.

à influência da frequência cardíaca (FC) sobre o IP, apesar de IA comparáveis. O IP e a FC média diferiram entre os cães com TV e os sem, mas não discriminam adequadamente os grupos como variáveis isoladas. A sobrevida global foi menor nos cães com TV (463 dias *vs* 1645 dias, HR=4,31, P=0,03). Os parâmetros da VFC, SDNN e SDANN, foram associados ao prognóstico. O IA e o IP não possuem valor prognóstico em Boxers com arritmias ventriculares. A FC média ≥112bpm é 100% sensível, mas apenas 46% específica para detectar Boxers com TV no Holter de 24 horas. A presença de TV, SDNN≤245ms, ou SDANN≤134ms no momento do primeiro Holter de 24 horas estão associados a menor sobrevida global no Boxer.

TERMOS DE INDEXAÇÃO: Prognóstico, intervalo de acoplamento, índice de prematuridade, frequência cardíaca, raça Boxer, arritmias, sistema nervoso simpático, morte súbita, cães.

INTRODUCTION

Arrhythmogenic right ventricular cardiomyopathy (ARVC) was first described in Boxer dogs in the early 1980's (Harpster 1983) as a myocardial disease in which cardiomyocytes are replaced by fibrofatty tissue, making affected dogs prone to developing ventricular arrhythmias (VA). Boxer ARVC is a familial disease apparently inherited as an autosomal dominant trait (Meurs et al. 1999). Three clinical presentations have been proposed: 1) asymptomatic dogs with occasional ventricular premature complexes (VPC), 2) Boxer dogs with tachyarrhythmias and syncope or exercise intolerance, and 3) least commonly diagnosed, animals with myocardial systolic dysfunction and ventricular dilation, sometimes with evidence of congestive heart failure - CHF (Harpster 1991a).

There is no single specific test to diagnose Boxer ARVC (Meurs 2017), and 24-hour Holter monitoring plays a fundamental role as part of screening, diagnosis, and monitoring of treatment in this condition. The severity of VA is usually described by the number, pattern, morphology and occurrence. Although many affected animals live a normal life, Boxer dogs with ARVC are at risk of sudden cardiac death (SCD), thus, the study of additional electrocardiographic characteristics may help stratify risk. The prognosis of Boxers with ARVC without CHF is widely variable and predicting outcome is usually challenging (Meurs 2017).

The coupling interval (CI) and the prematurity index (PI) of VA have been extensively studied in human beings, although the results are still controversial (Igarashi et al. 2012, Kim et al. 2014, Lee et al. 2014). In dogs with degenerative mitral valve disease, our group recently demonstrated that PI differs between symptomatic and pre-clinical stages, but the prognostic value of such indices has not been evaluated (Carvalho et al. 2018). However, heart rate variability (HRV), a non-invasive way to assess the interaction between the sympathetic and parasympathetic autonomic nervous system, is a well-established method to identify people at risk of developing CHF or SCD (Fauchier et al. 1997, 1999, Bikkina et al. 1998, Stein & Kleiger 1999). Curiously, in Boxer dogs the prognostic value of HRV has not yet been completely characterized.

Therefore, the aims of this study were twofold: 1) to investigate the characteristics of VA, including the CI and PI, and the HRV variables in Boxer dogs with respect to clinical status and the presence/absence of ventricular tachycardia - previously established as an independent predictor of mortality in Boxer ARVC (Mõtskula et al. 2013); and 2) to evaluate the impact of such characteristics on the overall survival time.

MATERIALS AND METHODS

Study design and Ethics Statement. This was a retrospective study, approved by the institutional Animal Care and Use Committee (protocol 012277/17).

Animals. Boxer dogs investigated at the Cardiology section of a referral teaching practice were identified by searching the medical record database. The database was searched to identify all boxers which met the following inclusion criteria: ≥4 years old, examined from 2012 to 2017, with an available conventional electrocardiography (ECG) (ECGPCVet, Tecnologia Eletrônica Brasileira, São Paulo, Brazil) a three-channel 24-hour Holter recording (CardioLight, Cardios Sistemas, São Paulo, Brazil) and echocardiography (My Lab 30, Esaote, Italy), performed by the attending cardiologist within seven days of one another. For animals in which more than one Holter recording was available, only the first examination was used for analysis. Dogs found to have any heart disease other than ARVC diagnosed by echocardiography, CHF, a systemic disease process that could explain the observed arrhythmias (*e.g.*, ehrlichiosis (Ferreira et al. 2017), neoplasia (Marino et al. 1994), as well as those with syncope due to neurocardiogenic bradycardia, and those receiving antiarrhythmics were excluded. Baseline clinical details obtained from medical records included age, gender, body weight, and history of syncope at the time of the first 24-hour Holter Recording.

Electrocardiographic evaluation. One investigator (E.R.C), who was blinded to the clinical status of dogs, manually reviewed Holter records checking the QRS templates, including possible misinterpretation for horizontal and vertical resolution, and checked the digital 24-hour editing program (CardioManenger S540, Cardios Sistemas, São Paulo, Brazil). Measurements, as well as analyses, were made on channel 1 of the Holter recording (positive pole toward the apex of the left ventricle).

Ventricular arrhythmias over a 24 hour period were characterized according to: 1) presence or absence of ventricular premature complexes (VPC); 2) number of VPCs; 3) site of origin of VPCs: right ventricle (left bundle branch block morphology - LBBB), left ventricle (right bundle branch block morphology - RBBB), or both; 4) VPC morphology: monomorphic (unifocal – same QRS morphology and polarity throughout the exam) or polymorphic (multifocal – QRS morphology changing with regard to amplitude and/or polarity); and 5) VPC complexity: isolated VPC, repeating patterns (*e.g.,* couplets, triplets, bigeminy, trigeminy) or ventricular tachycardia (VT). VT was defined as a sequence of more than three consecutive large QRS complexes, with heart rate (HR) >180 beats/min and atrioventricular dissociation. Holter recordings are not appropriate for accurate identification of true supraventricular arrhythmias, for this reason, detection of this arrhythmia was not included in analyses.

Boxers were further classified according to the degree of ventricular ectopy, based on the following criteria: 0 to 20 VPCs per 24 hours were interpreted as normal; 21 to 300 VPCs per 24 hours were interpreted as indeterminate; and animals with more than 300 VPCs/24h were considered likely to be affected by ARVC (Meurs 2017).

In addition, the CI of VPC, defined as the R-R interval between the VPC and the preceding sinus complex, and the sinus cycle length (SCL), defined as the R-R interval of the sinus cycle just before the VPC, were determined as described elsewhere (Igarashi et al. 2012, Lee et al. 2014, Carvalho et al. 2018). The PI of VPCs was calculated

as the ratio of CI to SCL. Five VPCs per dog were manually chosen at random, all measurements were made five times, the highest and lowest values were excluded and a mean of three measurements was used for statistical analysis. When the randomization included a run of VT, the first VPC of the run and the SCL from the sinus beats before the episode were used. Boxers with fewer than 5 VPCs recorded had all ventricular premature beats measured, and a mean was obtained.

The 24-h HRV was assessed at the time domain, and included the following parameters: 1) the standard deviation of the R-R intervals (SDNN); 2) the standard deviation of the mean R-R intervals obtained at 5 minute intervals (SDANN); 3) the mean of the standard deviation of the R-R intervals obtained at 5 minute intervals (SDNNidx); 4) the square root of the mean squared differences of successive R-R intervals (rMSSD); 5) percentage of adjacent R-R intervals differing in duration by more than 50 milliseconds (pNN>50); 6) minimum HR over 24-hour recording (HRmin); 7) mean HR (HRme); and 8) maximum HR (HRmax).

Survival analyses. Was performed using an end-point of all-causes of death. Follow-up information was documented from medical records or a telephone call with owners, in up to six attempts over a number of days. If survival data were still incomplete, referring veterinarians were contacted. Outcome data included patient status (alive or dead), date of death or euthanasia, and the probable cause of death. Additionally, owners were asked whether there was a family history of ARVC. SCD was defined as an unexpected death without apparent clinical signs during the preceding 24 hours (Meurs et al. 2011). For dogs that were still alive at the end of the study, or those lost to follow-up, the date that were last seen alive was recorded and then they were censored of the analysis.

Statistical analyses. The Shapiro-Wilk test was used to investigate the normal distribution of data. Syncopal and non-syncopal animals and dogs that presented with VT and those without were compared using either Mann–Whitney test or Student's t-test according to distribution. Parametric data are represented as mean ± standard deviation, while non-parametric variables are shown as median (interquartile range). The contingency was evaluated using either Fisher's exact test or the Chi-square when three or more categories were compared. Receiver operating characteristic (ROC) curves were constructed to investigate sensitivity and specificity of CI, PI and HRV parameters to distinguish dogs with VT from those without, as VT has previously been established as an independent predictor of mortality in Boxer ARVC (Mõtskula et al. 2013). Kaplan-Meier survival curves were constructed and compared using the log-rank test to identify factors that significantly influenced mortality, and to estimate median survival time. Statistical processing was performed using commercially available statistical software (Prism Windows 6.0, GraphPad Software, California, USA), and significance was defined as $P<0.05$.

RESULTS

A total of 36 dogs met the inclusion/exclusion requirements and were included in this study. The sample comprised 17 females and 19 males, with a mean of 8 (6-10.8 y) years old, weighing 27.9kg (24.3-32kg). Fourteen animals (38.9%) had ≤ than 20 VPCs/24-h and were considered normal, seven (19.4%) had between 21 and 300 VPCs/24-h being considered as indeterminate to ARVC, and fifteen (41.7%) had more than 300 VPCs/24-h and were considered likely to be affected by ARVC. Ten dogs (27.8%) had only VPCs with first deflection positive, suggesting the right ventricle as site of origin, five (13.9%) animals had only VPCs with the first deflection negative, suggesting the left ventricle as site of origin, and twenty-one (58.3%) dogs showed both.

Seven dogs (18.9%) experienced syncope at the time of first 24-h Holter recording, and no animal had clinical signs of CHF (ascites, pulmonary edema, and pleural effusion). Characteristics evaluated in syncopal and non-syncopal Boxer dogs are summarized in Table 1. Age and weight did not differ between groups; however, male dogs were overrepresented in the syncopal group (P=0.001). In addition, symptomatic patients were found to have more polymorphic VPCs (P=0.0002), as well as a higher SDNN (P=0.02) and SDNNidx (P=0.009).

After the first 24-h Holter recording, Boxer dogs that had syncope, and/or VPCs in couplets, triplets, bigeminy or VT, regardless of the number of VPCs, were given antiarrhythmic therapy, a total of 18 animals (48.6%). The most commonly prescribed drug was sotalol (77.8%) at a dose of 30-60mg per dog orally every 12 hours. Amiodarone was prescribed to four animals (22.2%), at a dose of 150-200mg per dog orally every 12 hours. Twelve dogs (66.7%) received omega-3 fish oil (500mg orally daily) in addition to sotalol or amiodarone.

Boxers were categorized according to the presence of VT, and the CI, SCL, PI, and HRV features were compared, as shown in Table 2. The CI was considered similar between dogs with or without VT (P=0.09), however, the SCL was shorter in the VT group (280 *vs* 410ms; P=0.008), therefore, the PI was higher (less premature) in these patients (0.85 *vs* 0.68, P=0.02). Furthermore, among HRV parameters, only the HRme differed between non-VT and VT dogs, being higher in the latter (89 *vs* 104bpm, P=0.01).

Areas under the curve (AUC) above 0.5 were obtained for CI, PI and HRV parameters to try to differentiate dogs with VT from others at the time of the first 24-h Holter (Table 3), but only PI (AUC=0.73, P=0.02) and HRme (AUC=0.72, P=0.03) were considered relevant. For this reason, two proposed cut-off values to PI and HRme, based on the better match between specificity and sensitivity, are presented in Table 4.

Median follow-up period for the whole population was 700 days (354-934 days). Sixteen dogs (44.4%) reached the end-point. Among them, three animals were euthanized due to end-stage cancer (18.75%), SCD occurred in two (12.5%), and eleven died of unknown causes (68.75%). Among dogs that died suddenly, one had 8 isolated monomorphic VPCs over 24-h, and another 2 isolated monomorphic VPCs and a run of VT of 8 seconds over 24-h at the time of first Holter.

A family history of ARVC was known in five animals (13.9%, 7-11 years old), with three dogs having ARVC parents that died suddenly, and two with healthy parents. The three dogs with family history of ARVC showed more than 300 polymorphic VPCs/24-h. Also, two of these dogs had VT and syncope, and the third had isolated VPCs and was asymptomatic at the time of the first Holter monitoring.

Median survival time was significantly shorter for dogs with VT compared to those with isolated or repeated patterns of VPC (463 *vs* 1173 days, HR=4.25, 95% CI=1.23-14.64, P=0.02). The presence of couplets, triplets and bigeminy were not statistically significant (P=0.057). There was no significant difference in survival time between the dogs in the monomorphic or polymorphic VPCs group (P=0.26). The presence of syncope (P=0.78), number of VPCs over 24-h (P=0.18), as well as CI (P=0.29) and PI (P=0.62) was

Table 1. Sample characterization, characteristics of ventricular arrhythmias and time domain heart rate variability in 36 adult Boxer dogs according to the presence of syncope

	Non-syncopal (n=30)	Syncopal (n=6)	P
Age (years)	8.1±2.8[a]	7.7±2.8	0.77
Body weight (kg)	26.5 (24-30.4)[b]	32.8 (22.4-40)	0.42
Gender (%)			
Female	53	28	0.001
Male	47	72	
Number of VPC	25 (3-2538)	1023 (47-3517)	0.22
Morphology (%)			0.0002
Monomorphic	38	14	
Polymorphic	62	86	
Occurrence			0.11
Isolated	45	42	
Repeated patterns	17	29	
VT	38	29	
Coupling interval (ms)	320 (275-370)	300 (270-360)	0.49
Prematurity index	0.75±0.19	0.70±0.24	0.52
HRmin (bpm)	39 (36-45)	34 (31-38)	0.06
HRme (bpm)	93 (85-105)	90 (83-92)	0.30
HRmax (bpm)	235 (226-250)	250 (223-250)	0.38
SDNN (ms)	300 (245-352)	369 (327-429)	0.02
SDANN (ms)	170±55	210±43	0.09
SDNNidx	236 (178-282)	297 (269-353)	0.009
rMSSD (ms)	135.5±62.4	151.4±70	0.56
pNN>50 (%)	45.8±14.7	57.5±8.9	0.05

[a,b] Parametric data are represented as mean ± standard deviation, while non-parametric variables are shown as median (interquartile range); Repeated patterns: couplets, triplets, bigeminy, trigeminy; VT = ventricular tachycardia, HRmin = minimum heart rate over 24 hours, HRme = mean heart rate over 24h, HRmax = maximum heart rate over 24h, SDNN = the standard deviation of the RR intervals, SDANN = the standard deviation of the mean RR intervals obtained at 5 minute intervals, SDNNidx = mean of the standard deviation of the RR intervals obtained at 5 minute intervals, rMSSD = the square root of the mean squared differences of successive RR intervals, pNN>50 = percentage of adjacent RR intervals differing by more than 50 milliseconds in duration.

Table 2. Electrocardiographic features of ventricular arrhythmias and time domain heart rate variability in 36 adult Boxer dogs according to the presence of ventricular tachycardia

	CI (ms)	SCL (ms)	PI	HRmin (bpm)	HRme (bpm)	HRmax (bpm)	SDNN (ms)	SDANN (ms)	SDNNidx	rMSSD (ms)	pNN>50 (%)
VT (n=13)	280 (260-340)[a]	360 (280-475)	0.85±0.12[b]	43±12	104±21	231 (229-248)	292 (140-386)	158±68	223 (103-333)	114±69	45±17
Non-VT (n=23)	320 (300-380)	490 (410-600)	0.68±0.20	38±6	89±10	245 (224-250)	317 (278-374)	186±42	252 (201-300)	150±56	50±13
P	0.09	0.008	0.02	0.11	0.01	0.29	0.37	0.14	0.38	0.09	0.34

[a,b] Parametric data are represented as mean ± standard deviation, while non-parametric variables are shown as median (interquartile range); VT = ventricular tachycardia, CI = coupling interval, SCL = sinus cycle length, PI = prematurity index, HRmin = minimum heart rate over 24 hours, HRme = mean heart rate over 24h, HRmax = maximum heart rate over 24h, SDNN = the standard deviation of the RR intervals, SDANN = the standard deviation of the mean RR intervals obtained at 5 minute intervals, SDNNidx = mean of the standard deviation of the RR intervals obtained at 5 minute intervals, Rmssd = the square root of the mean squared differences of successive RR intervals, pNN>50 = percentage of adjacent RR intervals differing by more than 50 milliseconds in duration.

Table 3. Receiver operating characteristic of coupling interval, prematurity index and time domain heart rate variability parameters to distinguish adult Boxer dogs with ventricular tachycardia from those without VT

	CI	PI	HRmin	HRme	HRmax	SDNN	SDANN	SDNNidx	rMSSD	pNN>50
AUC	0.67	0.73	0.60	0.72	0.60	0.59	0.62	0.59	0.68	0.58
95% CI	0.46-0.87	0.56-0.89	0.39-0.81	0.53-0.90	0.41-0.80	0.38-0.80	0.40-0.83	0.37-0.80	0.48-0.87	0.36-0.78
P	0.09	0.02	0.31	0.03	0.30	0.36	0.23	0.37	0.08	0.45

AUC = area under the curve, CI = coupling interval, PI = prematurity index, HRmin = minimum heart rate over 24 hours, HRme = mean heart rate over 24h, HRmax = maximum heart rate over 24h, SDNN = the standard deviation of the RR intervals, SDANN = the standard deviation of the mean RR intervals obtained each 5 minutes, SDNNidx = mean of the standard deviation of the RR intervals obtained each 5 minutes, rMSSD = the square root of the mean squared differences of successive RR intervals, pNN>50 = percentage of adjacent RR intervals differing by more than 50 milliseconds in duration.

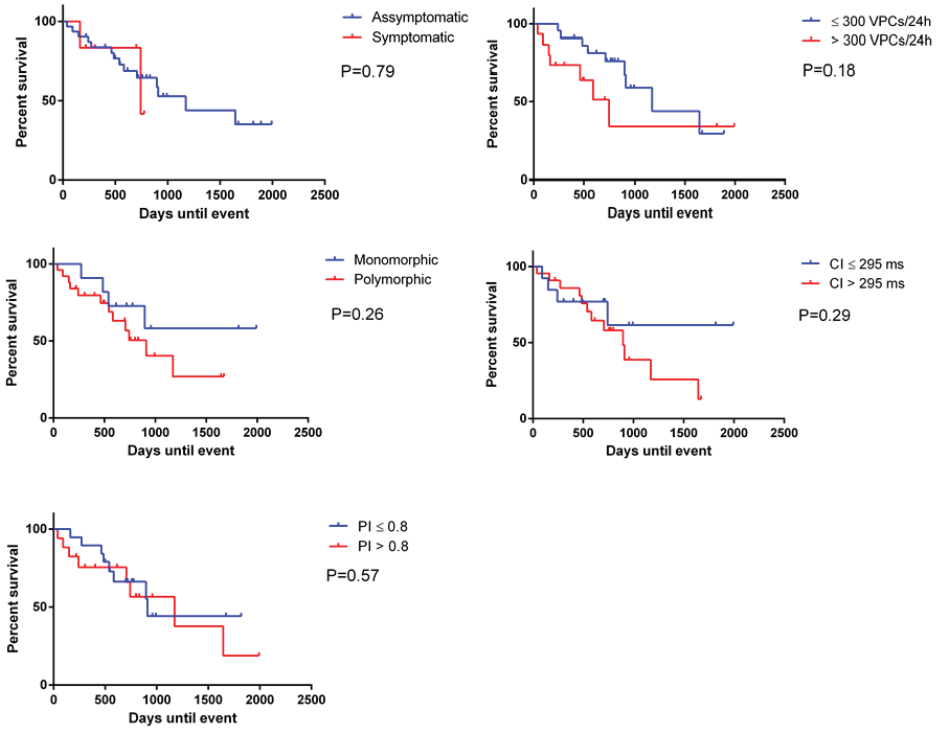


Fig.1. Kaplan-Meier analyses used to assess the prognostic value of several characteristics of ventricular arrhythmias and syncope in 36 adult Boxer dogs. The end-point was all-cause of death. VPCs = ventricular premature complexes, CI = coupling interval, PI = prematurity index.

Table 4. Cut-off values to prematurity index (PI) and mean heart rate (HRme) that better distinguished Boxer dogs with ventricular tachycardia (VT) from others at the time of the first 24h Holter recording

	Cut-off	Sensitivity (%)	Specificity (%)	Likelihood ratio
PI	0.79	65.22	69.23	2.12
	0.83	65.22	53.85	1.41
HRme (bpm)	94	76.16	53.85	1.65
	112	100	46.15	1.85

not associated with overall survival at the proposed cut-off values, as shown in Figure 1.

Of the HRV features, SDNN was shown to be associated with prognosis (SDNN>245ms = 1645 days *vs* SDNN≤245ms = 271 days, HR=19.97, 95% of CI= 3.91-101.8, P=0.0003), also SDANN (SDANN>134ms = 1645 days *vs* SDANN≤134ms = 463 days, HR=3.95, 95% CI=2.13–38.79, P=0.01) (Fig.2). Survival analyses according to HRV features are summarized in Table 5.

DISCUSSION

In this study, the impact on overall survival of some characteristics of ventricular arrhythmias and heart rate variability on the first 24-h Holter monitoring in thirty-six Boxer dogs was assessed. In these analyses, both the dogs with syncope and those examined for routine breed screening were included. The mean age in the whole population was similar, approximately 8 years old, although only animals' ≥4 years old were actually accepted into the study. Boxer ARVC is usually of adult-onset, more frequently seen in animals above 5 years

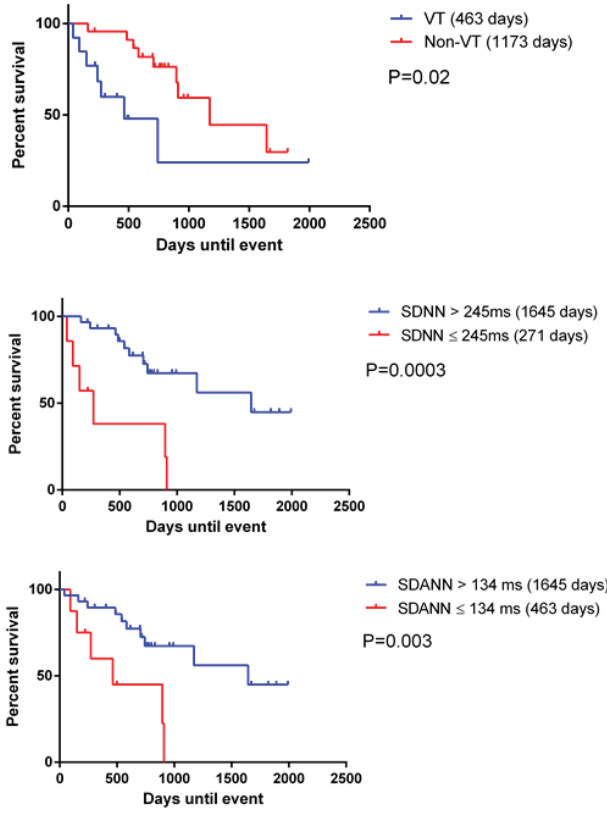


Fig.2. Kaplan-Meier analyses with prognostic value at the first 24-Hour Holter monitoring in 36 adult Boxer dogs. The end-point was all-cause of death. VT = ventricular tachycardia, SDNN = the standard deviation of the RR intervals, SDANN = the standard deviation of the mean RR intervals obtained at 5 minutes intervals, over 24-hours.

Table 5. Log-rank comparison of Kaplan-Meier survival curves analyses of heart rate variability features to the proposed cut-off values (in parentheses), in 36 Boxer dogs monitored by 24h Holter

Parameter	P
SDNN (245 ms)	0.0003
SDANN (134 ms)	0.003
SDNNidx (192 ms)	0.16
rMSSD (173 ms)	0.14
pNN>50%	0.26
HRmin (37 bpm)	0.34
HRme (95 bpm)	0.07
HRmax (247 bpm)	0.16

SDNN = the standard deviation of the RR intervals, SDANN = the standard deviation of the mean RR intervals obtained at 5 minutes intervals, over 24 hours, SDNNidx = mean of the standard deviation of the RR intervals obtained each 5 minutes, rMSSD = the square root of the mean squared differences of successive RR intervals, pNN>50% = percentage of adjacent RR intervals differing by more than 50 milliseconds in duration, HRmin = minimum heart rate over 24 hours, HRme = mean heart rate over 24h, HRmax = maximum heart rate over 24h.

of age. Among symptomatic dogs, a male predominance was observed, similarly to previously reported data (Harpster 1991b, Palermo et al. 2011).

The diagnosis of ARVC is best based on a combination of findings, that may include a middle age Boxer dog with syncope, ventricular arrhythmias with left bundle branch block morphology in caudoventral leads (II, III and aVF) (Kraus et al. 2002) without other documentable causes for the arrhythmia, and a family history of disease (Meurs et al. 1999, Meurs 2017). Although in man the familial history of ARVC is considered a major criterion for diagnosis (McKenna et al. 1994), in this study the family history was known in only three dogs, 13.9% of cases.

The number of VPCs over 24-h was not statistically different between asymptomatic and symptomatic dogs. In addition, the complexity of VA did not differ between symptomatic and asymptomatic dogs. The small number of symptomatic dogs enrolled in our study may have affected these findings. Palermo et al. (2011) found that symptomatic Boxers with ventricular dilation (form 3 of ARVC) usually had a higher number of ventricular ectopic beats, in addition to more complex arrhythmias (couplets, triplets, VT) in comparison to those without ventricular dilation (forms 1 and 2). However, only a small proportion of dogs in Palermo's study had a Holter recording available, making it difficult to draw conclusions regarding the association between the different ARVC forms and either severity or frequency of arrhythmias.

Although 24-h Holter monitoring provides a better assessment of presence, overall frequency and complexity of VA and should be used as part of the diagnosis of Boxer ARVC (Meurs 2004, Palermo et al. 2011), there is no established cut-off for the number of VPCs over 24-hour that would classify a Boxer as ARVC affected. Ventricular arrhythmias have a spontaneous daily variability, which can be as high as 80% in Boxer dogs with ARVC (Spier & Meurs 2004a). The proposed cut-off value of 300 VPCs over 24-h was not associated with a worse prognosis in this study, contrasting with another investigation in which more than 50 VPCs/24-h was associated with a shorter survival (Mõtskula et al. 2013).

Symptomatic Boxer dogs are more likely to have polymorphic VPCs, in concordance with what has been previously reported in Boxer dogs with CHF due to ARVC (Mõtskula et al. 2013), and symptomatic dogs with degenerative mitral valve disease (Carvalho et al. 2018). Although survival was considered similar between Boxer dogs exhibiting either monomorphic or polymorphic VA (P=0.057), the difference in median survival between these groups has clinical significance (1993 *vs* 910 days). Other authors have previously reported that polymorphism is associated with cardiac mortality in Boxer dogs (Mõtskula et al. 2013). The impact of polymorphic ventricular arrhythmias on mortality has not been clearly elucidated in veterinary studies. In the human medical literature, however, it is recognized as a negative prognostic indicator, especially polymorphic VT, which is considered a rhythm with a potential to progress to ventricular fibrillation, a major cause of sudden cardiac death (Haissaguerre et al. 2016). None of animals on this study had polymorphic VT.

The presence of VT was associated with an increased all-cause mortality in this study, similar to previously studies, in which Boxer dogs with VT were about 3 times more likely to suffer cardiac death (Mõtskula et al. 2013, Chamas et al. 2016). In people, many studies have reported that more frequent VPCs are closely associated with ventricular dilation, systolic dysfunction, malignant forms of VT, as well as a poor prognosis (Duffee et al. 1998, Shiraishi et al. 2002, Takemoto et al. 2005, Rhee et al. 2006, Sheldon et al. 2010, Del Carpio Munoz et al. 2011, Igarashi et al. 2012, Kim et al. 2014). Although the PI and HRme were the two evaluated variables that better distinguished dogs with VT from the remaining population, the specificity and sensitivity obtained for PI were low, and none of them were associated with prognosis. However, the HRme = 112bpm was 100% sensitive but only 46% specific for VT, meaning that all dogs without VT had an HRme <112 bpm. This finding suggests that if VT is not observed in a 24-hour Holter record in an adult Boxer but HRme is above 112bpm, it could be reasonable to pursue further Holter monitoring.

Boxer dogs with ARVC can experience SCD regardless of presence/absence of clinical signs (Meurs 2017). Our findings suggested that syncope at the time of the first 24-h Holter monitoring was not associated with a worse prognosis, however our population was heterogenous and only 41.7% was considered likely to be affected by ARVC according to the degree of ventricular ectopy. Palermo et al. 2011 found that the presence of syncope was associated with shorter survival times in Boxers affected with ARVC. Drawing conclusions about the significance of syncope and survival in Boxer dogs is difficult, as the majority of symptomatic animals received antiarrhythmic therapy after the first Holter, which may skew the survival analyses.

There has been interest in the study of VPC CI and PI as an aid to better stratification of risk in patients with VA, although these variables have been poorly investigated in veterinary patients. In human patients, results are conflicting, likely reflecting heterogeneous study populations (Haissaguerre et al. 2002, Viskin et al. 2005, Knecht et al. 2011, Igarashi et al. 2012). The CI and PI were not statistically significant different between symptomatic and asymptomatic Boxer dogs, in contrast with our own study in dogs with mitral

endocardiosis, in which the VPCs in symptomatic animals were less premature (Carvalho et al. 2018). However, the VPCs of dogs with clinical mitral endocardiosis could be considered relatively less premature, mainly because the influence of heart rate, or sinus cycle length, at PI. In addition, these two variables were not associated with overall mortality, and, at least to the best of our knowledge, this was the first time that the prognostic value of CI and PI has been evaluated in Boxer dogs. The VPCs in dogs with VT were found to be less premature (higher PI), due to a shorter sinus cycle length, despite CI being comparable to those without VT. The authors believe these findings could be explained by the effect of heart rate on the prematurity index, since dogs with VT had higher mean heart rate and thus higher PI. Moreover, PI at the proposed cut-off values does not adequately distinguish patients with and without VT.

The HRV is determined by autonomic nervous system activity. The propensity for lethal arrhythmias associated with either increased sympathetic or reduced vagal activity, reflected as decreased HRV, have been extensively studied in people (Bikkina et al. 1998, Obias-Manno & Wijetunga 2004, Frigy et al. 2018). From our data, some HRV variables, such as SDNN and SDNNidx, in symptomatic Boxer dogs were higher than in the asymptomatic group. When looking at animals with VT, only HRme was different from the other parameters, suggesting that elevated HRme (≥112bpm) could be associated with an increased risk of developing VT, although with a poor specificity. Previous studies of HRV in Boxer dogs did not identify a lower HRV in patients with complex and frequent VA (Spier & Meurs 2004b, Zacché et al. 2017). Although HRV was not evaluated in relation to syncope in such investigations, Boxer dogs with CHF had a lower HRV (Spier & Meurs 2004a, Haissaguerre et al. 2016, Chamas et al. 2016). Our findings suggest that symptomatic Boxer dogs, as well as those with VT and no CHF, did not have a persistently high sympathetic tone, or HRV changes have reflected subclinical diseases processes at the time that the first 24-hour Holter was made.

Nevertheless, the SDNN and SDANN, estimates of long-term components of HRV, which are thought to be more sympathetically mediated of HRV (Malliani et al. 1991, Montano et al. 1994), were found to have prognostic value in Boxer dogs, as a SDNN ≤245ms, or SDANN ≤134ms were associated with worse outcome. Conversely, a reduced HRV has already been associated with CHF and risk of SCD in people with VA (Fauchier et al. 1999, Stein & Kleiger 1999). Chamas et al. (2016) found no association between HRV variables and outcome in Boxer dogs, even though the lowest SDANN (58±13ms) was documented in animals with CHF, as previously reported by Spier & Meurs (2004a) (82±21ms).

The results of this study should be interpreted in the context of its limitations. This was a retrospective investigation and accepts the inherent criticisms of the methodology. The unequal groups and small sample size with regard to syncope, limits the stratification of VA characteristics and HRV between such individuals. The total sample size was small, including Boxer dogs considered normal, equivocal and likely affected by ARVC. In addition, at the end of follow-up period fewer than 50% of population had reached the end-point. Although any cause of mortality is of clinical relevance, a study to asses cardiac mortality would be preferable. Finally, the influence of antiarrhythmic therapy after the first 24-h Holter potentially played a major role in overall survival, and this was not standardized due to the retrospective methodology. Despite all the limitations, these preliminary results add new information about the risk stratification in Boxer dogs with VA, and may contribute to prospective studies.

CONCLUSIONS

Symptomatic Boxer dogs are more likely to have polymorphic VPCs than asymptomatic ones.

The VPCs of dogs with ventricular tachycardia are relatively less premature, probably due to the heart rate influence over the prematurity index in spite of a comparable coupling interval.

The PI and HRme were significant different between VT and non-VT dogs, whilst these parameters alone did not discriminate adequately between groups, HRme may provide additional information regarding the presence of VT, in a population known to have significant day-to-day variation of VA.

The presence of VT, SDNN≤245ms, or SDANN≤134ms in an adult Boxer dog at the time of the first 24-h Holter recording is associated with a shorter overall survival time.

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

REFERENCES

Bikkina M., Alpert M.A., Mukerji R., Mulekar M., Cheng B.Y. & Mukerji V. 1998. Diminished short-term heart rate variability predicts inducible ventricular tachycardia. Chest 113(2):312-316. <http://dx.doi.org/10.1378/chest.113.2.312> <PMid:9498944>

Carvalho E.R., Ampuero R.A.N., Tuleski G.L.R., Camacho A.A. & Sousa M.G. 2018. Polymorphism, coupling interval and prematurity index in dogs with degenerative mitral valve disease and ventricular arrhythmias. Vet. Res. Commun. 42(2):1-8. <http://dx.doi.org/10.1007/s11259-018-9718-0> <PMid:29536334>

Chamas P.P.C., Oliveira V.M.C., Yamaki F.L., Goldfeder G.T. & Larsson M.H.M.A. 2016. Valor prognóstico da variabilidade da frequência cardíaca e da eletrocardiografia ambulatorial em cães Boxer com cardiomiopatia arritmogênica do ventrículo direito. Arq. Bras. Med. Vet. Zootec. 68(5):1219-1227. <http://dx.doi.org/10.1590/1678-4162-8383>

Del Carpio Munoz F., Syed F.F., Noheria A., Cha Y.M., Friedman P.A., Hammill S.C., Munger T.M., Venkatachalam K.L., Shen W.K., Packer D.L. & Asirvatham S.J. 2011. Characteristics of premature ventricular complexes as correlates of reduced left ventricular systolic function: study of the burden, duration, coupling interval, morphology and site of origin of PVCs. J. Cardiovasc. Electrophysiol. 22(7):791-798. <http://dx.doi.org/10.1111/j.1540-8167.2011.02021.x> <PMid:21332870>

Duffee D.F., Shen W.K. & Smith H.C. 1998. Suppression of frequent premature ventricular contractions and improvement of left ventricular function in patients with presumed idiopathic dilated cardiomyopathy. Mayo Clin. Proc. 73(5):430-433. <http://dx.doi.org/10.1016/S0025-6196(11)63724-5> <PMid:9581582>

Fauchier L., Babuty D., Cosnay P., Autret M.L. & Fauchier J.P. 1997. Heart rate variability in idiopathic dilated cardiomyopathy: characteristics and prognostic value. J. Am. Coll. Cardiol. 30(4):1009-1014. <http://dx.doi.org/10.1016/S0735-1097(97)00265-9> <PMid:9316532>

Fauchier L., Babuty D., Cosnay P. & Fauchier J.P. 1999. Prognostic value of heart rate variability for sudden death and major arrhythmic events in patients with idiopathic dilated cardiomyopathy. J. Am. Coll. Cardiol. 33(5):1203-1207. <http://dx.doi.org/10.1016/S0735-1097(99)00021-2> <PMid:10193717>

Ferreira G.B., Filippi M.G. & Paes A.C. 2017. Electrocardiographic evaluation in dogs with monocytic ehrlichiosis. Revta Educ. Contin. Med. Vet. Zootec. CRMV-SP 15:38-44.

Frigy A., Csiki E., Carașca C., Szabó I.A. & Moga V.D. 2018. Autonomic influences related to frequent ventricular premature beats in patients without structural

heart disease. Medicine, Baltimore 97(28):1-10. <http://dx.doi.org/10.1097/MD.0000000000011489> <PMid:29995813>

Haissaguerre M., Shoda M., Jais P., Nogami A., Shah D.C., Kautzner J., Arentz T., Kalushe D., Lamaison D., Griffith M., Cruz F., de Paola A., Gaïta F., Hocini M., Garrigue S., Macle L., Weerasooriya R. & Clémenty J. 2002. Mapping and ablation of idiopathic ventricular fibrillation. Circulation 106(8):962-967. <http://dx.doi.org/10.1161/01.CIR.0000027564.55739.B1> <PMid:12186801>

Haissaguerre M., Vigmond E., Stuyvers B., Hocini M. & Bernus O. 2016. Ventricular arrhythmias and the His-Purkinje system. Nat. Rev. Cardiol. 13(3):155-166. <http://dx.doi.org/10.1038/nrcardio.2015.193> <PMid:26727298>

Harpster N.K. 1983. Boxer cardiomyopathy, p.329-337. In: Kirk R. (Ed), Current Veterinary Therapy VIII. W.B Saunders, Philadelphia, USA.

Harpster N.K. 1991a. Boxer cardiomyopathy. Vet. Clin. N. Am., Small Anim. Pract. 21(5):989-1004. <http://dx.doi.org/10.1016/S0195-5616(91)50107-8>

Harpster N.K. 1991b. Boxer cardiomyopathy: a review of the long-term benefits of antiarrhythmic therapy. Vet. Clin. N. Am., Small Anim. Pract. 21(5):989-1004. <http://dx.doi.org/10.1016/S0195-5616(91)50107-8> <PMid:1949503>

Igarashi M., Tada H., Kurosaki K., Yamasaki H., Akiyama D., Sekiguchi Y., Kuroki K., Machino T., Murakoshi N., Nakata Y., Kuga K., Nogami A. & Aonuma K. 2012. Electrocardiographic determinants of the polymorphic QRS morphology in idiopathic right ventricular outflow tract tachycardia. J. Cardiovasc. Electrophysiol. 23(5):521-526. <http://dx.doi.org/10.1111/j.1540-8167.2011.02232.x> <PMid:22136173>

Kim Y.R., Nam G.B., Kwon C.H., Lee W.S., Kim Y.G., Hwang K.W., Kim J., Choi K.J. & Kim Y.H. 2014. Second coupling interval of nonsustained ventricular tachycardia to distinguish malignant from benign outflow tract ventricular tachycardias. Hear Rhythm 11(12):2222-2230. <http://dx.doi.org/10.1016/j.hrthm.2014.08.012> <PMid:25111325>

Knecht S., Sacher F., Wright M., Hocini M., Nogami A., Arentz T., Petit B., Franck R., De Chillou C., Lamaison D., Farré J., Lavergne T., Verbeet T., Nault I., Matsuo S., Leroux L., Weerasooriya R., Cauchemez B., Lellouche N., Derval N., Narayan S.M., Jaïs P., Clementy J. & Haïssaguerre M. 2011. Long-term follow-up of idiopathic ventricular fibrillation ablation a multicenter study. J. Am. Coll. Cardiol. 54(6):522-528. <http://dx.doi.org/10.1016/j.jacc.2009.03.065> <PMid:19643313>

Kraus M.S., Moïse N.S., Rishniw M., Dykes N. & Erb H.N. 2002. Morphology of ventricular arrhythmias in the boxer as measured by 12-lead electrocardiography with pace-mapping comparison. J. Vet. Intern. Med. 16(2):153-158. <http://dx.doi.org/10.1111/j.1939-1676.2002.tb02347.x> <PMid:11899030>

Lee Y.H., Zhong L., Roger V.L., Asirvatham S.J., Shen W.K., Slusser J.P., Hodge D.O. & Cha Y.M. 2014. Frequency, origin, and outcome of ventricular premature complexes in patients with or without heart diseases. Am. J. Cardiol. 114(9):1373-1378. <http://dx.doi.org/10.1016/j.amjcard.2014.07.072> <PMid:25205629>

Malliani A., Pagani M., Lombardi F. & Cerutti S. 1991. Cardiovascular neural regulation explored in the frequency domain. Circulation 84(2):482-492. <http://dx.doi.org/10.1161/01.CIR.84.2.482> <PMid:1860193>

Marino D.J., Matthiesen D.T., Fox P.R., Lesser M.B. & Stamoulis M.E. 1994. Ventricular arrhythmias in dogs undergoing splenectomy: a prospective study. Vet. Surg. 23(2):101-106. <http://dx.doi.org/10.1111/j.1532-950X.1994.tb00453.x> <PMid:8191668>

McKenna W.J., Thiene G., Nava A., Fontaliran F., Blomstrom-Lundqvist C., Fontaine G., Camerini F., & Task Force of the Working Group Myocardial and Pericardial Disease of the European Society of Cardiology and of the Scientific Council on Cardiomyopathies of the International Society and Federation of Cardiology. 1994. Diagnosis of arrhythmogenic right ventricular dysplasia/cardiomyopathy. Brit. Heart J. 71(3):215-218. <http://dx.doi.org/10.1136/hrt.71.3.215> <PMid:8142187>

Meurs K.M. 2004. Boxer dog cardiomyopathy: an update. Vet. Clin. N. Am., Small Anim. Pract. 34(5):1235-1244. <http://dx.doi.org/10.1016/j.cvsm.2004.05.003> <PMid:15325480>

Meurs K.M. 2017. Arrhythmogenic right ventricular cardiomyopathy in the boxer dog: an update. Vet. Clin. N. Am., Small Anim. Pract. 47(5):1103-1111. <http://dx.doi.org/10.1016/j.cvsm.2017.04.007> <PMid:28647112>

Meurs K., Lahmers S. & Keene B. 2011. C-11: Characteristics of ARVC Boxers with sudden death. ACVIM Forum Research Abstracts Program. Denver, Colorado. American College of Veterinary Internal Medicine. (Resume)

Meurs K.M., Spier A.W., Miller M.W., Lehmkuhl L. & Towbin J.A. 1999. Familial ventricular arrhythmias in boxers. J. Vet. Intern. Med. 13(5):437-439. <http://dx.doi.org/10.1111/j.1939-1676.1999.tb01460.x> <PMid:10499727>

Montano N., Ruscone T.G., Porta A., Lombardi F., Pagani M. & Malliani A. 1994. Power spectrum analysis of heart rate variability to assess the changes in sympathovagal balance during graded orthostatic tilt. Circulation 90(4):1826-1831. <http://dx.doi.org/10.1161/01.CIR.90.4.1826> <PMid:7923668>

Mõtsküla P.F., Linney C., Palermo V., Connolly D.J., French A., Dukes McEwan J. & Fuentes V.L. 2013. Prognostic value of 24-hour ambulatory ECG (Holter) monitoring in Boxer dogs. J. Vet. Intern. Med. 27(4):904-912. <http://dx.doi.org/10.1111/jvim.12107> <PMid:23679064>

Obias-Manno D. & Wijetunga M. 2004. Risk stratification and primary prevention of sudden cardiac death: sudden death prevention. AACN Clin. Issues 15(3):404-418. <http://dx.doi.org/10.1097/00044067-200407000-00008> <PMid:15475814>

Palermo V., Stafford Johnson M.J., Sala E., Brambilla P.G. & Martin M.W. 2011. Cardiomyopathy in Boxer dogs: a retrospective study of the clinical presentation, diagnostic findings and survival. J. Vet. Cardiol. 13(1):45-55. <http://dx.doi.org/10.1016/j.jvc.2010.06.005> <PMid:21306968>

Rhee K.H., Jung J.Y., Rhee K.S., Kim H.S., Chae J.K., Kim W.H. & Ko J.K. 2006. Tachycardiomyopathy induced by ventricular premature complexes: Complete recovery after radiofrequency catheter ablation. Korean J. Intern. Med. 21(3):213-217. <http://dx.doi.org/10.3904/kjim.2006.21.3.213> <PMid:17017676>

Sheldon S.H., Gard J.J. & Asirvatham S.J. 2010. Premature ventricular contractions and non-sustained ventricular tachycardia: association with sudden cardiac death, risk stratification, and management strategies. Indian Pacing Electrophysiol. J. 10(8):357-371. <PMid:20811538>

Shiraishi H., Ishibashi K., Urao N., Tsukamoto M., Hyogo M., Keira N., Hirasaki S., Shirayama T. & Nakagawa M. 2002. A case of cardiomyopathy induced by premature ventricular complexes. Circulation J. 66(11):1065-1067. <http://dx.doi.org/10.1253/circj.66.1065> <PMid:12419942>

Spier A.W. & Meurs K.M. 2004a. Evaluation of spontaneous variability in the frequency of ventricular arrhythmias in Boxers with arrhythmogenic right ventricular cardiomyopathy. J. Am. Vet. Med. Assoc. 224(4):538-541. <http://dx.doi.org/10.2460/javma.2004.224.538> <PMid:14989546>

Spier A.W. & Meurs K.M. 2004b. Assessment of heart rate variability in Boxers with arrhythmogenic right ventricular cardiomyopathy. J. Am. Vet. Med. Assoc. 224(4):534-537. <http://dx.doi.org/10.2460/javma.2004.224.534> <PMid:14989545>

Stein P.K. & Kleiger R.E. 1999. Insights from the study of heart rate variability. Ann. Rev. Med. 50:249-261. <http://dx.doi.org/10.1146/annurev.med.50.1.249> <PMid:10073276>

Takemoto M., Yoshimura H., Ohba Y., Matsumoto Y., Yamamoto U., Mohri M., Yamamoto H. & Origuchi H. 2005. Radiofrequency catheter ablation of premature ventricular complexes from right ventricular outflow tract improves left ventricular dilation and clinical status in patients without structural heart disease. J. Am. Coll. Cardiol. 45(8):1259-1265. <http://dx.doi.org/10.1016/j.jacc.2004.12.073> <PMid:15837259>

Viskin S., Rosso R., Rogowski O. & Belhassen B. 2005. The "short-coupled" variant of right ventricular outflow ventricular tachycardia: A not-so-benign form of benign ventricular tachycardia? J. Cardiovasc. Electrophysiol. 16(8):912-916. <http://dx.doi.org/10.1111/j.1540-8167.2005.50040.x> <PMid:16101636>

Zacché E., Assumpção T.C.A., Corsini T.B. & Camacho A.A 2017. Time domain heart rate variability in Boxer dogs with arrhythmogenic right ventricular cardiomyopathy. Ciência Rural 47(5):1-6. <http://dx.doi.org/10.1590/0103-8478cr20160740>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(8):643-648, August 2019
DOI: 10.1590/1678-5150-PVB-5908

Original Article
Small Animal Diseases

Acetabular ventroversion using the sacroiliac wedge, with or without pelvic osteotomies in dogs: an *ex vivo* study[1]

Fernanda M. Ikenaga[2], Jessé R. Rocha[2], Leonardo L. Carvalho[2], Cristiane S. Honsho[2], Fernanda G.G. Dias[2], Rodrigo C. Costa[3], Thiago A.S.S. Rocha[3] and Luís G.G.G. Dias[3]*

ABSTRACT.- Ikenaga F.M, Rocha J.R., Carvalho L.L., Honsho C.S., Dias F.G.G., Costa R.C., Rocha T.A.S.S. & Dias L.G.G.G. 2019. **Acetabular ventroversion using the sacroiliac wedge, with or without pelvic osteotomies in dogs: an *ex vivo* study.** *Pesquisa Veterinária Brasileira 39(8):643-648*. Universidade de Franca, Avenida Dr. Armando Salles de Oliveira 201, Parque Universitário, Franca, SP 14404-600, Brazil. E-mail: gustavogosuen@gmail.com

Canine hip dysplasia (CHD) is a common condition observed in the surgical clinics for small animals. Among the surgical techniques for management of CHD, triple pelvic osteotomy and sacroiliac wedge promote acetabular lateral axial rotation (ventroversion), increasing acetabular coverage and joint stability. The present study aimed to evaluate radiographically, by measuring the Norberg angle (NA) and the acetabular coverage percentage (ACP), the acetabular ventroversion induced by the sacroiliac wedge technique, with or without pelvic osteotomies; we also checked the feasibility of wedges made of polyamide with an angulation of 20° and 30°. The software used to measure NA and ACP was AutoCAD® 2009. Pelves from 10 canine corpses were evaluated radiographically at four time-points: M0 (Control Group), M1 (wedges of 20° and 30°), M2 and M3 (wedges associated with bilateral pubis and ischium osteotomies, respectively). There was no significant increase in the acetabular ventroversion at M1, M2, and M3. The polyamide sacroiliac wedge technique proved to be feasible, stable, and easy to apply. Further, the software proved to be efficient and easy to use for NA and ACP measurements. In the present study, even in the cases of non-dysplasic adult canine corpses, it was concluded that the sacroiliac wedge technique does not require to be accompanied by pubis and ischial osteotomies because they did not significantly increase the NA and ACP.

INDEX TERMS: Acetabular ventroversion, canine hip dysplasia, sacroiliac wedge, pelvic osteotomies, *ex vivo*, dogs, surgery, clinics.

RESUMO.- [**Ventroversão acetabular associada ou não a osteotomias pélvicas por meio da utilização de cunha sacroilíaca em cães: estudo ex-vivo.**] A displasia coxofemoral (DCF) é afecção comum na clínica cirúrgica de pequenos animais. Entre as técnicas cirúrgicas para controle da DCF, a osteotomia pélvica tripla (OPT) e a cunha sacroilíaca (CSI), promovem rotação lateral acetabular no eixo axial (ventroversão), aumentando a cobertura acetabular e a estabilidade da articulação. Desta forma, o presente estudo objetivou avaliar radiograficamente, por meio da aferição do ângulo de Norberg (NA) e da porcentagem de cobertura acetabular (PCA), a ventroversão acetabular induzida pela técnica da cunha sacroilíaca, associada ou não às osteotomias pélvicas, além de verificar a exequibilidade das cunhas confeccionadas de poliamida com angulação de 20º e 30º. O software utilizado para aferir o AN e o PCA foi o AutoCAD® 2009. Dez pelves de cadáveres caninos foram avaliadas radiograficamente em quatro momentos: MO (Grupo Controle), M1 (cunhas de 20º e 30º), M2 e M3 (cunhas associadas à osteotomia bilateral do púbis e ísquio, respectivamente). Não houve aumento significativo da ventroversão em M1, M2 e M3. A técnica de cunha sacroilíaca de poliamida mostrou-se exequível, estável e de fácil aplicação. Não obstante, o software utilizado mostrou-se eficiente e de fácil utilização nas aferições do AN e PCA. Neste estudo, mesmo tratando-se de cadáveres

[1] Received on March 19, 2019.
Accepted for publication on April 9, 2019.
[2] Universidade de Franca (Unifran), Avenida Dr. Armando Salles de Oliveira 201, Parque Universitário, Franca, SP 14404-600, Brazil.
[3] Departamento de Clínica e Cirurgia Veterinária, Universidade Estadual Paulista "Júlio de Mesquita Filho" (Unesp), Jaboticabal, SP 14884-900, Brazil.
*Corresponding author: gustavogosuen@gmail.com

de cães adultos e de maioria não displásicos, concluiu-se que a utilização da técnica de cunha sacroilíaca não necessita de associação à ostectomia púbica e a osteotomia do ísquio por não promoverem aumento significativo do AN e da PCA.

TERMOS DE INDEXAÇÃO: Cunha sacroilíaca, displasia coxofemoral canina, osteotomias pélvicas, ventroversão acetabular, cães, *ex vivo*, caninos, cirurgia, clínica.

INTRODUCTION

Canine hip dysplasia (CHD) is frequent in dogs, especially in the fast-growing large breeds (Minto et al. 2012, Rocha et al. 2013); CHD occurs due to joint incongruence between the femoral head and the acetabulum, causing soft tissue looseness and instability, as well as degenerative joint disease (Rocha et al. 2013).

The diagnosis of CHD is based on the review, history, clinical signs, and orthopedic and radiographic examination (Piermattei et al. 2015, Rocha et al. 2013). The measurement of Norberg angle (NA) and acetabular coverage percentage (ACP) are effective methods for the detection of CHD in dogs, which can be calculated using specific software (Lopez et al. 2008, Regonato et al. 2009). Both assess the structural and functional conditions that influence the hip joint, such as joint laxity, hip joint subluxation, and acetabular configuration, but they are not evaluated by the same method since the craniolateral acetabular border influences the NA and the dorsal acetabular border the ACP measurement (Ohlerth et al. 2001).

Treatment of CHD may involve conservative or surgical techniques; however, it is recommended that it be instituted as early as possible, regardless of the technique chosen. Surgical techniques for treating CHD aim to relieve the pain during ambulation and reduce the increased acetabular coverage, improving joint stability (Schachner & Lopez 2015). In this context, Slocum & Slocum (1992) reported triple pelvic osteotomy (TPO), and Conzemius et al. (1999) and Regonato et al. (2009) reported the sacroiliac wedge (SW), as techniques to increase the surface contact between the femoral head and acetabulum, by increasing the acetabular ventroversion, indicated in 5–12-month-old animals of large and giant breeds, restoring the joint function (David & Kasper 1992, Vezzoni 2007).

There is no consensus in the scientific literature regarding the degree of optimal rotation of the pelvis to allow greater acetabular coverage, although rotation from 20° to 30° is accepted for dogs without hip joint subluxation (Slocum & Devine 1986, Tomlinson & Cook 2002). It is known that a lower degree of rotation does not lead to improvement, and excessive rotations may compromise the extension and abduction of the hip joint due to collision of the femoral neck with the acetabular border (Schrader 1981, Slocum & Devine 1986).

In a study with canine corpse pelves, Conzemius et al. (1999) proposed the use of SW, producing an effect similar to that of TPO. Regonato et al. (2009) demonstrated the surgical approach, and proved its efficiency for acetabular ventroversion, using castor bean (*Ricinus communis*) polyurethane wedges in canine corpses. This technique, besides promoting acetabular ventroversion, overcame the disadvantages of TPO, reducing surgical time, risks, and costs. The SW technique involves the application of a wedge between the sacroiliac junction, promoting rotation of the acetabular segment, being associated with ischial and pubis osteotomies (Regonato et al. 2009). The wedge stabilization at the sacroiliac junction is achieved using orthopedic screws applied to the lateral side of the ilium, passing through the wedge, and penetrating the sacral body (Conzemius et al. 1999, Regonato et al. 2009).

The aim of this study was to radiographically evaluate NA and ACP in order to compare the effectiveness of the acetabular ventroversion achieved by the polyamide SW technique with angulations of 20° and 30°, with or without pelvic osteotomies. In addition, we sought to assess the feasibility and possible advantages of the polyamide wedge and the actual need for pelvic osteotomies, with a view to minimizing the surgical time and possible post-operative complications.

MATERIALS AND METHODS

Ethics statement. The present study was carried out under the agreement and surveillance of the Ethics Committee on the Use of Animals of the "Universidade de Franca" (Unifran), under protocol number 027/12.

Animals. The use of corpses is advocated for ethical considerations as the well-being of animals. Such specimens were donated by the animal tutors for the Veterinary Hospital. No preliminary screening was performed for the presence of hip dysplasia.

For this study, the hip joints from 10 canine corpses of medium to large size (body weight above 15kg) were used.

Radiographic evaluation. The hip joints were radiographically evaluated (ventrodorsal and laterolateral projections) at four time-points. In the first time-point, M0 (control), no surgical intervention was performed in order to obtain the NA and ACP. The second time-point (M1) was after the implantation of SW, right and left, with 20° and 30° angulation, respectively, without any pelvic osteotomy. In the third time-point (M2), after SW removal and bilateral pubis ostectomy (Fig.1B,C), the SW were reassigned, fixed, and the radiographic examination was performed sequentially. In the fourth time-point (M3), the SW were removed again, and ischial osteotomy was performed bilaterally (Fig.1D). Simultaneously, the SW were relocated and fixed to perform the last radiographic examination. For all four time-points (M0 to M3), 10 right and 10 left hip joints were assessed (Fig.2).

Implants. The wedges were made using nylon (polyamide), angled at 20° and 30°, 2.5cm high, 3cm long, and 1cm wide. The stainless steel orthopedic screws used for stabilizing the wedges at the sacroiliac joint were 3.5-mm thick, and of appropriate length, as required.

Surgical technique. The operative procedures were performed in M1 to M3, in both hemipelves, for the implantation of the wedges with 20° and 30° angulation in the left and right sacroiliac junctions, respectively. Initially, a wide trichotomy was performed from the second lumbar vertebra to the beginning of the coccygeal vertebrae, and enlarged in both lateral directions.

The corpses were positioned in the ventral decubitus, and the sacral bones were technically approached, as proposed by Piermattei (1993). For placement of the wedge at the sacroiliac junction, the dorsal sacroiliac ligament was sectioned, and the access/removal of the synchondrosis (sacroiliac joint) was performed using a periosteal elevator, osteotome, and hammer lift.

Once the sacroiliac joint was sectioned, a 2.5-mm bore was made on the lateral surface of the sacrum using a surgical drill, with the depth being 50% to 60% of that of the sacral body. Simultaneously, the wedge was positioned at the sacroiliac junction, with its base level with the dorsal plane of the sacrum, as described by Regonato et al. (2009) (Fig.1A).

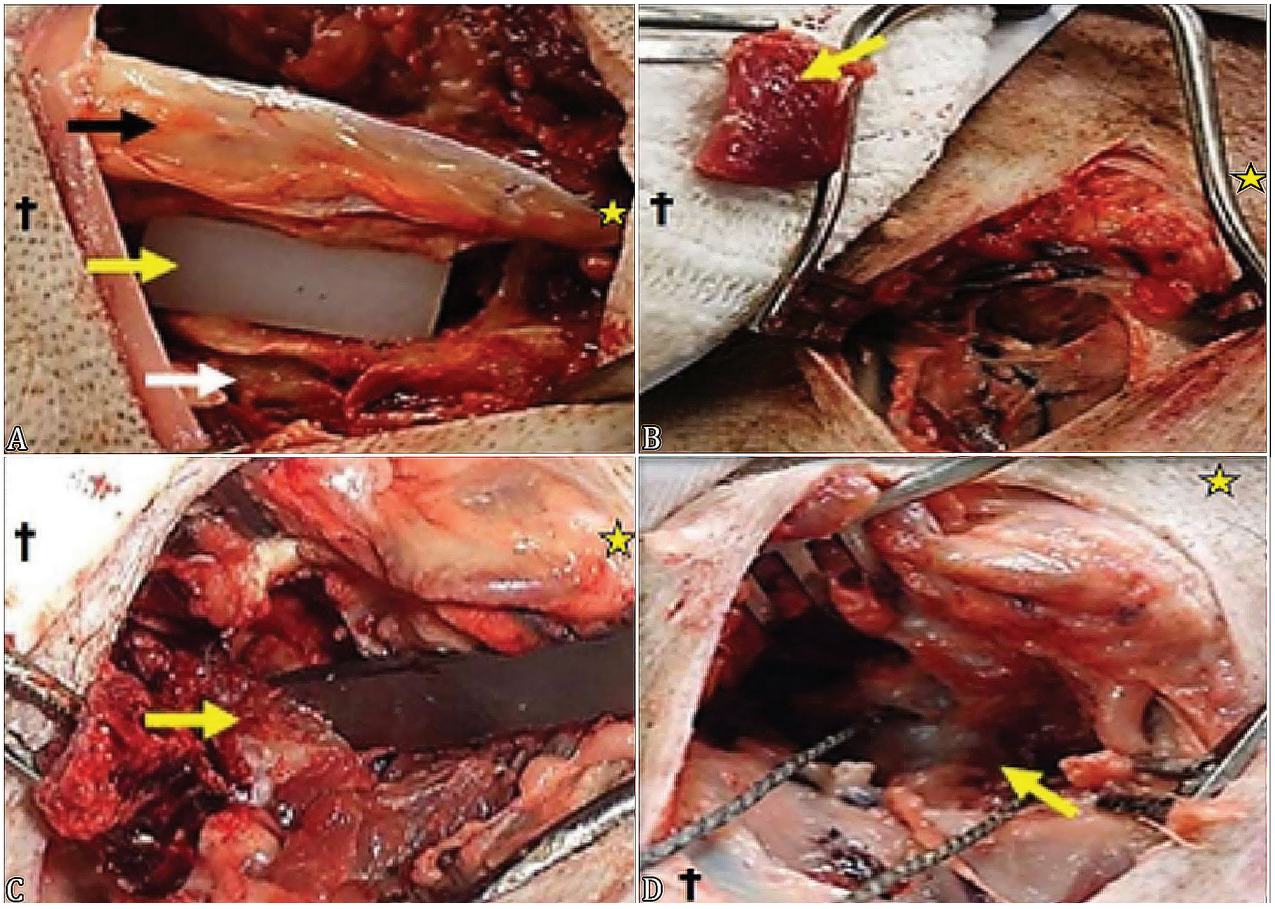


Fig.1. Surgical time-points studied. (**A**) Placement of the 20° polyamide wedge implant (yellow arrow) with its base level with the dorsal plane of the sacrum (white arrow) on the medial aspect of the right ilium wing (black arrow). (**B**) Ventral incision on the pubis, with removal of a fragment of the pectineus muscle (yellow arrow). (**C**) Performing ostectomy of the pubis (yellow arrow) using hammer and osteotome. (**D**) Passage of the Gigli saw for osteotomy of the ischium (yellow arrow).

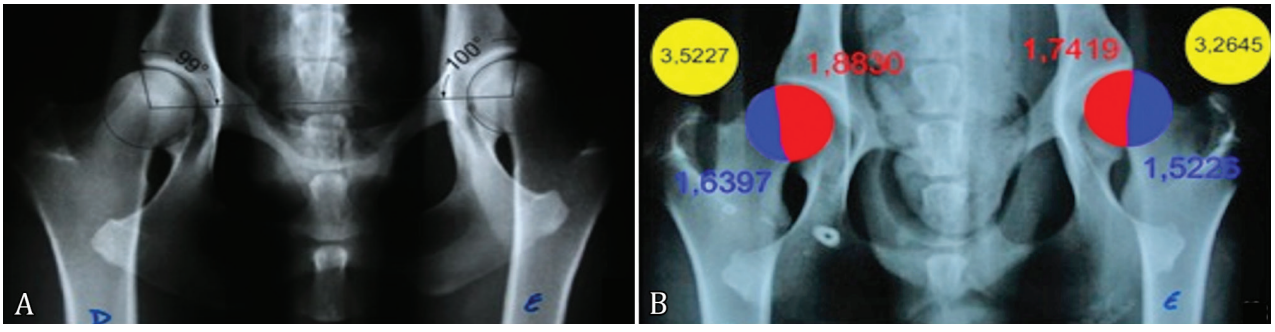


Fig.2. Radiographic images of the canine cadaver pelvis in the ventrodorsal projection. (**A**) Norberg angle gauging. (**B**) Measurement of the area of the femoral head covered by the acetabulum (red semicircle) and not covered by the acetabulum (blue semicircle), used to calculate the ACP (acetabular coverage percentage). The yellow circle represents the total area of the femoral head.

The iliac crest and wedge were then drilled using a pneumatic surgical drill, with an attempt to combine this opening with the one previously made in the sacrum. A stainless steel screw of 3.5mm diameter with compatible length was used for stabilization. This procedure was performed on both hemipelves. After radiographic evaluation (M1), the wedges were removed, and the pubis was osteotomized bilaterally using an osteotome and orthopedic hammer. To facilitate this, the cadaver was positioned in dorsal decubitus, the pectin muscle was removed, and then, using the osteotome, a 1-cm area of the pubis was removed (Fig.1B,C). Thereafter, the SW were reassigned and fixed, as previously described.

After a new radiographic evaluation (M2), the wedges were removed again, and the animal was positioned in lateral decubitus (right and left) to perform ischial osteotomy using the lateral approach. After elevation and removal of the musculature, the Gigli saw was used for the ischial osteotomy (right and left) (Fig.1D). Subsequently, the SW was reassigned and stabilized (M3).

Radiographic positioning and evaluation of acetabular ventroversion. The ventrodorsal (VD) position of the corpses was

adopted, as recommended by the Orthopedic Foundation for Animals. Right and left laterolateral (LL) projections were also performed to validate the correct positioning of the screws in the sacral body.

Radiography was performed at all time-points (M0 to M3) to verify which angulation of SW (20° or 30°), with or without pelvic osteotomies, provided adequate acetabular ventroversion, and consequently, increased acetabular coverage. Two parameters, NA and ACP, were measured. To achieve this, the radiographs were photographed using a digital camera (Sony Cyber-shot, 16.1 megapixels) and the images were exported to AutoCAD® 2009 software - Autodesk. Measurement of NA was performed as described by Brass et al. (1978) (Fig.2A), and the ACP was measured based on the formula (a/b)x100=ACP, as described by Tomlinson & Johnson (2000), where: "a" represents the area of the femoral head covered by the acetabulum (red area, Fig.2B) and "b" represents the total area of the same femoral head (yellow circumference, Fig.2B).

Statistical analysis. The variables were tested for normality using the Shapiro-Wilk test. For the normally distributed data, a single-variance analysis with multiple replications was performed, followed by the Tukey test, and the data were reported as mean ± standard deviation. The means of the tested groups (M1, M2, and M3) were compared to the means of the Control Group (M0) for the same side of the limb (hemipelvis). The means for the right and left sides of each group were also compared. The level of statistical significance was set at P≤0.05.

RESULTS

Ten canine corpses (7 males, 70% and 3 females, 30%) unidentified breed were used, with the weight ranging from 17.4kg to 42.8kg (mean 27.32kg). Regarding the presence of hip dysplasia, 3 (30%) and 5 (50%) of the cadavers included in this experiment were diagnosed with dysplasia (based on NA) in the right and left hips, respectively.

After radiographic evaluations (M0, M1, M2, and M3) and NA and ACP measurements for each time-point, we observed in both groups an increase in the NA values at M1 and a decrease at M2 and M3, but without statistically significance (Table 1).

Table 1. Values (in degrees) and standard deviations of the NA (Norberg angle) and ACP (acetabular coverage percentage) of the hip joints of the 10 dogs at the time-points studied

Surgical technique/ Measurement method	SW	M0	M1	M2	M3
NA (°)	Right 20°	$106±4^{aA}$	$111±6^{aA}$	$112±5^{aA}$	$110±8^{aA}$
	Left 30°	$103±5^{aA}$	$109±3^{aA}$	$108±5^{aA}$	$108±7^{aA}$
ACP (%)	Right 20°	$56±3^{aA}$	$57±2^{aA}$	$58±4^{aA}$	$56±2^{aA}$
	Left 30°	$54±8^{aA}$	$57±2^{aA}$	$57±1^{aA}$	$56±2^{aA}$

M0 = corresponds to the preoperative time-point, M1 = corresponds to the time-point after placement of polyamide sacroiliac wedge (SW) with an angulation of 20° and 30° in the right and left sacroiliac junctions, respectively, M2 = corresponds to the time-point after bilateral pubis ostectomy and repositioning and stabilization of SW, M3 = corresponds to the time-point after bilateral ischial osteotomy and repeated stabilization of SW; [a] similar lower case letters indicate that there is no statistical difference in the data between the time-points, [A] similar capital letters indicate that there is no statistical difference between the data for each hemipelvis at each time-point studied.

DISCUSSION

The proportion of males and females in the experimental animals studied (70:30, respectively) corroborate the findings of Minto et al. (2012), who mentioned that there is no sexual predisposition to hip dysplasia. The dysplastic animals observed in the study included 3 (30%) and 5 (50%) corpses affected in the right and left hips, respectively. This non-uniformity between the groups may be due to the absence of preliminary screening, since the animals cadavers were donated to the institution; moreover, these animals were adults and some were non-dysplastic, which, in clinical practice, would not be considered candidates for the technique.

In this study, at one of the time-points, no osteotomy was performed for the wedge placement (M1), as proposed by Conzemius et al. (1999), the developer of this technique, who believed that SW technique without osteotomy was feasible. We performed an osteotomy at M2 and two osteotomies at M3, avoiding the third osteotomy of the ilium performed in the TPO technique, based on the study by Regonato et al. (2009), who cited the advantages of SW over TPO.

The ventral rotation of the acetabulum between 20° and 30° is, according to Slocum & Devine (1987), sufficient for most patients. The authors produced polyamide wedges with an angulation of 20° and 30° in order to test whether there is a difference in the resulting ventroversion, since the 30° angulation, according to Slocum & Devine (1986) and Regonato et al. (2009) can impair pelvic limb abduction, adversely affecting the patient's ambulation. Polyamide was selected due to its easy acquisition, low cost, ease of sterilization, and ability to be modeled; it allows the drilling and allocation of orthopedic screws and is biocompatible (Spadeto-Junior et al. 2010).

The placement of the wedges at the right and left sacroiliac junctions was performed as described by Conzemius et al. (1999), and reproduced by Regonato et al. (2009). In this study, no intercurrences were found in the surgical technique between the groups. However, the most difficult part of the procedure, requiring the greatest attention, also cited by the aforementioned authors, was the correct placement of the screw in the sacral body.

The polyamide wedges could not be seen on radiographic examination because they were radiolucent, as shown in the study by Regonato et al. (2009), who used castor bean wedges. The VD and LL projections were essential for the verification of the correct placement of the implants in the sacrum; in particular, the DV projection was essential for verification of NA and ACP measurements.

Both ACP and NA were measured at all the time-points studied, since, according to Lopez et al. (2008), such combined assessments can correctly classify pelvic conformation in up to 98% of the cases, thus increasing the accuracy of the results.

Using the same methodology proposed by Lopez et al. (2008), the radiographic images obtained were photographed and exported to AutoCAD® 2009 software, and sequentially, NA was measured in accordance with the method proposed by Regonato et al. (2009), and ACP was measured according to the method described by Tomlinson & Johnson (2000) and Regonato et al. (2009).

Rasmussen et al. (1998) reported that in the immediate postoperative period of TPO, there was an increase in the NA and ACP values, and that in the majority of animals, the values increased by an average of 20° in the case of NA. In the present study, there was an increase in M1 in relation to M2 and M3, but the difference was not significant (Table 1). This

was probably due to the fact that some of the animals were non-dysplasic; moreover, according to Regonato (2010), there may be a gradual increase in the acetabular coverage in the first 6 weeks following the surgical procedure. The changes that occur in the pelvic structure during the postoperative period, such as healing and soft tissue remodeling, cannot be observed using this type of experimental paradigm.

There was an increase in the NA at M1 (SW only) and a decrease at M2 and M3, but the differences were not statistically significant. This is possibly due to ostectomy of the pubis and ischium, which may have reduced the pressure caused by the wedge placement at the sacroiliac junction, which, in turn, increases the acetabular coverage. The same observation was made with respect to ACP, which was higher at M1 than at the other time-points, corroborating the findings of Lopez et al. (2008) and Regonato (2010), who mentioned that with the increase or decrease in NA, the ACP increases or decreases proportionally. It is believed that the reasoning used to explain the changes in the NA can also be used to explain the changes in ACP for this experimental model.

Another factor possibly contributing to the small increases in NA and ACP is the methodology used: the removal and replacement of the wedges at each time-point may have impaired the correct positioning and ideal stabilization of the wedges, but could not be observed in the intraoperative examination and in the radiographic images of the groups.

Assessment of differences in the values obtained at the different time-points revealed a 4.7%, 5.6%, and 3.7% increase at M1, M2, and M3, respectively, for the use of the 20° wedge, compared with the value at M0. For the wedge of 30°, there was an increase of 5.8% at M1 and 4.8% at M2 and M3 compared with M0. Subjectively, since there was no statistical significance, the performance of both osteotomies seems to have impaired the increase of NA, except at M2 for the wedge of 20°.

Regarding ACP, when we evaluated the percentage difference in the values, we observed the same trend as that for NA, with an increase of 1%, 2%, and 0% at M1, M2, and M3 for the 20° wedge, compared with the value at M0. When using a 30° wedge, a 3% increase was observed in the ACP at M1 and M2 and 2% at M3, compared with M0. Therefore, SW technique performed with osteotomies reduced the ACP.

CONCLUSIONS

From the surgical point of view, the placement of the polyamide wedge at the sacroiliac junction of dogs proved to be feasible, easy to apply, and stable, with the application of the compressive screw, at the different time-points studied.

The scanning of the radiographic images and the use of AutoCAD® 2009 software for measuring the NA and ACP of the studied groups at the desired time-points proved to be efficient and easy to use.

In the present study, in the case of cadavers of adult and mostly non-dysplasic dogs, it was concluded that the SW technique modified the acetabular coverage without osteotomies, but not significantly, as shown by the measured NA and ACP, mainly for the wedges with a 20° angle than those with 30° angle. The osteotomies did not enhance the operative technique studied, and a small reduction in ACP was detected.

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

REFERENCES

Brass W., Freudiger U., Muller L.F., Paatsama S., Van Der Velden N.A. & Van De Watering C.C. 1978. Bericht der huftgelenkdysplasie-komission. Kleintierpraxis 23:169-180.

Conzemius M.G., Aper R.L. & Browm M.D. 1999. Evaluation of sacroiliac wedge rotation to incrase acetabular ventroversion - a canine cadaver study. Vet. Comp. Orthop. Traumatol. 12(4):173-177. <http://dx.doi.org/10.1055/s-0038-1632486>

David T.H. & Kasper M. 1992. Triple pelvic osteotomy with axial acetabular rotation in canine hip dysplasia. Eur. J. Companion Anim. Pract. 2(2):23-38.

Lopez M.J., Lewis B.P., Swaab M.E. & Markel M.D. 2008. Relationships among measurements obtained by use of computed tomography and radiography and scores of cartilage microdamage in hip joints with moderate to severe joint laxity of adults dogs. Am. J. Vet. Res. 69(3):362-370. <http://dx.doi.org/10.2460/ajvr.69.3.362> <PMid:18312135>

Minto B.W., Souza V.M., Brandão C.V.S., Mori E.S., Morishin Filho M.M. & Ranzani J.J.T. 2012. Avaliação clínica da denervação acetabular em cães com displasia coxofemoral atendidos no Hospital Veterinário da FMVZ, Botucatu, SP. Vet Zootec. 19(1):91-98.

Ohlerth S., Lang J., Busato A. & Gaillard C. 2001. Estimation of genetic population variables for six radiographic criteria of hip dysplasia in a colony of Labrador Retrievers. Am. J. Vet. Res. 62(6):846-852. <http://dx.doi.org/10.2460/ajvr.2001.62.846> <PMid:11400839>

Piermattei D.L. 1993. An Atlas of Surgical Approaches to the Bones and Joints of the Dogs ans Cats. 3rd ed. W.B. Saunders, Philadelphia, p.324.

Piermattei D.L., Flo G.L. & Decamp C.E. 2015. Handbook of Small Animal Orthopedics and Fracture Repair. 5th ed. Saunders, Philadelphia, p.880.

Rasmussen L.M., Kramek B.A. & Lipowitz A.J. 1998. Preoperative variables affecting long-term outcome of triple plevic osteotomy for treatment of naturally develping hip dysplasia in dogs. J. Am. Vet. Med. Assoc. 213(1):80-85. PMid:9656029.

Regonato E. 2010. Avaliação clínica e radiográfica da cobertura acetabular à cabeça femoral, após aplicação de cunha sacroilíaca em cães. Doctoral Dissertation, Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal, SP. 74p.

Regonato E., Canola J.C., Chierice G.O. & Padilha Filho J.G. 2009. Avaliação radiográfica da cobertura acetabular à cabeça femoral, após osteotomia tripla e aplicação de cunha sacroilíaca, em pelve de cadáveres de cães. Pesq. Vet. Bras. 29(8):625-631. <http://dx.doi.org/10.1590/S0100-736X2009000800005>

Rocha L.B., Tudury E.A., Roehsig C., Baraúna D., Chioratto R., Araújo F.P. & Kemper B. 2013. Denervação articular coxofemoral em cães com doença articular degenerativa secundária à displasia. Ciênc. Anim. Bras. 14(1):120-134. <http://dx.doi.org/10.5216/cab.v14i1.3528>

Schachner E.R. & Lopez M.J. 2015. Diagnosis, prevention, and management of canine hip dysplasia: a review. Vet. Med. Res. Rep. 6:181-192. <PMid:30101105>

Schrader S.C. 1981. Triple pelvic osteotomy of the pelvis as a treatment for canine hip dysplasia. J. Am. Vet. Med. Assoc. 178(1):39-44. <PMid:7204220>

Slocum B. & Devine T. 1986. Pelvic osteotomy technique for axial rotation of the acetabular segment in dogs. J. Am. Anim. Hosp. Assoc. 22(1):331-338.

Slocum B. & Devine T. 1987. Pelvic osteotomy in the dog as treatment for hip dysplasia. Semin. Vet. Med. Surg., Small Anim. 2(2):107-116. <PMid:3454998>

Slocum B. & Slocum T.D. 1992. Pelvic osteotomy for axial rotation of the acetabular segment in dogs with hip dysplasia. Vet. Clin. N. Am., Small Anim. Pract. 22(3):645-682. <http://dx.doi.org/10.1016/S0195-5616(92)50061-4> <PMid:1604778>

Spadeto Junior O., Faleiros R.F., Alves G.E.S., De Las Casas E.B., Rodrigues L.B. & Loiacono B.Z. 2010. Falhas na utilização de poliacetal e poliamida

em forma de haste intramedular bloqueada para imobilização de fratura femural induzida em bovinos jovens. Ciência Rural 40(4):907-912. <http://dx.doi.org/10.1590/S0103-84782010005000038>

Tomlinson J.L. & Johnson J.A. 2000. Quantification of measurement of femoral head coverage and Norberg angle within and among four breeds of dogs. Am. J. Vet. Res. 61(12):1492-1500. <http://dx.doi.org/10.2460/ajvr.2000.61.1492> <PMid:11131587>

Tomlinson J.L. & Cook J.L. 2002. Effects of degree of acetabular rotation after triple pelvic osteotomy on the position of the femoral head in relationship to the acetabulum. Vet. Surg. 31(1):398-403. <http://dx.doi.org/10.1053/jvet.2002.33598> <PMid:12094355>

Vezzoni A. 2007. Defenition and clinical diagnosis of canine hip dysplasia: early diagnosis and treatment options. Eur. J. Companion Anim. Pract. 17(1):126-132.

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(8):649-654, August 2019
DOI: 10.1590/1678-5150-PVB-6083

Original Article
Small Animal Diseases

Canine monocytic ehrlichiosis in Buenos Aires, Argentina: comparison of serological and molecular assays[1]

Paula L. Martin[3], María N. De Salvo[2], Gabriel L. Cicuttin[2]* and María S. Arauz[3]

ABSTRACT.- Martin P.L., De Salvo M.N., Cicuttin G.L. & Arauz M.S. 2019. **Canine monocytic ehrlichiosis in Buenos Aires, Argentina: comparison of serological and molecular assays.** *Pesquisa Veterinária Brasileira 39(8):649-654.* Laboratorio de Zoonosis Bacterianas y Parasitarias Transmitidas por Vectores, Instituto de Zoonosis Luis Pasteur, Av. Díaz Velez 4821, CABA, CP C1405DCD, Argentina. E-mail: gcicuttin@gmail.com

Canine monocytic ehrlichiosis (CME) is an infectious disease caused by the bacterium *Ehrlichia canis* and transmitted by *Rhipicephalus sanguineus* sensu lato, a tick with worldwide distribution. When not diagnosed and treated early, disease can be severe. Currently, the disease is confirmed by serological or molecular assays. The objective of this study was to compare a serological assay based on immunochromatography (SPEED® EHRLI immunochromatographic test; BVT, France) and a molecular assay (a screening PCR followed by a nested PCR specific for *E. canis*) for the diagnosis of *E. canis* in suspected dogs from Buenos Aires city and southern Greater Buenos Aires, Argentina. Blood samples from 20 clinically healthy dogs (Control Group) and from 80 sick dogs suspected of having CME (Groups 1 to 4) were tested in parallel. Neither the immunochromatographic test nor the PCR assay was able to detect the presence of *E. canis* in the Control Group. In the group which had been previously tested by serology, the agreement between the tests was low (kappa: 0.200), whereas in the group which had been previously tested by PCR, the concordance between the tests was adequate (kappa: 0.650). The concordance between the tests evaluated in the total population studied was moderate (kappa: 0.496). The results of our study suggest that the use of rapid serological tests as a first approach, together with subsequent confirmation by PCR, will improve the diagnosis of CME.

INDEX TERMS: Canine, monocytic ehrlichiosis, Buenos Aires, Argentina, serology, molecular assay, dogs, diagnosis, ehrlichiosis, PCR, bacterioses.

RESUMO.- [Ehrlichiose monocítica canina em Buenos Aires: comparação de testes serológicos e moleculares.] A ehrlichiose monocítica canina (CME) é uma doença infecciosa transmitida pelo carrapato *Rhipicephalus sanguineus* sensu lato com distribuição mundial causada por *Ehrlichia canis*, que pode produzir uma doença grave se não foi diagnosticada e tratada precocemente. A confirmação da doença é feita diretamente pela detecção do DNA fazendo a reação em cadeia da polimerase (PCR) ou indiretamente por métodos sorológicos. O objetivo deste estudo foi comparar o método sorológico baseado na imunocromatografia e a técnica de PCR para o diagnóstico de *E. canis* em cães suspeitos da Cidade de Buenos Aires e da região sul da Grande Buenos Aires. As amostras de sangue de 20 cães clinicamente saudáveis (Grupo Controle) e de 80 cães com suspeita clínica de CME (Grupo 1-4) foram avaliadas em paralelo. O diagnóstico serológico foi feito pelo teste imunocromatográfico SPEED® EHRLI (BVT, França). Para a detecção molecular, foi utilizada uma PCR de triagem para amplificar um fragmento de 345 pb do gene que codifica a subunidade 16S do rRNA da família Anaplasmataceae. As amostras positivas depois foram processadas pela PCR aninhada específica para *E. canis*. No Grupo Controle, a presença de *E. canis* não foi detectada por PCR ou anticorpos específicos com o teste imunocromatográfico. No grupo em que a sorologia foi solicitada inicialmente (1 e 2), a concordância entre os testes foi baixo (kappa: 0,200) enquanto que no grupo onde o teste inicialmente solicitado foi a PCR, a concordância

[1] Received on March 12, 2019.
Accepted for publication on April 7, 2019.

[2] Laboratorio de Zoonosis Bacterianas y Parasitarias Transmitidas por Vectores, Instituto de Zoonosis Luis Pasteur, Av. Díaz Velez 4821, Ciudad Autónoma de Buenos Aires, CP C1405DCD, Argentina. *Corresponding author: gcicuttin@gmail.com

[3] Servicio Central, Laboratorio del Hospital Escuela, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata (UNLP), Av. 60 y 118, CC 296, La Plata, CP 1900, Buenos Aires.

entre os testes era adequado (kappa: 0,650). A concordância entre os testes avaliados na população total estudada foi moderada (kappa: 0,496). Em conclusão, os resultados do nosso estudo sugerem que o uso de testes serológicos rápidos inicialmente, juntamente com a confirmação subsequente por PCR, permitirá melhorar o diagnóstico de CME.

TERMOS DE INDEXAÇÃO: Ehrlichiose monocítica, caninos, Buenos Aires, teste serológico, teste molecular, cães, diagnóstico, ehrlichiose, PCR, bacterioses.

INTRODUCTION

Canine monocytic ehrlichiosis (CME) is a tick-borne disease caused by *Ehrlichia canis*, an obligate intracellular bacterium of the family Anaplasmataceae. This bacterium is mainly transmitted by the tick *Rhipicephalus sanguineus* sensu lato (Dumler et al. 2001, Bremer et al. 2005). In dogs, the clinical presentation of CME can range from mild to severe depending on the immune status of the host, virulence of the strain and co-infection with other microorganisms (Klag et al. 1991, Unver et al. 2009, Rotondano et al. 2015). The course of CME can be divided into three phases: acute, subclinical and chronic. The acute phase is characterized by high fever, depression, lethargy, anorexia, lymphadenomegaly, splenomegaly, hemorrhagic tendencies and ophthalmological signs (Neer et al. 2002, Leiva et al. 2005, Harrus et al. 2012). Untreated dogs and those treated inappropriately may recover clinically but then enter the subclinical phase. In this phase, dogs show no clinical signs but may remain persistent carriers of *E. canis*. In the chronic phase, signs are more severe and infected dogs may be less responsive to therapy (Neer et al. 2002, Harrus et al. 2012). Therefore, early and accurate diagnosis of suspected cases in dogs is essential to alter the course of this disease (Neer et al. 2002).

Currently, diagnosis of CME includes direct (e.g. blood smears and PCR) and indirect (serology) methods (Harrus & Waner 2011). The evaluation of blood smears has low sensitivity and specificity (Mylonakis et al. 2003, Ramos et al. 2009, Harrus et al. 2012). Although this method may be optimized by the examination of multiple buffy coat smears, the presence of platelets, lymphocytic azurophilic granules, and phagocytosed nuclear material may all be confused with ehrlichial inclusions (Harrus & Waner 2011). In contrast, the PCR assay is a sensitive method to detect *E. canis* DNA. Thus, several assays based on different target genes, such as 16S rRNA, *p28*, *p30*, *dsb*, *VirB9*, and *groESL*, have been published (Stich et al. 2002, Labruna et al. 2007, Baneth et al. 2009, Cicuttin et al. 2016). Both conventional and real time PCR have the advantage over serology that they can detect active infection in a single sample (Harrus & Waner 2011, Maggi et al. 2014). However, in animals with subclinical infection, in which *E. canis* persists in the bone marrow or spleen but is below the limit of PCR detection in peripheral blood, these assays could give negative results (Harrus et al. 1998, Otranto et al. 2010). Regarding indirect methods, the indirect immunofluorescence antibody assay is considered the 'gold standard' for detection and titration of *E. canis* antibodies (Waner et al. 2001, Harrus et al. 2002). However, this assay usually presents cross-reactivity between *E. canis* and other ehrlichiae and to confirm recent infection, it should be repeated in 2-3 weeks to demonstrate seroconversion (i.e. a four-fold change in the patient's antibody titer between acute and convalescent serum samples). There are also several commercial serological assays, such as rMAP2 ELISA, InDx Canine Multiassay Dip-S-Ticks (PanBio, InDx), Snap Combo, Snap3Dx (IDEXX Laboratories), Snap 4Dx (IDEXX Laboratories), and SPEED® EHRLI (BVT, France), which have been designed for in-clinic use. However, serological assays are not appropriate to confirm active infection because antibodies can persist for variable intervals after a pathogen is eliminated (Harrus et al. 1998). For a suitable diagnosis of CME and to detect acutely infected dogs before seroconversion and/or sub clinically infected dogs with blood negative PCR, it is thus recommended to use serology in conjunction with molecular detection methods (Çetinkaya et al. 2016).

In Argentina, *E. canis* has only been confirmed in dogs with compatible signs of EMC from Buenos Aires (Eiras et al. 2013, Cicuttin et al. 2016) and *R. sanguineus* ticks from Formosa and Buenos Aires (Cicuttin et al. 2015, Cicuttin et al. 2017). Using molecular assays, Eiras et al. (2013) have detected *E. canis* in 6/86 (7.0%) dogs with suspected blood smear evidence and/or thrombocytopenia from southern Greater Buenos Aires. Cicuttin et al. (2016) in samples of canines with symptoms compatible with EMC of the Metropolitan Area of Buenos Aires described a prevalence of 6.7% (15/223) to *E. canis*. On the other hand, in clinical healthy dogs from Buenos Aires, San Luis, Córdoba and Santa Fe, only the presence of *Anaplasma platys* DNA, but not *E. canis*, was demonstrated (Cicuttin et al. 2011, 2014a, 2014b, 2017a, Mascarelli et al. 2016). Serological studies published in our country are scarce. Mera y Sierra & Neira (2014) found that 46.6% of canines from Mendoza with symptoms compatible were seropositive for *E. canis* with Speed-Ehrli serological assay.

Since comparison between these direct and indirect assays may provide valuable information for the diagnosis of CME in clinical practice, the objective of this study was to compare a serological assay based on immunochromatography with a molecular assay for diagnosis of CME by using samples from healthy dogs and dogs suspected of being infected with CME from Buenos Aires, Argentina.

MATERIALS AND METHODS

Sample collection. Whole blood samples with EDTA anticoagulant received for the diagnosis of CME were kindly provided by two diagnostic centers: "Laboratorio Diagnóstico Veterinario Sur", Quilmes, southern Buenos Aires (a center where dogs are diagnosed by means of a serological assay), and "Instituto de Zoonosis Luis Pasteur", Buenos Aires, Argentina (a center where dogs are diagnosed by means of a molecular assay). Only samples from animals with presumptive clinical diagnosis of CME were selected. In all cases, the samples were stored at -20°C until their processing. In addition, whole blood samples with EDTA from clinically healthy dogs from the same locations were included to establish background exposure or infection (Control Group). The samples from sick dogs were divided into four groups taking into account the result of the serology or PCR assay previously performed at the diagnostic center: Group 1, which consisted of 20 samples in which the serological SPEED® EHRLI assay had been negative for *Ehrlichia canis*; Group 2, which consisted of 20 samples in which the serological SPEED® EHRLI assay had been positive for *E. canis*; Group 3, which consisted of 20 samples in which the PCR assay (screening PCR targeted to the 16S ribosomal RNA and nested PCR targeted to a specific fragment

of the 16S rRNA gene from *E. canis* assays) had been negative for *E. canis*; and Group 4, which consisted of 20 samples in which the PCR assay had been positive for *E. canis*.

Serological assay. The serological diagnosis of the samples obtained from the two diagnostic centers was also performed by the SPEED® EHRLI assay. This commercial assay is based on the detection of anti-*E. canis* antibodies by using an immunochromatographic membrane. The cut-off value from which the assay detects specific antibodies is not specified by the manufacturer. The sensitivity and specificity of the assay is 87% and 95% respectively (Martin 2004).

Whole blood was used according to the manufacturer's instructions. This method was used for the detection of anti-*E. canis* antibodies in the 60 samples corresponding to Groups 3 and 4 and the controls.

Molecular assay. For the molecular assays, DNA was extracted from 200µL of whole blood samples with EDTA anticoagulant, using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. Initial screening for the family Anaplasmataceae was performed with a screening PCR assay targeted to the 16S ribosomal RNA (16S rRNA) gene (Table 1). Reactions were performed in a final volume of 25µL, containing 12.5pmol of each primer. The thermocycling conditions for the reactions were 95°C for 5min, followed by 34 cycles at 95°C for 30 seconds, with annealing at 55°C for 30s, and extension at 72°C for 90s; a final extension step at 72°C for 5min was used. In each PCR reaction, an endogenous control was included to amplify a 289bp region of exon III of the beta-actin gene to evaluate the presence of inhibitors. *Anaplasma centrale* was used as a positive control and nuclease-free water was used as a negative control.

Positive DNAs by screening PCR assay were then analyzed by nested PCR assay to amplify a specific fragment of the 16S rRNA gene from *E. canis* described by Breitschwerdt et al. (1998). Moreover, another nested PCR assay was performed to amplify a fragment of the 16S rRNA gene from *Anaplasma platys* (Kordick et al. 1999). The sequences of these primers are shown in Table 1. These methods were used for the detection of *E. canis* or *A. platys* DNA in the 60 samples corresponding to Groups 1 and 2 and the Controls.

Statistical analysis. Statistical analysis was carried out using Epi Info 7.1.2.0 (Centers for Disease Control and Prevention, Atlanta, USA) and WinEpi (Facultad de Veterinaria, Universidad de Zaragoza, Spain). The concordance between the SPEED®EHRLI serological assay and the PCR was determined by calculating kappa values with 95% confidence intervals. According to the kappa values, the concordance between assays was classified as follows: 0-0.20 indicated poor agreement, 0.21-0.40 indicated fair agreement, 0.41-0.60 indicated moderate agreement, 0.61-0.80 indicated strong agreement, and 0.81-1 indicated almost perfect agreement (Landis & Koch 1977).

RESULTS

The results of the SPEED®EHRLI serological assay versus the PCR-positive samples within each group and the agreement between both assays are summarized in Table 2. The internal control used in the screening PCR assay was positive in all reactions.

The 20 healthy dog blood samples (Control Group) were negative both for the detection of antibodies by the SPEED®EHRLI serological assay and for the detection of DNA by the screening-PCR assay.

In Groups 1 and 2 (patients with presumptive diagnosis of CME in which the assay initially performed was the serology and the PCR assay was retrospectively performed), the concordance between the assays was poor (kappa value: 0.200, 95%, CI -0.021-0.421). In Group 1, the nested PCR specific to *Ehrlichia canis* showed one positive result, whereas one of the samples was positive by the nested PCR specific to *Anaplasma platys*. Five out of the 20 dogs in Group 2 were PCR-positive to *E. canis*.

In Groups 3 and 4 (patients with presumptive diagnosis of CME in which the assay initially performed was the PCR assay and then the serological assay was retrospectively performed), the agreement between the assays was strong (kappa value: 0.650, 95%, CI 0.340-0.960).

Table 1. Primer pairs used in the present study to detect *Ehrlichia canis* and *Anaplasma platys*

Primer pairs	Primer	Sequence (5´3´)	Reference
Family Anaplasmataceae	EHR16S-D	GGTACCYACAGAAGAAGTCC	Parola et al. (2000)
	EHR16S-R	TAGCACTCATCGTTTACAGC	
Outside primers for family Anaplasmataceae	EHR-OUT1	CTGGCGGCAAGCYTAACACATGCCAACATCTCACGAC	Breitschwerdt et al. (1998)
	EHR-OUT2	GCTCGTTGCGGGACTTAACCCAACATCTCACGAC	
Inner primers for *E. canis*	HE3-R	CTTCTATAGGTACCGTCATTATCTTCCCTAT	Breitschwerdt et al. (1998)
	E. canis	CAATTATTTATAGCCTCTGGCTATAGGAA	
Inner primers for *A. platys*	*E. platys*	GAT TTTTGTCGTAGCTTGCTA	Kordick et al. (1999)
	Ehrl3-IP2	TCATCTAATAGCGATAAATC	

Table 2. Results of serology and PCR assays

Groups	No. of dogs	CI positive (%)	PCR positive (%)	Concordance (%)
Control	20	0(0%)	0(0%)	-
1	20	0 (0)	1(5%)	0/1 (0)
2	20	20(100%)	5(25%)	5/20 (25)
3	20	4(20%)	0(0%)	0/4 (0)
4	20	17(85%)	20(100%)	17/20 (85)

DISCUSSION

In South America, the clinical suspicion of CME is increasingly common. However, there is no single method to reach definitive diagnosis of CME and several factors must be taken into account in the interpretation of the results (Waner et al. 2001, Otranto et al. 2009). The moment of sample collection, for example, as well as the sensitivity and diagnostic specificity of the assay used directly influence the final interpretation (Otranto et al. 2009, Harrus et al. 2012). Moreover, the presence of other tick-borne pathogens such as *Hepatozoon* spp. or *Babesia* spp. should be considered together with possible infection with *Ehrlichia canis* (Gal et al. 2007).

In our study, the concordance observed between the SPEED®EHRLI serological assay and the PCR assay used in the total dog population was moderate, in agreement with that reported by other authors (Maggi et al. 2014). All dogs included in the Control Group (exposed, without CME signs) were negative both by PCR and the SPEED®EHRLI serological assay. These findings coincide with previous reports from other authors in which no DNA of *E. canis* was detected in samples obtained from clinically healthy dogs (Cicuttin et al. 2015, Mascarelli et al. 2016). However, some studies carried out in Buenos Aires city have reported a prevalence value of 13.5 for *Anaplasma platys* (Cicuttin et al. 2011, 2014a). These differences in the prevalence of *A. platys* are probably due, among other factors, to the area from which the samples were obtained, the time of year in which the study was conducted, and the number of animals sampled. The absence of *E. canis* in dogs of the Control Group can be explained if we consider that only *Rhipicephalus sanguineus* of the temperate lineage has been detected in the area in which the sampling was carried out and as mentioned, the epidemiological and experimental studies relate *E. canis* with the tropical lineage and not with the temperate one (Moraes-Filho et al. 2015). The finding of animals with active infection (detected by PCR) raises the question of the mode of transmission in these cases. A possible explanation that could clarify this topic is that the dogs could have acquired the infection in other areas or that *R. sanguineus* of the temperate lineage would present low, but not null, vector capacity to transmit *E. canis* (Cicuttin et al. 2016).

With respect to the groups of patients in which the assay initially performed was the serology and the PCR assay was retrospectively performed (Groups 1 and 2), we observed poor concordance between the assays evaluated. The finding of a positive dog by PCR and negative by the SPEED®EHRLI serological assay could reflect the onset of the disease with presence of bacteremia and absence of detectable antibodies (Iqbal et al. 1994). It should be noted that the use of PCR for screening (combined with the specific nested PCR) in this group of samples also allowed detecting infection with *A. platys* in a patient in whom the serology had been negative. On the other hand, only five dogs of Group 3 were also positive by PCR, which may be due to several factors. First, the SPEED®EHRLI serological assay may give false positive results in animals that maintain detectable antibody titers subsequent to recovery from infection or that have been exposed to the agent (Iqbal et al. 1994, Wen et al. 1997, Harrus et al. 1998, Çetinkaya et al. 2016). It should also be considered that there are cross reactions between *E. canis* and other strains of *Ehrlichia* (which circulate in ticks of our country), as well as between *E. canis* and *A. platys*, although to a lesser extent (Dumler et al. 2001, Harrus et al. 2012). Secondly, the analysis of samples from patients who had received treatment with antibiotics (which is not discriminated in the protocol for sending samples to our lab) decreases or eliminates the number of circulating microorganisms in the blood, causing false negative results by the PCR assay. Likewise, some authors have mentioned that the persistence of *E. canis* in bone marrow or spleen (chronic phase of CME) with low levels of microorganisms in peripheral blood could result in DNA concentrations below the limit of detection of the PCR (Otranto et al. 2010, Harrus et al. 2012). Finally, the presence of inhibitors of the enzyme polymerase in whole blood samples can affect the amplification and efficiency of the PCR assay, causing false negative results (Harrus & Waner 2011). In our study, an endogenous amplification control, which allowed us to monitor if the reaction was inhibited, was included in each PCR reaction.

Groups 3 and 4, in which the assay initially performed was the PCR and the serological assay was retrospectively performed, showed a strong concordance between both assays. Samples negative for PCR but positive for the SPEED®EHRLI serological assay obtained in Group 4 may be due to the mentioned factors, whereas samples positive for PCR and negative for SPEED®EHRLI may have been extracted in the acute stage of the disease before the immune system developed a detectable antibody response (Harrus et al. 1998). In this context, when rapid qualitative serological assays are used, it is important to consider the cut-off point defined by the manufacturer. For example, the Snap 4D plus kit (IDEXX) was standardized for the detection of an antibody titer above 1/160. Therefore, any sample that shows antibody levels below that value will give a negative result (Harrus et al. 2012). The cut-off value for the SPEED®EHRLI serological assay is not specified; however, it is possible that it is in a similar range. According to previous studies, the methods based on ELISA or immunochromatography are able to differentiate seronegative and seropositive animals when the antibody titer is ≥1/320, while lower titers could cause false negative results (Waner et al. 2000).

CONCLUSIONS

The results of our study suggest that the combination of molecular and serological methods increases certainty in the diagnosis of CME. However, it is important to note that serological diagnosis is not conclusive in an endemic area for CME because these assays do not differentiate between infection and exposure to the microorganism. Therefore, the use of rapid serological assays as initial screening together with the subsequent confirmation by PCR seem to be useful diagnostic tools in the confirmation of EMC in dogs.

Moreover, to suitable diagnosis of EMC clinicians should take into account epidemiological data, clinical signs, laboratory test results and possible co-infections with other tick-borne pathogens transmitted by *Rhipicephalus sanguineus* ticks.

Acknowledgements.- We thank Dr. Fontana Lorena from the "Laboratorio Veterinario Diagnóstico Sur" and colleagues at "Instituto de Zoonosis Luis Pasteur and Hospital Escuela (Facultad de Ciencias Veterinarias) Universidad Nacional de La Plata" for submitted samples.

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

REFERENCES

Baneth G., Harrus S., Ohnona F.S. & Schlesinger Y. 2009. Longitudinal quantification of *Ehrlichia canis* in experimental infection with comparison to natural infection. Vet. Microbiol. 136(3/4):321-325. <http://dx.doi.org/10.1016/j.vetmic.2008.11.022> <PMid:19128893>

Breitschwerdt E.B., Hegarty B.C., Hancock S.I. & Carolina N. 1998. Sequential evaluation of dogs naturally infected with *Ehrlichia canis, Ehrlichia chaffeensis, Ehrlichia equi, Ehrlichia ewingii*, or *Bartonella vinsonii.* J. Clin. Microbiol. 36(9):2645-2651. <PMid:9705408>

Bremer W.G., Schaefer J.J., Wagner E.R., Ewing S.A., Rikihisa Y., Needham G.R., Jittapalapong S., Moore D.L. & Stich R.W. 2005. Transstadial and intrastadial experimental transmission of *Ehrlichia canis* by male *Rhipicephalus sanguineus.* Vet. Parasitol. 131(1/2):95-105. <http://dx.doi.org/10.1016/j.vetpar.2005.04.030> <PMid:15941624>

Çetinkaya H., Matur E., Akyazi İ., Ekiz E.E., Aydin L. & Toparlak M. 2016. Serological and molecular investigation of *Ehrlichia* spp. and *Anaplasma* spp. in ticks and blood of dogs, in the Thrace Region of Turkey. Ticks Tick Borne Dis. 7(5):706-714. <http://dx.doi.org/10.1016/j.ttbdis.2016.02.021> <PMid:26961274>

Cicuttin G.L., Navarro O., Connor M., Lobo B. & Jado I. 2011. Evidencia molecular de *Anaplasma platys* en caninos domésticos de la Ciudad Autónoma de Buenos Aires. Revta FAVE Ciênc. Vet. 10(2):19-23.

Cicuttin G.L., Brambati D.F., Rodríguez Eugui J.I., Lebrero C.G., Salvo M.N., Beltrán F.J., Gury Dohmen F.E., Jado I. & Anda P. 2014a. Molecular characterization of *Rickettsia massiliae* and *Anaplasma platys* infecting *Rhipicephalus sanguineus* ticks and domestic dogs, Buenos Aires (Argentina). Ticks Tick Borne Dis. 5(5):484-488. <http://dx.doi.org/10.1016/j.ttbdis.2014.03.001> <PMid:24907186>

Cicuttin G.L., Vidal P., Nazarena De Salvo M., Beltrán F.J. & Gury Dohmen F.E. 2014b. Detección molecular de *Rickettsia massiliae* y *Anaplasma platys* en garrapatas *Rhipicephalus sanguineus* y caninos domésticos del municipio de Bahía Blanca (Argentina). Revta Chil. Infectol. 31(5):563-568. <http://dx.doi.org/10.4067/S0716-10182014000500008> <PMid:25491455>

Cicuttin G.L., Tarragona E.L., De Salvo M.N., Mangold A.J. & Nava S. 2015. Infection with *Ehrlichia canis* and *Anaplasma platys* (Rickettsiales: Anaplasmataceae) in two lineages of *Rhipicephalus sanguineus* sensu lato (Acari: Ixodidae) from Argentina. Ticks Tick Borne Dis. 6(6):724-729. <http://dx.doi.org/10.1016/j.ttbdis.2015.06.006> <PMid:26100492>

Cicuttin G.L., De Salvo M.N. & Gury Dohmen F.E. 2016. Molecular characterization of *Ehrlichia canis* infecting dogs, Buenos Aires. Ticks Tick Borne Dis. 7(5):954-957. <http://dx.doi.org/10.1016/j.ttbdis.2016.04.017> <PMid:27236582>

Cicuttin G.L., De Salvo M.N. & Nava S. 2017a. Two novel *Ehrlichia* strains detected in *Amblyomma tigrinum* ticks associated to dogs in peri-urban areas of Argentina. Comp. Immunol. Microbiol. Infect. Dis. 53:40-44. <http://dx.doi.org/10.1016/j.cimid.2017.07.001> <PMid:28750866>.

Cicuttin G.L., De Salvo M.N., Silva D.A., Brito M. & Nava S. 2017b. *Ehrlichia canis* (Rickettsiales: Anaplasmataceae) en garrapatas *Rhipicephalus sanguineus* sensu lato del linaje templado (Acari: Ixodidae), provincia de Buenos Aires, Argentina. Revta FAVE. Ciênc. Vet. 16:93-96.

Dumler J.S., Barbet A.F., Bekker C.P.J., Dasch G.A., Palmer G.H., Ray S.C., Rikihisa Y. & Rurangirwa F.R. 2001. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma, Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, description of six new species conmbinations and designation of *Ehrlichia equi* and "HE agent" as subjective synonyms of *Ehrlichia phagocytophila.* Int. J. Syst. Evol. Microbiol. 51(6):2145-2165. <http://dx.doi.org/10.1099/00207713-51-6-2145> <PMid:11760958>

Eiras D.F., Craviotto M.B., Vezzani D., Eyal O. & Baneth G. 2013. First description of natural *Ehrlichia canis* and *Anaplasma platys* infections in dogs from Argentina. Comp. Immunol. Microbiol. Infect. Dis. 36(2):169-173. <http://dx.doi.org/10.1016/j.cimid.2012.11.008> <PMid:23273677>

Gal A., Harrus S., Arcoh I., Lavy E., Aizenberg I., Mekuzas-Yisaschar Y. & Baneth G. 2007. Coinfection with multiple tick-borne and intestinal parasites in a 6-week-old dog. Can. Vet. J. 48(6):619-622. <PMid:17616060>

Harrus S. & Waner T. 2011. Diagnosis of canine monocytotropic ehrlichiosis (*Ehrlichia canis*): an overview. Vet. J. 187(3):292-296. <http://dx.doi.org/10.1016/j.tvjl.2010.02.001> <PMid:20226700>

Harrus S., Waner T. & Neer T.M. 2012. *Ehrlichia canis* infection, p.227-238. In: Greene C.E. (Ed.), Infectious Diseases of the Dog and Cat. Elsevier, Missouri.

Harrus S., Waner T., Aizenberg J., Foley J., Poland A. & Bark H. 1998. Amplification of ehrlichal DNA from dogs 34 months after infection with *Ehrlicia canis.* J. Clin. Microbiol. 36(1):73-76. <PMid:9431923>

Harrus S., Alleman A.R., Bark H., Mahan S.M. & Waner T. 2002. Comparison of three enzyme-linked immunosorbant assays with the indirect immunofluorescent antibody test for the diagnosis of canine infection with *Ehrlichia canis.* Vet. Microbiol. 86(4):361-368. <http://dx.doi.org/10.1016/S0378-1135(02)00022-6> <PMid:11955786>

Iqbal Z., Chaichanasiriwithaya W. & Rikihisa Y. 1994. Comparison of PCR with other tests for early diagnosis of canine ehrlichiosis. J. Clin. Microbiol. 32(7):1658-1662. <PMid:7929754>

Klag A.R., Dunbar L.E. & Girard C.A. 1991. Concurrent ehrlichiosis and babesiosis in a dog. Can. Vet. J. 32(5):305-307. <PMid:17423787>

Kordick S.K., Breitschwerdt E.B., Hegarty B.C., Southwick K.L., Colitz C.M., Hancock S.I., Bradley J.M., Rumbough R., Mcpherson J.T. & MacCormack J.N. 1999. Coinfection with multiple tick-borne pathogens in a Walker Hound kennel in North Carolina. J. Clin. Microbiol. 37(8):2631-2638. <PMid:10405413>

Labruna M.B., McBride J.W., Camargo L.M., Aguiar D.M., Yabsley M.J., Davidson W.R., Stromdahl E.Y., Williamson P.C., Stich R.W., Long S.W., Camargo E.P. & Walker D.H. 2007. A preliminary investigation of *Ehrlichia* species in ticks, humans, dogs, and capybaras from Brazil. Vet. Parasitol. 143(2):189-195. <http://dx.doi.org/10.1016/j.vetpar.2006.08.005> <PMid:16962245>

Landis J.R. & Koch G.G. 1977. The measurement of observer agreement for categorical data. Biometrics 33(1):159-174. <http://dx.doi.org/10.2307/2529310> <PMid:843571>

Leiva M., Naranjo C. & Peña M.T. 2005. Ocular signs of canine monocytic ehrlichiosis: a retrospective study in dogs from Barcelona, Spain. Vet. Ophthalmol. 8(6):387-393. <http://dx.doi.org/10.1111/j.1463-5224.2005.00409.x> <PMid:16359361>

Maggi R.G., Birkenheuer A.J., Hegarty B.C., Bradley J.M., Levy M.G. & Breitschwerdt E. 2014. Comparison of serological and molecular panels for diagnosis of vector-borne diseases in dogs. Parasit. Vectors 7(1):127. <http://dx.doi.org/10.1186/1756-3305-7-127> <PMid:24670154>

Martin C. 2004. Les Ehrlichioses du chien, étude bibliographique, diagnostic et comparaison de trois kits de diagnostic serologique rapide de l'ehrlichiose monocytaire. Doctoral Dissertation, Université Claude-Bernard, Lyon.

Mascarelli P.E., Tartara G.P., Pereyra N.B. & Maggi R.G. 2016. Detection of *Mycoplasma haemocanis, Mycoplasma haematoparvum, Mycoplasma suis* and other vector-borne pathogens in dogs from Córdoba and Santa Fe, Argentina. Parasit. Vectors. 9(1):642. <http://dx.doi.org/10.1186/s13071-016-1920-8> <PMid:27978844>

Mera y Sierra R. & Neira G. 2014. Ocurrencia de *Ehrlichia canis* en caninos de la provincia de Mendoza. III Congreso Panamericano de Zoonosis and VIII Congreso Argentino de Zoonosis, La Plata, Argentina.

Moraes-Filho J., Krawczak F.S., Costa F.B., Soares J.F. & Labruna M.B. 2015. Comparative evaluation of the vector competence of four south American populations of the Rhipicephalus sanguineus group for the bacterium *Ehrlichia canis*, the agent of canine monocytic ehrlichiosis. PLoS One 10(9):e0139386. <http://dx.doi.org/10.1371/journal.pone.0139386> <PMid:26414283>

Mylonakis M.E., Koutinas A.F., Billinis C., Leontides L.S., Kontos V., Papadopoulos O., Rallis T. & Fytianou A. 2003. Evaluation of cytology in the diagnosis

of acute canine monocytic ehrlichiosis (*Ehrlichia canis*): a comparison between five methods. Vet. Microbiol. 91(2/3):197-204. <http://dx.doi.org/10.1016/S0378-1135(02)00298-5> <PMid:12458168>

Neer T.M., Breitschwerdt E.B., Greene R.T. & Lappin M.R. 2002. Consensus statement on ehrlichial disease of small animals from the infectious disease study group of the American College of Veterinary Internal Medicine. J. Vet. Intern. Med. 16:309-315. <PMid:12041661>

Otranto D., Dantas Torres F. & Breitschwerdt E. 2009. Managing canine vector-borne diseases of zoonotic concern: part one. Trends Parasitol. 25(4):157-163. <http://dx.doi.org/10.1016/j.pt.2009.01.003> <PMid:19269898>

Otranto D., Testini G., Dantas-Torres F., Latrofa M.S., Diniz P.P., Caprariis D., Lia R.P., Mencke N., Stanneck D., Capelli G. & Breitschwerdt E.B. 2010. Diagnosis of canine vector-borne diseases in young dogs: a longitudinal study. J. Clin. Microbiol. 48(9):3316-3324. <http://dx.doi.org/10.1128/JCM.00379-10> <PMid:20660218>

Parola P., Roux V., Camicas J.L., Baradji I., Brouqui P. & Raoult D. 2000. Detection of ehrlichiae in African ticks by polymerase chain reaction. Trans. R. Soc. Trop. Med. Hyg. 94(6):707-709. <http://dx.doi.org/10.1016/S0035-9203(00)90243-8> <PMid:11198664>

Ramos C.A.N., Ramos R.A.N., Araújo F.R., Guedes Junior D.S., Souza I.I.F., Ono T.M., Vieira A.S., Pimentel D.S., Rosas E.O., Faustino M.A.G. & Alves L.C. 2009. Comparação de nested-PCR com o diagnóstico direto na detecção de *Ehrlichia canis* e *Anaplasma platys* em cães. Revta Bras. Parasitol. Vet. 18(Suppl.1):58-62. <http://dx.doi.org/10.4322/rbpv.018e1011> <PMid:20040193>

Rotondano T.E., Almeida H.K., Krawczak F.S., Santana V.L., Vidal I.F., Labruna M.B., Azevedo S.S., Adelmeida A.M. & Melo M.A. 2015. Survey of *Ehrlichia canis, Babesia* spp. and *Hepatozoon* spp. in dogs from a semiarid region of Brazil. Revta Bras. Parasitol. Vet. 24(1):52-58. <http://dx.doi.org/10.1590/S1984-29612015011> <PMid:25909253>

Stich R.W., Rikihisa Y., Ewing S.A., Needham G.R., Grover D.L. & Jittapalapong S. 2002. Detection of *Ehrlichia canis* in canine carrier blood and in individual experimentally infected ticks with a p30-based PCR assay. J. Clin. Microbiol. 40(2):540-546. <http://dx.doi.org/10.1128/JCM.40.2.540-546.2002> <PMid:11825969>

Unver A., Rikihisa Y., Karaman M. & Ozen H. 2009. An acute severe ehrlichiosis in a dog experimentally infected with a new virulent strain of *Ehrlichia canis.* Clin. Microbiol. Infect. 15(Suppl.2):59-61. <http://dx.doi.org/10.1111/j.1469-0691.2008.02634.x> <PMid:19548986>

Waner T., Strenger C. & Keysary A. 2000. Comparison of a clinic-based ELISA test kit with the immunofluorescence test for the assay of *Ehrlichia canis* antibodies in dogs. J. Vet. Diagn. Invest. 12(3):240-244. <http://dx.doi.org/10.1177/104063870001200307> <PMid:10826837>

Waner T., Harrus S., Jongejan F., Bark H., Keysary A. & Cornelissen A.W. 2001. Significance of serological testing for ehrlichial diseases in dogs with special emphasis on the diagnosis of canine monocytic ehrlichiosis caused by *Ehrlichia canis.* Vet. Parasitol. 95(1):1-15. <http://dx.doi.org/10.1016/S0304-4017(00)00407-6> <PMid:11163693>

Wen B., Rikihisa Y., Mott J.M., Greene R., Kim H.Y., Zhi N., Couto G.C., Unver A. & Bartsch R. 1997. Comparison of nested PCR with immunofluorescent-antibody assay for detection of *Ehrlichia canis* infection in dogs treated with doxycycline. J. Clin. Microbiol. 35(7):1852-1855. <PMid:9196207>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(8):655-662, August 2019
DOI: 10.1590/1678-5150-PVB-5096

Original Article
Wildlife Medicine

Influence of social stress on value of minimal anesthetic concentration of isuflurano in guan (*Penelope obscura*) captured in wildlife[1]

Bruno Lunardeli[2]*, Aury N. Moraes[3], Eloisa C. Bach[3], Cristiana C. Kuci[3], Átila Costa[4], Adson Costa[3], Maria H.M. Baldini[3] and Marzia Antonelli[3]

ABSTRACT.- Lunardeli B., Moraes A.N., Bach E.C., Kuci C.C., Costa A., Costa A., Baldini M.H.M. & Antonelli M. 2019. **Influence of social stress on minimal anesthetic concentration value of isuflurano in guan (*Penelope obscura*) captured in wildlife.** *Pesquisa Veterinária Brasileira 39(8):655-662.* Universidade do Estado de Santa Catarina, Av. Luiz de Camões 2090, Conta Dinheiro, Lages, SC 88520-000, Brazil. E-mail: brunoo_lunardeli@hotmail.com

This study aimed to determine and evaluate whether there are differences in values of MAC in wildlife captured guan (*Penelope obscura*) under different conditions of social stress. This study used 27 bird species guan (*P. obscura*), divided into two groups: animals kept in the same enclosure (Collective Group) and animals kept in individual cages (Individual Group). The research was conducted at the Advanced Research Base of IBAMA, Painel/SC, and at the Clinical Veterinary Hospital of the "Universidade do Estado de Santa Catarina", Lages/SC. The birds were captured using network trap with manual disarmament and transported to the Veterinary Hospital in cages. The animals were fasted for 2 to 6 hours before the procedure; anesthetic induction was performed with isoflurane for instrumentation. The isoflurane CAM was placed in the target value 1.3v% in the first animal of each group, and waited 15 minutes for the nociceptive (electric) stimulus, in the value of 50 hertz and 50mA, held in faradic form (3 consecutive simple stimuli, followed by 2 continuous stimuli). The stimulus was performed on the lateral side of the left pelvic limb in the tibiotarsal region, and the electrodes were fixed with a 22G needle at a distance of 5cm between them. The bird's responses to the stimulus were considered positive (wing movements, head or vocalization) or negative (not presented movements of wings, head or vocalization) and the MAC value of the animal was recorded. Negative responses reduced next birds' MAC value by about 10%, and MAC positive responses increased by about 10%. Statistical analysis was done by methods up and down and analyze quantal for MAC and paired t-test for equivalent or t-test for variances not equal variances for the physiological variables. At the end of the experiment, the birds were reintroduced in the same capture area. The MAC value of isoflurane in the Collective Group was 1.4v% and the Individual Group 1.9v% to 0.903atm. It is observed that guan (*P. obscura*) in the Collective Group showed lesser anesthetic resistance to isoflurane than the birds in the Individual Group, showing that some levels of social stress can influence the MAC values of the isoflurane.

INDEX TERMS: Social stress, minimal anesthetic concentration, isuflurano, guan, *Penelope obscura*, life free, MAC, anesthesia, wildlife animals, conservation.

[1] Received on October 26, 2018.
Accepted for publication on April 9, 2019.

[2] Departamento de Medicina Veterinária, Universidade Federal do Paraná (UFPR), Rua dos Funcionários 1540, Juveve, Curitiba, PR 80035-050, Brazil. *Corresponding author: brunoo_lunardeli@hotmail.com

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

[4] Departamento de Medicina Veterinária, Centro Universitário Unifacvest, Av. Marechal Floriano 947, Centro, Lages, SC 88501-103.

RESUMO.- [**Influência do estresse social sobre o valor da concentração anestésica mínima de isuflurano em jacus (*Penelope obscura*) capturados em vida livre.**] Este estudo teve como objetivo determinar e avaliar se há diferença nos valores de CAM em jacus (*Penelope obscura*) capturados em vida livre e submetidos a diferentes condições de estresse social. Foram utilizadas 27 aves da espécie jacu (*P. obscura*) de vida livre, que depois de capturados foram alocados em dois grupos: 10 animais que permaneceram em grupo no mesmo

recinto (Grupo Coletivo) e 17 animais que permaneceram em gaiolas individuais (Grupo Individual). A pesquisa foi realizada na Base de Pesquisa Avançada do IBAMA, Painel/SC, e no Hospital de Clínica Veterinária da Universidade do Estado de Santa Catarina, Lages/SC. A captura foi realizada utilizando armadilha de rede com desarmamento manual e as aves foram transportadas para o Hospital Veterinário em gaiolas. Os animais foram submetidos a um jejum alimentar de 2 a 6 horas antes do procedimento, a indução anestésica foi realizada com isoflurano para instrumentação. A CAM de isoflurano foi colocada no valor alvo 1,3v% no primeiro animal de cada grupo, e esperado 15 minutos para realização do estímulo nociceptivo (elétrico), no valor de 50 hertz e 50mA, realizado de forma farádica (3 estímulos simples consecutivos, seguidos de 2 estímulos contínuos). O estímulo foi realizado na face lateral do membro pélvico esquerdo na região tibiotársica, e os eletrodos fixados com agulha 22G a uma distância de 5cm entre elas. A resposta da ave ao estímulo foi considerada positiva (movimentos de asas, cabeça ou vocalização) ou negativa (não apresentou movimentos de asas, cabeça ou vocalização) e o valor de CAM do animal foi registrado. Para resultados negativos, a CAM da próxima ave foi reduzida em torno de 10%, para positivos a CAM foi aumentada em torno de 10%. A análise estatística foi feita pelos métodos *up and down* e análise quantal para a CAM e teste t de pareado para variâncias equivalentes ou teste t para variâncias não equivalentes para as variáveis fisiológicas. Ao final do experimento as aves utilizadas foram reintroduzidas na mesma área de captura. O valor da CAM de isoflurano no Grupo Coletivo foi de 1,4v% e no Grupo Individual a CAM de 1,9v% a 0,903atm, sendo o valor do Grupo Coletivo significativamente menor que o Grupo Individual. Observa-se assim que os jacus (*P. obscura*) que permaneceram em recinto coletivo apresentaram uma menor resistência anestésica ao isoflurano que as aves que permaneceram em recintos individuais, mostrando que alguns níveis de estresse social como os observados aqui podem influenciar sobre os valores da CAM do isoflurano.

TERMOS DE INDEXAÇÃO: Estresse social, concentração anestésica mínima, isuflurano, jacu, *Penelope obscura*, vida livre, CAM, anestesia, animais silvestres, conservação.

INTRODUCTION

Guan (*Penelope obscura* Temminck, 1815) belongs to the Galliform order, which includes 62 genera and 215 species and its representatives are known as fowl having cosmopolitan distribution (except Antarctica). It is divided into five families: Cracidae, Megapodiidae, Phasianidae, Numinidae, and Odontophoridae (Marques 2014). The Cracidae family will be addressed in this study.

Generally, in the capture and anesthesia of free-living animals, the biological materials collection (blood) for laboratory analysis and a pre-anesthetic physical evaluation is not possible. Patients' physical condition is not correctly assessed, and animals are usually assumed to be healthy (Caulkett & Arnemo 2015). Given the challenges found during wildlife animal capture, their morbidity and mortality can be high and harmful for the people involved in the capture process. The most common problem is the weather condition, which dictates whether capture is possible or not (Caulkett & Arnemo 2015).

The anatomy and physiology of birds have some considerable differences, such as their respiratory system, which is composed of two separate and distinct functional components: a ventilation component (airways, air sacs, pneumatic skeleton, and breathing muscles), and gas exchange (pulmonary parabronchi) (Ludders & Matthews 2007). Airflow through the lungs is unidirectional unlike mammalian "in and out" (Scheid & Piiper 1972). Birds have central carbon dioxide (PCO2) sensitive central chemoreceptors, and many peripheral chemoreceptors similar to mammalians (Gleeson 1989). For bird anesthesia, it is important to obtain information on the respiratory and cardiovascular systems, as they tend to be sources of frequent problems in the design and implementation of anesthetic protocols for them (Ludders 2015a, 2015b).

The term "minimal alveolar concentration" (MAC) is not appropriate for discussions about inhaled anesthesia in birds because it presupposes the absence of an alveolar lung. Therefore, MAC in birds has been defined as the minimum anesthetic concentration to avoid intentional and crude movement in a bird subjected to painful stimuli (Ludders et al. 1989). Isoflurane is considered the most volatile anesthetic for use in poultry, but sevoflurane may be used because of its faster induction and recovery characteristics (Granone et al. 2012).

Electrical stimulation was first described by Eger 2nd et al. (1965) to determine the MAC. Currently, electrical stimulation has been used more frequently, varying its intensity in 50Hz, 50mA and 10ms (Costa 2009, Escobar 2010, Pavez et al. 2011). It is considered a nociceptive stimulus of higher intensity (supramaximal) than the clamping method as demonstrated by Costa (2009), who compared the two stimuli in chickens and observed that in the digital clamping method, the isoflurane MAC was 1.11v%. With electrical stimulation with hypodermic needles, MAC was 1.47v%, and repeated mechanical stimuli at the same site may lead to hyperalgesia (Hellebrekers 2002, Hellyer et al. 2002).

The aim of this study was to determine the MAC value of isoflurane in free-living captured guans (*Penelope obscura*) and to assess whether there is a difference in MAC values in individuals of this species subjected to two different situations of social stress, housed individually or in groups.

MATERIALS AND METHODS

This study was analyzed and approved by the Animal Experimentation Ethics Committee of the Center for Agricultural Sciences of "Universidade do Estado de Santa Catarina" (CETEA-CAV-UDESC), protocol number 04/2013, and by the Chico Mendes Institute for Biodiversity Conservation (ICMBio), through the Biodiversity Authorization and Information System (SISBIO), protocol number 38776-1. Part of the project was carried out at IBAMA's Advanced Research Base in Panel, Santa Catarina state, and at the Veterinary Clinics Hospital (HCV-CAV-UDESC) in the city of Lages, Santa Catarina state.

Animals. The animals were captured in the IBAMA Advanced Research Base, after a period of conditioning of the birds using cornmeal for two months. The day before the experiment they were transported to the HCV in cages, where the animals were evaluated through biological material collection (blood, feathers, and feces) and physical examination (heart rate, respiratory rate, temperature, weight, level of hydration) to confirm their health.

A total of 27 birds were captured with the aid of a handcrafted 12 mm mesh trap and manual disarming and divided into two groups.

The captures were taken in the twilight period, when the birds are most active and used to receiving cornmeal.

Collective Group. It was performed in the first phase of the study. In this group, 10 birds were used. They were captured and left in a group in the same enclosure of dimensions 2×3×3 meters (width × length × height) at IBAMA Advanced Research Base during one day and transported individually to the CAV-UDESC Veterinary Clinic Hospital, where they were placed in a group again in a room of the same size and isolated by a visual barrier.

Individual Group. It was performed in the second phase of the study. There were 17 birds used in this group. After capture, each of them was accommodated in a cage of 0.7×1×0.5 meters (width × length × height) with a visual barrier, not allowing them to see other birds and the environment. They remained there until the time of the experiment, which ranged from 24 to 48 hours.

The feathers taken from the birds were used for ectoparasite research and for sex evaluation. The sexing was performed by polymerase chain reaction (PCR) of the feather samples with the cannons and epithelial tissue for the presence of nucleated cells, and sent to a private laboratory (São Camilo Laboratory, Maringá, Paraná state).

Blood samples were collected under physical restraint by the puncture of the right jugular vein, right or left brachial vein. Approximately 3 ml of blood from each animal were collected, stored in heparin tubes and 3% EDTA tubes, for hematimetric analyzes (erythrogram, leukogram, thrombocyte count and total plasma protein).

Stool samples were collected at the bottom of the enclosures and sent for co-parasitological exams at the institution's parasitology laboratory.

All birds were held captive for a total period of 3 to 4 days in both groups. The experiment period was from June 7, 2014 to August 8, 2014. The experiment was always performed on weekends, when the presence of people at HCV was low. The period was chosen because it was outside the breeding season of the birds and without dependent parent chicks.

Unhealthy birds had pre-experiment physical and laboratory examination (blood count) values outside the reference values and were excluded from the study.

Experimental design. The animals went through fasting that ranged from 2 to 6 hours. This variation in fasting time occurred because the food was taken from the animals at the same time, but they were randomly selected for the procedure, so the first animals had a shorter fasting time than the last birds. After evaluating the pre-anesthetic parameters of animals under physical restraint (heart rate - HR, respiratory rate - ƒ, temperature in degrees celsius - ToC, degree of hydration), the anesthesia was induced with the aid of a calibrated vaporizer[1] for isoflurane and neonatal mask adapted to a latex glove to prevent leakage, with isoflurane[2] initially at 2 v% and increasing 1 v% per minute to 4 v%, and solubilized at 100 v% oxygen at a flow rate of 3 L min^{-1}. After the loss of ocular and laryngotracheal reflexes was detected, the bird was intubated with a slightly inflated Pean Murphy 3.0 endotracheal tube cuff.

Anesthesia was maintained through an open circuit without gas rebreathing. A gas analyzer[3] was used to determine end-expiratory isoflurane concentration (EtISO) and end-expiratory carbon dioxide concentration (EtCO2), and assisted ventilation was performed on birds with $EtCO_2$ values below 35 or above 45, with an average of 6 to 10 movements per minute, aiming to keep the birds in normocapnia.

After induction, the isoflurane concentration was reduced to 2.5 v% for poultry instrumentation (pacing needle for stimulation, vascular Doppler[4], blood pressure cuff[5], multiparameter monitor pulse oximeter[6] placed on the barb). Then, it was reduced to isoflurane target MAC, which in the first animal of each group was 1.3 v%.

After the instrumentation, there was a waiting period of 15 minutes to obtain the balance between parabronchial concentration, blood and cerebral circulation of the anesthetic. At this moment (M0), the following parameters were noted: systolic blood pressure (SBP), HR, ƒ, T °C, arterial blood bicarbonate ion concentration (HCO^{-3}), arterial blood oxygen pressure (PaO_2), carbon dioxide pressure in arterial blood ($PaCO_2$), $EtCO_2$ and EtISO, to perform nociceptive stimulation. After the last stimulus or movement of the bird, the parameters at M1 were reevaluated: SBP, HR, ƒ, T °C, $EtCO_2$, EtISO and tabulated.

The nociceptive stimulus used was the electrical stimulus. It was performed using a neurostimulator[7] in the pharydic mode, being three simple stimuli with a 5-second interval followed by two 5-second long and 5-second long stimuli. Its intensity was 50mA, 50 hertz, and it was performed on the lateral face of the leg (tibiotarsus), with 25×0.7mm hypodermic needles transfixed on the skin, at the level of the fibular and superficial fibular nerve, at a distance of 5cm from each other.

The answer to the stimulus was considered positive or negative. The negative answer was considered when there was no head lift, wing flap or vocalization, and a positive answer was considered when one of the described events occurred. If not, the isoflurane concentration was reduced by about 10v% of the initial concentration and the procedure was repeated in the next bird. This procedure was performed until one bird answered to the painful stimulus (positive answer), in which the isoflurane concentration was increased by about 10v% in the next bird.

Systolic blood pressure was noninvasively measured by spectral Doppler, manometer and cuff (2.5 or 3). The HR was measured using a Doppler on the medial face of the contralateral pelvic limb stimulated above the tibiotarsicametatarsal joint, at 5-minute periods and at the pre-stimulus moment. The ƒ, $EtCO_2$, and EtISO were evaluated by the gas analyzer and the PaO_2, $PaCO_2$, HCO^{-3} were evaluated by collecting dorsal metatarsal artery arterial blood with a heparinized 1mL syringe with sodium heparin[8] and processed in a blood gas device[9].

The temperature was measured full time using an esophageal thermometer connected to a multiparametric monitor maintained from 39°C to 41.5°C using a thermal blanket[10]. The room temperature was kept close to 22°C with a room climate control.

At the end of the study, all birds were released at the same place where they were caught.

Statistical analysis. The values of the pre-stimulus and post-stimulus physiological variables were paired and submitted to the paired t-test, and presented as mean ± standard deviation (SD). The differences between the Collective and Individual Groups were submitted to the

1 Calibrated vaporizer. Fortec®, Cyprane, Keighle Yorkshire, England.
2 Isoflurane. Isoflurine® Cristália, São Paulo, Brazil.
3 Gas analyzer. Poet® IQ2, Criticare, USA.
4 Spectral Doppler. Doppler Parks Medical Eletronics® 812, Oregon, USA.
5 Cuff. Latex free®, Guandong, China.
6 Multiparameter Monitor. Instramed®, Porto Alegre/RS, Brazil.
7 Neurostimulator. Medcir MGF II®.
8 Sodium heparin. Cristália, São Paulo/SP, Brazil.
9 Hemogasometry device. Cobas B121, Roche, Mannhein, Germany.
10 Thermal blanket. Estec®, São Paulo/SP, Brazil.

f test and then paired t-test for samples of the same variance or the paired t-test for samples with different variances. Minimum anesthetic concentration (MAC) was calculated using Dixon's up-and-down technique and quantal analysis (Dixon 1965, Sonner 2002), using a software[11] for each analysis. A confidence interval of 95 v% was considered for physiological variables. The confidence interval for MAC was calculated based on the binomial distribution. Associating this with logistic regression, a probability curve of negative response to stimulus in relation to anesthetic concentration (EtISO) can be found. Differences were considered statistically significant when $p<0.05$. Only animals belonging to crossover events were analyzed.

RESULTS

The average weight of the birds used in the Collective Group was 1.3+0.35kg and the total anesthetic period (induction until stimulation) averaged 72±15 minutes. The average weight of the 17 birds used in the Individual Group was 1.35±0.2kg and the total anesthetic period (induction until stimulation) averaged 65±12 minutes.

Three birds from the Collective Group and five birds from the Individual Group needed assistance ventilation, the other birds kept $EtCO_2$ values within normal range.

The isoflurane MAC for birds in the Collective Group obtained by the quantal analysis was 1.4v% at 0.903atm. The isoflurane MAC for birds in the Individual Group obtained by the quantal analysis was 1.9v% at 0.903atm, presenting a statistically significant difference between the groups.

In birds of the Collective Group, crossover events (positive answer followed by negative or vice versa) occurred four times (Fig.1), and the same bird was not included in more than one crossover. Eight out of 10 birds were used for quantal analysis and it was possible to obtain the logistic regression curve with less than 5v% error ($p<0.05$) (Fig.2). The individual number 8 was excluded from the study due to the value $PaCO_2$ was above the acceptable limit.

For Individual Group birds, crossover events (positive answer followed by negative or vice versa) occurred five times (Fig.3), and the same bird was not included in more than one crossover. Ten of the 17 birds were used for quantal analysis and it was possible to obtain the logistic regression curve with less than 5v% error ($p<0.05$) (Fig.4).

From the physiological variables measured before and after nociceptive stimulation (HR, f, $EtCO_2$, SpO_2, T°C), a statistical difference was observed only in systolic blood pressure between the birds of the Collective Group and the birds of the Individual Group (Table 1). The birds of the Collective Group had a higher SBP value than the birds of the Individual Group.

Hematimetric evaluations were performed only in the pre-anesthetic period (Table 2) and pre-stimulus hemogasometric evaluations (Table 3) remained within the reference values for the species.

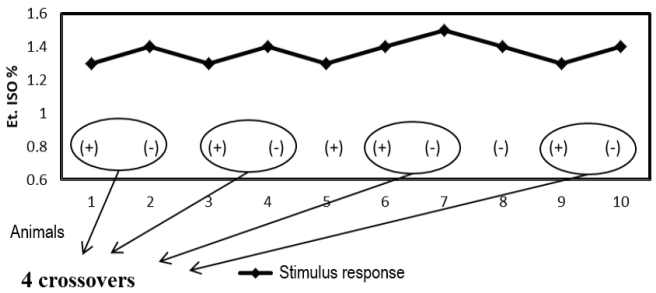


Fig.1. The variation of the expired isoflurane concentration (Et.ISO) values observed in each crossover event (circulated), using the up-and-down method in the Collective Group of guans (*Penelope obscura*).

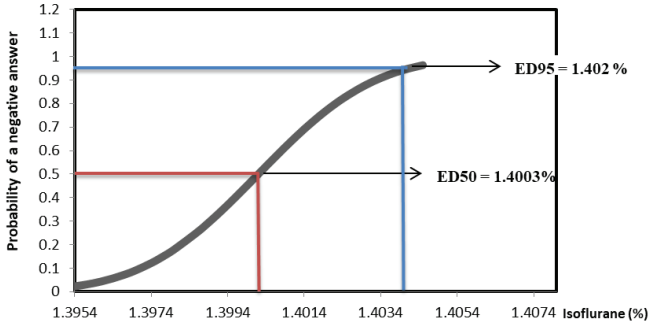


Fig.2. Curve adjusted for determination of the isoflurane minimum anesthetic concentration (MAC) of the Collective Group by the electrical stimulation method with hypodermic needles in the guans (*Penelope obscura*), which demonstrates the probability of the animal presenting a negative answer to the stimulus in the increase of MAC, indicating the effective dose for 50% of the population and MAC for 95% of the population.

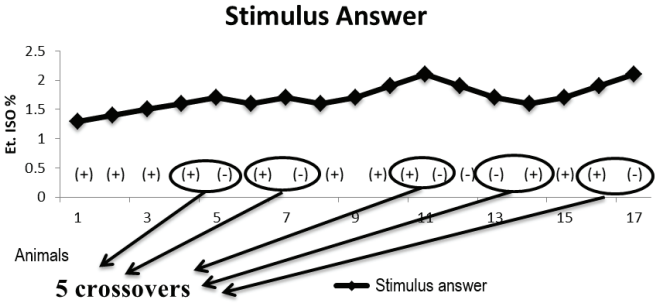


Fig.3. The variation of isoflurane expired concentration (Et.ISO) values observed in each crossover event (circulated), using the up-and-down method in the Individual Group of guans (*Penelope obscura*).

DISCUSSION

This bird species was chosen because it is from our region and because it is one of the species that indicates the quality of the environment since it is used to help programs for the management and conservation of protected areas (Strahl & Grajal 1991). Isoflurane is a safe drug, currently the most widely used in human and veterinary medicine, being the most suitable for critically ill patients and for having the best cost-benefit (Oliveira et al. 2014) and currently the most suitable for use in poultry (Granone et al. 2012).

There are several methods described for capturing small and medium-sized birds. In this study, the manual disarming trap method was opted due to the practice and training of the equipment handler. As observed in most capture programs,

11 Software. Microsoft, Exel®, and SAS®.

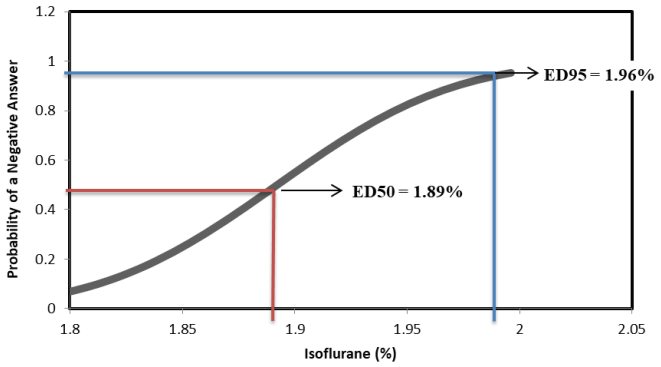


Fig.4. Adjusted curve for determination of the minimum anesthetic concentration (MAC) of Individual Group isoflurane by the electrical stimulation method with hypodermic needles in the guans (*Penelope obscura*), which demonstrates the probability of the animal presenting a negative response to the stimulus in the increase of MAC, indicating the effective dose for 50% of the population and MAC for 95% of the population.

their success is a result of the efforts of seasoned professionals and methods studied and tested before starting any new projects (Schemnitz et al. 2009).

In this study, the sex of the birds was not considered for the determination of MAC. Since the birds were randomly captured and sexing was performed in the post-experiment period, it was not possible to perform groups with a specific sex. As quoted by Fantoni et al. (2002), normally the MAC does not vary in animals of the same species, not suffering major interferences due to sex.

It took 10 birds to obtain the MAC in the Collective Group while 17 birds were used for the Individual Group. This difference is because the minimum acceptable number for MAC determination is four crossovers using the up and down method and each bird cannot be present in more than one of these events. Animals that did not participate in crossover events did not enter the analyses. Consequently, the total number always ends up differing from the actual number (Dixon 1965, Mercado et al. 2008). In this case, there was

Table 1. Values of physiological variables presented as mean (+ standard deviation), heart rate beats per minute (HR btm^{-1}), respiratory rate (ƒ), systolic blood pressure (SBP - mmHg), carbon dioxide concentration at end-expiratory ($EtCO_2$ - mmHg), partial oxygen saturation in arterial blood (SpO_2) and central temperature in Celcius degrees (T °C), before and after electrical stimulation, in determining the minimum anesthetic concentration (CAM) of guans (*Penelope obscura*) in the Collective and Individual Groups

Physiological variables	Groups			
	Collective		Individual	
	Pre	Post	Pre	Post
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
FC beat/min	176 + 48	187 + 49	188 + 52	210 + 59
ƒ mov/min	7 + 2	9 + 2	7 + 3	9 + 2
BPS mmHg	131 + 24[a]	130 + 20[a]	109 + 20[a]	108 + 19[a]
$EtCO_2$ mmHg	42 + 11	38 + 14	41 + 11	39 + 15
$SpO_{2\%}$	98 + 1	98 + 1	99 + 1	99 + 1
T°C	39,8 + 0,6	39,8 + 0,6	40,2 + 0,5	40,1 + 0,5

[a] The difference of SBP after paired t-test between two groups assuming equivalent variables.

Table 2. Presentation of values (mean + standard deviation) of blood counts (erythrocytes, hemoglobin, hematocrit (g dL^{-1}), mean globular volume (MGV), mean globular hemoglobin concentration (MGHC - %), thrombocytes, total plasma protein, total leukocytes, heterophile, lymphocytes, eosinophils, basophils, and monocytes) collected in the pre-anesthetic period, from guans (*Penelope obscura*) in the Collective and Individual Groups

Blood count	Groups	
	Collective	Individual
	Mean ± SD	Mean ± SD
Erythrocytes	2,35 + 0,4 x 10^6/µL	2,56 + 0,3 x 10^6/µL
Hemoglobin	9,37 + 0,99 g/dL	11 + 2 g/dL
Hematocrit	36 + 3%	41 + 5%
MGV	161,55 + 40,43fL	161 + 14,59fL
MGHC	25,5 + 1,4%	27 + 3,1%
Thrombocytes	14500 + 5032/µL	13771 + 5499/µL
Plasma Protein	3,91 + 0,22 g/dL	3,6 + 0,4 g/dL
Leukocytes	12121 + 4954/µL	9224+ 4997/µL
Heterophiles	7465 + 3796/µL	5354 + 2997/µL
Lymphocytes	2566 + 1587/µL	2293 + 1642/µL
Eosinophils	524 + 318/µL	174 + 181/µL
Basophils	125 + 139/µL	202 + 166/µL
Monocytes	1562 + 796/µL	1249 + 945/µL

Table 3. Presentation of values (mean + standard deviation) of blood gas (pH), arterial oxygen partial pressure (PaO_2 - mmHg), arterial carbon dioxide partial pressure ($PaCO_2$ - $mmHg^{-1}$) and bicarbonate (HCO_{-3} - $mmol\ L^{-1}$) performed at the time of electrical stimulation during the determination of the minimum anesthetic concentration (MAC) of the Collective and Individual Groups

Hemogasometry	Groups	
	Collective	Individual
	Mean ± SD	Mean ± SD
pH	7.4 + 0.04	7.4 + 0.04
PaO_2	137 + 72	185 + 46
$PaCO_2$	42.1 + 5.4	43.4 + 7.3
HCO^{-3}	29 + 3.26	28.2 + 3.8

a total of 10 birds and an effective number of eight birds in the Collective Group, obtaining four crossovers. In the birds of the Individual Group, there were 17 birds and an effective number of 10 birds, obtaining five crossovers, being possible in both groups to perform the quantal analysis and formation of the logistic regression curve.

Positive stimulus was considered, that is, with one or more of the following responses: vocalization, flapping, sudden movement of the contralateral to stimulated limb, head and tail raising (Nicolau et al. 2002, Mercado et al. 2008, Escobar 2010, Kim et al.2011, Pavez et al.2011, Chan et al.2013). This was well determined and standardized in this study because the lack of this standardization is a major cause of MAC value variability (Oliveira et al. 2014).

The initial MAC value for baseline was determined at 1.3v% isoflurane in both groups, which was obtained from a pilot study conducted earlier. The pilot study was very important because no reference was found for this species in the literature for isoflurane MAC values or the description of anesthetic techniques. Only Costa (2009) citation using chickens (*Gallus gallus domesticus*) with electrical stimulation obtained the MAC value of 1.47v%.

A 15 min period for the drug to stabilize between lung, blood, and brain was established. This time was based on the main studies that performed MAC determination in birds (Ludders et al. 1989, Mercado et al. 2008, Costa 2009, Escobar 2010, Kim et al. 2011, Chan et al. 2013).

Body temperature is one of the most important physiological variables to be controlled, as decreasing body temperature reduces animal metabolism and thereby decreases anesthetic consumption; increasing body temperature elevates animal enzymatic metabolism, increasing anesthetic consumption (Quasha et al. 1980).

The temperature of the birds in the research was maintained from 39.2 to 40.5°C, a value considered basal for birds of this order and observed as ideal in most studies (Costa 2009, Escobar 2010, Kim et al. 2011, Pavez et al. 2011). Nicomedus et al. (1969) and Ludders et al. (1989) state that to be considered MAC, besides the control of some physiological parameters such as body temperature, it is necessary to perform a logistic probability analysis, which in this study was performed by quantal analysis.

The quantal analysis is based on data obtained by the up-and-down method, unlike the bracketing study design method, which is one of the most widely used in animals (Sonner 2002). Although it is the most suitable method for animals in general, it was not chosen due to the long anesthetic time and because it is not considered a logistic probability analysis, as mentioned by Ludders et al. (1989) and Sonner (2002). Furthermore, in this method individual MAC is obtained and then performed for the group (Hellebrekers 2002, Hellyer et al. 2002), so we wanted to avoid possible hyperalgesia caused by repeated nociceptive stimuli at the same site since. In human and dog studies no differences were observed in the value of MAC obtained from the Bracketing study design and the up-and-down method (Sonner 2002).

The physiological parameters (HR, ƒ, T°C, PAS, SpO_2, $EtCO_2$), blood gas values (pH, PO2, PCO2 and HCO_{-3}) measured in birds during the experiment and hematological values are within the values observed in other studies with Galliformes (Naganobu & Hagio 2000, Costa 2009, Escobar 2010).

The isoflurane MAC in healthy adult guans (*Penelope obscura*) determined by the up-and-down method was 1.4v% for the Collective Group and 1.9v% for the birds of the Individual Group. In this study, electrical stimulation was chosen as a supramaximal nociceptive stimulus, as this type of stimulation has been evaluated as superior to other methods, even in birds described by other authors (Costa 2009, Escobar 2010, Pavez et al. 2011). Electrical stimulation has shown a higher standardization and more accurate techniques, as observed in all studies cited, such as the same frequency, voltage and time (50Hz, 50mA, and 10ms) and location (tibiotarsal lateral face). This is not observed in the digital or interdigital clamping methods, as there is no standardization of the clamping location, type of clamping and the amount of pressure applied at the point. It is only known that it is done at the level of the clamping rack, and in the other points the authors made their own adaptations (Ludders et al. 1988, 1989, Naganobu & Hagio 2000, Nicolau et al. 2002, Mercado et al. 2008, Kim et al. 2011, Chan et al. 2013, Oliveira et al. 2014).

The MAC value for the Collective Group was lower than the Individual Group, but its value of 1.4v% resembles the MAC value for birds of the same order, being 1.47+0.10v% in chickens (*Gallus gallus*) (Costa 2009). The MAC value of 1.9v% for the Individual Group is similar to the birds of Pavez et al. (2011), where the MAC of isoflurane in red-tailed buttocks (*Buteo jamaicensis*) was 2.05±0.45v%.

Blood collection for blood gas analysis was recommended only before the stimulus to confirm that $PaCO_2$ was not outside the expected values, so as not to influence the MAC value. As quoted by Brosnan et al. (2007), in which the increase in $PaCO_2$ may lead to a decrease in the final MAC value, a small increase in the $PaCO_2$ value of the birds of the Individual Group was observed. The birds of the Collective Group remained in

normocapnia, and the difference in MAC value between the two groups should not be attributed to this factor. This slight increase in $PaCO_2$ in the Individual Group may be justified because it has been suggested that responses to systemic stressors associated with immediate survival, such as hypoxia and hypercapnia, are likely to be directly transmitted from brain stem nuclei and are not associated with higher-order CNS processing and conscious experiences (Herman & Cullinan 1997). These factors can be considered stress determinant.

Spontaneous ventilation was used in this study, and the animal was ventilated only when $PaCO_2$ and $EtCO_2$ did not have normocapnia values in the pre-stimulation analyses (Nicolau et al. 2002, Mercado et al. 2008, Costa 2009, Chan et al. 2013). Unlike, other authors who opted for controlled ventilation with or without gas re-inhalation system (Escobar 2010, Kim et al. 2011). Chan et al. (2013) stated that spontaneous ventilation becomes more reliable without a gas rebreathing system because air does not go through a CO_2 filtration system and reuse of the anesthetic agent. Not knowing whether mild hypercapnia can alter MAC in guans (*Penelope obscura*), it was decided to keep $PaCO_2$ and $EtCO_2$ levels within normal limits because in some studies involving birds with resulting relative hypoventilation, known to decrease the requirement for inhalant agents to varying levels observed in different species, changing the final MAC value to less than it should be (Brosnan et al. 2007). In rats, the increase in end-tidal CO_2 concentration at MAC of halothane, isoflurane, and desflurane decreased dose-dependent (Brosnan et al. 2007). Chemonges (2014) observed that intermittent pressure ventilation in cockatoos (Cacatua) leads to an increase in isoflurane-dependent dose anesthetic depth.

Within the evaluated parameters, a statistical difference was observed only in systolic blood pressure between the birds of the Collective Group and the Individual Group, in which the SBP value was 131±24mmHg in the Collective Group and 109±20mmHg in the Individual Group. This difference between the two groups may be explained by the fact that MAC in the birds of the Individual Group was significantly higher (1.35 times higher), so a higher dose of inhaled isoflurane is required, leading to mild hypotension. This side effect was observed in studies in which after MAC determination, the isoflurane dose was increased by 1.5 and 2 times to assess its cardiovascular effect. In this case, only the dose-dependent decrease in blood pressure was observed, with no change in heart rate (Ludders et al. 1989, Naganobu & Hagio 2000, Kim et al. 2011). The same result was observed in this study.

In the determination of the isoflurane MAC, a statistical difference was observed between the birds of the Collective Group and those of the Individual Group, with the highest MAC value observed in the Individual Group. One of the causes of this difference can be the stress intensified by the separation of individuals (social stress), which alters the natural behavior of the species, considering that these birds have a habit of staying in groups in the wild (Marques 2014). The influence of social behavior on pain and stress levels was also described by Gentle & Tilston (1999), who observed that chickens that remained in the group after application of sodium urate in the left ankle had a higher pain threshold than birds that remained isolated in the enclosure, attributing this behavioral change to social stress. Massone (2011) mentioned that accommodations in the pre-anesthetic period are very important, as they interfere with animal behavior and it is known that the environment (boxes, stalls, kennels, and stables) where animals stay before surgical interventions may cause a high level of stress to the point of being affected to the anesthetic act. According to Sharp et al. (2006), simply placing mice in an unknown exposure chamber containing ambient air produces excitement lead to distress. This may justify the higher stress in the birds of the Individual Group because, in addition to being removed from their natural environment, they were allocated so they did not observe the other individuals and the rest of the environment.

The Individual Group was considered the one with the greatest influence of social stress because it had a higher MAC value. What characterize the influence of stress are the increase of blood glucose, lactate free fatty acids, and the increase of metabolism rates, oxygen uptake, and accelerated pharmacological metabolism (Muir 2007).

In birds, the anesthetic potency of an inhaled drug is determined by its minimum anesthetic concentration (MAC), a method similar to the one used for mammals (Ludders et al. 1989). Apparently, this was the first paper reporting the determination of isoflurane MAC in guans (*Penelope obscura*) and the observation of the influence of social stress on MAC.

CONCLUSIONS

The isoflurane MAC value for these birds was close to other galiforms, but increased significantly when birds were subjected to stress, such as being kept individually.

Based on the MAC results of this study (Collective Group vs. Individual Group), the ideal acclimatization is fundamental for animals that will undergo clinical, surgical and anesthetic treatments.

This information becomes important for future studies in determining MAC in birds of this species or other species that can be easily influenced if subjected to social conditions outside the habitat, especially for birds that live in groups.

Acknowledgments.- We thank "Universidade do Estado de Santa Catarina" for the opportunity of conducting the research, IBAMA for providing the staff and the capture and storage of animals, to all the people who participated in this research, and for the Santa Catarina Research Support Foundation (FAPESC).

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

REFERENCES

Brosnan R.J., Eger 2nd E., Laster M.J. & Sonner J.M. 2007. Anesthetic properties of carbon dioxide in the rat. Anesth. Analg. 105(1):103-106. <http://dx.doi.org/10.1213/01.ane.0000265556.69089.78> <PMid:17578964>

Caulkett N.A. & Arnemo J.M. 2015. Comparative anesthesia and analgesia of zoo animals and wildlife, p.764-776. In: Grimm K.A., Lamont L.A., Tranquilli W.J., Greene S.A. & Robertson S.A. (Eds), Lumb and Jones' Veterinary Anesthesia and Analgesia. 5th ed. John Wiley and Sons, Iowa. <http://dx.doi.org/10.1002/9781119421375.ch40>.

Chan F.T., Chang G.R., Wang H.C. & Hsu T.H. 2013. Anesthesia with isoflurane and sevoflurane in the crested serpent eagle (*Spilornis cheela hoya*): minimum anesthetic concentration, physiological effects, hematocrit, plasma chemistry and behavioral effects. J. Vet. Med. Sci. 75(12):1591-1600. <http://dx.doi.org/10.1292/jvms.13-0161> <PMid:23955396>

Chemonges S. 2014. Effect of intermittent positive pressure ventilation on depth of anaesthesia during and after isoflurane anaesthesia in sulphur-

crested cockatoos (*Cacatua galerita galerita*). Vet. Med. Int. 2014:1-7. <http://dx.doi.org/10.1155/2014/250523> <PMid:24587938>

Costa A. 2009. Pinçamento digital e estímulo elétrico na determinação da concentração anestésica mínima (CAM) de isoflurano em galinhas (*Gallus gallus*) pré tratadas ou não com Meloxicam. Master's Thesis, Universidade do Estado de Santa Catarina, Florianópolis, SC. 75p.

Dixon W.J. 1965. The up-and-down method for small samples. J. Am. Stat. Assoc. 60(312):967-978. <http://dx.doi.org/10.1080/01621459.1965.10480843>

Eger 2nd E.I., Saidman L.J. & Brandstater B. 1965. Minimum alveolar anesthetic concentration: a standard of anesthetic potency. Anesthesiology 26(6):756-763. <http://dx.doi.org/10.1097/00000542-196511000-00010> <PMid:5844267>

Escobar A. 2010. Efeitos do butorfanol na concentração anestésica mínima do sevofluorano em galinha d'angola (*Numida meleagris*). Doctoral Dissertation, Faculdade de Ciências Agrária e Veterinárias, Universidade Estadual Paulista, Jaboticabal. 85p.

Fantoni D.T., Cortopassi S.R.G. & Bernardi M.M. 2002. Anestésicos inalatórios, p.132-142. In: Spinosa H.S., Górniak S.L. & Bernardi M.M. (Eds), Farmacologia Aplicada a Medicina Veterinária. 3ª ed. Guanabara Koogan, Rio de Janeiro.

Gentle M.J. & Tilston V.L. 1999. Reduction in peripheral inflammation by changes in attention. Physiol. Behav. 66(2):289-292. <http://dx.doi.org/10.1016/S0031-9384(98)00297-2> <PMid:10336156>

Gleeson M. 1989. Control of breathing, p.439-484. In: King AS & McLelland J. (Eds), Form and Function in Birds. Academic Press, London.

Granone T.D., Francisco O.N., Killos M.B., Quandt J.E., Mandsager R.E. & Graham L.F. 2012. Comparison of three different inhalant anesthetic agents (isoflurane, sevoflurane, desflurane) in red-tailed hawks (*Buteo jamaicensis*). Vet. Anest. Analg. 39(1):29-37. <http://dx.doi.org/10.1111/j.1467-2995.2011.00668.x> <PMid:22103452>

Hellebrekers L.J. 2002. Dor em Animais. Ed. Manole, São Paulo. 166p.

Hellyer P.W., Robertson S.A. & Fails A.D. 2002 Pain and its management, p.38-66. In: Tranquilli J.C., Thurmon J.C. & Grinn K.A. (Eds), Lumb and Jones' Veterinary Anesthesia and Analgesia. 4th ed. Blackwell Publishing, Iowa.

Herman J.P. & Cullinan W.E. 1997. Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. Trends Neurosci. 20(2):78-84. <http://dx.doi.org/10.1016/S0166-2236(96)10069-2> <PMid:9023876>

Ludders J.W. 2015a. Respiration in birds, p.245-262. In: Reece W.O., Erickson H.H. Goff J.P. & Uemura E.E. (Eds), Dukes' Physiology of Domestics Animals. 13th ed. John Wiley and Sons, Iowa.

Ludders J.W. 2015b. Comparative anesthesia and analgesia of birds, p.800-818. In: Grimm K.A., Lamont L.A. Tranquilli W.J., Greene S.A. & Robertson S.A. (Eds), Lumb and Jones' Veterinary Anesthesia and Analgesia. 5th ed. John Wiley and Sons, Iowa.

Ludders J.W. & Matthews N.S. 2007. Birds, p.923-952. In: Tranquilli J.C., Thurmon J.C. & Grinn K.A. (Eds), Lumb and Jones' veterinary anesthesia and analgesia. 4th ed. Blackwell Publishing, Iowa.

Ludders J.W., Mitchell G.S. & Schaefer S.L. 1988. Minimum anesthesic dose and cardiopulmonary response for halotanein chickens. Am. J. Vet. Res. 49(6):929-932. <PMid:3135771>

Ludders J.W., Rode J. & Mitchell G.S. 1989. Isoflurane anesthesia in sandhill cranes (*Grus canadensis*): minimal anesthetic concentration and cardiopulmonary dose-response during spontaneous and controlled breathing. Anest. Analg. 68(4):511-516. <http://dx.doi.org/10.1213/00000539-198904000-00016> <PMid:2929984>

Kim Y.K., Lee S.S., Suh E.H., Lee L., Lee H.C., Lee H.J. & Yeon S.C. 2011. Minimum anesthetic concentration and cardiovascular dose-response relationship of isoflurane in cinereous vultures (*Aegypius monachus*). J. Zoo Wild. Med. 42(3):499-503. <http://dx.doi.org/10.1638/2010-0151.1> <PMid:22950326>

Marques M.V.R. 2014. Galiformes (aracuã, jacu, jacutinga, mutum e uru), p.354-383. In: Cubas Z.S., Silva J.C. R. & Catão-Dias J.L. (Eds), Tratado de Animais Selvagens. 2ª ed. Roca, São Paulo.

Massone F. 2011. Considerações gerais, p.1-16. In: Ibid. (Ed.), Anestesiologia Veterinária: farmacologia e técnicas. 6ª ed. Guanabara Koogan, Rio de Janeiro.

Mercado J.A., Larsen S., Wack R.F. & Pypendop B.H. 2008. Minimum anesthetic concentration of isoflurane in captive thick-billed parrots (*Rhynchopsitta pachyrhyncha*). Am. J. Vet. Res. 69(2):189-194. <http://dx.doi.org/10.2460/ajvr.69.2.189> <PMid:18241014>

Muir W.W. 2007. Considerations for general anesthesia, p.7-37. In: Tranquilli J.C., Thurmon J.C. & Grinn K.A. (Eds), Lumb and Jones' Veterinary Anesthesia and Analgesia. 4th ed. Blackwell Publishing, Iowa.

Naganobu K. & Hagio M. 2000. Doserelated cardiovascular effects of isoflurane in chickens during controlled ventilation. J. Vet. Med. Sci. 62(4):435-437. <http://dx.doi.org/10.1292/jvms.62.435> <PMid:10823732>

Nicolau A.A., Fantoni D.T., Auler J.O. & Ambrósio A. 2002. O sevoflurano em psitacídeos (*Amazona aestiva*) determinação da Dose Anestésica Mínima (DAM) para a produção da anestesia geral. Ciência Rural 32(5):781-786. <http://dx.doi.org/10.1590/S0103-84782002000500007>

Nicomedus H.F., Nassiri-Ralimi C. & Bachman L. 1969. Median effective doses (ED50) of halotane in adults and children. Anesthesiology 31(4):344-348. <http://dx.doi.org/10.1097/00000542-196910000-00011> <PMid:5811601>

Oliveira M.G.C., Moraes A.M.L., Lima A.G.A., Paiva A.L.C., Nunes T.L., Passos Y.D.B., Oliveira M.F. & Paula V.V. 2014. Determinação da concentração alveolar mínima do isoflurano em catetos (*Tayassu tajacu*). Pesq. Vet. Bras. 6(34):576-581. <http://dx.doi.org/10.1590/S0100-736X2014000600012>

Pavez J.C., Hawkins M.G., Pascoe P.J., Knych H.K.D. & Kass P.H. 2011. Effect of fentanyl target-controlled infusions on isoflurane minimum anaesthetic concentration and cardiovascular function in red-tailed hawks (*Buteo Jamaicensis*). Vet. Anesth. Analg. 38(4):344-351. <http://dx.doi.org/10.1111/j.1467-2995.2011.00627.x> <PMid:21672126>

Quasha A.L., Eger 2nd E.I. & Tinker J.H. 1980. Determination and applications of MAC. Anesthesiology 53(4):315-334. <http://dx.doi.org/10.1097/00000542-198010000-00008> <PMid:6107067>

Sharp J., Azar T. & Lawson D. 2006. Comparison of carbon dioxide, argon, and nitrogen for inducing unconsciousness or euthanasia of rats. J. Am. Assoc. Lab. Anim. Sci. 45(2):21-25. <PMid:16542038>

Scheid P. & Piiper J. 1972. Cross current gas exchange in avian lungs: effect of reversed parabronchial air flow in ducks. Respiratory Physiol. 16(3):304-312. <http://dx.doi.org/10.1016/0034-5687(72)90060-6> <PMid:4644057>

Schemnitz S.D., Batcheller G.R., Lovallo M.J., White H.B. & Fall M.W. 2009. Capturing and handling wild animals. Natl Wildl. Res. Center 1:232-269.

Sonner J.M. 2002. Issues in the design and interpretation of minimum alveolar anesthetic concentration (MAC) studies. Anest. Analg. 95(3):609-614. <PMid:12198046>

Strahl S.D. & Grajal A. 1991. Conservation of large avian fugivores and the management of neotropical protected areas. Oryx 25(1):50-55. <http://dx.doi.org/10.1017/S0030605300034074>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(8):663-667, August 2019
DOI: 10.1590/1678-6160-PVB-6228

Original Article
Animal Morphophysiology

B-mode ultrasonography and gray scale histogram for evaluation of the nuchal ligament in Quarter horse[1]

Wildemberto A. Santos[2], Michel C. Vettorato[2], Jéssica L. Fogaça[2], Nayara M.G. Mazzante[2], Fernanda G. Oliveira[2], Guilherme P. Nogueira[3], Maria C.R. Luvizotto[3] and Vânia M.V. Machado[2]*

ABSTRACT.- Santos W.A., Vettorato M.C., Fogaça J.L., Mazzante N.M.G., Oliveira F.G., Nogueira G.P., Luvizotto M.C.R. & Machado V.M.V. 2019. **B-mode ultrasonography and gray scale histogram for evaluation of the nuchal ligament in Quarter horse.** *Pesquisa Veterinária Brasileira 39(8):663-667.* Departamento de Reprodução Animal e Radiologia Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Julio de Mesquita Filho", Rua Prof. Dr. Walter Mauricio Correra s/n, Rubião Junior, Botucatu, SP 18618-970, Brazil. E-mail: vania.mv.machado@unesp.br

The gray scale histogram (GSH) makes it possible to measure the amount and distribution of gray shade frequencies, providing quantitative information on both echogenicity and echotexture of tissues. There is a need to diminish the subjectivity of the ultrasound images of the nuchal ligament (NL). This work proposes to evaluate the NL by ultrasound B-mode GSH images in different ages of Quarter horses. It used 15 healthy Quarter horses, which were classified by age into three different groups: "baby" (1), "sobreano" (2) and "adult" (3). The animals were submitted to chemical restraint for the ultrasound examination. Subsequently, the GSH tool was used in each image for statistical analysis. There was a significant difference between Mean and Mode between groups. Group 1 differed significantly when compared to Group 2 and 3. Group 2 presented superior echogenicity to Group 1 and 3. The height of the NL did not vary considerably between groups. GSH indicated that the echogenicity of NL in Quarter horses varies with age.

INDEX TERMS: B-mode, ultrasonography, gray scale histogram, nuchal ligament, histogram, equine, Quarter Horse, morphology.

RESUMO.- [**Ultrassonografia modo-B e histograma em escala de cinza para avaliação do ligamento nucal em equinos da raça Quarto de Milha.**] O histograma em escala de cinza (HEC) possibilita a mensuração da quantidade e distribuição da frequência de tonalidades de cinza, fornecendo informações quantitativas, tanto sobre a ecogenicidade quanto a ecotextura dos tecidos. Havendo a necessidade de diminuir a subjetividade das imagens ultrassonográficas do ligamento nucal (LN), esse trabalho propôs avaliar o LN por imagens ultrassonograficas modo-B pelo HEC em diferentes idades de cavalos da raça Quarto de Milha. Utilizou 15 cavalos da raça Quarto de Milha saudáveis os quais foram classificados por idade em três grupos diferentes: "baby" (1), "sobreano" (2) e "adulto" (3). Os animais foram submetidos a contenção química para a realização do exame ultrassonográfico. Posteriormente, a ferramenta HEC foi empregada em cada imagem para análise estatística. Houve diferença significativa entre as variáveis "Mean" e "Mode" entre os grupos. O Grupo 1 diferenciou significativamente quando comparado ao Grupo 2 e 3. O Grupo 2 apresentou ecogenicidade superior ao Grupo 1 e 3. Quanto à altura do LN não teve variação considerável entre os grupos. O HEC indicou que a ecogenicidade do LN em cavalos Quarto de Milha varia conforme a idade.

TERMOS DE INDEXAÇÃO: Ultrassonografia, modo-B, histograma escala de cinza, avaliação, ligamento nucal, equinos, Quarto de Milha, morfologia.

[1] Received on February 12, 2019.
Accepted for publication March 20, 2019.

[2] Departamento de Reprodução Animal e Radiologia Veterinária, Faculdade de Medicina Veterinária e Zootecnia (FMVZ), Universidade Estadual Paulista "Julio de Mesquita Filho" (Unesp), Distrito de Rubião Junior s/n, Botucatu, SP 18618-970, Brazil. *Corresponding author: vania.mv.machado@unesp.br

[3] Faculdade de Medicina Veterinária de Araçatuba, Unesp, Rua Clóvis Pestana 793, Dona Amélia, Araçatuba, SP 16050-680, Brazil.

INTRODUCTION

Equines are animals with great physical ability and well-developed bone and muscle structures. Their dynamics result from the combined movement of each individual vertebra and is limited by the presence of different adjacent structures, such as muscles, ligaments, invertebral joints, and ribs (Getty 1975, König & Liebich 2013).

Traumatic head injuries are common in horses, especially in foals and younger individuals, as several health conditions cause traumas in this region (Ragle 1993, Ramirez et al. 1998, Swarbrick et al. 2018). In such circumstances, a clinical examination must be carried out to diagnose the conditions affecting this region at different ages.

The nuchal ligament (NL) is an elastic structure located in the posterior midline of the spinous process between the occipital crest and cervical vertebrae (Gellman & Bertram 2002). This structure can be affected by health conditions such as desmitis, calcification, and spondylosis. In addition, the morphology and distribution of these lesions support a pathogenesis where developmental abnormalities with potential biomechanical impact on the cervical spine contribute to cervical stenotic myelopathy in horses (Janes et al. 2015). Two-dimensional ultrasound imaging (B-mode) allows for a precise evaluation of this region (Henson 2013).

Detailed anatomy of equines has received little attention, especially ligaments. Thus the need for studies that aim to broaden the knowledge on the normal and pathological characterization of the neck ligaments, allowing for early and accurate diagnosis (Ragle 1993, Ramirez et al. 1998, Gollob et al. 2002).

Ultrasound imaging combined with grayscale histogram (GSH) allows the measurement of the amount and distribution of the frequency of gray levels, providing quantitative information (Osawa & Mori 1996). According to Ferreira & Rasband (2012), the ultrasound images are made up of pixels numerically represented on a scale of 255 gray levels varying according to their brightness, where 0=black (hypoechoic) and 255=white (hyperechoic).

Tsukiyama et al. (1996) pointed out that GSH analysis is efficient for evaluation of tendons, and Hsu et al. 2017), of ligaments. Ultrasound findings are subjective and produce many discrepancies either due to the ultrasound system variability or to the operator's experience. The GSH can help in diagnostics interpretation and has become an increasingly used tool both in clinical routine and in research (Osawa & Mori 1996, Ferreira & Rasband 2012, Silva et al. 2015, Fogaça 2018, Castiglioni et al. 2018).

The GSH tool can help evaluate this structure and reduce the subjectivity of NL ultrasound images, as studies on equines are scarce in the literature reviewed. Therefore, this work aims to assess the NL by means of B-mode ultrasound and GSH images in American Quarter horses of different ages.

MATERIALS AND METHODS

This study has been approved by the Animal Research Ethics Committee (CEUA) of "Faculdade de Medicina Veterinária e Zootecnia" (FMVZ) of "Universidade Estadual Paulista 'Júlio de Mesquita Filho'" (Unesp), Botucatu campus, under protocol number 0182/2016.

This study included 15 healthy horses of the American Quarter horse breed. The animals were randomized and classified into three different groups by age. Group 1 included five post-weaning animals under one year of age (foals). Group 2 included five halter-broke animals above one year of age (colts), and Group 3, five animals above three years of age undergoing taming (adults). Groups 1, 2 and 3 were 8, 20 and 39 months old, on average, and presented a mean body mass of 238kg, 389kg and 462kg, respectively.

The inclusion criteria were based on the age and body weight. The procedures were performed in a private property located in the city of Bilac, State of São Paulo, Brazil. The horses were placed in an individual constraint pen, where good animal welfare was observed. The only group requiring anesthesia was Group 1, as its members were not halter-broke yet.

Animals in Group 1 were sedated with alpha 2-adrenoceptor agonists xylazine and detomidine. The sedation was reverted after the ultrasound scan using the alpha 2-adrenoceptor antagonist, yohimbine. The following dosages were administered: 0.1-0.2 milligrams per kilogram of body weight (mg/kg) of xylazine, 0.02mg/kg of detomidine, and 0.125-0.250mg/kg of yohimbine, in accordance with the literature (Guedes & Natalini 2002, Melo et al. 2007, Marques et al. 2009).

The ultrasonography was performed on a SonoScape ultrasound system model A6 and a linear transducer with a frequency bandwidth of 7.5-8.5 megahertz (MHz) and gain at 180. 50% isopropyl alcohol (50% water, 50% alcohol) was used prior to the application of the ultrasound gel.

The animals in Group 1 had not experienced the interference of haltering--their only contact with men was for feeding, hoof trimming (in case of congenital defects affecting balance), pulverization and administration of vermifuges. The foals were guided through a handling pen (corridor placed inside the corral to take the animal to the examination pen) and constrained by the keeper. Following constraint, they were weighted and sedated.

The animals in Group 2 and 3 were taken to the examination pen by a guide. They were neither sedated nor physically immobilized in addition to the pen and halter. The most comfortable head position for the animals was allowed; however, all of the animals kept their head above the withers, which became a standard position.

Three images were taken for each animal and assessed by three technicians. The interobserver correlation was evaluated by means of an analysis of the Mean values found. Afterwards, a GSH analysis was performed on the ultrasound images on software ImageJ (National Institutes of Health), in order to quantitatively represent echogenicity in each image.

The GSH measurements were performed on an area of 2x5cm circumscribed at the center of the NL image. An interval from 1,000 to 1,500 pixels was considered for sample collection within the area circumscribed.

The Mean and Mode values were obtained (Fig.1). Following this procedure, the NL height was measured by the same software, where 35 pixels per centimeters was the area considered for measurement (Fig.2). The data were inserted into a Microsoft Excel 2013 spreadsheet for statistical analysis.

Statistical analysis was performed using GraphPad Prism 7 software to evaluate the variables of interest. An ANOVA was applied with a level of significance of 5% ($p<0.05$) for differences among groups with post-hoc Tuckey.

RESULTS

Table 1 shows the mean and standard deviation of the GSH variables (Mean and Mode) followed by the ANOVA p-value for the NL of the three groups evaluated.

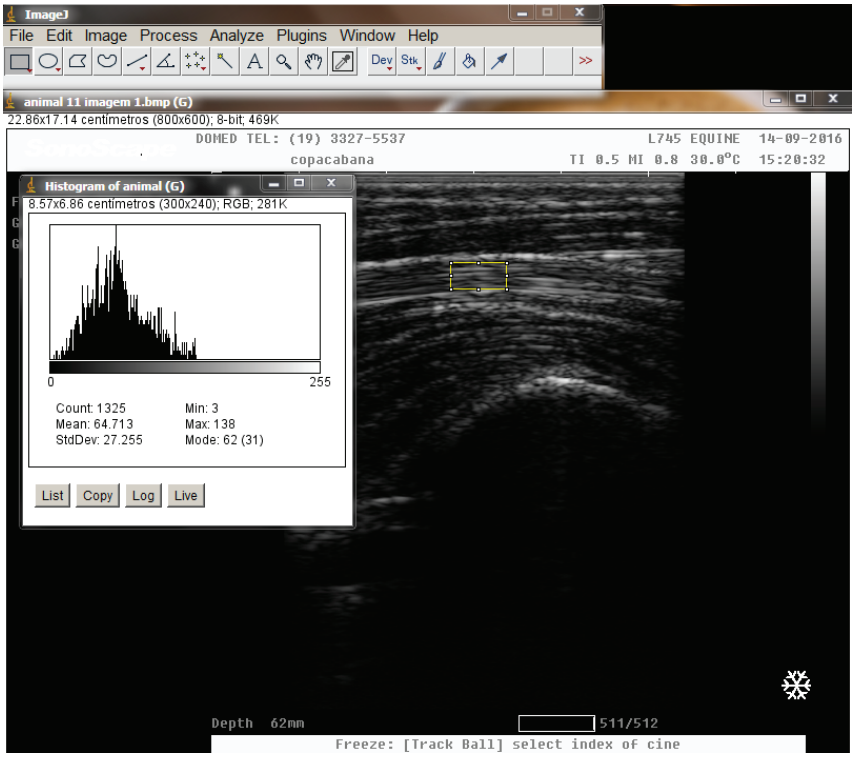


Fig.1. Grayscale histogram performed on the nuchal ligament of a horse to obtain the Mean and Mode variables.

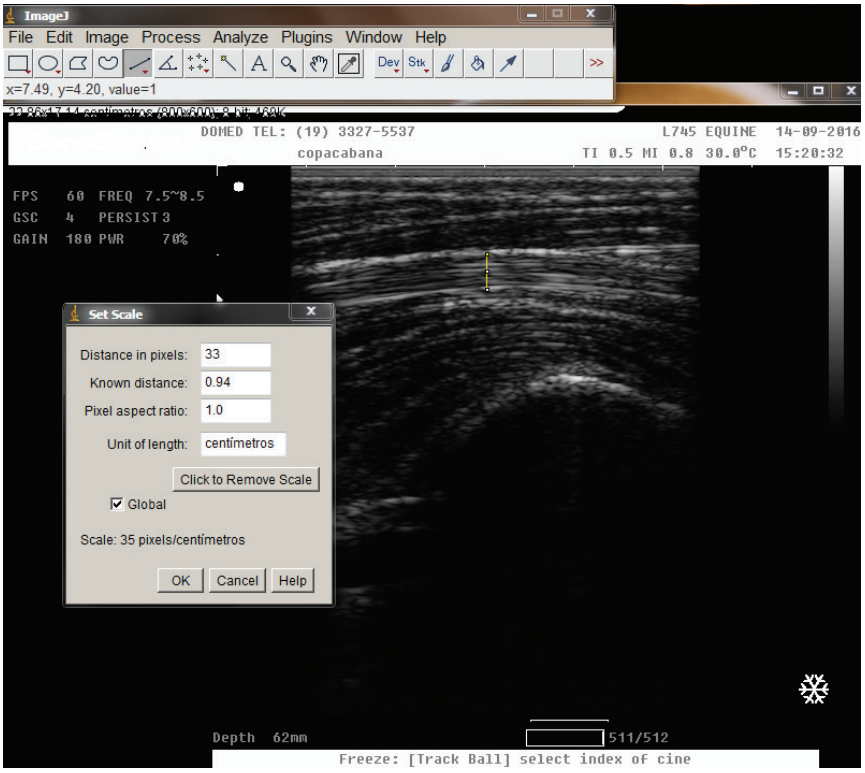


Fig.2. Measurement of the nuchal ligament height on the ultrasound image of a horse to obtain the measurements in centimeters (cm) and distance in pixels.

According to Table 1, Group 2 presented a mean of variables Mean and Mode higher than Group 1 and 3; i.e. echogenicity was higher in this sample. A significant difference was found between the Mean (p=0.053) and Mode (p=0.014) of the groups.

Table 2 shows the mean and standard deviation of the NL height (cm) ANOVA for each group (1 to 3).

As shown in Table 2, we observed no statistical difference among the groups.

Table 1. Mean and standard deviation of the GSH variables (Mean and Mode) followed by the ANOVA p-value for the NL of the three groups

Group	Mean	Mode
1	49.67±23.86	34.80±20.91
2	77.42±10.83	69.04±10.04
3	64.86±8.93	60.80±13.18
p-value	0.0533*	0.014*

*p-value<0.05.

Table 2. Mean and standard deviation of the NL height (cm) ANOVA for Group 1 to 3

Group	Average and standard deviation (cm)
1	0.984±0.1128
2	0.834±0.1258
3	0.864±0.0357
p-value	0.0786

*p-value<0.05.

DISCUSSION

The GSH analysis has been reported as a means to overcome variations due to observers' subjectivity (Osawa & Mori 1996). According to Maeda et al. (1998) and Monteiro et al. (2010), the depth of the region evaluated, the ultrasound system's marks and the gain used in the test do not cause a statistically significant impact on the results obtained by the GSH. In this study, gain was standardized and the same machine was used for all of the exams.

Micklethwaite et al. (2001) have described the efficiency of pixel tone analysis in ultrasound scans, as it allows veterinarians to monitor healing of lesions in tendons and ligaments and to investigate the efficacy of different treatments.

The ultrasound scan was efficient in characterizing the NL echogenicity, which corroborates Langevin et al. (2009) in their evaluation and diagnosis of ligaments and soft tissues adjacent to the spine in humans.

According to Gollob et al. (2002), the NL is a bilobular structure with well-defined edges, spindle-shaped points, and higher echogenicity than the adjacent tissues when examined by ultrasound, characteristics that are similar to those found in this research.

Most studies involving the GSH Mode and Mean variables have found a similarity between the two (Castiglioni 2018, Fogaça 2018). Nevertheless, the present study found a significant difference between these variables among the groups. The Mode did not present confidence in relation to the Mean, which means that the most recurrent gray levels in the image, according to the GSH, did not correspond to the total mean of pixels selected in the image. In view of the difference between the Mode and the Mean, it is preferable to use the Mean to quantify the GSH.

According to Ferreira & Rasband (2012), the GSH allows for an accurate evaluation of the gray levels found in an area of interest in any image. The analysis of the results of this study in the ultrasound images showed that the NL echogenicity (Mean and Mode) was higher in Group 2 compared to Groups 1 and 3. The echogenicity was probably influenced by the attenuation coefficient of the soft subjacent tissues, such as the surrounding muscles, subcutaneous fatty tissues, and skin, which corroborates Yu et al. (2012) and Hsu et al. (2017). According to Collinger et al. (2010), body weight is indirectly proportional to the echogenicity of the supraspinatus tendon--an observation similar to the findings of this study with regard to the echogenicity of the NL in Group 3. Thus, this correlation is attributed to Group 2.

According to Yu et al. (2012), in human beings, age can have an indirect influence on the echogenicity of the supraspinatus tendon since, older people are more susceptible to lesions, more hypoechogenic and thicker, similar to the findings in Group 3 with regard to echogenicity.

According to Martinoli et al. (1993), the linear echoes of the tendons depend on the acoustic interfaces between the boundaries of the collagen bundles and the endotendinous septum, based on their different histological composition (Martinoli et al. 1993). Extrapolating to the ligaments, we must also consider the histological characteristics that are closely related to the ultrasound image. In this way, as collagen content increases from birth to adulthood, when its contents are relatively stable, and gradually decreases with age (Parry et al. 1978), this might justify the differences in echogenicity found between Group 1 and the others.

The GSH has been shown to be a promising tool for detecting the hypoechogenic aspect of supraspinatus tendinopathy (Hsu et al. 2017), so that it may be strongly recommended for several ligaments of Quarter horses, particularly the NL, for which the literature is scarce.

Free movement of the horses' head and neck would result in measurable changes in the nuchal ligament dimensions (Nestadt et al. 2015). For this reason, the animals in Groups 2 and 3 were haltered and all of them kept their head above the withers. The animals in Group 1, however, were submitted to anesthesia for immobilization during the ultrasound scans.

According to Collinger et al. (2010), there is no correlation between the height of the supraspinatus ligament and health status in humans, and this study did not find a significant difference among the NL heights within the groups. Considering the absence of studies in the literature reviewed on NL GSH in horses and human beings, it is not possible to determine if this difference in echogenicity related to age is present in other breeds and species, which makes it important to conduct further research.

CONCLUSION

The grayscale histogram (GSH) showed that the nuchal ligament (NL) echogenicity in Quarter horses varies according to age and underlines the efficacy of this tool added to B-mode ultrasound to evaluate the NL.

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

REFERENCES

Castiglioni M.C.R., Vettorato M.C., Fogaça J.L., Puoli-Filho J.N.P. & Machado V.M.V. 2018. Quantitative ultrassound of kidneys, liver, and spleen: a comparison between mules and horses. J. Equine Vet. Sci. 70:71-77. <http://dx.doi.org/10.1016/j.jevs.2018.07.011>

Collinger J.L., Fullerton B., Impink B.G., Koontz A.M. & Boninger M.L. 2010. Validation of greyscale-based quantitative ultrasound in manual wheelchair

users: relationship to established clinical measures of shoulder pathology. Am. J. Phys. Med. Rehab. 89(5):390-400. <http://dx.doi.org/10.1097/PHM.0b013e3181d8a238> <PMid:20407304>

Ferreira T. & Rasband W.S. 2012. ImageJ User Guide: ImageJ/Fiji. 1.46. 198p. Available at <https://imagej.nih.gov/ij/docs/guide/user-guide.pdf> Accessed on May 10 2018

Fogaça J.L. 2018. Estudo comparativo de ultrassonografia modo-B, Doppler e histograma em escala de cinza na avaliação das artérias carótidas comuns em equinos e muares. Master's Thesis, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista de Botucatu. 136p.

Gellman K.S. & Bertram J.E.A. 2002. The equine nuchal ligament 1: structural and material properties. Vet. Comp. Orthop. Traumatol. 15(1):1-6. <http://dx.doi.org/10.1055/s-0038-1632705>

Getty R. 1975. Sisson and Grossman's, The Anatomy of the Domestic Animal. 5th ed. W.B. Saunders, Philadelphia, 2095p.

Gollob E., Edinger H., Stanek C. & Wurnig C. 2002. Ultrasonographic investigation of the atlanto-occipital articulation in the horse. Equine Vet. J. 34(1):44-50. <http://dx.doi.org/10.2746/042516402776181196> <PMid:11817551>

Guedes A.G.P. & Natalini C.C. 2002. Anesthesia horses with colic syndrome: analysis of 48 cases and literature review. Ciência Rural 32(3):535-542. <http://dx.doi.org/10.1590/S0103-84782002000300028>

Henson F.M.D. 2013. Equine Back Pathology: diagnosis and treatment. John Wiley Blackwell, Ames, 280p.

Hsu J.C., Chen P.H., Huang K.C., Tsai Y.H. & Hsu W.H. 2017. Efficiency of quantitative echogenicity for investigating supraspinatus tendinopathy by the gray-level histogram of two ultrasound devices. J. Med. Ultrason. 44(4):297-303. <http://dx.doi.org/10.1007/s10396-017-0777-6> <PMid:28197744>

Janes J.G., Garrett K.S., McQuerry K.J., Waddell S., Voor M.J., Reed S.M., Williams N.M. & MacLeod J.N. 2015. Cervical vertebral lesions in equine stenotic myelopathy. Vet. Pathol. 52(5):919-927. <http://dx.doi.org/10.1177/0300985815593127> <PMid:26169385>

König H.E. & Liebich H.G. 2013. Veterinary Anatomy of Domestic Mammals: textbook and colour atlas. Schattauer Verlag, Stuttigart. 681p.

Langevin H.M., Stevens-Tuttle D., Fox J.R., Badger G.J., Bouffard N.A., Krag M.H., Wu J. & Henry S.M. 2009. Ultrasound evidence of altered lumbar connective tissue structure in human subjects with chronic low back pain. BMC Musculoskeletal Disorders 10(1):151. <http://dx.doi.org/10.1186/1471-2474-10-151> <PMid:19958536>

Maeda K., Utsu M. & Kihaile P.E. 1998. Quantification of sonographic echogenicity with grey-level histogram width: a clinical tissue characterization. Ultrasound Med. Biol. 24(2):225-234. <http://dx.doi.org/10.1016/S0301-5629(97)00266-4> <PMid:9550181>

Marques J.A., Pereira D.A. & Marques I.C.S. 2009. A combination study of midazolam and detomidine in thepremedication anestesia for the induction of general anestesia with ketamine in foals. Arq. Bras. Med. Vet. Zootec. 61(6):1290-1296. <http://dx.doi.org/10.1590/S0102-09352009000600006>

Martinoli C., Derchi L.E., Pastorino C., Bertolotto M. & Silvestri E. 1993. Analysis of echotexture of tendons with US. Radiology 186(3):839-843. <http://dx.doi.org/10.1148/radiology.186.3.8430196> <PMid:8430196>

Melo U.P., Palhares M.S. & Ferreira C. 2007. Íleo adinâmico em eqüinos: fisiopatologia e tratamento. Arq. Ciênc. Vet. Zool. UNIPAR 10(1):49-58.

Micklethwaite L., Wood A.K., Sehgal C.M., Polansky M., Dowling B.A., Dart A.J., Rose R.J. & Hodgson D.R. 2001. Use of quantitative analysis of sonographic brightness for detection of early healing of tendon injury in horses. Am. J. Vet. Res. 62(8):1320-1327. http://dx.doi.org/10.2460/ajvr.2001.62.1320. PMid:11497458.

Monteiro J.N.M., Santos W.G., Oliveira D.C., Borlini D.C., Martins Filho S., Machado F.M. & Costa F.S. 2010. Ultrassonografia quantitativa do fígado de gatos hígidos: nota prévia. Braz. J. Vet. Res. Anim. Sci. 2038(5):367-370. <http://dx.doi.org/10.11606/issn.1678-4456.bjvras.2010.26817>

Nestadt C.L., Lusi C.M. & Davies H.M.S. 2015. Effect of different head-and-neck positions on nuchal ligament dimensions in fetal foals. J. Equine Vet. Sci. 35(2):153-160. <http://dx.doi.org/10.1016/j.jevs.2014.12.013>

Osawa H. & Mori Y. 1996. Sonographic diagnosis of fatty liver using a histogram technique that compares liver and renal cortical echo amplitudes. J. Clin. Ultrasound 24(1):25-29. <http://dx.doi.org/10.1002/(SICI)1097-0096(199601)24:1<25::AID-JCU4>3.0.CO;2-N> <PMid:8655663>

Parry D.A.D., Craig A.S. & Barnes G.R.G. 1978. Tendon and ligament from the horse: an ultrastructural study of collagen fibrils and elastic fibres as a function of age. Proc. R. Soc. London B 203(1152):293-303. <http://dx.doi.org/10.1098/rspb.1978.0106> <PMid:33394>

Ragle C.A. 1993. Head trauma. Vet. Clin. N. Am., Equine Pract. 9(1):171-183. <http://dx.doi.org/10.1016/S0749-0739(17)30422-4>

Ramirez O.J., Joan S. & Thrall D.E. 1998. Imaging basilar skull fractures in the horse: a review. Vet. Radiol. Ultrasound 39(5):391-395. <http://dx.doi.org/10.1111/j.1740-8261.1998.tb01624.x> <PMid:9771589>

Silva E.G., Gonçalves M.T., Pinto S.C., Soares D.M., Oliveira R.A., Alves F.R., Araújo A.V.C. & Guerra P.C. 2015. Análise quantitativa da ecogenicidade testicular pela técnica do histograma de ovinos da baixada ocidental maranhense. Pesq. Vet. Bras. 35(3):297-303. <http://dx.doi.org/10.1590/S0100-736X2015000300014>

Swarbrick M.T., Powell S.E. & Haggett E.F. 2018. Computed tomography of nuchal ligament and semispinalis capitis tendon avulsions in a foal. Equine Vet. Educ. 30(2):70-75. <http://dx.doi.org/10.1111/eve.12627>

Tsukiyama K., Acorda J.A. & Yamada H. 1996. Evaluation of superficial digital flexor tendinitis in racing horses through gray scale histogram analysis of tendon ultrasonograms. Vet. Radiol. Ultrasound 37(1):46-50. <http://dx.doi.org/10.1111/j.1740-8261.1996.tb00811.x>

Yu T.Y., Tsai W.C., Cheng J.W., Yang Y.M., Liang F.C. & Chen C.H. 2012. The effects of aging on quantitative sonographic features of rotator cuff tendons. J. Clin. Ultrasound 40(8):471-478. <http://dx.doi.org/10.1002/jcu.21919> <PMid:22508403>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(8):668-671, August 2019
DOI: 10.1590/1678-5150-PVB-6218

Original Article
Animal Morphophysiology

Serum amyloid A and muscle activity biomarkers in horses submitted to equestrian show jumping[1]

Wilson P. Carvalho Filho[2], Leandro A. Fonseca[2]*, Fabricia M. Girardi[2], Lucas D. Bento[3], Pollyanna C. Souto[2] and Andres M.O. Orozco[2]

ABSTRACT.- Carvalho Filho W.P., Fonseca L.A., Girardi F.M., Bento L.D., Souto P.C. & Orozco A.M.O. 2019. **Serum amyloid A and muscle activity biomarkers in horses submitted to equestrian show jumping.** *Pesquisa Veterinária Brasileira* 39(8):668-671. Departamento de Medicina Veterinária, Universidade Federal de Viçosa, Campus Viçosa, Avenida Peter Henry Rolfs s/n, Campus Universitário, Viçosa, MG 36570-900, Brazil. E-mail: leandroabreu@ufv.br

The aim of this study was to evaluate the serum amyloid A (SAA) and biomarkers of muscle activity of horses submitted to show jumping activity. To do this, the variables SAA, glucose, lactate and the biomarkers creatine kinase (CK) and aspartate amino transferase (AST) were evaluated in 10 horses submitted to the show jumping exercise in a tournament for beginners. The evaluations occurred before exercise (T0), immediately after (T1), 30 minutes (T2), 60 minutes (T3) and 24 hours after the end (T4). Data were evaluated using analysis of variance for repeated measures. The statistical software SAEG 9.1 was used to verify the level of significance between the moments for $P<0.05$. Glucose presented a difference between the moments T0 (97.7±13.3mg/dL) and T1 (79.7±14.1mg/dL). Lactate presented elevation in T1 (15.3±6.1mmol/L) compared to the others T0 (3.8±0.8mmol/L), T2 (6.5±3.9mmol/L), T3 (5.3±2.2mmol/L) and T4 (5.1±1.6mmol/L). The CK showed a significant difference between T0 (82.8±51.2U/L) and T1 (140.1±58.5U/L) and between T4 (74.4±43.1U/L) with T1 (140.1±58.5U/L). The AST presented no difference between moments. The show jumping activity with one-meter obstacles did not induce changes in the SAA protein between the moments

INDEX TERMS: Serum amyloid A, muscle activity, biomarkers, horses, equestrian show jumping, acute-phase protein, inflammation, exercise, equine, morphology.

RESUMO.- [**Biomarcadores de amilóide sérica A e de atividade muscular em cavalos submetidos a salto equestre.**] O objetivo deste estudo foi avaliar a amilóide sérica A (SAA) e biomarcadores de atividade muscular de equinos submetidos a atividade de salto, ou hipismo clássico. Para tanto, foram avaliadas as variáveis SAA, glicose, lactato e os biomarcadores creatina quinase (CK) e aspartatoaminotransferase (AST) em 10 equinos submetidos ao exercício de saltos em torneio para iniciantes. As avaliações ocorreram antes do exercício (T0), imediatamente após (T1), 30 minutos (T2), 60 minutos (T3) e 24 horas após o término (T4). Os dados foram avaliados utilizando análise de variância para medidas repetidas. O software estatístico SAEG 9.1 foi utilizado para verificar o nível de significância entre os momentos para $P<0,05$. A glicose diferenciou-se entre os momentos T0 (97.7±13.3mg/dL) e T1 (79.7±14.1mg/dL). O lactado apresentou elevação comparada com o momento T1(15.3±6.1mmol/L) e os demais T0 (3.8±0.8mmol/L), T2 (6.5±3.9mmol/L), T3 (5.3±2.2mmol/L) e T4 (5.1±1.6mmol/L). A CK mostrou diferença significativa entre T0 (82.8±51.2U/L) e T1 (140.1±58.5U/L) e entre T4 (74.4±43.1U/L) com T1 (140.1±58.5U/L). A AST não apresentou diferença entre os momentos. A atividade de hipismo clássico com obstáculos de um metro não induziu alterações na proteína SAA entre os momentos.

TERMOS DE INDEXAÇÃO: Biomarcadores, amilóide sérica A, atividade muscular, cavalos, salto equestre, proteínas de fase aguda, biomarcadores musculares, inflamação, exercício, hipismo, equinos, morfologia.

INTRODUCTION

Equestrian sporting activities are increasingly evident in the world and the show jumping is the sport that stands out most among sports with horses. According to the International

[1] Received on February 22, 2019.
Accepted for publication on April 9, 2019.
[2] Departamento de Medicina Veterinária, Universidade Federal de Viçosa (UFV), Campus Viçosa, Avenida Peter Henry Rolfs s/n, Campus Universitário, Viçosa, MG 36570-900, Brazil. *Corresponding author: leandroabreu@ufv.br

[3] Universidade Federal de Minas Gerais (UFMG), Avenida Pres. Antônio Carlos 6627, Pampulha, Belo Horizonte, MG 31270-901, Brazil.

Equestrian Federation (Fédération Equestre Internationale 2013), almost 65 thousand animals are registered in the entity and more than 50 percent belong to the modality of show jumping. However, this type of exercise induces intense structural and metabolic changes and adaptations, which depend on adequate stimulus and must be monitored (Fazio et al. 2014). It is a sport that involves the largest number of horses in the world. Like any sport, in show jumping there are two important challenges that are to ensure the animal's health and to improve athletic performance.

The acute phase response presents great complexity involving local and systemic reactions. Part of these effects is the expression of the acute phase proteins (APPs) (Jain et al. 2011). The production of these proteins is triggered by inflammatory mediators, among them interleukin 1b, interleukin 6 and tumor necrosis factor (Leclere et al. 2015). Changes in APR can occur induced by exercise with aerobic or anaerobic pathways (Gondin et al. 2013).

Elevations of APPs concentration in serum demonstrate a peculiar acute phase reaction and indicate the clinical condition of the animal. C-reactive protein (CRP), haptoglobin (Hp) and serum amyloid A (SAA) are the most sensitive proteins in humans and animals. However, the manifestation of these proteins varies in different animal species. In horses Hp has medium manifestation and can be used as a marker of hemolysis during exercises (Gondin et al. 2013). The SAA is the one with the highest pronouncement in response to an acute phase reaction, followed by moderate manifestation of Hp and CRP (Cywińska et al. 2012).

SAA has low or undetectable concentration in clinically normal animals. On the other hand, it can increase from 10 to 1000 times within 24 hours after tissue damage, cell necrosis, inflammation and/or infection occur. It presents biological half-life of approximately 20 to 35 hours (Westerman et al. 2016).

Enzymes such as CK and AST are commonly used as biomarkers of muscle activity and the amount of these enzymes released from muscle depends on cell location, molecule size and injury (Fisher et al. 2014, Buzala et al. 2015, Noleto et al. 2016). It is important to use the measurement of CK and AST activity in the diagnosis of human muscle injury, equine as well as greyhound racing dogs (Lucas et al. 2015). It is also used as exercise intensity monitoring and is typical of muscle physiology (Cerqueira et al. 2018).

Considering that show jumping is a high intensity sport and of great impact, the possibility of injury is relevant. It is important to monitor the behavior of the animal's organism in situations such as that described.

The aim of this study was to verify the hypothesis that SAA, CK, AST, glucose and lactate are responsive in horses submitted to show jumping with obstacles of one meter.

MATERIALS AND METHODS

Ethics committee. The experimental design was submitted to the Ethics Committee on the Use of Animals (CEUA-UFV) and approved (CEUA-UFV 77/2015).

Animals. Ten horses, three females and seven males, Hanoverian mixed breed and Belgian saddle mixed breed, aged between seven and fifteen years old, with a mean weight of 570±95kg were used. The horses were experienced and trained in the show jumping, with training routine of three times weekly and were regular participants of regional competitions (National Show Jump Concourse - CNS) of one meter high obstacles. They had similar training routine suggesting belonging to the same physical and technical level. The animals were submitted to exercise in healthy conditions, under previous physical and clinical exams, without indication of lameness or apparent sign of another disease, signaling perfect health at the moment of the competition.

Experimental design. Data were collected during a show jumping competition. The exercise consisted in making a course of approximately 350 meters, on sand floor and twelve vertical obstacles arranged along the path, with height of one meter, protocol characteristic of the sport (Fazio et al. 2014). The animals performed individual 15 minutes warm up. The horses were conducted all the time by their usual rider. After the warm-up period the heart rate monitor was installed (Polar Equine RS800CX G3®) and started the exercise.

Blood collection. All materials used were sterile. Blood collection was performed by venipuncture of the jugular, with previous antisepsis, using vacuum tubes containing 10% EDTA plus sodium fluoride for glucose and lactate and plain vacuum tube without any additional to the tubes for other biochemistry parameters. The samples were centrifuged in the laboratory near the site at 3000 revolutions per minute for 10 minutes and then frozen at -20°C for further analysis. The blood was collected in five moments, namely: T0 - animal in the bay, at rest immediately before being prepared for the exercise; T1 - immediately after the end of the journey; T2 - 30 minutes after completion; T3 - 60 minutes after the end and T4 - 24 hours after the end of the activity.

Laboratory analysis. Commercial kits were used with CK and AST being enzymatic kinetic assay; lactate by enzymatic method and glucose by the colorimetric method (Humastar 300, Human, In Vitro®) before freezing. SAA concentrations were analyzed by enzyme-linked immunosorbent assay (Nori® Equine SAA Elisa Kit). The samples were processed in the Veterinary and Biochemistry Departments, "Universidade Federal de Viçosa", Brazil.

Statistical analysis. Results were expressed as mean ± standard deviation. The Cochran and Bartlett test were used to verify the variance homogeneity of the data. The effect of exercise on physiological variables was evaluated by ANOVA for repeated measures. Then, the Tukey test was applied, in order to determine significant differences for each sampling time, when normality was verified. Kruskal - Wallis test was used for non-parametric analysis of variance. A $p<0.05$ value was established as significant.

RESULTS

The results of the studied variables are presented in Table 1. The animals had a mean resting heart rate of 34±3bpm; 161±20bpm during activity and peak of 190±16bpm. The activity caused alteration in the glucose, lactate and CK. The AST and acute phase protein SAA were not influenced by exercise.

Table 1.Mean values ± standard deviation of glucose concentration (GLU), lactate (LAC), aspartate aminotransferase (AST), creatine phosphokinase (CK) and serum amyloid A (SAA) at different times under the influence of show jumping activity

	GLU (mg/dL)	LAC (mmol/L)	AST (U/L)	CK (U/L)	SAA (ng/mL)
T0	97.7±13.3^{a}	3.8±0.8^{b}	206.9±39.0^{a}	82.8±51.2^{ac}	74.2±78.1^{a}
T1	79.7±14.1^{b}	15.3±6.1^{a}	207.4±28.9^{a}	140.1±58.5^{b}	78.7±78.6^{a}
T2	93.1±14.9^{ab}	6.5±3.9^{b}	209.2±25.7^{a}	135.0±85.5^{ab}	64.2±76.0^{a}
T3	90.8±8.4^{ab}	5.3±2.2^{b}	210.4±25.4^{a}	121.4±54.0^{ab}	84.5±74.9^{a}
T4	91.7±10.6^{ab}	5.1±1.6^{b}	184.9±31.1^{a}	74.4±43.1^{c}	72.6±84.6^{a}

T0 = Results at rest, T1 = immediately after exercise, T2 = 30 minutes after, T3 = 60 minutes after, T4 = 24 hours after exercise; ^{a,b,c} different letters on the same column indicate significant difference between moments, for $p<0.05$.

DISCUSSION

This study shows that behavior of the heart rate was similar to other study with show jumping horses (Fazio et al. 2014) and represents 64.76% of the maximum, characterizing the event as of submaximal to moderate intensity (Hinchcliff et al. 2008, Gomes 2009). The average of the speed was 282.8 meters per minute.

The exercise did not cause significant change in SAA between moments. Some horses presented values at all times very close to zero. This phenomenon has raised the standard deviation and may have contributed to the irregularity of the data. The absence of homogeneity of our studied group may be based an argument suggesting possible previous different conditions. Even the animals having similar technical level and the same training center they belong to different owners as well as their trainers. The community of competitive sport in Brazil still holds the right to keep secrets that may involve some ethical questioning. Investigation with standardbred trotters in races from 1600 to 2500 meters also did not find variation in SAA (Kristensen et al. 2014). The authors state that their results do not corroborate the hypothesis that exercise causes elevation in this acute phase protein. However, an experiment with 25 thoroughbred horses in maximum speed of 1200 and 1600 meters and found a significant difference between rest and 24 hours after the effort (Turlo et al. 2016). In our study of the 10 horses only three had high SAA in T4 and they were neither winners nor the last ones placed. The most experienced and less experienced animals showed no change. The behavior of SAA in our study can be understood by the fact that in the exercise it seems that its elevation of concentration is linked to long duration efforts (Nolen-Walston 2015).

The exercise induced a difference between T0 and T1 on glucose concentration, characterizing a depletion of glycemia but no difference between the other moments. After the exercise the values show that a similar behavior was found in a study with show jumping horses, where it is clear that the effort induced muscle glycogenolysis, which caused a migration of blood glucose into the muscle cell (Fazio et al. 2014). However, the body seeks to reestablish the glycolytic balance through high activity of sympathetic system and release of adrenaline which activate hepatic glycogenolysis (Hassan et al. 2015). Muscle glycogen recovery depends on each animal as well as individual diet and this period can take days (Marlin & Nakervis 2004). This may be an explanation that glycemic levels, even after 24 hours, are still not the initials.

Lactate showed significant elevation only at moment T1. This hyperlactacidemia occurs parallel the glucose depletion demonstrated previously. In this case the muscle cell in intense activity and of short duration that characterizes the anaerobic effort (Roberts et al. 2014). A study with show jumping horses and intense treadmill exercise also found significant elevation in lactate levels (Fazio et al. 2014, Santos et al. 2015). Quarter horses after barrel race showed raise of 9.35±0.40mmo/L (Souza et al. 2018). The high concentration of lactate after the activity in this study may be due to inadequate physical conditioning of the horses evaluated.

The CK of this study found similar behavior in thoroughbred horse after maximal exercise of running, significant, but with a value of much higher mean, 559.8±203.6U/L (Octura et al. 2014) in contrast to our study (140.1±58.5U/L).This effect is expected because the rapid degradation of this compound in the CK reaction occurs because the released energy can be used to resynthesize ATP and thereby maintain the elevated intracellular ATP: ADP ratio. This activity is only possible with the elevation of the enzymatic activity of the CK (Maughan et al. 2000).This elevation of CK concentration after exertion due to phosphocreatine demand occurs by increasing the permeability of the muscle membrane in response to exercise also found in study with racehorses. (Hassan et al. 2015). It can be seen that the CK values is decaying after the peak post exertion. This phenomenon suggests that the body is reestablishing homeostasis, since energy is no longer being requested for intense work (Octura et al. 2014). Other important fact is the CK halflife is between two to six hours (Cerqueira et al. 2018); this can be the reason of declining. Monitoring CK activity can be an important indicator to evaluate the impact of training on the animal, as it responds to the duration and intensity of the exercise (Buzala et al. 2015).

The behavior of AST are in agreement with other study that did not find a significant difference in thoroughbred horses after fast running with a peak of 269.25±63.87U/L (Octura et al. 2014). They believe that the effort was not enough to induce sufficient permeability for the enzyme to manifest. Another study with show jumping horses also did not reveal significant variation of AST before and after exercise. The authors attribute the result to the good physical fitness of the animals (Santos et al. 2015). Study with quarter horses during vaquejada exercise also did not find AST alteration (Sousa et al. 2018).

CONCLUSIONS

The results refute the hypothesis that activity of show jumping with obstacles of one meter induces an acute phase reaction by raising SAA concentration as well as the biomarker of muscle activity AST. However it triggered expected metabolic change of glucose, lactate and CK.

These results do not change the position of the acute phase proteins as an important marker of inflammation. They can serve as an additional biomarker in monitoring the health of animals. It is suggested new investigation seeking answer with different height of the obstacles.

Acknowledgements.- This study was financed in part by the "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior", Brasil (CAPES) - Finance Code 001, CNPq e FAPEMIG.

Conflict of interest.- There is no conflict of interest.

REFERENCES

Buzala M., Krumrych W. & Janick B. 2015. Usefulness of creatine kinase activity determination for assessing the effects of physical effort in horses. Pakistan Vet. J. 35(3):267-273.

Cerqueira J.A., Restan W.A.Z., Fonseca M.G., Catananti L.A., De Almeida M.L.M., Junior W.H.F., Pereira G.T., Carciofi A.C. & De Camargo Ferraz G. 2018. Intense exercise and endurance-training program influence serum kinetics of muscle and cardiac biomarkers in dogs. Res. Vet. Sci. 121:31-39. <http://dx.doi.org/10.1016/j.rvsc.2018.10.004> <PMid:30316014>

Cywińska A., Szarska E., Górecka R., Witkowski L., Hecold M., Bereznowski A., Schollenberger A. & Winnicka A. 2012. Acute phase protein concentrations after limited distance and long distance endurance rides in horses. Res. Vet.

Sci. 93(3):1402-1406. <http://dx.doi.org/10.1016/j.rvsc.2012.02.008> <PMid:22390917>

Fazio F., Casella S., Assenza A. & Arfuso F. 2014. Blood biochemical changes in show jumpers during a simulated show jumping test. Veterinarski Arhiv 84:143-152.

Fédération Equestre Internationale 2013. Annual Report. Fédération Equestre Internationale, Lausanne, p.92.

Fisher A.L., Greene H.M., Talmadge R.J. & Nout Y.S. 2014. Effect of a lactate-guided conditioning program on fitness markers in the equine athlete. Annals Southern California Conference for Undergraduate Research (SCCUR), California State Polytechnic University, Pomona.

Gomes A.C. 2009. Treinamento Desportivo: estruturação e periodização. Artmed, Porto Alegre, p.89.

Gondin M.R., Foz N.S.B., Pereira M.C., Flagliari J.J., Orozco C.A.G., D'Angelis F.H.F., Queiroz-Neto A. & Ferraz G.C. 2013. Acute phase responses of different positions of high-goal (elite) polo ponies. J. Equine Vet. Sci. 33(11):956-961. <http://dx.doi.org/10.1016/j.jevs.2013.02.005>

Hassan H.Y., Aly M.A., Elseady Y.M., Nayel M.A., Elsify A.M., Salama A.A., Hassan M.S., Elbarody E.F. & Kamar A.B. 2015. The effect of race in the clinical, hematological and biochemical biomarkers in thoroughbred horses. Alexandria J. Vet. Sci. 46(1):161-169. <http://dx.doi.org/10.5455/ajvs.190592>

Hinchcliff K., Geor R. & Kaneps A. 2008. Equine Exercise Physiology: the science of exercise in the athletic horse. W.B. Saunders, Philadelphia, p.2-11. <http://dx.doi.org/10.1016/B978-070202857-1.50003-2>.

Jain S., Gautam V. & Naseem S. 2011. Acute-phase proteins: as diagnostic tool. J. Pharm. Bioallied Sci. 3(1):118-127. <http://dx.doi.org/10.4103/0975-7406.76489> <PMid:21430962>

Kristensen L., Buhl R., Nostell K., Bak L., Petersen E., Lindholm M. & Jacobsen S. 2014. Acute exercise does not induce an acute phase response (APR) in Standardbred trotters. Can. J. Vet. Res. 78(2):97-102. <PMid:24688170>

Leclere M., Lavoie-Lamourex A. & Lavoi J.P. 2015. Acute phase protein in race horses with inflammatory airway disease. J. Vet. Intern. Med. 29(3):940-945. <http://dx.doi.org/10.1111/jvim.12587> <PMid:25857218>

Lucas V., Barrera R., Duque F.J., Ruiz P. & Zaragoza C. 2015. Effect of exercise on serum markers of muscle inflammation in Spanish greyhounds. Am. J. Vet. Res. 76(7):637-643. <http://dx.doi.org/10.2460/ajvr.76.7.637> <PMid:26111094>

Marlin D. & Nakervis K.J. 2004. Equine Exercise Physiology. Blackwell Science, Oxford. 304p.

Maughan R.J., Gleeson M. & Greenhaff P.L. 2000. Bioquímica do Exercício e Treinamento. Manole, São Paulo, p.47-63.

Nolen-Walston R. 2015. How to interpret serum amyloid A concentrations. AAEP Proceedings 61:130-137.

Noleto P.G., Dos Santos J.B.F., Rocha F.M., Fasano P.E., Guimarães E.C. & Mundim A.V. 2016. Effect of a 130 km endurance ride on the serum biochemical profiles of Mangalarga Marchador horses. J. Equine Vet. Sci. 39:7-11. <http://dx.doi.org/10.1016/j.jevs.2015.08.010>

Octura J.E.R., Lee K.J., Woo C.H. & Vega R.S.A. 2014. Elevation of blood creatine kinase and selected blood parameters after exercise in thoroughbred race horses. J. Res. Agricult. Anim. Sci. 2:7-13.

Roberts C., Harris P., Murray R., Cnockaert R. & Roberts C. 2014. The relationship between blood lactate, serum muscle enzymes, jumping performance and muscle soreness in show-jumping horses. Equine Vet. J. 46(Suppl. 46):9-9. <http://dx.doi.org/10.1111/evj.12267_27>

Santos V.P.S., Gonzales F.D.G., Castro Junior F.C. & Correio T.F.C. 2015. Hemato-biochemical response to exercise with ergometric treadmil, mount training and competition in jumping horses. Arch. Vet. Sci. 20(Suppl.1):1-8.

Sousa R.A., Silva G.A., Rêgo G.M.S., Gonçalves Neto J.R., Gottardi F.P. & Machado L.P. 2018. Effect of vaquejada exercise on the physiological and biochemical profiles of sporadic competitors and atletic horses. Acta Vet. Bras. 12(1):17-23. <http://dx.doi.org/10.21708/avb.2018.12.1.7231>

Souza L.A., Hunka M.M., Nery P.C.R., Coelho C.S., Manso H.E.C.C.C. & Manso Filho H.C. 2018. The effect of repeated barrel racing on blood biomarkers and physiological parameters in Quarter Horses. Comp. Exerc. Physiol. 14(1):47-54. <http://dx.doi.org/10.3920/CEP170019>

Turlo A., Cywinska A., Czopowicz M., Witkowski L., Jaśkiewicz A. & Winnicka A. 2016. Racing induces changes in the blood concentration of serum amyloid A in thoroughbred racehorses. J. Equine Vet. Sci. 36:15-18. <http://dx.doi.org/10.1016/j.jevs.2015.09.008>

Westerman T.L., Foster C.M., Tornquist S.J. & Poulsen K.P. 2016. Evaluation of serum amyloid A and Haptoglobin concentrations as prognostic indicators of horses with colic. J. Am. Vet. Med. Assoc. 248(8):935-940. <http://dx.doi.org/10.2460/javma.248.8.935> <PMid:27031421>

Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

INSTRUÇÕES AOS AUTORES

A submissão de artigos à revista "Pesquisa Veterinária Brasileira" (PVB) deve ser feita em Word, através do Sistema ScholarOne, *link* <https://mc04.manuscriptcentral.com/pvb-scielo>

A tramitação somente pode ter início se o seu artigo estiver rigorosamente dentro das normas de apresentação da revista, de acordo com as Instruções aos Autores, o modelo no site da revista e os últimos fascículos publicados (www.pvb.com.br). Na verificação de falhas de apresentação, o artigo será devolvido aos autores para as devidas correções.

Os autores podem submeter seus artigos em **Inglês** ou em **Português**, mas sempre com um Resumo em português. No caso de artigos aceitos escritos em **Português**, estes serão traduzidos para o **Inglês** pela Editora Cubo; pois todos os artigos publicados na PVB serão em inglês. Para os artigos já submetidos em **Inglês**, os autores devem apresentar via ScholarOne um Certificado de Tradução de uma empresa habilitada ou de um Tradutor Nativo. **Essa regra vale para artigos submetidos a partir de 1 de janeiro de 2018.**

Os pagamentos da taxa de publicação (*Paper Charge*) serão cobrados na ocasião do envio da comunicação de aceite por e-mail:

(1) **Artigos submetidos em inglês, R$ 1.500,00 (*US$ 480.00*) por artigo;**

(2) **Artigos submetidos em português, R$ 1.500,00 (*US$ 800.00*) mais R$ 0,32 por palavra** (o valor total cobrado por será enviado por e-mail pela tesouraria do CBPA).

A partir de 2019, **todos os autores deverão criar um registro no ORCID** (Open Researcher and Contributor ID - https://orcid.org/register) e vinculá-lo ao seu perfil no ScholarONE. O vínculo pode ser feito editando o perfil do usuário no ScholarONE na opção **Associate your existing ORCID iD**. Os identificadores ORCID contribuem para a identificação única dos autores e, portanto, para os processos interoperacionais e bibliométricos que envolvem autores. Usando um ORCID, os pesquisadores são fácil e corretamente conectados com seus resultados de pesquisa, publicações e afiliações.

O **texto** deve ser formatado, em todos os pormenores, de acordo com as normas de apresentação da revista (www.pvb.com.br).
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.

Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

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

- Livros:

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

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

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

- Capítulos de livros:

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

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

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

- Dissertações e Teses:

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

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

- Resumos publicados em eventos:

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

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

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

Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

GUIDE FOR AUTHORS

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

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

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

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

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

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

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

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

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

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

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

The author rights of the accepted papers are preserved.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3. **Figure presentation:**

a) Save images at 300 dpi, TIF files.

b) Send each figure separately.

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

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

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

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

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

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

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

Exemples for References:

➢ Articles published in scientific journals:

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

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

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

➢ Books:

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

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

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

➢ Chapters of books:

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

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

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

➢ Dissertations and Theses:

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

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

➢ Abstracts published in Events:

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

Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

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

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

(Note: Consult entire papers instead of only Abstracts)

Pesquisa Veterinária Brasileira

Brazilian Journal of Veterinary Research

CONTENTS Volume 39, Number 8, August 2019

editora cubo
soluções para o universo acadêmico

ISSN 0100-736X *(Print)*
ISSN 1678-5150 *(Online)*