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Figura da capa: *Ipomoea bahiensis* em fase de florescimento durante estação chuvosa de 2017, no município de Manari, PE, Brasil. (Mendonça et al., p. 2047)

Cover illustration: Ipomoea bahiensis *in flowering phase during the rainy season of 2017, municipality of Manari, PE, Brazil.* (Mendonça et al., p. 2047)

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Original Article
Animais de Produção/*Livestock Diseases*
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Cryopreservation of lymphocytes for immunological studies in horses[1]

Renata F. Siqueira[2]* and Wilson Roberto Fernandes[2]

ABSTRACT.- Siqueira R.F. & Fernandes W.R. 2018. **Cryopreservation of lymphocytes for immunological studies in horses.** *Pesquisa Veterinária Brasileira 38(11):2019-2022*. Departamento de Clínica Médica, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Dr. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP 05508-270, Brazil. E-mail: refarinelli@yahoo.com.br

The use of frozen cells allows studies on diseases and other immunological assays, since it facilitates the logistics of collecting and transporting, including laboratories located in different cities or other countries. The objectives of this study were to verify if the storage in the refrigerator after collection at different times changes the viability of total leukocytes after months of freezing and the ratio of CD4/CD8 is affected by the freezing process. Venous blood of 15 healthy horses was used and the experiment was divided into 2 stages. In the first, the viability of the leukocytes before and after freezing was verified, as well as different storage times in the refrigerator (fresh blood, stored for 24 and 48 hours) before the freezing process. In the second part, the immunophenotyping of the T lymphocytes was performed, in order to observe if after thawing the relationship between LT CD4 and LT CD8 undergoes change. There was no difference between the amounts of viable leucocytes from frozen fresh blood compared to fresh blood before freezing, nor difference between the viability of blood left in the refrigerator (4°C) for 24 hours and fresh blood and fresh frozen blood. There was a decrease in viability of frozen leukocytes after 48 hours left in the freezer for other samples; however, the recovery was 10^7x cells. Regarding the immunophenotyping of CD2CD4+ and CD2CD8+ double-labeled T lymphocytes in the blood stored in the refrigerator for 24 hours before freezing, no difference was observed between before and after 6 months of freezing. It is concluded that cryopreservation of equine total leukocytes is possible and, although there was a difference between freezing times, even in the less viable sample, sufficient numbers of cells were recovered for other immunological assays.

INDEX TERMS: Cryopreservation, lymphocytes, immunology, horses, immunophenotyping, mononuclear cells.

RESUMO.- [Criopreservação de linfócitos para estudos imunológicos em equinos.] A utilização de células congeladas possibilita estudos sobre doenças e outros ensaios imunológicos, pois facilita a logística de coleta e transporte, inclusive para laboratórios localizados em cidades diferentes ou outros países. Os objetivos desse estudo foram verificar se o armazenamento sobre refrigeração em diferentes tempos e a criopreservação alteram a viabilidade de leucócitos totais e se a relação entre LT CD4/CD8 é afetada pelo processo de congelamento. Utilizou-se sangue venoso de 15 cavalos hígidos e o experimento foi dividido em 2 etapas. Na primeira foi analisado se houve alteração na viabilidade dos leucócitos provenientes de amostras de sangue armazenadas em diferentes tempos em geladeira antes e depois de 6 meses de congelamento a -80°C. Na segunda parte, realizou-se a imunofenotipagem dos linfócitos T, com a finalidade de observar se após o descongelamento a relação entre LT CD4 e LT CD8 sofre alteração. Não houve diferença entre a quantidade de leucócitos viáveis da amostra de sangue fresco descongelado em relação ao sangue fresco antes do congelamento, nem diferença entre a viabilidade do sangue deixado em congelador (4°C) por 24 horas e do sangue fresco. Houve uma diminuição da viabilidade dos leucócitos, após o descongelamento de 6 meses (-80°C), das amostras de sangue deixado em geladeira por 48 horas antes do congelamento em relação às outras amostras, porém, a recuperação foi de células x10^7. Quanto à imunofenotipagem de linfócitos T com dupla marcação CD2CD4+ e CD2CD8+, no sangue armazenado

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[2] 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. *Corresponding author: refarinelli@yahoo.com.br

em geladeira por 24 horas antes do congelamento, e não foi observada diferença entre antes ou depois de 6 meses de congelamento. Conclui-se que a criopreservação de leucócitos totais de equinos é possível e, embora tenha havido diferença entre os tempos de congelamento, mesmo na amostra menos viável, houve recuperação de uma quantidade de células suficientes para outros ensaios imunológicos.

TERMOS DE INDEXAÇÃO: Criopreservação, linfócitos, imunologia, equinos, imunofenotipagem, células mononucleares.

INTRODUCTION

The use of frozen blood cells, mainly mononuclear leukocytes, is a routine procedure very well established in human laboratories for both diagnostic and therapeutic purposes (Sputtek et al. 2007). Cryopreservation allows late assays of both cellular function and phenotyping, besides centralizing in a single laboratory samples collected in several places, thus minimizing possible interlaboratory variability (Aziz et al. 2013).

Cell freezing and thawing processes can cause cell death or irreversible damage due to water crystallization and organelle modifications and DNA damage. Lymphocytes and monocytes are more resistant to such damage because they have fewer granules. However, the use of solutions with cryoprotective substances such as dimethylsulfoxide (DMSO), fetal bovine serum, RPMI can minimize damages if the freezing and thawing time is respected (Svedentsov et al. 2007).

Cryopreservation of human mononuclear leukocytes maintains the ability to express histocompatibility antigens and cellular immunity parameters such as lymphocyte proliferation in response to mitogens, cytokine expression and surface antigens, and NK cell cytotoxicity (Truax et al. 1990). CD4 and CD8 T lymphocytes are crucial components of the antiviral immune response, since limiting virus replication and monitoring the function of these cells is prerequisite for the development of antiviral therapies and vaccines (Kutscher et al. 2013).

Although the use of frozen cells in Veterinary Medicine is not as routine as in Medicine there are numerous possibilities of application in the study of vaccines, therapies and in the knowledge of all stages of development of some viral diseases of animals that are in great distances of the laboratories, facilitating logistics. In horses, in the acute phase of Equine Infectious Anemia the immune response is mediated by the major histocompatibility complex (MHC) and cytotoxic CD8 T lymphocytes (Craigo & Montelaro 2013). In West Nile virus infection the CD8 T lymphocyte also plays an important role in the control of infection through perforins and interleukins such as tumor necrosis factor (TNF) and interferon gamma (IFNγ), but the importance of LT CD4 (Netland & Bevan 2013). Equine Viral Arteritis virus has tropism by monocytes and CD3 LT, especially by CD4 (Go et al. 2010). Immunity of neonates is not fully understood and the mechanism of passive transfer by colostrum in several mammalian species suggests that the maternal lymphocyte plays key roles in the development of the immune system of foals (Perkins et al. 2014).

The use of frozen cells would allow more studies on these diseases and other tests, as it facilitates collection and transport logistics, including for laboratories located in different cities or other countries.

The objectives of this study were to verify if refrigeration storage at different times and cryopreservation alter the viability of total leukocytes and whether the relationship between CD4/CD8 LT is affected by the freezing process.

MATERIALS AND METHODS

This study was approved by the CEUA (Ethics Committee for the Use of Animals) of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo, under the protocol number 2606/2012.

Fifteen healthy, vaccinated and vermifugated horses, aged between 3 and 5 years, weighing between 400 and 450 kg, were fed with commercial concentrate and Coast-cross hay.

The experiment was divided into two stages. In the first one, it was verified if different storage times of the blood at 4°C alter the viability of the leukocytes before and after the freezing at -80°C. For this, nine heparinized tubes of peripheral blood of the jugular vein of each horse were collected. Three samples from each animal were immediately processed and frozen (fresh blood). Three samples were stored for 24 hours at 4°C and only then were processed and frozen (24h blood). The last three samples were stored for 48 hours at 4°C and then processed and frozen (48h blood).

Both fresh and blood samples stored in the refrigerator were centrifuged at 800g for 10 minutes, the leucocyte bud removed and deposited in PBS (phosphate buffered saline) and the erythrocytes lysed three times. Total leukocytes were stained with Trypan blue and counted. Then suspended in heat-inactivated fetal bovine serum solution (FCS, Invitrogen, Karlsruhe, Germany) and 10% DMSO (dimethylsulfoxide, Sigma-Aldrich, Munich, Germany) and gradually frozen at -80°C. Six months then all samples were thawed and the leucocytes counted.

In the second part, the immunophenotyping of the T lymphocytes was performed, in order to observe whether after the thawing the relationship between LT CD4 and LTCD8 changes. Immunophenotyping of the leukocytes before freezing the blood stored in the refrigerator was carried out 24 hours before the freezing and after 6 months frozen in a freezer -80°C. Leukocytes were thawed rapidly in a water bath at 37°C, centrifuged at 800g for 5 minutes, resuspended in 1ml of PBS, pH 7.4, and counted. 1x10 6 cells were deposited in 1 ml PBS in each tube. For each sample, 3 tubes were used, the first as white, the second for monoclonal anti-CD2 mice (IgG2a anti-horse CD2, Serotec MCA1278) and CD4 (IgG1 mouse anti-horse CD4, Serotec MCA1078) tube for monoclonal anti-CD2 and CD8 (anti-horse CD8 mouse IgG1, Serotec MCA 2385) monoclonal antibodies. After a half-hour incubation at room temperature and second wash, goat anti-rat IgG RPE (Serotec 303009) and Goat anti-mouse IgG FITC (Serotec 103002) secondary antibodies were labeled, another half-hour incubation in the dark and last wash. The labeled cells were resuspended in 1ml of PBS and subjected to analysis on the flow cytometer (BD FACS Calibur, United States) for identification and enumeration of lymphocyte subpopulations.

The data read by flow cytometry were analyzed by a computer program (Flow Jo software), which provided the histogram, and the table with the number of cells identified by immunophenotyping.

The data were analyzed using SPSS statistical software v.9.0. The behavior of the studied variables, both in the comparison between viability before and after freezing, and in the comparison between lymphocyte subpopulations, was shown to be non-parametric. Therefore, the Wilcoxon test was paired with 5% significance ($P<0,05$).

RESULTS AND DISCUSSION

The cryopreserved human mononuclear leukocytes are separated by the Ficoll-Hypaque separation gradient where only the target cells of the study are obtained. Because it is a more laborious and time consuming technique, it was decided to work with total leukocytes obtained from the leukocyte bud after centrifugation, as suggested by Truax et al. (1990), where a single person can process larger numbers of samples.

Initially, we tested whether the permanence of whole blood in the refrigerator at different times (24 and 48 hours) alter the viability of total leukocytes compared to fresh blood, or which has been processed immediately after harvest. The results are shown in Table 1.

There was no difference between the amounts of viable leukocytes from fresh frozen blood relative to fresh blood before freezing, which suggests that the freezing process used was adequate. There was also no difference between the viability of blood left in the refrigerator (4°C) for 24 hours and fresh blood and fresh frozen blood. There was a decrease in the viability of the frozen leukocytes after 48 hours left in the freezer relative to the other samples, however, the recovery was of x107 cells, which means that even if a significant decrease occurred, a sufficient amount of cells were recovered for the accomplishment of some tests. According to Sputtek et al. (2007), if the purpose of the freezing is laboratory assays, quantities of 0.5x10 6 are sufficient, especially if 5 to 10% DMSO is used as a cytoprotectant. Pereira-Cunha et al. (2014) worked with human umbilical cord mesenchymal stem cells and other mononuclear cells stored at room temperature for 96 hours prior to freezing and found that despite the decrease in the number of live cells there was still sufficient T and B lymphocyte viability to perform assays.

In the second part of this study the immunophenotyping of CD2CD4+ and CD2CD8+ double-labeled T lymphocytes was performed in the blood stored in the refrigerator for 24 hours before freezing, and no difference was observed between before and after 6 months of freezing, as illustrated by Table 2.

Weinberg et al. (2009) performed the immunophenotyping and cell proliferation of peripheral blood mononuclear cells from 104 human volunteers (49 seropositive for HIV and 55 healthy) using blood samples frozen at -70°C and in liquid nitrogen and concluded that fresh and frozen blood samples of the two forms, present a good correlation, since they did not find difference in the amount of CD4 and CD8 LT before and after freezing and between fresh and frozen blood. Other researchers (Reimann et al. 2000, Alam et al. 2012, Aziz et al. 2013) also analyzed the difference in the number of the same cells after a long freezing period (6 months to 6 years) and found no alteration, as in the present study. Duthie et al. (2002) evaluated antioxidant status, DNA damage and the ability to repair fresh and frozen human lymphocytes for more than seven months. They concluded that the thawing process does not cause DNA damage and that the antioxidant capacity is also preserved.

Kutscher et al. (2013) report that rest of a few hours or all night after lymphocyte thawing at 37°C is a common practice in laboratories that work with human blood and aims to increase the functional activity of these cells. The same authors tested the effect of overnight rest on in vitro stimulation, cytokine production and the antiviral capacity of CD4 and CD8 T lymphocytes. They concluded that although there was a decrease in cell viability after rest, there was a significant increase in the antiviral capacity of both cells. CD8 T lymphocytes increased expression of IFNγ, IL-2, TNFα and MIP 1β. The findings of this study and others cited by these authors, who use the rest of the cells, reinforce the idea of this present work that it is possible to use frozen cells and to use the blood stored for 24 hours in many immunological tests in Equine Veterinary Medicine also, where transporting the blood to the laboratory can be time consuming.

Table 1. Median values, first and third quartiles of the variables fresh blood, fresh frozen blood, 24 hours frozen blood and 48 hours frozen blood

	Fresh blood	Fresh frozen blood	24 hour frozen blood	48 hour frozen blood
Median (1 and 3 Quartiles)	7.250[A] (5.950-15.70)	7.225[A] (5.819-9.282)	7.388[A] (3.850-8.560)	3.850[B] (2.863-7.388)

Different letters on the same line represents difference between variables (P<0.05).

Table 2. Median values, first and third quartiles of the LT CD4 / CD8 ratio pre and post freezing

	CD4 / CD8 Pre Freezing	CD4 / CD8 Post Freezing
n	15	15
Median (1 and 3 Quartiles)	0.9664 (0.9009 - 0.9906)	0.8803 (0.9420 - 0.8395)
P	0.1055	

CONCLUSIONS

Cryopreservation of total leukocytes from equines is possible, and although there was a difference between freezing times even in the less viable sample, sufficient numbers of cells were recovered for other immunological assays.

Blood may be stored for up to 24 hours in a refrigerator prior to freezing for immunological assays using mononuclear cells, as up to that time there has been no change in cell viability or the relationship between CD4/CD8 T lymphocytes.

Further studies should be carried out in order to understand the action of storage time on the activity of these cells.

REFERENCES

Alam I., Goldeck D., Larbi A. & Pawelec G. 2012. Flow cytometric lymphocyte subset analysis using material from frozen whole blood. J. Immunoassay Immunochem. 33(2):128-139. <http://dx.doi.org/10.1080/15321819.2011.604370> <PMid:22471603>

Aziz N., Margolick J.B., Detels R., Rinaldo C., Phair J., Jamieson B.D. & Butch A. 2013. Value of quality assessment program in optimizing cryopreservation of peripheral blood mononuclear cell in a multicenter study. Clinic. Vac. Immunol. 20(4):590-595. <http://dx.doi.org/10.1128/CVI.00693-12> <PMid:23408528>

Craigo J.K. & Montelaro R.C. 2013. Lessons in AIDS vaccine development learned from studies of equine infectious, anemia virus infection and immunity. Virus 5(12):2963-2976. <http://dx.doi.org/10.3390/v5122963> <PMid:24316675>

Duthie S.J., Pirie L., Jenkinson A.M. & Narayanan S. 2002. Cryopreserved versus freshly isolated lymphocytes in human biomonitoring: endogenous and induced DNA damage, antioxidant status and repair capability.

Mutagenesis 17(3):211-214. <http://dx.doi.org/10.1093/mutage/17.3.211> <PMid:11971991>

Go Y.Y., Zhang J., Timoney P.J., Cook R.F., Horohov D.W. & Balasuriya U.B.R. 2010. Complex interactions between the major and minor envelope proteins of equine arteritis virus determine its tropism for equine CD3 T lymphocytes and CD14 monocytes. J. Virol. 84(10):4898-4911. <http://dx.doi.org/10.1128/JVI.02743-09> <PMid:20219931>

Kutscher S., Dembek C., Deckert S., Russo C., Korber N., Bogner J.R., Geisler F., Umgelter A., Neuenhahn M., Albrecht J., Cosma A., Protzer U. & Bauer T. 2013. Overnight resting of PBMC changes functional signatures of antigen specific T-cell responses: impact for immune monitoring with clinical trials. Plos One 8(10):1-13. <http://dx.doi.org/10.1371/journal.pone.0076215> <PMid:24146841>

Netland J. & Bevan M.J. 2013. CD8 and CD4 T cells in West Nile Virus immunity and pathogenesis. Viruses 5(10):2573-2584. <http://dx.doi.org/10.3390/v5102573> <PMid:24153060>

Pereira-Cunha F.G., Duarte A.S.S., Reis-Alves S.C., Olalla Saad S.T., Metze K., Lorand-Metze I. & Luzo Â.C.M. 2014. Umbilical cord blood CD34+ stem cells and other mononuclear cell subtypes processed up to 96 h from collection and stored at room temperature maintain a satisfactory functionality for cell therapy. Vox Sanguinis 108(1):72-81. <http://dx.doi.org/10.1111/vox.12199> <PMid:25333825>

Perkins G.A., Goodman L.B., Wimer C., Freer H., Babasyan S. & Wagner B. 2014. Maternal T-lymphocytes in equine colostrum express a primarily inflammatory phenotype. Vet. Immunol. Immunopathol. 161(3-4):141-150. <http://dx.doi.org/10.1016/j.vetimm.2014.07.009> <PMid:25174977>

Reimann K.A., Chernoff M., Wilkening C.L., Nickerson C.E. & Landay A.L. 2000. Preservation of lymphocyte immunophenotype and proliferative responses in cryopreserved peripheral blood mononuclear cells from human immunodeficiency virus type-1 infected donors: implication for multicenter clinical trials. Clin. Diagn. Lab. Immunol. 7(3):352-359. <PMid:10799445>

Sputtek A., Kühnl P. & Rowe A.W. 2007. Cryopreservation of erythrocytes, thrombocytes and lymphocytes. Transfus. Med. Hemother. 34(4):262-267. <http://dx.doi.org/10.1159/000104136>

Svedentsov E.P., Tumanova T.V., Zaytseva O.O., Solomina O.N. & Utemov S.V. 2007. Preservation of leukocytes in conditions of cryoanabiosis. Cell Preserv. Tech. 5(2):144-150. <http://dx.doi.org/10.1089/cpt.2007.0508>

Truax R.E., Powell M.D., Montelaro R.C., Issel C.J. & Newman M.J. 1990. Cryopreservation of equine mononuclear cells for immunological studies. Vet. Immunol. Immunopathol. 25(2):139-153. <http://dx.doi.org/10.1016/0165-2427(90)90031-M> <PMid:2378055>

Weinberg A., Song L.Y., Wilkening C., Sevin A., Blais B., Louzao R., Stein D., Defechereux P., Durand D., Reidel E., Raftery N., Jesser R., Brown B., Keller M.F., McFarland E. & Fenton T. 2009. Optimization and limitations of use of cryopreserved peripheral blood mononuclear cells for functional and phenotypic T-cell characterization. Clin. Vac. Immunol. 16(8):1176-1186. <http://dx.doi.org/10.1128/CVI.00342-08> <PMID: 19515870>

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Cattle trade between and within biomes in the state of Mato Grosso, Brazil[1]

Rísia L. Negreiros[2], José H.H. Grisi-Filho[3], Ricardo A. Dias[3], Fernando Ferreira[3], Valéria S.F. Homem[4], José S. Ferreira Neto[3], Raul Ossada[3], and Marcos Amaku[3]*

ABSTRACT.- Negreiros R.L., Grisi-Filho J.H.H., Dias R.A., Ferreira F., Homem V.S.F., Ferreira Neto J.S., Ossada R. & Amaku M. 2018. **Cattle trade between and within biomes in the state of Mato Grosso, Brazil**. *Pesquisa Veterinária Brasileira 38(11):2023-2028.* Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Avenida Prof. Dr. Orlando Marques de Paiva 87, São Paulo, SP 05508-270, Brazil. E-mail: amaku@vps.fmvz.usp.br

The analysis of animal movement patterns may help identify farm premises with a potentially high risk of infectious disease introduction. Farm herd sizes and bovine movement data from 2007 in the state of Mato Grosso, Brazil, were analyzed. There are three different biomes in Mato Grosso: the Amazon, Cerrado, and Pantanal. The analysis of the animal trade between and within biomes would enable characterization of the connections between the biomes and the intensity of the internal trade within each biome. We conducted the following analyses: 1) the concentration of cattle on farm premises in the state and in each biome, 2) the number and relative frequency of cattle moved between biomes, and 3) the most frequent purposes for cattle movements. Twenty percent (20%) of the farm premises had 81.15% of the herd population. Those premises may be important not only for the spread of infectious diseases, but also for the implementation of surveillance and control strategies. Most of the cattle movement was intrastate (97.1%), and internal movements within each biome were predominant (88.6%). A high percentage of movement from the Pantanal was to the Cerrado (48.6%), the biome that received the most cattle for slaughter, fattening and reproduction (62.4%, 56.8%, and 49.1% of all movements for slaughter, fattening, and reproduction, respectively). The primary purposes for cattle trade were fattening (43.5%), slaughter (31.5%), and reproduction (22.7%). Presumably, movements for slaughter has a low risk of disease spread. In contrast, movements for fattening and reproduction purposes (66.2% of all movements) may contribute to an increased risk of the spread of infectious diseases.

INDEX TERMS: Cattle movement, herd size, biomes, Mato Grosso, infectious disease.

RESUMO.- [**Trânsito de bovinos entre e dentro dos biomas no estado de Mato Grosso, Brasil.**] A análise dos padrões de trânsito animal pode ajudar a identificar estabelecimentos sob um risco potencialmente maior de introdução de doença infecciosa. Foram analisadas a rede de trânsito de bovinos no estado do Mato Grosso, Brasil, e uma base de dados com tamanho de rebanho, ambas de 2007. Há três biomas diferentes em Mato Grosso: Amazônia, Cerrado e Pantanal. A análise do trânsito animal entre e dentro dos biomas nos permitiu caracterizar quão conectados são os biomas e quão intenso é o trânsito interno dentro de cada bioma. Conduzimos as seguintes análises: 1) se o rebanho bovino está concentrado em alguns estabelecimentos no estado todo e em cada bioma; 2) sobre o número absoluto e a frequência relativa de bovinos movimentados entre os biomas; e 3) quais são os propósitos mais frequentes para o trânsito animal. Encontramos que 20% dos estabelecimentos possuíam 81,15% do rebanho total do estado. Estes estabelecimentos podem ser importantes para o espalhamento de doenças infecciosas, mas também para a implementação de estratégias de vigilância e controle. A maior parte do trânsito foi dentro do estado (97,1%), e o trânsito interno dentro de cada bioma foi predominante

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[2] Instituto de Defesa Agropecuária do Estado de Mato Grosso (Indea/MT), Av. Jorn. Arquimedes Pereira Lima 1000, Boa Esperança, Cuiabá, MT 78068-635, Brazil.

[3] Departamento de Medicina Veterinária Preventiva e Saúde Animal, Universidade de São Paulo (USP), Avenida Prof. Dr. Orlando Marques de Paiva 87, São Paulo, SP 05508-270, Brazil. *Corresponding author: amaku@vps.fmvz.usp.br

[4] Ministério da Agricultura Pecuária e Abastecimento, Brasília, DF 70632-100, Brazil.

(88,6%). Um percentual alto de movimentos do Pantanal (48,6%) foi para o Cerrado, o bioma que recebeu mais bovinos para abate, engorda e reprodução (62,4%, 56,8% e 49,1% de todos os movimentos para abate, engorda e reprodução, respectivemente). Os principais propósitos para o comércio de bovinos foram engorda (43,5%), abate (31,5%) e reprodução (22,7%). Movimentos para abate presumivelmente oferecem um baixo risco para espalhamento de doença. Em contraste, movimentos para engorda e reprodução (66,2% de todos os movimentos) podem contribuir para o aumento do risco de espalhamento de doenças infecciosas.

TERMOS DE INDEXAÇÂO: Trânsito de bovinos, tamanho de rebanho, biomas, Mato Grosso, doença infecciosa, bovinos.

INTRODUCTION

The movement of animals between farm premises may contribute to the spread of infectious diseases. Hence, an understanding of the characteristics of the network of animal movements may help prevent or control the spread of diseases. In a scenario with no control measures in place, one could assume that the risk of disease spread between farm premises increases as the number of animals moved increases (Amaku et al. 2015). Thus, the analysis of animal movement may help identify farm premises with a potentially high risk of infectious disease introduction.

The state of Mato Grosso (MT) had the largest cattle herd (25.7 million cattle) in Brazil in 2007 (IBGE 2015b). There are three different biomes in the state (Fig.1): the Amazon (primarily rainforest), Cerrado (savannah), and Pantanal (floodplain). These biomes cover, respectively, 49%, 24%, and 2% of the country (IBGE 2015a). Approximately 8% of the Cerrado and 40% of the Amazon are protected areas (Bustamante 2015). Thus, the intensity of livestock production and trade raises economic and environmental concerns. The analysis of the animal trade within and between biomes would enable the characterization of the connections between biomes and the intensity of the internal trade within each biome.



Fig.1. Map of the state of Mato Grosso, Brazil and the areas of the Amazon, Cerrado, and Pantanal biomes.

Cipullo et al. (2016) reported an association between herd size and cattle trade in the state of Mato Grosso in 2007, and that larger size herds traded more animals than smaller herds. Herd size was also identified as a risk factor for bovine brucellosis in Mato Grosso (Negreiros et al. 2009). Thus, the analysis of the distribution of herd size, in the state and by biome, would contribute to the characterization of the herd distribution within the state.

Cipullo et al. (2016) analyzed the network for reproduction purposes and the network without abattoirs in Mato Grosso in 2007. They found a statistically significant difference between brucellosis, positive and negative farms, from a survey carried out in 2002-2003 (Negreiros et al. 2009) in the total number of bovines and batches of bovines moved, in the number of bovines and batches sold, and in the average herd size. These findings raise the question of whether the purposes for cattle movement are different in different biomes.

Our intention is to characterize the cattle trade between and within biomes in Mato Grosso. Using the 2007 data regarding network of bovine movements and premises herd sizes in Mato Grosso, we analyzed if the cattle herd is concentrated on some farm premises in the state and within each biome, the intensity of cattle movements between biomes in the state, and the most frequent purposes for cattle movements.

MATERIALS AND METHODS

Data on farm herd sizes on December 31, 2007 and on the cattle movement network in the state of Mato Grosso in 2007 were analyzed. Both databases (herd size and cattle movement network) were provided by the local veterinary agency (Instituto de Defesa Agropecuária do Estado de Mato Grosso, Indea/MT). Movements with origin and destination on the same premises were excluded from the analysis.

There were 112,924 premises registered with the 141 Indea/MT offices; 88,451 of those premises moved bovines in 2007. In addition, data on herd size were available for 100,216 premises, and biome identification (Amazon, Cerrado or Pantanal) was available for 67,945 farm premises.

The following analyses were performed: 1) if the cattle herd is concentrated on some farm premises, 2) the number and relative frequency of cattle moved between biomes, and 3) the most frequent purposes for cattle movement (reproduction, fattening, slaughter, exhibition, markets, or sports and work).

The median herd sizes of the three biomes were compared using the Kruskal-Wallis test. The analyses were performed in R (R Core Team 2017).

RESULTS AND DISCUSSION

Descriptive data on the area of the biomes, number of slaughterhouses, number of premises in the animal movement database, number of premises in each biome, and cattle moved from/to a given biome are shown in Table 1. Note that the biome Pantanal consistently (lower percentages) has the smallest area and the lowest number of slaughterhouses, premises, and cattle moved. In a comparison between the Cerrado and the Amazon, the Cerrado, which is a biome with an intense economic activity in Mato Grosso, has the smaller area and number of premises, but a higher number of slaughterhouses

Table 1. Descriptive data regarding the biomes

	Amazon	Cerrado	Pantanal
Area in square kilometers (% of state area) (CMT 2018)	480,251	354,823	60,885
	(53.6%)	(39.6%)	(6.8%)
Number of slaughterhouses in the animal movement database	19	43	5
	(28.3%)	(64.2%)	(7.5%)
Number of premises in Mato Grosso analyzed in the animal movement database	42,914	39,661	5,108
	(48.9%)	(45.2%)	(5.8%)
Cattle moved from the biome	6,475,775	7,931,595	1,098,725
	(41.8%)	(51.1%)	(7.1%)
Cattle moved to the biome	5,854,965	8,880,314	770,816
	(37.7%)	(57.3%)	(5.0%)

and cattle moved. On the other hand, the Amazon has the larger area and number of premises, but a lower number of slaughterhouses and animals moved. The cover extent of Amazon forest (Morton et al. 2011) could partially explain the lower volume of animal trade in comparison to the Cerrado.

Other cattle movement studies were performed in the Brazilian states of Pernambuco (Silva Júnior et al. 2017), Mato Grosso and Mato Grosso do Sul (Capanema et al. 2012), and Paraná and Santa Catarina (Felipe et al. 2013). Capanema et al. (2012) and Felipe et al. (2013) analyzed the cattle movement for slaughter and identified the slaughter poles. As mentioned by Silva Júnior et al. (2017), in the cattle movement of Mato Grosso, Negreiros (2010) observed that slaughterhouses were important hubs of the cattle trade network, followed by markets and big farms. Beef production for national and international consumer markets is an important economic activity in Mato Grosso (Silva Júnior et al. 2017). In contrast, in Pernambuco the majority of cattle production is in small scale and livestock markets were important hubs (Silva Júnior et al. 2017).

The distribution of herd size by biome is shown in Figure 2. The Kruskal-Wallis test revealed a significant difference among the median herd sizes of the three biomes ($p<0.001$). The median herd sizes and the corresponding interquartile ranges (IQR) for the Amazon, Cerrado, and Pantanal were 98 (IQR: 39-269), 110.5 (IQR: 43-333), and 148 cattle (IQR: 50-494.2), respectively (Table 2).

The analyzed database included 26.9 million cattle. An analysis of herd size revealed that 20% of the farm premises had between 1 and 19 cattle (0.75% of the state herd); 20% of the farm premises had between 19 and 42 cattle (2.20% of the state herd); 20% of the farm premises had between 42 and 90 cattle (4.70% of the state herd); 20% of the farm premises had between 90 and 248 cattle (11.20% of the state herd); and 20% of the farm premises had between 248 and 136,005 cattle (81.15% of the state herd).

Figure 3 shows the percentage of the total herd size as a function of the percentage of the largest herds in the three biomes and in the state. The curves for the three biomes are similar, and the 20/80 rule applies approximately to all three biomes: the largest 20% of the herds included approximately 77% of the total population. Statewide, the largest 20% of the herds included 81.15% of the total population, as mentioned above. Biome identification was available for only 68% of all herds in the dataset, which may explain the discrepancy between the ratio observed for the state and for each biome.

This finding approximately obeys the 20/80 Pareto Principle and indicates that roughly 80% of the state herd is

Table 2. Descriptive statistics of herd size by biome, showing the minimum, the first quartile (Q1), the median, the mean, the third quartile (Q3), and the maximum value

Parameter	Amazon	Cerrado	Pantanal
Minimum	1	1	1
Q1	39	43	50
Median	98	110.5	148
Mean	350.1	415.6	545.5
Q3	269	333	494.2
Maximum	94,664	47,839	35,954

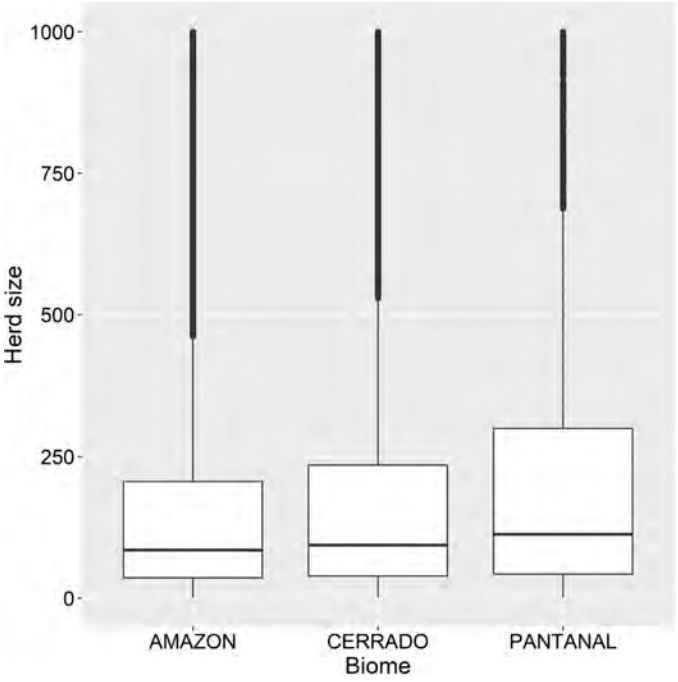


Fig. 2. Distribution of herd size by biome.

concentrated on 20% of the farm premises. Cipullo et al. (2016) analyzed the same database and observed that herd size and animal trade were positively associated, and also that farms positive for bovine brucellosis in the 2003 survey were trading and selling more than the negative farms in 2007, which presumably increased the risk of disease dissemination. Hence, the primary cattle traders are potential spreaders of infectious diseases through the animal trade network and deserve special attention from the veterinary surveillance system.

Table 3 shows the number and percentage of cattle moved intrastate and interstate for different purposes. Most cattle movement was intrastate (97.1%). Of the 15,965,825 bovines moved within the state, 6,944,103 (43.5%), 5,031,387 (31.5%), and 3,623,514 (22.7%) were moved for fattening, slaughter, and reproduction, respectively. In the state of Pernambuco, most cattle movement (91.3%) was also intrastate (Silva Júnior et al. 2017).

Analysis of bovine movement by biome revealed that most bovines were moved within the same biome (Fig.4). The Cerrado received the most bovines for slaughter, fattening, and reproduction (Table 4); it was the destination of 62.4%, 56.8%, and 49.1% of all movements for slaughter, fattening, and reproduction, respectively.

Analysis of movements between biomes (Table 4 and Fig.4) revealed the percentage of movements from the Pantanal (48.6%) or the Amazon (12.6%) to the Cerrado. In absolute figures, the number of bovines (398,430) moved from the Cerrado to the Pantanal or the Amazon was less than the number (1,347,149) moved from the other two biomes to the Cerrado. Of the 15,506,095 bovines moved within the state, 1,762,305 (11.4%) were moved between biomes. Thus, internal movement within each biome was predominant (Fig.4). When cattle purchases were analyzed, including movements within each biome, the Pantanal had the lowest rate of cattle purchased (5.0%). As presented in Figure 1, the Pantanal is also the smallest of the three biomes. Although the Pantanal had the least intense trade, an important extension of the Brazilian international frontier is in the Pantanal area.

Transport within each biome was predominant (88.6% of all movements). This finding is partially related to the community structure observed in cattle movement among the counties in Mato Grosso in 2007 (Grisi-Filho et al. 2013), and

Table 3. Cattle moved for different purposes in the state of Mato Grosso

Purpose	Intrastate (% of total)	Interstate (% of total)	Total
Slaughter	4,776,943 (29.9%)	254,444 (1.6%)	5,031,387 (31.5%)
Fattening	6,762,209 (42.4%)	181,894 (1.1%)	6,944,103 (43.5%)
Reproduction	3,600,964 (22.6%)	22,550 (0.14%)	3,623,514 (22.7%)
Exhibition	16,838 (0.11%)	435 (0.0027%)	17,273 (0.11%)
Market	342,406 (2.1%)	398 (0.0025%)	342,804 (2.1%)
Sports/Work	6,735 (0.04%)	9 (0.000056%)	6,744 (0.04%)
TOTAL	15,506,095 (97.1%)	459,730 (2.9%)	15,965,825

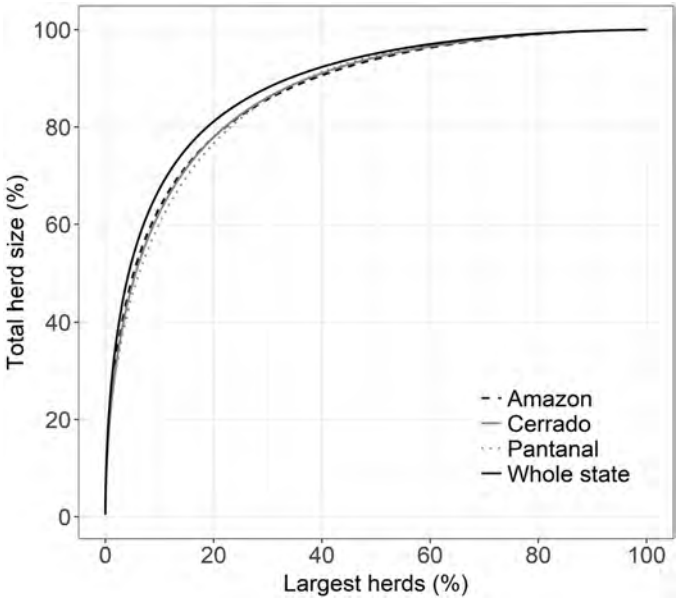


Fig. 3. Percentage of total herd size as a function of the percentage of the largest herds in each biome and in Mato Grosso.

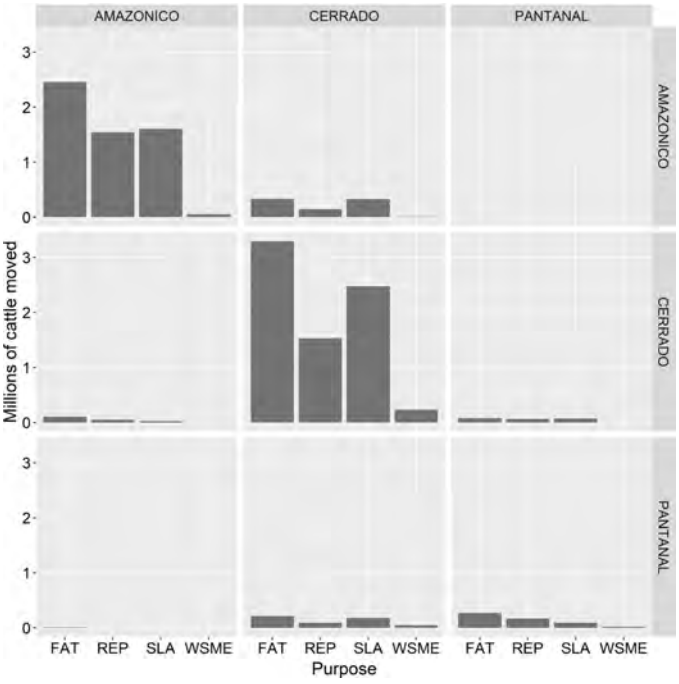


Fig. 4. Number of cattle moved from a point of origin (row) to a destination (column) biome by purpose. FAT = fattening, REP = reproduction, SLA = slaughter, WSME = work, sport, market, or exhibition.

Table 4. Cattle moved for slaughter, fattening, and reproduction by biome

		Destination			
		Amazon	Cerrado	Pantanal	TOTAL
Purpose	Origin	Cattle	Cattle	Cattle	Cattle
		(%)	(%)	(%)	(%)
		Total	*Total*	*Total*	*Total*
Slaughter	Amazon	1,604,448	326,650	0	1,931,098
		(28.4%)	(40.2%)	0%	(29.8%)
		5,658,146	*813,198*	*4,431*	*6,475,775*
	Cerrado	25,750	2,476,116	71,773	2,573,639
		(14.0%)	(32.9%)	(33.6%)	(32.4%)
		184,524	*7,533,165*	*213,906*	*7,931,595*
	Pantanal	0	178,615	93,591	272,206
		0%	(33.5%)	(16.9%)	(24.8%)
		12,295	*533,951*	*552,479*	*1,098,725*
	TOTAL	1,630,198	2,981,381	165,364	4,776,943
		(27.8%)	(33.6%)	(21.5%)	(30.8%)
		5,854,965	*8,880,314*	*770,816*	*15,506,095*
Fattening	Amazon	2,456,131	332,129	3,696	2,791,956
		(43.4%)	(40.8%)	(83.4%)	(43.1%)
		5,658,146	*813,198*	*4,431*	*6,475,775*
	Cerrado	103,444	3,295,499	77,474	3,476,417
		(56.1%)	(43.7%)	(36.2%)	(43.8%)
		184,524	*7,533,165*	*213,906*	*7,931,595*
	Pantanal	9,065	214,840	269,931	493,386
		(73.7%)	(40.2%)	(48.8%)	(44.9%)
		12,295	*533,951*	*552,479*	*1,098,725*
	TOTAL	2,568,640	3,842,468	351,101	6,762,209
		(43.9%)	(43.3%)	(45.5%)	(43.6%)
		5,854,965	*8,880,314*	*770,816*	*15,506,095*
Reproduction	Amazon	1,542,251	145,987	735	1,688,973
		(27.3%)	(18.0%)	(16.6%)	(26.1%)
		5,658,146	*813,198*	*4,431*	*6,475,775*
	Cerrado	52,490	1,531,014	63,706	1,647,210
		(28.4%)	(20.3%)	(29.8%)	(20.8%)
		184,524	*7,533,165*	*213,906*	*7,931,595*
	Pantanal	3,150	92,501	169,130	264,781
		(25.6%)	(17.3%)	(30.6%)	(24.1%)
		12,295	*533,951*	*552,479*	*1,098,725*
	TOTAL	1,597,891	1,769,502	233,571	3,600,964
		(27.3%)	(19.9%)	(30.3%)	(23.2%)
		5,854,965	*8,880,314*	*770,816*	*15,506,095*

potentially related to a preference for short- or medium-distance (a median less than 100 kilometers) movements (Negreiros 2010). When we consider only intrastate movements, the median geodesic spatial distance (in kilometers) for the batches moved from the biomes Amazon, Cerrado, and Pantanal were 73 (IQR: 35-159), 77 (IQR: 36-153), and 97 (IQR: 59-143), respectively. The interquartile ranges were similar for the Amazon and the Cerrado, but the median distance for the Pantanal was longer. However, the distance distribution for all biomes is compatible with a short- or medium-distance movement pattern, suggesting that spatial closeness, potentially related to an underlying network of cattle trade, is a determinant factor for animal movement.

The percentages of animals moved for slaughter within the Amazon, Cerrado, and Pantanal biomes were 27.8%, 33.6%, and 21.5%, respectively, with regard to all movements to that biome (Table 4). The percentages of animals moved for fattening were 43.9%, 43.3%, and 45.5%, respectively. The percentages of animals moved for reproduction were 27.3%, 19.9%, and 30.3%, respectively. Thus, in all biomes, movements for fattening prevailed over movements for slaughter or reproduction. Interestingly, in the Pantanal, we observed a higher percentage of animals moved for reproduction (30.3%) than for slaughter (21.5%). Historically, the Pantanal has been considered a breeding area (Santos et al. 2002). There was a low number of slaughterhouses in the Pantanal (Table 1) and

the slaughtering of most cattle from the Pantanal occurred in the Cerrado (Table 4). The percentage of animals moved for slaughter from the Amazon was similar to the percentage moved for reproduction.

Cattle movement for slaughter has a low risk of disease dissemination. However, movement for fattening or reproduction may contribute to the spread of certain diseases, such as bovine brucellosis, which had a herd prevalence of 41.2% in the state according to a survey performed in 2002 and 2003 (Negreiros et al. 2009). The herd prevalence was reduced to 24.0% in 2014 (Bardall et al. 2016).

Although the Amazon was the biome with the largest number of premises in Mato Grosso in the analyzed animal movement network (Table 1), probably related to its large area, it was not the biome with the most intense cattle trade pattern (Fig.4). Our findings suggest that, regarding cattle trade, all biomes were anthropized. The economic activity in Mato Grosso is intense in the Cerrado area. Amongst the 11 Mato Grosso counties with more than 50,000 inhabitants (Wikipedia 2018), 8 are located in the Cerrado, 2 in the Amazon and 1 in the Pantanal biome. Cerrado was the biome with the most intense cattle trade pattern (Fig.4), what presumably explains the fact that the largest number of slaughterhouses in Mato Grosso was in the Cerrado. Capanema et al. (2012) found four counties that were important poles in the cattle movement for slaughter in Mato Grosso, three located in the Cerrado and one in the Amazon. Although the median herd size in the Pantanal was the largest (Table 2), the least intense cattle trade pattern was observed there, which could be related to the smallest area, the lowest number of farm premises and slaughterhouses, and also to the environmental constraints since it is a floodplain.

CONCLUSIONS

Twenty percent (20%) of the farm premises had 81.15% of the Mato Grosso state herd in 2007. Those farms may be important for the spread of infectious diseases and, conversely, for the implementation of surveillance and control strategies.

Most cattle movement was intrastate (97.1%); within the state, internal movements within each biome were predominant (88.6%).

The primary purposes for the cattle trade were fattening (43.5%), slaughter (31.5%), and reproduction (22.7%). Movement for slaughter has a low risk of disease spread. However, movement for fattening or reproduction (66.2%) may contribute to an increased risk of the spread of certain infectious diseases, such as bovine brucellosis.

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REFERENCES

Amaku M., Grisi-Filho J.H.H., Negreiros R.L., Dias R.A., Ferreira F., Ferreira Neto J.S., Cipullo R.I., Marques F.S. & Ossada R. 2015. Infectious disease surveillance in animal movement networks: an approach based on the friendship paradox. Prev. Vet. Med. 121(3/4):306-313. <http://dx.doi.org/10.1016/j.prevetmed.2015.08.002> <PMid:26277202>

Bardall J.E.I., Quixabeira-Santos J.C., Lopes I.F., Ferreira Neto J.S., Ferreira F., Amaku M., Dias R.A., Telles E.O., Grisi-Filho J.H.H., Heinemann M.B., Gonçalves V.S.P. & Aguiar D.M. 2016. Effect of vaccination in lowering the prevalence of bovine brucellosis in the state of Mato Grosso, Brazil. Semina, Ciênc. Agrárias 37(5 Suppl. 2):3479-3492.

Bustamante M. 2015. Política de clima negligencia o Cerrado: mais uma vez. Observatório do Clima, November 24, 2015. Available at <http://www.observatoriodoclima.eco.br/politica-de-clima-negligencia-o-cerrado-mais-uma-vez/> Accessed on Dec. 3, 2015.

Capanema R.O., Haddad J.P.A. & Felipe P.L.S. 2012. Trânsito de bovinos nos estados do Mato Grosso e Mato Grosso do Sul, Brasil. Arq. Bras. Med. Vet. Zootec. 64(2):253-262. <http://dx.doi.org/10.1590/S0102-09352012000200002>

Cipullo R.I., Dias R.A., Ferreira F., Ferreira Neto J.S., Gonçalves V.S.P., Marques F.S., Negreiros R.L., Ossada R. & Amaku M. 2016. Cattle movement network, herd size, and bovine brucellosis in the State of Mato Grosso, Brazil. Semina, Ciênc. Agrárias 37(5 Suppl. 2):3777-3792.

CMT 2018. Biomas. Coisas de Mato Grosso, Mato Grosso. Available at <http://www.coisasdematogrosso.com.br/mato-grosso/biomas/> Accessed on Mar. 2, 2018.

Felipe P.L.S., Nicolino R.R., Capanema R.O. & Haddad J.P.A. 2013. Caracterização do trânsito bovino no estado do Paraná e Santa Catarina, Brasil, 2008. Arq. Bras. Med. Vet. Zootec. 65(3):659-668. <http://dx.doi.org/10.1590/S0102-09352013000300007>

Grisi-Filho J.H.H., Amaku M., Ferreira F., Dias R.A., Ferreira Neto J.S., Negreiros R.L. & Ossada R. 2013. Detecting livestock production zones. Prev. Vet. Med. 110(3-4):304-311. <http://dx.doi.org/10.1016/j.prevetmed.2012.12.013> <PMid:23434375>

IBGE 2015a. Mapas de biomas e de vegetação. Instituto Brasileiro de Geografia e Estatística. Available at <http://www.ibge.gov.br/home/presidencia/noticias/21052004biomashtml.shtm> Accessed on Dec. 3, 2015.

IBGE 2015b. Pesquisa pecuária municipal. Instituto Brasileiro de Geografia e Estatística. Available at <http://www.sidra.ibge.gov.br/bda/tabela/protabl.asp?c=73&z=p&o=29&i=P> Accessed on Nov. 24, 2015.

Morton D.C., Sales M.H., Souza Junior C.M. & Griscom B. 2011. Historic emissions from deforestation and forest degradation in Mato Grosso, Brazil: 1) source data uncertainties. Carbon Balance Manag. 6(1):18. <http://dx.doi.org/10.1186/1750-0680-6-18> <PMid:22208947>

Negreiros R.L. 2010. Caracterização e análise da rede de movimento de bovinos no Estado de Mato Grosso. Tese de Doutorado em Epidemiologia Experimental Aplicada às Zoonoses, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo. 121p.

Negreiros R.L., Dias R.A., Ferreira F., Ferreira Neto J.S., Gonçalves V.S.P., Silva M.C.P., Figueiredo V.C.F., Lôbo J.R., Freitas J. & Amaku M. 2009. Situação epidemiológica da brucelose bovina no Estado de Mato Grosso. Arq. Bras. Med. Vet. Zootec. 61(Suppl. 1):56-65. <http://dx.doi.org/10.1590/S0102-09352009000700008>

R Core Team 2017. R: a language and environment for statistical computing. Vienna, Austria, Available at <http://www.r-project.org> Accessed on Jul. 7, 2017.

Santos S.A., Pellegrin A.O., Moraes A.S., Barros A.T.M., Comastri Filho J.A., Sereno J.R.B., Silva R.A.M.S. & Abreu U.G.P. 2002. Sistema de produção de gado de corte no Pantanal. Embrapa Pantanal, Corumbá. 82p.

Silva Júnior J.L., Almeida E.C., Corrêa F.N., Lima P.R.B., Ossada R., Marques F.S., Dias R.A., Ferreira F., Ferreira Neto J.S., Grisi-Filho J.H.H., Amaku M., Manso Filho H.C. & Silva J.C.R. 2017. Livestock markets play an important role in the cattle movement network in Pernambuco, Brazil. Braz. J. Vet. Res. Anim. Sci. 54(3):225-237. <http://dx.doi.org/10.11606/issn.1678-4456.bjvras.2017.124303>

Wikipedia 2018. Lista de Municípios de Mato Grosso por População. Available at <https://pt.wikipedia.org/wiki/Lista_de_munic%C3%ADpios_de_Mato_Grosso_por_popula%C3%A7%C3%A3o> Accessed on Mar. 8, 2018.

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Frequency of *Staphylococcus aureus* virulence genes in milk of cows and goats with mastitis[1]

Atzel C. Acosta[2]*, Pollyanne Raysa F. Oliveira[2], Laís Albuquerque[2], Isamara F. Silva[3], Elizabeth S. Medeiros[2], Mateus M. Costa[3], José Wilton Pinheiro Junior[2] and Rinaldo A. Mota[2]

ABSTRACT.- Acosta A.C., Oliveira P.R.F., Albuquerque L., Silva I.F., Medeiros E.S., Costa M.M., Pinheiro Junior J.W. & Mota R.A. 2018. **Frequency of *Staphylococcus aureus* virulence genes in milk of cows and goats with mastites.** *Pesquisa Veterinária Brasileira 38(11):2029-2036.* Laboratório de Bacterioses dos Animais Domésticos, Departamento de Medicina Veterinária, Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. E-mail: acabad80@gmail.com

The present study determined the frequency of *Staphylococcus aureus* virulence genes in 2,253 milk samples of cows (n=1000) and goats (n=1253) raised in three different geographical regions of the state Pernambuco, Brazil. The presence of genes of virulence factors associated to adhesion to host cells (*fnbA, fnbB, clfA* and *clfB*), toxinosis (*sea, seb, sec, sed, seg, seh, sei, tsst, hla* and *hlb*), and capsular polysaccharide (*cap5* and *cap8)* was evaluated by PCR. A total of 123 and 27 *S. aureus* strains were isolated from cows' and goats' milk, respectively. The *sec* and *tsst* genes were detected exclusively in goats' isolates, while the *seh* gene was only identified in cows' isolates. The number of toxin genes per strain showed that goats' isolates are likely more toxic than bovines' isolates. The *cap5* genotype predominated in both host species, especially in strains collected from cows raised in the *Agreste* region. The *cap8* genotype is likely more virulent due to the number of virulence genes per strain. The results of the present study demonstrate that *S. aureus* may pose a potential threat to human health in Brazil, and, therefore, these results should support actions related to mastitis control programs.

INDEX TERMS: *Staphylococcus aureus*, virulence genes, milk, cows, goats, mastitis, molecular typing, genotyping, staphylococcal enterotoxin, capsular polysaccharide, cattle, bacterioses.

RESUMO.- [**Frequência de genes fatores de virulência de *Staphylococcus aureus* em leite de vacas e cabras com mastite.**] O presente estudo determinou a frequência de genes de virulência de *Staphylococcus aureus* em 2253 amostras de leite, sendo de vacas n=1000 e de cabras n=1253, procedentes das três regiões geográficas do estado de Pernambuco, Brasil. A presença de genes de fatores de virulência associados à adesão às células hospedeiras (*fnbA, fnbB, clfA* e *clfB*), toxinosis (*sea, seb, sec, sed, seg, seh, sei, tsst, hla* e *hlb*) e polissacarídeo capsular (*cap5* e *cap8)* foram avaliadas por PCR. Um total de 123 e 27 cepas de *S. aureus* foram isoladas do leite de vacas e cabras, respectivamente. Os genes *sec* e *tsst* foram detectados exclusivamente em isolados de cabras, enquanto o gene *seh* foi identificado apenas em isolados de vaca. O número de genes de toxina por cepa mostrou que os isolados de cabras são potencialmente mais tóxicos do que os isolados obtidos de bovinos. O genótipo *cap5* predominou em ambas as espécies hospedeiras, especialmente em cepas coletadas de vacas criadas na região Agreste. O genótipo *cap8* é potencialmente mais virulento devido ao número de genes de virulência por isolado. Os resultados do presente estudo demonstram que *S. aureus* pode representar uma ameaça potencial para a saúde humana no Brasil e, portanto, estes resultados devem subsidiar ações relacionadas aos programas de controle de mastite.

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[2] Laboratório de Bacterioses dos Animais Domésticos, Departamento de Medicina Veterinária, Universidade Federal Rural de Pernambuco (UFRPE), Rua Dom Manoel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. *Corresponding author: acabad80@gmail.com

[3] Universidade Federal do Vale do São Francisco (Univasf), Campus Ciências Agrárias, Rod. BR-407 Km 12, Lote 543, Projeto de Irrigação Senador Nilo Coelho, Petrolina, PE 56300-990, Brazil.

TERMOS DE INDEXAÇÃO: Genes, virulência, *Staphylococcus aureus*, leite, cabras, mastite, tipagem molecular, genotipagem, enterotoxina estafilocócica, polissacarídeo capsular, bovinos, caprinos, bacterioses.

INTRODUCTION

Intramammary infections (IMI) are the main cause of economic loss in the dairy industry worldwide (Halasa et al. 2007). The average cost of clinical mastitis in high-yielding cows was estimated in $71 per cow per year (Bar et al. 2008). *Staphylococcus aureus* is an important etiological agent of ruminant IMI in Brazil (Mota et al. 2012), and its eradication from dairy cattle and dairy small ruminants has proven to be difficult (Bergonier et al. 2003).

Several characteristics contribute for the pathogenesis and spread of *S. aureus* including virulence factors, the host and the environment (Roberson et al. 1998, Barkema et al. 2006). Previous studies suggest that existing phenotypic and genotype diversity between groups of *S. aureus* would make that some of these strains are better adapted to live in hostile environment (Bardiau et al. 2014, 2016). This bacterium can cause a broad range of diseases due to an abundance of virulence factors that facilitate attachment, colonization, tissue invasion, toxinosis, and immune evasion, including adhesion proteins, enterotoxins and capsular polysaccharides (Zecconi et al. 2006, Piccinini et al. 2010).

Adhesion is hypothesized to be a prerequisite and crucial early step for IMI development (Kerro et al. 2002). Two fibronectin-binding proteins (FnBPs), FnBPA and FnBPB, are involved in not only adhesion to cells but also internalization by cells (Garzoni & Kelley 2009). Other two important adhesion factors involved in the pathogenesis of *S. aureus* are the clumping factors, ClfA and ClfB (Ní Eidhin et al. 1998, Garzoni & Kelley 2009).

Staphylococcal enterotoxins (SEs) are an important group of virulence factors. They play a significant role in modulating the host immune response and may contribute to maintaining a suitable environment for colonization. In addition, SEs and toxic shock syndrome toxin 1 (TSST1) are superantigens, which have the ability to stimulate large populations of T cells that have a particular Vβ element of the T-cell receptor (Omoe et al. 2003). Other important toxins are hemolysins, which can negatively affect a wide range of host cells including erythrocytes, epithelial cells, endothelial cells, T cells, monocytes and macrophages (Berube & Wardenburg 2013).

Capsular polysaccharide (cap) is a cell wall bacterial component that protects bacterium from phagocytic uptake and enhances microbial virulence (Sutra et al. 1990). *Cap5* and *cap8* were the predominant capsular types in *S. aureus* isolated from clinical bovine mastitis in different countries (Gogoi-Tiwari et al. 2015).

The strong pathogenicity of *S. aureus* strains is driven by multifactorial and complex virulence factors. Appropriate molecular typing methods and information about the genetic diversity of *S. aureus* strains in a particular region may contribute to the development of effective strategies for epidemiological control. The present study aimed to determine the frequency of virulence factors genes in *S. aureus* strains isolated from cow and goat herds raised in three different geographical regions of the state of Pernambuco, Brazil.

MATERIALS AND METHODS

Ethical statement. The present study was approved by the Institutional Animal Care and Use Committee of the School of Veterinary Medicine, Federal Rural University of Pernambuco (Universidade Federal Rural de Pernambuco - UFRPE), under the protocol number 079/2014-CEUA.

Sample collection and presumptive identification of *Staphylococcus aureus*. The samples were collected from 24 bovine and 13 goat herds raised in three different geographical regions of state of Pernambuco, Brazil: the Atlantic Forest (five bovine and two goat herds), the Agreste (nine bovine and three goat herds), and the Sertão regions (ten bovine and eigth goat herds). The Atlantic Forest region (Paulista, Recife, Camaragibe and Paudalho) has a hot and humid climate, while the Agreste region (Gravatá, Caruaru and Garanhuns) and Sertão region (Sertânia, Custódia, Serra Talhada, Floresta, Salgueiro, Cabrobó, Santa Maria da Boa Vista and Petrolina) both have a semi-arid climate (Fig.1) (Nóbrega et al. 2015).

A total of 1000 and 1253 cows' and goats' milk samples were collected from individual mammary glands (udder quarters for cows or udder halves for goats) after disinfection of the ostium with 70% ethanol following the recommendations of the National Mastitis Council (National-Mastitis-Council 1999), and transported to the laboratory under refrigeration (4-10°C).

In cows, subclinical mastitis was diagnosed by the California Mastitis Test (CMT, (Schalm & Noorlander 1957); while in goats, IMI was defined as the presence of three or more colonies of the same type after primary culture (Pantoja et al. 2009).

Primary culture of milk samples (10μL) was performed in 5% ovine blood agar plates incubated aerobically at 37°C for 72h. Milk samples were considered contaminated when three or more dissimilar colony types were founded (Hiitiö et al. 2015). The presumptive identification of *S.* aureus was based on the following parameters: Gram-positive cocci, hemolytic on blood agar, catalase positive, and coagulase positive in 4-18h (Zecconi et al. 2006). The *S. aureus* ATCC 29213 strain was used as positive control.

Confirmation of the presumptive identification of *Staphylococcus aureus.* Species confirmation was performed by PCR amplification of the *nuc* gene, as previously described by Brakstad et al. (1992). The isolates presumptively identified as *S. aureus* were freshly cultured in brain infusion broth (Merck) and incubated overnight at 37°C. Subsequently, DNA was extracted using a commercial kit (Promega) following the manufacturer's instructions and stored at -20°C. The nuc_F and nuc_R primers, which amplify a single amplicon with an expected size of approximately 296bp (nt 862768-863064, HF937103.1), were specifically designed for the present study (Table 1).

PCR testing for virulence factors genes. The primers used for amplification of the virulence factors genes are shown in Table 1. The PCR was performed in 0.2mL tubes with a total volume of 25μL containing: PCR buffer (10mM Tris-HCl, pH 9.0; 50mM KCl; 0.1% Triton X-100), 1.5mM $MgCl_2$, 250μM each dNTP, 0.5μM each gene-specific primers, 1.5U Taq DNA polymerase (Promega), 20ng DNA, and distilled water.

PCR conditions for the amplification of the *nuc* gene and the virulence factors genes were as follows: 94°C for 5min (initial denaturation), followed by 32 cycles of 94°C for 1min, annealing temperature specific for each fragment (Table 1) for 1min and 72°C for 1min, and a final extension of 72°C for 5min. The PCR products were visualized by electrophoresis in 2% agarose gels stained with Blue Green Loading Dye I (LGC Biotecnologia) and photographed under UV illumination (Molecular Imaging L.PIX Loccus biotecnologia).

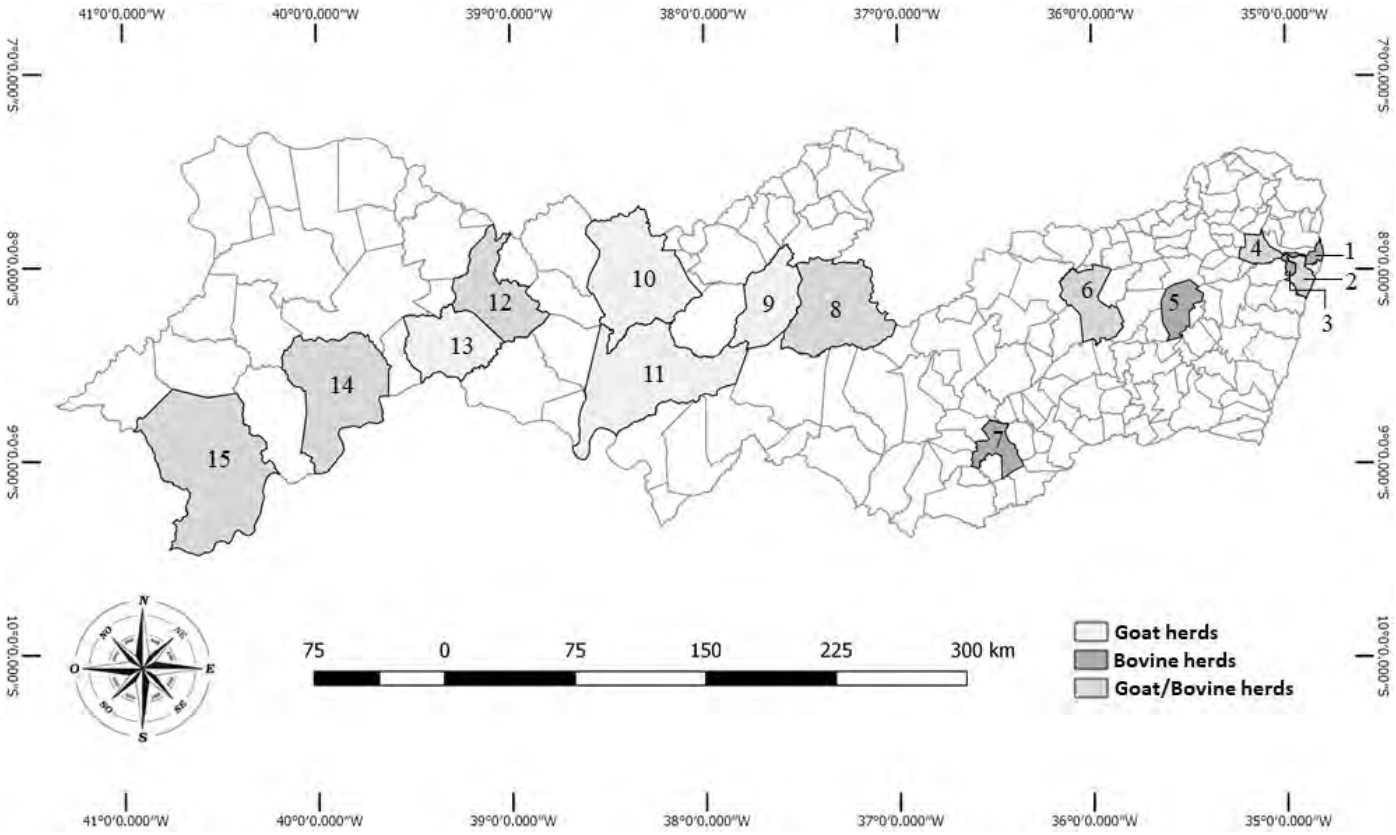


Fig.1. Map of Pernambuco state, Brazil. Distribution of the bovine and goat herds by municipal districts where the *Staphylococcus aureus* isolates were recovered. Each number represents municipal districts studied of the Atlantic Forest region: Paulista (1), Recife (2), Camaragibe (3), Paudalho (4). Agreste region: Gravatá (5), Caruaru (6), Garanhuns (7). Sertão region: Sertânia (8), Custódia (9), Serra Talhada (10), Floresta (11), Salgueiro (12), Cabrobó (13), Santa Maria da Boa Vista (14), Petrolina (15).

Three amplicons of each virulence factor were purified using the Wizard® SV gel and PCR clean-up system (Promega) and bidirectionally sequenced by standard protocols using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City/CA, USA) in an automated sequencer ABI-PRISM 3130 (Applied Biosystems, Foster City/CA, USA). Sequences were aligned using the BioEdit v.7.0.9 software (Hall 1999) and compared with those available in the GenBank database by Basic Local Alignment Search Tool (BLAST- http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Data analysis. Each *S. aureus* strain was considered a experimental unit in all analyses. Gene frequencies were calculated as categorical (binary) variables using Microsoft Excel. The frequency of each virulence gene by host species was compared using the chi-square test (Statgraphics Centurion XVI version 16.1.15, Statpoint Technologies Inc, Warrenton, Virginia). Statistical significance was set at $p<0.05$ for interpretation and discussion.

RESULTS

All the *Staphylococcus aureus* strains isolated in the present study were identified by a genotypic method in which the *nuc* gene was targeted using specific primers, a well-accepted method that has been used for confirmation of *S. aureus* infection worldwide (Rall et al. 2014). Three amplicons of the *nuc* gene and each virulence factors (*fnbA, fnbB, clfA, clfB, sea, seb, sec, sed, seg, seh, sei, tsst, hla, hlb, cap5* and *cap8*) were sequenced, and the partial sequences were confirmed by BLAST searches.

S. aureus strains were recovered from 12.3% (123/1000) and 2.15% (27/1253) of the cows' and goats' samples, respectively. Table 2 shows the number of strains recovered by geographical area, species and animals' disease status.

Table 3 shows the frequency of the virulence factors by host species and geographical area. The frequency of the virulence factors according to capsular genotyping is shown in Table 4. The fibronectin-binding proteins, *fnbA* and *fnbB*, were detected in 141 (94%) and 122 strains (81.33%), respectively. *S. aureus* strains isolated from bovines had a higher frequency of *fnbB* (91.06%, p=0.0000) than those isolated from goats (Table 3). Strains positive for *cap5* had a higher frequency of the *fnbB* gene (88.68%, p=0.0012) than *cap* (-) strains (Table 4).

The clumping factor A (*clfA*) and clumping factor B (*clfB*) were detected in 114 (76%) and 115 strains (76.67%), respectively. The lowest frequency of *clfA* (62.79%, p=0.0267) and *clfB* (65.12% p=0.0323) were observed in samples collected from animals raised in the Atlantic forest (Table 3). *Cap* (-) strains had the lowest frequency of *clfA* (47.06%, p=0.0000) (Table 4). *S. aureus* adhesion cannot be explained by the action of a single virulence determinant, and likely a number of factors act in combination during the infective process.

Table 1. Nucleotide sequences and their characteristics for the *Staphylococcus aureus* gene-specific oligonucleotide primers used in this study

Target gene	Name	Oligonucleotide sequence (5´- 3´)	Expected size	At (°C)[a]
nuc	nuc_F	GGTTCTGAAGATCCAACAGTAT	296pb	61
	nuc_R	GCTAAGCCACGTCCATATTTA		
fnbA	fnbA _F	ACTTCACCTGTCGCCATTAC	539pb	57
	fnbA _R	GCAGTACAAGCACCACAAAC		
fnbB	fnbB _F	AGGCGACGGCAAAGATAAA	317pb	60
	fnbB _R	TAGTAACCTGACCACCACCT		
clfA[b]	clfA_F	GATTCTGACCCAGGTTCAGA	945pb	55
	clfA_R	CTGTATCTGGTAATGGTTCTTT		
clfB[b]	clfB_F	ATGGTGATTCAGCAGTAAATCC	880pb	55
	clfB_R	CATTATTTGGTGGTGTAACTCTT		
sea[c]	sea_F	GGTTATCAATGTGCGGGTGG	102pb	65
	sea_R	CGGCACTTTTTTCTCTTCGG		
seb	seb_F	CCCGTTTCATAAGGCGAGTT	314pb	57
	seb_R	ACGTAGATGTGTTTGGAGCTAAT		
sec[c]	sec_F	AGATGAAGTAGTTGATGTGTATGG	451pb	57
	sec_R	CACACTTTTAGAATCAACCG		
sed	sed_F	GTCACTCCACACGAAGGTAATAA	255pb	55
	sed_R	GAGACTTTAGACCCATCAGAAGAA		
see[c]	see_F	GCTGGAGGCACACCAAATA	301pb	57
	see_R	CATAACTTACCGTGGACCCTTC		
seg	seg_F	GCCAGTGTCTTGCTTTGTAATC	491pb	56
	seg_R	GAATGCTCAACCCGATCCTAA		
seh	seh_F	CACATCATATGCGAAAGCAGAAG	365pb	60
	seh_R	CCCAAACATTAGCACCAATCAC		
sei	sei_F	AGGCAGTCCATCTCCTGTATAA	568pb	55
	sei_R	TGCTCAAGGTGATATTGGTGTAG		
tsst[c]	tsst_F	ACCCCTGTTCCCTTATCATC	326pb	57
	tsst_R	TTTTCAGTATTTGTAACGCC		
hla	hla_F	CTGTAGCGAAGTCTGGTGAAA	293pb	62
	hla_R	CGGCCTTATTGGTGCAAATG		
hlb	hlb_F	GCCAAAGCCGAATCTAAGAAAG	495pb	60
	hlb_R	ATCATGTCCAGCACCACAA		
cap5	cap5_F	CGAACCGATGATTGATGCTATTG	555pb	61
	cap5_R	TGCTATGACTGCACCAGTATTT		
cap8	cap8_F	GGAGGAAATGACGATGAGGATAG	608pb	61
	cap8_R	TAGCTTCTGTAGCGGTGAATG		

[a] Annealing temperature °C, [b] PCR primers designed by Sabat et al. (2003), [c] PCR primers designed by Mehrotra et al. (2000).

Table 2. Numbers of strains recovered by regions of the herd, species and disease status of the animal

		Animal species		Disease status of the animal	
		Bovine	Goat	Subclinical	Clinical
Regions of the herd	Atlantic Forest	36	7	34	9
	Agreste	52	5	57	0
	Sertão	35	15	49	1
Animal species	Bovine			120	3
	Goat			20	7

Of the 150 strains isolated, 78 (52%) were positive for one or more SEs genes, which were found in 46.34% (57/123) and 77.78% (21/23) of the bovines' and goats' isolates, respectively (p=0.0000). There was no pattern observed in the frequency of the virulence factors by geographical area. The SEs genes most frequently detected were *seg* (25.33%), *seh* (18%), *sei* (13.33%), *sed* (10.67%), *sec* (7.33%), *sea* (6.67%) and *seb*, which was present in only one strain (0.67%).

S. aureus strains isolated from goats had a statistically significant higher frequency of *sea, seg* and *sei* than bovines' isolates (Table 3). The *sec* and the *seh* genes were detected exclusively in goats' and bovines' isolates, respectively. The *sec* gene was most frequently found in isolates from goats raised in the *Sertão* region (p=0.0061); while the smallest frequency of the *seh* gene was detected in bovines isolates from the Atlantic forest (p=0.0206) (Table 3). Goats' isolates had a higher frequency of the SEs genes than bovines (p=0.0005). The *sea* (p=0.0011), *seb* (p=0.0007), *sec* (p=0.0040) and *seh* (p=0.0000) genes were more frequently found in *cap8* (+) strains (Table 4). The *tsst* gene was exclusively detected in goats' isolates (5.33%) (Table 3), and *cap5* (+) strains had the lowest frequency of this gene (1.89%, p=0.0115) (Table 4).

The *hla* and *hlb* genes were detected in 132 (88%) and 128 strains (85.33%), respectively. Both genes were more frequent in bovine isolates than in goats isolates (95.93%, p=0.0000 and 93.5%, p=0.0000 respectively), and less frequent in strains isolated from animals raised in the *Agreste* region (78%, p=0. 0233 and 72%, p=0.0042) (Table 3). Strains that did not carry neither *cap5* nor *cap8* genes had the lowest frequency of *hla* and *hlb* genes (58.82%, p=0.0000 and 55.88%, p=0.0000) (Table 4).

The capsular gene most frequently detected among the isolated strains was *cap5* (70.67%), while *cap8* was detected in only ten strains (6.67%). The *cap5* gene was more frequently found in bovines isolates (81.3%, p=0.0000) and in samples from animals raised in the *Agreste* region (87.72%, p=0.0002) (Table 3).

DISCUSSION

Staphylococcus aureus has been detected in 10.2% of cows diagnosed with mastitis and 17.5% of cows with subclinical mastitis in the state of São Paulo, Brazil (Silva et al. 2013, Rall et al. 2014). *S. aureus* has also been identified in 16.2% of sheep and goats from the states of Pernambuco and Bahia, Brazil, (Peixoto et al. 2010) and in 2.72% of samples from lactating goats from the state of Paraíba, Brazil (Ferreira et al. 2014). The results of the present study corroborates with previous claims that *S. aureus* is one of the main pathogens that causes mastitis in small and large ruminants (Mota et al. 2012).

The *see* gene was not present in any of the strains evaluated in the study. This gene is carried by a defective phage, and *sea* and *see* have 84% nucleotide homology (Couch et al. 1988). In Italy, studies have shown a low frequency of the *see* gene in mastitis-related strains (Zecconi et al. 2006) and have not identified the *see* gene in *S. aureus* isolated from milk or dairy products (Carfora et al. 2015) similarly to what have been reported in Brazil (Ferreira et al. 2014).

Intramammary infections caused by *S. aureus* may represent a potential risk for human health not only by the risk of pathogen transmission but also by the presence of enterotoxins in milk or dairy products, especially in goats'

Table 3. Absolute and relative frequency of virulence factors in relation to species and regions of origin

	Species					Regions						
	Bovine (n=123)		Goat (n=27)		p	Atlantic Forest (n=43)		Agreste (n=57)		Sertão (n=50)		p
	AF[a]	RF[b]	AF	RF		AF	RF	AF	RF	AF	RF	
Virulence factors involved in adhesion to host cells												
fnbA	115	93.50	26	96.30	0.8400	38	88.37	54	94.74	49	98.00	0.1451
fnbB	112	91.06	10	37.04	**0.0000**	33	76.74	51	89.47	38	76.00	0.1357
clfA	96	78.05	18	66.67	0.3454	27	62.79 [A*]	49	85.96 [B]	38	76.00 [AB]	**0.0267**
clfB	93	75.61	22	81.48	0.4316	28	65.12 [A]	43	75.44 [AB]	44	88.00 [B]	**0.0323**
Virulence factors: toxins												
sea	5	4.07	5	18.52	**0.0062**	6	13.95	3	5.26	1	2.00	0.0610
seb	1	0.81	0	0.00	0.6624	0	0.00	1	1.75	0	0.00	0.4452
sec	0	0.00	11	40.74	**0.0000**	3	6.98 [AB]	0	0.00 [A]	8	16.00 [B]	**0.0061**
sed	14	11.38	2	7.41	0.2574	4	9.30	7	12.28	5	10.00	0.8789
see	0	0.00	0	0.00		0	0.00	0	0.00	0	0.00	
seg	23	18.70	15	55.56	**0.0034**	9	20.93	14	24.56	15	30.00	0.6013
seh	27	21.95	0	0.00	**0.0240**	2	4.65 [A]	12	21.05 [B]	13	26.00 [B]	**0.0206**
sei	10	8.13	10	37.04	**0.0076**	5	11.63	6	10.53	9	18.00	0.4923
tsst	0	0.00	8	29.63	**0.0000**	3	6.98	0	0.00	5	10.00	0.0611
hla	118	95.93	14	51.85	**0.0000**	39	90.70 [AB]	54	94.74 [A]	39	78.00 [B]	**0.0233**
hlb	115	93.50	13	48.15	**0.0000**	39	90.70 [A]	53	92.98 [A]	36	72.00 [B]	**0.0042**
Virulence factors to evade innate immune defenses												
cap5	100	81.30	6	22.22	**0.0000**	30	69.77 [A]	50	87.72 [B]	26	52.00 [C]	**0.0002**
cap8	8	6.50	2	7.41	0.7048	1	2.33	5	8.77	4	8.00	0.4014

[a] Absolute frequency, [b] relative frequency, [A, B, C] relative frequency followed by the same capital letters on the lines do not differ significantly; statistical significance p<0.05.

Table 4. Absolute and relative frequency of virulence factors in relation to capsular genotyping

	Capsular genotyping						
	cap - (n=34)		*cap5* (n=106)		*cap8* (n=10)		p
	AF[a]	RF[b]	AF	RF	AF	RF	
Virulence factors involved in adhesion to host cells							
fnbA	31	91.18	100	94.34	10	100.00	0.5706
fnbB	21	61.76 [A*]	94	88.68 [B]	7	70.00 [AB]	**0.0012**
clfA	16	47.06 [A]	89	83.96 [B]	9	90.00 [B]	**0.0000**
clfB	24	70.59	81	76.42	10	100.00	0.1556
Virulence factors: toxins							
SEs	19	55.88 [A]	49	46.23 [A]	10	100.00 [B]	**0.0005**
sea	6	17.65 [A]	2	1.89 [B]	2	20.00 [A]	**0.0011**
seb	0	0.00 [A]	0	0.00 [A]	1	10.00 [B]	**0.0007**
sec	6	17.65 [AB]	3	2.83 [A]	2	20.00 [B]	**0.0040**
sed	3	8.82	12	11.32	1	10.00	0.9185
see	0	0.00	0	0.00	0	0.00	
seg	10	29.41	24	22.64	4	40.00	0.4032
seh	0	0.00 [A]	21	19.81 [A]	6	60.00 [B]	**0.0000**
sei	7	20.59	11	10.38	2	20.00	0.2586
tsst	5	14.71 [A]	2	1.89 [B]	1	10.00 [AB]	**0.0115**
hla	20	58.82 [A]	102	96.23 [B]	10	100.00 [B]	**0.0000**
Hlb	19	55.88 [A]	100	94.34 [B]	9	90.00 [B]	**0.0000**

[a] Absolute frequency, [b] relative frequency, [A, B] relative frequency followed by the same capital letters on the lines do not differ significantly; statistical significance p<0.05.

milk. Considering the high frequency of enterotoxin genes found in the present study and the fact that in Brazil the production of artisanal cheese with unpasteurized milk is a common practice (Shinohara et al. 2015), *S. aureus* infection can pose a threat to public health in the country.

SEs genes have different genetic structures, most of which are mobile genetic elements. These genes can be located in chromosomes, plasmids, pathogenicity islands and phages (Guimarães et al. 2013). The enterotoxins encoded by pathogenicity islands support the hypothesis that they

could play an important role in the evolution of *S. aureus* as a pathogen (Omoe et al. 2003), but their role in mastitis pathogenesis is still to be elucidated (Piccinini et al. 2010).

Staphylococcal enterotoxins and TSST-1 may act as super antigens for cells of the bovine immune system (Farahmand-Azar et al. 2016). These super antigenic toxins play an important role in modulating the host immune response and may therefore contribute to maintain a suitable environment for colonization (Omoe et al. 2003).

Yadav et al. (2015) reported high frequencies of *hla* (93.75%) and *hlb* genes (81.25%) in bovines isolates. The pathogenicity of *S. aureus* is related to the production of a wide variety of exoproteins including alpha and beta hemolysins that contribute to its ability to cause diseases in different mammalian species (Silva et al. 2005).

It is known that IMI caused by *S. aureus* have different patterns in different herds, which vary according to the bacterial strain, geographic location, as well as host- and tissue-related characteristics (Gilot & Van Leeuwen 2004, Van Leeuwen et al. 2005, Zecconi et al. 2005).

Capsular polysaccharides are important virulence factors of *S. aureus* because they confer resistance to phagocytosis (Sutra et al. 1990) and prolong the pathogen persistence in the host blood stream (O'Riordan & Lee 2004). However, Tuchscherr et al. (2005) hypothesized that the lack of capsule expression might permit intracellular persistence of *S. aureus* and promote subclinical mammary gland infection.

The distribution of the different *cap* genotypes of *S. aureus* around the world may differ (Ote et al. 2011, Gogoi-Tiwari et al. 2015). The CP serotype 5 (CP5) or 8 (CP8) are the target of conjugate vaccines. Therefore, it is important to know their prevalence in our geographic regions.

A characterization of 87 *S. aureus* strains from individual animals of 23 farms located in six different municipal districts of two geographical regions of the state of São Paulo found *cap5* and *cap8* in 11 (12.64%) and 76 (87.36%) strains, respectively (Cabral et al. 2004). Similar results were found in a molecular characterization of *S. aureus* strains isolated from small and large ruminants in 12 regions of France, Brazil, USA, and Belgium, which showed that the *cap8* gene was predominant and accounted for 65.4% of *S. aureus* strains, while *cap5* and the non-typeable CP types accounted for 30.7% and 3.9%, respectively. When considering the host species, *cap8* was predominant in small ruminants, with an overall prevalence of 83.1% in ovine-caprine isolates. In contrast, *cap5* was slightly predominant in bovine isolates (56.3%) (Alves et al. 2009).

In our study, 34 (22.67%) strains did not carry neither *cap5* nor *cap8* genes. *S. aureus* strains that do not express capsule induce chronic mastitis in mice, suggesting that the absence of capsule synthesis may help the bacteria to persist in the mammary glands (Tuchscherr et al. 2005).

The existence of two groups of *S. aureus* strains isolated from bovine mastitis based on capsular typing, intracellular survival and *agr*-typing was confirmed by Bardiau et al. (2016). They hypothesized that the first group "*cap5-agrI*" may correspond to strains adapted to the intracellular niche leading to chronic infection and that the second group "*cap8-agrII*" may correspond to strains better adapted to the extracellular niche leading to acute infection. The existence of these two groups is highly important as they may represent two clusters that are adapted differently to the host and/or the surrounding environment (Bardiau et al. 2016).

CONCLUSIONS

According to the results obtained in the present study, *Staphylococcus aureus* strains isolated from goats are more toxic than those isolated from bovines.

Our data also show that the *cap5* genotype is predominant in both bovines' and goats' isolates.

The *cap8* genotype is likely more virulent due to the number of virulence genes per strain.

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REFERENCES

Alves P.D.D., McCulloch J.A., Even S., Le Maréchal C., Thierry A., Grosset N., Azevedo V., Rosa C.A., Vautor E. & Le Loir Y. 2009. Molecular characterisation of *Staphylococcus aureus* strains isolated from small and large ruminants reveals a host rather than tissue specificity. Vet. Microbiol. 137(1-2):190-195. <http://dx.doi.org/10.1016/j.vetmic.2008.12.014> <PMid:19157725>

Bar D., Tauer L.W., Bennett G., González R.N., Hertl J.A., Schukken Y.H., Schulte H.F., Welcome F.L. & Gröhn Y.T. 2008. The cost of generic clinical mastitis in dairy cows as estimated by using dynamic programming. J. Dairy Sci. 91(6):2205-2214. <http://dx.doi.org/10.3168/jds.2007-0573> <PMid:18487643>

Bardiau M., Detilleux J., Farnir F., Mainil J.G. & Ote I. 2014. Associations between properties linked with persistence in a collection of *Staphylococcus aureus* isolates from bovine mastitis. Vet. Microbiol. 169(1/2):74-79. <http://dx.doi.org/10.1016/j.vetmic.2013.12.010> <PMid:24444863>

Bardiau M., Caplin J., Detilleux J., Graber H., Moroni P., Taminiau B. & Mainil J.G. 2016. Existence of two groups of *Staphylococcus aureus* strains isolated from bovine mastitis based on biofilm formation, intracellular survival, capsular profile and *agr*-typing. Vet. Microbiol. 185:1-6. <http://dx.doi.org/10.1016/j.vetmic.2016.01.003> <PMid:26931384>

Barkema H.W., Schukken Y.H. & Zadoks R.N. 2006. Invited review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. J. Dairy Sci. 89(6):1877-1895. <http://dx.doi.org/10.3168/jds.S0022-0302(06)72256-1> <PMid:16702252>

Bergonier D., De Crémoux R., Rupp R., Lagriffoul G. & Berthelot X. 2003. Mastitis of dairy small ruminants. Vet. Res. 34(5):689-716. <http://dx.doi.org/10.1051/vetres:2003030> <PMid:14556701>

Berube B.J. & Wardenburg J.B. 2013. *Staphylococcus aureus* α-toxin: nearly a century of intrigue. Toxins 5(6):1140-1166. <http://dx.doi.org/10.3390/toxins5061140> <PMid:23888516>

Brakstad O., Aasbakk K. & Maeland J.A. 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. J. Clin. Microbiol. 30(7):1654-1660. <PMid:1629319>

Cabral K., Lämmler C., Zschöck M., Langoni H., De Sá M.E.P., Victória C. & Da Silva A. 2004. Pheno and genotyping of *Staphylococcus aureus*, isolated from bovine milk samples from São Paulo State, Brazil. Can. J. Microbiol. 50(11):901-909. <http://dx.doi.org/10.1139/w04-082> <PMid:15644907>

Carfora V., Caprioli A., Marri N., Sagrafoli D., Boselli C., Giacinti G., Giangolini G., Sorbara L., Dottarelli S., Battisti A. & Amatiste S. 2015. Enterotoxin genes, enterotoxin production, and methicillin resistance in *Staphylococcus aureus* isolated from milk and dairy products in Central Italy. Int. Dairy J. 42:12-15. <http://dx.doi.org/10.1016/j.idairyj.2014.10.009>

Couch J.L., Soltis M.T. & Betley M.J. 1988. Cloning and nucleotide sequence of the type E staphylococcal enterotoxin gene. J. Bacteriol. 170(7):2954-2960. <http://dx.doi.org/10.1128/jb.170.7.2954-2960.1988> <PMid:3384800>

Farahmand-Azar S., Ahmadi M., Dastmalchi Saei H. & Anassori E. 2016. Identification of Toxic Shock Syndrome Toxin-1 (TSST-1) gene in *Staphylococcus aureus* isolated from bovine mastitis milk. Arch. Razi Inst. 68:17-22.

Ferreira D.H., Carvalho M.G.X., Nardelli M.J., Sousa F.G. & Oliveira C.J. 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>

Garzoni C. & Kelley W.L. 2009. *Staphylococcus aureus*: new evidence for intracellular persistence. Trends Microbiol. 17(2):59-65. <http://dx.doi.org/10.1016/j.tim.2008.11.005> <PMid:19208480>

Gilot P. & Van Leeuwen W. 2004. Comparative analysis of agr locus diversification and overall genetic variability among bovine and human *Staphylococcus aureus* isolates. J. Clin. Microbiol. 42(3):1265-1269. <http://dx.doi.org/10.1128/JCM.42.3.1265-1269.2004> <PMid:15004090>

Gogoi-Tiwari J., Babra Waryah C., Sunagar R., Veeresh H.B., Nuthanalakshmi V., Preethirani P.L., Sharada R., Isloor S., Bhat A., Al-Salami H., Hegde N.R. & Mukkur T.K. 2015. Typing of *Staphylococcus aureus* isolated from bovine mastitis cases in Australia and India. Aust. Vet. J. 93(8):278-282. <http://dx.doi.org/10.1111/avj.12349> <PMid:26220320>

Guimarães F.F., Nóbrega D.B., Richini-Pereira V.B., Marson P.M., Figueiredo Pantoja J.C. & Langoni H. 2013. Enterotoxin genes in coagulase-negative and coagulase-positive staphylococci isolated from bovine milk. J. Dairy Sci. 96(5):2866-2872. <http://dx.doi.org/10.3168/jds.2012-5864> <PMid:23477822>

Halasa T., Huijps K., Østerås O. & Hogeveen H. 2007. Economic effects of bovine mastitis and mastitis management: a review. Vet. Quart. 29(1):18-31. <http://dx.doi.org/10.1080/01652176.2007.9695224> <PMid:17471788>

Hall T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41:95-98.

Hiitiö H., Riva R., Autio T., Pohjanvirta T., Holopainen J., Pyörälä S. & Pelkonen S. 2015. Performance of a real-time PCR assay in routine bovine mastitis diagnostics compared with in-depth conventional culture. J. Dairy Res. 82(2):200-208. <http://dx.doi.org/10.1017/S0022029915000084> <PMid:25704849>

Kerro D.O., Van Dijk J. & Nederbragt H. 2002. Factors involved in the early pathogenesis of bovine *Staphylococcus aureus* mastitis with emphasis on bacterial adhesion and invasion. a review. Vet. Quart. 24(4):181-198. <http://dx.doi.org/10.1080/01652176.2002.9695135> <PMid:12540135>

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>

Mota R.A., Medeiros E.S., Santos M.V., Pinheiro Júnior J.W., Moura A.P.B. & Coutinho L.C.A. 2012. Participação dos *Staphylococcus* spp. na etiologia das mastites em bovinos leiteiros no Estado de Pernambuco (Brasil). Ciênc. Anim. Bras. 13(1):124-130. <http://dx.doi.org/10.5216/cab.v13i1.3790>

National-Mastitis-Council 1999. Laboratory and field handbook on bovine mastitis. National Mastitis Council Madison, WI. 222p.

Ní Eidhin D., Perkins S., Francois P., Vaudaux P., Höök M. & Foster T.J. 1998. Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of *Staphylococcus aureus.* Mol. Microbiol. 30(2):245-257. <http://dx.doi.org/10.1046/j.1365-2958.1998.01050.x> <PMid:9791170>

Nóbrega R.S., Farias R. & Santos C. 2015. Variabilidade temporal e espacial da precipitação pluviométrica em Pernambuco através de índices de extremos climáticos. Revta Bras. Meteorol. 30(2):171-180. <http://dx.doi.org/10.1590/0102-778620130624>

O'Riordan K. & Lee J.C. 2004. *Staphylococcus aureus* capsular polysaccharides. Clin. Microbiol. Rev. 17(1):218-234. <http://dx.doi.org/10.1128/CMR.17.1.218-234.2004> <PMid:14726462>

Omoe K., Hu D.-L., Takahashi-Omoe H., Nakane A. & Shinagawa K. 2003. Identification and characterization of a new staphylococcal enterotoxin-related putative toxin encoded by two kinds of plasmids. Infect. Immun. 71(10):6088-6094. <http://dx.doi.org/10.1128/IAI.71.10.6088-6094.2003> <PMid:14500536>

Ote I., Taminiau B., Duprez J.-N., Dizier I. & Mainil J.G. 2011. Genotypic characterization by polymerase chain reaction of *Staphylococcus aureus* isolates associated with bovine mastitis. Vet. Microbiol. 153(3-4):285-292. <http://dx.doi.org/10.1016/j.vetmic.2011.05.042> <PMid:21708435>

Pantoja J.C.F., Hulland C. & Ruegg P. 2009. Somatic cell count status across the dry period as a risk factor for the development of clinical mastitis in the subsequent lactation. J. Dairy Sci. 92(1):139-148. <http://dx.doi.org/10.3168/jds.2008-1477> <PMid:19109272>

Peixoto R.M., França C.A., Souza Júnior A.F., Veschi J.L.A. & Costa M.M. 2010. Etiologia e perfil de sensibilidade antimicrobiana dos isolados bacterianos da mastite em pequenos ruminantes e concordância de técnicas empregadas no diagnóstico. Pesq. Vet. Bras. 30(9):735-740. <http://dx.doi.org/10.1590/S0100-736X2010000900005>

Piccinini R., Borromeo V. & Zecconi A. 2010. Relationship between *S. aureus* gene pattern and dairy herd mastitis prevalence. Vet. Microbiol. 145(1-2):100-105. <http://dx.doi.org/10.1016/j.vetmic.2010.03.005> <PMid:20413230>

Rall V.L.M., Miranda E.S., Castilho I.G., Camargo C.H., Langoni H., Guimarães F.F., Araújo Júnior J.P. & Fernandes Júnior A. 2014. Diversity of *Staphylococcus* species and prevalence of enterotoxin genes isolated from milk of healthy cows and cows with subclinical mastitis. J. Dairy Sci. 97(2):829-837. <http://dx.doi.org/10.3168/jds.2013-7226> <PMid:24359821>

Roberson J., Fox L., Hancock D., Gay J. & Besser T. 1998. Sources of intramammary infections from *Staphylococcus aureus* in dairy heifers at first parturition. J. Dairy Sci. 81(3):687-693. <http://dx.doi.org/10.3168/jds.S0022-0302(98)75624-3> <PMid:9565871>

Sabat A., Krzyszton-Russjan J., Strzalka W., Filipek R., Kosowska K., Hryniewicz W., Travis J. & Potempa J. 2003. New method for typing *Staphylococcus aureus* strains: multiple-locus variable-number tandem repeat analysis of polymorphism and genetic relationships of clinical isolates. J. Clin. Microbiol. 41(4):1801-1804. <http://dx.doi.org/10.1128/JCM.41.4.1801-1804.2003> <PMid:12682193>

Schalm O. & Noorlander D. 1957. Experiments and observations leading to development of the California mastitis test. J. Am. Vet. Med. Assoc. 130(5):199-204. <PMid:13416088>

Shinohara N.K.S., Santos M.C.G., Medeiros S.T.C., Padilha M.R.D.F. & Matsumoto M. 2015. Cultural importance of cheese type for kingdom pernambucano. Holos 1:62-71. <http://dx.doi.org/10.15628/holos.2015.2488>

Silva E.R., Boechat J.U.D., Martins J.C.D., Ferreira W.P.B., Siqueira A.P. & Silva N. 2005. Hemolysin production by *Staphylococcus aureus* species isolated from mastitic goat milk in Brazilian dairy herds. Small Rumin. Res. 56(1/3):271-275. <http://dx.doi.org/10.1016/j.smallrumres.2004.04.011>

Silva N., Guimarães F., Manzi M., Budri P., Gómez-Sanz E., Benito D., Langoni H., Rall V. & 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>

Sutra L., Rainard P. & Poutrel B. 1990. Phagocytosis of mastitis isolates of *Staphylococcus aureus* and expression of type 5 capsular polysaccharide are influenced by growth in the presence of milk. J. Clin. Microbiol. 28(10):2253-2258. <PMid:2229349>

Tuchscherr L.P.N., Buzzola F.R., Alvarez L.P., Caccuri R.L., Lee J.C. & Sordelli D.O. 2005. Capsule-negative *Staphylococcus aureus* induces chronic experimental mastitis in mice. Infect. Immun. 73(12):7932-7937. <http://dx.doi.org/10.1128/IAI.73.12.7932-7937.2005> <PMid:16299284>

Van Leeuwen W.B., Melles D.C., Alaidan A., Al-Ahdal M., Boelens H.A., Snijders S.V., Wertheim H., Van Duijkeren E., Peeters J.K., Van der Spek P.J., Gorkink R., Simons G., Verbrugh H.A. & Van Belkum A. 2005. Host- and tissue-specific pathogenic traits of *Staphylococcus aureus*. J. Bacteriol. 187(13):4584-4591. <http://dx.doi.org/10.1128/JB.187.13.4584-4591.2005> <PMid:15968069>

Yadav J., Bhati T. & Kataria A.K. 2015. Phenotypic and genotypic haemolysin properties of *Staphylococcus aureus* obtained from milk of cattle and buffalo with clinical mastitis. J. Pure Appl. Microbiol. 9:349-355.

Zecconi A., Binda E., Borromeo V. & Piccinini R. 2005. Relationship between some *Staphylococcus aureus* pathogenic factors and growth rates and somatic cell counts. J. Dairy Res. 72(2):203-208. <http://dx.doi.org/10.1017/S0022029905000841> <PMid:15909686>

Zecconi A., Cesaris L., Liandris E., Daprà V. & Piccinini R. 2006. Role of several *Staphylococcus aureus* virulence factors on the inflammatory response in bovine mammary gland. Microb. Pathogenesis 40(4):177-183. <http://dx.doi.org/10.1016/j.micpath.2006.01.001> <PMid:16517115>

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Survey of *Salmonella* spp. in beef meat for export at slaughterhouses in Brazil[1]

Daniele Bier[2], Jalusa D. Kich[4], Sabrina C. Duarte[4], Márcio R. Silva[5], Luiza M. Valsoni[2], Carlos A.N. Ramos[6], Dália P. Rodrigues[7] and Flábio R. Araújo[3]*

ABSTRACT.- Bier D., Kich J.D., Duarte S.C., Silva M.R., Valsoni L.M., Ramos C.A.N., Rodrigues D.P. & Araújo F.R. 2018. **Survey of *Salmonella* spp. in beef meat for export at slaughterhouses in Brazil.** *Pesquisa Veterinária Brasileira 38(11):2037-2043*. Setor de Sanidade Animal, Embrapa Gado de Corte, Av. Rádio Maia 830, Zona Rural, Campo Grande, MS 79106-550, Brazil. E-mail: flabio.araujo@embrapa.br

The aim of the present study was to investigate the presence of *Salmonella* spp. in samples collected from beef meat at three points of the slaughter line (after skinning, washing and cooling) at three slaughterhouses in Brazil that export meat. Detection was based on ISO 6579:2002 and confirmed by PCR and qPCR. The isolates were typified using slide agglutination tests and PFGE. The antibiotic sensitivity profile was determined using the disk diffusion method. Contamination was detected in only one slaughterhouse. The overall frequency of contamination by *Salmonella* spp. was 6.7% of carcasses (6/90) and 2.6% of carcass surface samples (7/270). All isolates were confirmed by PCR and qPCR. The serological analysis and the PFGE showed a single profile: Typhimurium. The strains demonstrated 100% susceptibility to ampicillin, cefotaxime, ciprofloxacin, chloramphenicol, gentamicin and tetracycline. Positive carcasses after cooling pose a direct risk to consumers, since the meat is considered ready to be marketed after this process.

INDEX TERMS: *Salmonella* spp., beef meat, slaughterhouses, Brazil, serotype, antibiotic resistance, salmonellosis, skinning, cooling, carcass, cattle.

RESUMO.- [**Pesquisa de *Salmonella* spp. em carcaças bovinas durante o processamento em abatedouros-frigoríficos exportadores.**] O objetivo deste trabalho foi investigar a presença de *Salmonella* spp. em amostras coletadas de carcaças de bovinos, em três pontos da linha de abate (após a esfola, lavagem e refrigeração) de três frigoríficos exportadores no Brasil. A detecção foi realizada pela ISO 6579:2002, e confirmada por PCR e qPCR. Os isolados foram tipificados por testes de soroaglutinação e PFGE e avaliado o perfil de sensibilidade aos antibióticos pelo método de difusão em disco. A contaminação foi detectada em apenas um abatedouro-frigorífico. As contaminações das carcaças (n=90) e amostras de carne (n=270) por *Salmonella* spp. foram 6 (6,7%) e 7 (2,6%), respectivamente. Todos os isolados foram confirmados por PCR e qPCR. A análise sorológica e o PFGE mostraram um único perfil: Typhimurium. As cepas apresentaram 100% de suscetibilidade à ampicilina, cefotaxima, ciprofloxacina, cloranfenicol, gentamicina e tetraciclina. As carcaças positivas após a refrigeração apresentam um risco direto para o consumidor, uma vez que, após este processo, a carne está pronta para ser comercializada.

TERMOS DE INDEXAÇÃO: *Salmonella* spp., carcaças bovinas, abatedouros-frigoríficos, sorotipo, resistência a antibióticos, salmonelose, carne bovina, esfola, resfriamento, bovinos.

INTRODUCTION

Beef is one of the most important foods in the human diet and has a considerable impact on the economies of different countries (Sans & Combris 2015). Brazil has been the largest

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[2] Curso de Medicina Veterinária, Universidade Católica Dom Bosco (UCDB), Av. Tamandaré 6000, Jardim Seminário, Campo Grande, MS 79117-900, Brazil.

[3] Grupo de Produção Animal, Embrapa Gado de Corte, Av. Rádio Maia 830, Zona Rural, Campo Grande, MS 79106-550. *Corresponding author: flabio.araujo@embrapa.br

[4] Núcleo Temático de Sanidade de Suínos, Embrapa Suínos e Aves, Rodovia BR-153 Km 110, Concórdia, SC 89715-899, Brazil.

[5] Setor de Saúde Animal e Qualidade do Leite, Embrapa Gado de Leite, Eugênio do Nascimento 610, Juiz de Fora, MG 36038-330, Brazil.

[6] Faculdade de Medicina Veterinária e Zootecnia (FAMEZ), Universidade Federal de Mato Grosso do Sul (UFMS), Av. Senador Felinto Muller 2443, Pioneiros, Campo Grande, MS 79070-900.

[7] Laboratório de Enterobactérias, Instituto Oswaldo Cruz, Av. Brasil 4365, Rio de Janeiro, RJ 21040-900, Brazil.

exporter of beef in the world since 2008, and reached an export volume has reached 1.6 million tons, with gross revenues of approximately US$ 7.1 billion, which represents 28% of the international trade (Brasil 2015). Brazil exports to more than 170 countries in various regions of the world, such as Latin America, Middle East, Russia, the European Union and Africa (ABIEC 2016).

The state of Mato Grosso do Sul in the central western region of the country is the fourth largest cattle producer in Brazil and second in the number of slaughtered cattle, with an annual harvest of 3.9 million heads of cattle and an accumulated carcass weight of 849,000 tons (IAGRO 2018). With regard to beef exports, Mato Grosso do Sul represents 9.4% of the country's total, exporting mainly to Hong Kong, China, Egypt, Russia, Iran, Venezuela, Chile, Italy, Vietnam and the Netherlands (Brasil 2016).

Despite being one of the most important items in the human diet and an industry with considerable potential for growth, meat and meat products are considered one of the main vehicles of pathogens to humans (Rhoades et al. 2009), causing foodborne illnesses. Microbiological contamination of cattle carcasses occurs mainly during processing and handling, such as skinning, gutting, cutting, packaging, storage and distribution (Jay 2000, Borch & Arinder 2002, Madden et al. 2004). Cross-contamination by pathogenic bacteria through utensils used during the handling of meat is an important factor in the development of foodborne illnesses (Perez-Rodriguez et al. 2010, Papadopoulou et al. 2012). According to the World Health Organization, *Salmonella* sp. is one of the most relevant pathogens in beef and its presence poses a risk to consumers (WHO 2005). *Salmonella* ser. Typhimurium has been reported to be the serovar involved foodborne illnesses most frequently associated with the consumption of contaminated poultry, pork and beef (EFSA 2015).

The import market sets standards of quality and hygiene to be achieved by producing countries. In general, meat destined for foreign markets should be analyzed for the presence of pathogenic microorganisms and constraints to the marketing of these products may occur when microbiological criteria set by importing countries are not met (Wilhelm et al. 2011). The Rapid Alert System for Food and Feed (RASFF) of the European Union (EU) was created for the regulation of such products. When a risk to public health is identified, necessary measures are established, such as withholding, notification, seizure or rejection of these dangerous products. Through this warning system, health authorities in the EU have confiscated shipments of meat from Brazil, Argentina, Australia and the United States as the result of stiff control measures directed at pathogens, such as *Salmonella*, which has led to a degree of uncertainty among exporters and importers. From February 2016 to February 2018, two shipments of Brazilian beef were confiscated and notifications were issued regarding the presence of *Salmonella* spp. (RASFF 2018).

The United States requires daily testing for *Escherichia coli* and *Salmonella* spp., while the EU requires the enumeration of aerobic mesophilic microorganisms and enterobacteria in addition to the detection of *Salmonella* spp. (Commission Regulation-EC 2007). According to current microbiological standards of the Brazilian Health Regulatory Agency, chilled or frozen carcasses must be free of *Salmonella* spp. (Brasil 2001).

Quantitative and qualitative studies on microbiological safety are very important to the production of high quality meat products. The identification of potentially pathogenic microorganisms, such as *Salmonella,* in beef meat during slaughter operations contributes significantly to the implementation of quality monitoring programs and preventive measures, consequently reducing the risks to public health (Martínez-Chávez et al. 2015).

The aim of the present study was to investigate the presence of *Salmonella* spp. in cattle carcasses at different points in the slaughtering process at slaughterhouses in the state of Mato Grosso do Sul, Brazil, that produce beef for export. For such, different diagnostic methods were employed and the antibiotic sensitivity of the isolated strains was determined.

MATERIALS AND METHODS

Samples. Samples from beef meat were collected from three slaughterhouses registered with the Brazilian Federal Inspection Service and located in the state of Mato Grosso do Sul, Brazil.

At each slaughterhouse, samples were taken from five carcasses per week for six consecutive weeks, as established by the EU for microbiological testing on carcasses (Commission Regulation-EC 2007) and stipulated in Circular 463 of the Brazilian International Trade Control Division, Department of Animal Product Inspection, which establishes control programs for slaughterhouses that export to the United States and EU member states (Brasil 2004).

Samples were taken from each animal using a non-destructive method at three different points of the slaughter line: after skinning, after washing and after cooling. Dehydrated, sterilized sponges (Speci-Sponge - Nasco, Fort Atkinson, Wisconsin, USA) measuring 11.5x23.0cm and individually packed in sterile plastic bags (Whirl Pak, Nasco, Fort Atkinson, Wisconsin, USA) were used for the collection of the samples. The sponges were hydrated with 10mL of 1% buffered peptone water (1% BPW) (HiMedia Laboratories, Mumbai, India) and rubbed onto the chest (100 cm^2), flank (100cm^2) and rump close the occlusion of the rectum (200cm^2) using a sterile stainless steel mold measuring 10 x 10cm (total sampled surface: 400cm^2). The same carcass was tracked through each of the three stations at which samples were collected. The sponges were transferred to the plastic bags (Nasco, Fort Atkinson, Wisconsin, USA) and transported to the laboratory under refrigeration.

Strains from the microorganism reference collection of the Oswaldo Cruz Institute (FIOCRUZ-INCQS), Rio de Janeiro, Brazil, were used as negative and positive controls for all techniques: *Escherichia coli* INCQS 00033 (ATCC 25922), *Citrobacter freundii* INCQS 00576 (ATCC 43864), *Salmonella enterica* subsp. *enterica* serovar Enteritidis INCQS 00258 (ATCC 13076) and *S. enterica* subsp. *enterica* serovar Typhimurium INCQS 00150 (ATCC 14028).

Bacterial isolation. A total of 200mL of 1% BPW were added to each plastic bag containing the sponges. The mixture was homogenized in a stomacher (Lab-blender 400BA 6021, Seward Laboratory, London, England) for 60 sec and placed in Erlenmeyer flasks, which were incubated at 37±1°C for 18±2h. Detection of *Salmonella* spp. was performed according to the method recommended by the International Standardization Organization (ISO 2002), with modifications. After enrichment (with BPW), selection (with Muller-Kauffmann Tetrathionate-Novobiocin Broth and Rappaport-Vassiliadis Soya Peptone Broth) and differentiation (with Xylose-Lysine-Desoxycholate and *Salmonella-Shigella* agar) steps, suspected colonies isolated from standard cultivation media were subjected to further biochemical tests, which included indole,

urea, motility, lysine decarboxylation, H_2S production, methyl red, Voges-Proskauer, carbohydrate fermentation (Triple Sugar Iron), citrate and β-galactosidase.

DNA extraction. Prior to DNA extraction, the isolates were incubated in nutrient agar. The extraction of bacterial DNA for polymerase chain reaction (PCR) and real-time polymerase chain reaction (qPCR) analyses was performed using the DNeasy Blood and Tissue kit (QIAGEN, Valencia/CA, USA), following the manufacturer's instructions. The isolates of the following samples were used for DNA extraction:

- Samples biochemically compatible with *Salmonella* spp.
- Samples with inconclusive biochemical identification for *Salmonella* spp. (doubtful results for β-galactosidase and citrate)
- A random sample (n=85) selected from a total of 259 samples biochemically incompatible with *Salmonella* spp.

End-point PCR. End-point PCR for the confirmation of *Salmonella* spp. was performed based on the method described by Myint, et al. (2006) using *invA* primers (Skyberg et al. 2006) targeting *invA* gene. Amplicons were separated by 1.5% agarose gel electrophoresis in TAE buffer with GelRed (Biotium, Hayward/CA, USA). The image was then recorded with the L-PIX Image EX photo documenter (Loccus Biotechnology, Loccus Brazil, Cotia/SP, Brazil).

Real-time PCR. For RT-PCR, primers and DNA probes for the TaqMan MGB system were designed with the Primer Express program (Applied Biosystems, Foster City/CA, USA) targeting *invA* (Table 1). DNA detection was performed using the StepOne Plus system (Applied Biosystems, Foster City/CA, USA).

Serotyping. The *Salmonella* spp. isolates identified by biochemical methods, PCR and qPCR were sent to the National Reference Laboratory for Cholera and Bacterial Entero-Infections of the Oswaldo Cruz Institute (Fiocruz), Rio de Janeiro/RJ, Brazil, for serotyping by slide agglutination using the Kauffmann-White scheme with O and H antisera.

Pulsed-field gel electrophoresis. The *Salmonella* spp. isolates identified by biochemichal methods, PCR and qPCR were analyzed using pulsed-field gel electrophoresis (PFGE). The bacterial suspension was embedded in agarose, lysed, washed and digested with the restriction enzyme XbaI (New England Biolabs, Beverly, MA, USA) overnight (12-16h) at 37°C, essentially as described in the "One-Day (24-28h) Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli* O157:H7, non-typhoidal *Salmonella* serotypes, and *Shigella sonnei* by pulsed field gel electrophoresis (PFGE)" of the Centers for Disease Control and Prevention (Atlanta/GA, USA, http://www.cdc.gov/pulsenet/protocols.htm) (Ribot et al 2006). Electrophoresis was performed in 1% agarose gel using 0.5x Tris-borate-EDTA buffer on a Chef Mapper XA (BioRad Laboratories, Hercules/CA) at 6 V/cm for 19h at 14°C with an initial switch time of 2 min 16 sec and a final switch time of 63.8 sec. Gels were stained for 30 min at room temperature with ethidium bromide (Invitrogen, Carlsbad/CA), destained and photographed. *Salmonella* Braenderup (ATCC BAA-664) was included as reference. Pattern images were acquired using a Kodak Gel Logic 2200 system and analyzed using the BioNumerics software program, version 2.0 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Similarities between isolate fingerprints were determined based on the Dice correlation coefficient (Hunter & Gaston 1988). A band position tolerance of 1.7% was used for the analysis of PFGE patterns (Carriço et al. 2005). Dendrograms were generated by unweighted pairwise grouping with mathematical averaging (UPGMA). Isolates were considered as having the same pulsotype when the number and location of the bands were indistinguishable. Isolates with one band difference were considered to be of distinct pulsotypes.

Table 1. Primers and DNA probes for TaqMan MGB system targeting *invA*

Target gene	Primers and probe (5´-3´)	Expected amplicon size (bp)
invA	Forward: GCG AGC AGC CGC TCA GT Reverse: CGA GAT CGC CAA TCA GTC CTA Probe: NED-TGA GGA AAA AGA AGG GTC GT-MGBNFQ	63

Antimicrobial susceptibility profile. The antimicrobial susceptibility of the *Salmonella* spp. strain isolates was determined using the disk diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI 2011), employing the following antibiotics: ampicillin, cefotaxime, ciprofloxacin, chloramphenicol, gentamicin and tetracycline.

Statistical analyses. The rates of positive samples in each evaluation period (after skinning, after washing and after cooling) were compared using the OpenEpi software program (Dean et al. 2013). Fisher's exact test with the mid-p method for matched pairs was used to determine associations between each two evaluation periods and the presence/absence of *Salmonella*.

RESULTS

Among the 90 samples from 30 carcasses at Slaughterhouse I, seven (7.7%) isolates from six carcasses were biochemically compatible with *Salmonella* spp.: three carcasses were positive after cooling, two were positive after skinning and after washing and one carcass had two positive samples (after skinning and after cooling). All carcasses analyzed at Slaughterhouses II and III were negative for *Salmonella* spp. (Table 2).

Fisher's exact with the mid-p method for matched pairs revealed no statistically significant associations ($p>0.05$) between each two evaluation periods and the presence/absence of *Salmonella* spp. However, a tendency toward an increase in differences regarding positivity was found in the comparisons after skinning/after washing, after skinning/after cooling and after washing/after cooling, as demonstrated by the decrease in p-values (Table 3).

Among 270 samples collected from the three slaughterhouses, seven isolates biochemically compatible with *Salmonella*, four inconclusive isolates and 85 isolates biochemically incompatible with *Salmonella* were tested using end-point PCR and qPCR. Four samples with inconclusive biochemical profiles and 85 samples biochemically incompatible for *Salmonella* spp. were all negative in the end-point PCR and qPCR. The seven isolates biochemically compatible with *Salmonella* spp. were also confirmed by conventional PCR and pPCR and exhibited one profile after serotyping. Table 4 displays the positive carcasses, points of contamination and serotype of *Salmonella* spp.

These strains of *Salmonella* spp. were also analyzed by PFGE and exhibited 100% similarity (Fig.1).

Regarding the sensitivity profile to antimicrobials, the strains of *Salmonella* spp. isolated from cattle carcasses demonstrated 100% sensitivity to gentamicin, ampicillin,

Table 2. Samples from beef meat contaminated with *Salmonella* spp. after skinning, washing and cooling at three slaughterhouses that export meat, Mato Grosso do Sul, Brazil

Source	Number of carcasses	Positive carcasses	Positive samples after skinning (%)	Positive samples after washing (%)	Positive samples after cooling (%)
Slaughterhouse I	30	6 (20%)	2 (6.6%)	1 (3.3%)	4 (13.3%)
Slaughterhouse II	30	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Slaughterhouse III	30	0 (0%)	0 (0%)	0 (0%)	0 (0%)
TOTAL	90	6 (6.7%)	2 (2.2%)	1 (1.1%)	4 (4.4%)

Table 3. Comparison of evaluation periods and presence/absence of *Salmonella* using matched pairs of same carcasses

Pair-matched comparisons		p-values (Fisher's exact test - mid-p method)	
Pair 1 (% of positive)	Pair 2 (% of positive)	1-tail	2-tail
After skinning (2.2)	After washing (1.1)	0.3125	0.6250
After skinning (2.2)	After cooling (4.4)	0.1875	0.3750
After washing (1.1)	After cooling (4.4)	0.1094	0.2188

Table 4. Serotyping of seven strains of *Salmonella* spp. isolated from 270 beef samples in three different steps of slaughter line destined for export, Mato Grosso do Sul, Brazil

Sample	Skinning	Washing	Cooling	Serotype
Carcass 37	Negative	Positive	Negative	Typhimurium
Carcass 44	Positive	Negative	Positive	Typhimurium
Carcass 48	Negative	Negative	Positive	Typhimurium
Carcass 57	Negative	Negative	Positive	Typhimurium
Carcass 60	Negative	Negative	Positive	Typhimurium
Carcass 64	Positive	Negative	Negative	Typhimurium

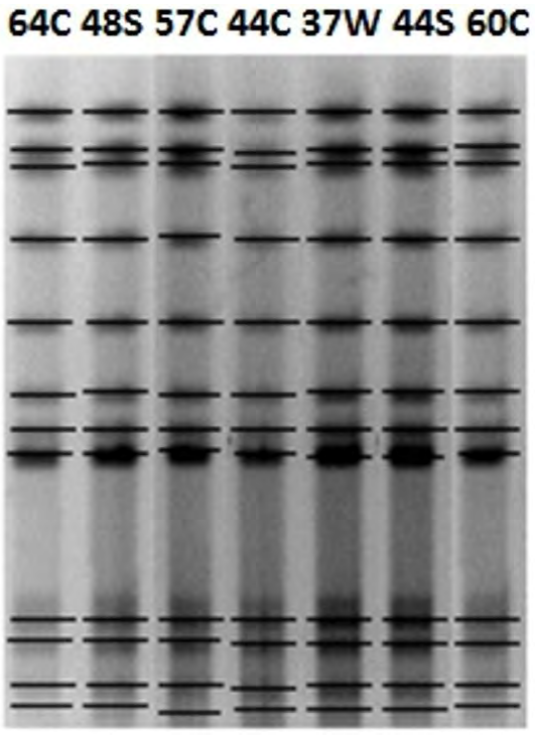


Fig.1. Profile of macrorestriction genomic DNA analysis of *Salmonella* spp. isolated from 90 bovine carcasses in slaughterhouse that exports meat, Mato Grosso do Sul, Brazil. S = Skinning, W = washing, C = cooling.

tetracycline, chloramphenicol, ciprofloxacin and cefotaxime (Table 5).

DISCUSSION

In the present study, seven isolates of *Salmonella* spp. were recovered from beef meat. Two isolates (2.2%) were found after skinning, which is a critical point of the slaughtering process due to the possibility of contamination of the substrate surface by microorganisms present on the skin, hair and hooves of the animal (Lambert et al. 1991, Penney et al. 2007). After washing, only one carcass (1.1%) exhibited *Salmonella* spp. Indeed, the purpose of this step is to reduce the microbial load in slaughterhouses (Dickson 1988). According to Brazilian legislation, maintenance of a minimum of 0.2mg/L and a maximum of 2mg/L of free residual chlorine in water is mandatory (Brasil 2011). Surprisingly, however, four isolates (4.4%) were found after cooling, which may have been due to contamination during the movement of the carcasses into the cooling chamber. Low temperatures reduce the number of microorganisms, but do not cause complete obliteration (Michener & Elliott 1964). This contamination may have occurred indirectly through the instruments used for slaughtering, workers' clothes (Prasai et al. 1995, Rahkio & Korkeala 1996, Pordesimo et al. 2002, Podpečan et al. 2007) and the hands of meat handlers (Bell 1997, Gill & McGinnis 2003). The floor can also be an important source of contamination through the transfer of organic matter to the shoes of workers or the spreading of microorganisms by cleaning with water under high pressure (Barros et al. 2007).

Positive carcasses for *Salmonella* after cooling pose a direct risk to consumers, since the meat is considered ready to be marketed after this process. It is well established that a high percentage of foodborne illnesses are caused by the failure of consumers to prepare food in a hygienic manner. Indeed, a common practice in households is to use the same kitchen equipment for both raw meat and fresh raw salads and fruits. Such a practice may lead to the cross-contamination of pathogenic microorganisms from raw meat to fruits and vegetables, which are mainly consumed without further processing. More seriously, washing or disinfecting kitchen equipment may not be sufficient to avoid the cross-contamination

Table 5. Sensitivity to antimicrobials of *Salmonella* strains isolated from bovine carcasses at slaughterhouse that exports meat, Mato Grosso do Sul, Brazil

Strain	Ampicillin	Gentamicin	Ciprofloxacin	Cefotaxime	Chloramphenicol	Tetracycline
37W	S	S	S	S	S	S
44S	S	S	S	S	S	S
44C	S	S	S	S	S	S
48S	S	S	S	S	S	S
57C	S	S	S	S	S	S
60C	S	S	S	S	S	S
64S	S	S	S	S	S	S
S. Typhimurium[11]	S	S	S	S	S	S

S = Skinning, W = Washing, C = Cooling; S = sensitive, I = intermediate, R = resistant; [1] *S.* Typhimurium INCQS 00150 (ATCC 14028).

of *S. enterica* Serovar Typhimurium to ready-to-eat foods (Gkana et al. 2016).

Among the seven strains serotyped in the present study, all were Typhimurium and exhibited a single pattern in PFGE. This result demonstrates a clonal relationship among these strains, indicating the presence of the same serovar, Typhimurium, probably from a same clone, and suggesting local contamination restricted to the slaughter room or a handler.

In the present investigation, some strains exhibited an inconclusive biochemical pattern for *Salmonella* spp. after culturing, pre-enrichment, selective enrichment differential plating and biochemical methods and were confirmed negative for *Salmonella* by the molecular tests. Biochemical tests identify *Salmonella* by the phenotypic profile, which, however, can exhibit variability due to the effect of environmental factors on gene expression and may lead to false-negative reactions or misinterpretations (Farber et al. 2001, Malorny et al. 2003, Settanni & Corsetti 2007).

Due to the short life of meat products, faster diagnostic methods are needed. There is considerable discussion about the applicability of PCR for the direct detection of *Salmonella* in meat products or carcasses, since the technique detects all viable, injured and dead bacteria (Malorny et al. 2003, Ibrahim et al. 2014) and the results may therefore not be valid, as injured or dead bacteria would not cause infection in the host. Thus, complementary diagnostic methods are often required, considering the possibility of false negatives due to inhibitory substances as well as false positives due to the detection of injured or dead bacteria (Wilson 1997). However, PCR from the isolates of *Salmonella* minimizes this error and increases the sensitivity of the technique, with the association of two or more enrichment broths with plating media resulting in a greater number of isolates of *Salmonella* (Busse 1995, Rall et al. 2005). Furthermore, PCR with isolates on selective plating saves two days in comparison to the traditional culture method as well as minimizes the labor expended and the possible misinterpretation of biochemical tests.

The increase in multi-antimicrobial-resistant *Salmonella* strains isolated from humans has been associated with the widespread use of antimicrobial agents in food animal production (Brown et al. 2017). The spread of antimicrobial resistance through the food chain is regarded as a major public health issue (Raufu et al. 2014). Thus, the analysis of antimicrobial susceptibility is crucial in the clinical and epidemiological context (Silley 2012). The antimicrobial sensitivity profile allows tracing the spread of multidrug-resistant strains (Olsen et al. 1993, Oueslati et al. 2016). In the case of *Salmonella*, Hur et al. (2012) reported an increase in Typhimurium and Newport multidrug-resistant serotypes. The multidrug-resistant *S. enterica* serovar Typhimurium definitive phage type harbors a chromosomally encoded genomic island (*Salmonella* Genomic Island 1), which is typically responsible for resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide and tetracycline (Hur et al. 2012) and has been isolated from human and pork samples, suggesting the transfer of resistance to humans via meat consumption (Van Boxstael et al. 2012). The strains of *S. enterica* serovar Typhimurium isolated in the present study demonstrated 100% susceptibility to ampicillin, chloramphenicol and tetracycline, suggesting that these isolates do not harbor the penta-resistant genomic island.

The prevalence of *Salmonella* spp. in beef is quite variable and depends on many factors, such as weather conditions, type of management, slaughter conditions and the storage and transportation of carcasses. Cross-contamination and incorrect handling practices are often associated with the contamination of beef, making it particularly difficult to identify the primary source of contamination (Perez-Rodriguez et al. 2010).

CONCLUSIONS

Salmonella spp. was found in beef meat at only one of the slaughterhouses investigated and the frequency was low.

The occurrence of bovine carcasses contaminated with *Salmonella* spp. after cooling imposes a direct risk to consumers, since the meat is considered ready to be marketed after this process.

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REFERENCES

ABIEC 2016. Exportações Brasileiras de Carne Bovina, janeiro a dezembro de 2015. Associação Brasileira das Indústrias Exportadoras de Carne. Available at <http://www.abiec.com.br/download/relatorio-anual-2015.pdf> Accessed on Nov. 5, 2016.

Barros M.F.A., Nero L.A., Monteiro A.A. & Beloti V. 2007. Identification of main contamination points by hygiene indicator microorganisms in beef processing plants. Food Sci. Technol. 27(4):856-862. <http://dx.doi.org/10.1590/S0101-20612007000400028>

Bell R.G. 1997. Distribution and sources of microbial contamination on beef carcasses. J. Appl. Microbiol. 82(3):292-300. <http://dx.doi.org/10.1046/j.1365-2672.1997.00356.x> <PMid:12455892>

Borch E. & Arinder P. 2002. Bacteriological safety issues in red meat and ready-treat meat products, as well as control measures. Meat Sci. 62(3):381-390. <http://dx.doi.org/10.1016/S0309-1740(02)00125-0> <PMid:22061614>

Brasil 2001. Resolução RDC 12 de 2 de janeiro de 2001. Regulamento Teórico Sobre Padrões Microbiológicos Para Alimentos nº 7-E, Agência Nacional de Vigilância Sanitária (ANVISA), Diário Oficial da República Federativa do Brasil, Brasil, p.46-53.

Brasil 2004. Programas de autocontroles de estabelecimento produtor de carne bovina habilitados para os Estados Unidos (EUA) e para Estados-Membros da União Europeia (UE). Circular Nº463/DCI/DIPOA/2004, Divisão de Controle do Comercio Internacional (DCI), Departamento de Inspeção de Produtos de Origem Animal (DIPOA), Secretaria de Defesa Agropecuária (DAS), Ministério da Agricultura, Pecuária e Abastecimento.

Brasil 2011. Dispõe sobre os procedimentos de controle e de vigilância da qualidade da água para consumo humano e seu padrão de potabilidade. Portaria nº 2914 de 12 de dezembro de 2011, Diário Oficial da República Federativa do Brasil, Ministério da Saúde, Brasília. Brazil 2015. Resultados de 2015, perspectivas para 2016. Estatísticas de Comercio Exterior do Agronegócio Brasileiro (Agrostat), Secretaria de Relações Internacionais do Agronegócio (SRI), Ministério da Agricultura, Pecuária e Abastecimento. Available at <http://www.agricultura.gov.br/arq_editor/file/acs/apresentacao1.pdf> Accessed on Jun. 1, 2016.

Brasil 2015. Resultados de 2015, perspectivas para 2016. Estatísticas de Comércio Exterior do Agronegócio Brasileiro (Agrostat), Secretaria de Relações Internacionais do Agronegócio (SRI), Ministério da Agricultura, Pecuária e Abastecimento. Available at <http://www.agricultura.gov.br/arq_editor/file/acs/apresentacao1.pdf> Accessed on Sep. 6, 2016.

Brasil 2016. Estatística da produção pecuária, março de 2016. Indicadores IBGE, Comissão Especial de Planejamento, Controle e Avaliação das Estatísticas Agropecuárias, (Cepagro), Instituto Brasileiro de Geografia e Estatística. Available at <http://www.ibge.gov.br/home/estatistica/indicadores/agropecuaria/producaoagropecuaria/abate-leite-couro-ovos_201504_publ_completa.pdf> Accessed on Jun. 1, 2016.

Brown A.C., Grass J.E., Richardson L.C., Nisler A.L., Bicknese A.S. & Gould L.H. 2017. Antimicrobial resistance in *Salmonella* that caused foodborne disease outbreaks: United States, 2003-2012. Epidemiol. Infect. 145(4):766-774. <http://dx.doi.org/10.1017/S0950268816002867> <PMid:27919296>

Busse M. 1995. Media for *Salmonella.* Int. J. Food Microbiol. 26(1):117-131. <http://dx.doi.org/10.1016/0168-1605(93)E0030-U> <PMid:7662516>

Carriço J.A., Pinto F.R., Simas C., Nunes S., Sousa N.G., Franzão N., de Lencastre H. & Almeida J.S. 2005. Assessment of band-based similarity coefficients for automatic type and subtype classification of microbial isolates analyzed by pulsed-field gel electrophoresis. J Clin. Microbiol. 43(11):5483-5490. <http://dx.doi.org/10.1128/JCM.43.11.5483-5490.2005> <PMid:16272474>

CLSI 2011. Twenty-first informational supplement Performance Standards for Antimicrobial Susceptibility Testing, Vol.1, Clinical and Laboratory Standards Institute.

Commission Regulation-EC 2007. Microbiological criteria for foodstuffs. European Commission N° 1441/2007 (ameding Regulation N° 2073/2005). Oficial Journal of the European Union L322:12-29.

Dean A.G., Sullivan K.M. & Soe M.M. 2013. OpenEpi: open source epidemiologic statistics for public health. Available at <www.OpenEpi.com> Accessed on Jul. 21, 2016.

Dickson J.S. 1988. Reduction of bacteria attached to meat surfaces by washing with selected compounds. J. Food Protect. 51(11):869-873. <http://dx.doi.org/10.4315/0362-028X-51.11.869>

EFSA. 2015. EU summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2013. European Food Safety Authority J. 13:3991.

Farber J.M., Gendel S.M., Tyler K.D., Boerlin P., Landry W.L., Fritschel S.C. & Barrett T.J. 2001. Molecular typing and differentiation, p.127-156. In: Ibid. (Eds), Compendium of Methods for the Microbiological Examination of Foods. 4th ed. American Public Health Association, Washington, D.C. <http://dx.doi.org/10.2105/9780875531755ch11>.

Gill C.O. & McGinnis J.C. 2003. Microbiological effects of hand washing at a beef carcass-breaking facility. J. Food Protect. 66(3):493-496. <http://dx.doi.org/10.4315/0362-028X-66.3.493> <PMid:12636307>

Gkana E., Lianou A. & Nychas G.J. 2016. Transfer of *Salmonella* enterica Serovar Typhimurium from Beef to Tomato through Kitchen Equipment and the Efficacy of Intermediate Decontamination Procedures. J. Food Protect. 79(7):1252-1258. <http://dx.doi.org/10.4315/0362-028X.JFP-15-531> <PMid:27357047>

Hunter P.R. & Gaston M.A. 1988. Numerical Index oh the discriminatory ability of typing systems: an application of Simpson's Index of Diversity. J. Clin. Microbiol. 26(11):2465-2466. <PMid:3069867>

Hur J., Jawale C. & Lee J.H. 2012. Antimicrobial resistance of *Salmonella* isolated from food animals: a review. Food Res. Int. 45(2):819-830. <http://dx.doi.org/10.1016/j.foodres.2011.05.014>

IAGRO 2018. Rebanho bovino de MS cresce, superando 21,8 milhões de cabeças. Agência Estadual de Defesa Sanitária Animal e Vegetal. Available at <http:// http://www.iagro.ms.gov.br/rebanho-bovino-de-ms-cresce-superando-218-milhoes-de-cabecas/> Accessed on Jun. 10, 2018.

Ibrahim W.A., Abd El-Ghany W.A., Nasef S.A. & Hatem M.E. 2014. A comparative study on the use of real time polymerase chain reaction (RT-PCR) and standard isolation techniques for the detection of *Salmonellae* in broiler chicks. Int. J. Vet. Sci. Med. 2(1):67-71. <http://dx.doi.org/10.1016/j.ijvsm.2013.11.001>

ISO 2002. ISO 6579:2002: microbiology of food and animal feeding stuffs, horizontal method for the detection of *Salmonella* spp. International Organization for Standardization, Switzerland. 20p.

Jay J.M. 2000. Fresh meats and poultry, p.59-85. In: Jay J.M. (Ed.), Modern Food Microbiology. Aspen Publishers Inc., USA. <http://dx.doi.org/10.1007/978-1-4615-4427-2_4>.

Lambert A.D., Smith J.P. & Dodds K.L. 1991. Shelf life extension and microbiological safety of fresh meat: a review. Food Microbiol. 8(4):267-297. <http://dx.doi.org/10.1016/S0740-0020(05)80002-4>

Madden R.H., Murray K.A. & Gilmour A. 2004. Determination of the principal points of product contamination during beef carcass dressing processes in Northern Ireland. J. Food Protect. 67(7):1494-1496. <http://dx.doi.org/10.4315/0362-028X-67.7.1494> <PMid:15270508>

Malorny B., Tassios P.T., Rådström P., Cook N., Wagner M. & Hoorfar J. 2003. Standardization of diagnostic PCR for detection of foodborne pathogens. Int. J. Food Microbiol. 83(1):39-48. <http://dx.doi.org/10.1016/S0168-1605(02)00322-7> <PMid:12672591>

Martínez-Chávez L., Cabrera-Diaz E., Pérez-Montaño J.A., Garay-Martínez L.E., Varela-Hernández J.J., Castillo A., Lucia L., Ávila-Novoa M.G., Cardona-López M.A., Gutiérrez-González P. & Martínez-Gonzáles N.E. 2015. Quantitative distribution of *Salmonella* spp. and *Escherichia coli* on beef carcasses and raw beef at retail establishments. Int. J. Food Microbiol. 210(1):149-155. <http://dx.doi.org/10.1016/j.ijfoodmicro.2015.06.016> <PMid:26125489>

Michener H.D. & Elliott R.P. 1964. Minimum growth temperatures for food-poisoning, fecal-indicator, and psychrophilic microorganisms. Adv. Food Res. 13:349-396. <http://dx.doi.org/10.1016/S0065-2628(08)60103-2> <PMid:14288887>

Myint M.S., Johnson Y.J., Tablante N.L. & Heckert R.A. 2006. The effect of pre-enrichment protocol on the sensivity and specifity of PCR for detection of naturally contaminated *Salmonella* in raw poultry compared to conventional culture. Food Microbiol. 23(6):599-604. <http://dx.doi.org/10.1016/j.fm.2005.09.002> <PMid:16943057>

Olsen J.E., Brown D.J., Skov M.N. & Christensen J.P. 1993. Bacterial typing methods suitable for epidemiological analysis applications in investigations of salmonellosis among livestock. Vet. Quartely 15(4):125-135. <PMid:8122347>

Oueslati W., Rjeibi M.R., Mhadhbi M., Jbeli M., Zrelli S. & Ettriqui A. 2016. Prevalence, virulence and antibiotic susceptibility of *Salmonella* spp. strains, isolated from beef in Greater Tunis (Tunisia). Meat Sci. 119:154-159. <http://dx.doi.org/10.1016/j.meatsci.2016.04.037> <PMid:27183540>

Papadopoulou O.S., Chorianopoulos N.G., Gkana E.N., Grounta A.V., Koutsoumanis K.P. & Nychas G.J. 2012. Transfer of foodborne pathogenic bacteria to non-inoculated beef fillets through meat mincing machine. Meat Sci. 90(3):865-869. <http://dx.doi.org/10.1016/j.meatsci.2011.11.008> <PMid:22119672>

Penney N., Bigwood T., Barea H., Pulford D., LeROUX G.U.I.L.L., Cook R., Jarvis G. & Brightwell G. 2007. Efficacy of a peroxyacetic acid formulation as an antimicrobial intervention to reduce levels of inoculated *Escherichia coli* O157:H7 on external carcass surfaces on hot-boned beef and veal. J. Food Protect. 70(1):200-203. <http://dx.doi.org/10.4315/0362-028X-70.1.200> <PMid:17265881>

Perez-Rodriguez F., Castro R., Posada-Izquierdo G.D., Valero A., Carrasco E., García-Gimeno R.M. & Zurera G. 2010. Evaluation of hygiene practices and microbiological quality of cooked meat products during slicing and handling at retail. Meat Sci. 86(2):479-485. <http://dx.doi.org/10.1016/j.meatsci.2010.05.038> <PMid:20573456>

Podpečan B., Pengov A. & Vadnjal S. 2007. The source of contamination of ground meat for production of meat products with bacteria *Staphylococcus aureus.* Slov. Vet. Res. 44(1/2):25-30.

Pordesimo L.O., Wilkerson E.G., Womac A.R. & Cutter C.N. 2002. Process engineering variables in the spray washing of meat and produce. J. Food Protect. 65(1):222-237. <http://dx.doi.org/10.4315/0362-028X-65.1.222> <PMid:11808801>

Prasai R.K., Phebus R.K., Zepeda C.M.G., Kastner C.L., Boyle A.E. & Fung D.Y.C. 1995. Effectiveness of trimming and/or washing on microbiological quality of beef carcasses. J. Food Protect. 58(10):1114-1117. <http://dx.doi.org/10.4315/0362-028X-58.10.1114>

Rahkio M. & Korkeala H. 1996. Microbiological contamination of carcasses related to hygiene practice and facilities on slaughtering lines. Acta Vet. Scand. 37(3):219-228. <PMid:8996868>

Rall V.L.M., Rall R., Aragon L.C. & Silva M.G. 2005. Evaluation of three enrichment broths and five plating media for *Salmonella* detection in poultry. Braz. J. Microbiol. 36(2):147-150. <http://dx.doi.org/10.1590/S1517-83822005000200009>

RASFF 2018. Notifications by product category and notifying country. Rapid Alert System for Food and Feed, European Commission. Available at <https://webgate.ec.europa.eu/rasff-window/portal/?event=searchResultList> Accessed on Mar. 2, 2018.

Raufu I.A., Lawan F.A., Bello H.S., Musa A.S., Ameh J.A. & Ambali A.G. 2014. Occurrence and antimicrobial susceptibility profiles of *Salmonella* serovars from fish in Maiduguri, sub-Saharah, Nigeria. Egypt. J. Aquat. Res. 40(1):59-63. <http://dx.doi.org/10.1016/j.ejar.2014.01.003>

Rhoades J.R., Duffy G. & Koutsoumanis K. 2009. Prevalence and concentration of verocytotoxigenic *Escherichia coli, Salmonella enterica* and *Listeria monocytogenes* in the beef production chain: a review. Food Microbiol. 26(4):357-376. <http://dx.doi.org/10.1016/j.fm.2008.10.012> <PMid:19376457>

Ribot E.M., Fair M.A., Gautom R., Cameron D.N., Hunter S.B., Swaminathan B. & Barrett T.J. 2006. Standardization of Pulsed-Field Gel Electrophoresis Protocols for the Subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. Foodborne Pathog. Dis. 3(1):59-67. <http://dx.doi.org/10.1089/fpd.2006.3.59> <PMid:16602980>

Sans P. & Combris P. 2015. World meat consumption patterns: An overview of the last fifty years (1961-2011). Meat Sci. 109:106-111. <http://dx.doi.org/10.1016/j.meatsci.2015.05.012> <PMid:26117396>

Settanni L. & Corsetti A. 2007. The use of multiplex PCR to detect and differentiate food and beverage-associated microorganisms: a review. J. Microbiol. Methods 69(1):1-22. <http://dx.doi.org/10.1016/j.mimet.2006.12.008> <PMid:17280731>

Silley P. 2012. Susceptibility testing methods, resistance and breakpoints: what do these terms really mean? Rev. Sci. Tech. OIE 31(1):33-41. <http://dx.doi.org/10.20506/rst.31.1.2097> <PMid:22849266>

Skyberg J.A., Logue C.M. & Nolan L.K. 2006. Virulence Genotyping of *Salmonella* spp. with Multiplex PCR. Avian Dis. 50(1):77-81. <http://dx.doi.org/10.1637/7417.1> <PMid:16617986>

Van Boxstael S., Dierick K., Van Huffel X., Uyttendaele M., Berkvens D., Herman L., Bertrand S., Wildemauwe C., Catry B., Butaye P. & Imberechts H. 2012. Comparison of antimicrobial resistance patterns and phage types of *Salmonella* Typhimurium isolated from pigs, pork and human in Belgium between 2001 and 2006. Food Res. Int. 45(2):913-918. <http://dx.doi.org/10.1016/j.foodres.2011.05.025>

WHO 2005. Drug-resistant *Salmonella*. Fact sheet n. 139, Food Safety Department, Word Health Organization. Available at <http://www.who.int/mediacentre/factsheets/fs139/en/> Accessed on Jun. 17, 2016.

Wilhelm B., Rajić A., Greig J.D., Waddell L. & Harris J. 2011. The effect of hazard analysis critical control point programs on microbial contamination of carcasses in abattoirs: a systematic review of published data. Foodborne Pathog. Dis. 8(9):949-960. <http://dx.doi.org/10.1089/fpd.2010.0809> <PMid:21568689>

Wilson I.G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63(10):3741-3751. <PMid:9327537>

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Detection of swainsonine and calystegines in Convolvulaceae species from the semiarid region of Pernambuco[1]

Fábio S. Mendonça[2]*, Givaldo B. Silva Filho[2], Hisadora A.S. Chaves[2], Lorena D.A. Aires[2], Thaiza C. Braga[2], Dale R. Gardner[3], Daniel Cook[3] and Maria T. Buril[4]

ABSTRACT. - Mendonça F.S., Silva Filho G.B., Chaves H.A.S., Aires L.D.A., Braga T.C., Gardner D.R., Cook D. & Buril M.T. 2018. [**Detection of swainsonine and calystegines in Convolvulaceae species from the semiarid region of Pernambuco].** *Detecção de swainsonina e calisteginas em espécies de Convolvulaceae da região semiárida de Pernambuco. Pesquisa Veterinária Brasileira 38(11):2044-2051.* Laboratório de Diagnóstico Animal, Departamento de Morfologia e Fisiologia Animal, Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. E-mail: fabio.mendonca@pq.cnpq.br

Numerous plant species worldwide including some *Ipomoea* (Convolvulaceae) and *Sida* (Malvaceae) species in Brazil cause lysosomal storage disease in herbivores and are known to contain swainsonine and calystegines as the main toxic compounds. The aim of this work was to determine swainsonine and calystegines concentrations in species of Convolvulaceae from the semiarid region of Pernambuco. Seven municipalities in the Moxotó region were visited and nine species were collected and screened for the presence of swainsonine and calystegines using an HPLC-APCI-MS method. The presence and concentration of these alkaloids within the same and in different species were very variable. Seven species are newly reported here containing swainsonine and/or calystegines. *Ipomoea subincana* contained just swainsonine. *Ipomoea megapotamica*, *I. rosea* and *Jacquemontia corymbulosa* contained swainsonine and calystegines. *Ipomoea sericosepala*, *I. brasiliana*, *I. nil*, *I. bahiensis* and *I. incarnata* contained just calystegines. The discovery of six *Ipomoea* species and one *Jacquemontia* species containing toxic polyhydroxy alkaloids reinforces the importance of this group of poisonous plants to ruminants and horses in the semiarid region of Pernambuco. Epidemiological surveys should be conducted to investigate the occurrence of lysosomal storage disease associated to these new species.

INDEX TERMS: Poisonous plant, swainsonine, calystegines, Convolvulaceae species, Pernambuco, alkaloids, lysosomal storage disease, plant poisoning, herbivores, toxicoses.

RESUMO.- [**Detecção de swainsonina e calisteginas em espécies de Convolvulaceae da região semiárida de Pernambuco.**] Numerosas espécies de plantas em todo o mundo, incluindo algumas espécies de *Ipomoea* (Convolvulaceae) e *Sida* (Malvaceae) no Brasil, causam doença de armazenamento lisossomal em herbívoros e são conhecidas por conterem swainsonina e calisteginas como princípios tóxicos. O objetivo deste trabalho foi determinar a concentração de swainsonina e calisteginas em espécies de Convolvulaceae da região semiárida de Pernambuco. Sete municípios na região do Sertão do Moxotó foram visitados, onde foram coletadas amostras das folhas de nove espécies de Convolvulaceae para avaliação da presença de swainsonina e calisteginas utilizando-se cromatografia líquida com espectrometria de massa. A presença e concentração destes alcaloides nas folhas de plantas da mesma espécie e dentre as espécies foram muito variáveis. Seis novas espécies de *Ipomoea* e uma espécie de *Jacquemontia* contendo swainsonina e/ou calisteginas são relatadas neste

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[2] Laboratório de Diagnóstico Animal, Departamento de Morfologia e Fisiologia Animal (DMFA), Universidade Federal Rural de Pernambuco (UFRPE), Rua Dom Manuel Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. *Corresponding author: fabio.mendonca@pq.cnpq.br

[3] Poisonous Plant Research Laboratory, ARS, USDA, 1150 E. 1400 N., Logan, UT 84341, USA.

[4] Departamento de Biologia, UFRPE, Rua Dom Manuel Medeiros s/n, Dois Irmãos, Recife, PE 52171-900.

estudo. *Ipomoea subincana* continha apenas swainsonina. *Ipomoea megapotamica*, *I. rosea* e *Jacquemontia corymbulosa* continham swainsonina e calisteginas. *Ipomoea sericosepala*, *I. brasiliana*, *I. nil*, *I. bahiensis* e *I. incarnata* continham apenas calisteginas. A descoberta de novas espécies de *Ipomoea* e *Jacquemontia* contendo alcaloides polihidroxílicos tóxicos reforçam a importância deste grupo de plantas tóxicas para ruminantes e equinos na região semiárida de Pernambuco. Pesquisas epidemiológicas devem ser realizadas para investigar a ocorrência de doença de depósito lisossomal associada a essas novas espécies.

TERMOS DE INDEXAÇÃO: Plantas tóxicas, swainsonina, calisteginas, Convolvulaceae, Pernambuco, alcaloides, doença de depósito lisossomal, intoxicação por plantas, herbívoros, toxicoses.

INTRODUCTION

Swainsonine-containing plants comprises a group of several toxic weeds that impair the development of pasture and livestock worldwide. These plants belong to three genera of the Fabaceae family (*Astragalus*, *Oxytropis* e *Swainsona*) (Burrows & Tyrl 2012, Cook et al. 2009, 2014), one genus of the Convolvulaceae family (*Ipomoea*) and one genus of the Malvaceae family (*Sida*) (Oliveira Júnior et al. 2013) and are distributed mainly in the United States, Canada, Mexico, Brazil, Russia, Spain, Ireland, Morocco, Egypt, Australia and China (Jung et al. 2011, Cook et al. 2014, Oliveira Júnior et al. 2013).

Swainsonine is a polyhydroxy alkaloid, a potent inhibitor of lysosomal α-mannosidase and α-mannosidase II of Golgi apparatus of neurons and epithelial cells. Some calystegines are potent glycosidases inhibitors and is suggested to disrupt intestinal glycosidases, lysosomal function and glycoprotein processing causing enzymatic dysfunction and accumulation of complex oligosaccharides in lysosomes (Colegate et al. 1979, Dorling et al. 1980, Stegelmeier et al. 2008). Recent research has shown that swainsonine is a secondary metabolite resulting from endophytic fungi and is not therefore produced by the host plant. The presence of endophytic fungi was demonstrated in *Astragalus*, *Oxytropis*, *Swainsona* and *Ipomoea* species (Braun et al. 2003, Pryor et al. 2009, Hao et al. 2012, Cook et al. 2014). However, it is suggested that calystegines are secondary metabolites resulting from plants as it has been demonstrated in a previous study when these toxins remained present even in plants derived from seeds treated with fungicides (Cook et al. 2013).

Clinical signs presented by ruminants and horses grazing swainsonine-containing plants consists mainly in neurological disorders, although endocrine and reproductive alterations may also occur (Oliveira et al. 2011, Tokarnia et al. 2012). Neurological changes observed are mainly of cerebellar origin, evidenced by loss of balance, followed by falls when the animals are stressed, ataxia, hypermetria, dysmetria, nystagmus, lateral gait and head and neck tremors. After the falls, the animals have difficulty into raising up and may present spasticity of the hindlimbs. Other alterations, such as somnolence, progressive emaciation and bristly, brittle and opaque hair may be observed (Tokarnia et al. 2012, Mendonça et al. 2011, 2012, Lima et al. 2013, Oliveira Júnior et al. 2013, Rocha et al. 2016).

Convolvulaceae family has a wide distribution worldwide, comprises 60 genera and at least 1.900 species. *Ipomoea* and *Jacquemontia* are being the most representative genera of this family with most of their species endemic in Brazil (Staples 2012, Bianchinni & Ferreira 2013, Buril et al. 2015). During epidemiological investigations of neurological diseases in ruminants, especially goats in the semiarid region of Pernambuco, our research team has observed outbreaks of lysosomal storage disease in which several species of *Ipomoea* could be associated to the disease, some of them being known to be toxic but in another hand, most species remains unknown about their alkaloid contents and toxicity. For this reason, the aim of this study was to determine swainsonine and calystegines concentrations in species of Convolvulaceae from the semiarid region of the State of Pernambuco.

MATERIALS AND METHODS

This study was performed in the microregion of the Sertão do Moxotó, Pernambuco, in the municipalities of Arcoverde, Sertânia, Betânia, Ibimirim, Custódia, Inajá and Manari. The weather in these municipalities is semiarid, with high temperatures and scarce and badly distributed raining. The characteristic vegetation is the Brazilian Caatinga Savannah. This study was performed during the rainy season in 2017, which comprehends the months from March to June. The main highways of those municipalities and bordering roads throughout rock outcrops and livestock farms and were car-driven with an average speed of 18 mph making a minimum distance of 62 miles and a maximum of 124 miles in each municipality, to collect and determine the occurrence of Convolvulaceae species. Those species observed during the rout were collected for botanical identification and vouchers were deposited in Vasconcelos Sobrinho Herbarium of the Federal Rural University of Pernambuco. Samples containing 500g of leaves from each species (five individuals by species) were collected, dry shaded, crushed, mixed to create a pool of samples and analyzed by high-performance liquid chromatography–atmospheric pressure chemical ionization–tandem mass spectrometry (HPLC-APCI-MS) method, according to the procedures described by Gardner et al. (2001).

Samples (100g) of the dry vegetal material were used to obtain extract and posterior analysis for presence of swainsonine by ion exchange resin insulation. The aqueous extract was then analyzed by liquid chromatography coupled to mass spectrometry using a HP 1100 binary solvent pump, an automatic sampler, a HPLC column of Betasil C18 reverse phase (100x2mm) and a Finnigan LCQ mass spectrometer. Swainsonine was eluted using an isocratic mixture of 5% methanol in 20 mM ammonium acetate at a flow rate of 0.5mL/min. The size of the sample injection was 20 μL. Ionization was achieved using an atmospheric pressure chemical ionization (APCI) source with a vaporization temperature of 450°C and a corona discharge current of 5 μA. The mass spectrometer was conducted in a MS/MS mode, examining the ions of the products in a mass range of 70-300 amu after the fragmentation of the protonated swainsonine molecule using a relative collision energy setting of 25%.

The area of the swainsonine peak was measured from the reconstructed-ion chromatogram (m/z 156) and the quantification was based in an external standard calibration. The presence of swainsonine was verified through gas chromatography - mass spectrometry (GC/MS) after a portion from the aqueous extract was dried and the residue derived by the addition of N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) and pyridine to convert swainsonine into its timesilil ether derivative. GC/MS analysis was performed using an electronic therm GC/MS system installed with an Agilent DB-5MS capillary column (30m×0.25mm). Helium was used as a carrier gas

at a constant flow rate of 1.5 mL/min. The samples (2.0µL) were injected using a split/splitless injector with a temperature of 250 °C. The column temperature was programmed at 120°C for 1 min, increased from 120 to 200°C at 5°C/min and from 200 to 300°C at 20°C/min and then maintained at 300°C for 8 min. The presence of calystegines was determined using the same GC/MS data to verify the existence of swainsonine.

The plants were photographed and the data on their occurrence were compared with the pluviometric indexes of each municipality, according to data from the Instituto Agronômico de Pernambuco (IPA).

RESULTS

In the municipalities that comprise the Sertão do Moxotó region, eight species of *Ipomoea* and one species of *Jacquemonthia* (Fig.1) were identified containing variable concentrations of swainsonine and calystegines (Table 1). Most species were found in Sertânia (seven in total). In this municipality the average rainfall in the last three months that preceded the collection of plants was 36.6mm and the annual average rainfall was 34mm, with more significant rains occurring from March to July. The species most observed and in higher amounts was *Ipomoea sericosepala*, which contained only calystegines (B1 0.013%, B2<0.001% and C1 0.002%) and *I. brasiliana*, which also contained only calystegines (B1 0.019%, B2 0.005% and C1 0.031%). Two other species were also observed frequently, however one contained both swainsonine and calystegines in its composition, identified as *I. megapotamica* (swainsonine 0.016%, calystegines B1 0.024%, B2 0.001%, B3 0.002% and C1 0.003%) and the other one, identified as *I. subincana* contained only swainsonine, in the concentration of 0.011%. *Ipomoea sericosepala* and *I. brasiliana* were observed both on the main highways and bordering roads, covering fences of the farms and shrubs in most of rock outcrops. At the collection sites, numerous sprouts were observed, as well as well-developed adult individuals up to 2.5m in height and abundant leaf mass. In the rock outcrops, where usually goats and sheep gather for pasture it was common to observe these animals ingesting three or four species of *Ipomoea* that vegetated simultaneously, some covering the others (Fig.2). In this situation it was possible to observe specially *I. sericosepala*, *I. brasiliana*, *I. megapotamica* and *I. subincana*. The other species of *Ipomoea* were not so abundant and were only seen occasionally; however, they contained concentrations of both swainsonine and calystegines.

In Manari, three species of *Ipomoea* and one of *Jacquemontia* were observed. In this municipality the average rainfall of the last three months previous the collection of plants was 81.3mm and the annual rainfall was 67.5mm, with significant rains occurring from April to August (Fig.3). The main species observed was *I. sericosepala*. The mean concentration of swainsonine in this species was 0.011% and the concentration of calystegines B1, B2, B3 and C1 was 0.012%, 0.001%, 0.002% and 0.003% respectively. *Ipomoea bahiensis* was the second more frequent species observed; the analyses identified only calystegines B2 (0.061%) and C1 (0.002%) in this plant composition. *Jacquemontia corymbulosa* was the third more common species found both on the roads and near the fences of the farms, forming an abundant leaf mass, sometimes covering extensive areas of soil and pasture. The mean concentration of both swainsonine and calystegine B2 in these species was 0.01%. No concentrations of indolizidine alkaloids were detected in *Ipomoea rosea* collected from this region.

Table 1. Eight species of *Ipomoea* and one specie of *Jacquemonthia* identified containing variable concentrations of swainsonine and calystegines from Sertão do Moxotó region, Pernambuco, Brazil

Month of collection	Municipality	Total rainfall[a]	Voucher number	Species	Swainsonine[b]	Calystegines			
						B1	B2	B3	C1
April	Sertânia	36,6mm	60018	*Ipomoea sericosepala*	-[c]	0.013	<0.001	-	0.002
			60019	*Ipomoea brasiliana*	-	0.019	0.005	-	0.031
			60020	*Ipomoea subincana*	0.011	-	-	-	-
			60021	*Ipomoea megapotamica*	0.016	0.024	0.001	0.002	0.003
			60022	*Ipomoea rosea*	0.001	-	-	-	-
			60023	*Ipomoea bahiensis*	-	-	0.001	-	-
			60024	*Ipomoea incarnata*	-	0.003	0.022	0.001	0.006
May	Betânia	250mm	60025	*Ipomoea nil*	-	0.003	0.007	-	0.001
			60026	*Ipomoea sericosepala*	0.012	0.013	0.001	-	0.002
June	Ibimirim	229mm	60027	*Ipomoea rosea*	0.007	0.001	0.003	-	<0.001
June	Manari	81,3mm	60028	*Ipomoea rosea*	-	-	-	-	-
			60029	*Jacquemontia corymbulosa*	0.001	-	0.001	-	-
			60030	*Ipomoea brasiliana*	-	0.029	0.014	-	0.052
			60031	*Ipomoea bahiensis*	-	-	0.061	-	0.002
			60032	*Ipomoea sericosepala*	0.011	0.012	0.001	0.002	0.003
July	Custódia	414mm	60033	*Ipomoea nil*	-	-	-	-	-
July	Inajá	179mm	60034	*Ipomoea brasiliana*	-	-	<0.001	-	0.001
July	Arcoverde	412mm	60035	*Ipomoea nil*	-	0.002	0.008	-	0.001

[a] Total rainfall of the month and the last three months that preceded the collection of plants, [b] concentrations of swainsonine equal to or greater than 0.001% of dry matter are considered toxic to herbivores (Molyneux et al. 1995), [c] species in which the presence of swainsonine has not been detected are symbolized by a dash (-).

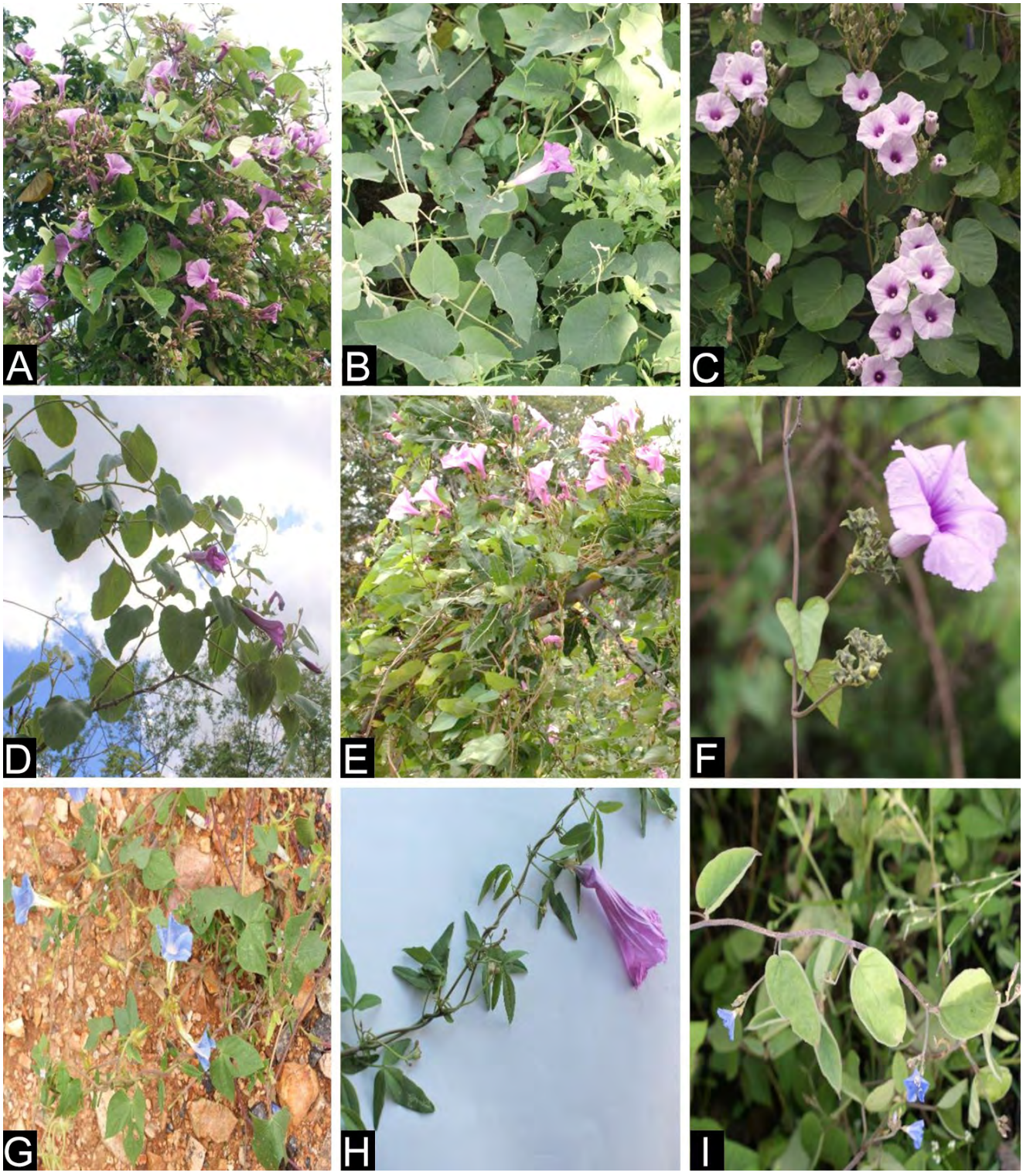


Fig.1. Species of *Ipomoea* in flowering phase during the rainy season of 2017, Sertão do Moxotó/PE, Brazil. (**A**) *Ipomoea sericosepala*, (**B**) *Ipomoea brasiliana*, (**C**) *Ipomoea megapotamica*, (**D**) *Ipomoea subincana* and (**E**) *Ipomoea incarnata* in April, municipality of Sertânia. (**F**) *Ipomoea bahiensis* in May, municipality of Manari. (**G**) *Ipomoea nil* in May, municipality of Betânia. (**H**) *Ipomoea rosea* in June, municipality of Manari. (**I**) *Jacquemontia corymbulosa* in May, municipality of Manari.

In Betânia, the most species observed was *I. sericosepala* and *I. nil* was seen occasionally, occurring only at borders of highways. The average rainfall in the period before the collection was 73.7mm with intense raining indexes from March to April. Swainsonine concentration in *I. sericosepala* was 0.012% and calystegines B1, B2 and C1 were 0.013%, 0.001% and <0.002%, respectively. Only calystegines B1, B2 and C1 were detected in *I. nil*, the average concentrations were



Fig.2. Pasture areas of goats in Sertão do Moxotó/PE, Brazil. (**A**) Pasture mainly formed by *Froelichia humboldtiana* and shrubs of *Ipomoea brasiliana* in sprouting phase (arrows), Inajá, July of 2017. (**B**) Caatinga area in the rainy season, with available forage and despite this, goats present predilection for *Ipomoea sericosepala* and (**C**) goat showing preference for *Ipomoea megapotamica* instead of other forages during the rainy season.

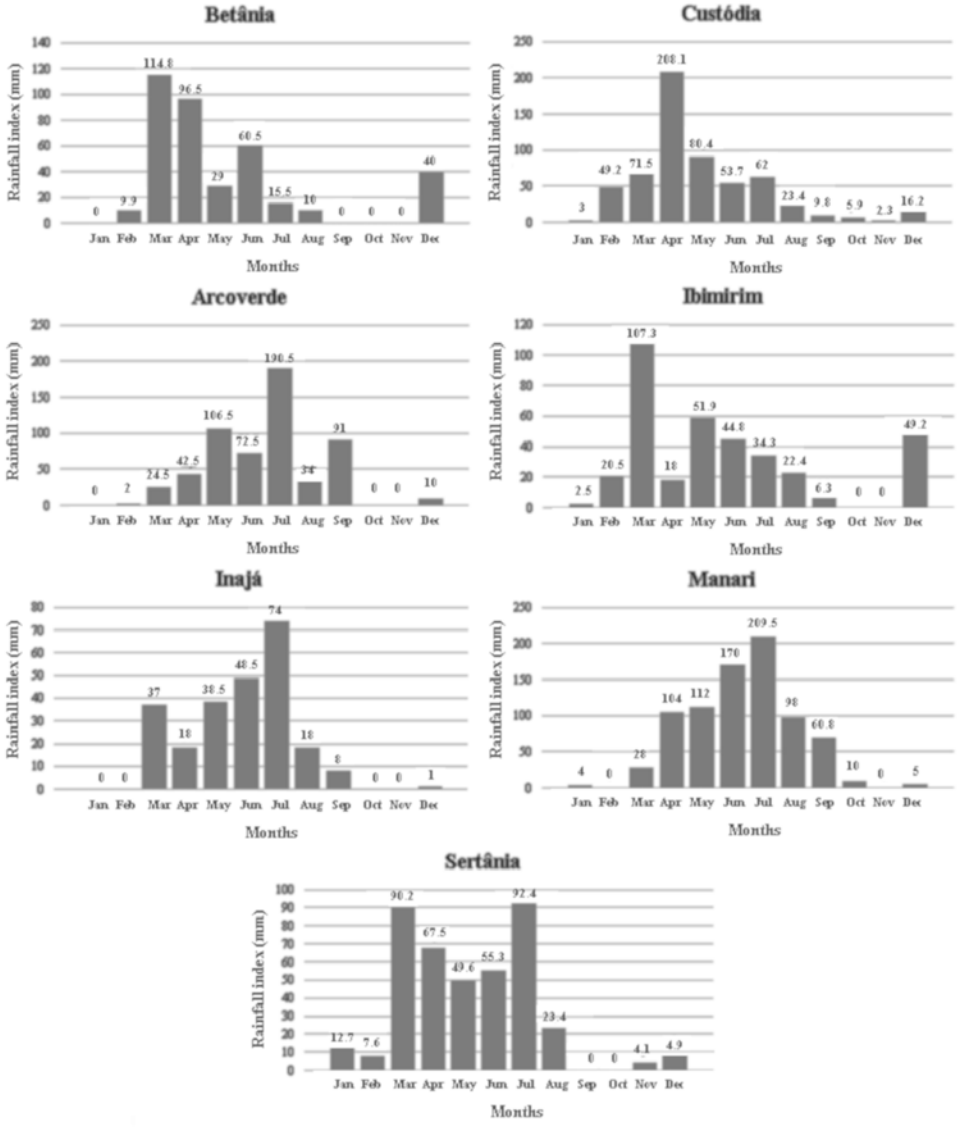


Fig.3. Pluviometric index in the municipalities that comprehend the Sertão do Moxotó region, Brazil. Notice March, April, May, June and July months had higher levels of rainfall, these periods could be associated with presence of Convolvulaceae in the studied region and when cases of lysosomal storage disease may occur.

0.003%, 0.007% and 0.001%, respectively. In Custódia and Ibimirim only two species of *Ipomoea* were observed, one in each municipality. The mean rainfall in Custódia in the period before the collection was 73.8mm with intense raining indexes from May to July; In Ibimirim the mean rainfall was 61.4mm with intense raining period from March to May. In Custódia only the occurrence of *I. nil* was observed and the presence of indolizidine alkaloids was not detected. The mean rainfall in the period before the collection of *I. nill* in Custódia was 117.4mm with rains from April to May. In *I. rosea* collected from Ibimirim, swainsonine concentration was 0.07% and calystegines B1, B2 and C1 were 0.001%, 0.003% and <0.001% respectively. In both municipalities amounts of these plants were not expressive and the observation was occasional.

In Arcoverde only *I. nil* was observed; swainsonine was not detected in this plant and concentrations of calystegines B1, B2 and C1 were 0.002%, 0.008% and 0.001%. In Inajá only *I. brasiliana* was observed, in which contained calystegines B2 (<0.001) and C1 (0.001). In Arcoverde, the average rainfall was 73.8mm with more intense rains from May to July. In Inajá the average was 35mm, with pluviometric indexes with below-average rainfall, compared to other municipalities. The distribution of *Ipomoea* species in the studied municipalities are displaced in Figure 4.

DISCUSSION

In this study six new species of *Ipomoea* (*I. subincana*, *I. megapotamica*, *I. rosea*, *I. bahiensis*, *I. incarnata* and *I. nil*) and one species of *Jacquemontia* (*J. corymbulosa*) are presented, containing toxic concentrations of swainsonine and/or calystegines. All these species are endemic from the Brazilian caatinga biome (Oliveira et al. 2013) and their importance as toxic plants for livestock must be investigated in all the Brazilian semiarid region. Two species most founded were *I. sericosepala* (previously *Turbina cordata)* (Wood et al. 2015) and *I. brasiliana* (previously reported as *I. marcellia* and *I.* aff. *verbascoidea*) (Mendonça et al. 2012, Rocha et al. 2016, Simão-Bianchini & Ferreira 2015). *Ipomoea sericosepala* has been reported as toxic to goats, cattle and horses (Dantas et al. 2007, Assis et al. 2010, Oliveira Júnior et al. 2013) and *I. brasiliana* so far has only been reported as important to goats (Mendonça et al. 2012, Lima et al. 2013). In previous studies, in both *I. sericosepala* and *I. brasiliana* significant amounts of swainsonine and calystegines were detected. Nonetheless, several samples of both plants were negative for swainsonine. This occurs because the swainsonine concentration varies considerably among species, since the production of this toxin is dependent on the presence of endophytic fungi. Calystegines, on the other hand, are secondary metabolites produced by plants (Cook et al. 2013).

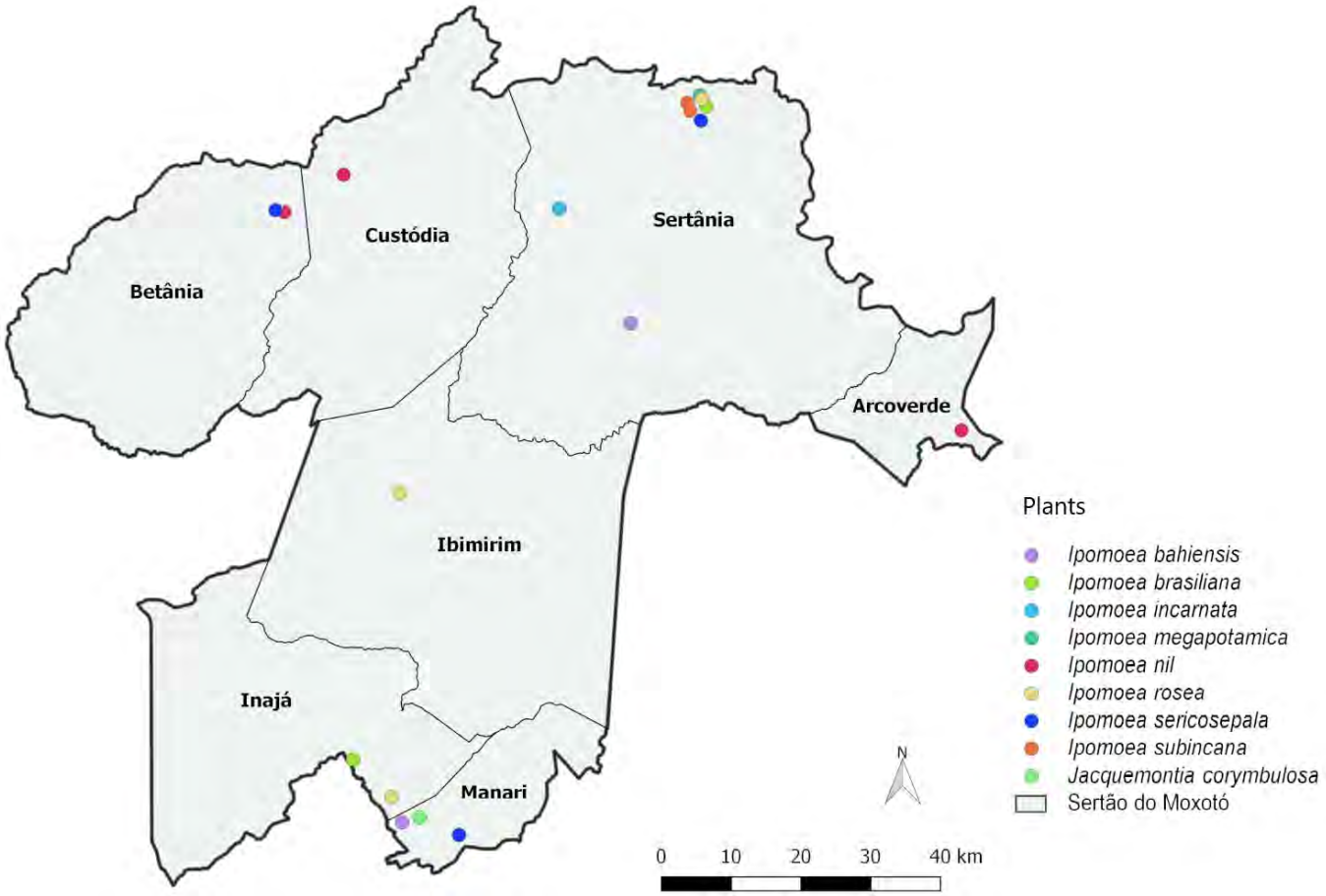


Fig.4. Distribution of the *Ipomoea* species found during the study in Sertão do Moxotó/PE, Brazil. It is observed that some regions have larger variability and predominance of species.

It is important to emphasize these species presented here grows during rainy season and its leaves dry and fall at the beginning of the drought, after the fructification, resprouting again at the beginning of the the rains (Barbosa et al. 2006, Mendonça et al. 2011, 2012). For this reason, it is necessary the study of pluviometric indexes, because high raining's could indicate the most favorable months for poisoning by these plants in ruminants and horses. Thus, considering the average rainfall of 2017 in the Sertão do Moxotó region, the most favorable months to occur outbreaks of lysosomal storage disease in herbivores are from March to July (Fig.3).

Several species of the Convolvulaceae family are non-toxic and are important fodder that should be used as animal feed (Cook et al. 2013), especially in northeastern semiarid region due to the animal food scarcity that frequently occurs in this region. However, several species are toxic to ruminants, horses and wildlife (Cook et al. 2013) and should therefore be studied. Thus, according to the results presented in this study, *I. subincana, I. megapotamica, I. rosea* and *Jacquemontia corymbulosa* presented a significative toxicity potential to herbivores, since they had swainsonine concentrations equal or higher than 0.001% (Molyneux et al. 1995). So far, the toxicity determination of these species still must be proven experimentally. For species containing only calystegines, such as *I. bahiensis, I. incarnata* and *I. nil*, additional studies should be conducted to prove the toxicity of these compounds in ruminants (without simultaneous action of swainsonine). *In vitro*, calystegines are potent inhibitors of hydrolases, but studies in rats have shown negative results on the development of lysosomal storage disease in this species. Another aspect to be considered is that *I. bahiensis, I. incarnata* and *I. nil* may present swainsonine in their composition, similarly to what was observed with *I. sericosepala* and *I. brasiliana* which sometimes presented or not swainsonine concentrations.

Considering our field observations over the past 10 years in the Sertão do Moxotó region about plant poisoning in ruminants, it is important to be noted that, unlike what occurs with *I. carnea* subsp. *fistulosa*, most of the species presented in this study have good palatability and be one of the feeding choices for goats, even when other forages are present. Perhaps because of that, in addition to the wide variety of species that contain swainsonine and calystegines, poisoning by plants containing these alkaloids are so frequent in the northeastern semiarid region, if considered the reported case numbers. Another factor that contributes to the occurrence of poisonings is the social facilitation mechanism, in which animals that start the ingestion of these plants develop the habit of compulsively ingesting them and, by influence, induce other animals of the same species to ingest them (Tokarnia et al. 1960, Driemeier et al. 2000, Colodel et al. 2002, Dantas et al. 2007, Barbosa et al. 2007, Oliveira et al. 2009, Mendonça et al. 2012).

CONCLUSIONS

The discovery of new species of *Ipomoea* and *Jacquemontia* containing toxic polyhydroxy alkaloids reinforces the importance of this group of poisonous plants for ruminants and horses in the Pernambuco semiarid region.

Epidemiological research must be conducted to investigate the occurrence of lysosomal storage disease associated with these new species.

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REFERENCES

Assis T.S., Medeiros R.M.T., Riet-Correa F., Galiza G.J.N., Dantas A.F.M. & Oliveira D.M. 2010. Intoxicações por plantas diagnosticadas em ruminantes e equinos e estimativa das perdas econômicas na Paraíba. Pesq. Vet. Bras. 30(1):13-20. <http://dx.doi.org/10.1590/S0100-736X2010000100003>

Barbosa R.C., Riet-Correa F., Medeiros R.M.T., Lima E.F., Barros S.S., Gimeno E.J., Molyneux R.J. & Gardner D.R. 2006. Intoxication by *Ipomoea sericophylla* and *Ipomoea riedelii* in goats in the state of Paraíba, northestern Brazil. Toxicon 47(4):371-379. <http://dx.doi.org/10.1016/j.toxicon.2005.11.010> <PMid:16488457>

Barbosa R.C., Riet-Correa F., Lima E.F., Medeiros R.M.T., Guedes K.M.R., Gardner D.R., Molyneux R.J. & Melo L.E.H. 2007. Experimental swainsonine poisoning in goats ingesting *Ipomoea sericophylla* and *Ipomoea riedelii* (Convolvulaceae). Pesq. Vet. Bras. 27(10):409-414. <http://dx.doi.org/10.1590/S0100-736X2007001000004>

Bianchinni R.S. & Ferreira P.P.A. 2013. Convolvulaceae. Lista de Espécies da Flora do Brasil. Available at <http://reflora.jbrj.gov.br/jabot/listaBrasil/ConsultaPublicaUC/BemVindoConsultaPublicaConsultar.do> Accessed on Jun. 10, 2013.

Braun K., Romero J., Liddell C. & Creamer R. 2003. Production of swainsonine by fungal endophytes of locoweed. Mycol. Res. 107(Pt 8):980-988. <http://dx.doi.org/10.1017/S095375620300813X> <PMid:14531620>

Buril M.T., Simões A.R., Carine M. & Alves M. 2015. Daustinia, a replacement name for *Austinia* (Convolvulaceae). Phytotaxa J. 197(1):60-60. <http://dx.doi.org/10.11646/phytotaxa.197.1.8>

Burrows G.E. & Tyrl R.J. 2012. Toxic Plants of North America. 2nd ed. Wiley-Blackwell, Ames, Iowa. <http://dx.doi.org/10.1002/9781118413425>.

Colegate S.M., Dorling P.R. & Huxtable C.R. 1979. A spectroscopic investigation of swainsonine: an alpha-mannosidase inhibitor isolated from *Swainsona canescens.* Aust. J. Chem. 32(10):2257-2264. <http://dx.doi.org/10.1071/CH9792257>

Colodel E.M., Driemeier D., Loretti A.P., Gimeno E.J., Traverso S.D., Seitz A.L. & Zlotowski P. 2002. Aspectos clínicos e patológicos da intoxicação por *Sida carpinifolia* (Malvaceae) em caprinos no Rio de Grande do Sul. Pesq. Vet. Bras. 22(2):51-57. <http://dx.doi.org/10.1590/S0100-736X2002000200004>

Cook D., Gardner D.R. & Pfister J.A. 2014. Swainsonine-containing plants and their relationship to endophytic fungi. J. Agric. Food Chem. 62(30):7326-7334. <http://dx.doi.org/10.1021/jf501674r> <PMid:24758700>

Cook D., Ralphs M.H., Welch K.D. & Stegelmeier B.L. 2009. Locoweed poisoning in livestock. Rangelands J. 31(1):16-21. <http://dx.doi.org/10.2111/1551-501X-31.1.16>

Cook D., Beaulieu W.T., Mott I.W., Riet-Correa F., Gardner D.R., Grum D., Pfister J.A., Clay K. & Marcolongo-Pereira C. 2013. Production of the alkaloid swainsonine by a fungal endosymbiont of the ascomycete order chaetothyriales in the host *Ipomoea carnea.* J. Agricult. Food Chem. 61(16):3797-3803. <http://dx.doi.org/10.1021/jf4008423> <PMid:23547913>

Dantas A.F.M., Riet-Correa F., Gardner D.R., Medeiros R.M.T., Barros S.S., Anjos B.L. & Lucena R.B. 2007. Swainsonine-induced lysosomal storage disease in goats caused by the ingestion of *Turbinata cordata* in Northeastern Brazil. Toxicon 49(1):111-116. <http://dx.doi.org/10.1016/j.toxicon.2006.08.012> <PMid:17030054>

Dorling P.R., Huxtable C.R. & Colegate S.M. 1980. Inhibition of lysosomal alpha-mannosidase by swainsonine, an indolizidine alkaloid isolated

from *Swainsona canescens.* Biochem. J. 191(2):649-651. <http://dx.doi.org/10.1042/bj1910649> <PMid:6786280>

Driemeier D., Colodel E.M., Gimeno E.J. & Barros S.S. 2000. Lysossomal storage disease caused by *Sida carpinifolia* poisoning in goats. Vet. Pathol. 37(2):153-159. <http://dx.doi.org/10.1354/vp.37-2-153> <PMid:10714644>

Gardner D.R., Molyneux R.J. & Ralphs M.H. 2001. Analysis of swainsonine: extraction methods, detection and measurement in populations of locoweeds (Oxytropis spp.). J. Agricult. Food Chem. 49(10):4573-4580. <PMID: 11599990>

Hao L., Chen J.P., Lu W., Ma Y., Zhao B.Y. & Wang J.Y. 2012. Isolation and identification of swainsonine-producing fungi found in locoweeds and their rhizosphere soil. Afr. J. Microbiol. Res. 6(23):4959-4969.

Jung J.-K., Lee S.-U., Kozukue N., Levin C.E. & Friedman M. 2011. Distribution of phenolic compounds and antioxidative activies in parts of sweet potato (*Ipomoea batata* L.) plants and in home processed roots. J. Food Comp. Analysis 24(1):29-37. <http://dx.doi.org/10.1016/j.jfca.2010.03.025>

Lima D.D.C.C., Albuquerque R.F., Rocha B.P., Barros M.E.G., Gardner D.R., Medeiros R.M.T., Riet-Correa F. & Mendonça F.S. 2013. Doença de depósito lisossomal induzida pelo consume de *Ipomoea verbascoidea* (Convolvulaceae) em caprinos no semiárido de Pernambuco. Pesq. Vet. Bras. 33(7):867-872. <http://dx.doi.org/10.1590/S0100-736X2013000700007>

Mendonça F.S., Albuquerque R.F., Evêncio-Neto J., Freitas S.H., Dória R.G.S., Boabaid F.M., Driemeier D., Gardner D.R., Riet-Correa F. & Colodel E.M. 2012. Alpha-mannosidosis in goats caused by the swainsonine-containing plant *Ipomoea verbascoidea.* J. Vet. Diagn. Invest. 24(1):90-95. <http://dx.doi.org/10.1177/1040638711425948> <PMid:22362938>

Mendonça F.S., Evêncio-Neto J., de Albuquerque R.F., Driemeir D., Camargo L.M., Dória R.G.S., Boabaid F.M., Caldeira F.H.B. & Colodel E.M. 2011. Spontaneous poisoning by *Ipomoea sericophylla* (Convolvulaceae) in goats at semi-arid region of Pernambuco, Brazil: a case report. Acta Vet. Brno 80(2):235-239. <http://dx.doi.org/10.2754/avb201180020235>

Molyneux R.J., McKenzie R.A., O'Sullivan B.M. & Elbein A.D. 1995. Identification of the glycosidase inhibitors swainsonine and calystegine B2 in Weir vine (*Ipomoea* sp. Q6 [aff. *calobra*]) and correlation with toxicity. J. Nat. Prod. 58(6):878-886. <http://dx.doi.org/10.1021/np50120a009> <PMid:7673932>

Oliveira C.A., Riet-Correa F., Dutra M.D., Cerqueira D.V., Araújo C.V. & Riet-Correa G. 2011. Sinais clínicos, lesões e alterações produtivas e reprodutivas em caprinos intoxicados por *Ipomoea carnea* subsp. *fistulosa* (Convolvulaceae) que deixaram de ingerir a planta. Pesq. Vet. Bras. 31(11):953-960. <http://dx.doi.org/10.1590/S0100-736X2011001100003>

Oliveira C.A., Barbosa J.D., Duarte M.D., Cerqueira V.D., Riet-Correa F., Tortelli F.P. & Riet-Correa G. 2009. Intoxicação por *Ipomoea carnea* subsp. *fistulosa* (Convolvulaceae) em caprinos na Ilha do Marajó, Pará. Pesq. Vet. Bras. 29(7):583-588. <http://dx.doi.org/10.1590/S0100-736X2009000700014>

Oliveira D.G., Prata A.P. & Ferreira R.A. 2013. Herbáceas da Caatinga: composição florística, fitossociologia e estratégias de sobrevivência em uma comunidade vegetal. Agraria. 8(4):623-633. <http://dx.doi.org/10.5039/agraria.v8i4a2682>

Oliveira Júnior C.A., Riet-Correa G. & Riet-Correa F. 2013. Intoxicação por plantas que contêm swainsonina no Brasil. Ciência Rural 43(4):653-661. <http://dx.doi.org/10.1590/S0103-84782013000400014>

Pryor B.M., Creamer R., Shoemaker R.A., McLain-Romero J. & Hambleton S. 2009. Undifilum, a new genus for endophytic *Embellisia oxytropis* and parasitic *Helminthosporium bornmuelleri* on legumes. Botany 87(2):178-194. <http://dx.doi.org/10.1139/B08-130>

Rocha B.P., Reis M.O., Diemeier D., Cook D., Camargo L.M., Riet-Correa F., Evêncio-Neto J. & Mendonça F.S. 2016. Biópsia hepatica como método diagnóstico para intoxicação por plantas que contém swainsonina. Pesq. Vet. Bras. 36(5):373-377. <http://dx.doi.org/10.1590/S0100-736X2016000500003>

Simão-Bianchini R. & Ferreira P.P.A. 2015. *Ipomoea* in Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro. Available at <http://floradobrasil.jbrj.gov.br/jabot/floradobrasil/FB17002> Accessed on Jan. 10, 2018.

Staples G. 2012. Convolvulaceae: the morning glories and bindweeds. Available at <http://convolvulaceae.myspecies.info/node/9> Accessed on Feb. 17, 2018.

Stegelmeier B.L., Molyneux R.J., Asano N., Watson A.A. & Nash R.J. 2008. The comparative pathology of the glycosidase inhibitors swainsonine, castanospermine, and calystegines A3, B2, and C1 in mice. Toxicol. Pathol. 36(5):651-659. <http://dx.doi.org/10.1177/0192623308317420> <PMid:18497426>

Tokarnia C.H., Döbereiner J. & Canella C.F.C. 1960. Estudo experimental sobre a toxidez do "canudo" (*Ipomoea fistula* Mart.) em ruminantes. Arqs Inst. Biol. Animal, Rio de Janeiro, 3:59-71.

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.

Wood J.R.I., Carine M.A., Harris D., Wilkin P., Williams B. & Scotland R.W. 2015. *Ipomoea* (Convolvulaceae) in Bolivia. Kew Bulletin 70(3):30-154. <http://dx.doi.org/10.1007/s12225-015-9592-7>

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Original Article
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Prevalence of mutations responsible for glycogen storage disease type-II and congenital myasthenic syndrome in Brazilian Brahman cattle[1]

Anelize S. Trecenti[2], André V. Fernandes[2], Danilo G.A. Andrade[2], Andreza Pimenta-Oliveira[2], Alexandre S. Borges[2] and José P. Oliveira-Filho[2]*

ABSTRACT.- Trecenti A.S., Fernandes A.V., Andrade D.G.A., Pimenta-Oliveira, A. Borges A.S. & Oliveira-Filho J.P. 2018. **Prevalence of mutations responsible for glycogen storage disease type-II and congenital myasthenic syndrome in Brazilian Brahman cattle.** *Pesquisa Veterinária Brasileira 38(11):2052-2055*. Departamento de Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Rua Prof. Dr. Walter Mauricio Correa s/n, Cx. Postal 560, Botucatu, SP 18618-681, Brazil. E-mail: jose.oliveira-filho@unesp.br

Glycogen storage disease type II (GSD-II) and congenital myasthenic syndrome (CMS) are important autosomal recessive disorders in Brahman cattle. The objective of this study was to investigate the presence of mutations responsible for GSD II (E7, c.1057_1058delTA; and E13, c.1783C>T) and CMS (c.470del20) in purebred Brazilian Brahman cattle and in purebred Brahman bulls that were routinely used in breeding programs in Brazil. A total of 276 purebred Brahman cattle (167 females and 109 males, with ages ranging from 12-24 months) and 35 frozen semen samples taken from purebred Brahman bulls (22 bulls from the USA, 11 Brazilian bulls, one Argentine bull and one Australian bull) were used in this study. Genomic DNA was purified from hair root samples and from semen samples. Purified DNA was used in PCR genotyping to mutations c.1057_1058delTA (E7) and c.1783C>T (E13) in the *GAA* gene and c.470del20 in the *CHRNE* gene. The PCR products were purified and sequenced. The genotypic frequencies per polymorphism were estimated separately. Of the 276 Brahman cattle tested, 7.3% were identified as heterozygous for E7. All Brahman cattle studied were homozygous for the wild-type E13 allele. The E7 mutations was identified as heterozygous in 8.6% (3/35) of the commercial semen samples, whereas the E13 mutations was not identified. The c.470del20 mutation was identified as heterozygous in 0.73% of the hair root samples, but this mutation was not present in any semen sample assessed. No study had previously evaluated the prevalence of mutations responsible for GSD II or CMS in Brazilian Brahman cattle. In summary, the E7 and c.470del20 mutations are present in the Brazilian Brahman herd, and control measures should be adopted to prevent an increase in the incidence of GSD-II and CMS in Brahman cattle in Brazil.

INDEX TERMS: Mutation, glycogen storage disease, congenital myasthenic syndrome, Brazilian Brahman cattle, generalised glycogenosis, Pompe disease, Brazil.

RESUMO.- [**Prevalência de mutações responsáveis pela estocagem de glicogênio tipo II e síndrome miastênica congênita em bovinos brasileiros da raça Brahman.**] A doença de armazenamento de glicogênio tipo II (DAG-II) e a síndrome miastênica congênita (SMC) são importantes doenças autossômicas recessivas no gado Brahman. O objetivo deste estudo foi investigar a presença das mutações responsáveis pela DAG-II (E7, c.1057_1058delTA; e E13, c.1783C>T) e pela SMC (c.470del20) em bovinos da raça Brahman e em touros Brahman que são rotineiramente utilizados em programas de reprodução no Brasil. Um total de 276 amostras de bulbo piloso de bovinos Brahman (167 fêmeas e 109 machos, com idade variando de 12 a 24 meses) e 35 amostras de sêmen congeladas de touros Brahman (22 touros americanos, 11 touros brasileiros, um touro argentino e um touro australiano) foram usados neste estudo. O DNA genômico foi purificado, das amostras de bulbo

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

piloso e de sêmen, e utilizado na genotipagem por PCR das mutações c.1057_1058delTA (E7) e c.1783C>T (E13) no gene GAA e c.470del20 no gene CHRNE. Os produtos de PCR foram purificados e sequenciados. A frequência genotípica para cada polimorfismo foi estimada separadamente. Dos 276 Brahman testados, 7,3% foram identificados como heterozigotos para E7. Todos os Brahman foram homozigotos wild-type para o alelo E13. A mutação E7 foi identificada em homozigose em 8,6% (3/35) das amostras de sêmen comerciais, enquanto que a mutação E13 não foi identificada. A mutação c.470del20 foi identificada em heterozigose em 0,73% das amostras de bulbo piloso, mas esta mutação não estava presente nas amostras de sêmen avaliadas. Nenhum estudo prévio avaliou a prevalência das mutações responsáveis pela DAG-II ou SMC em bovinos Brahman brasileiro. Em suma, as mutações E7 e c.470del20 estão presentes no rebanho Brahman brasileiro, e medidas de controle devem ser adotadas para prevenir o aumento da incidência da DAG-II e SMC em bovinos da raça Brahman no Brasil.

TERMOS DE INDEXAÇÃO: Mutação, glicogênio, síndrome miastênica congênita, bovinos, raça Brahman, glicogenose generalizada, doença de Pompe, Brasil.

INTRODUCTION

Genetic disorders affecting breeds of cattle are relatively common and may be economically important in particular herds (Jolly 2002). Brazil is an important country for cattle breeding for milk and meat, having one of the largest herds in the world. Brazilian livestock production is characterised by large-scale extensive operations under grazing conditions using mainly natural mating or artificial insemination (AI) as reproductive strategies, although the use of in vitro embryo production (IVP) has increased each year for animals of high genetic value (Pimenta-Oliveira et al. 2011). In contrast, there have only been a few studies of genetic diseases in the Brazilian cattle herd.

The glycogen storage disease type II (GSD-II), also known as generalised glycogenosis or Pompe's disease, and congenital myasthenic syndrome (CMS) are important autosomal recessive disorders (Dennis et al. 2002, Thompson et al. 2003a). The GSD-II in Brahman cattle is caused by two independent mutation, c.1057_1058delTA (E7) and c.1783C>T (E13), in the gene coding the acidic α-glucosidase (GAA) enzyme (Dennis et al. 2000). Animals homozygous for any of these two polymorphisms will produce a truncated GAA, resulting in glycogen accumulation in lysosomes. Affected individuals show progressive muscle weakness, problems with motor coordination and abnormal growth (O'Sullivan et al. 1981, Reichmann et al. 1993). Usually, this condition is progressive and animals die before 12 months of age (Zlotowski et al. 2006).

Congenital myasthenic syndrome (CMS) in Brahman cattle is caused by a homozygous 20 base pair deletion (c.470del20) in the gene coding for the epsilon subunit of the acetylcholine receptor (CHRNE) (Kraner et al. 2002). This polymorphism, when homozygous, results in a non-functional allele and the inability to produce functional adult-type nicotinic acetylcholine receptor. This, in turn, results in progressive severe muscle weakness observed either at birth or within the first month of life, which leads to death shortly thereafter (Thompson 1998, Thompson et al. 2003b).

Brahman cattle is a cosmopolitan breed, found in more than 70 countries around the world. In Brazil, the breed was mainly derived from American Brahmans, being officially introduced in Brazil in 1994. Currently, this breed is the second major meat breed of zebuine origin in Brazil. Although the Brazilian Brahman herd is considered the third largest Brahman herd in the world, unlike in other countries (Dennis et al. 2002, Thompson et al. 2003a), no study has previously evaluated the prevalence of mutations responsible for GSD-II or CMS in Brazilian Brahman cattle. Therefore, the objective of this study was to investigate the mutation E7, E13 and c.470del20 in Brazilian Brahman cattle and commercial Brahman semen used in Brazil.

MATERIALS AND METHODS

Ethics statement. All procedures were approved by the Board of Ethics and Animal Experimentation of the institution (Protocol no. 048/2016 - CEUA).

Experimental samples. A total of 276 hair root samples were collected from purebred Brahman cattle (167 females and 109 males, with ages ranging from 12-24 months) from meat farms located in four geopolitical regions of Brazil, North+Midwest (28/276), Southeast (136/276) and Southern (112/276), under a strict confidentiality agreement to ensure the anonymity of establishments, owners and animals. The sampling was randomly performed by ABCZ technicians (Associação Brasileira dos Criadores de Zebu) during the certification of animals by ABCZ in farms. In addition, 35 frozen semen samples taken from purebred Brahman bulls (22 bulls from the USA, 11 Brazilian bulls, one Argentine bull and one Australian bull) and obtained from reproduction companies in Brazil were also used in this study.

DNA purification and genotyping analysis. Genomic DNA was purified from hair root samples using an in-house method and from semen samples using the GenElute™ Genomic Blood DNA Kit (Sigma-Aldrich®) according to the manufacturer's instructions. The DNA obtained was used to genotype the mutations c.1057_1058delTA (E7) and c.1783C>T (E13) in the GAA gene and c.470del20 in the CHRNE gene. Two specific primer pairs were designed with Primer Express software (Applied Biosystems, Grand Island, NY) to genotype the mutations E7 (258-bp) (CGGGATCCTGGACGTGTA, AGTAGGCCCTGGTCATATTCT), and E13 (323-bp) (AGCTATCCGGTCCTTGA, GACCTGGCTCTGGACAAAC) in the *GAA* gene. The specificity of the PCR primers was evaluated in silico with the Basic Local Alignment Search Tool (National Center for Biotechnology Information/USA). A previously described specific primer pair (Thompson et al. 2003b) was used to amplify a 211-bp fragment containing the polymorphism c.470del20 in the *CHRNE* gene. Purified DNA from the hair root and semen samples was used in PCR genotyping. Polymerase chain reactions (25μL) contained 12.5μL of GoTaq Green PCR Master Mix (Promega, Madison, WI), 0.3μM of each forward and reverse primer, 2.5μL of template DNA, and nuclease-free water up to the final volume. The amplification conditions were as follows: initial denaturation at 95°C for 2 minutes; followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 62°C (c.1057_1058delTA and c.1783C>T) or 60°C (c.470del20) for 30 seconds, and extension at 72°C for 1 minute; followed by a final extension at 72°C for 5 minutes. Amplicons were analysed by 1.5% agarose gel electrophoresis, purified, and subjected to direct sequencing.

Sequencing analysis. To sequence the DNA, 10μL of each PCR product, 5μL of the purified forward primer and the BigDye® Terminator Cycle Sequencing Kit were used (Life Technologies™, CA,

USA). The sequences were determined using the ABI 3500 Genetic Analyzer (Life Technologies™ CA, USA). The obtained sequences and the electropherograms were analysed using Sequencher™ 5.1 (Gene Codes, MI, USA). The sequences were compared with the normal *Bos taurus GAA* or *CHRNE* gene sequences using BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Data analysis. The genotypic frequencies per polymorphism were estimated separately. Prevalence estimates provided the basis for calculation of the number of heterozygous and homozygous recessive calves born annually, assuming random mating (Thompson et al. 2003a).

RESULTS

Hair root samples were collected during the year of 2,014 and all animals were apparently healthy, and no clinical signs of GSD-II or CMS were observed in animals during the sampling procedures. Of the 276 Brahman tested, 20 animals (7.3%; 10 females and 10 males) were identified as heterozygous for the E7 polymorphism (E7/N) and 256 (92.7%) were homozygous for the wild-type allele (N/N). The E7 polymorphism was observed as heterozygous in 9.6% (13/136) of the tested animals from the Southeast region, 5.4% (6/112) from the Southern region and 3.6% (1/28) from the North+Midwest regions. All Brahman cattle studied were homozygous for the wild-type allele E13. Two (0.73%) Brahman cattle were identified as heterozygous for the c.470del20 polymorphism, including one female from the Southern region and one male from the Midwest region. The other 274 (99.3%) Brahman cattle were homozygous for the wild-type allele of this polymorphism. No animal was found to be heterozygous for both polymorphism (E7 and c.470del20).

Considering the E7 and c.470del20 mutation frequencies and random mating, may be estimated that 7,034 E7 heterozygous calves and 727 c.470del20 heterozygous calves will be born in every 100,000 births, and 133 E7-affected calves and one c.470del20-affected calf will be born in every 100,000 births in Brazil.

Of the 35 semen samples assessed, three (8.6%) were identified as heterozygous to E7, including two bulls from the USA and one bull that was born in Brazil but was the son of parents imported from the USA. The E13 c.470del20 polymorphism was not identified in the studied bulls.

DISCUSSION

Glycogen storage disease type II and CMS are important autosomal recessive disorders in Brahman cattle herds from Australia (Dennis et al. 2002, Thompson et al. 2003a) and South Africa (Thompson et al. 2003a, 2007), and although the origin of these animals and the Brazilian herd are the same, the prevalence of these diseases in Brazil was unknow. In this study, we used purebred Brahman cattle registered with ABCZ, as the bulls were frequently used in several breeding programmes for beef cattle in Brazil and in other countries.

In the present study, the frequency of animals heterozygous for E7 GSD-II was similar to South Africa (7.3%) (Thompson et al. 2003a); and lower than Australia (12%) (Dennis et al. 2002). Assuming random breeding over the Australian Brahman herd, approximately 400 affected calves will be born for every 100,000 births. Because GSD-II is a fatal disease, the annual loss was predicted in 2002 to be USD $1,035,000 (Dennis et al. 2002), and proportional losses might also occur in Brazil. Unfortunately, these losses may be greater in both Australia and in Brazil because the standard practice of line breeding increases inbreeding, leading to an inevitable increase in the frequency of homozygous recessive alleles (Dennis et al. 2002).

The prevalence of E7 carriers was higher in the Southeast region than in the Southern and North+Midwest regions. Although the southeast region has the highest number of purebred Brahman cattle registered in ABCZ, animals heterozygous for E7 were found in all Brazilian regions studied, indicating that this polymorphism is widespread throughout Brazil. On the other hand, although the E13 GSD-II mutation provokes generalised glycogenosis in Australian Brahman cattle, it is less common and was restricted to descendants of one bull imported from the USA, which had arrived in Australia in 1982 (Dennis et al. 2002). This is probably why the 1783T allele was not detected in Brazilian Brahman cattle in the present study.

In the present study, the two animals identified as c.470del20 carriers were from two farms located in the Southern and Midwest regions of Brazil. The frequency of the c.470del20 carriers in Brazil was similar to South Africa (Thompson et al. 2003a). This low frequency probably does not have a significant economic impact on the Brazilian Brahman herd. However, because c.470del20 carriers are heavier at 600 days of life compared to wild-type animals, there is a selective advantage that tends to increase the frequency of the mutant allele in the population if the genotypes of bulls and/or cows are unknown (Thompson et al. 2007). This human interference may lead to artificial selection resulting in economic losses that may be difficult to compensate (Ciepłoch et al. 2017).

The 24 imported bulls together accounted for 50,000 descendants registered in ABCZ since the introduction of the breed in Brazil (1994-2014). The two American bulls identified as E7 carriers together produced 3,000 registered offspring in the same period. Unfortunately, data about the number of descendants of the 11 Brazilian bulls used in the present study was not available. However, all commercial semen samples assessed in the present study, including the 11 Brazilian bulls, belonged to bulls that were routinely used in breeding programmes in Brazil.

Calves affected by GSD-II usually have progressive muscular weakness and ill-thrift, with a life expectancy of less than 12 months (Reichmann et al. 1993). Only one report of E7 GSD-II was described in a farm located in the Southern region of Brazil (Zlotowski et al. 2005). However, the data of the present study, reinforce the hypothesis that affected animals (E7 GSD-II) are probably being born in Brazil and dying before weaning without having the cause of death correctly diagnosed. Additionally, non-detection of the alleles of the other two polymorphisms (E13 and c.470del20) in the cohort of the assessed bulls may allude to the lower relevance of these two mutations for the Brazilian Brahman herd compared to E7 mutation.

CONCLUSION

The E7 and c.470del20 mutations are present in Brazilian Brahman herd, and control measures, as have been adopted in Australia, should be adopted in Brazil to prevent an increase in the incidence of GSD II and CMS in Brazilian Brahman cattle.

Conflict of interest statement. - The authors declare that there are no conflicts of interest.

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REFERENCES

Ciepłoch A., Rutkowska K., Oprządek J. & Poławska E. 2017. Genetic disorders in beef cattle: a review. Genes Genomics 39(5):461-471. <http://dx.doi.org/10.1007/s13258-017-0525-8> <PMid:28458779>

Dennis J.A., Moran C. & Healy P.J. 2000. The bovine alpha-glucosidase gene: coding region, genomic structure, and mutations that cause bovine generalized glycogenosis. Mammalian Genome 11(3):206-212. <http://dx.doi.org/10.1007/s003350010038> <PMid:10723725>

Dennis J.A., Healy P.J. & Reichmann K.G. 2002. Genotyping Brahman cattle for generalised glycogenosis. Aust. Vet. J. 80(5):286-291. <http://dx.doi.org/10.1111/j.1751-0813.2002.tb10845.x> <PMid:12074310>

Jolly R.D. 2002. Screening for genetic diseases in cattle. Aust. Vet. J. 80(5):284-285. <http://dx.doi.org/10.1111/j.1751-0813.2002.tb10843.x> <PMid:12074309>

Kraner S., Sieb J.P., Thompson P.N. & Steinlein O.K. 2002. Congenital myasthenia in Brahman calves caused by homozygosity for a CHRNE truncating mutation. Neurogenetics 4(2):87-91. <http://dx.doi.org/10.1007/s10048-002-0134-8> <PMid:12481987>

O'Sullivan B.M., Healy P.J., Fraser I.R., Nieper R.E., Whittle R.J. & Sewell C.A. 1981. Generalised glycogenosis in Brahman cattle. Aust. Vet. J. 57(5):227-229. <http://dx.doi.org/10.1111/j.1751-0813.1981.tb02666.x> <PMid:6945845>

Pimenta-Oliveira A., Oliveira-Filho J.P., Dias A. & Gonçalves R.C. 2011. Morbidity-mortality and performance evaluation of Brahman calves from in vitro embryo production. BMC Vet. Res. 7(1):79. <http://dx.doi.org/10.1186/1746-6148-7-79> <PMid:22136315>

Reichmann K.G., Twist J.O. & Thistlethwaite E.J. 1993. Clinical, diagnostic and biochemical features of generalised glycogenosis type II in Brahman cattle. Aust. Vet. J. 70(11):405-408. <http://dx.doi.org/10.1111/j.1751-0813.1993.tb06073.x> <PMid:8280022>

Thompson P.N. 1998. Suspected congenital myasthenia gravis in Brahman calves. Vet. Rec. 143(19):526-529. <http://dx.doi.org/10.1136/vr.143.19.526> <PMid:9839364>

Thompson P.N., Van der Werf J.H., Heesterbeek J.A. & Van Arendonk J.A. 2007. The CHRNE 470del20 mutation causing congenital myasthenic syndrome in South African Brahman cattle: prevalence, origin, and association with performance traits. J. Anim. Sci. 85(3):604-609. <http://dx.doi.org/10.2527/jas.2006-379> <PMid:17121978>

Thompson P.N., Dennis J.A., Mercieca K.N., Harper C.K. & Guthrie A.J. 2003a. Screening for two genetic diseases affecting calf survival in South African and Australian Brahman cattle. 10th International Symposium on Veterinary Epidemiology and Economics, Vina del Mar, Chile, p.358.

Thompson P.N., Steinlein O.K., Harper C.K., Kraner S., Sieb J.P. & Guthrie A.J. 2003b. Congenital myasthenic syndrome of Brahman cattle in South Africa. Vet. Rec. 153(25):779-781. <PMid:14735994>

Zlotowski P., Nakazato L., Dutra V., Barros S.S., Gimeno E.J., Göcks M., Colodel E.M. & Driemeier D. 2005. Inherited glycogenosis in Brahman cattle in Brazil. Pesq. Vet. Bras. 25:210-214. <http://dx.doi.org/10.1590/S0100-736X2005000400004>.

Zlotowski P., Gimeno E.J., Diaz A., Barros R., Barros S.S., Cruz C.E. & Driemeier D. 2006. Lectin-histochemistry: glycogenosis in cattle. Vet. Res. Commun. 30(4):369-377. <http://dx.doi.org/10.1007/s11259-006-3237-0> <PMid:16502105>

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Original Article
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Lung lesions of slaughtered horses in southern Brazil[1]

Matheus V. Bianchi[2], Lauren S. Mello[2], Cíntia De Lorenzo[2], Bruna C. Lopes[2], Gustavo G.M. Snel[2], David Driemeier[2] and Saulo P. Pavarini[2]*

ABSTRACT.- Bianchi M.V., Mello L.S., De Lorenzo C., Lopes B.C., Snel G.G.M., Driemeier D. & Pavarini S.P. 2018. **Lung lesions of slaughtered horses in southern Brazil.** *Pesquisa Veterinária Brasileira 38(11):2056-2064.* 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: saulo.pavarini@ufrgs.br

Respiratory diseases cause significant veterinary costs, reduce performance and require withdrawal of horses. Yet, studies of the causes of pneumonia in horses are scant. This study aimed to describe the pathological and microbiological features of lung lesions in slaughtered horses in southern Brazil. In this study, 84 samples of lungs were examined, and a conclusive diagnosis was obtained in 74 cases. These were composed of bronchopneumonia in 50 cases, followed by granulomatous eosinophilic pneumonia (9/74), recurrent airway obstruction (7/74), lung fibrosis (4/74), lung hemorrhage (3/74) and pulmonary pythiosis (1/74). Bronchopneumonia had grossly firm focally extensive yellow to dark-red areas, which consisted microscopically of multifocal to coalescing infiltrate of degenerate neutrophils. *Streptococcus equi* subsp. *zooepidemicus* was identified in 21 of the 50 cases. Granulomatous eosinophilic pneumonia had multifocal pinpoint firm-hard yellow areas, which microscopically were composed of granulomas with a mineralized center surrounded by collagen fibers and severe infiltrate of eosinophils. Recurrent airway obstruction had mild multifocal pinpoint firm white areas that consisted microscopically of large amounts of mucus inside bronchi and bronchiole. Lung fibrosis had two patterns: focally extensive areas of consolidation and firm nodular areas. Microscopically, the first pattern had interstitial to peribronchial fibrosis, while the second had, in addition to the interstitial fibrosis, a severe pneumocyte hyperplasia and an alveolar infiltrate of neutrophils and macrophages with rare intranuclear inclusion bodies (equine herpesvirus 5, EHV-5). Pulmonary pythiosis presented a focal firm nodular area, with multiple kunkers observed in the cut surface, which corresponded microscopically to areas of necrosis surrounded by a mixed inflammatory infiltrate. At the periphery of the necrotic areas, multiple negatively stained hyphae were observed, which were evidenced through Grocott's stain and immunohistochemistry anti-*Pythium insidiosum*.

INDEX TERMS: Lung lesions, slaughtered horses, Brazil, equine multinodular pulmonary fibrosis, granulomatous eosinophilic pneumonia, pneumonia, pythiosis, recurrent airway obstruction, *Streptococcus equi*, horses, pathology.

RESUMO.- [**Lesões pulmonares de equinos de matadouro-frigorífico no Sul do Brasil.**] Doenças respiratórias causam em equinos custos significativos com tratamento veterinário, redução de performance e descarte de animais. No entanto, estudos que abordem as causas de pneumonia em equinos são escassos. O objetivo deste estudo foi descrever os aspectos patológicos e microbiológicos de lesões pulmonares em equinos abatidos em matadouro-frigorífico no Sul do Brasil. Neste estudo, 84 amostras de pulmões foram examinadas, e o diagnóstico conclusivo das condições foi obtido em 74 casos. Esses foram compostos por broncopneumonia em 50 casos, seguido por pneumonia granulomatosa eosinofílica (9/74), obstrução aérea recorrente (7/74), fibrose pulmonar (4/74), hemorragia pulmonar (3/74) e pitiose pulmonar (1/74). A broncopneumonia era caracterizada macroscopicamente por áreas focalmente extensas firmes de coloração amarelada a

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[2] Setor de Patologia Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Avenida Bento Gonçalves 9090, Agronomia, Porto Alegre, RS 91540-000, Brasil. E-mail: matheusviezzerb@hotmail.com; *Corresponding author: saulo.pavarini@ufrgs.br

vermelho-escuras, as quais consistiam microscopicamente em infiltrado multifocal a coalescente de neutrófilos degenerados. *Streptococcus equi* subsp. *zooepidemicus* foi identificado em 21 dos 50 casos. A pneumonia eosinofílica granulomatosa era caracterizada por áreas multifocais puntiformes firmes a duras e amareladas, que microscopicamente eram compostas por granulomas com área central mineralizada circundados por fibras de colágeno e infiltrado acentuado de eosinófilos. A obstrução aérea recorrente era caracterizada por discretas áreas puntiformes firmes e brancacentas que consistiam microscopicamente em grande quantidade de muco no interior de brônquios e bronquíolos. A fibrose pulmonar exibia dois padrões: áreas de consolidação focalmente extensas e áreas nodulares firmes. Microscopicamente, o primeiro padrão exibia fibrose intersticial a peribronquial, enquanto no segundo padrão havia, além da fibrose intersticial, intensa hiperplasia de pneumócitos e infiltrado alveolar de neutrófilos e macrófagos com raros corpúsculos de inclusão intranucleares (herpesvírus equino 5, EHV-5). A pitiose pulmonar exibia uma área nodular firme focal com múltiplos kunkers ao corte, os quais correspondiam microscopicamente a áreas de necrose circundadas por infiltrado inflamatório misto. À periferia das áreas necróticas, múltiplas imagens negativas de hifas eram observadas, as quais foram evidenciadas através da coloração de Grocott e imuno-histoquímica anti-*Pythium insidiosum*.

TERMOS DE INDEXAÇÃO: Lesões pulmonares, equinos, matadouro-frigorífico, Brasil, fibrose pulmonar multinodular equina, pneumonia eosinofílica granulomatosa, pneumonia, pitiose, obstrução aérea recorrente, *Streptococcus equi*, patologia.

INTRODUCTION

Brazil has a horse population estimated at a total of 5.5 million animals, which encompasses the fourth largest population worldwide after United States, China and Mexico (FAO 2016). Horses in Rio Grande do Sul state compose the second largest population in Brazil, with approximately 537,159 animals (IBGE 2016), and are widely employed for handling cattle herds (Bianchi et al. 2016), for sports (Costa et al. 2014), and occasionally they are slaughtered for meat consumption. Horse meat consumption became popular in Europe after World War II, among people with lower incomes, since cattle beef was scarce (Padalino 2015).

Knowledge of diseases that occur in the equine species is crucial for determining its economic importance as well as the establishment of control procedures. Numerous studies described the causes of death in horses in the United States (USDA 2017), Europe (Baker & Ellis 1981) and Brazil (Pierezan et al. 2009, Pereira et al. 2012, Marcolongo-Pereira et al. 2014). In these studies, the alimentary and locomotor systems were the most commonly affected (Baker & Ellis 1981, Pierezan et al. 2009, Pereira et al. 2012, USDA 2017), while the respiratory system has been indicated as one of the five leading causes of death in horses (Baker & Ellis 1981, Pierezan et al. 2009, Pereira et al. 2012, USDA 2017).

Respiratory diseases cause significant veterinary costs, reduce performance and require the withdrawal of racehorses (Wood et al. 2005, Carvallo et al. 2017). Bacterial pneumonia in foals is mainly caused by *Rhodococcus equi* and *Escherichia coli* (Lakritz et al. 1993), while in adult horses it has been associated to upper respiratory tract normal flora and opportunist pathogens that reach the lower respiratory tract, such as *Streptococcus equi* subsp. *zooepidemicus* and *S. equi* subsp. *equi* (Carvallo et al. 2017). Still, data for the causes of pneumonia in slaughtered horses have not yet been described.

This study aimed to describe the pathological and microbiological features of lung lesions observed in slaughtered horses in the southern Brazil, and to provide and discuss the differential diagnosis of these conditions.

MATERIALS AND METHODS

From January 2014 to December 2016, lung samples from adult horses (more than 5 years-old) submitted to slaughter were received refrigerated by the pathology laboratory for gross and histopathological analysis. Samples were collected at a slaughterhouse, which is located 350 km distant from the pathology laboratory in the municipality of São Gabriel (30°20'11" S; 54°19'12" W), only when the veterinary inspector of the abattoir had a suspicious of pneumonia due the presence of gross lesions, while respiratory clinical signs were not reported by the veterinary inspector. These samples were then sent refrigerated or frozen to the pathology laboratory, where the lung lesions were grossly described.

Bacterial culture was performed when a bacterial etiology was suspected and it consisted in the inoculation of tissue fragments and purulent contents in Blood agar plates (5% sheep blood, Mueller Hinton, Kasvi®, Brazil) and MacConkey agar (Kasvi®, Brazil) followed by aerobic incubation at 37°C for 72 hours. *Streptococcus equi* subsp. *zooepidemicus*, *Streptococcus equi* subsp. *equi* and *S. equi* subsp. *ruminatorum* were identified by Gram staining, microscopic appearance, and by epidemiology, cultural and biochemical characteristics (Table 1) (Fernández et al. 2004, Markey et al. 2013).

Samples were fixed in 10% neutral buffered formalin for 24-48h, trimmed, processed routinely for histopathology and stained by hematoxylin and eosin (HE). Microscopically, when a granulomatous response was observed, the slides were submitted to the Ziehl-Neelsen (ZN) histochemistry exam, in addition to Grocott's methenamine silver (GMS) stain to evidence fungal structures. Cases of recurrent airway obstruction were submitted to alcian blue stain. In the cases where fibrosis was microscopically observed, tissue sections were submitted to Masson's trichrome stain.

Fungal-suspected cases were submitted to the immunohistochemistry anti-*Pythium insidiosum*, according to a protocol previously described (Konradt et al. 2016) with a polyclonal antibody. The reaction was revealed with 3-amino-9-ethylcarbazole (AEC; Biocare Medical, Pacheco/CA, USA), followed by counterstaining with Harris hematoxylin.

RESULTS

From January 2014 to December 2016, 84 samples of lungs (containing 1 to 2 lung lobules) from slaughtered horses were examined. Horses slaughtered in this slaughterhouse were originated from multiple sites in the state and from the neighboring state of Santa Catarina, with a maximum travel distance of 750km. A conclusive diagnosis was obtained in 74 cases, which were mainly composed of suppurative inflammation involving the bronchi, bronchioles and alveolar spaces (bronchopneumonia; 50/74), followed by granulomatous eosinophilic pneumonia (9/74), recurrent airway obstruction (7/74), lung fibrosis (4/74), lung hemorrhage (3/74) and pulmonary pythiosis (1/74).

Suppurative bronchopneumonia

Grossly, the lungs with suppurative inflammation had firm (36/50) focally extensive to multifocal yellow (24/36) to dark-red (17/36) (Fig.1A,B) areas in addition to purulent content within the bronchi (22/36), occasionally extending to the parenchyma (7/36). Microscopically, a moderate to intense multifocal to coalescing inflammatory infiltrate of degenerate neutrophils involving the bronchi (36/50),

Table 1. Biochemical characteristics useful in distinguishing *Streptococcus equi* subsp. *equi* from *S. equi* subsp. *zooepidemicus* and *S. equi* subsp. *ruminatorum*

Test	Strain		
	S. equi subsp. *equi*	*S. equi* subsp. *zooepidemicus*	*S. equi* subsp. *ruminatorum*
CAMP reaction	-	-	+
Hydrolysis of hippurate	-	-	+
Hydrolysis of esculin	-	-	-
Production of acid from:			
Sorbitol	-	+	-
Trehalose	-	-	-
Lactose	-	+	+
Ribose	-	+	+
Maltose	+	+(-)	+
Sucrose	+	+	-

- Negative, + positive; (-) = a few strains are negative.

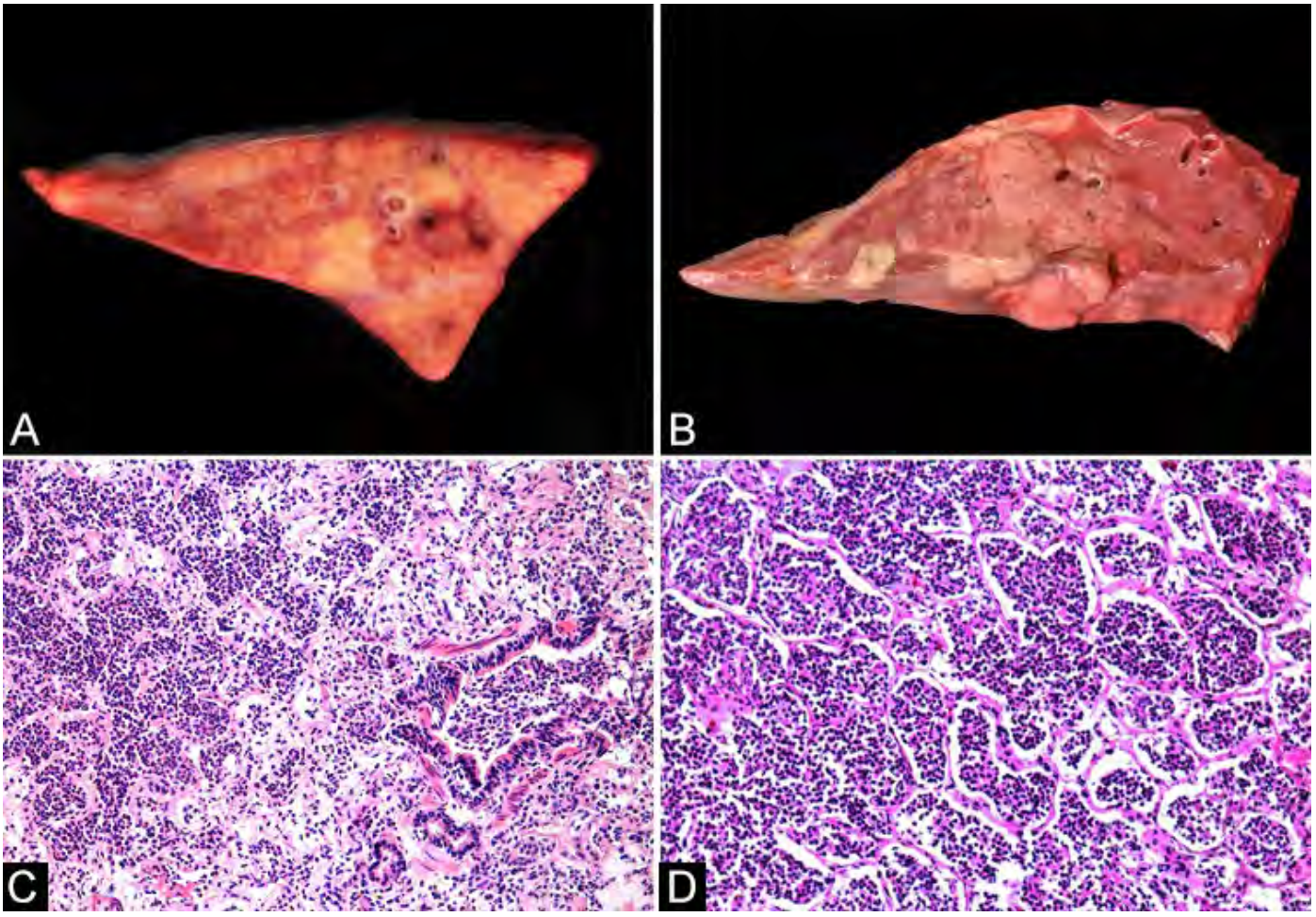


Fig.1. Suppurative inflammation involving the bronchi, bronchioles and alveolar spaces (bronchopneumonia) in slaughtered horses. (**A**-**B**) The lungs had focally extensive to multifocal yellow to dark-red areas in the parenchyma. (**C**) Intense multifocal to coalescent infiltrate of degenerate neutrophils involving the bronchioli and alveolar spaces. HE, obj.10x. (**D**) Severe infiltrate of neutrophils in the alveolar spaces. HE, obj.20x.

bronchioles (48/50) and alveolar spaces (40/50) characterized these lesions (Fig.1C,D), in addition to occasional coccoid bacterial myriad mainly in the bronchi (11/50) and a severe and diffuse alveolar edema (32/50), which rarely involved the lobular septum (7/50). The suppurative infiltrate was often accompanied by fibrin deposition in the alveolar spaces (32/50), occasionally by interstitial fibrosis (11/50) and rarely by areas of intra-alveolar hemorrhage (3/50).

Lung suppurative processes involved a bacterial cause in 26 cases (26/50). Strains showing beta-hemolytic mucoid colonies (1–3 mm in diameter), with gram-positive spherical or ovoid cocci in pairs or chains, that were catalase-negative, with no growth in 6.5% NaCl were identified as *S. equi*. According to the biochemical characteristics available in Table 1, 21 isolates were identified as *Streptococcus equi* subsp. *zooepidemicus*, followed by *S. equi* subsp. *equi* (2/26), and *S. equi* subsp. *ruminatorum* (1/26). The identification of two strains was not possible through cultural and biochemical methods, and, thus, these were identified as ß-hemolytic *Streptococcus* spp.

Granulomatous eosinophilic pneumonia

Granulomatous eosinophilic pneumonia was grossly characterized in all cases by multifocal pinpoint firm to hard yellow areas (0.1-0.3cm in diameter) involving mainly the lung parenchyma and occasionally the pleura (Fig.2A). Microscopically, these pinpoint areas had a central area of dystrophic mineralization surrounded by abundant collagen fibers, occasional epithelioid macrophages and foreign body-type multinucleated giant cells. At the periphery of these, there was a severe inflammatory infiltrate mainly composed of eosinophils, with a few plasma cells and lymphocytes (Fig.2B). The lumen of bronchi and bronchioli was filled by an amorphous amphophilic material intermixed by an intense inflammatory infiltrate of eosinophils. At the ZN and GMS, neither acid-fast bacilli nor fungi were identified. No bacterial agent was obtained through aerobic culture.

Recurrent airway obstruction

Recurrent airway obstruction (RAO) was characterized grossly by non-collapsed areas in the lung, which on the cut surface showed multifocal discrete pinpoint firm white areas with accumulation of mucopurulent content inside bronchi (7/74). Histologically, bronchi and bronchioles contained in the lumen large amounts of an amphophilic amorphous material (mucus) intermixed by a mild inflammatory infiltrate of eosinophils and the mucosa was often hyperplastic with large numbers of goblet cells (metaplasia) (Fig.2C). The smooth muscle of these bronchi and bronchioles was variably hypertrophic. Alcian blue histochemistry exam evidenced the mucus in the

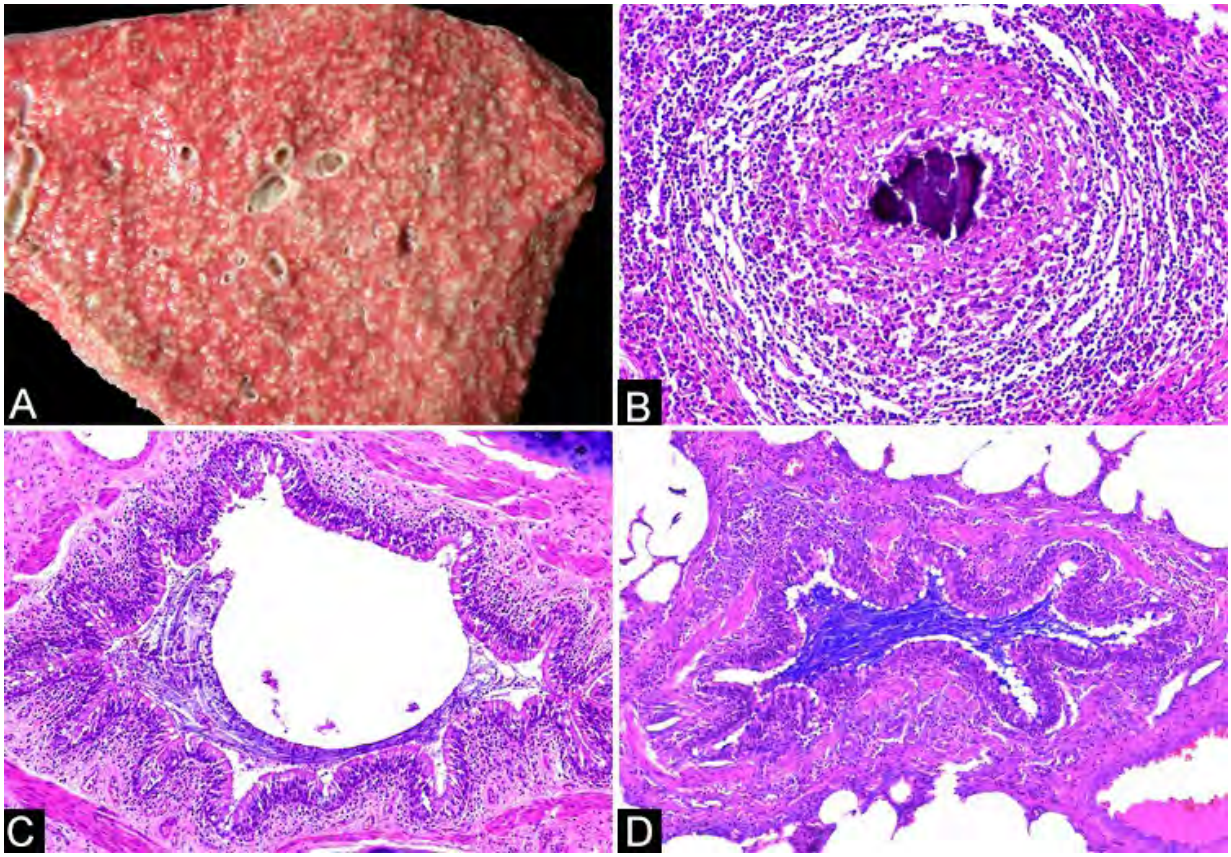


Fig.2. (**A**-**B**) Granulomatous eosinophilic pneumonia and (**C**-**D**) recurrent airway obstruction in slaughtered horses. (**A**) Multifocal pinpoint yellow areas (0.1-0.3cm in diameter) involving the lung parenchyma. (**B**) Microscopically these had a central area of mineralization surrounded by collagen fibers and occasional epithelioid macrophages, and at the periphery large numbers of eosinophils. HE, obj.20x. (**C**) Mucus filled the lumen of bronchi and the mucosa was hyperplasic. HE, obj.10x. (**D**) Mucus in the lumen and increased numbers of goblet cells in the mucosa of this bronchiole were evidenced. Alcian blue stain, obj.10x.

lumen of the bronchi and bronchioli, in addition to the large numbers of Goblet cells in the mucosa (Fig.2D).

Lung fibrosis

Lung fibrosis occurred in two distinct gross and microscopic patterns. The first one involved two cases and was grossly characterized by focally extensive whitish poorly limited areas of lung consolidation. Microscopically, these cases had interstitial to peribronchial fibrosis, in addition to moderate infiltrate of macrophages containing a brown granular pigment in the cytoplasm (hemosiderosis) in the alveolar septum. The second pattern occurred in two cases and was grossly characterized by a firm and whitish nodular area in each case, which measured 8x5x4cm and 4x3x2cm, and, on the cut surface, it was outlined by a red halo from the remaining of the lung parenchyma in one of the cases (Fig.3A). Microscopically, both cases had moderate to severe interstitial fibrosis and a severe type II pneumocyte hyperplasia in the alveolar spaces, which was associated to a moderate inflammatory infiltrate of degenerate neutrophils, macrophages and rare multinucleated giant cells. Multiple basophilic and round intranuclear inclusion bodies were observed in the macrophages in one of these cases (Fig.3B) and, thus, this was compatible with equine multinodular pulmonary fibrosis (EMPF) caused by equine herpesvirus 5 (EHV-5). Masson's trichrome stain evidenced the interstitial fibrosis in all cases.

Lung hemorrhage

Grossly, lungs with hemorrhage were reddened on gross examination, while microscopically there was a moderate to severe diffuse hemorrhage in the alveolar spaces (3/3), occasionally associated to a multifocal infiltrate of macrophages containing granular brownish pigment (hemosiderosis) in the alveolar septum (1/3).

Pulmonary pythiosis

Pulmonary pythiosis corresponded to a focal nodular and firm area in the lung parenchyma, measuring 11x9x5cm. On the cut surface, this nodular area had multifocal to coalescing yellow, irregular, firm, but friable masses (compatible with "kunkers"), which were surrounded by a moderate red halo and outlined by a thick white tissue (capsule) (Fig.3C). Microscopically, these consisted of multifocal to coalescing nodular structures which contained a central amorphous eosinophilic necrotic material surrounded by a marked inflammatory infiltrate of neutrophils, eosinophils, epithelioid macrophages and Langhans-type multinucleated giant cells, in addition to severe fibrous tissue proliferation. At the periphery of the necrotic

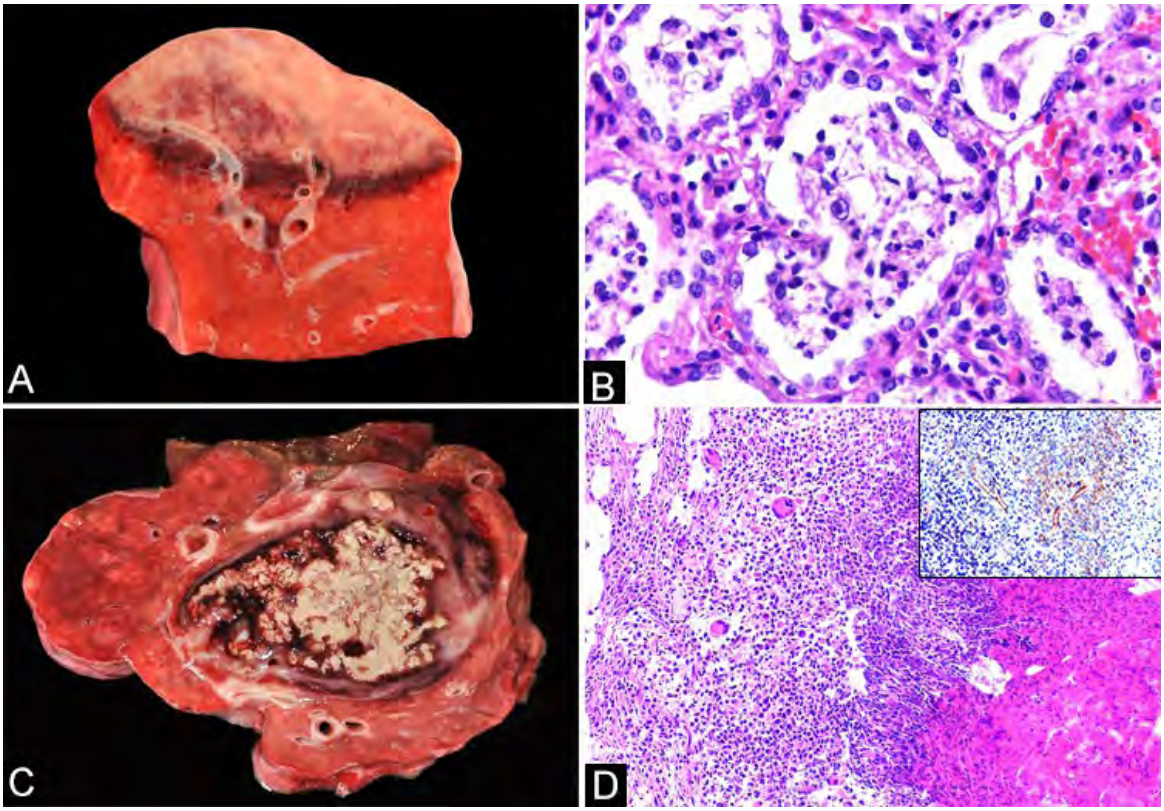


Fig.3. (**A**-**B**) Equine multinodular pulmonary fibrosis (EMPF) and (**C**-**D**) pulmonary pythiosis. (**A**) Focally extensive whitish nodule outlined by a red halo in the lung parenchyma. (**B**) Alveolar spaces had severe pneumocyte hyperplasia associated to infiltrate of degenerate neutrophils and macrophages, often containing intranuclear inclusion bodies (suggestive of EHV-5; arrow). HE, obj.40x. (**C**) Pythiosis corresponded to a focal nodule that on the cut surface had multifocal to coalescing "kunkers". (**D**) Microscopically, these had a central eosinophilic necrosis surrounded by a marked inflammatory infiltrate of neutrophils, eosinophils, epithelioid macrophages and Langhans-type multinucleated giant cells. Negatively stained sections of hyphae were observed at the periphery of the necrotic areas. HE, obj.10x. Inset: marked immunostaining of hyphae within the "kunkers". Immunohistochemistry anti-*Pythium insidiosum*. AEC, obj.40x.

areas, multiple negatively stained transverse and longitudinal sections of hyphae were observed (Fig.3D). Sections of lung stained by GMS evidenced abundant branching, rarely septate and 5-µm-in-diameter irregular hyphae, which were also stained by IHC for *P. insidiosum* (Fig.3D inset).

DISCUSSION

The diagnosis in all cases was obtained by the association of the gross, microscopical, immunohistochemical, histochemical and bacteriological features. Our findings indicated that suppurative bronchopneumonia was the main lung lesion in slaughtered horses. Primary pneumonia in adult horses may happen due to stressful events, such as transportation and long distance travel (Mair & Lane 1989), as we have observed in this study. Stress may predispose lower respiratory tract infection in horses by impairing the pulmonary defense mechanisms (Bayly et al. 1986), and, thereby, it facilitates colonization by *Streptococcus equi* subsp. *zooepidemicus*, which is considered an upper respiratory tract commensal bacterium. Still, it is one of the most frequently isolated bacterial pathogen of young (Lakritz et al. 1993) and adult horses (Bailey & Love 1991). Since horses of the present study were transported for long distances with variable duration of trips, we speculated that these were the main predisposing factors for the lesions observed. *S. equi* subsp*. zooepidemicus* was identified in 21 cases, followed by *S. equi* subsp*. equi* in two cases. When these pathogens are compared, *S. equi* subsp*. zooepidemicus* has a broader tissue tropism (Carvallo et al. 2017), being able to infect, in addition to the lungs, other reproductive organs (Erol et al. 2012). On the other hand, *S. equi* subsp*. equi*, the etiologic agent of strangles, predominantly involves the upper airways and associated lymph nodes with abscess formation, but spread to other organs may occur in "bastard strangles" through blood, lymphatic or aspiration of pus into the lungs (Timoney 2004, Caswell & Williams 2016), with the development of necrotizing pneumonia. In this study, however, both *S. equi* subsp*. zooepidemicus* and *S. equi* subsp*. equi* most likely spread through the airway system, affecting the lower respiratory tract with a suppurative bronchopneumonia. This lesion distribution is consistent with inhalation of aerolised bacteria and has been referred as acentric-acinar, corresponding, grossly, to well-defined areas of dark red consolidation (Oikawa et al. 1995, Racklyeft & Love 2000). Still, further evaluation of the pathogenesis of the condition was compromised by the lack of all lung lobes in the samples analyzed. A bacterial agent was not obtained in 24 cases, yet the gross and microscopical lesions were characteristic of a bacterial cause. However, culture may be unsuccessful during the incubation and early clinical phases of the condition (Timoney 2004).

Fibrosing granulomas involving the lungs were initially described as primary lesions of the liver and peritoneum of horses, and were grossly characterized by numerous firm and gritty nodules (Buergelt & Greiner 1995), similarly to the ones observed in the present study. Likewise, similar gross lesions in the lungs without systemic involvement have been described in horses, being referred as idiopathic eosinophilic pneumonia or disseminated eosinophilic pulmonary granulomas (Uhlhorn et al. 2006, Bell et al. 2008, Magi et al. 2016). Since lesions observed in the present study were both gross and microscopically similar to these studies, the condition was referred as granulomatous eosinophilic pneumonia, which was the second most common condition involving the lungs of slaughtered horses. Microscopically, the granulomas of the present study had large amounts of dense fibrous tissue, epithelioid macrophages and multinucleated giant cells surrounding a necrotic or mineralized center, and at the periphery there was a severe inflammatory infiltrate of eosinophils, corroborating with previous findings (Uhlhorn et al. 2006), but it differed from another description of associated vascular lesions (Magi et al. 2016).

After extensive search for parasitary structures in the lesions, these were absent. Previous studies have also failed to identify the cause of these lesions in the lungs (Buergelt & Greiner 1995, Uhlhorn et al. 2006, Magi et al. 2016). Yet, we believe that these were initially caused by a parasite migration, considering that while the chronic reaction progresses, the dead eggs or larvae may disappear through degeneration and calcification in these lesions (Buergelt & Greiner 1995). Migrating helminths parasites, as *Parascaris equorum* or *Dictyocaulus arnfield,* are possible causes of these lesions (Nicholls et al. 1978, Mackay & Urquhart 1979). Yet, the larvae of *P. equorum* usually undergo pulmonary migration when horses are young (Bell et al. 2008), whilst the horses of the present study were all adults, and the lesions are usually transitory (Nicholls et al. 1978). On the other hand, *Dictyocaulus arnfieldi* causes eosinophilic bronchitis in adult horses (Mackay & & Urquhart 1979), which was not observed in the present study. *Heterobilharzia americana*, a schistosome that causes canine schistosomiasis in the USA, is able to cause a similar granulomatous and eosinophilic pneumonia in llamas and horses, but is mostly an hepatic disease (Buergelt & Greiner 1995, Corapi et al. 2012, 2015). Still, this parasite has not been reported in Brazil, neither have the characteristic eggs and empty eggshells been observed in the center of the granulomas in the present study.

Granulomatous and eosinophilic lesions in the lungs of horses should be differentiated mainly from *Mycobacterium* spp. infections, fungi infections (Buergelt & Greiner 1995), multisystemic eosinophilic epitheliotropic disease (MEED), eosinophilic bronchiolitis and parasitic bronchopneumonia (Bell et al. 2008, Magi et al. 2016). *Mycobacterium* spp. and fungi infections were ruled out in these cases based on the absence of staining through ZN and GMS, as reported previously (Buergelt & Greiner 1995, Uhlhorn et al. 2006, Magi et al. 2016), as well as by the marked eosinophilic infiltrate, which are not typical of these conditions (Uhlhorn et al. 2006). MEED typically affects young horses (Uhlhorn et al. 2006), while the horses of the present study were all adults, and significant involvement of the respiratory tract is uncommon (Pucheu-Haston & Del Piero 2013).

RAO or "heaves" is an inflammatory airway disease of mature horses (Caswell & Williams 2016), formerly known as chronic obstructive pulmonary disease (Buergelt & Del Piero 2014). Similarly to the observed in the present study, the most characteristic gross lesions of this condition in horses are the lack of pulmonary collapse (Buergelt & Del Piero 2014) and the accumulation of mucopurulent secretions throughout the tracheobronchial tree (Pirie 2014). Lesions are usually more severe in the caudodorsal lung (Caswell & Williams 2016); however, since only fragments of lung lobes were evaluated in this study, this focal involvement was not analyzed, as well as

the clinical importance of this lesion may not be extrapolated from these cases. Microscopically, this condition presents the accumulation of mucus and neutrophils in bronchiolar lumens and hyperplasia of goblet cells (Caswell & Williams 2016), which were observed in all horses, in addition to a variable peribronchial and peribronchioli smooth muscle hypertrophy (Caswell & Williams 2016), which may explain the gross pinpoint white firm areas, as previously described (Buergelt & Del Piero 2014).

Interstitial lung fibrosis is a proliferative response to the damage of the alveolar walls and loss of functional alveolar-capillary in horses. A specific cause for this condition may not be recognized (Buergelt et al. 1986), as in three of the four cases in this study. Interstitial lung fibrosis was identified in six horses in a retrospective study in Brazil (Estima-Silva et al. 2017), which were microscopically similar to all the four cases described in the present study. In older horses, this condition may result in interalveolar septal thickening by fibrosis, in addition to fibrosis in the visceral pleura (Buergelt et al. 1986). This pattern of lesion was observed in two of the horses of the present study, while in the other two cases we observed a preservation of the "alveolar-like" architecture with the lumen of the alveoli delineated by type II pneumocyte hyperplasia, which have been described as characteristic of EMPF (Williams et al. 2007). This is a chronic infectious disease caused by EHV-5 in adult horses, which forms numerous coalescing nodules in the lungs (Williams et al. 2007). This condition has been previously described in Brazil in two horses (Panziera et al. 2014, Estima-Silva et al. 2017), and in this study a suggestive diagnosis of EMPF by EHV-5 in one of the cases was obtained by the association of the gross and the microscopic features. Similarly to the previously described changes in a race horse, the multiple intranuclear inclusion bodies associated to the interstitial fibrosis are characteristic of this condition (Williams et al. 2007, Carvallo et al. 2017). The histological features of EMPF are distinct from other causes of fibrosis (Williams et al. 2007), as it was observed in the remaining two cases of interstitial fibrosis, in which a specific cause could not be determined.

Pythiosis is a tropical to subtropical disease caused by *Pythium insidiosum* that mostly affects horses (Mendoza et al. 1996), and lesions are usually cutaneous to subcutaneous (Souto et al. 2016). This condition is considered endemic in the Brazilian Pantanal (Leal et al. 2001) and in the Rio Grande do Sul state (Weiblen et al. 2016). Pulmonary pythiosis has been described in a *Panthera onca* (Camus et al. 2004) and in horses (Goad 1984, Reis Junior et al. 2003, Souto et al. 2016), being mainly associated to the inhalation of the agent in water and/or ingestion with subsequent aspiration of infective material (Goad 1984). It is possible that the lesion observed was associated to a nasal infection, since it has been reported previously in horses (Reis Junior et al. 2003, Vaz et al. 2009, Galiza et al. 2014, Souto et al. 2016), but grossly the origin of this lesion was not determined. In addition to that, some authors have described previous cutaneous lesions that would allow the hematogenous dissemination of the pathogen to internal organs, such as the lungs, through detached hyphal segments of *P. insidiosum* (Reis Junior et al. 2003). This form of dissemination would lead to a multifocal distribution of these lesions (Goad 1984, Reis Junior et al. 2003), and seems less likely considering that the focal nodular was isolated from the remaining parenchyma. Sequestra in the lungs, which commonly develops with masses of necrotic lung parenchyma (Caswell & Williams 2016), has been described in a case of lung pythiosis in a horse (Goad 1984), similarly to the present case, suggesting that the response of the organism may be effective in isolating the lesion. On the other hand, the presence of "kunkers" seems to be a constant finding in horses both in cutaneous, nasal, alimentary and pulmonary lesions (Goad 1984, Reis Junior et al. 2003, Martins et al. 2012, Souto et al. 2016). Microscopical lesions were similar to those observed in nasal and lung lesions (Goad 1984, Souto et al. 2016), but distinct from cutaneous or subcutaneous lesions, in which an eosinophilic dermatitis associated with the formation of granulation tissue predominates (Martins et al. 2012). The gross and microscopic lesions, in addition to the immunohistochemical features observed, were characteristic of the condition, and allowed us to differentiate it from *Conidiobolus* spp. and *Basidiobolus* spp. infections, as previously described (Reis Junior et al. 2003).

CONCLUSIONS

Bacterial bronchopneumonia caused by *Streptococcus equi* subsp. *zooepidemicus* is the main cause of lung lesions in slaughtered horses.

No etiological agent was detected in granulomatous eosinophilic pneumonia cases, although microscopically these were characteristic of parasite induced lesions with a mineralized center surrounded by collagen and large numbers of eosinophils at the periphery.

Recurrent airway obstruction had mild gross lesions, while microscopically it presented an accumulation of mucus and eosinophils in the airways associated to hyperplasia of the epithelium and goblet cells.

Lung interstitial fibrosis was mostly of unknown cause, while in one of the cases the lesion was characteristic of EMPF caused by EHV-5.

Lung pythiosis occurred in one horse and shared similar gross and microscopic features to cutaneous, alimentary and nasal pythiosis. Immunohistochemistry is essential for pythiosis diagnosis in the absence of culture.

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

Animal welfare/ethical statement.- We authors of the article entitled "Lung lesions of slaughtered horses in Southern Brazil" declared, for all due purposes, the project that gave rise to the present data of this manuscript has been submitted for evaluation to the Ethics Committee of the Universidade Federal do Rio Grande do Sul (UFRGS), and has been approved under the number 33501 at December 7th, 2017.

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REFERENCES

Bailey G.D. & Love D.N. 1991. Oral associated bacterial infection in horses: studies on the normal anaerobic flora from the pharyngeal tonsillar surface and its association with lower respiratory tract and paraoral infections.

Vet. Microbiol. 26(4):367-379. <http://dx.doi.org/10.1016/0378-1135(91)90030-J> <PMid:2031304>

Baker J.R. & Ellis C.E. 1981. A survey of post mortem findings in 480 horses 1958 to 1980: causes of death. Equine Vet. J. 13(1):43-46. <http://dx.doi.org/10.1111/j.2042-3306.1981.tb03448.x> <PMid:7238480>

Bayly W.M., Liggit H.D., Huston L.J. & Laegreid W.W. 1986. Stress and its effect on equine pulmonary mucosal defenses. Proc. Am. Assoc. Equine Pract. 32:253-262.

Bell S.A., Drew C.P., Wilson W.D. & Pusterla N. 2008. Idiopathic chronic eosinophilic pneumonia in 7 horses. J. Vet. Intern. Med. 22(3):648-653. <http://dx.doi.org/10.1111/j.1939-1676.2008.0100.x> <PMid:18466259>

Bianchi M.V., Boos G.S., Mello L.S., Vargas T.P., Sonne L., Driemeier D. & Pavarini S.P. 2016. A retrospective evaluation of equine cutaneous lesions diagnosed in Southern Brazil. Acta Scient. Vet. 44:1388.

Buergelt C.D. & Greiner E.C. 1995. Fibrosing granulomas in the equine liver and peritoneum: a retrospective morphologic study. J. Vet. Diagn. Invest. 7(1):102-107. <http://dx.doi.org/10.1177/104063879500700117> <PMid:7779944>

Buergelt C.D. & Del Piero F. 2014. Color Atlas of Equine Pathology. John Wiley and Sons, Ames, p.115-116.

Buergelt C.D., Hines S.A., Cantor G., Stirk A. & Wilson J.H. 1986. A retrospective study of proliferative interstitial lung disease of horses in Florida. Vet. Pathol. 23(6):750-756. <http://dx.doi.org/10.1177/030098588602300614> <PMid:3811140>

Camus A.C., Grooters A.M. & Aquilar R.F. 2004. Granulomatous pneumonia caused by *Pythium insidiosum* in a central American jaguar *Panthera onca.* J. Vet. Diagn. Invest. 16(6):567-571. <http://dx.doi.org/10.1177/104063870401600612> <PMid:15586573>

Carvallo F.R., Uzal F.A., Diab S.S., Hill A.E. & Arthur R.M. 2017. Retrospective study of fatal pneumonia in racehorses. J. Vet. Diagn. Invest. 29(4):450-456. <http://dx.doi.org/10.1177/1040638717717290> <PMid:28681687>

Caswell J.L. & Williams K.J. 2016. Respiratory system, p.465-591. In: Maxie M.G. (Ed.), Jubb, Kennedy and Palmer's Pathology of Domestic Animals. 6th ed. Vol 2. Elsevier Saunders, St Louis. <http://dx.doi.org/10.1016/B978-0-7020-5318-4.00011-5>.

Corapi W.V., Eden K.B., Edwards J.F. & Snowden K.F. 2015. *Heterobilharzia americana* infection and congestive heart failure in a llama (*Lama glama*). Vet. Pathol. 52(3):562-565. <http://dx.doi.org/10.1177/0300985814540541> <PMid:24964922>

Corapi W.V., Snowden K.F., Rodrigues A., Porter B.F., Buote M.A., Birch S.M., Jackson N.D., Eden K.B., Whitley D.B., Mansell J., Edwards J.F., Hardy J. & Chaffin M.K. 2012. Natural *Heterobilharzia americana* infection in horses in Texas. Vet. Pathol. 49(3):552-556. <http://dx.doi.org/10.1177/0300985811432346> <PMid:22273574>

Costa E., Diehl G.N., Santos D.V. & Silva A.P.S. 2014. Panorama da equinocultura no Rio Grande do Sul. Informativo Técnico 5, Departamento de Defesa Agropecuária, Secretaria da Agricultura, Pecuária e Irrigação, Rio Grande do Sul.

Erol E., Locke S.J., Donahoe J.K., Mackin M.A. & Carter C.N. 2012. Beta-hemolytic *Streptococcus* spp. from horses: a retrospective study (2000-2010). J. Vet. Diagn. Invest. 24(1):142-147. <http://dx.doi.org/10.1177/1040638711434138> <PMid:22362945>

Estima-Silva P., Marcolongo-Pereira C., Santos B.L., Coelho A.C.B., Amaral L.A., Lim A., Bolin S.R. & Schild A.L. 2017. Equine multinodular pulmonary fibrosis in southern Brazil: pathology and differential diagnosis. Pesq. Vet. Bras. 37(11):1247-1252. <http://dx.doi.org/10.1590/s0100-736x2017001100009>

FAO 2016. Data of live animals: horses. Food and Agriculture Organization of the United Nations. Available at <http://www.fao.org/faostat/en/#data/QA> Access on Mar. 19, 2018.

Fernández E., Blume V., Garrido P., Collins M.D., Mateos A., Domínguez L. & Fernández-Garayzábal J.F. 2004. *Streptococcus equi* subsp. *ruminatorum* subsp. *nov.*, isolated from mastitis in small ruminants. Int. J. Syst. Evol. Microbiol. 54(6):2291-2296. <http://dx.doi.org/10.1099/ijs.0.63145-0> <PMid:15545473>

Galiza G.J.N., Silva T.M., Caprioli R.A., Barros C.S.L., Irigoyen L.F., Fighera R.A., Lovato M. & Kommers G.D. 2014. Ocorrência de micoses e pitiose em animais domésticos: 230 casos. Pesq. Vet. Bras. 34(3):224-232. http://dx.doi.org/10.1590/S0100-736X2014000300005.

Goad M.E.P. 1984. Pulmonary pythiosis in a horse. Vet. Pathol. 21(2):261-262. <http://dx.doi.org/10.1177/030098588402100224> <PMid:6730214>

IBGE 2016. Tabela 3939: efetivo dos rebanhos por tipo de rebanho. Instituto Brasileiro de Geografia e Estatística, Brasília, DF. Available at <https://sidra.ibge.gov.br/Tabela/3939#resultado> Accessed on Mar. 19, 2018.

Konradt G., Bassuino D.M., Bianchi M.V., Castro L., Caprioli R.A., Pavarini S.P., Santurio J.M., Azevedo M.I., Jesus F.P. & Driemeier D. 2016. Cutaneous pythiosis in calves: An epidemiologic, pathologic, serologic and molecular characterization. Med. Mycol. Case Rep. 14:24-26. <http://dx.doi.org/10.1016/j.mmcr.2016.11.004> <PMid:28050341>

Lakritz J., Wilson W.D., Berry C.R., Schrenzel M.D., Carlson G.P. & Madigan J.E. 1993. Bronchointerstitial pneumonia and respiratory distress in young horses: clinical, clinicopathologic, radiographic, and pathological findings in 23 cases (1984-1989). J. Vet. Intern. Med. 7(5):277-288. <http://dx.doi.org/10.1111/j.1939-1676.1993.tb01020.x> <PMid:8263846>

Leal A.B.M., Leal A.T., Santurio J.M., Kommers G.D. & Catto J.B. 2001. Equine pythiosis in the Brazilian Pantanal region: Clinical and pathological findings of typical and atypical cases. Pesq. Vet. Bras. 21(4):151-156. <http://dx.doi.org/10.1590/S0100-736X2001000400005>

Mackay R.J. & Urquhart K.A. 1979. An outbreak of eosinophilic bronchitis in horses possibly associated with *Dictyocaulus arnfieldi* infection. Equine Vet. J. 11(2):110-112. <http://dx.doi.org/10.1111/j.2042-3306.1979.tb01320.x> <PMid:157874>

Magi G.E., Mari S., Mariotti F. & Rossi G. 2016. Idiopathic eosinophilic pneumonia with associated pulmonary vasculitis in horse: a case series. Vet. Med. Open J. 1(1):18-21. <http://dx.doi.org/10.17140/VMOJ-1-104>

Mair T.S. & Lane J.G. 1989. Pneumonia, lung abscesses and pleuritis in adult horses: a review of 51 cases. Equine Vet. J. 21(3):175-180. <http://dx.doi.org/10.1111/j.2042-3306.1989.tb02135.x> <PMid:2731504>

Marcolongo-Pereira C., Estima-Silva P., Soares M.P., Sallis E.S.V., Grecco F.B., Raffi M.B., Fernandes C.G. & Schild A.L. 2014. Equine diseases in Southern Brazil. Pesq. Vet. Bras. 34(3):205-210. <http://dx.doi.org/10.1590/S0100-736X2014000300002>

Markey B., Leonard F., Archambault M., Cullinane A. & Maguire D. 2013. The streptococci and related cocci, p.121-134. In: Markey B., Leonard F., Archambault M., Cullinane A. & Maguire D. (Eds), Clinical Veterinary Microbiology. 2nd ed. Mosby Elsevier, USA.

Martins T.B., Kommers G.D., Trost M.E., Inkelmann M.A., Fighera R.A. & Schild A.L. 2012. A comparative study of the histopathology and immunohistochemistry of pythiosis in horses, dogs and cattle. J. Comp. Path. 146(2/3):122-131. <http://dx.doi.org/10.1016/j.jcpa.2011.06.006> <PMid:21824626>

Mendoza L., Ajello L. & McGinnis M.R. 1996. Infections caused by the oomycetous pathogen *Pythium insidiosum.* J. Med. Mycol. 6(4):151-164.

Nicholls J.M., Clayton H.M., Pirie H.M. & Duncan J.L. 1978. A pathological study of the lungs of foals infected experimentally with *Parascaris equorum.* J. Comp. Pathol. 88(2):261-274. <http://dx.doi.org/10.1016/0021-9975(78)90030-0> <PMid:659680>

Oikawa M., Takagi S., Anzai R., Yoshikawa H. & Yoshikawa T. 1995. Pathology of equine respiratory disease occurring in association with transport. J. Comp. Pathol. 113(1):29-43. <http://dx.doi.org/10.1016/S0021-9975(05)80066-0> <PMid:7490335>

Padalino B. 2015. Effects of the different transport phases on equine health status, behavior, and welfare: a review. J. Vet. Behav. 10(3):272-282. <http://dx.doi.org/10.1016/j.jveb.2015.02.002>

Panziera W., Giaretta P.R., Galiza G.J.N., Lim A., Bolin S.R., Borges C.H., Fighera R.A. & Barros C.S.L. 2014. Equine multinodular pulmonary fibrosis associated with equine hespesvirus 5 in a horse in Brazil. Braz. J. Vet. Pathol. 7(1):17-20.

Pereira M.C., Ferraz G.C., Ferraudo A.S., Quintelas A.R., Berkman C. & Queiroz-Neto A. 2012. Causes of death of Thoroughbred racehorses at Octavio Dupont Veterinary Hospital, Brazilian Jockey Club, Rio de Janeiro. Pesq. Vet. Bras. 32(3):189-193.

Pierezan F., Rissi D.R., Rech R.R., Fighera R.A., Brum J.S. & Barros C.S.L. 2009. Necropsy findings related to the cause of death in 335 horses: 1968-2007. Pesq. Vet. Bras. 29(3):275-280. <http://dx.doi.org/10.1590/S0100-736X2009000300015>

Pirie R.S. 2014. Recurrent airway obstruction: a review. Equine Vet. J. 46(3):276-288. <http://dx.doi.org/10.1111/evj.12204> <PMid:24164473>

Pucheu-Haston C.M. & Del Piero F. 2013. Equine multi-systemic eosinophilic epitheliotropic disease. Equine Vet. Educ. 25(12):614-617. <http://dx.doi.org/10.1111/eve.12107>

Racklyeft D.J. & Love D.N. 2000. Bacterial infection of the lower respiratory tract in 34 horses. Aust. Vet. J. 78(8):549-559. <http://dx.doi.org/10.1111/j.1751-0813.2000.tb11901.x> <PMid:10979512>

Reis Junior J.L.J., Carvalho E.C.Q., Nogueira R.H.G., Lemos L.S. & Mendoza L. 2003. Disseminated pythiosis in three horses. Vet. Microbiol. 96(3):289-295. <http://dx.doi.org/10.1016/j.vetmic.2003.07.005> <PMid:14559176>

Souto E.P.F., Maia L.A., Olinda R.G., Galiza G.J.N., Kommers G.D., Miranda-Neto E.G., Dantas A.F.M. & Riet-Correa F. 2016. Pythiosis in the nasal cavity of horses. J. Comp. Pathol. 155(2/3):126-129. <http://dx.doi.org/10.1016/j.jcpa.2016.06.005> <PMid:27406311>

Timoney J.F. 2004. The pathogenic equine streptococci. Vet. Res. 35(4):397-409. <http://dx.doi.org/10.1051/vetres:2004025> <PMid:15236673>

Uhlhorn M., Hurst M. & Demmers S. 2006. Disseminated eosinophilic pulmonary granulomas in a pony. Equine Vet. Educ. 18(4):178-181. <http://dx.doi.org/10.1111/j.2042-3292.2006.tb00441.x>

USDA 2017. Equine mortality in the United States, 2015. Veterinary Services: Center for Epidemiology and Animal Health, Animal and Plant Health Inspection Service (APHIS), United States Department of Agriculture. Available at <https://www.aphis.usda.gov/animal_health/nahms/equine/downloads/equine15/Equine15_is_Mortality.pdf> Accessed on Mar. 19, 2018.

Vaz B.B.U., Maia F.C.L., Rocha N.S. & Thomassian A. 2009. Nasal pythiosis in horse. Med. Vet. UFRPE 3(4):27-32.

Weiblen C., Machado G., Jesus F.P.K., Santurio J.M., Zanette R.A., Pereira D.I.B., Diehl G.N., Santos L.C., Corbellini L.G. & Botton S.A. 2016. Seroprevalence of *Pythium insidiosum* infection in equine in Rio Grande do Sul, Brazil. Ciência Rural 46(1):126-131. <http://dx.doi.org/10.1590/0103-8478cr20150056>

Williams K.J., Maes R., Del Piero F., Lim A., Wise A., Bolin D.C., Caswell J., Jackson C., Robinson N.E., Derksen F., Scott M.A., Uhal B.D., Li X., Youssef S.A. & Bolin S.R. 2007. Equine multinodular pulmonary fibrosis: a newly recognized herpesvirus-associated fibrotic lung disease. Vet. Pathol. 44(6):849-862. <http://dx.doi.org/10.1354/vp.44-6-849> <PMid:18039898>

Wood J.L., Newton J.R., Chanter N. & Mumford J.A. 2005. Association between respiratory disease and bacterial and viral infections in British racehorses. J. Clin. Microbiol. 43(1):120-126. <http://dx.doi.org/10.1128/JCM.43.1.120-126.2005> <PMid:15634959>

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Experimental poisoning in broiler chickens by *Senecio vernonioides, Senecio conyzaefolius* and *Senecio paulensis*[1]

Claudia Pies Biffi[2], Thaiza Savaris[3], Leíse Hermann Parizoto[3], Daiane Ogliari[3], Vanessa Boreli[3], Tiffany Emmerich[3], Dale Gardner[4] and Aldo Gava[3]*

ABSTRACT.- Biffi C.P., Savaris T., Parizoto L.H., Ogliari D., Boreli V., Emmerich T., Gardner D. & Gava A. 2018. **Experimental poisoning in broiler chickens by *Senecio vernonioides, Senecio conyzaefolius* and *Senecio paulensis*.** *Pesquisa Veterinária Brasileira 38(11):2065-2069.* Laboratório de Patologia Animal, Centro de Ciências Agroveterinárias, Universidade do Estado de Santa Catarina, Av. Luiz de Camões 2090, Conta Dinheiro, Lages, SC 88520-000, Brazil. E-mail: aldo.gava@udesc.br

Plants of the genus *Senecio* sp. are known to produce hepatic lesions in different animal species, including man. To evaluate the toxicity of three species of *Senecio* found in regions where cattle are bred in the state of Santa Catarina, experiments were conducted on broiler chickens (*Gallus domesticus*). Green leaves of *S. conyzaefolius*, *S. vernonioides* and *S. paulensis* were collected and dried in the shade. After drying, the leaves were ground, mixed into the feed and fed to the chickens divided into 3 groups. Group 1 and Group 2 received single doses of 5g/kg and 20g/kg, respectively. Group 3 was given daily doses of 1g/kg for 20 days (values corresponding to green plant) and Group 4 (control) received free feed of the plant. For each group, five chickens were submitted to necropsy at 30 days and five to 60 days after planting, and five chickens that consumed *S. vernonioides* and *S. conyzaefolius* at the dose of 1g/kg for 20 days were necropsied at 90 days after the beginning of the experiment. Macroscopic lesions were observed in chickens that consumed *S. vernonioides* at a dose of 1 g/kg for 20 days in birds necropsied at 30, 60 and 90 days and were characterized mainly by liver of firm consistency, diminished in size with yellowish coloration and evident lobular pattern, ascites and hydropericardium. In microscopy the main lesions observed were megalocytosis, hepatocyte swelling, fibrosis and biliary hyperplasia and were more intense at the dosage of 1 g/kg for 20 days for *S. vernonioides* and less severe in the chickens that ingested *S. conyzaefolius*. The chickens that received *S. paulensis* showed no macroscopic and microscopic lesions.

INDEX TERMS: Poisonous plants, broiler chickens, poisoning plants, *Senecio conyzaefolius, S. vernonioides*, *S. paulensis,* toxicoses.

RESUMO.- [**Intoxicação experimental em frangos de corte por *Senecio vernonioides, Senecio conyzaefolius* e *Senecio paulensis*.**] As plantas do gênero *Senecio* sp. são conhecidas por produzirem lesões hepáticas em diferentes espécies de animais, inclusive no homem. Para avaliar a toxicidade de três espécies de *Senecio* encontradas em regiões onde são criados bovinos no estado de Santa Catarina, foram conduzidos experimentos em frangos de corte (*Gallus domesticus*). Folhas verdes de *S. conyzaefolius, S. vernonioides* e *S. paulensis* foram coletadas e secadas a sombra. Após a secagem, as folhas foram trituradas, misturadas na ração e fornecidas aos frangos divididos em 3 grupos. O Grupo 1 e o Grupo 2 receberam doses únicas de 5g/kg e 20g/kg, respectivamente. Ao Grupo 3 foram fornecidas doses diárias de 1g/kg por 20 dias (valores correspondentes a planta verde) e o Grupo 4 (Controle) recebeu ração livre da planta. Para cada grupo, cinco frangos

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

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

[4] Poisonous Plant Research Laboratory, ARS, USDA, Logan, Utah, USA.

foram submetidos à necropsia aos 30 dias e cinco aos 60 dias após o início do fornecimento da planta e cinco frangos que consumiram *S. vernonioides* e *S. conyzaefolius* na dosagem de 1g/kg por 20 dias foram necropsiados aos 90 dias após início do experimento. Lesões macroscópicas foram observadas nos frangos que consumiram S. *vernonioides* na dose de 1g/kg por 20 dias nas aves necropsiadas aos 30, 60 e 90 dias e se caracterizaram principalmente por fígado de consistência firme, diminuído de tamanho com coloração amarelada e padrão lobular evidente, ascite e hidropericárdio. Na microscopia as principais lesões observadas foram megalocitose, tumefação de hepatócitos, fibrose e hiperplasia biliar e foram mais intensas na dosagem de 1g/kg por 20 dias para *S. vernonioides* e menos graves nos frangos que ingeriram *S. conyzaefolius*. Os frangos que receberam *S. paulensis* não mostraram lesões macroscópicas e microscópicas.

TERMOS DE INDEXAÇÃO: Plantas tóxicas, frangos de corte, intoxicação por plantas, *Senecio conyzaefolius, S. vernonioides*, *S. paulensis,* toxicoses.

INTRODUCTION

Plants of the genus *Senecio* are responsible for the death of cattle in Brazil, mainly in the Southern region (Riet-Correa & Medeiros 2001). However, since they are not very palatable plants, they are only consumed when cattle undergo periods of food restriction, whether due to drought, winter or prolonged transport (Riet-Correa et al. 1993, Pessoa et al. 2013). Another form of plant ingestion is its presence in silages, or, hay (Méndez et al. 1987, Barros et al. 2007). In the State of Rio Grande do Sul, the main species involved in cases of natural and experimental poisoning in production animals include: *Senecio brasiliensis* (Barros et al. 1987b, Driemeier et al. 1991, Barros et al. 1992, 2007, Karam et al. 2004), *S. madagascariensis* (Cruz et al. 2010), *S. oxyphyllus, S. cisplatinus, S. leptolobus, S. heterotrichius, S. selloi, S. tweediei* (Méndez 1993). In the State of Santa Catarina, although several *Senecio* species occur, only *Senecio brasieliensis* poisoning outbreaks have been reported.

Experimentally, broilers are sensitive to pyrrolizidine alkaloids (PAs) present in plants of the genus *Senecio* and develop lesions similar to those found in cattle (Cheeke & Pierson-Goeger 1983, Mendéz et al. 1987, 1990, Huan et al. 1992), and for being of low economic value, they are used as experimental model.

Due to the high availability of *Senecio vernonioides, S. conyzaefolius* and *S. paulensis* in the Highland and Midwestern regions of the State of Santa Catarina, and due to the lack of reports of poisoning in animals with these species, this study aims to verify the effect of the plant on broilers and its toxic potential for cattle.

MATERIALS AND METHODS

During the months from June to August 2014, *Senecio vernonioides* (Água Doce/SC), *S. conyzaefolius* (Lages/SC) and *S. paulensis* green leaves (Matos Costa/SC) were collected. After collection, leaves were dried in the shade and later crushed in a grinder. For each *Senecio* species, 100 grams were separated and dried separately for weight loss evaluation after drying. Values corresponding to the weight loss of each species are described in Table 1. The doses used in the experiment correspond to the green plant.

Table 1. *Senecio* species, group and doses corresponding to green plant and dry plant respectively

Plant used	Group	Dose Green plant (g/kg)	Dose corresponding to dry plant (g/kg)
Senecio vernonioides	1	5 s.d.	2 s.d.
	2	20 s.d.	8 s.d.
	3	1/ 20 days	0.4/20 days
Senecio conyzaefolius	1	5 s.d.	1.75 s.d..
	2	20 s.d.	7 s.d.
	3	1/ 20 days	0.35/20 days
Senecio paulensis	1	5 s.d.	1.75 s.d.
	2	20 s.d.	7 s.d.
	3	1/ 20 days	0.35/20 days

s.d. = Single dose.

For the experiment, 110 one-day-old chicks of the Coob lineage were housed in experimental aviary located in the Poultry Sector of the Agroveterinary Sciences Center of the State University of Santa Catarina (CAV-UDESC). Birds were kept in boxes where they received feed and water ad libitum. For the *S. vernonioides* and *S. conyzaefolius* species, groups of 35 birds were subdivided into lots of 10 chickens for single doses of 5g/kg/LW and 20g/kg/LW and of 15 birds for the dose of 1g/kg/LW for twenty days. For the *S. paulensis* species, 10 birds were used for each dose evaluated. In total, 100 chickens received *Senecio* in the feed plus control group with 10 birds.

From the 12 days of age, the groups of birds received the different types of *Senecio* mixed in the feed, except for the Control group that received only feed. The formation of groups, as well as the *Senecio* species tested and their respective doses are shown in Table 1.

During the experiment, birds were daily inspected and behavior and feed consumption were evaluated. Weekly, birds from Groups 1, 2 and Control were weighted. For Group 3, weighing was performed twice a week, during the first 20 days to readjust the daily *Senecio* dose to the increasing weight of birds.

For euthanasia, the cervical dislocation method, approved by Normative 1000 of the Federal Veterinary Medicine Council (CFMV) and by the ethics committee of animal experimentation of CAV-UDESC (Protocol 01.07.14) was used. All birds were necropsied to observe lesions and central nervous system, heart, kidney and liver samples were collected, which were fixed in 10% buffered formalin, routinely processed and stained with hematoxylin and eosin (HE). Selected sections were stained by Masson's trichomic histochemistry technique for the detection of fibrous connective tissue. Material processing and histological analysis were performed at the Laboratory of Animal Pathology of CAV/UDESC.

Samples of collected plants were sent to the Department of Botany of the State University of Santa Catarina for identification and classification. Dry plants were sent to USDA, ARS, Poisonous Plant Research Laboratory, Logan, Utah, USA, for analysis and quantification of pyrrolizidine alkaloid doses.

RESULTS

Analysis of APs of *Senecio* spp. samples used in this study revealed the presence of toxic alkaloids dehydrate pyrrolizidine ester (DHPA) and their quantification was performed with the dry weight of samples. *Senecio vernonioides* presented dose of 19,599µg/g, *S. conyzaefolius* 2,500µg/g and *S. paulensis*

157µg/g. For the three *Senecio* species tested, no clinical changes were observed.

For each group formed, five chickens were submitted to necropsy at 30 days and five at 60 days after the onset of plant consumption, five chickens that consumed *Senecio vernonioides* and *S. conyzaefolius* at the dose of 1g/kg for 20 days were also necropsied at 90 days after the start of the experiment. Necropsy showed lesions only in *S. vernonioides* poisoning, which are described in Table 2. The main macroscopic alterations occurred in the liver and were characterized by decreased size, firm consistency and whitish striations and/or reddish spots on the surface (Fig.1).

Microscopically, hepatic lesions were observed that varied from mild to severe in birds that received *S. vernonioides* (Table 3) and were demonstrated in Figure 2 and mild or absent in birds that received *S. conyzaefolius* (Table 4). In birds that received *S. paulensis*, no significant histological lesions were observed.

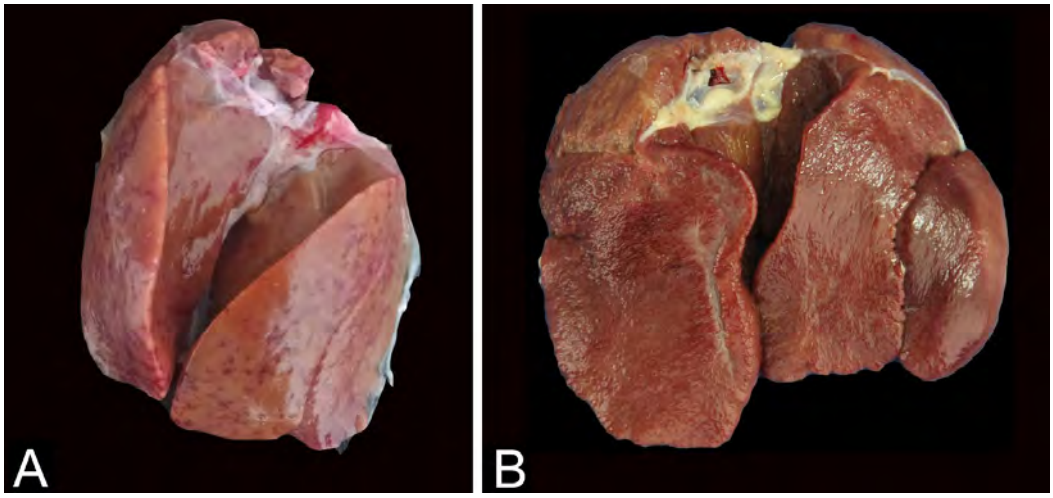


Fig.1. Livers of chicken from Group 3 (1g/kg for 20 days) necropsied at (**A**) 60 days and (**B**) 90 days after onset of the consumption of *Senecio vernonioides*. (**A**) Liver reduced in size and with reddish spots and (**B**) with whitish striations.

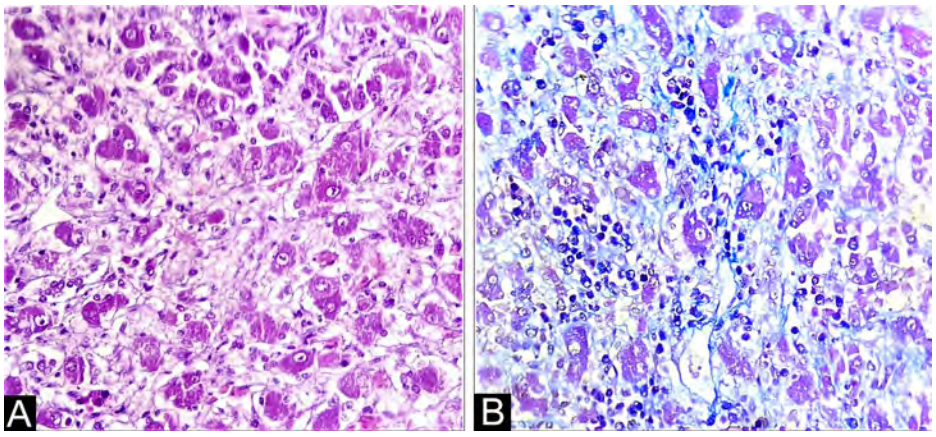


Fig.2. Histology of the liver of one of chickens from Group 3 (1g/kg for 20 days) necropsied 90 days after the onset of consumption of *Senecio vernonioides*. (**A**) Megalocytosis and fibrosis, which dissect the hepatocyte cords. HE, obj.40x. (**B**) Fibrous connective tissue. Masson's trichomic histochemistry staining, obj.40x.

Table 2. Macroscopic lesions found in chickens that received *Senecio vernonioides* at dose of 1g/kg for 20 days and sacrificed at 30, 60 and 90 days after consumption

Lesion	30 days	60 days	90 days
Ascite	1/5	1/5	1/5
Hydropericardium	1/5	3/5	3/5
Gallbladder with increased volume	-	1/5	-
Hepatic atrophy	2/5	3/5	5/5
Liver with evident lobular pattern	1/5	2/5	5/5
Yellowish liver	1/5	2/5	0/5

Table 3. Microscopic lesions observed in birds consuming *Senecio vernonioides* at single doses of 5g/kg (Group 1), 20g/kg (Group 2) and continuous dose of 1g/kg for 20 days (Group 3) at 30, 60 and 90 days after onset of plant consumption

Lesion	Group1		Group 2		Group 3		
	30 days	60 days	30 days	60 days	30 days	60 days	90 days
Hepatocyte swelling	+	+	+	-	++	++	+++
Megalocytosis	-	-	-	-	+	++	++
Fibrosis	-	+	-	-	+	+	++
Vacuolation hepatocytes	-	-	-	-	-	+	+
Biliary proliferation	-	-	-	-	-	+	+

+ Mild, ++ moderate, +++ severe, - absent.

Table 4. Microscopic lesions observed in birds consuming *Senecio conyzaefolius* at single doses of 5g/kg (Group 1), 20g/kg (Group 2) and continuous dose of 1g/kg for 20 days (Group 3) at 30, 60 and 90 days after onset of plant consumption

Lesion	Group 1		Group 2		Group 3		
	30 days	60 days	30 days	60 days	30 days	60 days	90 days
Hepatocyte swelling	+	+	+	+	+	+	+
Megalocytosis	+	+	+	+	+	+	+
Fibrosis	-	-	-	-	-	+	+
Vacuolation hepatocytes	+	+	+	+	+	+	+
Biliary proliferation	-	-	-	-	-	-	-

DISCUSSION

Macroscopic lesions characterized by ascites, hydropericardium, yellowish liver, firm consistency, evident lobular pattern and decreased size were observed at necropsy of chickens that received *Senecio vernonioides* at dose of 1g/kg for 20 days and are compatible with those reported by Méndez et al. (1990) in experimental poisoning with *S. brasiliensis, Senecio heterotrichius* and *S. cisplatinus*. In addition to birds, these lesions are also described in cattle (Tokarnia & Döbereiner 1984, Méndez et al. 1987, Barros et al. 1989), horses (Gava & Barros 1997, Pilati & Barros 2007) and sheep (Ilha et al. 2001, Schild et al. 2007, Grecco et al. 2011). The occurrence of lesions in other organs such as CNS (central nervous system) in cattle (Tokarnia & Döbereiner 1984, Méndez et al. 1987, 1990, Barros et al. 1987a), horses (Gava & Barros 1997, Pilati & Barros 2007) and sheep (Ilha et al. 2001, Grecco et al. 2011) was not observed in this study. These data are in agreement with Cheeke & Pierson-Goeger (1983) and Méndez et al. (1990).

S. conyzaefolius and S. *paulensis* species did not develop macroscopic lesions at the doss and consumption periods evaluated. This may be related to the time chickens consumed the plant and the PA concentration. In experiments performed by Méndez et al. (1990) with other *Senecio* species, birds received the plant for a longer period of time (60 consecutive days) and according to Tokarnia & Döbereiner (1984), lesions caused by PAs are progressive. Another factor involved could be the variation in the number of PAs present in these species when compared to *S. vernonioides*, which presented DPHA dose of 19,599µg/g, with *S. conyzaefolius* presenting 2,500µg/g and *S. paulensis* 157µg/g. Stigger et al. (2014) observed macroscopic lesions in bovines naturally poisoned by *S. madagascariensis* with PA doses of 500µg/g, lower than that found in *S. conyzaefolius*. The appearance of these lesions could be related to the period and amount of plant consumed, because in that case, animals were food restricted.

Lesions were also experimentally observed in birds and cattle that consumed different *Senecio* species with PA doses ranging from 50 to 3,100µg/g (Méndez et al. 1990). A small individual variation in the intensity of lesions within the same *Senecio* species evaluated was observed. This demonstrates the individual susceptibility to PAs (Hooper 1978). The administration of single and high doses is responsible for causing acute manifestations of the disease, with clinical signs and death of cattle (Tokarnia & Döbereiner 1984, Méndez et al. 1987, Riet-Correa et al. 2007). In this experiment, it was not possible to observe this result. A similar description is made by Tokarnia et al. (1990), with single *S. desiderabilis* doses of 20 and 40g/kg also failed to reproduce the acute disease, but with smaller and continuous doses, two cattle died. However, acute manifestation was experimentally demonstrated by Tokarnia & Döbereiner (1984) with a single *S. brasiliensis* dose of 20g/kg to a bovine that developed clinical symptoms and death 20 days after ingestion. Pilati & Barros (2007) also provided a single *S. brasiliensis* dose of 15g/kg to an equine that demonstrated neurological symptoms. Grecco et al. (2012) provided S. *brasiliensis* at a single dose of 60g/kg to sheep that showed no clinical signs or microscopic changes, demonstrating the resistance of this species to the single dose of the plant.

Regarding the dosages tested, the most evident lesions were at dose of 1g/kg for 20 consecutive days. Small doses over an extended period produced more significant lesions than high and single doses. This result corroborates Tokarnia & Döbereiner (1984) and Riet-Correa et al. (2007), who described lesions caused by PAs as progressive and irreversible. This progressive effect described by the authors may explain the lack of manifestations of clinical signs and death. However, the lesions found indicate liver function impairment, suggesting that if these animals remained alive, they could develop more serious lesions with the appearance of clinical signs and mortality. Histological lesions ranged from mild to moderate megalocytosis, fibrosis, biliary proliferation and hepatocyte swelling, and lesions were most evident in necropsied birds at 60 and 90 days after plant consumption, corroborating Tokarnia & Döbereiner (1984) and Riet Correa et al. (2007). Méndez et al. (1990) describe, megalocytosis lesions,

disorganization of the hepatic parenchyma, proliferation of biliary epithelium, necrosis for birds that consumed the plant for a period of 60 days and proliferation of connective tissue into the parenchyma from Glisson's capsule for birds experimentally poisoned by *S. heterotrichius*.

Regarding the single doses administered, the dose of 20g/kg was more toxic than the dose of 5g/kg, but this difference was not significant, as well as variations in lesion intensity among *Senecio* species. However, lesions were more significant in the necropsied chickens 60 days after plant consumption, which again proves the relationship between the period after plant consumption and the development of lesions (Tokarnia & Döbereiner 1984, Riet-Correa et al. 2007).

S. vernonioides and *S. conyzaefolius* species are possible sources of poisoning for cattle, as they have demonstrated toxic effect on broilers and are present in the in the Highland and Midwestern regions of the State of Santa Catarina.

CONCLUSIONS

Senecio vernonioides and *S. conyzaefolius* were toxic to chickens and morphological lesions varied according to the dose tested and PA concentration.

Senecio paulensis did not demonstrate toxicity to broilers at the doses tested.

REFERENCES

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

Barros C.L.S., Metzdorf L.L. & Peixoto P.V. 1987b. Ocorrência de surtos de intoxicação por *Senecio* spp. (Compositae) em bovinos no Rio Grande do Sul. Pesq. Vet. Bras. 7(1):101-107.

Barros C.L.S., Metzdorf L.L., Santos M.N., Barros S.S. & Peixoto P.V. 1989. Intoxicação experimental por *Senecio brasiliensis* (compositae) em ovinos. Pesq. Vet. Bras. 9(3/4):55-67.

Barros C.S.L., Driemeier D., Pilati C., Barros S.S. & Castilhos L.M. 1992. *Senecio* spp. poisoning in cattle in southern Brazil. Vet. Human Toxicol. 34(3):241-246. <PMid:1609498>

Barros C.S.L., Castilhos L.M.L., Rissi D.R., Kommers G.D. & Rech R.R. 2007. Biópsia hepática no diagnóstico da intoxicação por *Senecio brasiliensis* (Asteraceae) em bovinos. Pesq. Vet. Bras. 27(1):53-60. <http://dx.doi.org/10.1590/S0100-736X2007000100010>

Cheeke P.R. & Pierson-Goeger M.L. 1983. Comparative toxicity and metabolism of pyrrolizidine alcaloids in various laboratory animals and avian species. Toxicol. Let. 18(3):343-349. <http://dx.doi.org/10.1016/0378-4274(83)90116-9> <PMid:6665808>

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. 10:23-30.

Gava A. & Barros C.S.L. 1997. *Senecio* spp. poisoning in horses in southern Brazil. Pesq. Vet. Bras. 17(1):36-40. <http://dx.doi.org/10.1590/S0100-736X1997000100006>

Grecco F.B., Estima-Silva P., Marcolongo-Pereira C., Soares M.P., Collares G. & Schild A.L. 2011. Seneciose crônica em ovinos no sul do Rio Grande do Sul. Pesq. Vet. Bras. 31(4):326-330. <http://dx.doi.org/10.1590/S0100-736X2011000400009>

Grecco F.B., Estima-Silva P., Marcolongo-Pereira C., Soares M.P., Raffi M.B., Sallis E.S.V. & Schild A.L. 2012. Intoxicação experimental aguda por *Senecio brasiliensis* em ovinos e indução de resistência à intoxicação. Pesq. Vet. Bras. 32(9):912-916. <http://dx.doi.org/10.1590/S0100-736X2012000900015>

Hooper P.T. 1978. Pyrrolizidine alkaloid poisoning-pathology with particular reference to differences in animal and plant species, p.162-176. In: Keeler R.F., Van Kampen K.R. & James L.F. (Eds), Effects of Poisonous Plants on Livestock. Academic Press, New York. <http://dx.doi.org/10.1016/B978-0-12-403250-7.50022-4>.

Huan J., Cheeke P.R., Lowry R.R., Nakaue H.S., Snyder S.P. & Whanger P.D. 1992. Dietary pyrrolizidine *(Senecio)* alkaloids and tissue distribution of copper and vitamin A in broiler chickens. Toxicol. Letters 62(2/3):139-153. <http://dx.doi.org/10.1016/0378-4274(92)90017-E> <PMid:1412500>

Ilha M.R., Loretti A.P., Barros S.S. & Barros C.L.S. 2001. Intoxicação espontânea por *Senecio brasiliensis* (Asteraceae) em ovinos no Rio Grande do Sul. Pesq. Vet. Bras. 21(3):123-138. <http://dx.doi.org/10.1590/S0100-736X2001000300005>

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. 1993. Intoxicação por *Senecio* spp., p.43-57. In: Riet-Correa F., Méndez M.C. & Schild A.L. (Eds), Intoxicações por Plantas e Micotoxicoses em Animais Domésticos. Agropecuaria Hemisferio Sur, Montevideo.

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. & Mart W. 1990. Intoxicação experimental por cinco espécies de *Senecio* em bovinos e aves. Pesq. Vet. Bras. 10(3/4):63-69.

Pessoa C.R.M., Medeiros R.M.T. & Riet-Correa F. 2013. Importância econômica, epidemiologia e controle das intoxicações por plantas no Brasil. Pesq. Vet. Bras. 33(6):752-758. <http://dx.doi.org/10.1590/S0100-736X2013000600011>

Pilati C. & Barros C.S.L. 2007. Intoxicação experimental por *Senecio brasiliensis* (Compositae) em equinos. Pesq. Vet. Bras. 27(7):287-296. <http://dx.doi.org/10.1590/S0100-736X2007000700006>

Riet-Correa F. & Medeiros R.M.T. 2001. Intoxicação por plantas em ruminantes no Brasil e no Uruguai: importância econômica, controle e riscos para a saúde pública. Pesq. Vet. Bras. 21(1):38-42. <http://dx.doi.org/10.1590/S0100-736X2001000100008>

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

Riet-Correa F., Schild A.L., Lemos R.A.A. & Borges J.R. 2007. Doenças de Ruminantes e Equinos. 3ª ed. Palotti, Santa Maria, p.106-112.

Schild A.L., Ferreira J.L., Ladeira S.R.L., Soares M.P., Grecco F.B. & Guim T.N. 2007. Doenças diagnosticadas pelo Laboratório regional de Diagnóstico no ano 2006. Boletim do Laboratório Regional de Diagnóstico, Ed. Universitária, Pelotas, RS. 56p.

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.

Tokarnia C.H., Gava A., Peixoto P.V., Stolf L., Consorte L.B. & Dobereiner J. 1990. Intoxicação experimental por *Senecio desiderabilis* (Compositae) em bovinos. Pesq. Vet. Bras. 10:35-42.

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Original Article
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Biochemical responses, blood gas, oxidative stress and lipid peroxidation of goats transfused with fresh or stored whole homologous blood[1]

Nayanna B.S. Fonseca[2]*, Jucélio S. Gameleira[2], Jerson M. Cavalcante[2], Francisco L.C. Oliveira[3], Clara S. Mori[3], Rejane S. Sousa[3], Antonio H.H. Minervino[4] and Raimundo A. Barrêto-Júnior[2]

ABSTRACT.- Fonseca N.B.S., Gameleira J.S., Cavalcante J.M., Oliveira F.L.C., Mori C.S., Sousa R.S., Minervino A.H.H. & Barrêto-Júnior R.A. 2018. **Biochemical responses, blood gas, oxidative stress and lipid peroxidation of goats transfused with fresh or stored whole homologous blood.** *Pesquisa Veterinária Brasileira 38(11):2070-2079*. Laboratório de Medicina Interna Veterinária, Universidade Federal Rural do Semi-Árido, Av. Francisco Mota 572, Costa e Silva, Mossoró, RN 59625-900, Brazil. E-mail: nayanna_brunna@yahoo.com.br

Blood transfusion is a therapeutic procedure of great importance for veterinary medicine, in spite of only few studies in the literature on hemotherapy in goats. We aimed to evaluate the biochemical, blood gas, oxidative stress and lipid peroxidation of goats submitted to homologous transfusion of fresh whole blood or stored for 15 and 35 days. Eighteen adult male goats were submitted to a single phlebotomy to remove 30% of the blood volume, and we transfused 20mL/kg of whole blood stored in CPDA-1 bags according to the experimental group, being: G0 composed goats who received fresh blood, G15 and G35 goats that received blood stored for 15 and 35 days, respectively. For the biochemical evaluation, blood gas, oxidative stress and lipid peroxidation, blood samples were collected at the following moments: before the induction of anemia (TC0); 6 hours after phlebotomy and before transfusion (TC1); 1, 6, 12, 24 and 96 hours after transfusion (T1, T6, T12, T24 and T96 respectively); 8, 16 and 32 days after transfusion (T8d, T16d and T32d respectively). Before transfusion, blood samples were also withdrawn from the bags for the same analyzes. Statistical analyzes were performed in the statistical program GRAPHPAD PRISM 5.0, adopting a significance level of 5%. The bags of blood stored for 15 and 35 days showed more biochemical changes, blood gas, oxidative stress and lipid peroxidation than fresh blood bags. As for the biochemical analysis, after the transfusion was observed an increase of the total protein, albumin, glucose and creatine kinase in the 3 groups, and elevation of total bilirubin, direct bilirubin, and urea in G15 and G35. The changes observed in the blood gas analysis had no clinical significance, as they were within the reference values for the species. The goats that received stored blood showed disorder in their antioxidant system through alteration of the SOD activity. In the analysis of lipid peroxidation no difference between the groups for the concentration of malondialdehyde was found. Thus, it can be concluded that transfusion of whole fresh stored blood in goats did not compromise the blood gases, lipid peroxidation and liver and renal functions of the transfused animals. In addition, the method was proved to be efficient to restore, among other components, the total protein and albumin. The transfusion, as performed in this study, proved to be safe for used in the clinical practice of goats.

INDEX TERMS: Blood gas stress, oxidative stress, lipid peroxidation, goats, hemotherapy, blood transfusion, biochemistry, hemogasometry, clinics.

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[2] Laboratório de Medicina Interna Veterinária (LABMIV), Universidade Federal Rural do Semi-Árido (UFERSA), Av. Francisco Mota 572, Costa e Silva, Mossoró, RN 59625-900, Brazil. *Corresponding author: nayanna_brunna@yahoo.com.br

[3] Faculdade de Medicina Veterinária e Zootecnia (FMVZ), Universidade de São Paulo (USP), Avenida Prof. Dr. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP 05508 270, Brazil.

[4] Instituto de Biodiversidade e Floresta, Universidade Federal do Oeste do Pará (UFOPA), Rua Vera Paz, Unidade Tapajós, Salé, Santarém, PA 68035-110, Brazil.

RESUMO.- [**Respostas bioquímicas, hemogasométricas, estresse oxidativo e peroxidação lipídica de caprinos transfundidos com sangue homólogo total fresco ou armazenado.**] A transfusão de sangue é um procedimento terapêutico de grande importância para medicina veterinária, apesar disto, na literatura há poucos estudos sobre hemoterapia em caprinos. Este trabalho teve como objetivo avaliar as respostas bioquímicas, hemogasométricas, estresse oxidativo e peroxidação lipídica de caprinos submetidos à transfusão homóloga de sangue total fresco ou armazenado por 15 e 35 dias. Foram utilizados 18 caprinos adultos, machos, submetidos a uma única flebotomia para retirada de 30% do volume sanguíneo e transfundidos com 20mL/kg de sangue total armazenado em bolsas CPDA-1 de acordo com o grupo experimental, sendo: o G0 composto por animais que receberam sangue fresco; G15 e G35 animais que receberam sangue armazenado por 15 e 35 dias, respectivamente. Para a avaliação bioquímica, hemogasométrica, estresse oxidativo e peroxidação lipídica foram coletadas amostras de sangue nos seguintes momentos: antes da indução da anemia (TC0); 6 horas após a flebotomia e antes de transfusão (TC1); 1, 6, 12, 24 e 96 horas após a transfusão (T1, T6, T12, T24 e T96, respectivamente); 8, 16 e 32 dias após a transfusão (T8d, T16d e T32d, respectivamente). Antes da transfusão, amostras de sangue também foram retiradas das bolsas para realização das mesmas análises. As análises estatísticas foram realizadas no programa estatístico GRAPHPAD PRISM 5.0, adotando-se nível de significância de 5%. As bolsas de sangue armazenadas por 15 e 35 dias apresentaram mais alterações bioquímicas, hemogasométricas, estresse oxidativo e peroxidação lipídica do que as bolsas de sangue fresco. Quanto à análise bioquímica, após a transfusão observou-se aumento da proteína total, albumina, glicose e creatina quinase nos 3 grupos; e elevação da bilirrubina total, bilirrubina direta e ureia no G15 e G35. As alterações observadas na análise hemogasométrica não tiveram significado clínico, pois estavam dentro dos valores de referência para a espécie. Os animais que receberam sangue armazenado apresentaram desordem no seu sistema antioxidante demonstrada pela alteração da atividade da SOD. Na análise da peroxidação lipídica não houve diferença entre os grupos para a concentração de malondialdeído. Sendo assim, pode-se concluir que a transfusão de sangue homólogo total fresco ou armazenado em caprinos não comprometeu a gasometria, peroxidação lipídica e funções hepática e renal dos animais transfundidos. Além disso, mostrou-se eficiente em repor entre outros componentes, a proteína total e albumina. Desta forma, a transfusão, conforme realizada neste estudo, mostrou-se segura para ser utilizada na prática clínica desta espécie.

TERMOS DE INDEXAÇÃO: Estresse hemogasométricas, estresse oxidativo, peroxidação lipídica, caprinos, hemoterapia, transfusão de sangue, bioquímica, hemogasometria, clínica.

INTRODUCTION

Blood transfusion is an old therapeutic practice that throughout the years has been increasingly used, mainly due to important technological discoveries, such as the use of anticoagulants during the collection and analysis of differences among individuals to choose the donor (Lacerda 2005). In veterinary medicine, hemotherapy is widely used, and so it can be successful it is necessary to take care of the donor's choice, search standards for collection, blood bags processing and storage, as the establishment of parameters of quality control that indicates the blood's viability to be utilized (Lacerda 2005). In human medicine, there are many studies about standardization of quality control indicative parameters, in order to avoid storing lesions (Tomczak et al. 2010), however, in veterinary, these criteria are not well established yet. Studies on different animal species have demonstrated that the storing time can cause alteration in hematological, biochemical, blood gas, lipid peroxidation and antioxidants enzymes variables (Costa Júnior et al. 2008, Niinistö et al. 2008, Barros 2011, Bertoletti 2011, Sousa et al. 2012a, 2013, Tavares 2013). After transfusion, some of these alterations can be reflected in the animals' responses to the transfusion as observed by Sousa (2012) and Sousa et al. (2012b) while analyzing homologous and autologous blood transfusion in ovine, respectively. The blood bags used in veterinary medicine are the same used in human, being that in goat it is preferable the use of the CPDA-1 bags (Tavares 2013).

Total blood transfusion is still the most used in veterinary medicine (Lacerda 2005), and its indication or-one of its components when it is necessary the reestablishment of the oxygen transport capacity by the blood, disorders in the hemostasis, in passive immunity transfer, in hypoproteinemia or hypovolemia (Reichmann & Dearo 2001). In goat, the transfusion necessity occurs ordinarily justified by the anemia due to the presence of endo and ectoparasites or nutritional deficiencies. However, other causes as traumas and chirurgical interventions are observed in the veterinary medical care routine (Pugh 2004).

Although blood transfusion is a common practice in the small ruminant clinic, it is empirical because little information is available on the actual benefits or disorders generated by this therapeutic measure in goat. When it is considered the realization of this procedure using stored blood, these data are not found in literature, being necessary to study and evaluate the viability of the practice in this species. Thus, the present study aimed to evaluate the biochemical, blood gas, oxidative stress and lipid peroxidation responses of goat submitted to homologous blood transfusion of fresh or stored blood for 15 and 35 days.

MATERIALS AND METHODS

Study location. The study was performed in the Laboratório de Medicina Interna Veterinária (LABMIV) of the Universidade Federal do Semi-Árido (UFERSA), in Mossoró-RN, whose geographic coordinates are 5º11" of south latitude and 37º20" of west longitude. This study respected the ethical standards and animal care, being approved by the Ethics Committee on Animals Use (CEUA, Comisão de Ética no Uso de Animais) of this institution, under process number 23091.005234/2013-08 (Proposition 28/2013).

Animals. 18 adult male goat, of undefined breed and average weight of 21.75±3.48kg were used after considered healthy through physical exam, hemogram, and feces parasitological exam, accordingly to the normal parameters for the species. They were maintained in collective stalls and underwent an adaptation period of 30 days. During this period, they received an application of endectocide based on ivermectin (Ivermectan®, UCBVET, São Paulo, SP, Brazil), anthelminthic (Biozen®, Biofarm, São Paulo, SP, Brazil) and doses of coccidiostat (Trissulfin®, Ouro Fino, SP, Brazil). After this period, blood samples were collected for hemogram realization. The animals with

normal globular volume were used in this study. None of the animals had previously been submitted to any kind of blood transfusion.

The animals' diet was composed by 75% of dry matter (hay of Canarana grass and Elephant grass) and 25% of concentrated matter using commercial food (Rações Agromix®, Maracanaú, CE, Brazil). The goat also received mineral and vitaminic supplementation every day (Caprinofós®, Tortuga Companhia Zootécnica Agrária, Mairinque, SP, Brazil) (15g/animal) and they had free access to water.

Aiming to reduce the costs and to maximize the experimental logistic, all eighteen animals used for blood transfusion also served as donors, thus, in the moment of anemia induction, the blood was collected in bags avoiding waste of this important corporeal component. Six other animals were used in the experiment only as donors, whose have passed through the same adaptation, examination and feeding process.

Gathering and storage of the blood. Initially, the trichotomy and the asepsis of the jugular region were performed for the blood collection. Posteriorly, the animal was positioned in lateral decubitus for the jugular venous punction, and the needle placed in the caudocranial direction. The blood was stored in sterile plastic bags of the CPDA-1 type (Line CompoFlex®, Fresenius Kabi, Barueri, SP, Brazil), containing anticoagulant solution composed by citrate, phosphate, dextrose and adenine (CPDA-1). The bags were maintained under refrigeration at a temperature between 2ºC and 6ºC, during 15 and 35 days, depending on the group. During the storage period, the bags were homogenized daily through the blood bag homogenizer (BHB_800, Benfer, São Paulo/SP, Brazil), so all the blood would be mixed with all the preservative solution.

Anemia induction and blood transfusion. Acute anemia was induced in all 18 goats by the removal of 30% of their blood volume, measured according to the calculation of the total blood volume of the animals, which according to Liebich et al. (2016) corresponds to 8% of its corporal weight. The 30% percentage to induce acute anemia was based on previous studies with acute loss of blood in goat, but not yet published. Six hours after anemia induction, the total blood collected in CPDA-1 bags was transferred to the animals according to the experimental group: G0 (control group) received fresh blood transfusion (blood maintained in environment temperature for up to 6 hours after collection); G15 (15 days group) received transfusion of blood stored during 15 days; and G35 (35 days group) received transfusion of blood stored for 35 days. Each experimental group was composed by 6 animals presenting similar body weight.

Before the transfusion, the bags were removed from the fridge and left at environment temperature for about 30 minutes. In this moment, blood samples were taken from the bags to perform the microbiological, biochemical, blood gas, oxidative stress and lipid peroxidation analysis. For the hemoculture, the Pediatric Three-Phase Hemobac System (Probac®, São Paulo/SP, Brazil) composed by a two faced laminoculture containing chocolate agar, Sabouraud agar and MacConkey agar was used. After, the bag was connected to transfusion equipment (Sangodrop® B LLM, Fresenius Kabi, Barueri, SP, Brazil) and this to a catheter for blood administration, the blood was transfused into the jugular vein. Each animal received 20mL of total blood per kg of body weight during transfusion, volume considered safe to be administered (Hunt & Moore 1990).

In the first 15 minutes of transfusion, the blood was transfused more slowly (0.1mL of blood/kg) for verification of possible adverse reactions, posteriorly, the velocity was elevated to 20mL of blood per kilogram of body weight per hour (Reichmann & Dearo 2001). In order to minimize the animals' stress, the transfusion was performed in a climatized environment with ambiance temperature varying from 22ºC to 28ºC.

The biochemical parameters, oxidative stress and lipid peroxidation were evaluated from analysis of the blood samples collected in the following moments: before anemia induction (TC0); 6 hours after anemia induction and before the transfusion (TC1); 1, 6, 12, 24 and 96 hours after transfusion (T1, T6, T12, T24 and T96, respectively); 8, 16 and 32 days after the transfusion (T8d, T16d and T32d, respectively). The blood gas parameters were evaluated from TC0 to T96.

Biochemical analysis. For the biochemical analysis, 2 total blood samples were collected into tubes of 8.5mL without anticoagulant, which were centrifuged at 1,500g for 15 minutes to obtain the serum. One of these tubes was covered with aluminum paper to protect the serum from the contact with the ambience light, and it was used for determination of the bilirubin concentration. Besides, the total blood samples were collected in tubes with sodium fluoride for determination of the glucose and lactate concentrations.

The biochemical evaluation was performed from the determination of the total protein concentration (TP), albumin, urea, creatinine, total bilirubin (TB), direct bilirubin (DB), and activity of lactate dehydrogenase enzymes (LDH), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), and creatine kinase (CK) in serum samples. The glucose and lactate concentration were determined in plasma samples. The biochemical parameters were determined in an automatic biochemical analyzer (RX Daytona, Randox®, Antrim, UK) using commercial kits of the same brand, except for the concentrations of the sodium and potassium electrolytes, which were determined in serum, using a flame photometer (FC-130, Celm, São Paulo/SP, Brazil); and the indirect bilirubin concentration (IB), that was obtained by calculating the difference between TB and DB.

Blood gas analysis. The blood gas analysis was based in the analysis of the potential of hydrogen ion (pH), partial oxygen pressure (PO_2), partial carbon dioxide (PCO_2), oxygen saturation (SO_2), bicarbonate (HCO_3^-), base excess (BE), and total concentration of carbon dioxide (TCO_2). For this evaluation, total venous blood samples were used, collected in heparinized syringes and analyzed in portable hemogasometer (i-STAT®1, Abbott Point of Care Inc, Illinois, USA), using (i-STAT® CG8+ (Abbott Laboratories, Illinois, USA) cartridges. Each determination was corrected according to the rectal temperature of the animal.

Oxidative stress and lipid peroxidation analysis. The oxidative stress was evaluated from the activity of the superoxide dismutase (SOD) and the glutathione peroxidase (GPX). The total blood samples for determination of the SOD and GPX were collected in vacuo in two tubes of 8.5mL containing lytic heparin as anticoagulant. After, they were centrifuged at 1,050g for 10 minutes for the removal of plasma and *buffycoat.* Then, the red blood cells were washed three times with saline solution (NaCl 0,9%) and the pap of red blood cells obtained was conditioned in amber microtubes and frozen in a freezer at -80ºC for posterior analysis of SOD and GPX. The SOD and GPX concentrations were determined in an automatic biochemical analyzer (RX Daytona, Randox®, Antrim, UK) using the commercial kits (Ransod e Ransel - Randox®, Antrim, UK).

The lipid peroxidation was evaluated through the determination of the malondialdehyde (MDA) in serum samples using the method described by Esterbauer & Cheeseman (1990).

Statistical analysis. The statistical analysis was processed with help from the statistical program GRAPHPAD PRISM 5.0. The significance level adopted was of 5%. To verify the distribution of data in the diverse variable studied, these were submitted to the Kolmogorov Smirvov test. The statistical analysis for comparison

among the bags from the three groups was performed through analysis of variance followed by the Tukey test for comparison of averages. The evaluation of the animals in different moments was performed through analysis of variance of two ways with repeated measures in time (two-way repeated measure ANOVA), followed by the comparison test of averages of Tukey for comparison between the groups in different moments; and Bonferroni for comparison between the TC0 and the TC1 and of the subsequent times.

RESULTS

Bags analysis

In the biochemical analysis of the bags, no difference was observed among the B0, B15 and the B35 bags as for total protein, albumin and sodium concentrations. However, the bags differed as for the potassium, glucose, lactate and LDH concentrations, in which the bags with stored blood (B15 and B35) presented higher concentration of potassium (P<0,0001) and LDH when compared to the fresh blood bags. Besides that, it was observed higher lactate concentration (P<0,05) in the blood stored for 35 days e lower glucose content (P<0,05) in the blood stored for 15 days (Table 1).

No statistical difference was observed among the bags B0, B15 and B35 for TCO_2, HCO_3^- and BE. However, the bags differed as for pH, pO_2, pCO_2 and SO_2, in which the blood stored for 15 and 35 days presented lower values of pH (P<0.05) than the fresh blood; the blood stored for 15 days presented higher pCO_2 (P<0.01); and the blood stored for 35 days presented higher pO_2 e SO_2 (P<0.0001) than the fresh blood and the blood stored for 15 days (Table 1).

The bags oxidative stress evaluation showed that the activity of the SOD was lower (P<0.01) in the blood stored for 35 days, while it was not observed difference in the GPX activity for the stored or fresh blood (Table 1).

The bags lipid peroxidation evaluation showed higher MDA concentrations (P<0.01) in the blood stored for 35 days when compared to the fresh blood or to the blood stored for 15 days (Table 1).

In the microbiological analysis of the bags, all samples cultures performed during the experimental period were negative for any bacterial growth.

Biochemical analysis

No difference was observed between groups for total protein concentration, indirect bilirubin, glucose, lactate, potassium, and for the LDH, AST, GGT and CK enzymes.

The total protein reduced (P<0.0001) after anemia induction in TC1 in all groups. After transfusion, there was a TP increase (P<0.0001) from T1 in the G0 and from T6 in the G35 (Table 2).

In relation to the albumin concentration, there was difference among the groups (P<0.01), in which G0 present higher content of albumin than the animals from the G15 from the moments T6 to T96; and higher than the G15 and the G35 in T16d. In the time comparison, after anemia induction there was reduction (P<0.0001) of the concentration of serum albumin in the TC1 in all groups. After transfusion, in the evaluation within each group, it was observed a significative increase (P<0.0001) of the albumin in T1 and T16d of the G0 and T32d of the G15; and a numeric increase in T1 of the G15 and G35 (Table 2).

The concentration of total bilirubin was higher (P<0.05) from TC0 to T96 in the G15 and G35. Between times, after anemia induction, the concentration of total bilirubin reduced (P<0.0001) in the TC1 and from T24 to T32d of the G15, and from TC1 to T32d of the G35. Nevertheless, this reduction

Table 1. Mean values (X) and standard deviations (S) of biochemical, blood gas variables, lipid peroxidation and oxidative stress of fresh whole blood (B0) stored in CPDA-1 bags for 15 days (B15) or stored for 35 days

Variables		Blood bags (CPDA-1)		
		B0 (X±S)	B15 (X±S)	B35 (X±S)
Biochemistry	Sodium (mmol/L)	159.00 ± 3.03	155.00 ± 8.07	148.00 ± 15.85
	Potassium (mmol/L)	3.25 ± 0.30^{B}	8.05 ± 1.76^{A}	8.78 ± 1.41^{A}
	Glucose (mmol/L)	31.43 ± 3.21^{A}	27.63 ± 2.68^{B}	27.90 ± 1.08^{AB}
	Lactate (mmol/L)	3.01 ± 0.54^{B}	5.04 ± 1.16^{B}	8.40 ± 3.64^{A}
	LDH (U/L)	246.27 ± 60.46^{C}	441.33 ± 65.54^{A}	355.58 ± 40.61^{B}
	Total protein (g/L)	55.08 ± 4.25	55.70 ± 1.98	57.63 ± 3.75
	Albumin (g/L)	27.33 ± 1.51	27.00 ± 1.67	26.50 ± 2.66
Blood gas	pH	7.01 ± 0.08^{A}	6.89 ± 0.06^{B}	6.88 ± 0.08^{B}
	pCO_2 (mmHg)	78.78 ± 6.41^{B}	93.13 ± 5.85^{A}	85.02 ± 8.22^{B}
	pO_2 (mmHg)	42.67 ± 4.55^{B}	44.50 ± 4.64^{B}	121.17 ± 40.54^{A}
	SO_2 (%)	53.00 ± 8.15^{B}	47.00 ± 6.03^{B}	90.50 ± 7.31^{A}
	TCO_2 (mmol/L)	22.67 ± 3.33	21.33 ± 1.97	18.67± 3.61
	HCO_3^- (mmol/L)	20.35 ± 3.74	18.35 ± 1.95	16.13 ± 3.43
	BE (mmol/L)	-10.83 ± 4.36	-14.67 ± 2.88	-17.17 ± 4.83
Oxidative stress	SOD (U/g Hb)	3331.48 ± 466.30^{A}	4350.23 ± 957.11^{A}	1948.14 ± 777.62^{B}
	GPx (U/g Hb)	540.93 ±202.42	519.22 ± 104.71	359.63 ± 108.04
Lipid peroxidation	MDA (µmol/L)	0.83 ± 0.19^{B}	1.06 ±0.28^{B}	1.48 ± 0.35^{A}

LDH = Lactate dehydrogenase enzymes, BE = base excess, SOD = superoxide dismutase, GPx = glutathione peroxidase, MDA = malondialdehyde; $^{A, B, C}$ Statistical difference (p<0.05) between groups at the same time.

Table 2. Mean values (X) and standard deviations (S) of biochemical variation in goats undergoing blood loss and fresh blood transfusion (B0) stored in CPDA-1 bags for 15 days (B15) or stored for 35 days (G35)

Variables	G	Time									
		TC0 (X±S)	TC1 (X±S)	T1 (X±S)	T6 (X±S)	T12 (X±S)	T24 (X±S)	T96 (X±S)	T8d (X±S)	T16d (X±S)	T32d (X±S)
Total protein (g/L)	G0	66.17 ± 4.18	53.13 ± 3.94$^{b¥}$	58.45 ± 5.71$^{a¥}$	61.22 ± 5.27^{a}	61.22 ± 3.76^{a}	60.50 ± 2.57$^{a¥}$	61.48 ± 2.48^{a}	62.45 ± 2.26^{a}	65.68 ± 3.77^{a}	65.92 ± 3.73^{a}
	G15	61.27 ± 1.72	49.92 ± 2.38$^{b¥}$	53.93 ± 2.84$^{b¥}$	55.98 ± 2.58^{a}	55.68 ± 2.91$^{a¥}$	55.80 ± 2.76$^{a¥}$	58.25 ± 2.77^{a}	60.47 ± 4.74^{a}	61.92 ± 4.14^{a}	67.60 ± 4.51$^{a¥}$
	G35	66.77 ± 4.17	53.30 ± 3.42$^{b¥}$	57.85 ± 5.37$^{b¥}$	60.27 ± 3.21$^{a¥}$	60.13 ± 2.98$^{a¥}$	59.17 ± 2.31$^{a¥}$	61.07 ± 2.44$^{a¥}$	61.50 ± 2.24^{a}	61.60 ± 5.01^{a}	64.20 ± 6.31^{a}
Albumin (g/L)	G0	32.33 ± 1.37^{AB}	27.83 ± 1.17$^{b¥}$	29.67 ± 1.97^{a}	31.00 ± 1.79^{Ab}	30.50 ± 1.05^{Ab}	30.67 ± 1.03^{Ab}	30.50 ± 0.55^{Ab}	30.67 ± 0.82^{b}	31.67 ± 0.82^{Aa}	28.75 ± 2.67^{b}
	G15	30.33 ± 1.63^{B}	25.83 ± 1.72$^{b¥}$	27.83 ± 1.33^{b}	28.17 ± 1.72^{Bb}	27.83 ± 0.98^{Bb}	27.83 ± 0.98^{Bb}	28.00 ± 0.63^{Bb}	28.33 ± 1.75^{b}	27.50 ± 2.74^{Bb}	28.73 ± 2.42^{a}
	G35	32.67 ± 3.61^{A}	28.00 ± 1.26$^{¥}$	29.50 ± 1.76$^{¥}$	30.33 ± 1.21^{AB}	30.00 ± 1.41^{AB}	29.67 ± 1.51$^{AB¥}$	30.17 ± 1.83^{AB}	29.67 ± 1.37$^{¥}$	29.00 ± 1.26$^{B¥}$	28.17 ± 1.94$^{¥}$
Total bil. (µmol/L)	G0	9.21 ± 14.15^{B}	6.70 ± 11.67^{B}	8.41 ± 9.88^{B}	8.38 ± 11.39^{B}	8.41 ± 10.87^{B}	8.09 ± 10.21^{B}	6.75 ± 10.97^{B}	9.29 ± 9.14	8.46 ± 9.11	6.84 ± 8.35
	G15	25.71 ± 6.71^{A}	21.40 ± 5.92$^{Aa¥}$	22.60 ± 4.82^{Aa}	22.86 ± 4.91^{Aa}	22.57 ± 4.18^{Aa}	21.20 ± 5.02$^{Aa¥}$	21.18 ± 4.38$^{Aa¥}$	20.75 ± 4.74$^{a¥}$	18.24 ± 4.96$^{a¥}$	14.42 ± 5.71$^{b¥}$
	G35	25.85 ± 14.04^{A}	19.67 ± 10.07$^{Aa¥}$	18.21 ± 8.13$^{ABa¥}$	20.09 ± 8.82$^{ABa¥}$	19.78 ± 7.99$^{ABa¥}$	19.69 ± 8.80$^{ABa¥}$	18.67 ± 7.46$^{Aa¥}$	19.72 ± 8.96$^{a¥}$	16.10 ± 7.35$^{a¥}$	11.97 ± 4.46$^{b¥}$
Direct bil. (µmol/L)	G0	3.39 ± 6.41^{B}	2.34 ± 5.31^{B}	3.11 ± 5.40^{B}	2.59 ± 4.70^{B}	3.31 ± 5.76^{B}	2.91 ± 5.62^{B}	3.45 ± 5.92^{B}	4.05 ± 5.78^{B}	4.36 ± 5.31^{B}	3.31 ± 2.64
	G15	16.90 ± 3.55^{A}	13.48 ± 2.39$^{Aa¥}$	12.80 ± 1.36$^{Aa¥}$	14.79 ± 1.95^{Aa}	14.05 ± 1.80^{Aa}	13.79 ± 3.08$^{Aa¥}$	14.19 ± 1.14^{Aa}	13.25 ± 2.01$^{Aa¥}$	11.26 ± 2.44$^{Aa¥}$	5.67 ± 1.70$^{b¥}$
	G35	14.02 ± 8.44^{A}	11.17 ± 6.31^{A}	10.46 ± 5.57$^{A¥}$	11.60 ± 5.65^{A}	12.34 ± 5.78^{A}	11.97 ± 6.01^{A}	10.20 ± 5.03$^{A¥}$	11.34 ± 5.65^{A}	9.72 ± 4.30$^{AB¥}$	8.69 ± 4.62$^{¥}$
CK (U/L)	G0	33.13 ± 7.56	45.30 ± 21.33^{b}	195.68 ± 188.69$^{b¥}$	198.92 ± 167.10$^{a¥}$	101.07 ± 73.27^{b}	41.50 ± 11.71^{b}	57.40 ± 38.90^{b}	37.95 ± 12.82^{b}	43.63 ± 16.46^{b}	45.47 ± 14.36^{b}
	G15	59.78 ± 27.65	61.80 ± 16.85	125.55 ± 55.10	140.52 ± 97.42	115.13 ± 80.12	78.37 ± 39.26	74.72 ± 43.40	72.07 ± 49.14	58.47 ± 35.18	47.93 ± 5.27
	G35	85.57 ± 55.04	75.38 ± 27.65	191.03 ± 165.47	220.53 ± 285.51	95.87 ± 58.00	52.47 ± 15.46	59.27 ± 17.34	69.93 ± 52.39	53.48 ± 19.38	54.12 ± 22.49
Glucose (mmol/L)	G0	4.00 ± 0.20	4.83 ± 0.70^{b}	8.16 ± 3.41$^{a¥}$	4.75 ± 0.74^{b}	4.35 ± 0.73^{b}	3.76 ± 0.52^{b}	2.93 ± 1.26^{b}	3.21 ± 1.37^{b}	3.81 ± 0.51^{b}	3.72 ± 0.37^{b}
	G15	4.06 ± 0.43	5.94 ± 1.94^{a}	7.92 ± 0.92$^{a¥}$	4.15 ± 0.53^{a}	4.32 ± 0.52^{a}	3.78 ± 0.44^{b}	4.23 ± 0.44^{a}	4.11 ± 0.43^{a}	3.98 ± 0.66^{a}	3.07 ± 0.24^{b}
	G35	3.60 ± 0.42	4.46 ± 0.77^{b}	8.80 ± 3.72$^{a¥}$	3.89 ± 0.45^{b}	3.95 ± 0.45^{b}	3.59 ± 0.36^{b}	3.50 ± 0.17^{b}	3.22 ± 0.32^{b}	3.59 ± 0.50^{b}	3.90 ± 0.46^{b}
Lactate (mmol/L)	G0	1.67 ± 0.63	1.75 ± 0.46	2.19 ± 0.37	1.90 ± 0.75	1.36 ± 0.49	1.44 ± 1.07	0.73 ± 0.15	0.82 ± 0.20	1.51 ± 1.16	0.78 ± 0.37
	G15	1.46 ± 0.68	4.26 ± 1.83$^{a¥}$	3.44 ± 2.07$^{a¥}$	2.21 ± 1.16^{b}	2.14 ± 1.78^{b}	1.75 ± 1.03^{b}	1.27 ± 0.90^{b}	1.53 ± 0.46^{b}	1.92 ± 0.48^{b}	0.68 ± 0.28^{b}
	G35	1.74 ± 1.11	2.12 ± 1.15	2.72 ± 1.36	2.22 ± 0.82	1.48 ± 0.56	0.91 ± 0.48	1.01 ± 0.33	0.94 ± 0.25	1.72 ± 0.88	1.48 ± 0.66
Urea (mmol/L)	G0	7.48 ± 1.25^{B}	8.36 ± 1.94^{Ba}	7.81 ± 1.55^{Ba}	8.29 ± 1.25^{Ba}	8.16 ± 1.66^{a}	7.98 ± 1.30^{a}	7.36 ± 1.02^{a}	7.31 ± 0.67^{a}	5.64 ± 0.75^{b}	5.87 ± 1.11^{Bb}
	G15	8.59 ± 1.29^{AB}	10.46 ± 2.07^{Aa}	9.57 ± 1.69^{ABa}	8.10 ± 0.67^{Bb}	8.47 ± 1.02^{a}	7.83 ± 1.67^{b}	7.21 ± 0.47^{b}	6.19 ± 1.40$^{b¥}$	5.38 ± 2.59$^{b¥}$	5.35 ± 1.28$^{Bb¥}$
	G35	9.42 ± 1.28^{A}	10.71 ± 1.57^{Aa}	11.27 ± 1.25^{Aa}	10.43 ± 1.11^{Aa}	9.39 ± 1.08^{a}	9.14 ± 1.33^{a}	8.13 ± 1.14^{b}	8.03 ± 1.71^{b}	7.99 ± 1.72^{b}	6.77 ± 1.50$^{Ab¥}$
Potassium (mmol/L)	G0	3.99 ± 0.32	3.49 ± 0.61	3.62 ± 0.50	3.90 ± 0.65	4.18 ± 0.52	3.85 ± 0.25	4.07 ± 0.45	4.13 ± 0.23	4.17 ± 0.52	3.73 ± 0.61
	G15	4.20 ± 0.79	3.79 ± 0.42	3.20 ± 0.45$^{¥}$	4.50 ± 0.83	3.85 ± 0.83	3.75 ± 0.79	4.25 ± 0.40	4.27 ± 0.63	4.47 ± 0.50	3.62 ± 0.22
	G35	3.99 ± 0.33	4.16 ± 0.41^{a}	3.23 ± 0.30^{b}	3.72 ± 0.38^{a}	4.18 ± 0.41^{a}	4.12 ± 0.46^{a}	4.43 ± 0.30^{a}	4.40 ± 0.52^{a}	4.60 ± 0.38^{a}	4.27 ± 0.40^{a}

^{A,B} Statistical difference ($p<0.05$) between groups at the same time, ^{a,b} Statistical difference ($p<0.05$) between TC1 and other time at the same group, ¥ Statistical difference ($p<0.05$) between T0 and other time at the same group; G = groups

after transfusion was observed only in the T32d of these groups (Table 2).

The concentration of direct bilirubin was higher (P<0.01) from TC0 to T16d in the G 15 and G35 groups. Between times, after anemia induction, the concentration of direct bilirubin reduced (P<0.0001) in the TC1, T1, T24, T8d, T16d and T32d of the G15 and in T1, T96, T16d and T32d of the G35. After the transfusion, the reduction was observed only in T32d of the G15 (Table 2).

The concentration of indirect bilirubin after anemia induction reduced (P<0.0001) in T1, T12, T24, T16d and T32d of the G35, while after transfusion, this reduction was observed only in T32d of the G35 (data not shown).

As for the analyzed enzymes in the time evaluation after the anemia induction, there was an increase (P<0.0001) of the CK activity in the T1 and T6 of the G0 (Table 2) and reduction (P<0.0001) of the LDH activity in T32d of the G0 and the G15; and of the GGT in TC1 and T1 of the G35 (data not shown). After transfusion there was an increase (P<0.0001) of the LDH activity in T6, T12 and T8d of the G0, in T1, T6 and T12 of the G35; of the AST in T6 and T12 of the G0, in T1, T6 and T12 of the G15 and the G35; of the GGT in T8d, T16d and T32d in the G0, in T6 and T32d in the G35 (data not shown); significative of the CK in T6 of the G0 and numeric in the G15 and G35 from T1 (Table 2).

The glucose concentration after the anemia induction increased (P<0.0001) in T1 in all groups. After transfusion, an increase (P<0.0001) of the glucose in T1 of the G0 and G35 was observed, while in the G15 there was a reduction (P<0.0001) in T24 and T32d (Table 2).

The lactate concentration after anemia induction increased (P<0.0001) in TC1 and T1 of the G15, however, after transfusion, a reduction (P<0.0001) in the lactate from T6 to T32d of the G15 was observed. The higher glucose and lactate values post-transfusion were observed in the T1 of the three groups (Table 2).

In relation to the urea concentration, there was a difference (P<0.01) between the groups, in which in TC1 of the G15 and G35 and in T1, T6 and T32d of the G35 there was a higher urea concentration than the G0. In time comparison after anemia induction, there was a reduction of the urea from the T8d of the G15 and T32d of the G35. After transfusion a reduction (P<0.0001) of the urea from T16d of the G0; in-T6 and from T24 in the G15; and from T96 in the G35 was observed (Table 2).

In relation to the creatinine concentration, there was a difference (P<0.05) among the groups, in which from T96 to T16d, the G0 presented the highest creatinine values. In comparison among time, after the transfusion there was an increase (P<0.0001) of the creatinine only in T32d of the G35 (data not shown).

The sodium concentration did not differ among the groups, neither among the times studied (data not shown).

The potassium concentration reduced (P<0.0001) after anemia induction in T1 of the G15 and after transfusion in T1 of the G35 (Table 2).

Blood gas analysis

There was no difference among the groups and times studied for BE, HCO_3^- and TCO_2 (data not shown).

In relation to the blood pH, there was difference among the groups in TC1 and T96 moments, in which the G15 showed lower pH. In time comparison, an increase (P<0.01) in the pH in T1 of the G15 was observed when compared to the TC0 (Fig.1).

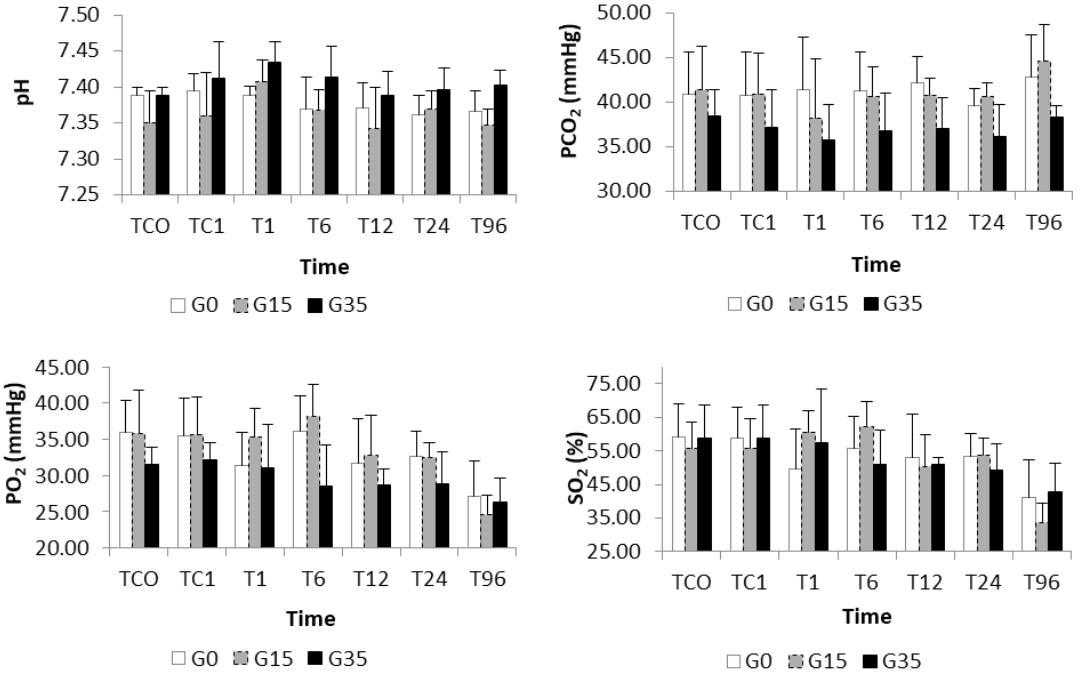


Fig.1. Blood gas analysis of goat submitted to blood loss, and posteriorly to the total homologue fresh blood transfusion (G0), stored in CPDA-1 bags for 15 days (G15) or 35 days (G35), in the analyzed times.

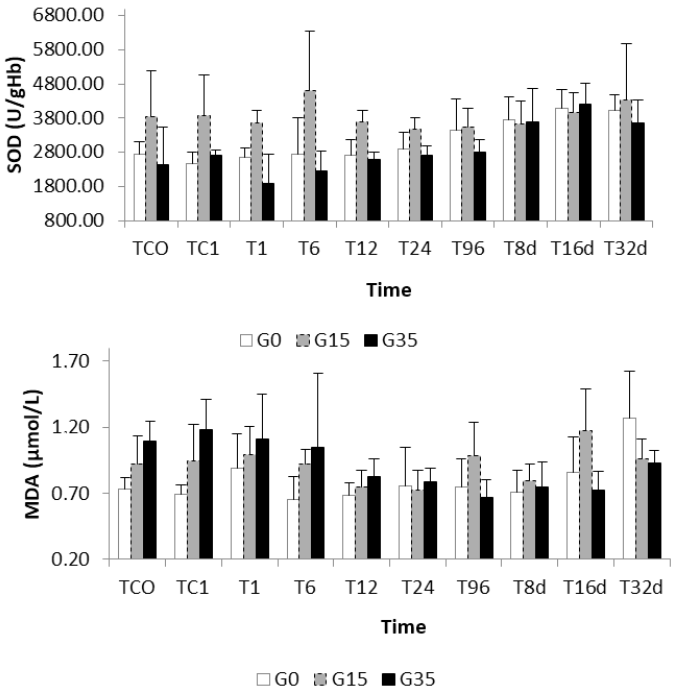


Fig.2. SOD activity and MDA concentration of goat submitted to blood loss, and posteriorly to the total homologue fresh blood transfusion (G0), stored in CPDA-1 bags for 15 days (G15) or 35 days (G35), in the analyzed times.

As for the PCO_2, the G0 presented higher values (P<0.05) in T1 and the G15 presented higher (P<0.05) PCO_2 in T96 (Fig.1).

The PO_2 was higher (P<0.05) in T6 of the G0 and G15.In time comparison after anemia induction and blood transfusion, a reduction (P<0.0001) in the PO_2 and SO_2 in T96 of the G0 and G15 was observed (Fig.1).

Oxidative stress and lipid peroxidation analysis

The SOD activity was higher (P<0.01) in the basal moment, Tc1, T1, T6 and T12 of the G15; whereas lower activity (P<0.01) was observed in the G35. In time comparison after anemia induction, an increase (P<0.0001) of the SOD in T16d of the G0 and G35 was observed, and after the transfusion in T16d and T32d of the G0 e in T16d of the G35 (Fig.2).

The GPX activity did not differ among the groups and times studied (data not shown).

The MDA concentrations did not differ among groups in none of the analyzed moments. However, in time comparison after anemia induction and blood transfusion, an increase (P<0.0001) of the MDA in T32d of the G0 was observed; and a reduction (P<0.0001) of the MDA of the G35 after anemia induction in T96 and after transfusion in T96, T8d and T16d (Fig.2).

DISCUSSION

In the bags' biochemical analysis, no difference was observed in the potassium and total protein concentration among the three periods of storage of the blood bags, corroborating with Tavares (2013) that, when analyzing the goat blood stored in CPDA-1 bags, also did not observed alteration in these parameters until the storage period of 35 days.

The bags stored for 15 and 35 days presented higher potassium values than the bags of fresh blood. This potassium elevation in the stored blood has already been found in other studies (Wallas 1979, Niinistö et al. 2008, Sousa 2012, Sousa et al. 2013) and occurs because during the storage, the temperature reduces the activity of the ATPase in the erythrocyte membrane, preventing the active transportation of Na^+/K^+ (sodium/potassium), with this there is the increase of intracellular sodium and reduction of intracellular potassium (Wallas 1979). According to Tomczak et al. (2010) the K^+ measurement in the extracellular environment is a useful marker for the quality control during the preservation.

The blood stored for 15 days presented lower glucose content when compared to the fresh blood. According to Hess & Greenwalt (2002), this glucose reduction occurs because during storage the glucose is used by the erythrocytes as energetic source for production of ATP. Besides that, Tavares (2013), when evaluating the goat blood stored in CPDA-1 bags, did not observe glucose content alteration throughout the storage period of 35 days, and associated it to the low consume of the same by goat red blood cells. That explains why the blood stored for 35 days in this study did not differ in the glucose content in relation to the fresh blood.

The blood stored for 35 days presented higher lactate content than the fresh blood, and the blood stored for 15 days, corroborating with other studies (Niinistö et al. 2008, Sousa et al. 2013, Tavares 2013). According to Högman & Meryman (1999) the lactate levels increase during the storage due to the glycolysis for the ATP production and it is associated to the pH changes, being that, for each molecule of metabolized glucose, two lactate molecules are produced. In this way, occurs the acidification of the mean, constituting one of the greatest problems in red blood cells preservation and limiting factor for its availability (Tomczak et al. 2010).

In the blood stored for 15 and 35 days, the LDH values were higher than the ones of the fresh blood. As this enzyme is present in high quantities in the red blood cells, studies with equine blood storage have used the activity of this enzyme to assist in the hemolysis evaluation (Mudge et al. 2004). Therefore, in the present study it is suggested that hemolysis has occurred in the stored blood.

In the blood gas analysis was not observed statistical difference among the bags for the TCO_2, HCO_3^- and BE. These three indexes are calculated from other parameters that were measured through the blood sample reading in the cartridge that is inserted in the hemogasometer. The HCO_3^- is responsible for approximately 95% of the TCO_2 measured (Carlson & Bruss 2008), and this has not altered in this study, thence the HCO_3^- also did not suffered alteration in its measurement. The BE is an indicator of the normal bicarbonate deviation (Carlson & Bruss 2008), in this way, as in this study there was no alteration in the bicarbonate, consequently there was also no alteration in the BE. Tavares (2013) and Sousa et al. (2013) when evaluating the goat and ovine blood stored in CPDA-1 bags respectively, did not observed bicarbonate alteration until the storage period of 35 days, corroborating with this study. However, Tavares (2013) observed an elevation in the

basis deficit starting in 21 days of storage, and reduction of the TCO_2 starting on 35 days of storage.

The blood bags stored for 15 and 35 days presented lower pH values when compared to the fresh blood bags, corroborating with other studies (Niinistö et al. 2008, Sousa et al. 2013, Tavares 2013). According to Hess (2009), this lower pH value in the stored blood occurs because during storage, the glucose degradation leads to the metabolites production, as lactate and H^+ ions, with consequent reduction of the pH and acidity elevation. However, this acidity elevation in the stored blood bags was not enough to increase the HCO_3^- consumption and consequently initiate a reduction on its levels.

The blood bags stored for 15 days presented higher PCO_2, corroborating with other studies (Sousa et al. 2013), in which the storage caused elevation of this gasometric variable. According to Sousa et al. (2012a), this CO_2 pressure elevation during storage occurs mainly to neutralize the lactic acid resulting from the cellular metabolism that results in the CO_2 synthesis.

The blood stored for 35 days presented higher PO_2 e SO_2, corroborating with the goat blood conservation study performed by Tavares (2013). The PO_2 increase can occur due to the oxygen passage from the external to the internal environment of the bag, since these are permeable (Högman & Meryman 1999). According to the hemoglobin dissociation curve that represents the oxygen affinity by the red blood cell, as higher the oxygen partial pressure values, higher will be the oxygen saturation values (Harvey 2008), thus this elevation in the oxygen partial pressure justifies the oxygen saturation increase in the bags stored for 35 days.

In the oxidative stress evaluation, there was lower activity of the SOD enzyme in the bags stored for 35 days. According to Hatherill et al. (1991), in oxidizing conditions, the presence of enzymes as SOD provides protection of the red blood cells against hemolysis. Thus, it is suggested in this study that the lower activity of SOD in the 35-day bags is related to the longer storage time that the red blood cells were exposed to the free radicals, resulting in a higher consumption of the SOD enzyme and consequently reducing its antioxidative potential.

In the lipid peroxidation evaluation, it was observed bigger formation of MDA in the 35-day bags. This parameter determination is used to estimate the intensity of the lipid peroxidation, in which higher the oxidative lesion of the red blood cell membrane and subsequent hemolysis, higher will be the formation of the MDA (Jóźwik et al. 1997). Thus, it is suggested in this study that the red blood cells of the 35-day bags suffered hemolysis by oxidative damage caused by the longer storage period.

In this study was observed that after acute blood loss, there was a reduction in the total protein in all groups for values below normal for the goat species (62-79g/L) (Jackson & Cockcroft 2002). However, after one hour of transfusion on the G0 and 6 hours of transfusion in the G15 and G35 was possible to observe the increase in this variable, and the return to the baseline values (TC0) occurred in the T6 of the G0 and G15 and T8d of the G35. Argôlo (2014) when analyzing the effects on acute blood loss in goat by the removal of 30% of the blood, also verified the reduction of the total protein, which returned to the baseline values on 24 days post challenge. Thus, it is evident that the fresh or stored blood transfusion restores the total protein, which maintained itself, unaltered during the storage period, and can be used in cases of hypoproteinemia.

After blood loss, there was albumin reduction in all groups for values under normal for the goat species (29-43g/L) (Jackson & Cockcroft 2002). Argôlo (2014) when analyzing the effects of acute blood loss in goat by the removal of 30% of the blood, also verified albumin reduction, which returned to baseline values only 8 days post challenge. In this study, after 1 hour of transfusion in the G0 and G15 and 6 hours of transfusion in the G35, baseline values returned (TC0). In this way, it can be observed that after the transfusion there was an increase of the albumin in the T1 of the G0 (significative), of the G15 and G35 (numeric), suggesting that the total fresh and stored blood restores the albumin in cases of hypoalbuminemia.

The bilirubin is the main product of the metabolization of the hemoglobin, and its level increase in case of hemolysis (Tennant & Center 2008). In this study, during comparison between groups, since the beginning was observed higher concentration of total bilirubin and direct bilirubin in the animals that received stored blood; the higher bilirubin concentration in these groups did not have clinical significance, because it did not exceed the reference values for the species of 1.71-29.24μmol/L (Pugh 2004). Thus, before the increase of the normal levels of bilirubin, it can be noticed that there was no hemolysis of the red blood cells transfused, and the fresh or stored blood transfusion did not cause hepatic lesion.

The AST, GGT, LDH and CK analysis was performed to verify possible post-transfusion hepatic and/or muscle lesions. The activity of these enzymes did not differ among groups. Nevertheless, between times was observed that after transfusion there was LDH and AST activity increase in the 3 groups studied; of the GGT in the G0 and G35 (data not shown); and the CK in the G0 (significative increase) and in the G15 and G35 (numeric increase) (Table 2). From these alterations observed, only CK rose above the reference values for the species of 0-100U/L (Jackson & Cockcroft 2002) in the 3 groups, returning to the normal values after 24 hours of the transfusion. The probable hypothesis is that during the period that the animals remained in decubitus for the transfusion fulfillment, micro muscle lesions occurred elevating the content of this enzyme. This finding was reported by Sousa (2012) in ovine.

After the transfusion a significant increase of the glucose was observed in T1 of the G0 and G35 and numeric in T1 of the G15 above reference values for the species of 2.4-4.0mmol/L (Jackson & Cockcroft 2002); yet, 6 hours after the transfusion in the G35 and 24 hours in the G0 and G15, the values returned to normal. Thus, nutrients (e.g., dextrose) contained in the blood bags were converted after the transfusion into energy source (glucose), so that initially there was a serum glucose increase of the animals. Besides that, it can also be observed that the hepatic function of the animals was not committed through the transfusion, since the liver promptly balanced the serum glucose levels.

The lactate concentration after acute blood loss, increased in the G15, Argôlo (2014) when analyzing the effects of acute blood loss in goat by removal of 30% of blood volume, also verified this lactate transitional increase, which derives from the tissue hypoperfusion provoked by the acute blood loss. Still about the lactate, in spite the elevation of its levels in the blood bags stored for 35 days, after the transfusion it was

not observed significant alterations in this group. Thus, it can be noticed that the hepatic function in animals was not committed through the transfusion, since the liver promptly balanced the serum lactate levels.

The urea concentration was higher after acute blood loss (TC1) in the G15 and G35, and after transfusion (T1, T6 and T32d) in the G35 than in the animals that received fresh blood. In these moments, there was a discreet elevation of the urea in relation to the reference values for the species of 4.0-8.6mmol/L (Jackson & Cockcroft 2002), but that 6 hours after the transfusion in the G15 and 96 hours after transfusion in the G35, these values had already returned to the species normal. Thus, it can be observed that the transfusion improved the renal perfusion even in animals that received stored blood.

The creatinine, despite being higher in the G0 from T96 to T16d, maintained itself inside the reference values for the species of 54-123µmol/L (Jackson & Cockcroft 2002).

The storage time despite having affected the Na^+/K^+ pump provoking greater efflux of potassium ions in the blood bags stored for 15 and 35 days, it was not observed post-transfusion relevant changes in the sodium and potassium levels of the transfused animals. It was observed only a light and transitory reduction of the potassium concentration in T1 of the G35, however in the T6, it had already returned to the reference values for the species of 3.4-6.1mmol/L (Jackson & Cockcroft 2002). Thus, in this study it became evident that the renal function of resorption of these electrolytes was occurring correctly, as for the activity of the Na^+/K^+ pump, which balanced the potassium levels promptly after the transfusion.

In relation to the blood gas analysis of the goats, it was found that, like the bags, the animals did not differ regarding the TCO_2, HCO_3^- and BE parameters. In comparison between groups, it was found that the animals of the G15 presented lower pH and higher PCO_2 in the T96, while in time comparison these animals presented lower PO_2 and SO_2 also in T96, as compensatory mechanism. These alterations are characteristics of respiratory acidosis, which develop due to the difficulty of oxygen transportation. In an attempt to correct this imbalance (pH reduction and PCO_2 increase), the hemoglobin releases higher oxygen quantities (Bohr's effect) (Carlson & Bruss 2008). However, despite the blood gas changes observed in this study, the analysis of these parameters showed that the acute blood loss and subsequent transfusion with homologue fresh or stored blood were not sufficient to provoke metabolic or respiratory disruption in the animals, because all the blood gas parameters stayed inside the referral values for the species (Nunes et al. 2014).

In the oxidative stress analysis of the animals, there was no difference in the GPX activity among groups and times studied, differently from the SOD activity, in which was observed in the comparison among groups that after the transfusion until the T12, there was higher activity in the G15 and lower in the G35. Sousa (2012) when evaluating the oxidative stress in ovine transfused with fresh and stored blood did not observe difference between the groups for SOD and GPX. According to Nazifi et al. (2009) these enzymes are part of the antioxidant system of the goat and act protecting them from the action of free radicals, being the SOD the frontline of defense against these radicals. In this study, the SOD alteration in the G15 and G35 shows that there was a disorder in the antioxidant system in the goat of these groups, suggesting then that the greater SOD activity in the G15 was due to the larger quantity of free radicals in the 15-day bags, while the 35-day stored bags, because they had greater SOD consumption during storage, presented less free radicals, consequently causing lower antioxidant activity in the G35.

The MDA is one of the products of the lipid peroxidation and its concentration is widely used as cellular lesion indicator (Esterbauer & Cheeseman 1990, Nazifi et al. 2009). In this study there was no difference among the groups for the MDA concentration, corroborating with Sousa (2012), which in a similar study with ovine, also did not observe difference in this parameter concentration among the animals that received stored and fresh blood. Thus, although the bags stored for 35 days presented higher MDA content, it is suggested that the cargo of reactive oxygen species contained in these bags was not enough to promote lipid peroxidation in the recipient animals.

CONCLUSIONS

The stored whole blood presented greater biochemical, blood gas, oxidative stress and lipid peroxidation changes when compared with fresh blood, but this did not impair its use as a therapeutic resource in goat.

The transfusion of whole fresh or stored blood in goat did not comprise the blood gas, lipid peroxidation, and hepatic and renal functions of the transfused animals; it has been shown to be effective in restoring total protein and albumin among other components, which can be used in animals with hypoproteinemia and hypoalbuminemia respectively.

The homologous transfusion of fresh or stored whole blood in goats as performed in this study has been shown to be safe for use in the clinical practice.

REFERÊNCIAS

Argôlo E.P. 2014. Respostas à perda sanguínea aguda em caprinos. Dissertação de Mestrado, Universidade Federal Rural do Semi-Árido, Mossoró, RN. 61p.

Barros I.O. 2011. Avaliação da conservação do sangue total de jumentos (*Equus asinus*) acondicionado em bolsas de sangue do tipo CPDA-1 e CPD/SAG-M. Dissertação de Mestrado, Universidade Federal Rural do Semi-Árido, Mossoró, RN. 79p.

Bertoletti B. 2011. Peroxidação lipídica e parâmetros bioquímicos do sangue total felino armazenado em bolsas plásticas contendo CPDA-1 e CPD/SAG-M. Dissertação de Mestrado, Universidade Federal de Santa Maria, Santa Maria, RS. 42p.

Carlson G.P. & Bruss M.L. 2008. Fluid, electrolyte and acid-base balance, p.529-559. In: Kaneko J.J., Harvey J.W & Bruss M.L. (Eds), Clinical Biochemistry of Domestic Animals. 6th ed. Academic Press, San Diego. <http://dx.doi.org/10.1016/B978-0-12-370491-7.00017-9>.

Costa Júnior J., Viana J.A., Ribeiro Filho J.D., Favarato E.S., Mata L.C. & Argôlo Neto N. 2008. Parâmetros bioquímicos e hemogasométricos do sangue total canino armazenado em bolsas plásticas contendo. CPDA-1 e CPD/SAG-M. Ciência Rural 38(2):378-383. <http://dx.doi.org/10.1590/S0103-84782008000200013>

Esterbauer H. & Cheeseman K.H. 1990. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods Enzymol. 186:407-421. <http://dx.doi.org/10.1016/0076-6879(90)86134-H> <PMid:2233308>

Harvey J.W. 2008. The erythrocyte: physiology, metabolism, and biochemical disorders, p.173-240. In: Kaneko J.J., Harvey J.W. & Bruss M.L. (Eds), Clinical

Biochemistry of Domestic Animals. 6th ed. Academic Press, San Diego. <http://dx.doi.org/10.1016/B978-0-12-370491-7.00007-6>.

Hatherill J.R., Till G.O. & Ward P.A. 1991. Mechanisms of oxidant-induced changes in erythrocytes. Agents Actions 32(3/4):351-358. <http://dx.doi.org/10.1007/BF01980898> <PMid:1862753>

Hess J.R. 2009. Red cell storage: when is better not good enough? Blood Transfusion 7(3):172-173. <PMid:19657479>

Hess J.R. & Greenwalt T.J. 2002. Storage of blood cells: new approaches. Transfus. Med. Rev. 16(4):283-295. <http://dx.doi.org/10.1053/tmrv.2002.35212> <PMid:12415514>

Högman C.F. & Meryman H.T. 1999. Storage parameters affecting red blood cell survival and function after transfusion. Transfus. Med. Revs 13(4):275-296. <http://dx.doi.org/10.1016/S0887-7963(99)80058-3> <PMid:10553271>

Hunt E. & Moore J.S. 1990. Use of blood and blood products. Vet. Clin. N. Am., Food Anim. Pract. 6(1):133-147. <http://dx.doi.org/10.1016/S0749-0720(15)30900-2> <PMid:2178738>

Jackson P.G.G. & Cockcroft P.D. 2002. Clinical Examination of Farm Animals. Blackwell Science, Oxford, p.304-305. <http://dx.doi.org/10.1002/9780470752425>.

Jóźwik M., Jóźwik M., Jóźwik M., Szczypka M., Gajewska J. & Laskowska-Klita T. 1997. Antioxidant defence of red blood cells and plasma in stored human blood. Clin. Chim. Acta 267(2):129-142. <http://dx.doi.org/10.1016/S0009-8981(97)00148-4> <PMid:9469248>

Lacerda L. 2005. Transfusão sanguínea em veterinária: desafios a vencer. Anais II Simpósio de Patologia Clínica Veterinária da Região Sul do Brasil, Porto Alegre, RS, p.62-81. (Resumo)

Liebich H.-G., Forstenpointner G., Pérez W. & König H.E. 2016. Introdução e anatomia geral, p.1-52. In: König H.E. & Liebich H.-G. (Eds). Anatomia dos Animais Domésticos: texto e atlas colorido. 6ª ed. Artmed, São Paulo.

Mudge M.C., MacDonald M.H., Owens S.D. & Tablin F. 2004. Comparison of 4 blood storage methods in a protocol for equine pre-operative autologous donation. Vet. Surg. 33(5):475-486. <http://dx.doi.org/10.1111/j.1532-950X.2004.04070.x> <PMid:15362986>

Nazifi S., Saeb M., Ghafari N., Razeghian I., Razavi S.M., Vosoughi F., Dehghani F. & Orangi H. 2009. Reference values of oxidative stress parameters in adult native iranian goats. Bulg. J. Vet. Med. 12(2):119-124.

Niinistö K., Raekallio M. & Sankari S. 2008. Storage of equine red blood cells as a concentrate. Vet. J. 176(2):227-231. <http://dx.doi.org/10.1016/j.tvjl.2007.02.015> <PMid:17449302>

Nunes T.L., Oliveira M.G.C., Paiva A.L.C., Bezerra T.C.G., Barrêto-Júnior R.A. & Paula V.V. 2014. Valores hemogasométricos e eletrolíticos de caprinos (*Capra Hircus*) da raça Canindé criados no semiárido nordestino. Revta Bras. Med. Vet. 36(3):255-260.

Pugh D.G. 2004. Clínica de Ovinos e Caprinos. Roca, São Paulo, p.403-502.

Reichmann P. & Dearo A.C.O. 2001. Transfusão de sangue e seus derivados em grandes animais. Semina, Ciênc. Agrárias 22(2):223-228.

Sousa R.S. 2012. Avaliação da transfusão sanguínea homóloga em ovinos. Dissertação de Mestrado, Universidade Federal Rural do Semi-Árido, Mossoró, RN. 168p.

Sousa R.S., Barros I.O., Tavares M.D., Sousa I.K.F., Oliveira G.B., Minervino A.H.H. & Barrêto-Júnior R.A. 2012a. Lesões de armazenamento durante a conservação de sangue nas diferentes espécies: uma revisão. Acta Vet. Bras. 6(2):68-79.

Sousa R.S., Chaves D.F., Barrêto-Júnior R.A., Sousa I.K.F., Soares H.S., Barros I.O., Minervino A.H.H. & Ortolani E.L. 2012b. Clinical, haematological and biochemical responses of sheep undergoing autologous blood transfusion. BMC Vet. Res. 8(1):61-67. <http://dx.doi.org/10.1186/1746-6148-8-61> <PMid:22607611>

Sousa R.S., Barrêto-Júnior R.A., Sousa I.K.F., Chaves D.F., Soares H.S., Barros I.O., Minervino A.H.H. & Ortolani E.L. 2013. Evaluation of hematologic, blood gas, and select biochemical variables in ovine whole blood stored in CPDA-1 bags. Vet. Clin. Pathol. 42(1):27-30. <http://dx.doi.org/10.1111/vcp.12014> <PMid:23278354>

Tavares M.D. 2013. Avaliação hematológica, bioquímica e hemogasométrica de sangue caprino armazenado em bolsas CPDA-1 e CPD/SAG-M. Dissertação de Mestrado, Universidade Federal Rural do Semi-Árido, Mossoró, RN. 73p.

Tennant B.C. & Center S.A. 2008. Hepatic function, p.379-412. In: Kaneko J.J., Harvey J.W & Bruss M.L. (Eds), Clinical Biochemistry of Domestic Animals. 6th ed. Academic Press, San Diego. <http://dx.doi.org/10.1016/B978-0-12-370491-7.00013-1>.

Tomczak A.C.T.Q., Grilo K.T.M., Castro J.M., Machado A.M.B., Leonart M.S.S. & Nascimento A.J. 2010. Estudo de métodos laboratoriais para o controle de qualidade de unidades transfusionais eritrocitárias no Centro de Hematologia e Hemoterapia do Paraná (Hemepar). Revta Bras. Hematol. Hemoter. 32(3):209-214. <http://dx.doi.org/10.1590/S1516-84842010005000076>

Wallas C.H. 1979. Sodium and potassium changes in blood bank stored human erythrocytes. Transfusion 19(2):210-215. <http://dx.doi.org/10.1046/j.1537-2995.1979.19279160297.x> <PMid:155333>

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Original Article
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Causes of death in goats in Rio Grande do Sul state, Brazil: analysis of 322 cases (2000-2016)[1]

Daniele M. Bassuino[2,3], Guilherme Konradt[2], Matheus V. Bianchi[2], Gustavo G.M. Snel[4], Luciana Sonne[2], Saulo P. Pavarini[2] and David Driemeier[2]*

ABSTRACT.- Bassuino D.M., Konradt G., Bianchi M.V., Snel G.G.M., Sonne L., Pavarini S.P. & Driemeier D. 2018. **Causes of death in goats in Rio Grande do Sul state, Brazil: analysis of 322 cases (2000-2016).** *Pesquisa Veterinária Brasileira 38(11):2080-2087*. Setor de Patologia Veterinária, Departamento de Patologia Clínica Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil. E-mail: davetpat@ufrgs.br

The diagnosis of the cause of death in goats submitted to necropsy from January 2000 to December 2016 by Setor de Patologia Veterinária from the Universidade Federal do Rio Grande do Sul was reviewed. Epidemiological features, such as the breed, sex and age, in addition to the clinical and pathological features were evaluated. During this period, 322 goats were necropsied, in which a conclusive diagnosis was obtained in 290 (90%) goats. Goats that were part of other experimental study were excluded from this study. From these 290 cases, 167 (57.6%) corresponded to diseases of infectious origin and toxinfectious diseases, while 123 (42.4%) were classified as non-infectious conditions. Infectious diseases included 55 cases of bacterial origin, 59 cases with parasitary involvement, 14 cases of viral origin, and 39 toxinfectious cases. Non-infectious conditions were grouped into metabolic diseases (44 cases), plants or chemical substances poisoning (36), mineral and nutritional deficiencies (20), and neoplasms and developmental disorders (5). In the remaining 18 cases, a conclusive diagnosis was obtained, however the conditions did not fit into those criteria and were classified as "others". The age range of the goats in this study was from 1 day-old to 10 years-old. Most of the goats were females (201), while 121 were males. Affected breeds included Boer, Saanen, Anglo-Nubian, Toggenburg and mixed breeds. Parasitic, infectious and toxin-infectious diseases were the main cause of deaths, especially haemonchosis, pleuropneumonia, eimeriosis and enterotoxemia. Among the non-infectious conditions, metabolic disorders, especially rumen acidosis, pregnancy toxemia and urolithiasis, were directly related to the management employed in the property. Plant poisoning diagnosis was also highlighted with locally present plants, such as *Sida carpinifolia*, as the most important.

INDEX TERMS: Causes of death, goats, Rio Grande do Sul, diagnosis, pathology, small ruminants, infectious diseases.

RESUMO.- [**Causas de morte em caprinos diagnosticadas no estado do Rio Grande do Sul: análise de 322 casos (2000-2016).**] Foram revisadas as causas de morte de caprinos submetidos ao exame de necropsia no período compreendido entre janeiro de 2000 e dezembro de 2016 pelo Setor de Patologia Veterinária da Universidade Federal do Estado do Rio Grande do Sul, Brasil. Avaliaram-se os dados epidemiológicos relacionados à raça, sexo e idade, assim como as alterações macroscópicas e microscópicas observadas. Durante esse período, foram recebidos para necropsia 322 caprinos, nos quais em 290 casos (90%) foi obtido o diagnóstico conclusivo. Caprinos destinados à experimentação animal foram excluídos deste estudo. Do total de 290 casos, 167 (57,6%) corresponderam a enfermidades

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[2] Setor de Patologia 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] Universidade de Cruz Alta (Unicruz), Rodovia Municipal Jacob Della Méa Km 5.6, Cruz Alta, RS 98005-972, Brazil.

[4] Fellowship CAPES/Brazil (Ciência sem Fronteiras Program, Italy-Brazil).

de origem infecciosa e toxi-infecciosa, enquanto 123 (42,4%) em causas não infecciosas. Entre as doenças infecciosas foram contabilizados 55 casos de origem bacteriana, 59 casos com envolvimento parasitário, 14 casos de origem viral, e 39 casos toxi-infecciosos. As doenças de caráter não infeccioso foram agrupadas em doenças metabólicas (44 casos), intoxicações por plantas ou substâncias tóxicas (36), deficiências minerais e nutricionais (20), neoplasias e distúrbios no desenvolvimento (5). Dezoito casos com diagnóstico conclusivo, mas que não se enquadravam nestas classificações foram agrupadas como "outros". A faixa etária dos caprinos neste estudo variou de um dia a 10 anos. A maior parte dos animais eram fêmeas (201), enquanto 121 eram machos. As raças dos caprinos necropsiados neste período incluíram Boer, Saanen, Anglo-Nubiano, Toggenburg e sem raça definida (SRD). As enfermidades de origem parasitárias, infecciosas e toxi-infecciosas, especialmente hemoncose, eimeriose, pleuropneumonias e a enterotoxemia acarretaram o maior número de mortes. Das causas não infecciosas, distúrbios metabólicos, como acidose lática ruminal, toxemia da prenhez e urolitíase, estiveram diretamente relacionadas com o manejo empregado nas propriedades. Evidenciou-se também a importância do diagnóstico de intoxicações por plantas relevantes da região, como *Sida carpinifolia*.

TERMOS DE INDEXAÇÃO: Causas de morte, caprinos, Rio Grande do Sul, diagnóstico, patologia, pequenos ruminantes, doenças infecciosas.

INTRODUCTION

Goat/sheep farming has been a prominent activity in the Brazilian agribusiness with a herd estimated at 14 million animals distributed on 436,000 agricultural establishments (Brasil 2015). In 2015, the national caprine herd reached the number of 9,614,722 with an increase of 8.6% compared to 2014 (IBGE 2015). The progressive growth of goat farming was accompanied by an increase in the productive capacity of this species, which has been achieved through several aspects, especially to the genetic improvement aiming an increase in milk and meat production (Resende et al. 2008). Goats are affected by several diseases, including some that are common to other livestock ruminants, while others are specific for this species and may present a zoonotic behavior (Kumar et al. 2003). Rosa et al. (2013) described the causes of death in caprine diagnosed in the central region of Rio Grande do Sul through a retrospective study in a 48-year period in which the infectious and parasitic causes were the most common causes of death, accounting for 40.35% of the conclusive diagnosis. On the other hand, Debien et al. (2013) diagnosed the causes of death of 152 necropsied goats in Quebec, and the most frequent diseases included enterotoxemia (17.1%), pneumonia (13.8%), Johne's disease (10.5%), listeriosis (6.6%), pregnancy toxemia (5.3%), caprine arthritis encephalitis (4.6%), and caseous lymphadenitis (3.9%). Kusiluka et al. (1998) highlighted the importance of ectoparasites and endoparasites in goat farming, and concluded that the helminths, diarrheas and respiratory diseases were the main causes of death and were directly influenced by the handling in the properties of Tanzania. The knowledge regarding the local and regional realities of goat farming is essential to elaborate preventive strategies, aiming the improvement in the sanitary, nutritional and productive aspects for this species. However, retrospective studies addressing the causes of death in goats in Brazil are scarce, making it hard to estimate the economic losses related to these deaths in this activity (Rosa et al. 2013). The aim of this study is to determine the frequency and the clinical-pathological features of the main causes of death diagnosed in goats in Rio Grande do Sul state, Brazil, from January 2000 to December 2016.

MATERIALS AND METHODS

A retrospective study aiming the causes of death in goats was conducted through the analysis of the necropsy reports database from the Setor de Patologia Veterinária of Universidade Federal do Rio Grande do Sul, SPV-UFRGS from January 2000 to December 2016. All goats were from Rio Grande do Sul state, mainly from the metropolitan area of Porto Alegre. Data regarding the epidemiological features, such as age, sex and breed were grouped and registered, as well as the clinical signs, pathological findings and the conclusive diagnosis. These were finally grouped into two main categories: infectious diseases and non-infectious diseases. Infectious diseases were further subdivided in bacterial, viral, parasitic, and toxic infectious diseases, while noninfectious diseases were subdivided as metabolic, plant and chemical substances poisonings, mineral and nutritional deficiency, developmental disorders and neoplasms. Cases that did fit in the above-mentioned etiologies were categorized as "others".

RESULTS

In the analyzed period, 322 goats were submitted to necropsy and evaluated in this study, from which 290 (90%) cases had a conclusive diagnosis, while 32 (10%) were inconclusive. From all the 290 cases, 167 (57.6%) corresponded to infectious and toxinfectious diseases, 123 (42.4%) to noninfectious diseases, and 18 (6.2%) corresponded to "others". From the infectious diseases, 59 of the cases were caused by parasites, 55 by bacteria, 39 toxinfectious, and 14 by virus. The frequency of occurrence of infectious and toxinfectious conditions is shown in Table 1.

The infectious parasitic diseases, mainly represented by haemonchosis and eimeriosis, were the main causes of death in goats in this study with 14.5% and 3.5% of the cases, respectively. *Haemonchus* sp. parasitism caused the death of 42 goats from four months-old to eight years-old, usually with an endemic occurrence on the herds. Eight of these goats were from properties in which *Haemonchus* sp. outbreaks occurred with a mortality rate varying from 8 to 12%. The other cases occurred individually on the evaluated herds. Clinically, there were weight loss, apathy, anorexia, submandibular edema, opaque and unruly skin, anemia and diarrhea in a clinical evolution of two to seven days. The necropsy findings were characterized by generalized pallor of the mucosa, blood with an aqueous aspect, ascites, hydropericardium, pallor of viscera in the abdominal cavity, epicardial serous atrophy, mesentery and mesocolon edema, and large amounts of adhered or free filiform and cylindrical reddish parasites (morphologically compatible with *Haemonchus* sp.) in the abomasum. The abomasum content had an aqueous aspect with a dark reddish color. Haemonchosis diagnosis was obtained through the epidemiological features, clinical signs, and pathological findings, and it was facilitated by the observation of large amounts of parasites compatible with *Haemonchus* sp. within the abomasum. Eimeriosis affected 10 goats from 30 days-old

Table 1. Infectious and toxinfectious diseases diagnosed in goats in Rio Grande do Sul state, Brazil

Infectious diseases	Number of cases
Bacterial	
Bacterial pleuropneumonia	26
Septicemias	11
Abscedative necrotic hepatitis	4
Listeriosis	3
Bacterial enteritis	2
Suppurative meningitis (*Escherichia coli*)	3
Caseous lymphadenitis	1
Suppurative meningoencephalomyelitis	1
Acute bacterial mastitis	1
Paratuberculosis	1
Salmonellosis	1
Rhodococcosis	1
Parasitic	
Haemonchosis	42
Eimeriosis	10
Toxoplasmosis	4
Verminous pneumonia (*Muellerius capillaris*)	2
Cenurosis	1
Toxinfectious	
Clostridium perfringens type D Enterotoxemia	35
Tetanus	3
Neurotoxicosis (*Aspergillus clavatus*)	1
Viral	
Caprine arthritis-encephalitis	7
Viral pneumonia	4
Contagious ectima	3
TOTAL	167

to four months-old, with death rates that varied from 8% to 15% in the attended herds. Two necropsied goats were from two outbreaks on distinct properties that had death rates from 11% to 13%. Environmental and handling factors, such as high stocking densities and a mixture of animals from different categories and ages were related to these cases. The clinical signs were characterized by weight loss, reduced growth, and chronic diarrhea, in a clinical course of 30 to 40 days. At necropsy, there were mild white nodular elevated lesions ranging from 0.5 to 1.5cm in diameter in the small intestine's mucosa, predominantly involving the jejunum and ileum segments, in addition to diffuse pale mucosae and submandibular subcutaneous edema. Histologically, villous hyperplasia and intestinal crypts associated with numerous protozoal structures in different developmental phases, including macrogametocytes and microgametocytes, morphologically compatible with *Eimeria* sp., as well as associated inflammatory infiltrate of lymphocytes, plasma cells and macrophages on the lamina propria.

Bacterial diseases accounted for 19% of the cases (55/290), of which pleuropneumonia was the most common diagnosis, with 9% of all the causes of death in goats of the present study. Goats had an age range of 10 months-old to 4 years-old, with a death rate of 8% within the herds, and a lethality of 90%. The outbreaks occurred in association to predisposing factors of stress, mainly due to prolonged transport and environmental changes. Clinically, there were apathy, fever, cough, mucopurulent nasal discharge, and dyspnea, with a clinical course of two to five days. At necropsy, there were extensive multifocal to coalescent areas of cranioventral consolidation of the lungs associated to fibrin deposits over the visceral pleura, as well as pleural adhesions, in addition to marked pulmonary edema. Microscopical lesions consisted of abundant inflammatory infiltrate involving the alveoli, bronchi and bronchioles of viable and degenerate neutrophils, as well as mild number of lymphocytes and macrophages frequently associated to fibrin deposits on the visceral pleura, in addition to marked and diffuse alveolar edema. *Mannheimia haemolytica* was identified in 22 cases through bacteriological and molecular analysis; *Escherichia coli* and *Streptococcus* sp. were isolated in one case each through microbiological culture.

Viral diseases occurred in 14 cases (4.8%), of which caprine arthritis encephalitis virus (CAEV) was diagnosed in seven cases (2.4%). Affected goats were females with an age range of 10 to 12 days-old, predominantly raised in semi-intensive system. The condition had mainly an endemic occurrence in properties that presented a history of neurological disorders in young and adult animals with pneumonia and lameness as well. Common pathological findings included multifocal lymphoplasmacytic meningoencephalitis with perivascular cuffs and areas of moderate microgliosis. Moreover, four goats also had pneumonia and interstitial mastitis, which were compatible with the infection by CAEV.

In the toxinfectious category, *Clostridium perfringens* type D enterotoxemia was the most frequent diagnosis, accounting for 35 (12%) cases of the conclusive diagnosis. The age ranged from 17 days-old to 4 years-old, with an occurrence in outbreaks reaching mortality rates of 5% to 20%, and a lethality of 100%. Predominantly, the goats were originated from unvaccinated herds, or previously vaccinated in less than four months. Clinical signs varied from sudden death to apathy, anorexia, fever, intense abdominal pain, and in some cases diarrhea with a clinical course of 12 hours to 3 days. At necropsy, there were intense congestion of the serous layer of the intestines, in addition to distension of the small and large intestine, which were filled by large amounts of reddish liquid occasionally containing filaments of fibrin content at the final segment of the ileum, cecum and colon. The kidneys had a gelatinous aspect and soft consistency (pulpy kidney). Histologically, there was a mild to moderate necrosis of the intestinal villi associated to fibrin deposition over the mucosa, in addition to basophilic bacillary bacteria and necrotic debris. The submucosa had also a diffuse inflammatory infiltrate composed of lymphocytes, macrophages and plasma cells. The kidneys had a diffuse and severe autolysis. The diagnosis was confirmed through clinical history, pathological aspects, and associated to the detection of the agent by microbiological culture, and by serum neutralization test in mice.

The noninfectious diseases were grouped into metabolic diseases, which represented 44 cases (15.2%); plants poisoning or chemical toxic substances with 36 cases (12.4%); mineral and nutritional deficiencies with 20 cases (6.9%); neoplasms and development disorders with 5 of the cases (1.7%). In this category, the following conditions were the most frequent: ruminal lactic acidosis with 17 cases (5.8%), pregnancy

toxemia in 13 cases (4.5%), and urolithiasis in 10 cases (3.4%). The frequency of the noninfectious conditions in goats is presented in Table 2.

Ruminal lactic acidosis affected goats with an age range of eight months-old to four years-old, and it was mainly associated to employment of highly fermentable diets (corn, rice, and wheat) administrated without a previous adaptation period of the animals. Most of the goats with ruminal lactic acidosis were from different properties which employed semi-intensive raising systems; however, most of the cases occurred individually. Yet, two goats were from properties in which outbreaks took place with mortality rates of up to 20%. Clinical signs consisted of depression, anorexia, prostration, dehydration, abdominal distention, occasionally bloat and death, with a clinical course of 12 hours to 2 days. Necropsy findings were characterized by moderate to severe abdominal distention, with abundant yellowish liquid content intermixed by feed content (high concentration of grains) in rumen and reticulum, aside from multifocal reddish and ulcerated areas in the rumen mucosa. Histological lesions consisted of hydrophobic degeneration of the rumen mucosa with foci of inflammatory infiltrate of neutrophils and multifocal ulcerative areas of the mucosa.

Table 2. Non infectious diseases diagnosed in goats in Rio Grande do Sul state, Brazil

Non infectious diseases	Number of cases
Metabolical diseases	
Ruminal lactic acidosis	17
Pregnancy toxemia	13
Urolithiasis	10
Fibrous osteodystrophy	3
Primary bloat	1
Poisonings	
Poisoning by *Sida carpinifolia*	11
Acute toxic hepatopathy of unknown cause	10
Poisoning by defatted rice bran	4
Poisoning by *Trema micrantha*	3
Poisoning by *Baccharis megapotamica*	2
Poisoning by *Prunus sphaerocarpa*	2
Poisoning by coumarin derivatives	2
Poisoning by *Amorimia exotropica*	1
Poisoning by *Dodonaea viscosa*	1
Nutritional and mineral deficiencies	
Vitamin E and selenium deficiency	10
Malnutrition	5
Iodine deficiency (goiter)	4
Copper deficiency	1
Developmental disorders and neoplasms	
Palatoschisis	2
Vulvar agenesis	1
Pheochromocytoma	1
Hypospadias	1
Others	18
TOTAL	123

Pregnancy toxemia was directly associated with the late stage of gestation of triplets and quadruplets, affecting goats with an age range of two to eight years-old. Predisposing factors included traumatic lesions, climate and environmental changes. Clinically, there were anorexia, locomotion problems, dehydration, blindness, staggered walk, decreased eyelid reflexes, recumbency, and death, with a clinical course of two to five days-old. At necropsy, the liver was slightly enlarged, yellowish, friable and, at the surface cut there was enhanced lobular pattern. Histologically, there was intense and diffuse microvacuolar hepatocellular degeneration (hepatic steatosis).

Urolithiasis affected 10 unneutered male goats (3.4%), with an age range of two to three months-old. Only one case was associated to an outbreak, with a concomitant occurrence in seven other goats out of 60 in a herd. The remaining cases occurred individually in the properties. In three cases, there was rupture of the urinary bladder with subsequent uroperitoneum. Common necropsy findings included marked hyperemia of the urinary bladder mucosa, with abundant sandy sediments, and partially or completely obstruction of the urethra involving the sigmoid flexure, ischiatic curvature, or the urethral process. In addition to that, occasionally there were hydronephrosis and/or hydroureter.

Plants poisoning and chemical toxic substances poisoning accounted for 12.4% of total of cases, and included poisoning by *Sida carpinifolia* (11), by *Trema micrantha* (3), by *Baccharis megapotamica* (2), by cyanogenic plants, by *Amorimia exotropica* (1), by *Dodonaea viscosa* (1), as well as other 10 cases of acute hepatic necrosis suggestive of a toxic cause from distinct properties where the cause was not determined. There were another four cases of poisoning by defatted rice bran and two goats poisoned by coumarin derivatives. In these cases, the diagnosis was established through the gross and microscopical findings associated mainly to the epidemiological factors, which were acquired through visits to the properties and visualization of the suspected toxic plant, as well as through epidemiological surveys conducted with the producer and/or veterinarian responsible for the properties.

In the mineral and nutritional deficiencies category, the lack of vitamin E and selenium was diagnosed in 3.4% of all diagnosed cases. Significant differences of age and sex of the affected goats were not observed. The goats necropsied were from two properties where outbreaks that affected several animals occurred, with mortality rates ranging from 8 to 10%, and lethality rates near 100%. The clinical signs included depression, muscle tremors, instability of thoracic and pelvic limbs with evolution to recumbency in a clinical course of one to five days. Grossly, extensive pale areas were observed in the cardiac and skeletal muscles, which histologically corresponded to coagulative necrosis of muscle fibers, characterized by fragmented and sometimes flocculate cytoplasm, with hypereosinophilic sarcoplasm and loss of striations, as well as pyknotic nuclei associated with an inflammatory infiltrate of macrophages and multifocal areas of mineralization.

DISCUSSION

Among the parasitic diseases, haemonchosis and eimeriosis were the main causes of death in this study, with 14.5% (42/290) and 3.5% (10/290), respectively. The epidemiological and clinical findings of both conditions were similar to the observed by other authors (Miller et al. 2012, Rosa et al. 2013), with a clinical condition of anemia and hypoproteinemia associated

with diarrhea (Uzal et al. 2016). Haemonchosis also was the main cause of death of goats in the retrospective study performed by Rosa et al. (2013) in the central region of Rio Grande do Sul state, as well as it was the main cause of death in sheep in this region (Rissi et al. 2010); in a study of the causes of death in goats in India related to the feeding system, haemonchosis represented the third most frequent condition. *Haemonchus contortus* is considered the most pathogenic nematode of goats due to its voracious hematophagous behavior (Smith & Sherman 2009), and triggers a clinical condition of severe anemia and consequent characteristics clinical signs (Costa et al. 2009). Eimeriosis affects mainly young goats (Uzal et al. 2016), similar to the observed in this study, in which the age ranged from 1 to 4 months-old. This condition leads to economic losses due to the high morbidity and mortality, reduced growth and high costs with treatments (Kaya 2004, Temizel et al. 2011). The main species of *Eimeria* that cause clinical disease in caprine are: *E. arloingi*, *E. christenseni* and *E. ninakohlyakimovae*. These cause a morbidity that varies from 38% to 100% (Smith & Sherman 2009), which is distinct from the observed in this study, in which goats were affected individually in the herds, and this may be directly influenced by the environmental and handling conditions of the species. The necropsy findings consisted of elevated white nodular lesions in the mucosa of the small intestine and large intestine, besides the histopathological lesions, which were similar to the described in the literature (Silva et al. 2014, Uzal et al. 2016).

Clostridium perfringens type D enterotoxemia represented the second infectious disease with higher impact in the mortality of goats in this study, accounting for 12% (35/290) of the cases of infectious and toxinfectious diseases. This is below than the observed in previous studies (Ershaduzzaman et al. 2007, Debien et al. 2013), and above from the found by Pawaiya et al. (2017). These differences may occur since casuistic is directly related with the different practices of handling employed in the properties. Predisposing factors include sudden modification of the gastrointestinal microbiota, which may occur with diets rich in carbohydrates, proteins and highly fermentable pastures, and are associated with the clinical disease, since it allows rapidly growth of *C. perfringens* and the production of its toxins (Kriek et al. 1994, Smith & Sherman 2009). These factors are epidemiologically related with the number of cases in each property and were compatible with the ones found in the present study. Enterotoxemia affects goats of variable ages and the pathogenesis is influenced by production of alpha and epsilon toxin by the bacteria (Uzal & Songer 2008, Nasir et al. 2013), however in the present study the diagnosis of the condition was based on the clinical, epidemiological and pathological aspects of the condition, sometimes associated to the detection of the agent by microbiological culture or through serum neutralization in mice (Colodel et al. 2003). Unvaccinated animals tolerate low levels of toxin without developing clinical signs (Uzal et al. 2002). Polyvalent vaccines provide smaller titers in goats when compared to the sheep (Uzal et al. 1998), as well as individuals may respond differently to the vaccine, depending on the vaccination intervals and on the adjuvants present in the vaccine. The higher mortality rate observed in this study was attributed to the absence of vaccination, as well as possible flaws in the process of immunization, aside from the short period between the vaccines and the challenge. Differential diagnosis for enterotoxemia included the conditions that cause sudden death in goats, such as other agents that cause diarrhea in adults (coccidiosis and salmonellosis) and also in young animals (cryptosporidiosis and colibacillosis) (Smith & Sherman 2009). A retrospective study of the causes of death in goats in India that specifically involved the gastrointestinal tract demonstrated that enteritis, paratuberculosis, haemonchosis, inanition, gastrointestinal parasites, bloat, and enterotoxemia were also the main causes of death diagnosed over a 25 years period of the study (Pawaiya et al. 2017).

Bacterial pleuropneumonias represented 9% of the cases (26/290). *Mannheimia haemolytica* was identified in 22 cases through bacteriologic and molecular analysis. In one case, *Escherichia coli* was identified, and in another *Streptococcus* sp. was identified through microbiological analysis. The gross findings of pleuropneumonia by *M. haemolytica* in goats are similar to the observed in other ruminants, and include cranioventral areas of consolidation occasionally followed by fibrinous pleuritis (Caswell & Williams 2016). Acute pulmonary infections caused by this agent are characterized by a necrotic and fibrinosuppurative inflammation that occurs in response to the release of *M. haemolytica* toxins, as well as the release of cytokines by neutrophils and other inflammatory cells (Yener et al. 2009). Microscopically, this condition is characterized by hemorrhage, alveolar necrosis, fibrin exudation, interlobular septal edema, as well as degenerate neutrophils with an 'oat' shape, which are characteristic of *M. haemolytica* lesions and essential for the pathological diagnosis (Smith & Sherman 2009, Woolums & Wilkins 2014, Caswell & Williams 2016). Predisposing factors such as climate and stressful situations related to the handling are correlated with the emergence of cases of acute pneumonia (Caswell & Williams 2016). Moreover, the mixture of animals of different origins increases the exposure to pathogenic agents. The cases of pleuropneumonia by *M. haemolytica* in goats of this study probably occurred due to the agglomeration of animals from different origins, which were also submitted to the prolonged transport as a stress factor. In addition to this condition, other possible common and concomitant diseases as cachexia and subclinical haemonchosis may increase the susceptibility of these animals to the bacteria.

CAEV infection was diagnosed in 2.4% of the cases of infectious origin (7/290). All goats necropsied in this study were young females. However, the infection by CAEV typically involves both males and females, of many breeds and age ranges (Rowe & East 1997), in a way that is difficult to establish a possible predisposing factor associated to the sex or age of the goats affected due to several factors that may be associated to handling in a property. The clinical and anatomopathological presentation of the CAEV infection manifests in four basic clinical manners: nervous, articular, respiratory and mammary (Peretz et al. 1993). The articular form is the most prevalent in adult goats, while the nervous is considered the less important, and both are observed mainly in 1 to 4 months-old goats, or sporadically in older animals associated with polyarthritis (East et al. 1993, Callado et al. 2001). The goats of the present study had a lymphoplasmacytic meningoencephalitis as the most frequent form observed in the

young females necropsied, which was occasionally associated to pneumonia and interstitial mastitis. The classic neurological lesion caused by CAEV is a demyelinating encephalomyelitis, though the myelinated fibers in the inflammatory focus stay intact in the initial phase of the disease (Cantile & Youssef 2016). This may explain the absence of demyelinating lesions in the cases of the present study, since the analyzed goats were mostly in the beginning of the clinical evolution. The farming handling methods are important factors that contribute to the viral dissemination. In this study, the goats were predominantly raised in a semi-intensive system, which provides the agglomeration of the animals and favors the viral infection and the contamination of the environment (Castro & Melo 2001). CAEV infections diagnosis is primarily based on the clinical findings and confirmed through the laboratorial tests for direct detection of the virus, its genetic material or by serological surveys (Pinheiro et al. 2009). The CAEV diagnosis in this study was based through the clinical and pathological findings associated to a previous history of endemic occurrence of the condition in the properties. Knowles (1997) reported that CAEV seroprevalence in a herd may reach 90%; however, most of the animals have no clinical signs and represent a potential source of infection.

The non-infectious diseases accounted for 42.41% (123/290) of the diagnosed cases. Ruminal acidosis was diagnosed in 5.8% (17/290) of the cases, and its occurrence was in according to previous descriptions of grain overload, with an abrupt change in the diet to high fermentation aliments that promote the growth of Gram-positive bacteria and the production of lactic acid (Gelberg 2013), which are common predisposing factors also observed in the goats of this study. The diagnosis in these cases was obtained through the observation of an aqueous ruminal content intermixed by large amounts of grains in the rumen, as well as detachment of ruminal mucosa associated with submucosal hyperemia., in addition to characteristic histological lesions, which included hydropic degeneration of the ruminal epithelium, with the formation of vesicles and areas of erosion and ulceration of the rumen mucosa (Afonso & Mendonça 2007, Gelberg 2013).

Ketosis in goats usually occurs at the end of gestation (pregnancy toxemia) and also may occur during the lactation peak due to the negative energy balance of these periods (Smith & Sherman 2009). In the present study, this condition was diagnosed in 4.5% (13/290) of the cases in which it was possible to observe a direct association of the condition in goats with gestations of triplets and quadruplets, aside to the predisposing stressful factors, such as periods of food restriction, feeding change at the end of gestation, transport, changes in the environment or climatic disturbances (Schild 2007), which were compatible with those reported in the historic by the producers in the cases of the present study.

Urolithiasis was diagnosed in 3.4% (10/290) of the cases. Uroliths may occur both in males and females, but obstruction is rarely observed in females due to the anatomical characteristics. All cases of urolithiasis involved 2 to 3 months-old males. This higher frequency observed in males is justified by a smaller urethral diameter with higher number of cases of obstructions verified in the sigmoid flexure, ischial curvature and urethral process (Riet-Correa et al. 2008), as observed in the cases of this study. Ruminants fed with grains or its sub products are more prone to develop urolithiasis due to a reduced saliva production and the high content of phosphorus in these aliments, as well as an increased formation of mucoproteins in the urine that contribute to form the organic core of calculi (Radostits et al. 2000).

The diagnosis of plant poisoning and chemical toxic substances poisoning accounted for 12.4% (36/290) of the cases. Toxic plants of animal interest lead to significant losses in the production of livestock, with direct losses related to animal mortality (Riet-Correa & Medeiros 2001). *Sida carpinifolia*, *Trema micrantha* and *Baccharis megapotamica* poisonings were the most prevalent in this study, which differs from other retrospective studies conducted in different regions of Brazil (Silva et al. 2006, Mello et al. 2010). These data reinforce the importance of these regional studies in determining the frequency of some diseases.

The vitamin E and selenium deficiency occurs in animals maintained in pastures where the soil is poor in vitamin E and selenium, or that are fed with deficient feed in these elements (Radostits et al. 2000). In this study, this deficiency represented 3.4% of the cases (10/290), which is below to the observed by Rosa et al. (2013) that described high mortality rates in the outbreaks, especially in young goats. In the Rio Grande do Sul state, mortality outbreaks in goats related to this deficiency have already been described by Loretti et al. (2001) in goats of many ages, similar to the one observed in the present study in which no breed, sex or age range predisposition was observed.

CONCLUSIONS

The parasitic infections and toxinfectious diseases, especially haemonchosis, eimeriosis, pleuropneumonias and enterotoxemia were the most frequently diagnosed diseases in the analyzed period.

Among the non-infectious conditions, metabolic disturbances were highlighted, especially ruminal lactic acidosis, pregnancy toxemia, and urolithiasis, which are diseases directly related to the handling in a property, while the relevant plant poisoning was represented by *Sida carpinifolia* poisoning.

REFERÊNCIAS

Afonso J.A.B. & Mendonça C.L. 2007. Acidose láctica ruminal, p.313-319. In: Riet-Correa F., Schild A.L., Lemos R.A.A. & Borges J.R.J. (Eds). Doenças de Ruminantes e Equídeos. Vol.2. 3ª ed. Palloti, Santa Maria. 691p.

Brasil. 2015. Ministério da Agricultura, Pecuária e Abastecimento. Caprinos e Ovinos. Available at <http://www.agricultura.gov.br/animal/especies/caprinos-e-ovinos> Accessed on Jan. 1, 2017.

Callado A.K.C., Castro R.S. & Teixeira M.F.S. 2001. Lentivírus de pequenos ruminantes (AEV e Maedi-Visna): revisão e perspectivas. Pesq. Vet. Bras. 21(3):87-97. <http://dx.doi.org/10.1590/S0100-736X2001000300001>

Cantile C. & Youssef S. 2016. Nervous System, p.251-406. In: Jubb K.V.F., Kennedy P.C. & Palmer's N. Pathology of Domestic Animals. Vol.1. 6th ed. Academic Press, New York. 796p.

Castro R.S. & Melo L.E.H. 2001. CAEV e Maedi-Visna: importância na saúde e produtividade de caprinos e ovinos e a necessidade de seu controle no Nordeste brasileiro. Ciênc. Vet. Trop. 4(2/3):315-320.

Caswell J.L. & Williams K.J. 2016. Respiratory system, p.465-592. In: Jubb K.V.F., Kennedy P.C. & Palmer's N (Eds), Pathology of Domestic Animals. Vol.2. 6th ed. Academic Press, New York. 592p.

Colodel E.M., Driemeier D., Schmitz M., Germer M., Nascimento R.A.P., Assis R.A., Lobato F.C.F. & Uzal F.A. 2003. Enterotoxemia em caprinos no Rio Grande do Sul. Pesq. Vet. Bras. 23(4):173-178. <http://dx.doi.org/10.1590/S0100-736X2003000400006>

Costa V.M.M., Simões S.V.D. & Riet-Correa F. 2009. Doenças parasitárias em ruminantes no semi-árido brasileiro. Pesq. Vet. Bras. 29(7):563-568. <http://dx.doi.org/10.1590/S0100-736X2009000700011>

Debien E., Hélie P., Buczinski S., Lebœuf A., Bélanger D. & Drolet R. 2013. Proportional mortality: a study of 152 goats submitted for necropsy from 13 goat herds in Quebec, with a special focus on caseous lymphadenitis. Can. Vet. J. 54(6):581-587. <PMid:24155449>

East N.E., Rowe W.J., Dahlberg J.E., Theilen G.H. & Pederson N.C. 1993. Models of transmission of caprine arthritis encefalites virus infection. Small Rumin. Res. 10(3):251-262. <http://dx.doi.org/10.1016/0921-4488(93)90130-A>

Ershaduzzaman M., Rahman M.M., Roy B.K. & Chowdhury S.A. 2007. Studies on the diseases and mortality pattern of goats under farm conditions and some factors affecting mortality and survival rates in black bengal kids. Bangl. J. Vet. Med. 5:71-76.

Gelberg H.B. 2013. Sistema Alimentar, Peritônio, Omento, Mesentério e Cavidade Peritonial, p.323-406. In: Zachary J. & McGavin M.D (Eds.), Bases da Patologia Veterinária. 5ª ed. Elsevier. Rio de Janeiro. 1344p.

IBGE 2015. Produção da Pecuária Municipal. Instituto Brasileiro de Geografia e Estatística. Available at <http://biblioteca.ibge.gov.br/visualizacao/periodicos/84/ppm_2015_v43_br.pdf> Accessed on Jan.1, 2017.

Kaya G. 2004. Prevalence of *Eimeria* species in lambs in Antakya province. Turk J. Vet. Anim. Sci. 28:687-692.

Knowles Junior J.R.D.P. 1997. Laboratory diagnostic tests for retrovirus infections of small ruminants. Vet. Clin. N. Am., Food Anim. Pract. 13(1):1-11. <http://dx.doi.org/10.1016/S0749-0720(15)30361-3> <PMid:9071743>

Kriek N.P.J., Odendaal M.W. & Hunter P. 1994. *Clostridium perfringens* type D enterotoxaemia, p.1315-1322. In: Coetzer J.A.W., Thomson G.R., Tustin R.C. (Eds), Infectious Diseases of Livestock with Special Reference to Southern Africa. Oxford: Oxford University Press, Cape Town. 874p.

Kumar S., Vihan V.S. & Deoghare P.R. 2003. Economic implication of diseases in goats in India with reference to implementation of a health plan calendar. Small Rumin. Res. 47(2):159-164. <http://dx.doi.org/10.1016/S0921-4488(02)00237-7>

Kusiluka L.J.M., Kambarage D.M., Harrison L.J.S., Daborn C.J. & Matthewman R.W. 1998. Causes of morbidity and mortality in goats in Morogoro district, Tanzania: the influence of management. Small Rumin. Res. 29(2):167-172. <http://dx.doi.org/10.1016/S0921-4488(97)00110-7>

Loretti A.P., Driemeier D., Traverso S.D. & Seitz A.L. 2001. Miopatia nutricional em caprinos no Rio Grande do Sul. Anais 10 Encontro Nacional de Patologia Veterinária, Pirassununga, SP, p.36. (Resumo)

Mello G.W.S., Oliveira D.M., Carvalho C.J.S., Pires L.V., Costa F.A.L., Riet-Correa F. & Silva S.M.M. 2010. Plantas tóxicas para ruminantes e equídeos no Norte Piauiense. Pesq. Vet. Bras. 30(1):1-9. <http://dx.doi.org/10.1590/S0100-736X2010000100001>

Miller J.E., Kaplan R.M. & Pugh D.G. 2012. Internal parasites, 125p. In: Pugh D.G. & Baird A.N. (Eds), Sheep and Goat Medicin. 2nd ed. Elsevier Saunders. <http://dx.doi.org/10.1016/B978-1-4377-2353-3.10006-X>.

Nasir A.A., Younus M., Rehman M.U., Lateef M., Khaliq S.A., Ahmad I. & Abbas M. 2013. Hematological and some biochemical alterations in sheep experimentally infected with *C. perfringens* type D infection. J. Anim. Plant Sci. 23:1553-1558.

Pawaiya R.V.S., Singh D.D., Gangwar N.K., Gururaj K., Kumar V., Paul S., Sharma N., Mishra A.K., Kumar A., Sharma D.K. & Singh S.V. 2017. Retrospective study on mortality of goats due to alimentary system diseases in an organized farm. Small Rum. Res. 149:141-146. <http://dx.doi.org/10.1016/j.smallrumres.2017.02.007>

Peretz G., Asso J. & Devillechaise P. 1993. Le C.A.E.V.: revue des connaissances actuelles et consequences pratiques. Rev. Méd. Vét. 144:93-98.

Pinheiro R.R., Ximenes L.J.F., Pinheiro A.A. & Teixeira M.F.S. 2009. Lentivírus de Pequenos Ruminantes: Diagnóstico, Prevenção e Vacinas. Embrapa Caprinos e Ovinos. Available at <https://www.alice.cnptia.embrapa.br/bitstream/doc/572791/1/PLRizaldo.pdf> Accessed on Mar. 1, 2018.

Radostits O.M., Gay C.C., Blood D.C. & Hinchcliff K.W. 2000. Clínica Veterinária: um tratado de doenças dos bovinos, ovinos, suínos, caprinos e equinos. 9ª ed. W.B. Saunders, London, p.441-447.

Resende K.T., Silva H.G.O., Lima L.D. & Teixeira I.A.M.A. 2008. Avaliação das exigências nutricionais de pequenos ruminantes pelos sistemas de alimentação recentemente publicados. Revta Bras. Zootec. 37(spe):161-177. <http://dx.doi.org/10.1590/S1516-35982008001300019>

Riet-Correa F. & Medeiros R.M.T. 2001. Intoxicações por plantas em ruminantes no Brasil e no Uruguai: importância econômica, controle e riscos para a saúde pública. Pesq. Vet. Bras. 21(1):38-42. <http://dx.doi.org/10.1590/S0100-736X2001000100008>

Riet-Correa F., Simões S.V.D. & Vasconcelos J.S. 2008. Urolitíase em caprinos e ovinos. Pesq. Vet. Bras. 28(6):319-322. <http://dx.doi.org/10.1590/S0100-736X2008000600010>

Rissi D.R., Pierezan F., Oliveira Filho J.C., Fighera R.A., Irigoyen L.F., Kommers G.D. & Barros C.S.L. 2010. Doença de ovinos da região Central do Rio Grande do Sul: 361 casos. Pesq. Vet. Bras. 30(1):21-28. <http://dx.doi.org/10.1590/S0100-736X2010000100004>

Rosa F.B., Caprioli R.A., Silva T.M., Galiza G.J.N., Barros C.S.L., Irigoyen L.F., Fighera R.A. & Kommers G.D. 2013. Doenças de caprinos diagnosticadas na região Central no Rio Grande do Sul: 114 casos. Pesq. Vet. Bras. 33(2):199-204. <http://dx.doi.org/10.1590/S0100-736X2013000200011>

Rowe J.D. & East N.E. 1997. Risk factors for transmission and methods for control of Caprine Arthritis-Encephalitis virus infection. Vet. Clin. N. Am., Food Anim. Pract. 13:34-53.

Schild A.L. 2007. Cetose, p.281-286. In: Riet-Correa F., Schild A.L., Lemos R.A.A. & Borges J.R.J. (Eds), Doenças de Ruminantes e Equídeos. Vol.2. 3ª ed. Palloti. Santa Maria. 691p.

Silva D.M., Riet-Correa F., Medeiros R.M.T. & Oliveira O.F. 2006. Plantas tóxicas para ruminantes e equídeos no Seridó Ocidental e Oriental no Rio Grande do Norte. Pesq. Vet. Bras. 26(4):223-236. <http://dx.doi.org/10.1590/S0100-736X2006000400007>

Silva L.M.R., Vila-Viçosa M.J.M., Nunes T., Taubert A., Hermosilla C. & Cortes H.C.E. 2014. *Eimeria* infections in goats in Southern Portugal. Braz. J. Vet. Parasitol. 23(2):280-286. <http://dx.doi.org/10.1590/S1984-29612014051> <PMid:25054515>

Smith M.C. & Sherman D.M. 2009. Goat Medicine. 2nd ed. Wiley-Blackwell, Iowa. 871p. <http://dx.doi.org/10.1002/9780813818825>.

Temizel E.M., Demir G., Selcuk O., Catık S., Senlik B. & Senturk S. 2011. Effect of treatment with cylindamycine in an outbreak of coccidiosis in goat kids in Turkey. J. Biol. Environ. Sci. 5(13):37-40.

Uzal F.A. & Songer J.G. 2008. Diagnosis of *C. perfringens* intestinal infections in sheep and goats. J. Vet. Diagn. Invest. 20(3):253-265. <http://dx.doi.org/10.1177/104063870802000301> <PMid:18460610>

Uzal F.A., Plattner B.L. & Hostetter J.M. 2016. Alimentary system, p.1-259. In: Jubb K.V.F., Kennedy P.C., & Palmer's N. (Eds), Pathology of Domestic Animals. Vol.2. 6th ed. Academic Press, New York. 592p.

Uzal F.A., Bodero D.A., Kelly W.R. & Nielsen K. 1998. Variability of serum antibody responses of goat kids to a commercial *Clostridium perfingens* epsilon toxoid vaccine. Vet. Rec. 143(17):472-474. <http://dx.doi.org/10.1136/vr.143.17.472> <PMid:9829304>

Uzal F.A., Kelly W.R., Morris W.E. & Assis R.A. 2002. Effects of intravenous injection of *Clostridium perfringens* type D epsilon toxin in calves. J. Comp. Pathol. 126(1):71-75. <http://dx.doi.org/10.1053/jcpa.2001.0514> <PMid:11814324>

Woolums A.R. & Wilkins P.A. 2014. Diseases of respiratory system, p.461-637. In: Smith B. (Ed.), Large Animal Internal Medicine. 5th ed. Mosby Elsevier. 1712p.

Yener Z., Ilhan F., Ilhan Z. & Saglam Y.S. 2009. Immunohistochemical detection of *Mannheimia* (*Pasteurella*) *haemolytica* antigens in goats with natural pneumonia. Vet. Res. Commun. 33(4):305-313. <http://dx.doi.org/10.1007/s11259-008-9178-z> <PMid:18972219>

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Original Article
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Skin burn and ocular damage by calcium oxide (virgin lime) in swines[1]

Juliana T.S.A. Macêdo[2], Jair A. Ferreira Júnior[2], Karla A. Nascimento[2], Maira S.C. Lacerda[2], Nathalia E.S. Pereira[2] and Pedro M.O. Pedroso[2]*

ABSTRACT.- Macêdo J.T.S.A., Ferreira Júnior J.A., Nascimento K.A., Lacerda M.S.C., Pereira N.E.S. & Pedroso P.M.O. 2018. **Skin burn and ocular damage by calcium oxide (virgin lime) in swines.** *Pesquisa Veterinária Brasileira 38(11):2088-2091*. Laboratório de Patologia Veterinária, Fundação Universidade de Brasília, Campus Universitário Darcy Ribeiro, Via L4 Norte s\n, Brasília, DF 70910-970, Brazil. E-mail: pedrosovet@yahoo.com.br

An outbreak of skin burn and ocular damage by calcium oxide in swines is reported. The outbreak occurred in a rural property located in the municipality of Iuiu, Western Bahia, during the transport of 60 pigs from a recreation farm to a fattening farm. The observed macroscopic lesions were erythematous areas; formation of papules, vesicles, and in some cases, there was the formation of firm, parched, high brown plaques and in other cases detachment and loss of skin. His eyes were dull. Histological analysis of the skin revealed areas of necrosis of focally extensive clotting of the epidermis, and dermis, delimited in the paniculus, by cellular debris and rare neutrophils. Corneal necrosis with perforated ulcer formation, superficial epithelial necrosis, edema, and neutrophil infiltration of the cornea adjacent to the ulcer were revealed in the eye.

INDEX TERMS: Skin, burn, ocular damage, calcium oxide, virgin lime, CaO, pigs, swine, clinics.

RESUMO.- [**Queimadura de pele e lesão ocular por óxido de cálcio (cal virgem) em suínos.**] Descreve-se um surto de queimadura de pele e lesão ocular por óxido de cálcio (cal virgem) em suínos. O surto ocorreu em uma propriedade rural localizada no município de Iuiu, região Oeste da Bahia, durante o transporte de 60 suínos de uma granja de recria para uma granja de engorda. Macroscopicamente foi evidenciado que na pele dos suínos havia áreas eritematosas, formação de pápulas, vesículas, e em alguns casos havia a formação de placas elevadas marrom firmes, ressequidas e em outros casos desprendimento e perda da pele. Os olhos estavam opacos. Na microscopia da pele observaram-se áreas de necrose de coagulação focalmente extensa da epiderme, e derme, delimitadas no panículo, por restos celulares e raros neutrófilos. No olho observou-se necrose da córnea com formação de úlcera perfurada, necrose do epitélio superficial, edema e infiltração de neutrófilos na córnea adjacente a úlcera.

TERMOS DE INDEXAÇÃO: Queimadura, pele, lesão ocular, óxido de cálcio, cal virgem, CaO, suíno, clínica.

INTRODUCTION

The chemical aggression to the skin can result from the local directly application or from the absorption of substances by the gastrointestinal tract, and from its subsequent distribution to the skin, resulting in contact dermatitis. The contact dermatitis may be subdivided in irritant and allergic contact dermatitis (Hargis & Ginn 2009). The irritant contact dermatitis occurs more commonly, being defined as cutaneous reaction to an irritant concentration of the aggressor agent (Smith 2006). Most cases of contact dermatitis are caused by direct contact with substances, as corporeal or wound secretions, drugs application or exposure to acids, alkalis, soaps, detergents or irritant plants (Hargis & Ginn 2009). The strong acids and bases produce lesion immediately after the contact and can be rightfully classified as chemical burns (Conceição & Santos 2011). Calcium oxide (CaO), known as virgin lime or burnt lime, is a chemical compound used for several purposes. In livestock is commonly used to promote the disinfection of the animal's accommodations. When virgin lime gets mixed with water and perspiration, it becomes calcium hydroxide (Ca (OH) 2), an alkaloid that exposed over the skin causes local irritation (Ng & Koh 2011).

Clinical lesions include erythema, edema and vesiculation, that may progress to erosion, ulcerations, rind formation,

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[2] Laboratório de Patologia Veterinária, Fundação Universidade de Brasília (UnB), Campus Universitário Darcy Ribeiro, Via L4 Norte s\n, Brasília, DF 70910-970, Brazil. *Corresponding author: pedrosovet@yahoo.com.br

and finally there is lichenification and hyperpigmentation (Smith 2006). In humans, among the chemical agents that cause ocular burns, the alkaline substances are more frequent and are responsible for serious sequelae to the ocular health (Moon & Robertson 1983, Makino et al. 1999). The lesions by alkalis have as most frequent agents: ammonia (NH3), caustic soda (NaOH), lime (Ca (OH) 2), and potassium hydroxide (KOH) (Morgan 1987). The objective of the present study is to characterize the cutaneous and ocular lesions by (CaO) in swine.

MATERIALS AND METHODS

The outbreak occurred in a rural property located in the municipality of Iuiu (14°24'50" S, 43°33'14" W), West region of Bahia, Brazil, during transportation of 60 swine from a recreation farm to a fattening farm. The clinical historic and epistemological data were obtained with the veterinarian that attended the cases. Seventeen pigs died spontaneously, and the remaining was slaughtered *in stremis* by the state inspection service. During the slaughter, skin fragments from 30 animals were collect, a tongue, an ocular globe, and fixed in 10% formalin, routinely processed for histology, imbibed in paraffin, cut 5 micrometers thick and stained by hematoxylin and eosin (HE).

RESULTS

The outbreak occurred in January 2014, affecting 60 swine of undefined race, of approximately 35kg and 70 days of age. According to informations from the veterinarian, before the boarding, the truck body was washed and sprinkled with virgin lime for disinfection. Right after the swine boarding and due to the high temperature of the day, the driver proceeded with water sprinkling over the animals, assuming it would ease the heat. Immediately after this procedure, all animals presented agitated behavior, several were tossing, and after the skin presented reddish with vesicles and some with detachment of it. The eyes presented diffuse cornea opacity (Fig.1). Macroscopically, multifocal areas to blackened coalescent were observed in the face (burns). The dorsum region and lateral surface of the pelvic and thoracic limbs were diffusely blackened and lichenified, in addition to erythematous areas in the skin (axillae and groin) (Fig.2). There was formation of papules, vesicles, and in some cases the formation of elevated, firm, dry brown plaques (Fig.3), and in others cases the detachment and loss of skin. Skin microscopy showed areas of intense and diffuse clotting necrosis of the epidermis and dermis (Fig.4), delimited by cellular debris and rare neutrophils. In the eye was observed cornea necrosis with perforated ulcer formation, necrosis of the superficial epithelium, edema and infiltration of neutrophils in the cornea adjacent to the ulcer.

DISCUSSION

The diagnosis of skin burns and cornea ulcer by virgin lime in pigs was performed by the historic and clinical-pathological alterations. Reports of chemical burns associated to virgin lime are little described in animals (Cowart et al. 2000) In swine is reported an outbreak with high mortality (63.6%) after exposure to the combination of hydrated lime and sodium bicarbonate (Cowart et al. 2000). In humans these accidents are frequent. The victims are usually of the male gender, young, and ordinarily with occurrence in the work or domestic place (Castellano et al. 2002). Epithelial necrosis



Fig.1. Ocular injury by calcium oxide in swine. Corneal opacity. Face with multifocal areas to blackened coalescent (burns) on the face.



Fig.2. Skin burn by calcium oxide in swine. Dorsum and lateral surface of diffusely blackened and liquefied limbs. Erythematous areas on the skin (axillae and groin).



Fig.3. Skin burn by calcium oxide in swine. Dorsum region with formation of papules, vesicles and high firm, parched and brown plates.

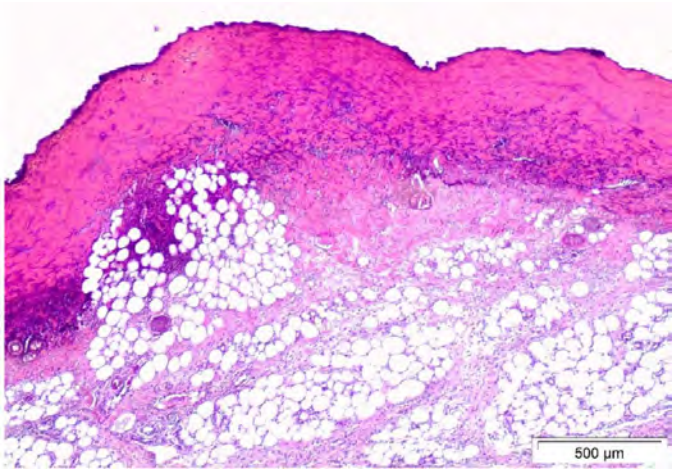


Fig.4. Skin burn by calcium oxide in swine. Areas of necrosis of intense and diffuse coagulation of the epidermis and dermis. HE, obj.10x.

occurs because the hydroxide anion saponifies fatty acids from cell membranes with resulting rupture and cell death, and cation is responsible for the free alkali (McCulley 1987, Freitas 1997). The cations react with the carboxyl groups of stromal collagens and glycosaminoglycans and the hydration of these, results in loss of transparency of the corneal stroma (Wagoner 1997).

Ocular irritation caused by disinfectants and insecticides inadvertently applied to the eye is not uncommon on farms. Chemical burns in the cornea and its attachments can have serious consequences, being the caused by alkalis more serious than the caused by acids. Corneal burns by acid tend to be acutely delimited rather than progressive, whereas alkaline burns cause progressive coagulation, dissolution, and detachment of the corneal stroma. Among the products used in disinfection of accommodations that frequently affect swine causing burns are caustic soda, virgin lime and hydrated lime, however, for its better use, one must have the appropriate knowledge in the manipulation of it to avoid accidents in humans and animals.

Both virgin lime and hydrated lime are used to paint maternity walls as a disinfection method or are scattered on the floor of the stall birthing as an aesthetic procedure in areas where piglets defecate in soft stools. When used the hydrated lime, the contact of it with water drinkers or urine produces an exothermic reaction with heat production capable of causing skin burns. When used virgin lime added with water, the formation of calcium hydroxide produces a highly alkaline suspension (pH 11-13) which does not stop the reaction immediately after drying. Thus, the biological activity of the product can maintain itself up to four days, period in which, when mixed with water, the reaction restarts and results in skin burns. In commercial farms, these problems tend to occur in the maternity causing lesions on the piglets' skin and on the mammary gland of matrices (Barcellos et al. 2007) Exceptionally, in this case, the event occurred in the transportation truck. The fact that swine have tossed explains the diffuse distribution of skin lesions, both by the action of heat and by the direct action of the irritating alkaline suspension in contact with the skin.

In the case of other differential diagnosis in domestic animals, allergic contact dermatitis (ACD) is added. Histological lesions such as vesicles, papules, spongiosis, edema and epidermal necrosis are variable, non-pathognomonic in both cases, and may be aggravated by self-trauma, being difficult to differentiate from ACD. On the other hand, lichenification, hyperpigmentation and alopecia are the result of chronic exposure to irritant agents (Gross et al. 2009, Mauldin & Peters-Kennedy 2016). Thus, minimal inflammation with intense necrosis of the epidermis, lesion distribution and number of affected animals were important for the differential. This was justified by the hyperacute nature of the process, by the self-trauma and the restricted exposure of the swine to the irritant, the concentration of the product and the nature of the caustic. In addition, a case of fatal necrolytic dermatitis of irritant character in swine related to direct contact with urine and feces, hypothetically containing tiamulin or one of its metabolites, was verified in the literature after administration of the drug (Mauldin & Peters-Kennedy 2016). However, there is no relation between the epidemiology and historic of the present outbreak with this antibiotic.

Burns in domestic animals are categorized as those that affect the partial or total thickness of the skin and the division is based on the depth of the lesion (Gross et al. 2009). Full thickness burns destroy all cutaneous structures and there is a lack of superficial blood supply and insensitivity to the touch. If full-thickness wounds cover more than 30 to 50 percent of the total surface area, then euthanasia should be considered (Rhodes 2014). In this case, because they were production animals, those animals that managed to reach the slaughterhouse were destined for emergency slaughter. Some animals died spontaneously, possibly due to the development of severe systemic shock. In severe burns, the release of inflammatory mediators may cause hemodynamic disturbances, including circulatory shock and ischemic reperfusion injury. Death can occur due to circulatory insufficiency or multiple organ failure. It is believed that multiple organ failure is the most common cause of death in patients with burns, even in the absence of uncontrolled infection (Cowart et al. 2000). Thus, the present outbreak represents an atypical condition of burn and cornea ulcer in swine, with serious lesions caused by the use of calcium oxide wet inadvertently.

CONCLUSIONS

It is recommended to avoid direct and prolonged contact of virgin lime or hydrated lime with the animals' skin to avoid irritation or possible burns.

The cases of strong alkali burns require continuous washing until the pH of the tissue returns to normal.

Neutralization of a chemical with an opposite weak acid is not recommended because the search for the neutralizing agent may delay immediate washing, and the neutralization reaction may be exothermic, causing further uncontrolled damage.

The importance of the history, clinical signs and the distribution of the lesions for the anatomopathological diagnosis of irritant contact dermatitis is also highlighted.

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REFERENCES

Barcellos D., Alberton G.C., Sobestiansky J., Donin D.G., Carvalho L.F.O.S. & Morés N. 2007. Doenças da pele, p.500-501. In: Sobestiansky J. & Barcellos D. (Eds), Doenças dos Suínos. Cânone Editorial, Goiânia.

Castellano A.G.D., Moreira H., Zago R.J. & Milicovsky F.S. 2002. Avaliação epidemiológica dos pacientes vítimas de queimadura ocular pelo agente químico cal no Serviço de Oftalmologia do Hospital Universitário Evangélico de Curitiba. Arq. Bras. Oftalmol. 65(3):311-314. <http://dx.doi.org/10.1590/S0004-27492002000300005>

Conceição L.G. & Santos R.L. 2011. Sistema tegumentar, p.423-524. In: Santos R.L. & Alessi A.C. (Eds), Patologia Veterinária. Roca, São Paulo.

Cowart R.P., Casteel S.W. & Pace L.W. 2000. Dermal necrosis and mortality from alkali exposure in a group of finisher pigs. J. Swine Health Prod. 8(5):225-227.

Freitas D. 1997. Queimadura ocular, p.35-39. In: Moreira Júnior C.A., Freitas D. & Kikuta H.S. (Eds), Trauma Ocular. Cultura Médica, Rio de Janeiro.

Gross T.L., Ihrke P.J., Walder E.J. & Affolter V.K. 2009. Doenças de Pele do Cão e do Gato: diagnóstico clínico e histopatológico. 2ª ed. Roca, São Paulo, p.91-98.

Hargis A.M. & Ginn P.E. 2009. O tegumento, p.1107-1261. In: McGavin M.D. & Zachary J.F. (Eds), Bases da Patologia em Veterinária. 4ª ed. Mosby Elsevier, Rio de Janeiro.

Makino M.C.A., Reggi J.R.A., Waetge R.T., Dantas M.C.N. & Dantas P.E.C. 1999. Perfil das queimaduras oculares na Santa Casa de São Paulo. Arq. Bras. Oftalmol. 62:472.

Mauldin E.A. & Peters-Kennedy J. 2016. Integumentary system, p.566-567. In: Maxie M.G. (Ed), Jubb, Kennedy and Palmer's Pathology of the Domestic Animals. Vol.1. 6th ed. Elsevier, St Louis. <http://dx.doi.org/10.1016/B978-0-7020-5317-7.00006-0>.

McCulley J.P. 1987. Chemical injuries, p.527-542. In: Smolin G. & Thoft R.A. (Eds), The Cornea: scientific foundation and clinical practice. 2nd ed. Little, Brown and Company, Boston.

Moon M.E. & Robertson I.F. 1983. Retrospective study of alkali burns of the eye. Aust. J. Ophthalmol. 11(4):281-286. <http://dx.doi.org/10.1111/j.1442-9071.1983.tb01094.x> <PMid:6421269>

Morgan S.J. 1987. Chemical burns of the eyes: cause and management. Brit. J. Ophthalmol. 71(11):854-857. <http://dx.doi.org/10.1136/bjo.71.11.854> <PMid:3689738>

Ng W.T. & Koh D. 2011. Occupational contact dermatitis in manual cloud seeding operations. Singapore Med. J. 52(5):e85-87. <PMid:21633757>

Rhodes V.N.C. 2014. Dermatological emergencies. Vet. Nursing J. 16(5):164-167. <http://dx.doi.org/10.1080/17415349.2001.11013178>

Smith B.P. 2006. Medicina Interna de Grandes Animais. 3ª ed. Malone, Barueri. 1728p.

Wagoner M.D. 1997. Chemical injuries of the eye: current concepts in pathophysiology and therapy. Surv. Ophthamol. 41(4):275-313. <http://dx.doi.org/10.1016/S0039-6257(96)00007-0> <PMid:9104767>

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Original Article
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Tuberculosis of the central nervous system in cattle in Paraíba, Brazil[1]

Aline M. Silveira[2]*, Eduardo M. Nascimento[2], Guilherme Konradt[3], Eldinê G. Miranda Neto[2], David Driemeier[3], Glauco José N. Galiza[2], Antonio F.M. Dantas[2] and Franklin Riet-Correa[2,4]

ABSTRACT: Silveira A.M., Nascimento E.M., Konradt G., Miranda Neto E.G., Driemeier D., Galiza G.J.N., Dantas A.F.M. & Riet-Correa F. 2018. **Tuberculosis of the central nervous system in cattle in Paraíba, Brazil.** *Pesquisa Veterinária Brasileira 38(11):2092-2098*. Laboratório de Patologia Animal, Hospital Veterinário, Universidade Federal de Campina Grande, Avenida Universitária s/n, Santa Cecília, Patos, PB 58700-970, Brazil. E-mail: silveira.a.monteiro@gmail.com

This paper describes six cases of tuberculosis in the central nervous system (CNS) of cattle in the state of Paraíba in northeastern Brazil. We reviewed the autopsy reports of 851 bovine necropsies performed from 2003 to 2016. Seventy-three (8.6%) cattle were diagnosed with tuberculosis and six showed lesions in the CNS. Three cases affected cattle up to two-year-old and other three affected adults. Three cattle presented exclusively nervous signs, two had respiratory signs and weight loss and one did not present any clinical signs. At necropsy, five cattle had thickening of the leptomeninges of the cerebellum, pons, obex, spinal cord and cortex, mainly, in the region near the brain basilar Willis´ circle. Another animal, presented a single focal lesion in the cerebellum. Microscopically we observed moderate to severe granulomatous meningitis and encephalitis. Five cattle presented lesions in the lungs and mediastinal lymph nodes and three of them had disseminated lesions in other organs. In all cattle acid-fast bacilli were observed in the lesions and marked positive for immunohistochemistry with polyclonal antibody anti-*Mycobacterium tuberculosis*. It is concluded that bovine tuberculosis of central nervous system occurs sporadically in Paraíba, in cattle of different ages, most of them with disseminate lesions in other organs. The location of the lesions suggests that the agent invaded the brain by hematogenous route through the circle of Willis.

INDEX TERMS: Tuberculosis, central nervous system, cattle, Paraíba, Brazil, *Mycobacterium* sp., acid-fast resistant bacteria, Ziehl Neelsen, pathology, bacterioses.

RESUMO.- [**Tuberculose no sistema nervoso central de bovinos na Paraíba.**] Descrevem-se seis casos de tuberculose no sistema nervoso central (SNC) em bovinos, no semiárido da Paraíba. Foram revisados os laudos de um total de 851 necropsias de bovinos realizadas no período de 2003 a 2016. Destes, 73 (8,6%) foram diagnosticados com tuberculose e seis apresentavam lesões no SNC. Três casos ocorreram em bovinos de até dois anos de idade e três em bovinos adultos. Três bovinos apresentaram exclusivamente sinais nervosos, dois tinham sinais respiratórios e perda de peso e um não apresentava nenhum sinal clínico. Macroscopicamente, em cinco bovinos, havia espessamento das leptomeninges do cerebelo, medula espinhal, ponte, obex, colículos e córtex, principalmente, na região basilar encefálica próxima ao polígono de Willis. Em apenas um bovino houve a presença de tubérculo único no cerebelo. Microscopicamente observou-se moderada a acentuada meningite e encefalite granulomatosa. Cinco bovinos apresentaram lesões pulmonares e nos gânglios mediastínicos e três deles tinham lesões disseminadas em outros órgãos. Em todos os bovinos foram encontrados bacilos ácido-álcool resistentes intralesionais e todos tiveram marcação

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[2] Programa de Pós-Graduação em Medicina Veterinária, Universidade Federal de Campina Grande (UFCG), Av. Universitária s/n, Bairro Santa Cecília, Patos, PB 58708-110, Brazil. *Corresponding author: silveira.a.monteiro@gmail.com

[3] Setor de Patologia Veterinária, Hospital Veterinário, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9090, Agronomia, Porto Alegre, RS 90540-000, Brazil.

[4] Instituto Nacional de Investigación Agropecuaria (INIA), Estación Experimental INIA La Estanzuela, Ruta 50 Km 11, Colonia del Sacramento, Colonia, Uruguay. E-mail: frcorrea@inia.org.uy

positiva na técnica de imuno-histoquímica com anticorpo policlonal anti-*Mycobacterium tuberculosis*. Conclui-se que a tuberculose do sistema nervoso central de bovinos ocorre de forma esporádica na Paraíba, principalmente em bovinos com lesões disseminadas em outros órgãos. Sugere-se que a disseminação do agente ocorre pela via hematógena, possivelmente através do polígono de Willis.

TERMOS DE INDEXAÇÃO: Tuberculose, sistema nervoso central, bovinos, Paraíba, *Mycobacterium* sp., bactérias ácido-álcool resistentes, Ziehl Neelsen, patologia, bacterioses.

INTRODUCTION

Tuberculosis is an important disease for the Brazilian farming and public health. It causes economic loss because of the low production of milk and meat and by the slaughter of the animals positive to the intradermal allergic test, implanted by the Brucellosis and Tuberculosis Control and Eradication National Program (Programa Nacional de Controle e Erradicação da Tuberculose e da Brucelose - PNCETB). Besides, the direct contact of the workers with the bovines and infected foods implicates in the possibility of infection of the human beings by *Mycobacterium* spp. (Roxo 1997, Riet-Correa & Garcia 2007).

Commonly, the bovine affected by the disease present progressive weight loss, and in some cases unproductive cough; these signs are associated to the aerial transmission way (Andreazza et al. 2015). Sporadically, young bovines present clinical neurological manifestation and granulomatous lesions in the leptomeninges of the encephalon and spinal cord (Oruç 2005, Riet-Correa & Garcia 2007, Del Fava et al. 2010). The lesions location in these cases is related to the transplacental transmission way or infection through ingestion of contaminated milk, being the dissemination through hematogenous way, which enables the ascendance to the central nervous system (CNS) (Roxo 1997, Oruç 2005, Riet-Correa & Garcia 2007, Del Fava et al. 2010, Borges et al. 2013, Domingo et al. 2014).

In Brazil there are few studies reporting the infection by *Mycobacterium* spp. affecting the CNS of bovine and mostly they report the infection in sporadic or isolated cases (Galiza et al. 2010, Konradt et al. 2016). This study aims the description of epidemiological, clinic-pathological, and histological findings of six tuberculosis cases in the CNS in bovine.

MATERIALS AND METHODS

Tuberculosis cases with CNS involvement were researched among bovine necropsy reports found in the archives of the Animal Pathology Laboratory of the Campina Grande Federal University (Laboratório de Patologia Animal da Universidade Federal de Campina Grande (LPA-UFCG), in a 14-year period (2003-2016). Of these reports, information about breed, gender and age, clinical signs, tuberculin test, and the macroscopic and histological alterations previously described were taken. Specimens of CNS and other organs from necropsies had been fixed in 10% formaldehyde and routinely processed for histopathology. Sections of 3-5µm, stained with the hematoxylin and eosin (HE) technique, were evaluated to characterize the inflammatory response in each case. In CNS sections, histochemical techniques such as Ziehl-Neelsen, Masson's Trichrome and Von Kossa were also performed for visualization of the agent, fibrous connective tissue and mineralization disclosure, respectively. CNS sections were selected to the immuno-histochemical exam, in which the anti-*Mycobacterium tuberculosis* polyclonal antibody in the dilution 1:200 in PBS were used (phosphate-buffered saline). To block the endogenous peroxidase, 10% hydrogen peroxide was used for 15 minutes. The antigenic recovery was performed with XIV protease for 15 minutes in ambience temperature. The block of the nonspecific reactions was performed with casein solution (skim powdered milk) at 5% for 15 minutes in ambience temperature. The primary antibody was incubated for 45 minutes at 37ºC. The secondary antibody biotinylated and the streptavidin-biotin-peroxidase complex (LSAB+System-HRP) were used consecutively, incubated in ambience temperature for 20 minutes each. EC^3 (3-Amino-9-ethylcarbazole) was the chromogen used, and after this process the sections were stained with Harri's hematoxylin for one minute, dehydrated and mounted with aqueous medium and cover slip. For positive control, a bovine lung fragment with *Mycobacterium bovis* positive diagnosis in the PCR technique was used (polymerase chain reaction). As negative controls, the same tested sections were used, with substitution of the primary antibody by antibody diluent.

RESULTS

From 851 bovine necropsies performed in the LPA-UFCG from 2003 to 2016, 73 (8.58%) had positive diagnosis for tuberculosis, and in six cases (8.2%) there was CNS involvement.

All six bovines with CNS lesion were from dairy farms with semi-extensive breeding regime. The epidemiological data, the six bovine's clinical signs, and the results of the tuberculin test performed in two animals are described in Table 1. Only Bovine 1 and 6 were necropsied with previous suspect of tuberculosis. In all other cases the presumptive diagnosis of tuberculosis was not considered, and Bovine 4 and 5 had clinical suspicion of rabies. Case 3 did not present clinical signs and euthanasia was performed due to the positivity of the brucellosis test. In Bovine 2 the suspicion of tuberculosis happened after accentuated lymphocytosis verification in the cerebrospinal fluid analysis.

The tuberculin test was performed in Bovine 1, which reacted positively to the test, and in Bovine 2 which was negative. The tuberculin test was not performed in the other animals. Bovine 1 and 2 died spontaneously due to the aggravation of their clinical condition. Bovine 4 and 5 were euthanized due to the severity of their neurological clinical signs.

Macroscopically, leptomeninges of the brain stem ventral region of Bovine 1, 2, 3 and 5 were observed gray and moderately thickened (Fig.1A) by many nodules of 0.2-1mm of diameter, yellowish and irregular with consistency varying from soft to hard. The meninges thickening involved the colliculi and extended until the leptomeninges of the sacral spinal cord in Bovine 2 and 5 (Fig.1B), and it affected the leptomeninges of the frontal cortex, falx cerebri, tentorium cerebelli, and cerebellum in Bovine 3. There was a minor cerebellar herniation in Bovine 4 associated to the yellow-whitish, rough, soft mass, with approximately 3cm of diameter in the fourth ventricle region (Fig.1C). In Bovine 6, a small area of thickening and little yellowish nodules with about 1mm of diameter near to the thickening of the frontal cortex meninge were observed.

In the encephalon serial cut of Bovine 2 projections in the lateral ventricles were evidenced (Fig.1D), and creaked when cut. In the spinal cord ventral portion, irregular whitish areas of up to 5mm were observed affecting the white and gray matter. In Bovine 3, multiple yellowish nodules of flat surface, with about 2mm of diameter were noticed in the ventral cerebellar vermis, and the lateral ventricles were

Table 1. Breed, sex, age, clinical signs and location of lesions in six cases of bovine tuberculosis with lesions in the nervous system

Cattle	Breed	Sex	Age	Clinical signs	Location of lesions
1	Red Sindhi	Male	Adult	Cough, exercise intolerance, weight loss and lymphadenomegaly	Central nervous system (CNS), lung and mediastinal lymph nodes
2	Brown Swiss	Male	One year	Incoordination, weakness, decreased sensitivity of the pelvic limbs, sitting dog posture and urinary incontinence	Lung and mediastinal lymph nodes, kidney
3	Crossbred Holstein	Female	Nine years	No clinical signs. Euthanized because brucellosis test was positive	CNS
4	Crossbred	Female	Two years	Loss of balance, discrete mandibular trismus, nystagmus, circling and falls	CNS, lung, liver
5	Girolando	Female	Six months	Lateral recumbence, opisthotonus, nystagmus, medial strabismus, reduced facial sensitivity and tetraparesis	CNS, lung and mediastinal lymph nodes
6	Crossbred Holstein	Female	Eight years	Weight loss, severe dyspnea, blindness with corneal opacity of the left eye	CNS, lung, mediastinal mesenteric and intramammary lymph nodes, kidney, liver, eyeball, diaphragm, spleen, mammary gland, uterus and heart

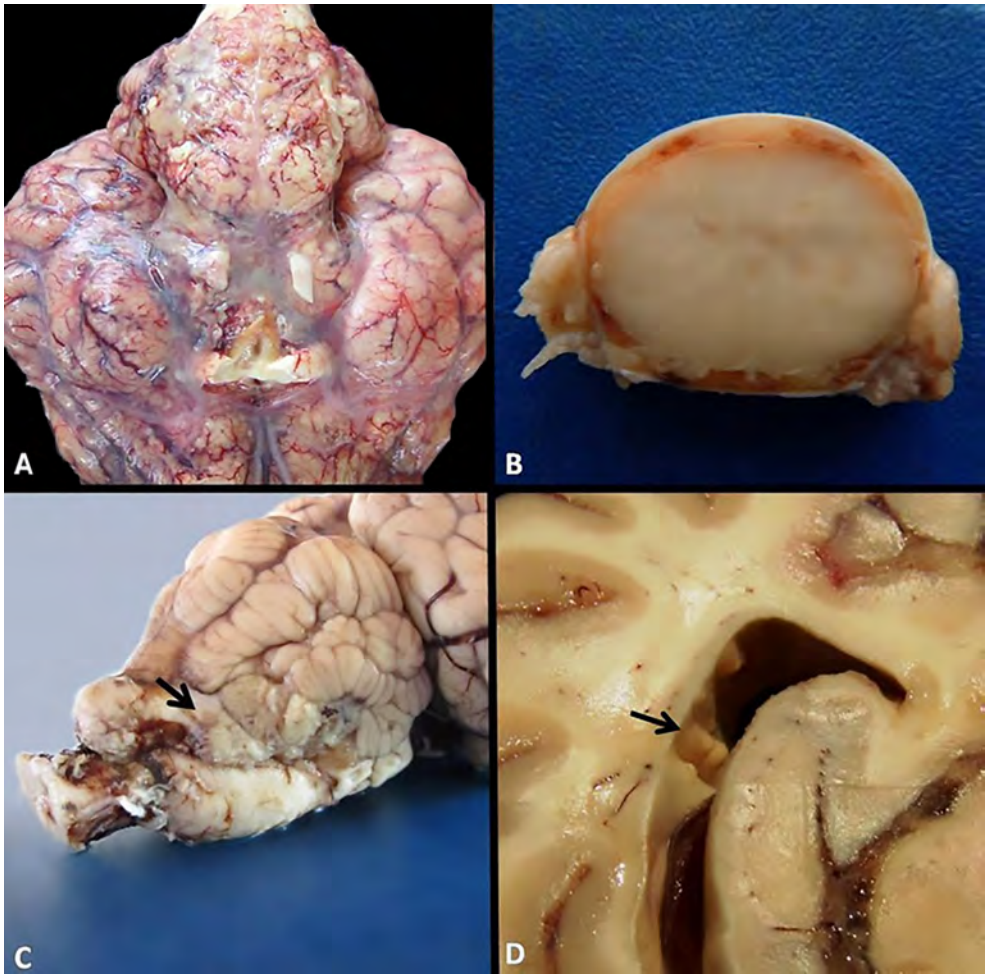


Fig.1. Tuberculosis in the central nervous system of bovine. (**A**) Encephalon of Bovine 2 presenting leptomeninges thickening in the basilar region. (**B**) Cervical spinal cord of Bovine 5 with thickening of the meninges. (**C**) Encephalon of Bovine 4 with cerebellar herniation and presence of a protruding mass in the cerebellum base (arrow). (**D**) Encephalon cut of Bovine 2 evidencing projections on the left lateral ventriculus wall (arrow).

dilated. In Bovine 4, besides the mass in the fourth ventricle, it presented dilatation of the mesencephalic aqueduct, third ventricle, and lateral ventricles. The gray matter adjacent to the cortex meninge thickening of Bovine 6 presented areas of malacia with softening and granular appearance.

Besides the CNS lesions, yellow-whitish, soft, well delimited nodulations that presented resistance to the cut and contained caseous material with mineralized center were observed in the lungs of all bovines, except Bovine 3. In Bovines 1, 2, 5 and 6, the mediastinal lymph nodes presented enlarged with nodulations similar to the visualized in the lungs. Multiple whitish nodules were also seen in the kidneys of Bovine 1 and 6, and in the liver of Bovine 4 and 6. Bovine 6 presented similar formations in the left ocular globe, mesenteric and intramammary lymph nodes, diaphragm, spleen, mammary gland, uterine horns, and pericardial sac. Yellowish nodulations and abundant amounts of fibrin were observed between the pericardium and the epicardium.

Microscopically, leptomeninges thickening was observed in all bovines (Fig.2A), except in Bovine 4. The degree of

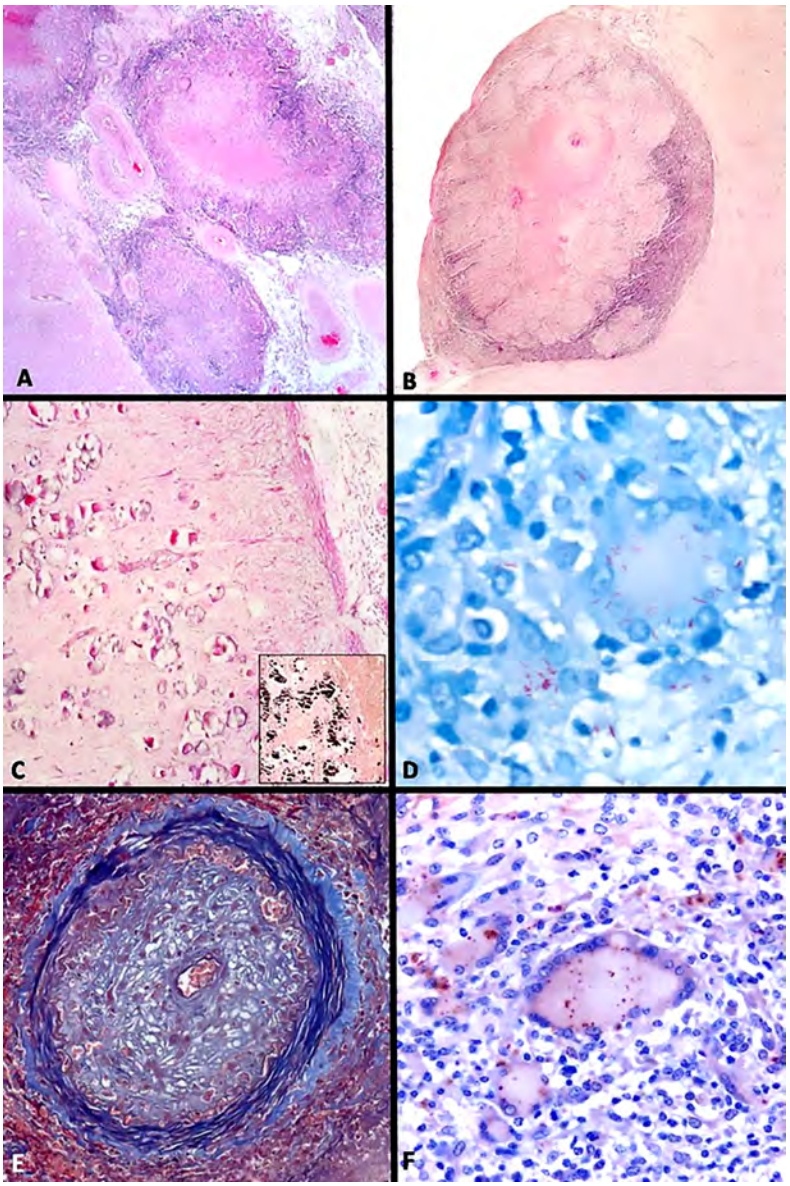


Fig.2. Tuberculosis in the central nervous system of bovine. (**A**) Cerebellar peduncle of Bovine 4. Thickening of the leptomeninges by multiple granulomas with central necrotic areas is observed. (**B**) Frontal cortex of Bovine 2 with meningeal granuloma compressing the adjacent neuropil. HE, obj.5x. (**C**) Pons of Bovine 2. Mineralization of the nervous tissue is observed. HE, obj.10x. In detail, Von Kossa stain demonstrating the mineralized areas in blackened tone. (**D**) Cerebral cortex of Bovine 5. Strong, intracytoplasmic alcohol-acid rods are observed in multinucleated giant cell of the Langerhans type and macrophages in the middle of the lesion. Ziehl-Neelsen, obj.100x. (**E**) Cerebral cortex of Bovine 6. Meningeal blood vessel evidencing tunica intima sharply proliferated in blue tone and with reduction in the lumen diameter. Masson's Trichome, obj.20x. (**F**) Cerebral cortex of Bovine 4. Positive immunostaining with anti-*Mycobacterium tuberculosis* is observed in the cytoplasm of epithelioid macrophages and multinucleated giant cells. Immuno-histochemical anti-*M. tuberculosis*, chromogen DAB, obj.40x.

thickened varied from moderate, in Bovines 1 and 3, to accentuated in Bovines 2, 5 and 6. The most affected regions were the cortex (all animals), colliculi, cerebellum, pons, obex (Bovine 1, 2, 3 and 5), and spinal cord (Bovine 2 and 5). This thickening was characterized by multiple granulomas with eosinophilic central areas of necrosis surrounded by mononuclear inflammatory infiltrate composed by lymphocytes, epithelioid macrophages, and giant multinucleated Langerhans cells associated to the slight proliferation of fibrous connective tissue (Fig.2B). In Bovines 2, 3 and 4, some granulomas presented mineralization in the necrotic central region. The granulomatous formation was accentuated around the blood vessels, resulting in the tunica intima thickening, with the presence of up to five layers of spindle cells with cytoplasm, and large and elongated nucleus. In some areas, the blood vessels observed in the middle of the inflammation presented eosinophilic walls with homogeneous and hyaline material deposition, similar to fibrinoid necrosis.

There was mild (Bovine 1, 3, 4 and 6) to moderate (Bovine 2 and 5) encephalitis, characterized by lymphocytic vasculitis in the neuropil adjacent to the granulomas. Furthermore, in Bovine 1, 2, 3 and 5 the leptomeninges of cortices, cerebellum, and cerebellar peduncle granulomas compressed the adjacent neuropil and in Bovine 5 were associated with vacuolization and axonal spheroids. The leptomeninges granulomas infiltrated the cortical neuropil of Bovine 6, which presented extensive area of malacia. In the spinal cord of Bovine 2, axonal spheroids and vacuoles of different sizes, sometimes containing Gitter cells ("digestion chambers"), were observed in the white matter. In this same animal there was granular material deposition foci, amorphous, strongly basophilic, identified as mineralization, in the granulous layer of the cerebellum and in the neuropil of the pons ventral region (Fig.2C).

Bovine 4 presented the same pattern of granulomatous inflammation around the vessels, however, the external layer of the granulomas had higher quantity of giant multinucleated cells and fibrous tissue in and around the inflammation. The inflammatory cells infiltrated the cerebellum and the cerebellar lateral peduncle provoking destruction of the adjacent tissue.

In other organs (lungs, liver, kidney, lymph nodes, spleen, peritoneum, uterus, and mammary gland) multifocal granulomatous areas were observed, composed by lymphocytes, macrophages and Langerhans giant multinucleated cells, with necrotic center, sometimes mineralized, and surrounded by a fibrous thick capsule. In the ocular globe, the granulomas invaded the uvea and occupied all anterior and posterior chambers. The uterus granulomas affected only the mucosa and there was mineralization of the muscle layer of the mucosa and submucosa vases.

In the Ziehl-Neelsen stain was evidenced few (Bovine 2 and 3) to numerous (Bovine 1, 4, 5 and 6) fine structures shaped like rods, reddish colored, localized in the macrophage's cytoplasm and Langerhans' multinucleated giant cells, and free in the granuloma (Fig.2D). With Masson's trichrome stain mild proliferation of collagenous was observed not only surrounding the granulomas, but also entering the lesion and interweaving the inflammatory cells. The wall of the blood vessels present in the middle and around the granulomas were thickened in up to seven times (Fig.2E). In the Von Kossa stain, mineralization was evidenced in the neuropil of Bovine 2 and in the middle of some granulomas of the leptomeninges of Bovines 2, 3 and 4.

In all bovines there was positive stain in the CNS by immuno-histochemical technique using the polyclonal antibody anti-*Mycobacterium tuberculosis*. The immunostaining was observed, predominantly, in the macrophages' cytoplasm and multinucleated giant cells; rarely free in the granulomatous lesions (Fig.2F). The bacillus disclosure in the immuno-histochemical technique varied from accentuated in Bovine 1, 2 and 4, moderated in Bovine 3 and 6, and mild in Bovine 5.

DISCUSSION

By this study's results, it is verified that tuberculosis of the central nervous system affects bovines of different breeds and ages, with variable intensity and extension. It matches with variable neurological clinical signs according to the lesion location; some animals may present other clinical signs associated to pulmonary lesions or disseminated lesions, or not even evidence any clinical sign, as occurred in one of the cases of this study. Consequently to this variation in the clinical signs, the diagnosis of encephalic tuberculosis must be based in the macroscopic and histopathological alterations, and be confirmed by the immuno-histochemical or agent isolation.

The tuberculin test performed in two of the six cases allowed the previous diagnosis of only one animal, implicating in its euthanasia. The negative reaction to the tuberculin test can occur in cases of recent infection or in very chronic cases, as for the performance of the test in the same animal with intervals shorter than 60 days; in these cases, other auxiliary tests are necessary (Tizard 2014). In spite the negative result observed in an animal, the observation that four bovines presented exclusively nervous signs, suggests that the tuberculin test may be useful as differential diagnosis of the diseases that cause nervous signs in bovines of several ages.

Only one from the six animals with central nervous system tuberculosis tested positively to the tuberculin test. Other bovines that were diagnosed in the routine of the Veterinary Hospital of the UFCG did not go through the tuberculin test, hence, they were not identified with tuberculosis by the Brucellosis and Tuberculosis Control and Eradication National Program. The frequency of animals with central nervous system tuberculosis (8.2% of the bovine with tuberculosis), as for the high frequency of the several forms of the disease (8.58% from the total of the cases received in LPV-UFCG) suggests that tuberculosis has high prevalence in dairy cattle in Paraíba and that efficient measures must be taken to control it.

In this study, the age of the six bovines with CNS tuberculosis was equal or superior to 12-months-old, differing from what is mentioned in the literature, which describes higher frequency of central nervous system tuberculosis in bovines with age under 12 months (Konradt et al. 2016), which is associated to transplacental infection, in animals born from cows with uterine tuberculosis, or through ingestion of milk from cows with lesions in the mammary gland (Roxo 1997, Oruç 2005, Riet-Correa & Garcia 2007, Del Fava et al. 2010, Borges et al. 2013, Domingo et al. 2014). On the other hand, in this study the cases occurred primarily in adult animals most with pulmonary or generalized lesions, suggesting that the infection in the CNS occurred by dissemination of the primary pulmonary lesion. After invading the organism,

Mycobacterium spp. infects macrophages that will circulate in the blood flow and reach several organs, as liver, lymph nodes, kidneys, spleen, uterus, mammary gland, eye and the CNS (Coutinho & Tarragô 1976, Oruç 2005, Del Fava et al. 2010, Domingo et al. 2014, Tizard 2014). In the CNS the agent is also capable to infect microglia cells (Rock et al. 2008).

The location of the lesions, preferably in the meninges of the encephalon basilar region, and the anatomical characteristics of the blood perfusion in the CNS suggest that the bacteria get in the nervous system through the Willis' circle vessels; in this structure there is concentration of the blood for initial distribution of the encephalic arterial blood, besides the alteration in the local blood flow (Oruç 2005, Rock et al. 2008, Dyce et al. 2010, Konradt et al. 2016).

The mechanisms determining the location of tuberculosis lesions in the different regions of the CNS are not known, however, three well-defined forms of presentation in humans are known: meningeal thickening, miliary distribution, and unique tuberculous formation (Coutinho & Tarragô 1976). Among the tuberculosis lesions observed in this report, the meningeal thickening was the most frequent, seen in five of the six cases studied in this study. The formation of a unique mass, observed only in one case (Bovine 4), occurs eventually (Konradt et al. 2016).

The fibrous tissue that covered the granulomas in the CNS, evidenced by the Masson's trichrome technique, was scarce, differing from the fibrosis observed commonly in other organs affected by the agent.. This fibrosis pattern, reported previously in the literature, maintains the inflammatory cells free and facilitates the infiltration of the tissues adjacent to the lesion (Andreazza et al. 2015).

The divergency in nervous clinical signs among the cases occurred possibly because of the different lesion intensity in the neuropil and its location (Coutinho & Tarragô 1976, Oruç 2005, Riet-Correa et al. 2002, Del Fava et al. 2010, Konradt et al. 2016), The chronicity may have influenced in the degree of the CNS lesion (Rock et al. 2008), since Bovines 1, 3 and 6 that presented granulomatous meningitis and compressive lesions, without significant alterations of the adjacent nervous tissue, did not show neurological clinical disturbances; while the bovines with diffuse and accentuated lesions in the meninges and brain tissues (Bovines 2, 4 and 5) presented nervous signs.

In the only case that the liquor was examined, lymphocytosis was confirmed, suggesting that the routine use of this technique, associated to the Ziehl-Neelsen stain, or to the isolation or molecular identification of the agent, may help in the diagnosis of the nervous form of tuberculosis. Humans with encephalic tuberculosis may present high lymphocyte cellular population in the liquor cytology; however, in the cases in which the infection is in the initial phase, neutrophils in greater quantity may be visualized (Coutinho & Tarragô 1976, Rock et al. 2008). Besides the evaluation of the inflammatory cells present in the liquor, the Ziehl-Neelsen technique can be performed to observe the agent in the cerebrospinal fluid, though the absence of bacillus does not imply in the exclusion of the disease (Wilkins et al. 1986).

The mineralization observed in the neuropil of the pons and cerebellum of Bovine 2 in areas where there were not granulomas may have occurred initially as a metastatic calcification of the blood vessels and latter expanding to the tissue, as it is observed in some chronic diseases, as tuberculosis and paratuberculosis (Zachary 2013). The same process may have developed the mineralization in the uterine vessels of Bovine 6.

The severe histologic lesions in the tunica intima of the meningeal vessels, described before in human encephalic tuberculosis cases (Coutinho & Tarragô 1976), suggest the invasion of the agent in the CNS through the hematogenous way. In humans, through angiographies, it is possible to identify tortuosity and aneurisms provoked by vascular compression, vasculitis and/or proliferation of the tunica intima of the blood vessels (Coutinho & Tarragô 1976). The meningeal vessels are, therefore, the first affected by the inflammation, and the proliferation of the tunica intima may provoke complete occlusion of the vascular lumen, causing encephalic infarcts (Oruç 2005, Zachary 2013).

CONCLUSIONS

The tuberculosis in the central nervous system in bovine of Paraíba affects young and old bovines, of different breeds, with different clinical signs.

The different ages in the affected bovines, the vascular lesions, and the presence of lesions in other organs, suggest that the infection occurs by dissemination from other organs by the hematogenous way.

The high relative frequency of tuberculosis in Paraíba (8.58% of all diagnosed cases in bovines) and the high frequency of cases with lesions in the nervous system (8.2% of all tuberculosis cases) indicate that encephalic tuberculosis must be included in the differential diagnosis of other diseases affecting the CNS in cattle in the state.

REFERENCES

Andreazza D., Boos G.S., Boabaid F.M., Wouters A.T.B., Wouters F., Souza S.O., Menegat M.B. & Driemeier D. 2015. Caracterização histologica e imuno-histoquímica das lesões de tuberculose em bovinos e de linfadenitegranulomatosa em suínos. Pesq. Vet. Bras. 35(2):129-136. <http://dx.doi.org/10.1590/S0100-736X2015000200006>

Borges P.C., Nakagaki K.Y.R., Lopes P.R.F., Souza B., Utiumi K. & Raymundo D.L. 2013. Tuberculose meningeana e na forma de tubérculos isolados no sistema nervoso central de uma novilha holandesa. Arch. Vet. Sci. 12(2):185-187.

Coutinho L.M.B. & Tarragô R.P. 1976. Tuberculose meningoencefálica na infância: estudo anatomo-patológico de 10 casos. Arq. Neuropsiquiatr. 34(2):127-134. <http://dx.doi.org/10.1590/S0004-282X1976000200003> <PMid:1084147>

Del Fava C., Roxo E., Baldassi L., Cunha E.M.S. & Cabral A.D. 2010. Granulomatous meningo myeloencephalitis in a calf caused by *Mycobacterium bovis*. Arqs Inst. Biológico, São Paulo, 77(2):335-338.

Domingo M., Vidal E. & Marco A. 2014. Pathology of bovine tuberculosis. Res. Vet. Sci. 97(Suppl.):20-29. <http://dx.doi.org/10.1016/j.rvsc.2014.03.017> <PMid:24731532>

Dyce K.M., Sack W.O. & Wensing C.J.G. 2010. O sistema nervoso, p.268-331. In: Ibid. (Eds), Tratado de Anatomia Veterinária. 4ª ed. Elsevier, Rio de Janeiro.

Galiza G.J.N., Silva M.L.C.R., Dantas A.F.M., Simões S.V.D. & Riet-Correa F. 2010. Doenças do sistema nervoso de bovinos no semiárido nordestino. Pesq. Vet. Bras. 30(3):267-276. <http://dx.doi.org/10.1590/S0100-736X2010000300014>

Konradt G., Bassuino D.M., Bianchi M.V., Bandinelli M.B., Driemeier D. & Pavarini S.P. 2016. Neurotuberculosis in cattle in southern Brazil. Trop. Anim. Health Prod. 48(5):1089-1094. <http://dx.doi.org/10.1007/s11250-016-1048-z> <PMid:27026233>

Oruç E. 2005. Meningoencephalitis tuberculosa in a holstein cow. Vet. Pathol. 42(6):856-858. <http://dx.doi.org/10.1354/vp.42-6-856> <PMid:16301587>

Riet-Correa F. & Garcia M. 2007. Tuberculose, p.432-442. 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.

Riet-Correa F., Riet-Correa G. & Schild A.L. 2002. Importância do exame clinico para o diagnóstico das enfermidades do sistema nervoso em ruminantes e eqüídeos. Pesq. Vet. Bras. 22(4):161-168. <http://dx.doi.org/10.1590/S0100-736X2002000400006>

Rock R.B., Olin M., Baker C.A., Molitor T.W. & Peterson P.K. 2008. Central nervous system tuberculosis: pathogenesis and clinical aspects. Clin. Microbiol. Rev. 21(2):243-261. <http://dx.doi.org/10.1128/CMR.00042-07> <PMid:18400795>

Roxo E. 1997. *Mycobacterium bovis* como causa de zoonose. Revta Bras. Ciênc. Farm. 18(1):101-108.

Tizard I.R. 2014. Hipersensibilidade do tipo IV: hipersensibilidade tardia, p.365-375. In: Ibid. (Eds), Imunologia Veterinária. 9ª ed. Elsevier, Rio de Janeiro.

Wilkins E.G.L., Griffiths R.J., Roberts C. & Green H.T. 1986. Tuberculous meningitis due to *Mycobaterium bovis*: a report of two cases. Postgrad. Med. J. 62(729):653-655. <http://dx.doi.org/10.1136/pgmj.62.729.653> <PMid:3529067>

Zachary J.F. 2013. Sistema nervoso, p.803-804. In: Zachary J.F. & McGavin M.D. (Eds) Bases da Patologia em Veterinária. 5ª ed. Elsevier, Rio de Janeiro.

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Topic of General Interest
Animais de Produção/*Livestock Diseases*
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Gross and histopathological pitfalls found in the examination of 3,338 cattle brains submitted to the BSE surveillance program in Brazil[1]

Raquel R. Rech[2], Paula R. Giaretta[2], Corrie Brown[3] and Claudio S. L. Barros[4]*

ABSTRACT.- Rech R.R., Giaretta P.R., Brown C. & Barros C.S.L. 2018. **Gross and histopathological pitfalls found in the examination of 3,338 cattle brains submitted to the BSE surveillance program in Brazil.** *Pesquisa Veterinária Brasileira 38(11):2099-2108*. Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul, Av. Senador Filinto Müller 2443, Campo Grande, MS 79074-460, Brazil. E-mail: claudioslbarros@uol.com.br

This study stems from the findings during the gross and histopathological exam of 3,338 cattle brains as part of the bovine spongiform encephalopathy (BSE) active surveillance program of the Brazilian Ministry of Agriculture, Livestock, and Supply from 2001 to 2005. The work was carried out in the Veterinary Pathology Laboratory of the Federal University of Santa Maria which at the time (2001-2007) was the national reference laboratory for the diagnosis of BSE and other transmissible spongiform encephalopathies. Both gross and histopathological aspects are described. Several gross aspects were annotated: anatomic normal structures not commonly recognized (non-lesions), lesions of no clinical significance, postmortem changes and artifacts; all these can amount to important pitfalls that distract the pathologist during the routine gross examination of the central nervous system (CNS). Accordingly, equivalent pitfalls were described in the histological examination. Non-lesions observed were the pineal body, embryo remnants such as the external germinal layer of the cerebellum, subependymal plates, and clusters of neuroblasts in the basal ganglia; or circumventricular structures such as area postrema, subcomisural organ, and melanosis in the leptomeninges and vessel walls. Lesions with little or no clinical importance included age-related changes as lipofuscin, hemosiderin, mineralization and hyalinization of vessel walls within the brain and meninges. *Corpora amylacea* and *corpora arenacea* were detected respectively in astrocyte processes and the pineal body. Cytoplasmic neuronal vacuolization was observed in the red nucleus and habenular nucleus. *Sarcocystis* sp. without a correspondent inflammatory reaction was rarely observed. Included within findings with no clinical manifestation were axonal spheroids and perivascular mononuclear cuffings. Changes in the CNS due to killing, sampling and fixation methods can obscure or distract from the more critical lesions. The ones related to the process of killing included hemorrhages caused in cattle destroyed by a captive bolt. Artifacts related to sampling and handling of CNS tissue consisted of inclusion of bone sand in the neural tissue from sawing the calvarium; dark neurons produced by excessive handling of the brain, and micro-organisms that contaminated the tissues during sampling or histological processing. Postmortem autolytic or putrefactive changes observed included vacuolar changes in the myelin sheath, clear halos surrounding neurons and oligodendrocytes, clusters of putrefaction bacilli within vessels or dispersed throughout the brain tissue associated or not to clear halos. One interesting, and somewhat frequent, postmortem autolytic change found in the bovine brain was the partial dissolution of the granule cell layer (GCL) of the cerebellum, also referred to as conglutination of the GCL

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[2] Department of Veterinary Pathobiology, College of Veterinary Medicine & Biomedical Sciences, Texas A&M University, College Station, TX 77843-4467, USA.

[3] University of Georgia College of Veterinary Medicine, Department of Pathology, 501 D.W. Brooks Drive Athens, Georgia 30602, USA.

[4] Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul (UFMS), Av. Senador Filinto Müller 2443, Campo Grande, MS 79074-460, Brazil. *Corresponding author: claudioslbarros@uol.com.br

or as the French denomination "état glace". Due to the shortage of comprehensive publications in the subject, this review is intended to address the main pitfalls that can be observed in the brain of cattle hoping to help other pathologists avoiding misinterpret them.

INDEX TERMS: Postmortem findings, histopathology, pitfalls, cattle, brain, Brazil, central nervous system, artifacts, histology, neuropathology.

RESUMO.- [**Ciladas macroscópicas e histopatológicas encontradas no exame de 3.338 encéfalos de bovinos submetidos ao programa de vigilância da EEB no Brasil.**] Os resultados deste estudo foram obtidos pelo exame macroscópico e histopatológico de 3.338 cérebros de bovinos examinados durante o programa de vigilância ativa da encefalopatia espongiforme bovina (BSE) do Ministério da Agricultura, Pecuária e Abastecimento (MAPA), de 2001 a 2005. O trabalho foi realizado no Laboratório de Patologia Veterinária (LPV) da Universidade Federal de Santa Maria (UFSM) que, de 2001 a 2007, foi o laboratório nacional de referência para o diagnóstico da BSE e de outras encefalopatias espongiformes transmissíveis. Macroscopicamente, foram descritas estruturas anatômicas normais (não-lesões), mas que são, com frequência, interpretadas como lesões; lesões sem significado clínico; alterações pós-mortais e artefatos. Esses achados podem confundir e desviar a atenção do patologista durante o exame de rotina do sistema nervoso central (SNC). Da mesma forma, estruturas equivalentes foram descritas no exame histológico. As não-lesões observadas foram corpo pineal, remanescentes embrionários, como a camada germinativa externa do cerebelo, placas subependimárias e aglomerados de neuroblastos nos gânglios da base; ou estruturas circunventriculares, como área de postrema, órgão subcomissural e melanose em leptomeninges e paredes dos vasos. Lesões com pouca ou nenhuma importância relacionadas ao envelhecimento incluíram lipofuscina, hemossiderina, mineralização, hialinização das paredes dos vasos do encéfalo e das meninges. *Corpora amylacea* foram detectados em processos astrocíticos e *corpora arenacea*, no corpo pineal. Adicionalmente, foi observada vacuolização no citoplasma de neurônios do núcleo vermelho e do núcleo habenular. *Sarcocystis* sp. sem reação inflamatória correspondente foi raramente observado. Incluídos nos achados sem manifestação clínica estavam esferóides axonais e manguitos mononucleares perivasculares. Alterações no SNC causadas pelo método de abate, amostragem e fixação podem simular ou obscurecer lesões mais importantes. Aquelas relacionadas ao método de abate incluíram hemorragias causadas em bovinos dessensibilizados pelo dardo cativo ou por punção por faca da medula na articulação atlanto-occipital. Artefatos relacionados à amostragem e manuseio de tecido do SNC consistiram na inclusão de pó de osso no tecido neural em consequência do uso de serra para abrir a caixa craniana; neurônios escuros produzidos pelo manuseio excessivo do cérebro e micro-organismos que contaminaram os tecidos durante a amostragem ou processamento histológico. Alterações autolíticas pós-mortais ou de putrefação incluíram vacuolizações na bainha de mielina, halos claros em torno dos neurônios e oligodendrócitos, aglomerados de bacilos de putrefação dentro dos vasos ou dispersos em todo o tecido cerebral, relacionados ou não a halos claros. Uma alteração autolítica pós-mortal intrigante e relativamente frequente encontrada foi a dissolução parcial da camada de células granulares (CCG) do cerebelo, também referida como conglutinação da CCG ou "état glacé". Devido à escassez de publicações abrangentes neste assunto, esta revisão pretende abordar as principais ciladas que possam aparecer no cérebro de bovinos, na esperança de ajudar outros patologistas a evitar interpretá-las erroneamente.

TERMOS DE INDEXAÇÃO: Achados de necrópsia, histopatologia, encéfalo, bovinos, Brasil, sistema nervoso central, artefatos, histologia, neuropatologia.

INTRODUCTION

Identification of artifacts, normal structures that resemble lesions, lesions that are not associated with clinical signs, and postmortem changes, are an important aspect of diagnosing central nervous system (CNS) diseases, allowing for correct interpretation of clinical features of the disease. These changes are grouped in four main categories: non-lesions, lesions of no clinical significance, postmortem changes, and artifacts. In the non-lesions category are included morphologic aspects of the CNS examination that might be interpreted as lesions when the pathologist is not thoroughly familiar with the CNS. Lesions of no clinical significance include those that do not contribute to the altered clinical state of the animal and so are only incidental at necropsy or histologic examination. *Postmortem* changes are not real lesions, but occur after death as a result of autolysis or putrefaction. Artifacts can be produced due to killing, sampling and fixation methods.

During the high of the worldwide concern with BSE, the examination of a large number of bovine brains (Wells et al. 1991) became indispensable to achieve reliable surveillance of the disease. This study stems from the alterations described above during the gross and histopathological exam of 3,338 cattle brains as part of the bovine spongiform encephalopathy (BSE) active surveillance program of the Brazilian Ministry of Agriculture, Livestock, and Supply (MAPA) from 2001-2005. Due to the shortage of comprehensive publications in the subject this review is intended to address the main pitfalls that can be observed in the brain of cattle hoping to help other pathologists avoiding misinterpret them.

MACROSCOPIC EXAMINATION OF THE BRAIN

Non-lesions

In the adult cow and sheep, leptomeningeal melanosis is a frequent normal finding (Innes & Saunders 1962a). Leptomeningeal melanosis occurs as a non-lesion in cattle in Brazil, in breeds with pigmented skin such as Nelore and pigmented skin and dark hair coats such as Angus. Melanosis is characterized by black or dark brown areas in the leptomeninges, especially over the frontal lobes (Fig.1A) where it is frequently mistaken by cortical necrosis. This normal finding is also frequently seen in the brains of sheep from the breeds Suffolk and

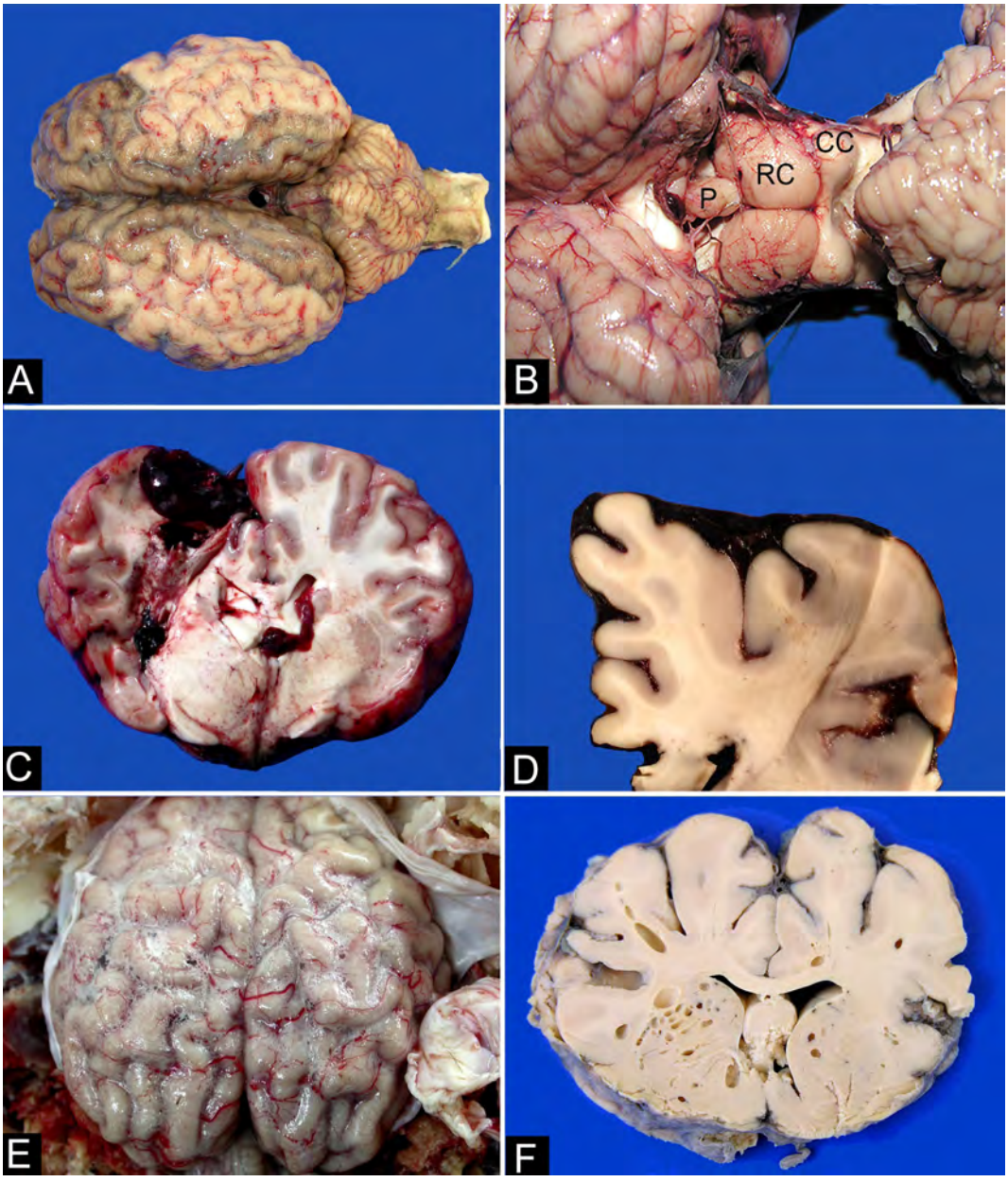


Fig.1. Macroscopic examination of the brain. (**A**) Frontal, parietal and occipital lobes of the brain with focally extensive leptomeningeal melanosis, a common normal finding in the bovine brain. See Figure 3F for histological appearance. (**B**) Location of the pineal gland (P) just rostral to the rostral colliculi (RC) of the midbrain and the thalamus. Caudal colliculi (CC). (**C**) Artifact due to the use of a captive bolt to insensibilize cattle before death by bleeding. Locally extensive area of subdural hemorrhage and a large fragment of bone is inserted in the brain parenchyma. (**D**) Artifact due to the use of a captive bolt to stun cattle before death by bleeding. Diffuse subdural hemorrhage is on the dorsal and lateral encephalic surfaces. (**E**) Artifact associated with the type of brain sampling. Air bubbles in the leptomeninges resulting from the ax's blows at the calvarium when removing the brain. (**F**) Postmortem putrefaction. Cross section of the brain at the level of the basal nuclei. Multiple variably-sized cavities in the brain parenchyma that resembles Swiss cheese.

Hampshire Down, where they can form impressive aggregates as to resemble a neoplasm (Summers et al. 1995). It occurs principally in the leptomeninges over the frontal lobes.

The *glandula pinealis* or pineal gland is a glandular structure (Gartner & Hiatt 2007) situated dorsally at the brainstem between the rostral colliculi of mesencephalic tectum and the thalamus (Fig.1B). It secretes melatonin and due to this glandular aspect that differs from the surrounding neural tissue, may be mistakenly interpreted as a neoplasm, especially on microscopic examination.

Artifacts due to the method of slaughter

The methods for killing and of removing the brain from the cranial cavity may create a variety of alterations in the bovine brain. Subdural hemorrhage is a lesion that can result from the method of euthanasia and is observed in brains from cattle that were killed by captive bolt (Fig.1C,D). A complete history in these cases is very important because it is difficult to differentiate these forms of hemorrhage from other causes of traumatic hemorrhage. Many times, when using a captive bolt for slaughter, fragments of bone can be found within brain

tissue. This can be attributed to the heavy pressure exerted by the captive bolt at the time of stunning (Coore et al. 2004). This heavy pressure can send emboli of brain tissue into the lungs and other organs such as liver (Garland et al. 1996). These emboli can assume several centimeters being promptly visible by the naked eye (Garland et al. 1996).

Artifacts due to sampling of the brain

The use of an axe for removal of the brain from the cranial vault produces bubbles in the leptomeninges. These bubbles are introduced in the leptomeningeal vessels while the prosector hits the bone with the axe (Barros et al. 2006). These bubbles are sometimes mistakenly interpreted as gas embolism (Fig.1E).

Postmortem changes

Postmortem changes include softening of the brain throughout, sometimes associated with the development of cavities caused by gases produced by the anaerobic bacilli of putrefaction. This may give the brain the appearance of a Swiss cheese (Fig.1F). It is important to distinguish Swiss cheese brain from necrosis or other true ante mortem lesion (Auer et al. 2008). The would-be neuropathologists may be trapped in diagnosing cystic necrosis when facing this type of postmortem change. However, the yellow color of necrosis is absent and, unlike in necrosis, the internal surface of the "holes" are smooth (Auer et al. 2008).

MICROSCOPIC EXAMINATION OF THE BRAIN

For optimal fixation for histopathologic examination, the bovine brain should be immersed in a volume of 10% neutral formalin fixative that is at least ten times greater than the volume of tissue to be fixed. Consequently, to adequately fix an entire bovine brain, 6 liters of 10% neutral formalin are needed. The time for fixation varies with the size of the brain. A brain from an adult bovine will require about 2 to 5 days before reaching full fixation. The sections, except the ones in Figure 2, are stained with hematoxylin-eosin (HE).

Microscopic examination of the brain should be carried out in a systematic way. To ensure representative analysis, diverse areas of brain are examined; it is recommended that both sides of serial transverse sections of 0.5 to 1cm be examined. The slides for histologic examination can be systematically processed from sections depicted in Figure 2. This procedure has the following advantages: permits detailed study of specific areas of the brain, offers consistent sections for comparison with control brains, and makes it easy to describe the distribution of lesions.

Non-lesions

Normal microscopic structures that might be mistaken for lesions include some remnants of embryonic structure (external layer of granule cells of the cerebellum, subependymal plaque), circumventricular organs or normal pigment in the vessels of the leptomeninges (melanin produced by melanocytes).

The external granule cell layer of the cerebellar cortex is normally found in fetuses, and calves up to a few months of age (Fig.3A). As the animal ages, these cells migrate toward the granule cell layer. In cattle, complete disappearance of this layer occurs between 12 and 18 months of age (Barros 1980). This external granule cell layer can be confused with

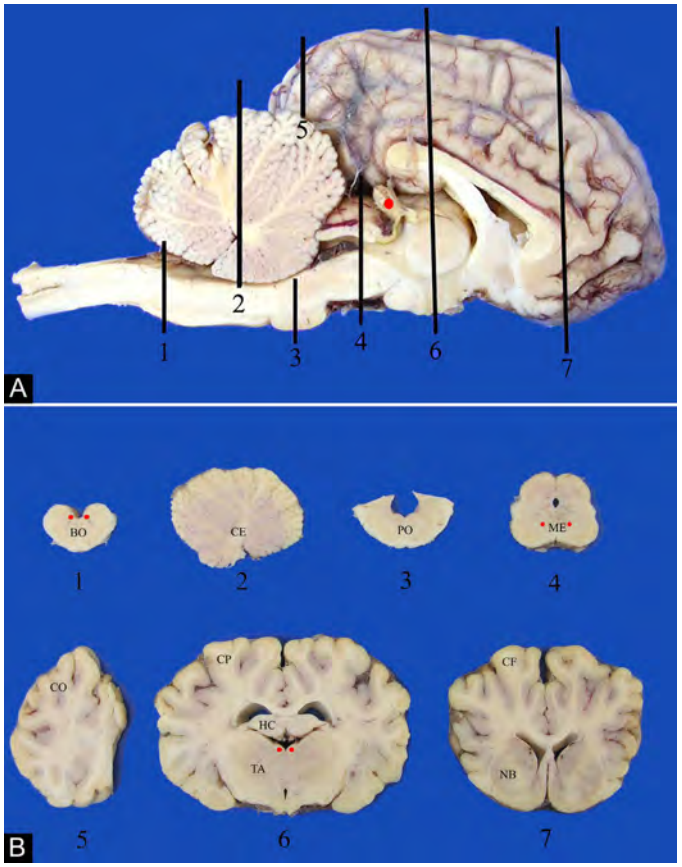


Fig.2. Bovine brain hemisphere showing a systematic way to obtain representative samples for histologic examination. (**A**) Medulla oblongata at the level of the obex, BO (1); Cerebellum, CE (2); Pons at the level of the cerebellar peduncles, PO (3); Midbrain at the level of the rostral colliculi, ME (4); Occipital lobe, CO (5); Diencephalon at the level of the thalamus: parietal lobe, CP, hippocampus, HC, and thalamus, TA (6); and frontal lobe, CF, at the level of the corpus callosum and basal nuclei, NB (7). Red dot = pineal gland. (**B**) Cross sections obtained from sampling of the brain in Figure 2A. Red dots = area postrema (section 1), red nuclei (section 4), habenular nuclei (section 6).

meningitis. In addition, these cells, as they migrate through the cerebellar cortex, can be confused with glial shruberries, a change seen when inflammatory cells accumulate in the molecular layer (Innes & Saunders 1962b).

Subependymal plaques can be localized in the lateral and ventral portions of the lateral ventricles and can be erroneously interpreted as ependymitis. Adjacent to the subependymal plaque and the olfactory bulb, neuroblast accumulations, known as residual glia, can be seen and these could be confused with areas of focal gliosis (Fig.3B).

Circumventricular organs are specialized centers that share two morphologic characteristics - periventricular location and vasculature lacking the typical blood-brain barrier organization (Duvernoy & Risold 2007, Vandevelde et al. 2012, Wohlsein et al. 2013). Circumventricular organs include: (a) subcommissural organ, a local modification of ependymal cells that projects into the mesencephalic aqueduct and which is located on the ventral surface of the posterior

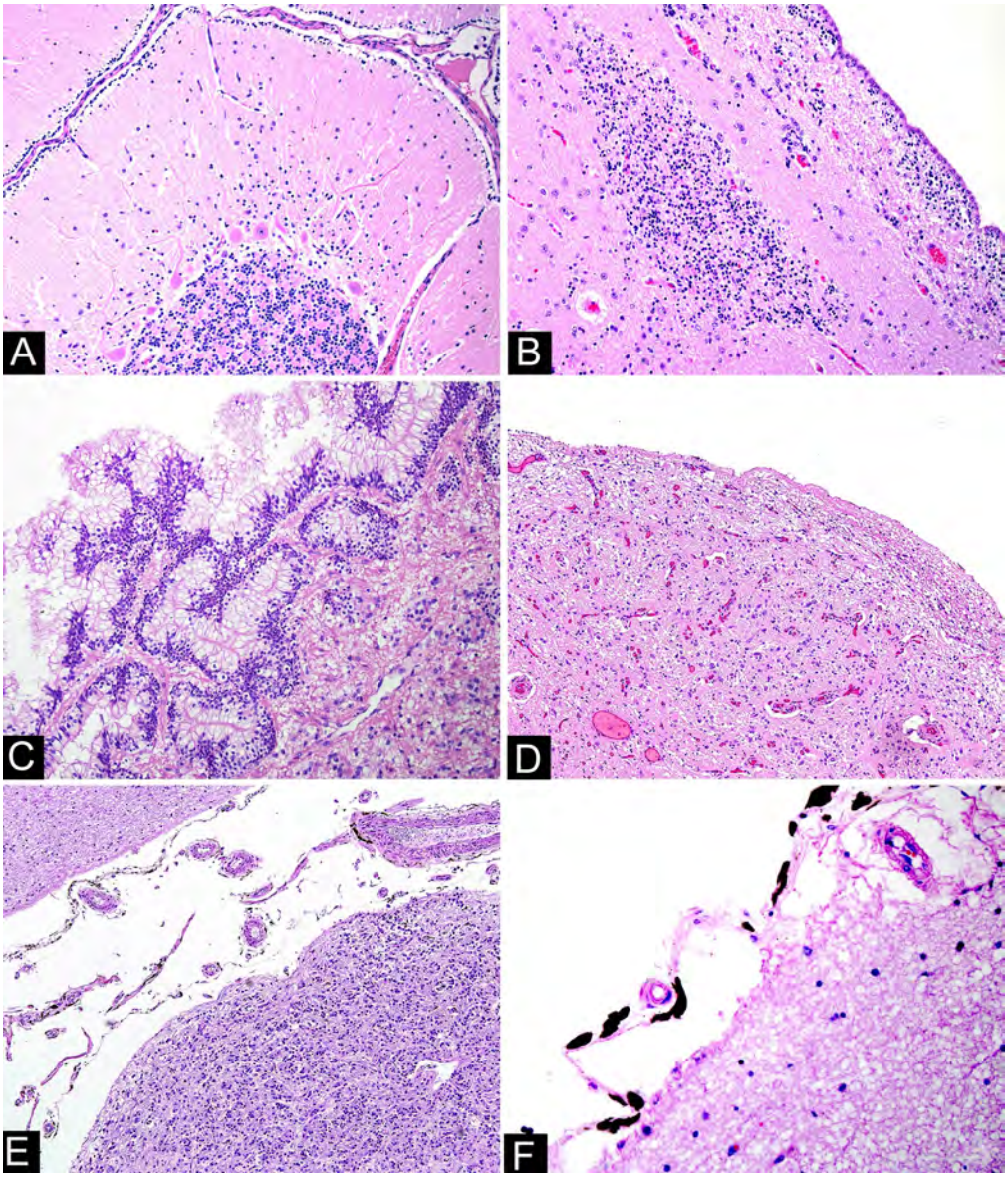


Fig.3. Non-lesions of the bovine brain. (**A**) The external granular cell layer is a mantle of germinative cells found in fetal, neonatal and young animals. Few germinative cells appear in the molecullar layer as they migrate towards the granular cell layer. HE, obj.20x. (**B**) The subependymal plaque consists of multifocal aggregates of germinative cells placed between the ependyma and the parenchyma of the brain at the level of the lateral ventricles. HE, obj.20x. (**C**) The subcomissural organ resembles a glandular arrangement with multiple acini formed by columnar cells with vacuolated cytoplasm and basally located nuclei. HE, obj.20x. (**D**) The area postrema is one of the circumventricular organs, located at the dorsomedial aspect of the medulla oblongata (see Fig.2B). It is composed of neuropil intermixed with the abundant vasculature. HE, obj.20x. (**E**) Histologically, the pineal gland or epiphysis is composed of pinealocytes intermixed with interstitial cells, giving a hypercellular appearance to the parenchyma. HE, obj.10x. (**F**) In leptomeningeal melanosis, the melanocytes are linearly distributed along the leptomeninges and can occasionally extend in the perivascular spaces of the peripheral areas of the cerebral parenchyma. HE, obj.40x.

commissure just caudal to the pineal gland; (b) area postrema, an agglomeration of glial cells and small capillaries located dorsally to parasympathetic nucleus of the vagus at the level of the medulla; and (c) the pineal gland. The subcommissural organ functions to secrete aldosterone (Duvernoy & Risold 2007). Because of the glandular structure seen histologically (Fig.3C), this organ could be erroneously interpreted as an epithelial neoplasm, especially when it is seen in an oblique section of the thalamus. The area postrema is located in the caudal portion of the roof of the fourth ventricle and it is observed microscopically when a section of the medulla oblogata is taken at level of the obex (Fig.3D). In humans, the function of the area postrema has been defined as the vomition center and it is there that chemoreceptors are located that will stimulate vomiting subsequent to intoxication (Borison 1974, Leslie & Osborne 1984). Also, the area postrema functions in cardiovascular control, as the cells here will respond to angiotensin II (Barnes & Ferrario 1980). In histologic sections,

the area postrema can be mistaken for gliosis, or when the capillaries are very prominent, it could be confused with a vascular neoplasm. The pineal gland is an evagination of the dorsocaudal aspect of the epithalamus of the diencephalon, which it is hold through a prolongation that contains the small recess of the third ventricle. The pineal gland is derived from neural epithelium, and consists of glial cells pinealocytes, and blood vessels but contains no true neurons. Pinealocytes synthesize melatonin from serotonine and also contain norepinephrine and thyrotropin releasing hormone (Gartner & Hiatt 2007, Garman 2011). In the lobes of the pineal gland, the pinealocytes are arranged around small lumina (Fig.3E). Neuropathologists prefer the term pineal body to pineal gland, because pinealocytes are neurosensory cells. The capsule and septa of the pineal gland are formed of pia mater and arachnoid, and are continuations of the leptomeninges of the diencephalon, and it is here that the blood vessels penetrate into the pineal (Vigh et al. 1998).

Melanin in the meninges is a non-lesion that can be grossly observed. Histologically, the melanocytes are flattened cells with dendritic cytoplasm and filled with granular dark-brown pigment. These cells can be found in the leptomeninges, or in the vessels within the neuropil, adjacent to the leptomeninges (Fig.3F). In transverse sections, melanocytes can be confused with hemosiderin-laden macrophages. In immunohistochemical studies that utilize diaminobenzidine (dark) as the chromogen, melanocytes can confuse the correct interpretation of the results (Summers et al. 1995).

Lesions of no clinical significance

Lipofuscin is a yellow-brown granular pigment that accumulates in the cytoplasm of neurons in aged animals and apparently does not alter the function of the neuron. It generally deposits in one pole of the perikaryon (Fig.4A), but can also be observed in the cytoplasm of glial cells or free in the neuropil. The extracellular occurrence of lipofuscin can be explained by the process of exocytosis or through rupture of glial cells that contained the pigment. This pigment can be engulfed by macrophages (Jahns et al. 2006a, Wohlsein et al. 2013). Other age-related alterations include mineralization and hyalinization of blood vessels in the brain and meninges (Jahns et al. 2006a, Mandara 2003), and *corpora amylacea* in astrocyte processes. Mineralization of blood vessels in the brain of cattle (Fig.4B) affects predominantly the vessels of the dentate nucleus, globus pallidus, internal capsule, and caudal nucleus, without any association with generalized vascular disease (Wohlsein et al. 2013). *Corpora amylacea* are common in the brains of aging mammals and consist of glucose polymers ("polyglucosan bodies"); they are located within the cytoplasm of astrocytes (Hirano 1985, Garman 2011) and should be differentiated from Lafora bodies, which can also be an age-related change (Yanai et al. 1994, Borras et al. 1999), but these are seen in within neuronal perikaryon and axon (Minassian 2001). Those that are in the axons are difficult to differentiate from *corpora amylacea* because both appear to be free in the neuropil (Wohlsein et al. 2013). Sometimes they can be mistaken for fungal organisms, since they have the same affinity for the special stains used to highlight these organisms.

Concretions of the pineal gland can occur in humans, cattle, horses, sheep, and donkeys, and can vary in size from several microns to several millimeters in diameter. These concretions are called *corpora arenacea*, *acervuli cerebri*, or brain sand (Fuller & Burger 2007). They are formed from concentric dark laminations rich in protein alternating with lighter layers rich in calcium. Mineral structure consists morphologically of hydroxyapatite and carbon apatite, with calcium and phosphorous as the principal constituents. These concretions generally localize between the nervous fibers (axons) and occasionally within glial cells (Fig.4C). The laminations could correspond to fluctuations of the pineal influenced by circadian rhythm or solar intensity (Vigh et al. 1998).

Lesions of no clinical significance can be confused with lesions specific to certain important diseases of the CNS. One of these is vacuolation of the cytoplasm of neurons in the red nucleus of the mesencephalon (Fig.4D) that could be mistaken for a lesion of BSE. It should be remembered that neuronal vacuolation is not always synonymous of BSE. Spongiform alterations of BSE occur in the neuropil (within neuronal processes, especially dendrites) and in the perikaryon of neurons from specific locations in the brainstem. However, spongiform alterations affecting the neuropil and neuronal bodies are also described in natural and experimental cases of rabies in opossums, foxes (Charlton et al. 1987), cattle (Foley & Zachary 1995); in ataxic Rottweiler puppies, (Jardim et al. 1999), and in goats with progressive paresis (Lancaster et al. 1987). Neuronal vacuolation rich in lipids was observed in one raccoon (Hamir & Fischer 1999). Incidental intracytoplasmic vacuolation of neurons in the red nucleus of the mesencephalon is found in 64% of normal adult cattle (Gavier-Widen et al. 2001), but has been seen even in cattle with only 12 months of age (Hamir et al. 2001). Vacuolation of the neuronal perikaryon in the habenular nucleus (Gavier-Widen et al. 2001) can be seen in 50% of normal cattle (Fig.4E). Sporadic neuronal vacuolation has been seen in sheep and goats (Hooper 1999), horses (Jahns et al. 2006a), and swine, (Jahns et al. 2006b), generally in neurons of the brainstem.

Sarcocystis spp. are usually seen distending myofibers of the heart, skeletal muscle, and Purkinje fibers, usually without inciting any inflammatory response (Fig.4F). Cattle become infected through ingestion of sporocysts excreted from the definitive host (carnivores, omnivores), that are in turn infected through ingestion of mature cysts (sarcocysts) in the muscles of the intermediate hosts. Infection by *Sarcocystis* spp. is common in cattle and does not produce lesions because it localizes within endothelial cells. Rupture of the cysts can cause the formation of small granulomas. Cattle are intermediate hosts for three species of *Sarcocysts*: *S. cruzi*, *S. hirsuta*, and *S. hominis*. (Dubey et al. 1988).

Axonal spheroids can be incidental findings, and are most frequently seen in the vestibular, cuneate, and accessory cuneate nuclei (Jahns et al. 2006a). In dogs, the presence of axonal spheroids is correlated with age and they are seen in 50% of dogs more than 12 years of age (Borras et al. 1999). Finding a few axonal spheroids in the brain also is a common incidental finding in sheep and goats, horses, and swine (Hooper 1999, Jahns et al. 2006a,b). Perivascular cuffs can be discrete and nonspecific. Inflammation of this type was seen in about 30% of normal adult cattle (Gavier-Widen et al. 2001). The cause of these inflammatory changes is controversial, but the clinical significance is minimal.

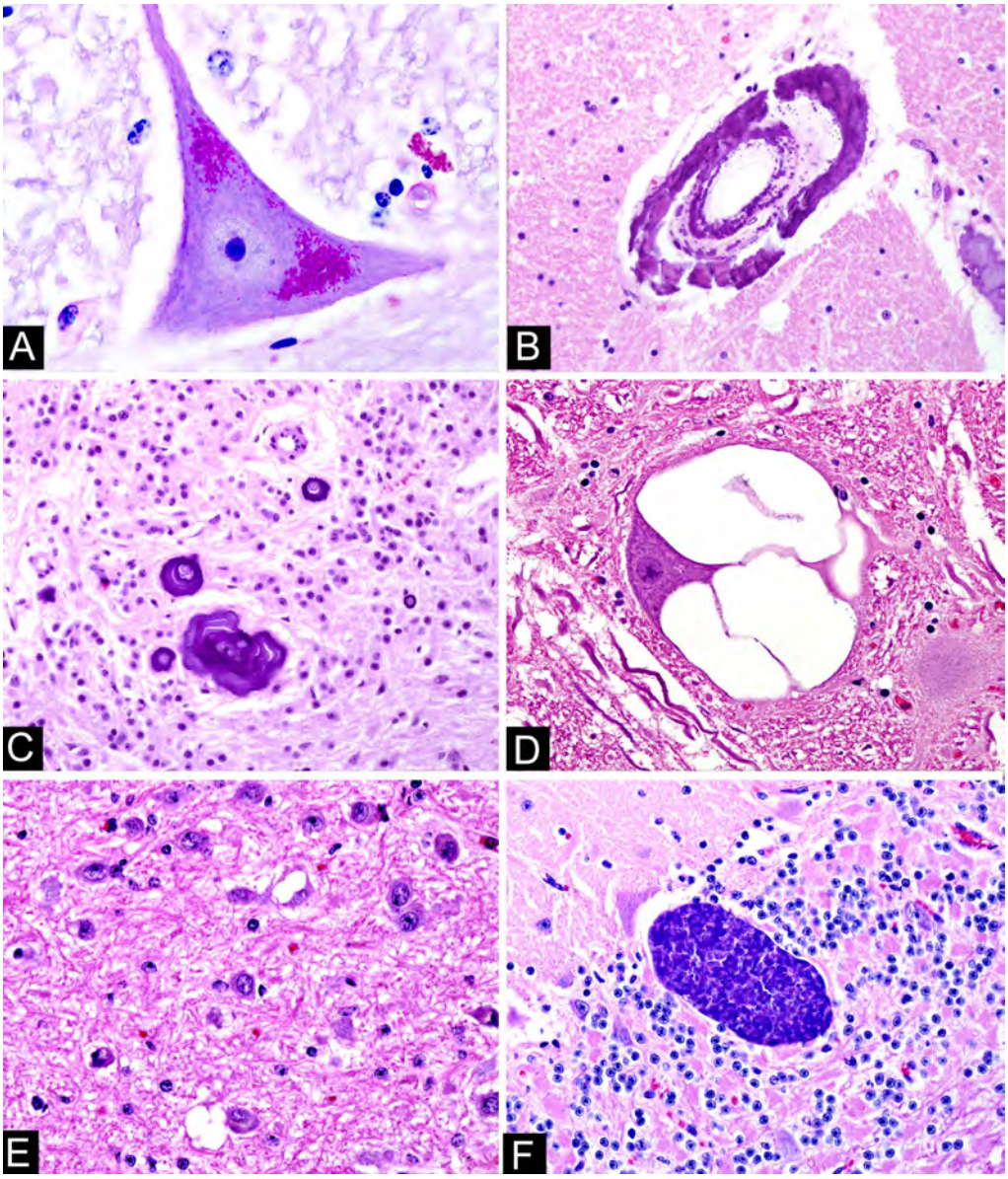


Fig.4. Incidental findings of the bovine brain. (**A**) Lipofuscin is a yellow-orange pigment that taccumulates within the perikaryon of neurons in aged animals. In Periodic acid-Schiff (PAS) stain, it appears as PAS-positive granules. PAS, obj.40x. (**B**) Vascular mineralization (siderocalcinosis) at the level of the internal capsule. HE, obj.20x. (**C**) Pineal gland concretions or corpora arenacea, brain sand or pineal (acervuli) are multiple concentric lamellar basophilic structures within the pineal gland. HE, obj.40x. (**D**) Multiple vacuolations of the perikaryon of a neuron of the red nucleus in the midbrain. HE, obj.40x. (**E**) Neuronal vacuolation of the habenular nucleus in the thalamus. HE, obj.40x. (**F**) Quiescent cyst of *Sarcocystis* sp. in the granular cell layer of the cerebellum. HE, obj.40x.

Artifacts due to the methods of slaughter, sampling, and fixation of the brain

Artifacts due to the method of slaughter, samping, or manipulation of fresh brain could be mistaken for significant antermortem changes. In most cases, these changes are not seen macroscopically. Lesions due to the method of euthanasia can be the cause of death, but often simply mask other alterations that might be a clue as to what was the underlying disease that led to the animals' euthanasia. These include submeningeal hemorrhages (Fig.5A). These lesions should be correctly interpreted as being inflicted on a brain that was normal prior to the decision to kill the bovine. Artifacts related to the collection and manipulation of the brain includes bone particles that lodge within the brain when it is removed from the cranial cavity with a saw, and can mimic pathologic mineralization (Fig.5B). Dark neurons or Cammermeyer neurons (Fig.5C) are a partially controversial subject (Cammermeyer 1978). However, it is generally accepted that it is an artifact caused by postmortem trauma to the nervous tissue caused by handling it prior to fixation (Jortner 2006, Wohlsein et al. 2013). This artifact can also be produced by improper fixation. Dark neurons should not be confused with red neurons, a hallmark neuronal necrosis

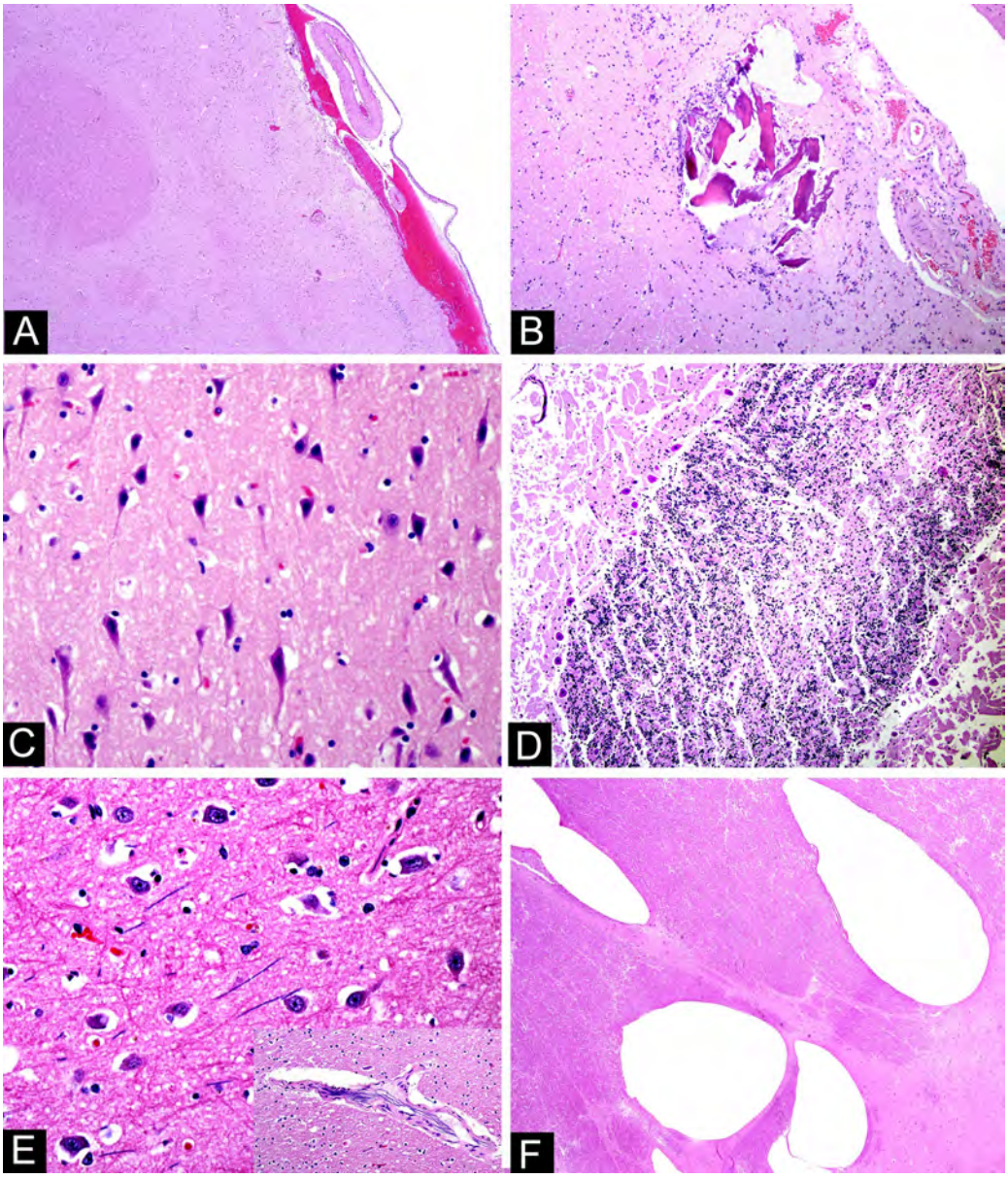


Fig.5. Artifacts in the bovine brain. (**A**) Leptomeningeal hemorrhage due to the use of a captive bolt to stun cattle before death by bleeding. HE, obj.10x. (**B**) A focal aggregate of bone sawdust is within the brain parenchyma. This artifact is commonly found at the lateral areas of the cerebrum when access to the brain is made by open the cranium vault with a handsaw or striker saw. HE, obj.20x. (**C**) Dark neurons or Cammermeyer neurons are neurons in the cerebral cortex with shrunken with dark basophilic cytoplasm and dark condensed nuclei. HE, obj.40x. (**D**) Freezing artifact. Linear interconnecting clefts crisscross the cerebellar cortex. Those clefts are the result of freezing and subsequently thawing of the neural tissue. HE, obj.10x. (**E**) Postmortem artifact. Multiple postmortem bacilli are in the cerebral cortex neuropil without inflammatory reaction. Inset: many bacilli obliterate the lumen of a blood vessel. HE, obj.40x. (**F**) Postmortem artifact. Multiple variable-sized cavities in the brain parenchyma, also called Swiss cheese brain, result from gas bubbles produced by clostridial bacilli. HE, obj.10x.

(Cantile & Youssef 2016). Linear clefts are made by crystals that form when unfixed nervous tissue is frozen (Fig.5D).

Autolysis and putrefaction

When the pathologist receives a brain to be examined in the diagnostic laboratory, there is usually little history regarding the time between the death of the animal and the necropsy and subsequent fixation of tissues. As an organ, the brain is relatively resistant to the process of autolysis, when compared with organs of the abdominal cavity. Autolysis of the CNS can mimic lesions associated with certain diseases, and be confusing to the pathologist. Also, some artefacts observed when the brain is not immediately fixed in formalin after death needs to be differentiated from ante mortem lesions.

Autolysis of gray matter can mimic lesions of trauma and autolytic vacuolar changes in myelin can mimic intramyelinic edema or Wallerian degeneration. Halos around the perikaryon of neurons, oligodendrocytes, and small vessels are common

findings in brains that are autolyzed and should not be confused with edema. Also, as autolysis sets in, glial cells can become small and hyperchromatic. Seeing cadaveric bacilli in the lumen of vessels and dispersed within the nervous tissue can help to determine the extent of autolysis. Clostridial bacilli proliferate extensively in the gastrointestinal tract after death, invade the circulatory system, and colonize various organs, including the brain (Fig.5E). These bacteria may, on occasion, produce gas that distends the nervous tissue making large multifocal vacuoles (Fig.5F).

Autolysis of the granular layer of the cerebellum is an artifact seen only in cattle (Rech et al. 2008) and humans (Albrechtsen 1977, Fuller & Burger 2007). This autolytic change is referred to as cerebellar conglutination, or "etát glace" (Fuller & Burger 2007), and it appears as a pale area in the granular layer. At higher magnification, one can see lysis of the granular neurons, with preservation of Purkinje cells (Rech et al. 2008). Although it is a postmortem phenomenon, it can be confused with necrosis of the granular cell layer caused by ischemia, and it was described in Japan as a specific bovine disease, named there as Kiriyoi disease (Goto et al. 1959, Yamagiwa & Goto 1959). Interestingly these publications (Goto et al. 1959, Yamagiwa & Goto 1959) are cited in some neuropathology texts (Fankhauser & Luginbühl 1968), thus unfortunately validating this true postmortem phenomenon as a real antemortem lesion.

REFERENCES

Albrechtsen R. 1977. The pathogenesis of acute selective necrosis of the granular layer of the human cerebellar cortex. Acta. Neuropathol. 37(1):31-34. <http://dx.doi.org/10.1007/BF00684537> <PMid:14472>

Auer R.N., Dunn J.F. & Sutherland G.R. 2008. Swiss chesee brain, p.94. In: Love S., Louis D.N. and Ellisonj D.W. (Eds), Greenfield's Neuropathology. 8th ed. Vol.1. Hodder-Arnold, London.

Barnes K.L. & Ferrario C.M. 1980. Angiotensin and CNS regulation of blood pressure. Clin. Exp. Hypertens. 2(3-4):465-477. <http://dx.doi.org/10.3109/10641968009037125> <PMid:7000458>

Barros C.S.L. 1980. Pathology of experimental infection of the bovine fetus with bovine parvovirus. PhD Thesis, Department of Pathology, Colorado State University, Fort Collins, Colorado. 199p.

Barros C.S.L., Drimeier D., Dutra I.S. & Lemos R.A.A. 2006. Exame macroscópico do sistema nervoso central, p.15-18. In: Ibid (Eds), Doenças do Sistema Nervoso de Bovinos no Brasil. Valée, Montes Claros.

Borison H.L. 1974. Area postrema: chemoreceptor trigger zone for vomiting, is that all? Life Sci. 14(10):1807-1817. <http://dx.doi.org/10.1016/0024-3205(74)90399-3> <PMid:4603263>

Borras D., Ferrer I. & Pumarola M. 1999. Age-related changes in the brain of the dog. Vet. Pathol. 36(3):201-211. <http://dx.doi.org/10.1354/vp.36-3-202> <PMid:10332828>

Cammermeyer J. 1978. Is the solitary dark neuron a manifestation of post-mortem trauma to the brain inadequately fixed by perfusion? Histochemistry 56(2):97-115. <http://dx.doi.org/10.1007/BF00508437> <PMid:97249>

Cantile C. & Youssef S. 2016. The nervous system, p.250-406. In: Maxie M.G. (Ed.), Jubb, Kennedy, and Palmer's Pathology of Domestic Animals, 6th ed. Vol.1. Elsevier, Saint Louis. <http://dx.doi.org/10.1016/B978-0-7020-5317-7.00004-7>.

Charlton K.M., Casey G.A., Webster W.A. & Bundza A. 1987. Experimental rabies in skunks and foxes, pathogenesis of the spongiform lesions. Lab. Invest. 57(6):634-645. <PMid:3695410>

Coore R.R., Love S., McKinstry J.L., Weaver H.R., Phillips A., Hillman T., Hiles M.J., Shand A., Helps C.R. & Anil M.H. 2004. Dissemination of brain emboli following captive bolt stunning of sheep: Capacity for entry into the systemic arterial circulation. J. Food Protect. 67(5):1050-1052. <http://dx.doi.org/10.4315/0362-028X-67.5.1050> <PMid:15151250>

Dubey J.P., Fayer R. & Speer C.A. 1988. Experimental *Sarcocystis hominis* infection in cattle: lesions and ultrastructure of *Sarcocystis.* J. Parasitol. 74(5):875-879. <http://dx.doi.org/10.2307/3282270> <PMid:3138399>

Duvernoy H.M. & Risold P.Y. 2007. The circumventricular organs: an atlas of comparative anatomy and vascularization. Brain Res. Rev. 56(1):119-147. <http://dx.doi.org/10.1016/j.brainresrev.2007.06.002> <PMid:17659349>

Fankhauser R. & Luginbühl H. 1968. Zentrales Nervensystem, p.191-436. In: Joest E. (Ed.), Handbuch der Speziellen Patologischen Anatomie de Hautiere. Vol.3. Verlag Paul Parey, Berlin.

Foley G.L. & Zachary J.F. 1995. Rabies-induced spongiform change and encephalitis in a heifer. Vet. Pathol. 32(3):309-311. <http://dx.doi.org/10.1177/030098589503200313> <PMid:7604498>

Fuller G.N. & Burger P.C. 2007. Central nervous system, p.273-319. In: Mills S.E. (Ed.), Histology for Pathologists. 3rd ed. Lippincott Williams and Wilkins, Philadelphia.

Garland T., Bauer N. & Bailey Junior M. 1996. Brain emboli in the lungs of cattle after stunning. Lancet 348(9027):610. <http://dx.doi.org/10.1016/S0140-6736(05)64821-0> <PMid:8774582>

Garman R.H. 2011. Histology of the central nervous system. Toxicol. Pathol. 39(1):22-35. <http://dx.doi.org/10.1177/0192623310389621> <PMid:21119051>

Gartner L.P. & Hiatt J.L. 2007. Pineal gland, p.324-325. In: Ibid (Eds), Color Textbook of Histology. Saunders Elsevier, Philadelphia.

Gavier-Widen D., Wells G.A.H., Simmons M.M., Wilesmith J.W. & Ryan J. 2001. Histological observations on the brains of symptomless 7-year-old cattle. J. Comp. Pathol. 124(1):52-59. <http://dx.doi.org/10.1053/jcpa.2000.0428> <PMid:11428189>

Goto M., Itagaki K., Yamane O., Fugihara H., Fujimoto Y., Ohshima K., Satoh H. & Yamagiwa S. 1959. Pathological studies on so-called "Kiriyoi disease". Jpn. J. Vet. Res. 7:156-170.

Hamir A.N. & Fischer K.A. 1999. Neuronal vacuolation in raccoons from Oregon. J. Vet. Diagn. Invest. 11(4):303-307. <http://dx.doi.org/10.1177/104063879901100401> <PMid:10424643>

Hamir A.N., Habecker P., Jenny A., Hutto D., Stack M.J., Chaplin M.J. & Stasko J. 2001. Idiopathic disseminated intracytoplasmatic neuronal vauolation in a neonatal Holstein calf born in the USA. J. Vet. Diagn. Invest. 13(4):349-351. <http://dx.doi.org/10.1177/104063870101300413> <PMid:11478610>

Hirano A. 1985. Neurons, astrocytes and ependyma p.1-91. In: Davis R.L. & Robertson D.M (Eds), Textbook of Neuropathology. Williams and Wilkins, Baltimore.

Hooper P.T. 1999. Incidental lesions in the brains of sheep and goats. Aust. Vet. J. 77(6):398-399. <http://dx.doi.org/10.1111/j.1751-0813.1999.tb10316.x> <PMid:10812408>

Innes J.R.M. & Saunders L.Z. 1962a. Pigmentation and depositions, p.658-667. In: Ibid (Eds), Comparative Neuropathology. Academic Press, New York.

Innes J.R.M. & Saunders L.Z. 1962b. Viral and rickettsial encephalomielidites, p.440. In: Ibid (Eds), Comparative Neuropathology. Academic Press, New York. <http://dx.doi.org/10.1016/B978-1-4832-3157-0.50015-4>.

Jahns H., Callanan J.J., McElroy M.C., Sammin D.J. & Bassett H.F. 2006a. Age-related and non-age-related changes in 100 surveyed horse brains. Vet. Pathol. 43(5):740-750. <http://dx.doi.org/10.1354/vp.43-5-740> <PMid:16966453>

Jahns H., Callanan J.J., Sammin D.J., McElroy M.C. & Bassett H.F. 2006b. Survey for transmissible spongiform encephalopathies in Irish pigs fed meat

and bone meal. Vet. Rec. 159(5):137-142. <http://dx.doi.org/10.1136/vr.159.5.137> <PMid:16877679>

Jardim L.L., Andrade-Neto J.P. & Alessi A.C. 1999. Neuronal vacuolation and spongiform lesions in young Rottweiler dogs. Arq. Bras. Med. Vet. Zootec. 51(5):449-452. <http://dx.doi.org/10.1590/S0102-09351999000500010>

Jortner B.S. 2006. The return of the dark neuron: a histological artifact complicating contemporary neurotoxicologic evaluation. Neurotoxicology 27(4):628-634. <http://dx.doi.org/10.1016/j.neuro.2006.03.002> <PMid:16650476>

Lancaster M.J., Gill I.J. & Hooper P.T. 1987. Progressive paresis in Angora goats. Aust. Vet. J. 64(4):123-124. <http://dx.doi.org/10.1111/j.1751-0813.1987.tb09652.x> <PMid:3619800>

Leslie R.A. & Osborne N.N. 1984. Amines and other transmitter-like compounds in the bovine area postrema. Brain Res. Bull. 13(3):357-362. <http://dx.doi.org/10.1016/0361-9230(84)90085-6> <PMid:6149796>

Mandara M.T. 2003. Meningeal blood vessel calcification in the brain of the cat. Acta Neuropathol. 105(3):240-244. <PMid:12557010>

Minassian B.A. 2001. Lafora's disease: Towards a clinical, pathologic, and molecular synthesis. Pediatr. Neurol. 25(1):21-29. <http://dx.doi.org/10.1016/S0887-8994(00)00276-9> <PMid:11483392>

Rech R.R., Rissi D.R., Pierezan F., Gabriel A.L. & Barros C.S.L. 2008. Autólise da camada de células granulares do cerebelo em bovinos. Ciência Rural 38(4):1181-1183. <http://dx.doi.org/10.1590/S0103-84782008000400048>

Summers B.A., Cummings J.F. & de Lahunta A. 1995. Examination of the central nervous system. p.40-50 In: Ibid (Eds), Veterinary Neuropathology. Mosby, Saint Louis.

Vandevelde M., Higgins R.J. & Oevermann A. 2012. General neuropathology, p.1-37. In: Ibid. (Eds), Veterinary Neuropathology: essentials of theory and practice. Wiley-Blackwell, Iowa.

Vigh B., Szél A., Debreceni K., Fejér Z., Manzano e Silva M.J. & Vigh Teichmann I. 1998. Comparative histology of pineal calcification. Histol. Histopathol. 13(3):851-870. <PMid:9690142>

Wells G.A.H., Wilesmith J.W. & McGill I.S. 1991. Bovine spongiform encephalopathy: a neuropathological perspective. Brain Pathol. 1(2):69-78. <http://dx.doi.org/10.1111/j.1750-3639.1991.tb00642.x> <PMid:1688299>

Wohlsein P., Deschl U. & Baumgärtner W. 2013. Nonlesions, unusual cell types, and postmortem artifacts in the central nervous system of domestic animals. Vet. Pathol. 50(1):122-143. <http://dx.doi.org/10.1177/0300985812450719> <PMid:22692622>

Yamagiwa S. & Goto M. 1959. Cortical cerebellar atrophy of granular type in Japanese cattle. Jpn. J. Vet. Res. 7:126-137.

Yanai T., Masegi T., Kawada M., Ishikawa K., Fukuda K., Yamazoe K., Iwasaki T., Ueda K. & Goto N. 1994. Spontaneous vascular mineralization in the brain of cows. J. Comp. Pathol. 111(2):213-219. <http://dx.doi.org/10.1016/S0021-9975(05)80053-2> <PMid:7806707>

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Original Article
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Low level of the immune response against rabies virus in dogs and cats, a cross-sectional study in sheltered animals, Santander, Colombia[1]

Lina María Trujillo-Rojas[2], Marlén Martínez-Gutierrez[3,4] and Julian Ruiz-Saenz[4]*

ABSTRACT.- Trujillo-Rojas L.M., Gutierrez-Gutiérrez M. & Ruiz-Saenz J. 2018. **Low level of the immune response against rabies virus in dogs and cats, a cross-sectional study in sheltered animals, Santander, Colombia.** *Pesquisa Veterinária Brasileira 38(11):2109-2116*. Universidad Cooperativa de Colombia, Bucaramanga, Colombia. E-mail: julianruizsaenz@gmail.com, julian.ruizs@campusucc.edu.co

Rabies is an incurable and fatal progressive viral encephalomyelitis that causes approximately 61,000 deaths a year globally mainly by dog bites, which show the importance of anti-rabies vaccination in dogs to achieve elimination of the disease. In Colombia, multiple cases of human rabies transmitted by dogs and cats have been reported since 1999, showing an increased significance of cats in the transmission of rabies, mainly in rabies of wild origin. Therefore, the aim of the present study was to evaluate the development of neutralizing antibodies in dogs and cats during the mass vaccination campaign of the second half of 2015 in the city of Bucaramanga. For this purpose, a descriptive cross-sectional study with convenience sampling was conducted in 382 dogs and cats (295 dogs, 87 cats), and an evaluation of the humoral immune response of the animals was performed by quantitative ELISA. The prevalence of optimal neutralizing antibodies (>0.5 IU/ml) was only 32.76% (95% CI=28.05-37.46%) in the entire population studied and most of the animals did not have an adequate response to the vaccination, or seroconversion was not detected on them (65.45-95% confidence interval, CI=60.68-70.21%). Significant difference was found between the neutralizing antibody titers in cats and dogs, with a higher neutralizing response in cats. In conclusion, although mass vaccination campaigns for dogs and cats are the most important measure to interrupt virus circulation among the animals, achievement of a good neutralizing immune response in the animals is useful to demonstrate that vaccination has been successful, allowing the maintenance of the required minimum levels of population immunity. These results will allow the implementation of corrective measures in Bucaramanga to achieve better seroconversion rates. Other cities are expected to implement similar seroconversion assessments to verify the quality of effective anti-rabies vaccination in animal populations.

INDEX TERMS: Immune response, rabies virus, dogs, cats, sheltered animals, Santander, Colombia, vaccination.

RESUMO.- [**Baixo nível de resposta imune contra o vírus da raiva em cães e gatos, um estudo transversal em animais abrigados, Santander, Colombia.**] A raiva é uma encefalomielite viral progressiva incurável e fatal que causa aproximadamente 61.000 mortes por ano no mundo, principalmente por mordidas de cães, que mostram a importância da vacinação anti-rábica em cães para conseguir a eliminação da doença. Na Colômbia, vários casos de raiva humana transmitida por cães e gatos foram relatados desde 1999, mostrando um aumento na importância de gatos na transmissão da raiva, principalmente em raiva de origem selvagem. Portanto, o objetivo do presente estudo foi avaliar o desenvolvimento de anticorpos neutralizantes em cães e gatos durante a campanha de vacinação em massa do segundo semestre de 2015 na cidade de Bucaramanga. Para este propósito, um estudo descritivo

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[2] Universitad Autònoma de Barcelona, Barcelona, España.

[3] Infettare, Facultad de Medicina, Universidad Cooperativa de Colombia, Medellín, Colombia.

[4] Grupo de Investigación en Ciencias Animales (GRICA), Universidad Cooperativa de Colombia, Calle 30A # 33-51, Bucaramanga, Colombia. *Corresponding author: julianruizsaenz@gmail.com, julian.ruizs@campusucc.edu.co; Phone: +57-7-685 45 00 ext 7037.

transversal com amostragem de conveniência foi realizado em 382 cães e gatos (295 cães, 87 gatos), e uma avaliação da resposta imune humoral dos animais foi realizada por ELISA quantitativo. A prevalência de anticorpos neutralizantes ótimos (>0,5 UI/ml) foi de apenas 32,76% (IC 95% = 28,05-37,46%) em toda a população estudada e a maioria dos animais não teve resposta adequada à vacinação, ou a soroconversão foi não detectado neles (65,45-95% intervalo de confiança IC = 60,68-70,21%). Diferença significativa foi encontrada entre os títulos de anticorpos neutralizantes em gatos e cães, com uma maior resposta neutralizante em gatos. Em conclusão, embora as campanhas de vacinação em massa para cães e gatos sejam a medida mais importante para interromper a circulação do vírus entre os animais, a obtenção de uma boa resposta imunológica neutralizante nos animais é útil para demonstrar que a vacinação foi bem sucedida, permitindo a manutenção necessária dos níveis mínimos de imunidade da população. Esses resultados permitirão que a implementação de medidas corretivas em Bucaramanga alcance melhores taxas de soroconversão. Outras cidades devem implementar avaliações similares de soroconversão para verificar a qualidade da vacinação anti-rábica efetiva em populações animais.

TERMOS DE INDEXAÇÃO: Resposta imune, vírus da raiva, caninos, felinos, animais abrigados, Santander, Colombia, vacinação.

INTRODUCTION

Rabies is an incurable and fatal progressive viral encephalomyelitis caused by a lyssavirus (Bourhy et al. 2008). Rabies virus (RABV) belongs to the order Mononegavirales, family *Rhabdoviridae*, and genus *Lyssavirus* (Afonso et al. 2016). Rabies transmission occurs in two epidemiological forms: a) urban, with the dog as the main reservoir and transmitter, and b) wild, with animals such as bats, foxes, and wolves as reservoirs and transmitters and cats as transmitters (Hampson et al. 2009, Páez et al. 2011).

Despite being fatal in 100% of cases, it can also be prevented in 100% of cases, either with massive pre-exposure vaccination or post-exposure prophylaxis measures, which include the use of immunoglobulins and vaccines, to prevent disease development in exposed patients (Abela-Ridder 2015, Cleaveland et al. 2006).

Countries that implement rabies elimination programs show a marked reduction in infections, often leading to the elimination of the disease (WHO 2013). To break the RABV transmission cycle, elimination programs center around dog vaccination campaigns, which must include at least 70% of the dog population in order to stop transmission (Belotto et al. 2005, Cleaveland et al. 2006, WHO 2013).

Of the 61,000 deaths per year caused by rabies in humans (WHO 2013), 99% are caused by dog bites, proving the importance of rabies vaccination in dogs to prevent human rabies (Abela-Ridder 2015). In Colombia, seven cases of rabies transmitted by dogs and nine by cats have been officially reported since 1999, showing that cats have acquired importance as a transmitter, due in large part to their nocturnal and hunting habits, that expose it to RABV by contact with wildlife rabies infected bats (Minsalud 2014, Castro Castro et al. 2016).

Colombia has historically been affected by urban and wildlife rabies outbreaks (Páez et al. 2003a, 2003b, 2005). The most recent outbreak of dog-transmitted rabies occurred in the years 2006-2008 in the Santa Marta Tourist District, Department of Magdalena, which claimed the lives of four humans and dozens of dogs (Páez et al. 2009a). Different case analyses show the ongoing occurrence of urban RABV outbreaks caused by wild variants and leading to multiple animal and human deaths in the country (Badillo et al. 2009, Mantilla et al. 2010, Páez et al. 2009b, Valderrama et al. 2006), and the increasing importance of cats as transmitter agents of these wild variants (Páez et al. 2009a, Castro Castro et al. 2016), which demonstrate the importance of maintaining high vaccination coverage also in cats, although the "Regional Program for the Elimination of Human Rabies" has been focused mainly on dogs (OPS 2005, Schneider et al. 2007).

To reduce rabies cases to zero, one essential point is to achieve sustained vaccination in dogs and cats. (Abela-Ridder 2015). In Colombia, canine and feline populations are increasing in urban areas; in Bucaramanga specifically, the estimated population of dogs and cats in 2010 was 39,582 individuals, of which 31,604 were canine and 7,978 feline (Arismendy et al. 2010), and although no clinical cases of canine rabies have been identified in the department of Santander since 2004, the arrival of wildlife rabies to urban populations is a constant threat (Badillo et al. 2009, Páez et al. 2009b, Mantilla et al. 2010).

In Colombia, the vaccination of canines and felines is mandatory. The national rabies vaccination program has achieved a national vaccination coverage that varied between 45% and 63% from 1994 to 2005 (Cediel et al. 2010). However, campaigns often do not have the expected impact because of multiple factors, including the dispersal of the vaccination strategy over the 12 months of the year and a likely underestimation of the canine population (OPS 2005), as well as the use of inactivated low potency vaccines, the number of doses, interval between vaccinations, age at vaccination, among others, all factors that can influence the immune response (Berndtsson et al. 2011). The impact of failures in the cold chain of vaccines, inadequate handling of biologicals, inappropriate vaccine inoculation in canines, use of expired vaccines, or dishonesty of the persons or entities that sell the vaccines (Paéz et al. 2007) has also been described. In Colombia, these problems have led to vaccine failures in which up to 35% of the vaccinated population does not develop an immune response or does so inadequately (Páez et al. 2011).

The aim of the present study was to evaluate the production of neutralizing antibodies in dogs and cats vaccinated during the mass vaccination campaign of the second half of 2015 in the city of Bucaramanga and its metropolitan area. These data should be used to study the outcome of mass vaccination campaigns and as a tool for decision-making about the implementation of control strategies to improve vaccination coverage by health authorities, at both the regional and national levels.

MATERIALS AND METHODS

Ethics statement. This study was approved by the Ethics Committee for Animal Experimentation of the Cooperative University of Colombia, Bucaramanga (Act 023 of July 2014). The owners and managers of the animal shelters signed an informed consent form endorsed by the above-mentioned ethics committee. In addition, the authors state that all scientific, technical, and administrative rules for animal research were followed on this study.

Type of study and samples. A descriptive cross-sectional study with convenience sampling was conducted in 382 dogs and cats (295 dogs, 87 cats) belonging to the metropolitan area of Bucaramanga (Santander), Colombia (Fig.1). The sample size was defined using WinEpi 2.0 software (available online at: http://www.winepi.net/), considering an estimated prevalence of 50% and a 95% confidence level and assuming an error of 5%. Taking into account pet ownership data, a ratio of 3:1 dogs to cats was sampled. The inclusion criterion for the patients was to have been vaccinated in a mass vaccination campaign and to have the card showing a vaccination date of no more than 10 months prior to the time of sampling.

In these patients, 1ml of blood was drawn into Vacutainer® tubes by puncturing the radial vein of the forearm or the cephalic vein (in dogs) or the jugular vein (in cats) using a 22 gauge needle. Samples were allowed to clot at room temperature and then centrifuged for 15 minutes at 5,000*x*g. Sera were separated and stored frozen at -80°C until the levels of neutralizing anti-rabies antibodies were measured.

ELISA screening of the samples. To evaluate the humoral response of the animals, the quantitative Enzyme-Linked Immunosorbent Assay (ELISA) "Platelia Rabies II Kit ad usum Veterinarium" (Bio-Rad® Laboratories-Marnes La Coquette, France) was used for the detection and quantification of anti-rabies glycoprotein antibodies in the serum of animals, following the methodology and precautions recommended by the manufacturer.

This ELISA allows the quantification of equivalent neutralizing antibody titers (EU/ml) by comparison with a calibration curve prepared with different dilutions of an OIE-approved International Standard Serum with neutralizing antibody titers measured in IU/ml. This ELISA has been previously validated, with viral neutralization tests (Servat et al. 2007) showing optimal results.

In order to increase sensitivity the Platelia Rabies II kit, we used a threshold of 0.3 EU/mL instead of 0.5 EU/mL as has been reported recently (Knoop et al. 2010, Wasniewski et al. 2014). Neutralizing antibody titers are presented in four international standard categories: undetectable titers at a level of <0.125 EU/ml, insufficient titers at 0.125-0.3 EU/ml, sufficient titers at 0.3-4 EU/ml, and high titers at >4 EU/ml. According to this modification, Sensitivity and Specificity for this test has been reported of 92.68 and 100% respectively (Wasniewski et al. 2014).

Statistical analysis. The computer programs WinEpi 2.0 (available online at: http://www.winepi.net/) and Prism® 7.01 for Windows™ (GraphPad Software, La Jolla/CA) were used for the statistical analysis and processing of data. Descriptive statistics of demographic data were generated. Categorical variables are summarized as frequencies and proportions, and continuous variables are summarized as means or medians with their dispersion measures. A bivariate analysis was performed to compare the positive and negative results of the vaccinated patients using the chi-square test or Fisher's exact test. Normality was verified by the Shapiro-Wilk test, and the differences between continuous variables were calculated with the Mann-Whitney test. Comparisons among the animal shelters were performed using the Kruskal-Wallis test with Dunn's multiple comparisons test. In all cases, the results were considered statistically significant at a level of $p<0.05$.

RESULTS

The study population consisted of 295 dogs and 87 cats belonging to animal shelters in the city of Bucaramanga and its metropolitan area (Fig.1). These animals were vaccinated with one dose of inactivated rabies vaccine between 5 and 8 months prior to sampling. The population of dogs studied consisted of 37.28% males and 62.72% females. A similar sex distribution was found in the cat population, in which 39.08% were males and 60.92% females. The mean age was 3.69 years for the dogs (range 1 to 8 years) and 3.81 for the cats (range 1 to 10 years) included in the study.

In the evaluation of neutralizing antibodies, most of the animals did not have an adequate response to the vaccination, or seroconversion was not detected on them (65.45%-95% confidence interval, CI=60.68-70.21%). To perform an optimal estimated of the antibody titers, we adjusted the prevalence according to the sensitivity and specificity data reported for the Platelia Rabies II ELISA (Wasniewski et al. 2014). The real prevalence of optimal neutralizing antibodies (≥0.3 EU/mL) was only 32.76% (95% CI=28.05-37.46%) in the entire

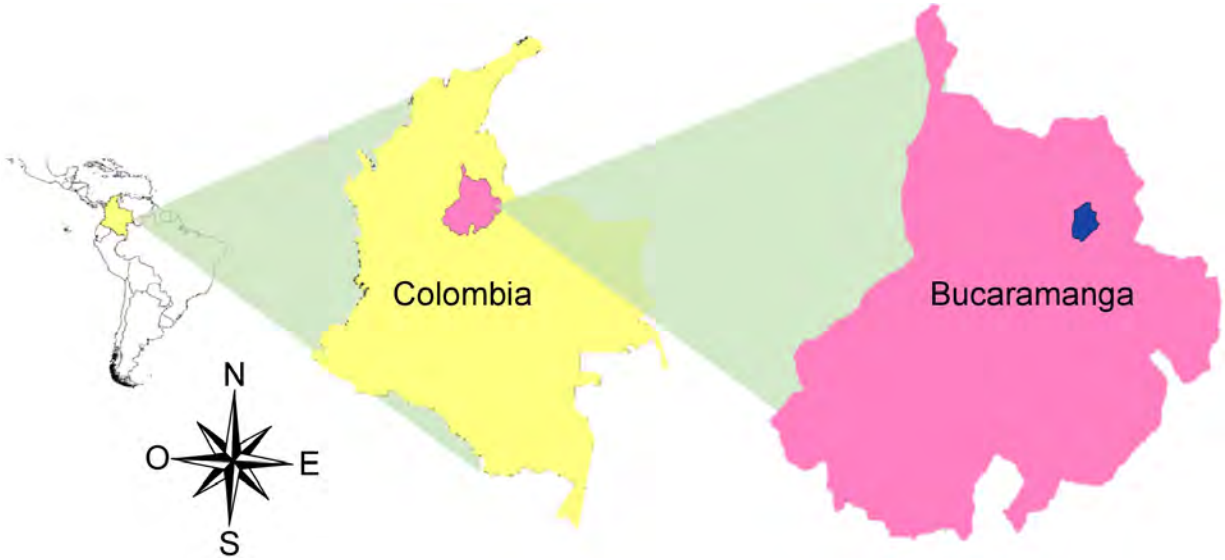


Fig.1. Geographical location of the sampling site in Bucaramanga, Colombia. The pink area denotes Santander Province, and the blue area denotes Bucaramanga City. The map was created using DIVA-GIS software version 7.5.0 for Windows™.

population studied. In addition, the prevalence of suboptimal or insufficient antibodies was 4.51% (95% CI=2.43-6.59%).

In the differential evaluation of neutralizing antibodies between dogs and cats, a highly significant difference (p<0.001) was found between the neutralizing antibody titers in cats and dogs when titers were measured in Equivalent Units/ml (EU/ml), with a higher neutralizing response shown in cats compared to dogs (Fig.2). Conversely, dogs had a statistically higher prevalence of non-reactors than cats (Fig.2A).

In the dog population, 26.69% (95% CI=21.64-31.74%) of the dogs sampled had a sufficient neutralizing serological response (between 0.3 and 4 EU/ml, Fig.2B). The highest proportion (77.17%-95% CI= 72.38-81.96%) did not showed serological evidence of seroconversion to vaccination and 4.01% (95% CI= 1.77-6.25%) of the dogs studied had an insufficient seroconversion rate.

The distribution in cats varied considerably to the dogs, with a prevalence of cats with sufficient antibody titers (52.08% - 95% CI=41.59-62.58%) similar to the proportion of cats in which seroconversion was not detected (48.36% - 95% CI=37.86-58.86%). Only 6.19% (95% CI=1.13-11.25%) of the cats sampled had an insufficient neutralizing serological response. Of the entire population evaluated, only one individual (1.23%-95% CI=0.00-3.55%) had a high level of neutralizing antibodies (>4 EU/ml, Fig.2B).

In the analysis of antibody distribution by sex in dogs, significant differences were found between the neutralizing serological response of male dogs compared to the response of females (Fig.3A), with a higher frequency of male dogs that did not produce an immune response compared to females (Fig.3B). In the case of cats, no difference was found between the antibody levels of males and females, nor was a difference observed in any of the seroconversion subgroups (Fig.3C,D)

Finally, to evaluate the occurrence of an anthropogenic effect in the vaccination process, a subgroup analysis was performed, evaluating 358 of the samples that belonged to the four main animal shelters. The assessment determined whether differences existed in the seroconversion level between the animals belonging to the different subgroups, with a highly significant difference being observed between them (p<0.001), with better seroconversion rates in some shelters (Fig.4A,D) compared to others (Fig.4B,C). However, as shown in the figure, the majority of the population is distributed with a low level of neutralizing humoral immunity.

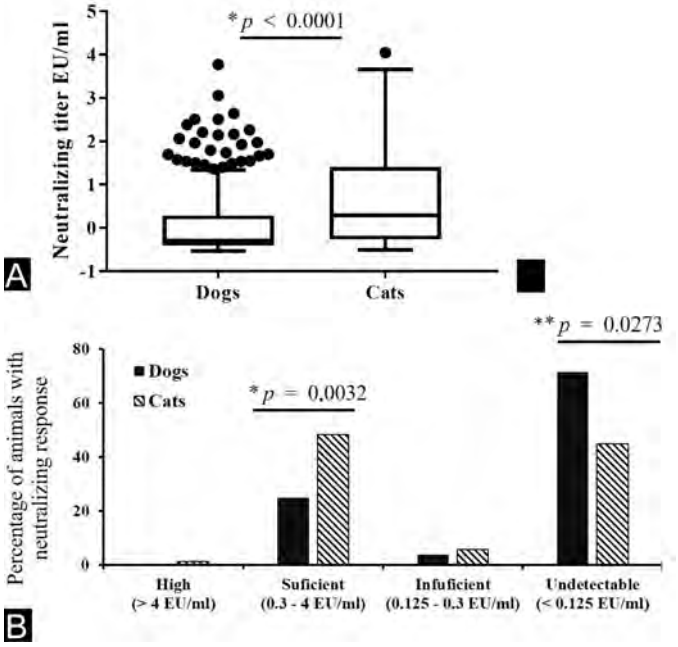


Fig.2. Neutralizing antibodies after rabies vaccination in dogs and cats of Bucaramanga. (**A**) Distribution range according to OIE standards. (**B**) Tukey's box comparing the distribution of antibodies between dogs and cats. *Statistically significant Chi-square test (X^2), ** Statistically significant Mann-Whitney test.

DISCUSSION

Although a reduction of more than 90% of the cases of human rabies transmitted by dogs in Latin America (OPS 2005, Schneider et al. 2007) has been achieved within the framework of the "Regional Program for the Elimination of Human Rabies Transmitted by Dogs", rabies is still considered the most important viral zoonosis (Dodet et al. 2008). In this endeavor, mass vaccination campaigns for dogs and cats are the most important measure to stop virus circulation among the animal population, and long-term sustained coverage is required to avoid the re-emergence of the disease (Schneider et al. 2007). In addition to good vaccination coverage, achievement of a good neutralizing immune response in the animals is necessary to demonstrate that vaccination has been successful, allowing the maintenance of the minimum levels of required population immunity (Hampson et al. 2009). Our results show that only a quarter of the population evaluated (25.92%) achieved an optimal neutralizing immune response (Fig.2). This undoubtedly shows a failure in urban rabies elimination programs and a latent risk to the animal and human populations.

Although most of human rabies cases in Latin America have been transmitted by dogs (Schneider et al. 2007), in the last decade, cats have become a major bridge of transmission of wildlife rabies to humans (Páez et al. 2009a). In fact, the last case of human rabies in the region studied was due to a wild-type virus transmitted by cats in an urban area (Mejía-Angarita & López-Martínez 2010), which shows the important impact that the inclusion of cats in the plans for control and elimination of rabies has on the epidemiology of the infection.

Notably, although 71.53% of the vaccinated dogs did not develop neutralizing humoral immunity, only 44.83% of the cats vaccinated in the mass vaccination campaigns of the second half of 2015 did not develop neutralizing antibodies. These results in dogs are far from the results reported by Paez et al (Páez et al. 2011), who demonstrated that 74.9% of dogs had an adequate humoral immune response, that is, at levels equal to or higher than 0.5 IU/ml. In the case of cats, the present study is the first one in Colombia that shows the results of the anti-rabies immune response in cats in the country.

In Bucaramanga, according to the data published by the Ministry of Health, rabies vaccination coverage in dogs and cats was 41.96% in 2014, (Minsalud 2015). When this coverage is added to the poor humoral response of vaccinated animals, a high risk level is shown for the re-emergence of both urban and wildlife rabies in animals and humans, as has already been reported (Mejía-Angarita & López-Martínez 2010).

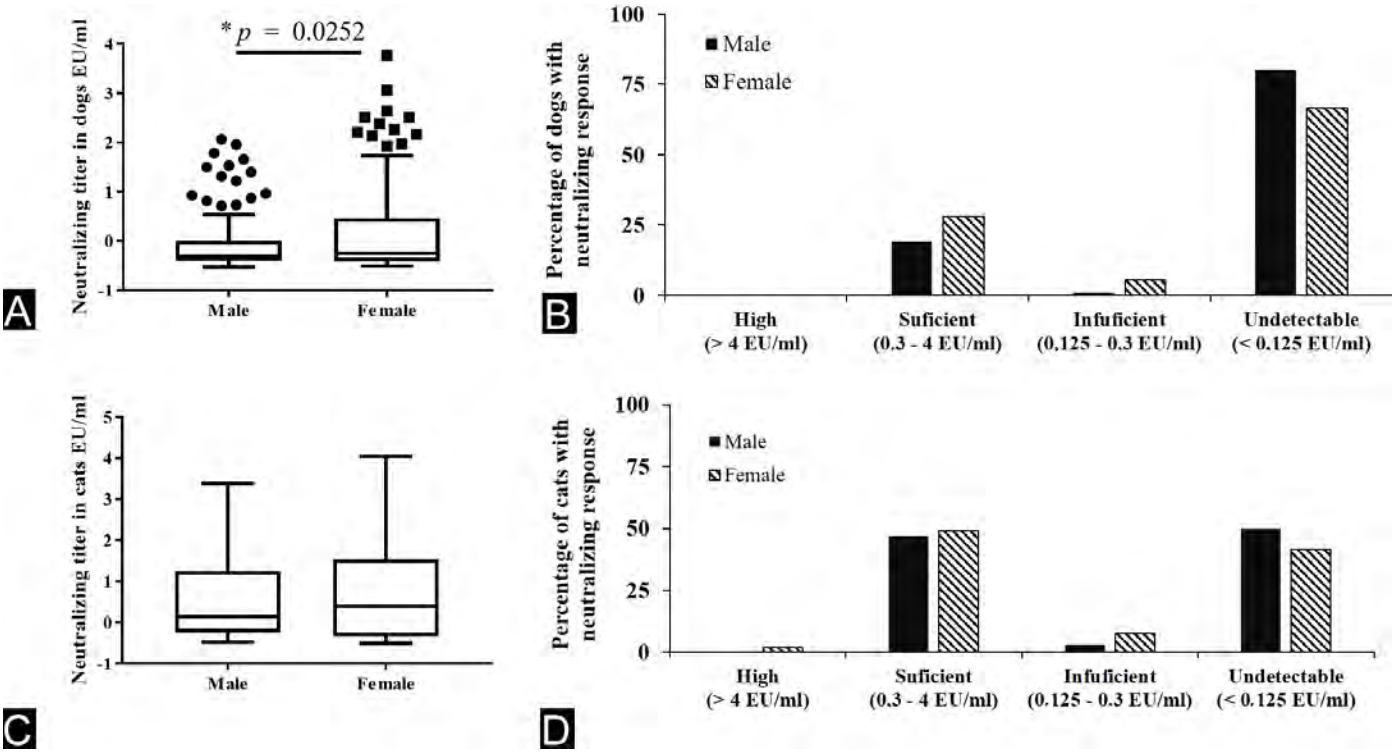


Fig.3. Sex distribution of rabies neutralizing antibodies after vaccination in dogs and cats Bucaramanga. (**A**) Tukey's box comparing the distribution of antibodies between male and female dogs. (**B**) Distribution range by sex according to OIE seroconversion standards in Dogs. (**C**) Tukey's box comparing the distribution of antibodies between male and female cats. (**D**) Distribution range by sex according to OIE seroconversion standards in cats. *Statistically significant Chi-square test (X^2).

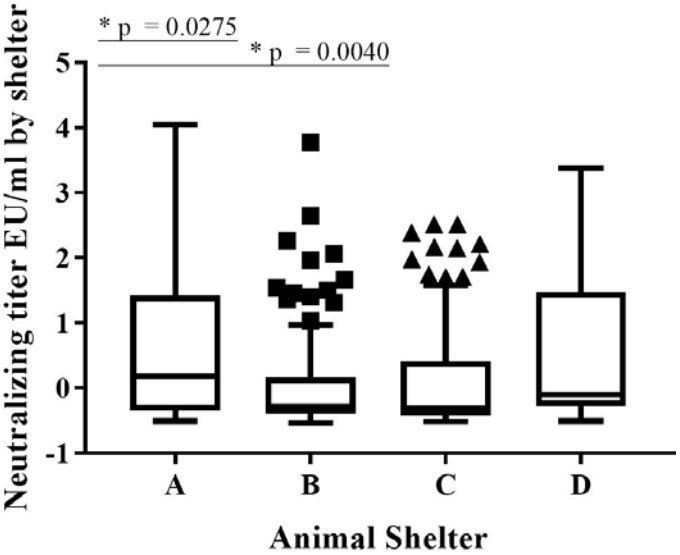


Fig.4. Distribution of neutralizing antibodies after anti-rabies vaccination in shelters. Tukey Box chart is presented comparing the distribution of neutralizing antibodies among the different shelters included in the study. *Statistically significant Kruskal-Wallis test with Dunn's multiple comparisons test.

Different factors might explain these low seroconversion rates. As an independent study, it was not possible to retrieve information from the manufacturer and/or type of vaccine used, batch, etc. However, although the vaccination card was reviewed (data not shown), the information provided in these cards by the personnel responsible for vaccinations was inconsistent and incomplete and did not allow the evaluation of vaccine-related variables that could explain the low rate of seroconversion. This information could be confirmed in the study by (Páez et al. 2011), demonstrating that there may be vaccines and/or batches that do not allow the production of effective antibody titers (Páez et al. 2011). The differences found in the level of seroconversion among animal shelters (Fig.4) allow us to assume that there are anthropogenic variables that influence the immune response of vaccinated animals. These occur in addition to the biological responses that affect the dogs and cats and are attributable to factors such as nutritional status or health status (Almeida et al. 1997, Rigo & Honer 2006, Tordo et al. 2005).

Most of the regional campaigns of vaccination of dogs and cats against rabies in Brazil (Fernandes et al. 2017) and Colombia (present data) used cell culture rabies inactivated virus and aluminum hydroxide as adjuvant. Similar to the analyzed data by (Fernandes et al. 2017), Vaccine quality, dose, age of primary vaccination, nutritional status, presence of concomitant infections, in addition to the individual immunological capacity could not be evaluated, it is not possible to attribute to any of them the observed unresponsiveness to vaccination in many animals.

We believe that the number of vaccine doses received by the animals is not confusing the results. Although it has been reported that around 14% of primo-vaccinated dogs

have presented neutralizing titers lower than the minimum threshold (0.5 IU/mL) (Cliquet et al. 2003), most of the animals belonging to the evaluated shelters has been previously vaccinated at least one time because vaccination at admittance of the shelter it is mandatory (Day et al. 2016) and it is well assumed that for multiple vaccinated dogs, antibody titers did not depend on the time elapsed since the last vaccination (Cliquet et al. 2003, Mansfield et al. 2004).

Also, it was previously demonstrated that the peak of rabies antibody titers initially rises and then from about 6 to 8 weeks onwards the titers slowly decreases, eventually resulting in an increase in the number of test failures (Jakel et al. 2008, Kennedy et al. 2007, Van de Zande et al. 2009). It could be possible that our evaluation lost the peak of seroconversion in the animals and that the level of antibodies goes below the protective threshold of 0.5 IU/mL, however this does not mean that they are not protected against rabies (Lakshmanan et al. 2006); in fact, the presence of detectable neutralizing antibodies against rabies at the time of a possible wild type virus challenge does not indicate protection for all animals (Aubert 1992, Hooper et al. 1998).

This is the first study evaluating the vaccine response that includes samples of both dogs and cats in Colombia. Considerably, our results (Fig.2A) demonstrate a significant difference with a higher prevalence of neutralizing antibodies in vaccinated cats compared to dogs. Similarly, in a study of samples of dogs and cats vaccinated in two cities in Southeastern Brazil, significant differences were found in the development of antibodies between dogs and cats in only one of the cities studied (Albas et al. 2013). This finding could explain that the differential response of dogs and cats is mainly mediated by anthropogenic factors such as the vaccination culture and the quality of the vaccination, etc., rather than by a differential biological response between the two species (Moore et al. 2015, Tordo et al. 2005).

In similar way to our results, it has been recently reported the frequency and magnitude of neutralizing antibodies to RABV in dogs with and without historic of vaccination in Santa Maria/RS, Brazil, during missive 2015 rabies vaccination campaign (Fernandes et al. 2017), the results shown that after single dose vaccination, between 30-58% of dogs do not contain neutralizing antibodies against RABV in adequate levels, highlighting current vaccine status in dogs that may not be sufficient to prevent an eventual introduction and transmission of the virus to unprotected dogs (Fernandes et al. 2017).

Since the development of the Platelia™ Rabies II kit *ad usum Veterinarium* by Bio-Rad in 2006 (Servat et al. 2007) and the certification by the OIE in 2007 for the determination of immune status postvaccination in individual dogs or cats (World Organisation for Animal Health 2007), this ELISA seemed to be a reliable alternative method to the current seroneutralisation tests (FAVN test: fluorescent antibody virus neutralisation test; RFFIT: rapid fluorescent focus inhibition test). However, many discordant results were observed when comparing the titers obtained by the Platelia Rabies II kit and those obtained with the FAVN or RFFIT. Compared to the FAVN test, this ELISA was not sensitive enough for rabies antibody detection with the current threshold of 0.5 EU/mL indicating that that this ELISA underestimates the rabies antibody titers obtained in vaccinated animals (even in high positive samples) compared to the gold standard tests (Knoop et al. 2010, Wasniewski et al. 2014).

In order to increase sensitivity of the Platelia Rabies II kit, results obtained from an international collaborative study of the proficiency of the test has shown that this ELISA could be used for research purposes with a threshold set at 0.3 EU/mL instead of 0.5 EU/mL (Wasniewski et al. 2014), recommendation that where take into account in this paper in order to have a more precise and less restrictive results.

As mention before, there is not outbreaks of urban rabies in Colombia since 2008 (Páez et al. 2009b); However multiple cases human rabies transmitted by cat or by bats has been recorded in urban areas (Valderrama et al. 2006, Páez et al. 2009a, Mantilla et al. 2010, Mejía-Angarita & López-Martínez 2010). Besides, we have recently showed that Livestock rabies transmitted by bats has an increasing trend of the disease and is present in 62.5% of Colombian provinces (Marin-Alvarez et al. 2014). Thus, considering the potential risk of introduction of the virus from nearby rural areas through bat or cat hunting bats, our present data indicated that the vaccine coverage in dogs/cat could be unable to respond efficiently to a possible challenge with field virus and could lead to possible outbreak of urban canine/feline rabies and possibility to human rabies cases.

CONCLUSIONS

Our results highlight the low real coverage of rabies vaccination and demonstrate that, after a mass vaccination campaign, the vast majority of animals in the study population would be unable to respond efficiently to a challenge with field virus.

A post-vaccination determination of antibody titers is recommended as a more objective measure to evaluate rabies vaccination campaigns in addition to traditional coverage measures.

These results will allow the implementation of corrective measures in the Bucaramanga dog and cat population to achieve better seroconversion rates.

Other cities are expected to implement similar seroconversion assessments to prove the quality of effective anti-rabies vaccination in animal populations.

Competing interests. The authors declare that they have no competing interests.

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REFERENCES

Abela-Ridder B. 2015. Rabies: 100 per cent fatal, 100 per cent preventable. Vet. Rec. 177(6):148-149. <http://dx.doi.org/10.1136/vr.h4196> <PMid:26251539>

Afonso C.L., Amarasinghe G.K., Bányai K., Bào Y., Basler C.F., Bavari S., Bejerman N., Blasdell K.R., Briand F.-X., Briese T., Bukreyev A., Calisher C.H., Chandran K., Chéng J., Clawson A.N., Collins P.L., Dietzgen R.G., Dolnik O., Domier L.L., Dürrwald R., Dye J.M., Easton A.J., Ebihara H., Farkas S.L., Freitas-Astúa J.,

Formenty P., Fouchier R.A., Fù Y., Ghedin E., Goodin M.M., Hewson R., Horie M., Hyndman T.H., Jiāng D., Kitajima E.W., Kobinger G.P., Kondo H., Kurath G., Lamb R.A., Lenardon S., Leroy E.M., Li C.X., Lin X.D., Liú L., Longdon B., Marton S., Maisner A., Mühlberger E., Netesov S.V., Nowotny N., Patterson J.L., Payne S.L., Paweska J.T., Randall R.E., Rima B.K., Rota P., Rubbenstroth D., Schwemmle M., Shi M., Smither S.J., Stenglein M.D., Stone D.M., Takada A., Terregino C., Tesh R.B., Tian J.H., Tomonaga K., Tordo N., Towner J.S., Vasilakis N., Verbeek M., Volchkov V.E., Wahl-Jensen V., Walsh J.A., Walker P.J., Wang D., Wang L.F., Wetzel T., Whitfield A.E., Xiè J.T., Yuen K.Y., Zhang Y.Z. & Kuhn J.H. 2016. Taxonomy of the order Mononegavirales: update 2016. Arch. Virol. 161(8):2351-2360. <http://dx.doi.org/10.1007/s00705-016-2880-1> <PMid:27216929>

Albas A., Picolo M.R., Soares C.N., Bachega H.V.U. & Tarumoto M.H. 2013. Humoral immune response in dogs and cats vaccinated against rabies in southeastern Brazil. J. Venom. Anim. Toxins incl. Trop. Dis. 19(1):17. <http://dx.doi.org/10.1186/1678-9199-19-17> <PMid:23899101>

Almeida M.F., Aguiar E.A., Martorelli L.A., Presotto D., Brandão M.M. & Pereira O.A. 1997. Resposta imune humoral de cães à vacina inativada, de cérebro de camundongos lactentes, utilizada nas campanhas anti-rábicas no Brasil. Revta Saúde Públ. 31(5):502-507. <http://dx.doi.org/10.1590/S0034-89101997000600009>

Arismendy A., García A., Gonzales C., Pinzón C., Delgado E. & Flóres F. 2010. Primer censo canino, felino del área urbana del municipio de Bucaramanga. Universidad Cooperativa de Colombia, Facultad de Medicina Veterinaria y Zootecnia. 130p.

Aubert M.F. 1992. Practical significance of rabies antibodies in cats and dogs. Rev. Sci. Tech. 11(3):735-760. <http://dx.doi.org/10.20506/rst.11.3.622> <PMid:1472723>

Badillo R., Mantilla J.C. & Pradilla G. 2009. Encefalitis rábica humana por mordedura de murciélago en un área urbana de Colombia. Biomédica 29(2):191-203. <http://dx.doi.org/10.7705/biomedica.v29i2.21> <PMid:20128344>

Belotto A., Leanes L., Schneider M., Tamayo H. & Correa E. 2005. Overview of rabies in the Americas. Virus Res. 111(1):5-12. <http://dx.doi.org/10.1016/j.virusres.2005.03.006> <PMid:15896398>

Berndtsson L.T., Nyman A.-K.J., Rivera E. & Klingeborn B. 2011. Factors associated with the success of rabies vaccination of dogs in Sweden. Acta Vet. Scand. 53(1):22. <http://dx.doi.org/10.1186/1751-0147-53-22> <PMid:21439029>

Bourhy H., Reynes J.-M., Dunham E.J., Dacheux L., Larrous F., Huong V.T.Q., Xu G., Yan J., Miranda M.E.G. & Holmes E.C. 2008. The origin and phylogeography of dog rabies virus. J. Gen. Virol. 89(Pt 11):2673-2681. <http://dx.doi.org/10.1099/vir.0.2008/003913-0> <PMid:18931062>

Castro Castro F.F., Rodríguez M. & Patricia M. 2016. Feline rabies in a domestic cat (*Felis silvestris catus*), Yumbo, Valle Del Cauca, Colombia. Revta UDCA Actual. Divers. Cient. 19:243-246.

Cediel N., De la Hoz F., Villamil L.C., Romero J. & Díaz A. 2010. Epidemiología de la rabia canina en Colombia. Revta Salud Publica 12(3):368-379. <http://dx.doi.org/10.1590/S0124-00642010000300003> <PMid:21311825>

Cleaveland S., Kaare M., Knobel D. & Laurenson M.K. 2006. Canine vaccination: providing broader benefits for disease control. Vet. Microbiol. 117(1):43-50. <http://dx.doi.org/10.1016/j.vetmic.2006.04.009> <PMid:16701966>

Cliquet F., Verdier Y., Sagne L., Aubert M., Schereffer J.L., Selve M., Wasniewski M. & Servat A. 2003. Neutralising antibody titration in 25,000 sera of dogs and cats vaccinated against rabies in France, in the framework of the new regulations that offer an alternative to quarantine. Rev. Sci. Tech. 22(3):857-866. <http://dx.doi.org/10.20506/rst.22.3.1437> <PMid:15005543>

Day M.J., Horzinek M.C., Schultz R.D. & Squires R.A. 2016. WSAVA Guidelines for the vaccination of dogs and cats. J. Small Anim. Pract. 57(1):E1-E45. <http://dx.doi.org/10.1111/jsap.2_12431> <PMid:26780857>

Dodet B., Fooks A.R., Müller T. & Tordo N. 2008. Towards the elimination of rabies in Eurasia. Proc. Joint OIE/WHO/EU International Conference. Develop. Biol., Basel 131:3-584.

Fernandes K.G., Martins M., Amaral B.P., Cargnelutti J.F., Weiblen R. & Flores E.F. 2017. Antibodies against rabies virus in dogs with and without history of vaccination in Santa Maria/RS, Brazil. Ciência Rural 47(11): e20170125. <http://dx.doi.org/10.1590/0103-8478cr20170125>

Hampson K., Dushoff J., Cleaveland S., Haydon D.T., Kaare M., Packer C. & Dobson A. 2009. Transmission dynamics and prospects for the elimination of canine rabies. PLoS Biol 7(3):e1000053. <http://dx.doi.org/10.1371/journal.pbio.1000053> <PMid:19278295>

Hooper D.C., Morimoto K., Bette M., Weihe E., Koprowski H. & Dietzschold B. 1998. Collaboration of antibody and inflammation in clearance of rabies virus from the central nervous system. J. Virol. 72(5):3711-3719. <PMid:9557653>

Jakel V., Konig M., Cussler K., Hanschmann K. & Thiel H.J. 2008. Factors influencing the antibody response to vaccination against rabies. Develop. Biol., Basel 131:431-437. <PMid:18634505>

Kennedy L.J., Lunt M., Barnes A., McElhinney L., Fooks A.R., Baxter D.N. & Ollier W.E. 2007. Factors influencing the antibody response of dogs vaccinated against rabies. Vaccine 25(51):8500-8507. <http://dx.doi.org/10.1016/j.vaccine.2007.10.015> <PMid:18006120>

Knoop E.V., Freuling C.M., Kliemt J., Selhorst T., Conraths F.J. & Muller T. 2010. Evaluation of a commercial rabies ELISA as a replacement for serum neutralization assays as part of the pet travel scheme and oral vaccination campaigns of foxes. Berl. Munch. Tierarztl. Wochenschr. 123(7-8):278-285. <PMid:20690539>

Lakshmanan N., Gore T.C., Duncan K.L., Coyne M.J., Lum M.A. & Sterner F.J. 2006. Three-year rabies duration of immunity in dogs following vaccination with a core combination vaccine against canine distemper virus, canine adenovirus type-1, canine parvovirus, and rabies virus. Vet. Ther. 7(3):223-231. <PMid:17039445>

Mansfield K.L., Burr P.D., Snodgrass D.R., Sayers R. & Fooks A.R. 2004. Factors affecting the serological response of dogs and cats to rabies vaccination. Vet. Rec. 154(14):423-426. <http://dx.doi.org/10.1136/vr.154.14.423> <PMid:15119893>

Mantilla J.C., Sierra J.C., García E. & Pradilla G. 2010. Forma paralitica de un caso de rabia humana trasmitida por murciélago, simulando Síndrome de Guillain Barre: presentación de un caso de autopsia. Revta Univ. Industr. Santander, Salud 42:139-151.

Marin-Alvarez L.M., Ruíz Buitrago J.D. & Ruiz-Saenz J. 2014. Analysis of a program for the prevention and control of wild rabies and its role in the number of cattle outbreaks during years 2001 to 2011. Revta CES Med. Zootec. 9:203-217.

Mejía-Angarita J. & López-Martínez L. 2010. Reporte de casos de rabia humana, Santander, 2008-2010. Informe Epidemiológico de Santander 4:1-12.

Minsalud 2014. Colombia Completa siete años sin casos de rabia humana transmitida por perro. Boletín de Prensa No 274 de 2014, Social MdSyP, Ministerio de Salud y Proteccion Social, Colombia. Available at <https://www.minsalud.gov.co/Paginas/Colombia-completa-siete-a%C3%B1os-sin-casos-de-rabia-humana-transmitida-por-perro.aspx> Accessed on Jul. 19, 2018.

Minsalud 2015. Cobertura municipal de vacunación antirrábica de perros y gatos 2014. Ministerio de Salud y Proteccion Social, Colombia.

Moore M.C., Davis R.D., Kang Q., Vahl C.I., Wallace R.M., Hanlon C.A. & Mosier D.A. 2015. Comparison of anamnestic responses to rabies vaccination in dogs and cats with current and out-of-date vaccination status. J. Am. Vet. Med. Assoc. 246(2):205-211. <http://dx.doi.org/10.2460/javma.246.2.205> <PMid:25554936>

OPS 2005. Eliminación de la Rabia Humana Transmitida por Perros en América Latina: análisis de la situación. Organización Panamericana de la Salud, Washington, D.C. 73p.

Páez A., Nuñez C., García C. & Boshell J. 2003a. Molecular epidemiology of rabies epizootics in Colombia, 1994-2002: evidence of human and canine rabies associated with chiroptera. Biomédica 23(1):19-30. <http://dx.doi.org/10.7705/biomedica.v23i1.1194> <PMid:12696396>

Páez A., Nũñez C., García C. & Bóshell J. 2003b. Molecular epidemiology of rabies epizootics in Colombia: evidence for human and dog rabies associated with bats. J. Gen. Virol. 84(Pt 4):795-802. <http://dx.doi.org/10.1099/vir.0.18899-0> <PMid:12655080>

Páez A., Saad C., Núñez C. & Boshell J. 2005. Molecular epidemiology of rabies in northern Colombia 1994-2003. Evidence for human and fox rabies associated with dogs. Epidemiol. Infect. 133(3):529-536. <http://dx.doi.org/10.1017/S0950268805003699> <PMid:15962560>

Paéz A., Gómez J., Calvo P. & Garzón P. 2007. Niveles de inmunidad humoral conferidos con la primera dosis de la vacuna antirrábica en caninos con dueño de la ciudad de Bogotá, Colombia. Revta Invest 7:191-197.

Páez A., Hernández C., Escobar H., Zapata J.J., Méndez J. & Rey-Benito G. 2011. Evaluaciôn de la seroconversion como respuesta a la vacunaciôn antirrabica en perros en el departamento del Valle del Cauca, Colombia, 2009. Biomédica 31(4):474-484. <http://dx.doi.org/10.7705/biomedica.v31i4.395> <PMid:22674358>

Páez A., Rey G., Agudelo C., Dulce A., Parra E., Díaz-Granados H., Heredia D. & Polo L. 2009a. Brote de rabia urbana transmitida por perros en el distrito de Santa Marta, Colombia, 2006-2008. Biomédica 29(3):424-436. <http://dx.doi.org/10.7705/biomedica.v29i3.14> <PMid:20436994>

Páez A., Polo L., Heredia D., Nuñez C., Rodriguez M., Agudelo C., Parra E., Paredes A., Moreno T. & Rey G. 2009b. Brote de rabia humana transmitida por gato en el municipio de Santander de Quilichao, Colombia, 2008. Revta Salud Publica 11(6):931-943. <http://dx.doi.org/10.1590/S0124-00642009000600009> <PMid:20379666>

Rigo L. & Honer M.R. 2006. Titulação de anticorpos contra o vírus da raiva em cães, em Campo Grande/MS, na Campanha anti-rábica de 2003. Revta Soc. Bras. Med. Trop. 39(6):553-555. <http://dx.doi.org/10.1590/S0037-86822006000600008>

Schneider M.C., Belotto A., Adé M.P., Hendrickx S., Leanes L.F., Rodrigues M.J.F., Medina G. & Correa E. 2007. Current status of human rabies transmitted by dogs in Latin America. Cad. Saúde Pública 23(9):2049-2063. <http://dx.doi.org/10.1590/S0102-311X2007000900013> <PMid:17700940>

Servat A., Feyssaguet M., Blanchard I., Morize J.L., Schereffer J.L., Boue F. & Cliquet F. 2007. A quantitative indirect ELISA to monitor the effectiveness of rabies vaccination in domestic and wild carnivores. J. Immunol. Methods 318(1-2):1-10. <http://dx.doi.org/10.1016/j.jim.2006.07.026> <PMid:17166510>

Tordo N., Bahloul C., Jacob Y., Jallet C., Perrin P. & Badrane H. 2005. Rabies: epidemiological tendencies and control tools. Develop. Biol., Basel 125:3-13. <PMid:16878455>

Valderrama J., García I., Figueroa G., Rico E., Sanabria J., Rocha N., Parra E., Saad C. & Páez A. 2006. Brotes de rabia humana transmitida por vampiros en los municipios de Bajo y Alto Baudó, departamento del Chocó, Colombia 2004-2005. Biomédica 26(3):387-396. <http://dx.doi.org/10.7705/biomedica.v26i3.357> <PMid:17176002>

Van de Zande S., Kaashoek M., Hesselink W., Sutton D. & Nell T. 2009. Comments to "Comparison of antibody responses after vaccination with two inactivated rabies vaccines" [Minke J.M. et al. 2009. Vet. Microbiol. 133:283-286]. Vet. Microbiol. 138(1/2):202-203. <http://dx.doi.org/10.1016/j.vetmic.2008.12.026> <PMid:19303227>

Wasniewski M., Labbe A., Tribout L., Rieder J., Labadie A., Schereffer J.L. & Cliquet F. 2014. Evaluation of a rabies ELISA as an alternative method to seroneutralisation tests in the context of international trade of domestic carnivores. J. Virol. Methods 195:211-220. <http://dx.doi.org/10.1016/j.jviromet.2013.10.021> <PMid:24161815>

WHO 2013. WHO Expert Consultation on Rabies: second report. World Health Organization. 150p.

World Organisation for Animal Health 2007. Resolution No. XXIX. Register of Diagnostic Tests Validated and Certified by the OIE World Organisation for Animal Health, Paris, France.

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Original Article
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Lung biopsy with guillotine cutting needle and biopsy forceps though transdiaphragmatic thoracoscopy in dogs with pulmonary alterations[1]

Letícia M. Fratini[2]*, Cristiano Gomes[3], Luciana Queiroga[4], Fabiane R. Santos[2], Alexandra P. Fantinatti[5], Saulo P. Pavarini[6], Daniel G. Gerardi[3] and Carlos Afonso C. Beck[3]

ABSTRACT.- Fratini L.M., Gomes C., Queiroga L., Santos F.R., Fantinatti A.P., Pavarini S.P., Gerardi D.G. & Beck C.A.C. 2018. **Lung biopsy with guillotine cutting needle and biopsy forceps though transdiaphragmatic thoracoscopy in dogs with pulmonary alterations.** *Pesquisa Veterinária Brasileira 38(11):2117-2123*. Programa de Pós-Graduação em Ciências Veterinárias, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Agronomia, Porto Alegre, RS 90540-000, Brazil. E-mail: lefratini@gmail.com

Lung diseases are common in small animal clinical routine. Diagnosis is usually affected due to nonspecific symptoms. Imaging features such as radiography and chest ultrasound are acceptable screening tests, although lung biopsy can provides a precise diagnosis. Thus thoracoscopy provides a minimally invasive diagnostic assessment for chest diseases and offers the benefits such as improved illumination and magnification of the image when compared with thoracotomy. In this study we evaluated the transdiaphragmatic thoracoscopic-assisted techniques of lung biopsy with a the guillotine cutting needle and biopsy forceps, in dogs presenting radiographic suspicion on pulmonary tumors. Fourteen dogs regardless of breed, gender, age and body weight admitted at the Hospital of Veterinary Clinics (HCV) of the Veterinary College (FAVET) of Universidade Federal do Rio Grande do Sul (UFRGS), were assessed. Inclusion criteria were presence of nodules on chest radiography and triage tests without changes that could hinder general anesthesia and surgical approach. The animals were positioned in dorsal recumbence and two thoracoscopic ports were established: the first port for working instruments; the second paraxyphoid port for the telescope. Three samples were collected using each sampling method from each lesion or from tumors macroscopically similar whenever their size was less than one centimeter. The samples were sent for histopathological examination in the Veterinary Pathology Laboratory of FAVET/UFRGS. Surgical time was recorded from first incision to wound closure and surgical complications were reported. The dogs were evaluated for the presence of subcutaneous emphysema, hematoma, seroma, local infection and dehiscence. No conversion to open surgery was necessary during the thoracoscopic procedure in any patient. Thoracoscopic assisted biopsy using guillotine needle and biopsy forceps was a safe and fast technique, without perioperative complications. Both devices provided good quality samples for histopathological analysis of lung abnormalities. However the cutting guillotine needle was more efficient especially in larger pulmonary nodules. The transdiaphragmatic access provided optimal approach for both hemithoraces.

INDEX TERMS: Lung biopsy, transdiaphragmatic thoracoscopy, dogs, video surgery, lung neoplasm, chest diseases, thoracic surgery.

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[2] Post-Graduated course in Veterinary Medicine (PhD), Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9090, Porto Alegre, RS 90540-000, Brazil. *Corresponding author: lefratini@gmail.com

[3] Departamento de Medicina Animal, Faculdade de Medicina Veterinária, UFRGS, Av. Bento Gonçalves 9090, Porto Alegre, RS 90540-000.

[4] Veterinarian, Hospital de Clínicas Veterinário, UFRGS, Av. Bento Gonçalves 9090, Porto Alegre, RS 90540-000.

[5] Autonomous Veterinarian, Hospital Veterinário Pet Support, Av. Plinio Brasil Milano 1135, Higienópolis, Porto Alegre, RS 90520-002.

[6] Departamento de Patologia Animal, Faculdade de Medicina Veterinária, UFRGS, Av. Bento Gonçalves 9090, Porto Alegre, RS 90540-000.

RESUMO.- [Biópsia pulmonar com agulha cortante guilhotinada e pinça de biopsia por toracoscopia transdiafragmática em cães com alterações pulmonares.] As afecções pulmonares são comuns na rotina clínica de pequenos animais, todavia, por apresentarem sintomas inespecíficos, muitas vezes o diagnóstico dessas doenças torna-se limitado. Recursos de imagem como a radiografia e a ultrassonografia torácica são válidos como exames de triagem, mas somente a biopsia pulmonar pode possibilitar um diagnóstico específico da doença. A toracoscopia fornece um meio minimamente invasivo de diagnóstico para as doenças torácicas e oferece os benefícios de melhor iluminação e ampliação da imagem, quando comparado com a toracotomia. O presente estudo teve como objetivo avaliar as técnicas de biópsia pulmonar por meio da agulha cortante guilhotinada e da pinça de biopsia, guiadas por toracoscopia, pelo acesso transdiafragmático em cães que apresentavam imagem sugestiva de nódulo pulmonar em exame radiográfico prévio. Foram utilizados 14 cães, independente de raça, sexo, idade e peso corporal. Somente caninos com nódulos visíveis na radiografia torácica e que apresentaram condições clínicas e laboratoriais de serem anestesiados foram incluídos no estudo. Os cães foram posicionados em decúbito dorsal e foram realizados dois acessos à cavidade torácica: um primeiro portal intercostal, para introdução dos dispositivos de biopsia; e outro portal paraxifoide transdiafragmático para introdução do endoscópio. Com cada instrumento de biopsia foram coletadas três amostras do mesmo nódulo ou de nódulos macroscopicamente semelhantes e próximos quando o tamanho destes era inferior a um centímetro. Posteriormente as amostras foram encaminhadas para exame histopatológico. O tempo cirúrgico foi cronometrado da incisão ao fechamento da ferida, etodas as informações foram registradas. No pós-operatório os cães foram avaliados quanto à presença de enfisema subcutâneo, hematoma, seroma, infecção local e deiscência de pontos. Não foi necessário converter os procedimentos toracoscópicos para cirurgia convencional em nenhum dos caninos. Concluiu-se tratar de uma técnica segura, rápida sem complicações trans e pós-operatórias. Ambos dispositivos permitiram aquisição de material suficiente para análise histopatológica das alterações pulmonares, no entanto a agulha cortante guilhotinada apresentou maior eficácia, principalmente, em nódulos pulmonares de maior diâmetro. O acesso transdiafragmático mostrou-se eficiente para exploração de ambos os hemitórax.

TERMOS DE INDEXAÇÃO: Biópsia pulmonar, toracoscopia transdiafragmática, caninos, videocirurgia, neoplasma pulmonar, afecções torácicas, cirurgia torácica.

INTRODUCTION

Pulmonary parenchymal conditions are common in dogs and cats. Pneumonias and neoplasias are some of the main parenchymal diseases. On the other hand, interstitial lung diseases are rarer (Reineiro & Cohn 2007).

The diagnosis of pulmonary diseases is through complementary examinations such as thoracic radiography, thoracic ultrasonography, computed tomography, magnetic resonance imaging, fine needle aspiration, tracheobronchial (LTB) and bronchioloalveolar lavage (BAL), and bronchoscopy. However, definitive diagnosis is often possible only by histological examination (Withrow 2013). Samples for this examination can be obtained by biopsy performed by thoracotomy, thoracoscopy or percutaneous with guillotine cutting needle (Wilson & Dungworth 2002, Baez & Sorenmo 2004, Basso et al. 2010).

Infirmities such as primary and metastatic neoplasm, inflammatory and infectious processes, circulatory disorders and degenerative conditions have their definitive diagnosis through histological examination (Chojniak et al. 2011). In order to perform the biopsy, video-assisted thoracoscopy is recommended because it causes less trauma to the patient when compared to thoracotomy (Saviano & Tazzioli 1998)

Thoracoscopic, pleuroscopic or video-assisted thoracoscopic surgery (VATS), when compared to thoracotomy, is a safe technique with minimal thoracic wall trauma, causing less pain, reduction of pneumothorax and subcutaneous emphysema, providing rapid recovery of the patient (Solli & Spaggiari 2007).

Pulmonary biopsy in dogs through thoracoscopy is still poorly studied and usually requires the use of two or three portals and the use of different types of instruments to obtain the tissue sample (Potter & Hendrickson 1999).

The aim of this study is to describe and evaluate techniques for pulmonary biopsy using guillotine cutting needle and biopsy forceps, guided by thoracoscopy for transdiaphragmatic and intercostal paraxiphoid accesses in dogs presenting a suggestive image of pulmonary nodule on previous radiographic examination.

MATERIALS AND METHODS

The present study was approved by the Animal Ethics Committees (AECs) of the Rio Grande do Sul Federal University (Universidade Federal do Rio Grande do Sul, UFRGS), through the approval document and under the protocol number 26239. The tutors were previously consulted about the interest in participating in the present study and, in cases of agreement, they were informed about the benefits and possible risks of the procedure.

Fourteen dogs from the routine care of the Veterinary Clinics Hospital (Hospital de Clínicas Veterinárias, HCV) of the Veterinary School (Faculdade de Veterinária, FAVET) of UFRGS, of different races, ages, body weight and both genders, being nine females and five males, were evaluated. The animals included presented radiographic alterations suggestive of pulmonary nodules, with or without clinical signs. Patients with clinical and laboratory abnormalities that prevented anesthetic and/or surgical procedures were excluded.

All dogs underwent general clinical examination and complementary preoperative examinations such as abdominal ultrasonography, thoracic X-rays in three projections, electrocardiogram, echocardiography, hemogram, total platelet count, total plasma proteins, ALT and creatinine.

After previous fasting, the patients received morphine sulfate ($0{,}3 mg.kg^{-1}$, IM) as preanesthetic medication. A wide trichotomy of the abdomen and thorax and intravenous administration of ampicillin sodium ($22 mg.kg^{-1}$) was performed. All animals were pre-oxygenated with O_2 at 100% for 15 minutes before general anesthesia. Anesthetic induction was performed with propofol ($4 mg.kg^{-1}$, IV), followed by the orotracheal intubation. For anesthetic maintenance, isoflurane was used, vaporized in oxygen at 100%. When necessary, the administration of fentanyl ($5 mcg.kg^{-1}$) in *bolus* in the transanesthetic period. Local blockade of intercostal innervation with lidocaine hydrochloride was performed.

All patients received multiparametric anesthesia monitoring and were placed in supine position, with antisepsis of the thorax and abdomen being performed. The site of choice to insert the trocar was based on the location of the lesion(s) in the thoracic radiograph, usually a caudal intercostal space to the alteration.

A cutaneous incision was made with approximately 0.5cm of extension, longitudinally to the ribs. Subsequently, Halsted hemostatic forceps was inserted for intercostal musculature divulsion and pleura perforation, with consequent induction of pneumothorax, in order to reduce the occurrence of iatrogenic lesions to the pulmonary parenchyma in the moment of insertion of the trocar.

A trocar with 5mm of diameter was inserted perpendicularly to the thoracic wall progressively. Once the obturator was removed, the insertion of the rigid endoscope with 4mm of diameter and 0° viewing angle was performed. A rapid exploration of the thoracic cavity was performed, and the endoscope was positioned to visualize the diaphragm region for video-assisted insertion of the second trocar, by the transdiaphragmatic paraxiphoid access.

A skin incision of approximately 0.5cm was made between the xiphoid appendix and the costal arch, through which a second trocar (2nd portal) of 5mm diameter was inserted. After the video-assisted perforation of the diaphragm, the endoscope was removed from its original position (inside the 1st portal) and repositioned through the 2nd portal. At that time the mediastinal membrane was perforated with the endoscope itself so that it was possible to visualize both hemithorax.

After exploring the thoracic cavity and identification, location and determination of the severity and characteristics of pulmonary nodulations, the pulmonary sampling instruments were inserted sequentially and alternately through the first portal: the biopsy forceps with teeth (1x1), 5mm of diameter and 36cm of length, and the guillotine cutting needle for soft tissue, semiautomatic Biomedical® of 14 G caliber and with 150mm of length (Fig.1). The order of attainment of the samples with each instrument was chosen randomly by the team at the time of the procedure.

Three samples of the same nodule were collected with each biopsy device. Each sample presented approximately 2mm of diameter with the forceps and 1mm of diameter with the guillotine needle. At the time of the harvest, possible technical difficulties related to the access to the pulmonary surface, handling of instruments and magnitude of the collected fragments were recorded.

Samples were kept in a 10% buffered formalin solution and then submitted for histological analysis. All samples were evaluated by the same pathologist through the Hematoxylin-Eosin staining technique.

Subsequently, the presence or absence of hemorrhage was verified, and trocars were removed, synthesis of wounds made, paraxiphoid and intercostal with Sultan suture pattern, with monofilament nylon 2-0 thread. The skin was sutured with simple isolated stitches with monofilament nylon 3-0 thread. The negative pressure of the thoracic cavity was reestablished through thoracocentesis.

The surgery was timed from the first incision to the wound closure and any surgical complications or technical difficulties were recorded if present.

The patients received as immediate postoperative medication tramadol hydrochloride (4mg.kg^{-1}, IV), dexamethasone (0,25mg.kg^{-1}, IV), and enrofloxacin (5mg.kg^{-1}, IM). All dogs were discharged after six hours of observation. Patients were submitted to clinical evaluation at the time of hospital discharge and at 48 hours after the procedure. In this moment, radiographic evaluation of the thorax was also performed. Rectal temperature was measured, possible respiratory changes observed, presence of subcutaneous emphysema, hematoma, seroma, local infection and dehiscence of stitches, which was not evidenced. The operated area was also palpated to evaluate the painful response of the animal.

Tutors were advised to clean surgical wounds with saline solution every 12 hours, and to maintain Elizabethan collar until the stitches were removed seven days later. Oral administration of tramadol hydrochloride (3mg.kg^{-1}, TID, for 3 days), meloxicam (0,1mg.kg^{-1}, SID, for 4 days), and enrofloxacin (5mg.kg^{-1}, BID, for 7 days) was advised. After seven days from the procedure the dogs were evaluated again, and the skin stitches were removed.

To verify the viability of the samples obtained with the different biopsy instruments, the qualitative analysis of the specimens was made. Surgical procedures were evaluated in terms of time to perform the procedure, frequency distribution of trans and postoperative complications, and technical difficulties in performing the procedure, if found. The quality of the samples obtained, and the data related to the surgical time, frequency distribution of the technical difficulties and possible complications were analyzed descriptively.

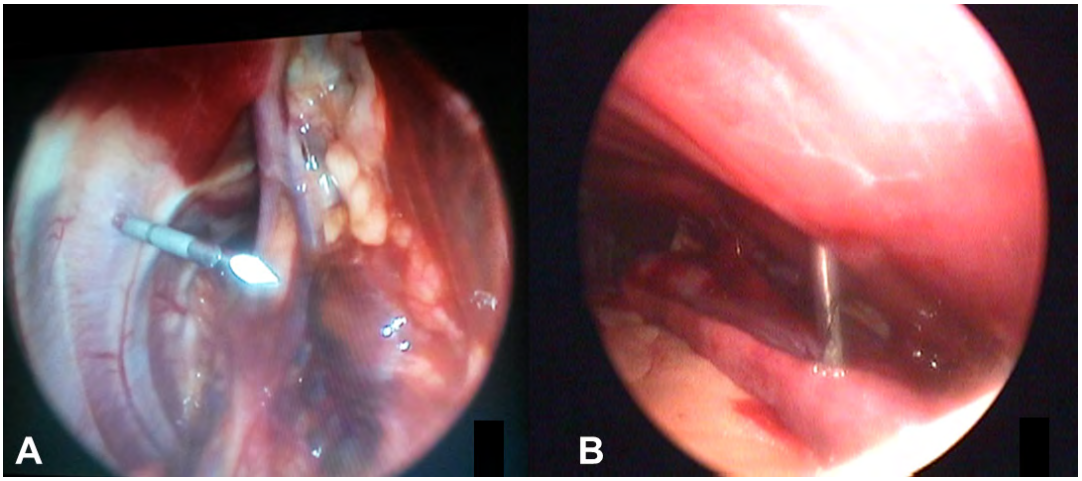


Fig.1. (**A**) Guillotine cutting needle positioned in the thoracic region to obtain a sample. (**B**) Biopsy forceps positioned inside the thoracic cavity to obtain a sample.

RESULTS

Twenty three dogs with suspicion of pulmonary nodules were evaluated in the period from April to December 2014, in which 9 were excluded because they presented alterations in the complementary exams, totaling 14 patients included in this study.

In pre-surgical thoracic radiography, three dogs (Dog 5, 7 and 14) presented effusion in small amount. None presented pre, trans and postoperative complications. No conversion was required for thoracotomy and all remained alive during the analyzed period.

The mean duration of the procedure was 26.5 minutes (Fig.2). In four dogs (Dog 3, 5, 6 and 7) the portal had to be reinserted into another intercostal space, different from the one planned based on radiography. The option to insert the second portal through the transdiaphragmatic paraxiphoid access guided by endoscopic intercostal visualization was safe, without the occurrence of iatrogenic lesion in any of the 14 animals, allowing wide exploration of the thoracic cavity in both hemithorax and excellent visualization of the pulmonary parenchyma.

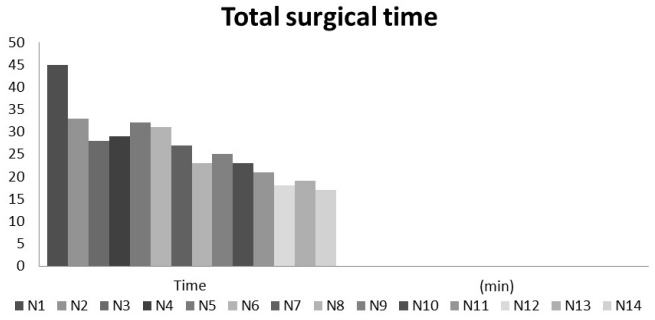


Fig.2. Graphic representation of the surgical time obtained to the biopsy guided by thoracoscopy using biopsy forceps and needle with guillotine cut, in 14 dogs. Evidencing the learning curve with the reduction of surgical time throughout the experiment.

Only one patient presented communication between the hemithorax; in the other 13 dogs (92.85%), the mediastinal membrane was intact and ruptured with the endoscope during thoracoscopy. Intercostal access for the introduction of biopsy devices was also adequate and made it possible to grasp the tissue without major difficulties.

Regarding the quality of the material collected by each biopsy device, the samples obtained were considered satisfactory, and it was possible to reach the same diagnosis with both the biopsy forceps and the cutting needle in 85.71% of the cases (Table 1). In two dogs the samples obtained by the biopsy forceps were not representative presenting only a focal area of necrosis, and artifact (atelectasis) by compression of the pulmonary parenchyma.

Little or no bleeding was observed after biopsy. The dogs did not present hematoma, subcutaneous emphysema, infection and pain during palpation at the surgical site within 48 hours until clinical reevaluation. After this period, the animals were submitted to thoracic radiography, and none presented pneumothorax. Dogs 5, 7 and 14 presented a small amount of effusion, which had already been observed in the radiography prior to the procedure.

Referring to the harvesting technique of the nodules sample, both devices tested were efficient in seizing and acquiring the material, obtaining histologically significant samples in 100% of the animals participating in the study.

DISCUSSION

Thoracic effusion found in three of the 14 dogs was probably due to pulmonary neoplasms (Fossum 2008). Thoracoscopy was a safe diagnostic tool, easy to perform and without major complications even in patients with pulmonary involvement, with a stable clinical picture corroborating with the literature (McCarthy & McDermaid 1999).

The mean duration of the procedure was considered short in relation to the conventional thoracotomy biopsy techniques reported in the literature. This is in part due to the good training and coordination of the surgical team, which

Table 1. Histopathological results of the biopsy with the guillotined cutting needle and the biopsy forceps and location of the lesions

N	Cutting needle sample	Biopsy forceps sample	Location
1	Papillary adenocarcinoma metastasis	Papillary adenocarcinoma metastasis	LCrD
2	Tubulopapillary carcinoma metastasis	Tubulopapillary carcinoma metastasis	LCdD
3	Leiomyosarcoma	Atelectasis, areas of necrosis	LCdE
4	Chronic pyogranulomatous inflammation	Chronic pyogranulomatous inflammation	LCdD
5	Adenosquamous carcinoma metastasis	Adenosquamous carcinoma metastasis	LCdD
6	Cholangiocarcinoma metastasis	Cholangiocarcinoma metastasis	LCdE
7	Hemangiosarcoma metastasis	Hemangiosarcoma metastasis	LCdE
8	Osteosarcoma metastasis	Osteosarcoma metastasis	LCrE
9	Complex carcinoma metastasis	Complex carcinoma metastasis	LCdE
10	Metastasis of carcinosarcoma	Metastasis of carcinosarcoma	LCdD
11	Metastasis myoepithelial carcinoma	Metastasis myoepithelial carcinoma	LCrD
12	Complex carcinoma metastasis	Atelectasis, areas of necrosis	LMD
13	Mesothelioma	Mesothelioma	LCdD, med.
14	Pulmonary carcinoma	Pulmonary carcinoma	LCdE

N = patient number, LCrD = right cranial lobe, LCdD = right caudal lobe, LCrE = left cranial lobe, LCdE = left caudal lobe, LMD = medium right lobe, med. = mediastinum.

according to Beck et al. (2004) is essential for performing the surgical procedure, although it is important to emphasize that both the correct positioning of the animals and the adequate technical execution of the procedures are indispensable factors for the execution of the surgery.

The learning curve in video-surgery makes operative time decrease as the surgeon gains experience with the technique and becomes more familiar and adapted to the instruments and equipment (Faraco 2013). This can be observed in this study in which surgical time was reduced throughout the experiment. This finding corroborates with Santos & Bravo Neto (2010), who affirmed that the success of a procedure requires, besides an accurate indication, technical ability, which is based on the repetition of movements and goes through a phase in which the learner acquires competence quickly, to then reach a plateau, after which no difference is observed in the gain of the abilities, although it continues to gain speed.

According to Beck et al. (2004), previous surgical planning, including the positioning of the tower, the surgical team and anesthetic is fundamental for the good progress of the procedures in the video-surgery. In the present experiment, this planning was established before all the procedures, so that the surgical team was able to carry out the surgical maneuvers in a comfortable and safe way. Regarding the positioning of the patients in the supine position, it was verified that this allowed the introduction of the trocars both through intercostal access and transdiaphragmatic paraxiphoid without major difficulties, besides allowing visualization of the mediastinum of the thoracic wall, pulmonary lobes except the dorsal face, pericardium, lymph nodes, large vessels, esophagus and diaphragm.

In all dogs included in the project, two trocars (portals) were used to access the thoracic cavity. The first portal was inserted in the intercostal region, and the second through transdiaphragmatic paraxiphoid access (Fig.3). The site of

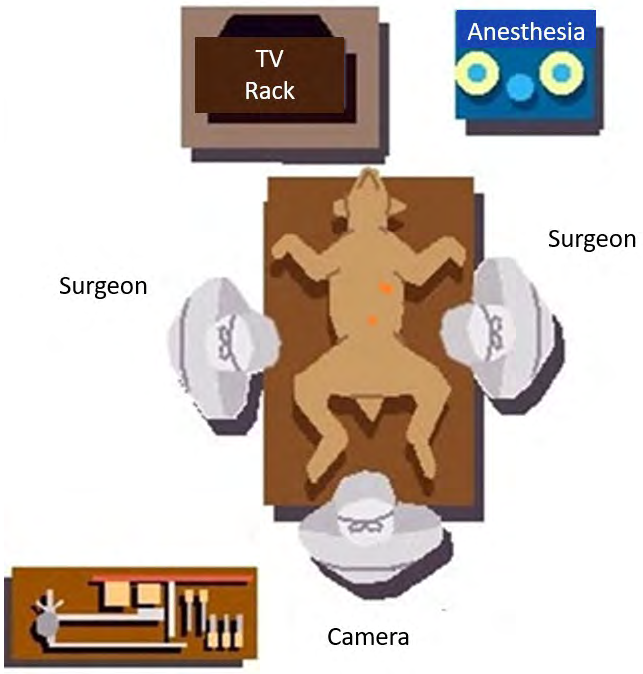


Fig.3. Schematic representation of the equipment, surgical team and positioning of the portals in the patient.

choice for the insertion of the first portal was based on the thoracic radiograph, which provided an approximate idea of the location of the tumor. The objective of the first access portal was to first access the thorax near the area(s) in which the tumors were identified by the radiographic images. At the same time, the intercostal access was used to perform the previous exploration of the corresponding hemithorax and to facilitate the insertion of the transdiaphragmatic paraxiphoid portal under endoscopic visualization.

The option to insert the second portal through transdiaphragmatic paraxiphoid access guided by endoscopic intercostal visualization was safe, without the occurrence of iatrogenic lesion in any of the 14 dogs of this study, corroborating the reports of Pigatto et al. (2008) and Basso et al. (2010). However, it is noteworthy that in these studies the pneumothorax was instituted by insufflation of the thoracic cavity with CO_2 which facilitated diaphragm distension and the transdiaphragmatic paraxiphoid trocar insertion safe.

In four dogs (Dog 3, 5, 6 and 7) the portal had to be reinserted into another intercostal space, different from the one planned based on radiography. It is believed that this mistake occurred due to image overlap in the radiographic examination. According to Norris et al. (2002), thoracic radiography is a good screening test, but because it does not detect lumps smaller than four millimeters and often present overlapping images it is a limited examination. Thus, the anesthetic block of two caudal and two cranial intercostal spaces was indispensable to the planned access site, in order to obtain a wider access margin.

Another fact that strengthens the radiographic examination, in relation to the identification of small pulmonary nodules and, at the same time, presents a quality of presentation of the surgical procedures, is related to the fact that in seven dogs (50%) the number of lesions were lower than those seen during thoracoscopy. Schmiedt (2009) and Radlinsky (2014) highlight the diagnostic character of the thoracoscopic procedures as one of the advantages that this access allows. It should be noted that, according to the findings of the present study, thoracic radiography characterizes an important stage as a screening test in patients with suspicion of thoracic malignancies. However, for greater sensitivity and specificity, the thoracoscopic approach is recommended.

In this study, since dogs presented respiratory compromise, undo the negative thoracic pressure, without the additional insufflation of CO_2, was the chosen option. For this, the initial approach to the thorax was performed through intercostal access. This provided greater safety, reducing the risks of iatrogenesis to the intrathoracic structures that could be caused by transdiaphragmatic paraxiphoid access, without the recommended previous pneumothorax.

The option for the initial intercostal approach, besides the safety provided in the endoscopic view of the insertion of the transdiaphragmatic trocar (2nd portal), also allowed to observe the entire diaphragm region, which cannot be performed through the cavitary visualization by the transdiaphragmatic paraxiphoid access. This maneuver was important because some dogs also had nodules on the diaphragmatic wall. The use of two 5mm diameter access portals, the first intercostal and the second transdiaphragmatic paraxiphoid, proved to be a good option, regarding also to the extent of thoracic cavity exploration. The possibility of a change in the position of the

4mm optic, alternating its insertion through the intercostal and paraxiphoid portal during the procedures allowed a greater reach both in the visual exploration as in the performance of the punctures and the pulmonary parenchyma.

The formation of pneumothorax is essential to prevent lesions of the pulmonary parenchyma at the time of the insertion of the trocar and to obtain full visualization of the intrathoracic structures (Walsh et al. 1999, Peroni et al. 2001). In this study, the divulsion of the intercostal muscles and thoracic cavity were performed with Halstead hemostatic forceps, thus reducing the negative pressure of the thorax. This maneuver provided insertion of trocars with little resistance, adequate pneumothorax and avoided possible iatrogenic lesions on the lung. These results corroborate with the study of Isakow et al. (2000) that produced pneumothorax only with musculature divulsion and perforation of the parietal pleura.

Landreneau et al. (1994) reported that intrathoracic inflation with carbon dioxide in thoracoscopic procedures is not essential and only assists the inspection of the pleural cavity by the total lung collapse that it produces and, in addition to the small distension of the cavity it promotes, enlarges the visual field of the thorax. But they can cause increased blood pressure and compromise circulatory dynamics.

The choice for transdiaphragmatic paraxiphoid access for the insertion of the endoscope allowed extensive exploration of the thoracic cavity in both hemithorax and excellent visualization of the pulmonary parenchyma, corroborating with the studies of Twedt (2002), Pigatto et al. (2008) and Basso et al. (2010). It is known that both dogs and cats may present communication between the hemithorax physiologically (Orton 1998).

Regarding the quality of the collected material, in general, satisfactory indexes of success in obtaining appropriate material for analyzes above 85% are considered satisfactory (Chojniak et al. 2011).

It is believed that in the two patients in which the samples collected were not significant, it is due to the fact that many tumors, regardless of their histological origin, present an area of peritumoral cell necrosis. Thus, larger diameter nodules tend to present their outermost layer composed only of cellular debris and necrosis (Jones et al. 2000).

The guillotined needle sample was more representative in the larger diameter neoplastic lung lesions. It is possible to observe in these the shape and the arrangement of the tumor cells, since the needle reached all the layers of the lesion. Chojniak et al. (2011) found specific histological results with a higher frequency (97%) in the use of cutting-type needles when compared to the thin needle and reported greater success in obtaining samples in lesions larger than 40mm in diameter.

Regarding the use of the biopsy forceps, Basso et al. (2010) found it difficult to section the lung tissue with forceps alone, and it was necessary to use a 40x12 needle. This was not observed in this study, since all the biopsied dogs had alterations in the pulmonary parenchyma, making it more friable and easily sectioned. Both Basso et al. (2010) and Lamounier (2006) concluded that the samples obtained by the biopsy forceps were considered satisfactory, according to the histopathological report, and it was possible to visualize the presence of lung tissue. This condition was also observed in this experiment. Regarding the healing of the biopsy site, it is believed that this occurred by second intention, probably by the deposition of fibrin buffer in the sectioned site, dispensing sutures in the pulmonary parenchyma. This can be evidenced by the absence of pneumothorax in the radiographic evaluation of the dogs 48 hours after the procedure. According to Lamounier (2006) there is a minimum air escape after the removal of pulmonary fragments of up to 0.5cm in diameter, because the clamp used promotes tissue crushing.

The use of guillotine cutting needle to obtain pulmonary biopsy in dogs has not been described in the literature yet. In humans it is a technique already consolidated and of everyday use in the routine. In this study, the acquisition of histologically significant samples occurred in 100% of the dogs participating in the study was observed. Even in lesions of reduced diameter it was possible to obtain material, corroborating with Chojniak et al. (2011), whom concluded that percutaneous cutting needle biopsy has become one of the main choices for nodule and mass investigation. Its versatility allows the access of lesions in different locations of the lung, being able to be used for peripheral and deep lesions even of small dimensions, achieving 97% success in obtaining viable samples.

After the end of the procedure, thoracentesis was chosen to restore the negative pressure in the thorax. The crushing caused by the forceps, the reduced diameter of the samples collected and alteration present in the pulmonary parenchyma avoided the formation of pneumothorax, making the use of the thoracic drain unnecessary. Fossum (2008) reports that despite a low incidence, formation of pyogranulomas, pleuritis and adhesions between lung and thoracic wall may occur when thoracic drainage is used.

CONCLUSIONS

We can conclude that both biopsy collection devices tested demonstrated efficacy, but the semiautomatic guillotine cutting needle provided a better diagnostic result when compared to the biopsy forceps, especially in the collection of larger tumor nodules.

Transdiaphragmatic paraxiphoid thoracoscopy was adequate for the bilateral assessment of the chest cavity and obtaining pulmonary biopsy in dogs from any lobe of both lungs and presented as a fast and safe technique even in animals with pulmonary involvement, with a stable clinical picture.

REFERENCES

Baez J.L. & Sorenmo K.U. 2004. Pulmonary and bronchial neoplasia, p.508-515. In: King L.G. (Ed.), Respiratory Disease in Dogs and Cats. W.B. Saunders, St Louis. <http://dx.doi.org/10.1016/B978-0-7216-8706-3.50073-6>.

Basso P.C., Raiser A.G., Brun M.V., Guizzo Junior N., Feranti J.P.S., Motta A.C., Bortolini C.E. & Muller D.C.M. 2010. Biópsia pulmonar incisional por toracoscopia paraxifoide transdiafragmática com dois portais em cães. Pesq. Vet. Bras. 30(7):566-572. <http://dx.doi.org/10.1590/S0100-736X2010000700010.

Beck C.A.C., Pippi N.L., Brun M.V., Contesini E.A., Cunha A.F., Stedile R., Bonfada A.T., Silva Filho A.P.F., Gomes K. & Colomé L.M. 2004. Toracoscopia nas hérnias diafragmáticas: estudo experimental em cães. Ciência Rural 34(6):1857-1863. <http://dx.doi.org/10.1590/S0103-84782004000600029>

Chojniak R., Pinto P.N.V., Ting C.J., Cohen M.P., Guimarães M.D., Yu L.S. & Bitencourt A.G.V. 2011. Biópsia transtorácica de nódulos e massas pulmonares dirigida por tomografia computadorizada. Radiol. Bras. 44(5):315-320. <http://dx.doi.org/10.1590/S0100-39842011000500010>

Faraco C.S. 2013. Avaliação do CentryPort® como acesso único (Less-Laparoendoscopic Single-Site Surgery) na realização de criptorquidectomia laparoscópica: estudo experimental em coelhos (*Oryctolagus cuniculus*). Dissertação de Mestrado, Universidade Federal do Rio Grande do Sul, Porto Alegre. 90p.

Fossum T.W. 2008. Cirurgia do sistema respiratório inferior: cavidade pleural e diafragma, p.752-785. In: Fossum T.W., Johnson A.L., Schulz K.S., Seim H.B., Willard M.D., Bahr A. & Carroll G.L. (Eds.), Cirurgia de Pequenos Animais. 3ª ed. Roca, São Paulo.

Isakow K., Fowler D. & Walsh P. 2000. Video-assisted thoracoscopic division of the ligamentum arteriosum in two dogs with persistent right aortic arch. J. Am. Vet. Med. Assoc. 217(9):1333-1336. <http://dx.doi.org/10.2460/javma.2000.217.1333> <PMid:11061385>

Jones T.C., Hunt R.D. & King N.W. 2000. Patologia Veterinária. 6ª ed. Manole, São Paulo, p.963-990.

Lamounier A.R. 2006. Biópsia pulmonar orientada por vídeo toracoscopia em cães: estudo experimental. Dissertação de mestrado, Universidade Federal de Minas Gerais, Belo Horizonte. 64p.

Landreneau R.J., Mack M.J., Hazelrigg S.R., Naunheim K., Dowling R.D., Ritter P., Magee M.J., Nunchuck S., Keenan R.J. & Ferson P.F. 1994. Prevalence of chronic pain after pulmonary resection by thoracotomy of video assistead thoracic surgery. J. Thorac. Cardiovasc. Surg. 107(4):1079-1066, discussion 1085-1086. <PMid:8159030>

McCarthy T.C. & McDermaid S.L. 1999. Thoracoscopy. Vet. Clin. N. Am., Small Anim.Pract. 20(5):1341-1352. <http://dx.doi.org/10.1016/S0195-5616(90)50308-3>

Norris C.R., Griffey S.M., Samii V.F., Christopher M.M. & Mellema M.S. 2002. Thoracic radiography bronchoalveolar lavage cytopathology and pulmonary parenchymal histopathology: acomparison of diagnostic results in 11 cats. J. Am. Vet. Anim. Hosp. Assoc. 38(4):337-345. <http://dx.doi.org/10.5326/0380337> <PMid:12118687>.

Orton E.C. 1998. Pleura e espaço pleural, p.469-489. In: Slatter D. (Ed.), Manual de Cirurgia de Pequenos Animais. Vol.1. 3ª ed. Manole, São Paulo.

Peroni J.F., Horner N.T., Robinson N.E. & Stick J.A. 2001. Equine thoracoscopy: normal anatomy and surgical technique. Equine Vet. J. 33(3):231-237. <http://dx.doi.org/10.2746/042516401776249688> <PMid:11352343>

Pigatto J., Brun M.V., Barcellos L.J.G., Rausch S.F., Phol V.H., Feranti J.P.S. & Guedes R.L. 2008. Produção de pneumotórax em cães e manejo por toracoscopia paraxifoide transdiafragmática. Ciência Rural 38(8):2210-2217. <http://dx.doi.org/10.1590/S0103-84782008000800019>

Potter L. & Hendrickson D.A. 1999. Therapeutic video-assisted thoracic surgery, p.169-187. In: Freeman L.J. (Ed.), Veterinary Endosurgery. Mosby, Philadelphia.

Radlinsky M. 2014. Thoracoscopy in the cat: a up-and-coming diagnostic and therapeutic procedure. J. Feline Med. Surg. 16(1):27-33. <http://dx.doi.org/10.1177/1098612X13516569> <PMid:24361947>

Reineiro C.R. & Cohn L.A. 2007. Interstitial lung diseases. Vet. Clin. N. Am., Small Anim. Pract. 37(5):937-947. <http://dx.doi.org/10.1016/j.cvsm.2007.05.008>

Santos E.G. & Bravo Neto G.P. 2010. Curva de aprendizado e lesões iatrogênicas em colecistectomias vídeo laparoscópicas. Revta Col. Bras. Cir. 37(3):184-189. <http://dx.doi.org/10.1590/S0100-69912010000300005>

Saviano M.S. & Tazzioli G. 1998. Video thoracoscopic surgery, diagnosis and treatment of lung nodules. World J. Surg. 15(6):21-24.

Schmiedt C. 2009. Small animal exploratory thoracoscopy. Vet. Clin. N. Am., Small Anim. Pract. 39(5):953-964. <http://dx.doi.org/10.1016/j.cvsm.2009.05.007> <PMid:19683654>

Solli P. & Spaggiari L. 2007. Indications and developments of video-assisted thoracic surgery in the treatment of lung cancer. Oncologist 12(10):1205-1214. <http://dx.doi.org/10.1634/theoncologist.12-10-1205> <PMid:17962614>

Twedt D.C. 2002. Diagnostic toracoscopy. Western Veterinary Conference, Denver, CO, p.34-39. (Resumo)

Walsh P.J., Remedios A.M., Ferguson J.F., Walker D.D., Cantwell S. & Duke T. 1999. Thoracoscopic versus open partialpericardectomy in dogs: comparison of postoperative pain and morbidity. Vet. Surg. 28(6):472-479. <http://dx.doi.org/10.1111/j.1532-950X.1999.00472.x> <PMid:10582745>

Wilson D.W. & Dungworth D.L. 2002. Tumors of the respiratory tract, p.365-399. In: Meuten D.J. (Ed), Tumors in Domestic Animals. 4th ed. Blackwell Publishing Company, Ames.

Withrow S.J. 2013. Lung cancer, p.432-453. In: Vail D.M. & Withrow S.J. (Eds), Withrow and MacEwen's Small Animal Clinical Oncology. 5th ed. Saunders Elsevier, Missouri.

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Original Article
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Acute phase proteins in bitches subjected to conventional and minimally invasive ovariohysterectomy[1]

Elizabeth M.S. Schmidt[2*], Camila P. Rubio[3], Funmilola Thomas[4], João C.P. Ferreira[2] and David P. Eckersall[5]

ABSTRACT.- Schmidt E.M.S., Rubio C.P., Thomas F., Ferreira J.C.P. & Eckersall D.P. 2018. **Acute phase proteins in bitches subjected to conventional and minimally invasive ovariohysterectomy**. *Pesquisa Veterinária Brasileira 38(11):2124-2128*. Departamento de Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Campus de Botucatu, Rua Prof. Dr. Walter Maurício Correa s/n, Cx. Postal 560, Botucatu, SP 18618-681, Brazil. E-mail: elizabeth.schmidt@unesp.br

The aim of this study was to evaluate and to compare the possible inflammatory changes by screening acute phase proteins concentrations in healthy bitches subjected to ovariohysterectomy. Minimally invasive and conventional (laparotomy) ovariohysterectomies were performed in 17 client-owned adult female mixed breed dogs. Nine animals were subjected to minimally invasive and eight animals to conventional ovariohysterectomy. Blood samples were taken before surgery, 24, 48 hours, and seven days postoperatively. Serum C-reactive concentration was determined by a commercial ELISA kit and serum haptoglobin concentration was measured via hemoglobin binding assay, both previously validated for use in dogs. As the data did not meet the normal distribution criteria, the nonparametric Kruskall-Wallis was performed to compare quantitative variables between groups. One-way ANOVA and the Friedman test were used for multiple comparisons between time points, with a $P<0.05$ considered significant. C-reactive protein concentration was significantly different ($P<0.0001$) at 24 hours postoperatively between groups. There was no significant difference in haptoglobin concentration between groups. C-reactive protein and haptoglobin concentrations were significantly different at 24 and 48 hours postoperatively for minimally invasive and conventional ovariohisterectomies. These findings provided an overview of the short-term inflammatory effects produced by minimally invasive and conventional ovariohysterectomies.

INDEX TERMS: Bitches, ovariohysterectomy, dogs, C-reactive protein, haptoglobin, surgery.

RESUMO.- [**Proteínas de fase aguda em cadelas submetidas à ovariohisterectomia convencional e minimamente invasiva.**] O objetivo deste trabalho foi avaliar e comparar o efeito inflamatório, por meio da determinação de proteínas de fase aguda, produzido por dois protocolos cirúrgicos distintos de ovariohisterectomia em cadelas. Para tanto, foram determinadas as concentrações de proteína C reativa (CRP) e haptoglobina (Hp) de 17 cadelas, sem raça definida, adultas,, sendo nove animais submetidos à ovariohisterectomia pela técnica convencional por laparotomia e oito a ovariohisterectomia pela técnica minimamente invasiva. Para avaliar a resposta de fase aguda causada pelo trauma cirúrgico, amostras de sangue foram obtidas antes dos procedimentos cirúrgicos e em quatro momentos distintos após as cirurgias (24, 48 horas e sete dias) para as dosagens de CRP e Hp, com kit comercial ELISA e via ensaio de ligação com a hemoglobina, respectivamente, validados para o uso em cães. Como não houve distribuição normal dos dados, utilizou-se o teste de Kruskall-Wallis

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[2] Departamento de Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Campus de Botucatu, Rua Prof. Dr. Walter Maurício Correa s/n, Cx. Postal 560, Botucatu, SP 18618-681, Brazil. *Corresponding author: elizabeth.schmidt@unesp.br

[3] Aluna Doutorado, Faculdad de Veterinaria, Universidad de Murcia, Avda. Teniente Flomesta 5, Murcia, 30003, Spain.

[4] Department of Veterinary Physiology and Pharmachology, College of Veterinary Medicine, Federal University of Agriculture Abeokuta, Abeokuta, Nigeria.

[5] Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, University Avenue, Glasgow G12 8QQ, United Kingdom.

para comparação das variáveis quantitativas entre os dois grupos. Os testes de Friedman e One-way ANOVA foram usados para comparações múltiplas entre os momentos avaliados, sendo considerado P<0,05 como significante. Houve diferença significativa (P<0,0001) para as concentrações de CRP 24 horas após o procedimento cirúrgico entre os dois protocolos utilizados. Não houve diferença significativa para as concentrações de Hp entre os dois protocolos cirúrgicos. Foram evidenciadas alterações significativas para as concentrações de CRP e Hp 24 e 48 horas após as ovariohisterectomias, independentemente da técnica cirúrgica utilizada. As concentrações de CRP e Hp demonstraram o efeito inflamatório imediato induzido pela ovariohisterectomia convencional e minimamente invasiva.

TERMOS DE INDEXAÇÃO: Cadelas, ovariohisterectomia, proteína C reativa, haptoglobina, cirurgia.

INTRODUCTION

The acute phase response is part of the body's innate defense system (Eckersall & Bell 2010), characterized by a systemic reaction caused by local or systemic disorders, promoted by infectious agents, trauma, and tissue injury, surgeries, neoplastic growth or immunological disorders (Ceron et al. 2005, Gruys et al. 2005, Eckersall 2008). During the acute phase response, there is increased synthesis and release of acute phase proteins (APP), induced by pro-inflammatory cytokines produced mainly by the liver and extrahepatic tissues such as adipose tissue, mammary gland and lungs. PFAs are classified according to changes in their concentrations in positive (C-reactive protein, haptoglobin, serum amyloid A) if they increase, or negative (albumin), if they decrease their concentrations during the acute phase response (Murata et al. 2004, Ceron et al. 2005).

Ovariohysterectomy is one of the most performed surgical procedures in companion animals and promotes the appearance of pain signals during the postoperative period in dogs (Lascelles et al. 1998). To minimize surgical trauma, minimally invasive techniques, such as endoscopic or laparoscopic surgeries, have been used as they cause less inflammatory reaction when compared to conventional surgical techniques (Suter et al. 2002, Kjelgaard-Hansen et al. 2013). In addition, the response to surgical stress is composed of a set of hormonal, metabolic and inflammatory reactions that occur after surgery, allowing the body to adapt to trauma and to recover damaged tissues (Jacobsen et al. 2009). In order to improve the surgical procedure and monitoring of the postoperative period, it is necessary to identify markers that are useful to evaluate the intensity of the trauma caused by the procedure. Recent investigations have demonstrated changes in APP concentrations in dogs related to the extent and progression of diseases, as well as their the inflammatory response in infectious, parasitic, trauma, degenerative diseases and neoplastic diseases (Bayramli & Ulutas 2008, Koster et al. 2009, Tecles et al. 2009, Méndez et al. 2014, Schmidt & Eckersall 2015, Hillström et al. 2016, Schmidt et al. 2016). However, although the acute phase response was investigated in dogs undergoing elective soft tissue surgeries (Serin & Ulutas 2010, Kjelgaard-Hansen et al. 2013, Kum et al. 2013), surgical correction of obstructive airway syndrome (Planellas et al. 2015), nephrectomy (Schmidt et al. 2016), and ovariohysterectomy as the treatment of pyometra (Dabrowski et al. 2007, 2009) few studies have evaluated the PFA concentrations and the acute phase response dynamics in dogs undergoing minimally invasive surgical procedures (Mathon et al. 2011, Tvarijonaviciute et al. 2011, Kjelgaard-Hansen et al. 2013).

Therefore, the objective of this work is to evaluate and to explore possible differences between two conventional and minimally invasive ovariohysterectomy protocols in bitches regarding the kinetics of the acute protein response of CRP (C-reactive protein) and Hp (haptoglobin), comparing the effects of inflammation caused by the two procedures in the postoperative period.

MATERIALS AND METHODS

The study population comprised of 17 client-owned adult female mixed breed dogs (2 to 7 years old; 10±1,2kg), admitted to the São Paulo State University Veterinary Teaching Hospital of the Faculty of Veterinary Medicine and Animal Science (FVMZ) for elective ovariohysterectomy. Inclusion criteria included the availability of complete clinical records as part of the routine care and ultrasound evaluation discarding pregnancy. The stage of oestrus cycle was not determined. The dogs included in the study showed no abnormal findings on physical and clinical examination or any other considerable change in hemograms, alanine aminotransferase activity, total serum protein, albumin, and fibrinogen. This approach was approved by the Faculty's Animal Experimentation Ethics Committee of the São Paulo State University, FMVZ-Unesp, Campus Botucatu (protocol number 194/2011-CEUA) with the consent form signed by the owners under the same protocol.

The animals were fasted (food and water) for 12 hours before the surgical procedure. All bitches received meloxican (0.2mg/kg, Maxicam 0.2%, Ourofino, Brazil) pre-surgery, 15 minutes before induction of anesthesia. At the same time, the preanesthetic medication used was the combination of acepromazine maleate (0.05mg/kg; Acepran 0.2%, Vetnil, Brazil) and morphine (0.5mg/kg; Dimorf, Cristalia, Brazil) via intramuscular. For anesthesia induction, the combination of ketamine (10mg/kg; Quetamina, Vetnil, Brazil) and diazepam (0.5mg/kg, Valium, Roche, Brazil) were used intravenously. The anesthetic protocol employed was adequate for performing the surgical procedure and lasted from 20 to 30 minutes, with no need for anesthetic complementation. After the surgical procedure, the animals received intravenous penicillin benzathine (24,000 IU, Veterinary Pentabiotic, Fort Dodge, Brazil) as a routine prophylactic measure.

The dogs were divided into two experimental groups. Group 1 (G1): 9 animals, submitted to ovariohysterectomy by the conventional technique. Group 2 (G2): 8 animals, submitted to ovariohysterectomy by the minimally invasive technique. The conventional technique was performed by laparotomy. After the procedures of trichotomy and asepsis of the abdominal region, a ventral midline incision was performed over the skin, subcutaneous tissue and the linea alba. Then the uterus was exposed and the ligatures of the ovarian pedicles and uterine body were performed with mononylon 2-0 yarn. Once the uterus and ovaries were removed, abdominal wall synthesis was performed with Sultan-type suture, and subcutaneous tissue using a simple continuous pattern of absorbable sutures and the skin was closed in a simple interrupted pattern. The stitches were removed seven days after the surgical procedure (Stone 2007).

For the minimally invasive technique, the animals were placed in dorsal decubitus position. After the trichotomy and asepsis of

the region of the abdomen, a midline skin incision of about two centimeters was performed caudal to the umbilical scar. Then the linea alba was incised and the abdominal cavity was accessed. The uterine horns and the ovarian pedicles were pulled into a midline portal with the aid of a spay hook and the ovarian ligament dissected, thus pulling the ovaries and ovarian pedicles which were ligated with a self-static nylon cable tie. Subsequently, the uterine horns were pulled into the midline incision and the cervix was ligated using the same nylon cable tie. Abdominal wall synthesis was performed with Sultan-type suture, and subcutaneous tissue using a simple continuous pattern of absorbable sutures and the skin was closed in a simple interrupted pattern as described by Silva et al. (2004) and Lima et al. (2010). All surgical procedures of G1 and G2 were performed by a single surgeon, but a different person for each group.

Blood samples were obtained by jugular vein puncture 24h preoperatively (M1) and at three time points postsurgery, i.e. 24h (M2), 48h (M3), and on day 7 after surgery (M4). Aliquots of the samples (3mL) were placed in plain tubes with gel separators (BD Vacutainer® Blood Collection Tube; Becton, Dickinson and Company, USA) were allowed to clot at room temperature, centrifuged (1,500×g for 5 min) and the harvest sera were stored in Eppendorf microtubes (Eppendorf, Hamburg, Germany), stored at -20°C with CRP and haptoglobin analyses performed within six months.

Serum CRP concentrations were determined by a commercial ELISA kit (cCRP; Avacta, York, UK) based on a previous assay validated for use in dogs (Eckersall et al. 1989). Serum HP concentrations were measured via a hemoglobin binding assay previously validated for use in dogs (Crawford et al. 2013). These assays showed a within run imprecision of less than 10%.

All statistics were performed using statistical software (GraphPad Version 6 for Windows, GraphPad Software Inc., San Diego/CA, USA). All variables were first assessed for normality using the Shapiro-Wilk test. The nonparametric Kruskal-Wallis test was used to compare the groups and One-way ANOVA and Friedman test was used for multiple comparisons between groups as the data was not normally distributed. Mann-Whitney U test was used for additional comparisons between groups. Statistical significance was set at $P<0.05$ for all analyses.

RESULTS

The preanesthetic and anesthetic protocols were adequate to perform the surgical procedures in both groups. Surgical procedures ranged from 20-30 minutes in G1 and 15-20 minutes in G2. There were no intercurrences or complication in all performed surgeries, regardless of the technique employed, until the stitches were removed.

Haptoglobin and CRP concentrations from all dogs of G1 and G2 are presented in Table 1. C-reactive protein concentration was significantly higher ($P<0.0001$) at 24h after the surgical procedures for both ovariohysterectomy techniques. There were no significant differences for Hp between both surgical protocols. The median concentrations of CRP were significantly higher when comparing 24 and 48h after surgery to before minimally invasive and conventional ovariohysterectomy. In our study, the median haptoglobin concentrations were significantly higher 48h after conventional ovariohysterectomy and at 24 and 48h after the minimally invasive surgery.

Significant differences for G1 and G2 were not observed for CRP and Hp on day 7 after surgeries (Table 1).

Table 1. Median and range (minimum - maximum) of CRP (µg/mL) and Hp (g/L) concentrations in female dogs subjected to conventional (n=9) and minimally invasive ovariohysterectomy (n=8) at four different time points

	CRP (µg/mL)		Hp (g/L)	
	G1	G2	G1	G2
Before surgery	5Aa (5 - 5.8)	5.4Aa (5 - 10)	1.3a (0.1 - 2.4)	1.0a (0.7 - 2.8)
24h after surgery	71.7Ab (26.1 - 94.7)	31.1Bb (12 - 57.3)	1.8ab (0.5 - 6.8)	4.7b (2.2 - 6.6)
48h after surgery	26.1Aab (18.6 - 56.2)	16.8Aab (5 - 31.1)	3.3b (0.5 - 9.4)	4.7b (1.9 - 7.9)
7 days after surgery	10.5Aa (5 - 22.7)	9.9Aa (5 - 24)	2.0ab (0.2 - 6.9)	1.7a (0 - 3)
P value	<0.0001	<0.0001	<0.0072	<0.0002

G1 = Conventional ovariohysterectomy, G2 = minimally invasive ovariohysterectomy. Different capital letters in the same line indicate significant difference between surgical protocols, lowercase letters in the same column indicate significant difference between time points.

DISCUSSION

CRP is the major acute phase protein in dogs and also a consistent marker of inflammation in this species, stimulated by the inflammatory response increasing up to 10- to 100-fold (Ceron et al. 2005, Eckersall 2008). Haptoglobin is considered a moderate acute phase protein in dogs and has also been used as a nonspecific biomarker of inflammation, but there is an APP pattern that has been described in dogs with hyperadrenocorticism (McGrotty et al. 2003, Caldin et al. 2009) and in dogs infected by the giant kidney worm (*Dioctophyme renale*) (Schmidt et al. 2016), with increased Hp concentration indicating a stress response due to increased endogenous cortisol concentration (Caldin et al. 2009).

The acute phase response observed in the dogs of this study consisted of increased CRP and Hp serum concentrations after ovariohysterectomy. These increases are related to the inflammatory response induced by pro-inflammatory cytokines interleukin-1, interleukin-6 and alpha tumor necrosis factor, which stimulate synthesis and release of APP by the liver (Gruys et al. 2005, Eckersall 2008). In our study, these results could evidence an acute inflammatory response as the median concentrations of CRP and Hp were above the reference intervals for dogs (CRP <12µg/mL, Hp<3 g/L) (Eckersall et al. 1999, Ceron et al. 2005), from 24 hours after the surgical procedure, similarly to those described previously for acute phase proteins after surgical trauma in dogs (Conner et al. 1988, Dabrowski et al. 2007, 2009, Tvarijonaviciute et al. 2011, Kjelgaard-Hansen et al. 2013, Schmidt et al. 2016).

The increased CRP concentrations at M2 found in dogs after conventional and minimally invasive ovariohysterectomy could reflect the systemic reaction of the surgical trauma. Bitches subjected to ovariohysterectomy by laparotomy demonstrated a cumulative increased CRP concentration postsurgery, peaking at 12h after the surgical procedure indicating that this could be the appropriate time point to evaluate the inflammatory impact of surgical trauma (Kjelgaard-Hansen et al. 2013). This response was also expected in our study; however, despite

increased CRP at 24h postsurgery, this result indicates a limitation of our investigation as it was not possible to obtain serum samples at the immediate postsurgical time points, i.e. at 4, 8, and 12h, as described by Kjelgaard-Hansen et al. (2013).

Conner et al. (1988), Dabrowski et al. (2009), and Kum et al. (2013) demonstrated that CRP concentration reaches a peak at 24h after the surgical trauma and there is a rapid decrease of CRP in the absence of postoperative complications. However, there was a different inflammatory response in bitches subjected to ovariohysterectomy by laparotomy with increased CRP concentrations from 24h until day 7 after surgery, demonstrating that although there could have been a decrease in inflammation, CRP remained increased (Serin & Ulutas 2010). No changes were observed in CRP concentration on day 7 postsurgery of the female dogs evaluated in this study. Despite the ovariohysterectomy technique, this indicates that the recovery from the inflammation associated with the surgery was complete.

In our study, no changes were observed for Hp concentrations between both surgical protocols. However, a hypothesis explaining why dogs subjected to conventional ovariohysterectomy present decreased serum Hp concentration at 24 and 48h post-surgery compared to dogs subjected to minimally invasive ovariohisterectomy would be that the highest degree of hemolysis immediately after surgery (24h) occurs due to a larger tissue injury and the decrease in Hp concentration could be caused by the monocyte/macrophage Hp-hemoglobin receptor expressed on Kupffer cells in the liver and the further removal of this complex (Conner et al. 1988, Eckersall 2008).

Haptoglobin is a moderate APP and usually peaks at 24 to 48h after a surgical trauma (McGrotty et al. 2003, Eckersall 2008). Haptoglobin concentrations were above reference intervals for the bitches (Hp<3g/L) (Eckersall et al. 1999) at 24h after the minimally invasive ovariohysterectomy as well as at 48h for both surgical procedures. These results could indicate a mild inflammatory response that could be associated with an inflammatory response of lower intensity after the initial surgical trauma. Dabrowski et al. (2009) and Serin & Ulutas (2010) demonstrated the same inflammatory response in bitches subjected to ovariohysterectomy by laparotomy.

Repeated measurements of CRP and Hp were useful in monitoring the short-term inflammatory process induced by the ovariohysterectomy that was associated with increases in serum concentration of both APPs. No further significant increases were recorded 7 days after conventional and minimally invasive ovariohysterectomy, when recovery from the inflammation associated with the surgery was complete.

CONCLUSIONS

Bitches were subjected to conventional and minimally invasive ovariohysterectomy that contributes towards an understanding of the postsurgical inflammatory response in dogs subjected to elective ovariohysterectomy.

Both surgical procedures increased CRP and Hp concentrations at different time points due to surgical trauma, demonstrating the usefulness of determining the concentrations of these proteins to monitoring the short-term inflammatory process induced by the ovariohysterectomy.

Conflict of interest.- None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence the content of the paper.

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REFERENCES

Bayramli G. & Ulutas B. 2008. Acute phase protein response in dogs with experimentally induced gastric mucosal injury. Vet. Clin. Pathol. 37(3):312-316. <http://dx.doi.org/10.1111/j.1939-165X.2008.00060.x> <PMid:18761525>

Caldin M., Tasca S., Carli E., Bianchini S., Furlanello T., Martinez-Subiela S. & Cerón J.J. 2009. Serum acute phase protein concentrations in dogs with hyperadrenocorticism with and without concurrent inflammatory conditions. Vet. Clin. Pathol. 38(1):63-68. <http://dx.doi.org/10.1111/j.1939-165X.2008.00087.x> <PMid:19228354>

Ceron J.J., Eckersall P.D. & Martýnez-Subiela S. 2005. Acute phase proteins in dogs and cats: current knowledge and future perspectives. Vet. Clin. Pathol. 34(2):85-99. <http://dx.doi.org/10.1111/j.1939-165X.2005.tb00019.x> <PMid:15902658>

Conner J.G., Eckersall P.D., Ferguson J. & Douglas T.A. 1988. Acute phase response in the dog following surgical trauma. Res. Vet. Sci. 45(1):107-110. <http://dx.doi.org/10.1016/S0034-5288(18)30902-0> <PMid:2464844>

Crawford K., Warman S.M., Marques A.I., Yool D.A., Eckersall P.D., McCulloch E., Lynn K., Mellanby R.J. & Gow A.G. 2013. Serum haptoglobin concentrations in dogs with liver disease. Vet. Rec. 173(23):579-582. <http://dx.doi.org/10.1136/vr.101306> <PMid:24158322>

Dabrowski R., Wawron W. & Kostro K. 2007. Changes in CRP, SAA and haptoglobin produced in response to ovariohysterectomy in healthy bitches and those with pyometra. Theriogenology 67(2):321-327. <http://dx.doi.org/10.1016/j.theriogenology.2006.07.019> <PMid:16962655>

Dabrowski R., Kostro K., Lisiecka U., Szczubiał M. & Krakowski L. 2009. Usefulness of C-reactive protein, serum amyloid A component, and haptoglobin determinations in bitches with pyometra for monitoring early post-ovariohysterectomy complications. Theriogenology 72(4):471-476. <http://dx.doi.org/10.1016/j.theriogenology.2009.03.017> <PMid:19477501>

Eckersall P.D. 2008. Proteins, proteomics and the dysproteinemias, p.117-155. In: Kaneko J.J., Harvey J.W. & Bruss M.L. (Eds), Clinical Biochemistry of Domestic Animals. 6th ed. Academic Press, Burlington. <http://dx.doi.org/10.1016/B978-0-12-370491-7.00005-2>.

Eckersall P.D. & Bell R. 2010. Acute phase proteins: biomarkers of infection and inflammation. Vet. J. 185(1):23-27. <http://dx.doi.org/10.1016/j.tvjl.2010.04.009> <PMid:20621712>

Eckersall P.D., Conner J.G. & Parton H. 1989. An enzyme-linked immunosorbent assay for canine C-reactive protein. Vet. Rec. 124(18):490-491. <http://dx.doi.org/10.1136/vr.124.18.490> <PMid:2750033>

Eckersall P.D., Duthie S., Safi S., Moffatt D., Horadagoda N.U., Doyle S., Parton R., Bennett D. & Fitzpatrick J.L. 1999. An automated biochemical assay for haptoglobin: prevention of interference from albumin. Comp. Haematol. Int. 9(3):117-124. <http://dx.doi.org/10.1007/BF02600369>

Gruys E., Toussaint M.J., Niewold T.A. & Koopmans S.J. 2005. Acute phase reaction and acute phase proteins. J. Zhejiand Univ., Sci. B. Biomed. Biotechnol. 11:1045-1056.

Hillström A., Bylin J., Hagman R., Björhall K., Tvedten H., Königsson K., Fall T. & Kjelgaard-Hansen M. 2016. Measurement of serum C-reactive protein concentration for discriminating between suppurative arthritis and osteoarthritis in dogs. BMC Vet. Res. 12(1):240-249. <http://dx.doi.org/10.1186/s12917-016-0868-4> <PMid:27793205>

Jacobsen S., Nielsen J.V., Kjelgaard-Hansen M., Toelboell T., Fjeldborg J., Halling-Thomsen M., Martinussen T. & Thoefner M.B. 2009. Acute phase response to surgery of varying intensity in horses: a preliminary study. Vet. Surg. 38(6):762-769. <http://dx.doi.org/10.1111/j.1532-950X.2009.00564.x> <PMid:19674420>

Kjelgaard-Hansen M., Strom H., Mikkelsen L.F., Eriksen T., Jensen A.L. & Luntang-Jensen M. 2013. Canine serum C-reactive protein as a quantitative marker of the inflammatory stimulus of aseptic elective soft tissue surgery. Vet. Clin. Pathol. 42(3):342-345. <http://dx.doi.org/10.1111/vcp.12063> <PMid:23899087>

Koster L.S., Van Schoor M., Goddard A., Thompson P.N., Matjila P.T. & Kjelgaard-Hansen M. 2009. C-reactive protein in canine babesiosis caused by *Babesia rossi* and its associations with outcome. J. S. Afr. Vet. Assoc. 80(2):87-91. <http://dx.doi.org/10.4102/jsava.v80i2.177> <PMid:19831269>

Kum C., Voyvoda H., Sekkin S., Karademir U. & Tarimcilar T. 2013. Effects of carprofen and meloxicam on C-reactive protein, ceruloplasmin, and fibrinogen concentrations in dogs undergoing ovariohysterectomy. Am. J. Vet. Res. 10(10):1267-1273. <http://dx.doi.org/10.2460/ajvr.74.10.1267> <PMid:24066910>

Lascelles B.D., Cripps P.J., Jones A. & Waterman-Pearson A.E. 1998. Efficacy and kinetics of carprofen, administered preoperatively or postoperatively, for the prevention of pain in dogs undergoing ovariohysterectomy. Vet. Surg. 27(6):568-582. <http://dx.doi.org/10.1111/j.1532-950X.1998.tb00533.x> <PMid:9845221>

Lima A.F.M., Luna S.P.L., Rodrigues M.M. & Quitzan J. 2010. Avaliação histological e videolaparoscópica de ligaduras de pedículos ovarianos realizados com mononáilon agulhado ou abraçadeiras auto-estáticas de náilon em cadelas submetidas a ovariossalpingohisterectomia pela técnica do gancho. ARS Vet. 2:66-70.

Mathon D.H., Palierne S., Meynaud-Collard P., Layssol-Lamour C., Dulaurent-Ferrieres A., Colson A., Lacroix M., Bousquet-Melou A., Delverdier M. & Autefage A. 2011. Laparoscopic-assisted colopexy and sterilization in male dogs: short-term results and physiologic consequences. Vet. Surg. 40(4):500-508. <http://dx.doi.org/10.1111/j.1532-950X.2011.00835.x> <PMid:21521243>

McGrotty Y.L., Knottenbelt C.M., Ramsey I.K., Reid S.W. & Eckersall P.D. 2003. Haptoglobin concentrations in a canine hospital population. Vet. Rec. 152(18):562-564. <http://dx.doi.org/10.1136/vr.152.18.562> <PMid:12751608>

Méndez J.C., Carretón E., Martínez S., Tvarijonaviciute A., Cerón J.J. & Montoya-Alonso J.A. 2014. Acute phase response in dogs with *Dirofilaria immitis*. Vet. Parasitol. 204(3/4):420-425. <http://dx.doi.org/10.1016/j.vetpar.2014.05.016> <PMid:24893697>

Murata H., Shimada N. & Yoshioka M. 2004. Current research on acute phase proteins in veterinary diagnosis: an overview. Vet. J. 168(1):28-40. <http://dx.doi.org/10.1016/S1090-0233(03)00119-9> <PMid:15158206>

Planellas M., Cuenca R., Tabar M.D., Bertolani C., Poncet C., Closa J.M., Lorente J., Cerón J.J. & Pastor J. 2015. Clinical assessment and C-reactive protein (CRP), haptoglobin (Hp), and cardiac troponin I (cTnI) values of brachycephalic dogs with upper airway obstruction before and after surgery. Can. J. Vet. Res. 79(1):58-63. <PMid:25673910>

Schmidt E.M.S. & Eckersall P.D. 2015. Acute phase proteins as markers of infectious diseases in small animals. Acta Vet., Belgrade, 65:149-161.

Schmidt E.M.S., Kjelgaard-Hansen M., Thomas F., Tvarijonaviciute A., Cerón J.J. & Eckersall P.D. 2016. Acute phase proteins in dogs naturally infected with the giant kidney worm (*Dioctophyme renale*). Vet. Clin. Pathol. 45(4):657-664. <http://dx.doi.org/10.1111/vcp.12400> <PMid:27748965>

Serin G. & Ulutas P.A. 2010. Measurement of serum acute phase proteins to monitor postoperative recovery in anoestrous bitches after ovariohysterectomy. Vet. Rec. 166(1):20-22. <http://dx.doi.org/10.1136/vr.b5585> <PMid:20045854>

Silva L.A.F., Araújo G.R.S., Miranda A.H., Rabelo R.E., Garcia A.M., Silva O.C., Araújo I.F.L., Macedo S.P., Sousa J.N., Fioravanti M.C.S., Oliveira K.S., Amaral A.V.C. & Silva E.B. 2004. Ovariohisterectomia em cadelas: uso da braçadeira de náilon na hemostasia preventiva. Ciênc. Anim. Bras. 5(Supl.):100-102.

Stone E.A. 2007. Ovário e útero, p.1487-1502. In: Slatter D. (Ed.), Manual de Cirurgia de Pequenos Animais. 3ª ed. Manole, São Paulo.

Suter M., Martinet O. & Spertini F. 2002. Reduced acute phase response after laparoscopic total extraperitoneal bilateral hernia repair compared to open repair with the Stoppa procedure. Surg. Endosc. 16(8):1214-1219. <http://dx.doi.org/10.1007/s00464-001-9164-9> <PMid:12189483>

Tecles F., Caldín M., Zanella A., Membiela F., Tvarijonaviciute A., Subiela S.M. & Cerón J.J. 2009. Serum acute phase protein concentrations in female dogs with mammary tumors. J. Vet. Diagn. Invest. 21(2):214-219. <http://dx.doi.org/10.1177/104063870902100206> <PMid:19286500>

Tvarijonaviciute A., Martinez-Subiela S., Carrillo-Sanchez J.D., Tecles F. & Ceron J.J. 2011. Effects of orchidectomy in selective biochemical analytes in Beagle dogs. Reprod. Domest. Anim. 6(6):957-963. <http://dx.doi.org/10.1111/j.1439-0531.2011.01765.x> <PMid:21366720>

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Original Article
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Histopathological and immunophenotypical assessment of canine primary splenic lymphoma according to the World Health Organization[1]

Cristiano P. Fracácio[2], Felipe A.R. Sueiro[3], Letícia A. Anai[4], Maiara B. Pucci[2], Igor L.S. Senhorello[4], Julielton S. Barata[4] and Paulo C. Jark[2,4]*

ABSTRACT.- Fracácio C.P., Sueiro F.A.R., Anai L.A., Pucci M.B., Senhorello I.L.S., Barata J.S. & Jark P.C. 2018. **Histopathological and immunophenotypical assessment of canine primary splenic lymphoma according to the World Health Organization.** *Pesquisa Veterinária Brasileira 38(11):2129-2132.* Departamento de Clínica Veterinária e Cirurgia, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Via de acesso Prof. Dr. Paulo Donato Castellane s/n, Jaboticabal, SP 14884-900, Brazil. E-mail: paulocjark@hotmail.com

Although there are several studies addressing multicentric lymphoma in dogs, data regarding splenic lymphoma remains scarce. The diagnosis of splenic lymphoma using the World Health Organization (WHO) classification system can aid prognostic characterization of splenic lymphoma. The aim of this study was to evaluate the most common histological types of splenic lymphoma in dogs from Brazil according to the WHO classification. We assessed 33 cases of splenic lymphoma diagnosed by histopathologic and immunohistochemical (IHC) analysis submitted to VETPAT- Pathology Laboratory, Campinas-SP, Brazil. IHC was performed using antibodies against CD3 for T-cell and CD79α for B-cell identification . Mean age of patients with splenic lymphoma was 9.8 years. The most affected breeds were mixed breed dogs (33%) followed by Pit bulls and Yorkshires (9.0%). The most prevalent histological type was marginal zone B-cell lymphoma (60.7%) followed by diffuse large B-cell lymphoma (12.1%) and lymphoblastic T-cell lymphoma (12.1%). Histological and immunohistochemical characterization of splenic lymphoma is important due to the high prevalence of indolent lymphomas such as marginal zone, which may be less aggressive and thus have different prognostic and distinct forms of treatment when compared to high-grade lymphomas.

INDEX TERMS: Histopathology, immunophenotypical, canine, splenic lymphoma, spleen, hematopoietic neoplasm, indolent lymphoma, pathology.

RESUMO.- [**Avaliação histopatológica e imunofenotípica segundo a classificação da Organização Mundial da Saúde do linfoma esplênico primário em cães.**] Embora existam diversos estudos a respeito do linfoma multicêntrico em cães, os dados sobre linfoma esplênico primário são escassos. O diagnóstico do linfoma esplênico utilizando a classificação da Organização Mundial da Saúde (OMS) pode melhorar a caracterização da doença. O objetivo do estudo foi avaliar os principais tipos de linfoma esplênico primário em cães no Brasil de acordo com a classificação da OMS. Foram avaliados 33 casos de linfoma esplênico diagnosticados por histopatologia e imuno-histoquímica submetidos ao Laboratório de Patologia Veterinária (VETPAT, Campinas/SP). A imuno-histoquímica foi realizada utilizando os anticorpos CD3 para linfomas T, CD79α para linfomas B. A média de idade dos pacientes com linfoma esplênico foi de 9,8 anos. Os animais sem raça definida (SRD) foram os mais acometidos (33%) seguidos de PitBulls e Yorkshire (9,0%). O tipo histológico mais comum foi o linfoma de zona marginal representando 60,7% dos casos seguido do linfoma difuso de grandes células B (12,1%) e linfoma linfoblástico T (12,1%). A caracterização histopatológica e imuno-histoquímica do linfoma esplênico é importante devido à alta prevalência de linfomas indolentes como o linfoma de zona marginal,

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[2] Departamento de Clínica Veterinária e Cirurgia, Av. Hilário da Silva Passos 950, Parque Universitário, Descalvado, SP 13690-000, Brazil.

[3] Laboratório VETPAT, Cel. Manuel de Morais 146, Jardim Brasil, Campinas, SP 13073-022, Brazil.

[4] Departamento de Clínica Veterinária e Cirurgia, Faculdade de Ciências Agrárias e Veterinárias (FCAV), Universidade Estadual Paulista (Unesp), Via de acesso Prof. Dr. Paulo Donato Castellane s/n, Jaboticabal, SP 14884-900.
* Corresponding author: paulocjark@hotmail.com

que devido ao seu comportamento indolente apresenta prognóstico e tratamento distintos quando comparado aos linfomas de alto grau.

TERMOS DE INDEXAÇÃO: Histopatologia, imunofenotípica, linfoma esplênico, caninos, baço, neoplasia hematopoiética, linfoma indolente, patologia.

INTRODUCTION

Lymphoma is a malignant neoplasm characterized by lymphoid cell proliferation. The most common presentation of lymphoma is multicentric, which usually involves peripheral lymph nodes and possibly liver, spleen, bone marrow and extranodal sites (Vail et al. 2013).

There are several studies addressing multicentric lymphoma in dogs, yet studies regarding primary splenic lymphoma remain scarce. The most widely used histological classification system for canine lymphoma is the World Health Organization (WHO) classification (Vezzali et al. 2009, Valli et al. 2011, 2013). Recent uses of this system with primary splenic lymphoma have demonstrated that the most common histological type is marginal zone B-cell lymphoma, which shows indolent progression and better prognosis compared to the most common multicentric type, the diffuse large B-cell lymphoma (O'Brien et al. 2013, Van Stee et al. 2015). Marginal zone lymphoma in the spleen has low mitotic rate and slow progression, which represents a challenge for pathologists to reach a diagnosis, due to its similarity with benign marginal zone hyperplasia. This warrants immunohistochemistry or other molecular techniques for correct diagnosis (O'Brien et al. 2013).

The aim of this study was to assess 33 cases of primary lymphoma of the spleen diagnosed through histopathology and immunohistochemistry according to the WHO classification in dogs, seeing that information regarding the epidemiology of this disease in Brazil is scarce.

MATERIALS AND METHODS

The research was performed through assessment of the database at VETPAT in Campinas, São Paulo State, Brazil. Samples from the eight regions of the country diagnosed as splenic lymphoma were classified according to the WHO histological and immunohistochemical classification from January 2012 to December 2016.

This study included only splenic lymphoma, and samples diagnosed as other forms of neoplasm such as multicentric, cutaneous, mediastinal, alimentary or extranodal lymphoma were excluded from further analyses. Data regarding patient identification, breed, gender and age were charted for analysis.

Immunohistochemistry (IHC) analysis for immunophenotyping of splenic lymphomas followed pre-stablished protocols, as follows. Immunohistochemical reactions were performed on paraffin-embedded samples sectioned at 4μm and positively charged for immunohistochemical reactions. A biotin-free detection reagent (REVEAL) was used for immunohistochemical analysis. Tissue sections were deparaffinized, rehydrated and incubated for 20 minutes in a 5% solution of hydrogen peroxide in absolute methanol (Synth) for endogenous peroxidase activity blocking. Heat-induced antigen retrieval was performed using a sodium citrate buffer of pH 6.0 in a pressure cooker. Sections were then washed three times in phosphate-buffered saline (PBS) at 0.01M (pH 7.2) for 5 minutes and nonspecific sites were blocked with a blocking buffer for nonspecific reactions (Reveal Spring kit reagent - Cod SPD-125). Slides were incubated overnight with anti-CD3, anti-CD79α and MUM-1 antibodies, as shown in Table 1.

If positive for CD79α and negative for CD3, lymphomas were deemed B-cell in origin and T-cell if positive for CD3 and negative for CD79α. Samples that were negative for MUM-1 were regarded as lymphomas and samples that were positive for MUM-1 were regarded as plasma cell tumor.

RESULTS

Thirty-three cases of primary splenic lymphoma were found in the study. Mean age of patients was 9.8 (5-16) years. Main breeds found were mongrels (11/33), Pit Bulls (3/33), Yorshire Terriers (3/33), Golden Retrievers (2/33), Labradors (2/33), Poodles (2/33), Shih Tzus (2/33), Lhasa Apso (1/33), Rottweiller (1/33), Bernese Mountain Dog (1/33), Pug (1/33), Fox Terrier (1/33), Cane Corso (1/33) and unspecified (1/33). With regard to gender, 18 were males, 14 females and one unspecified.

Immunophenotyping showed that 87.9% of splenic lymphomas were positive for CD79α (B-cell) and 12.1% for CD3 (T-cell). All cases were negative for MUM-1. According the WHO classification system, the most frequent histological type was marginal zone B-cell lymphoma, which accounted for 20 of the 33 cases (60.7%) (Fig.1), followed by diffuse large B-cell lymphoma (4/33, 12.1%) and lymphoblastic T-cell lymphoma (4/33, 12.1%) (Table2). One case (3%) of follicular lymphoma was found among the cases, which summed up to 63.7% of indolent splenic lymphomas in this study according to the WHO classification.

DISCUSSION

Lymphoma is a generic term to describe various diseases of unusual origin, which is lymphoid tissue. However, there are variations as to treatment and prognosis according to anatomic location, histological type and immunophenotype

Table 1. Clone, dilution and provenance of antibodies used for immunophenotyping of splenic lymphoma

Antibody	Clone	Dilution	Cod	Manufacturer
CD3	Polyclonal	1:300	A0452	DAKO
CD79α	HM47/A9	1:300	M7051	DAKO
MUM-1	MUM1p	1:100	M7259	DAKO

Table 2. Histopathological classification of primary lymphoma of the spleen in dogs, according to the WHO classification system

Histological type	Number of cases (N=33)	Percentage
Marginal zone B-cell lymphoma	20	60.7%
Diffuse large B-cell lymphoma	4	12.1%
Lymphoblastic T-cell lymphoma	4	12.1%
Lymphoplasmacytic lymphoma	3	9.1%
Follicular B-cell lymphoma	1	3%
Lymphocytic B-cell lymphoma	1	3%

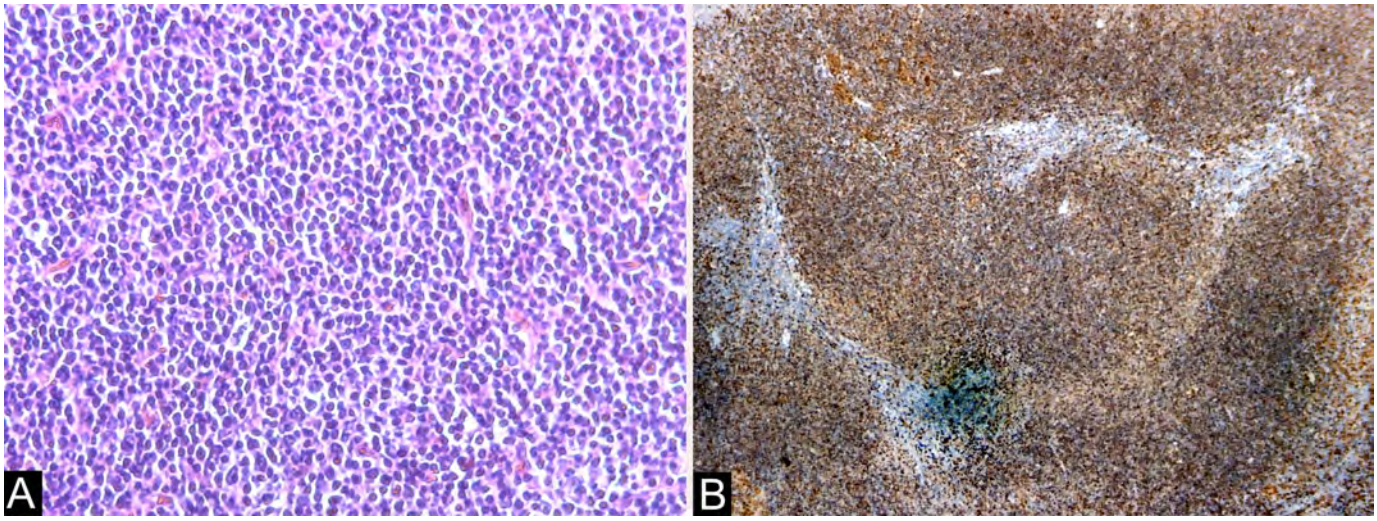


Fig.1. Marginal zone B-cell lymphoma in a dog. (**A**) Lymphoid neoplasm formed by dense lymphocyte clusters with dense chromatin, reniform nuclei and wide clear cytoplasm. Cells form a dense band that compresses follicular structures. HE, obj.20x. (**B**) Immunohistochemical analysis of marginal zone B-cell lymphoma in the spleen. Notice the bulk of neoplastic cells positive for CD79α. IHC, obj.4x.

(Rebhun et al. 2011, Marconato et al. 2011, Valli et al. 2013, Goodman et al. 2016). Information regarding treatment and prognosis of lymphoma in dogs is based on studies of various forms of presentation and distinct histological types, which results in heterogeneous groups and therefore difficulties when establishing individualized criteria (Chun 2009, Rebhun et al. 201, Vail et al. 2013, Goodman et al. 2016).

The study of histopathological characterization of splenic lymphoma in Brazil is somewhat important since current literature is scarce. One study showed low incidence of lymphoma (2.2%) in 179 cases of splenic diseases (Bandinelli et al. 2011).

Performing a morphological analysis along with immunohistochemistry is crucial to a more accurate classification of the disease and for differentiation of indolent and reaction hyperplasia that can affect the spleen. Although there are several classification systems for canine lymphoma, the WHO classification remains the most commonly used, because it correlates cytomorphological, immunophenotypical and clinical criteria, aside from its agreement among pathologists worldwide (Vezzali et al. 2010, Valli et al 2013). Anti-CD3 and anti-CD79α antibodies are used for immunophenotyping and described widely due to their high sensitivity and specificity (Valli et al. 2013). The main differential diagnosis for splenic lymphoma during histopathology is plasma cell tumor. One option to aid this distinction is the use of MUM-1, seeing that 93.5% of extramedullary plasmacytomas are positive for MUM-1, which most lymphomas are negative for (Ramos-Vara et al. 2007). In this study, all cases of splenic lymphomas were negative for MUM-1.

Indolent splenic lymphomas were more prevalent (63.7%) in this study, of which 60.7% of the cases were diagnosed as marginal zone B-cell lymphoma. These data corroborate the few studies from other countries, where the incidence of indolent lymphomas in the spleen was higher compared to high grade lymphomas (Van Stee et al. 2015). A study of 28 cases of primary splenic lymphoma showed 71.4% of indolent tumors and marginal zone B-cell lymphoma was also the most prevalent (39.3% of cases), followed by mantle cell lymphoma (32.1%) (Van Stee et al. 2015). Interestingly, although mantle cell lymphoma was the second most common in that study, no case of this type was seen in the present study.

The marginal zone B-cell lymphoma is characterized by small to medium size lymphocytes positive for CD79α, with low mitotic rate and slow progression, which are originated at the marginal zone of the lymphoid follicle. Since there are only a few reports in the current literature, the incidence of this tumor is unknown (Valli et al. 2006, Stefanello et al. 2011, Flood-Knapik et al. 2013). Valli et al. (2006) reported 66 cases of indolent lymphomas, of which 46 were marginal zone. However, 33 cases were extranodal and only 13 were splenic.

Despite the lack of information regarding the distribution of lymphoma lesions in this study, most of the cases reported by Valli et al. (2006) were characterized as solitary masses found occasionally during ultrasound exams. Other authors also report higher incidence of solitary masses over diffuse presentation or multiple nodes (O'Brien et al. 2013, Stefanello et al. 2011).

No predisposition was found in this study with regard to breed or gender for canine splenic lymphoma, which corroborates previous studies (O'Brien et al. 2013). Most patients were middle-aged to elder, which agrees with most studies of oncology where mean age is usually found to be >7 years (Stefanello et al. 2011, Flood-Knapik et al. 2013, O'Brien et al. 2013).

The distinction between indolent and high-grade lymphomas, such as lymphoblastic T-cell lymphoma (12.1% of the cases) is important, since the prognosis is quite different and they require individualized treatment (Van Stee et al. 2015). One study showed that 1-year survival time for marginal zone lymphoma represents 63.6% of the cases, whereas high-grade T-cell lymphomas are associated with shorter survival (Van Stee et al. 2015). Another study of 29 cases undergoing splenectomy reported mean survival time of 383 days, and patients that were asymptomatic at the time of surgery showed survival time of 1153 days, which corroborates other studies

reporting better prognosis with marginal zone lymphomas (O'Brien et al. 2013). Aside from the exact characterization of histological type, other features are important for choosing treatment, such as differing between localized forms or tumors that involve other organs and the associated clinical signs. These features can influence the prognosis of splenic lymphoma (Stefanello et al. 2011, O'Brien et al. 2013).

While this was a retrospective study that assessed the incidence of splenic lymphoid neoplasms rather than prognosis, nevertheless the results on prevalence of indolent forms are important to enhance the need for an exact characterization of histological type to aid choice of treatment and prognosis of these cases.

CONCLUSIONS

Indolent lymphoma represented by marginal zone B-cell lymphoma is the most frequent histological type of splenic lymphomas according to the WHO classification system in dogs.

The exact characterization is fundamental for establishing proper treatment and prognosis of the patients, since current literature show more favorable prognosis in indolent compared to high-grade T- or B-cell lymphomas.

REFERENCES

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>

Chun R. 2009. Lymphoma: which chemotherapy protocol and why? Top Companion Anim. Med. 24(3):157-162. <http://dx.doi.org/10.1053/j.tcam.2009.03.003> <PMid:19732735>

Flood-Knapik K.E., Durham A.C., Gregor T.P., Sánchez M.D., Durney M.E. & Sorenmo K.U. 2013. Clinical, histopathological and immunohistochemical characterization of canine indolent lymphoma. Vet. Comp. Oncol. 11(4):272-286. <http://dx.doi.org/10.1111/j.1476-5829.2011.00317.x> <PMid:22296667>

Goodman I.H., Moore A.S. & Frimberger A.E. 2016. Treatment of canine non-indolent T cell lymphoma using the VELCAP-TSC protocol: a retrospective evaluation of 70 dogs (2003-2013). Vet. J. 211:39-44. <http://dx.doi.org/10.1016/j.tvjl.2016.03.003> <PMid:27017053>

Marconato L., Stefanello D., Valenti P., Bonfanti U., Comazzi S., Roccabianca P., Caniatti M., Romanelli G., Massari F. & Zini E. 2011. Predictors of long-term survival in dogs with high-grade multicentric lymphoma. J. Am. Vet. Med. Assoc. 238(4):480-485. <http://dx.doi.org/10.2460/javma.238.4.480> <PMid:21320018>

O'Brien D., Moore P.F., Vernau W., Peauroi J.R., Rebhun R.B., Rodriguez Junior C.O. & Skorupski K.A. 2013. Clinical characteristics and outcome in dogs with splenic marginal zone lymphoma. J. Vet. Intern. Med. 27(4):949-954. <http://dx.doi.org/10.1111/jvim.12116> <PMid:23734665>

Ramos-Vara J.A., Miller M.A. & Valli V.E.O. 2007. Immunohistochemical detection of Multiple Myeloma 1/Interferon Regulatory Factor 4 (MUM1/IRF-4) in canine plasmacytoma: comparison with CD79a and CD20. Vet. Pathol. 44(6):875-884. <http://dx.doi.org/10.1354/vp.44-6-875> <PMid:18039900>

Rebhun R.B., Kent M.S., Borrofka S.A.E.B., Frazier S., Skorupski K. & Rodriguez C.O. 2011. CHOP chemotherapy for the treatment of canine multicentric T-cell lymphoma. Vet. Comp. Oncol. 9(1):38-44. <http://dx.doi.org/10.1111/j.1476-5829.2010.00230.x> <PMid:21303452>

Stefanello D., Valenti P., Zini E., Comazzi S., Gelain M.E., Roccabianca P., Avallone G., Caniatti M. & Marconato L. 2011. Splenic marginal zone lymphoma in 5 dogs (2001–2008). J. Vet. Intern. Med. 25(1):90-93. <http://dx.doi.org/10.1111/j.1939-1676.2010.0639.x> <PMid:21092007>

Vail D.M., Pinkerton M.E. & Young K.M. 2013. Withrow and MacEwen's Small Animal Clinical Oncology. 5th ed. W.B. Saunders, Missouri. 768p.

Valli V.E., Vernau W., de Lorimier L.-P., Graham P.S. & Moore P.F. 2006. Canine indolent nodular lymphoma. Vet. Pathol. 43(3):241-256. <http://dx.doi.org/10.1354/vp.43-3-241> <PMid:16672571>

Valli V.E., Myint M.S., Barthel A., Bienzle D., Caswell J., Colbatzky F., Durham A., Ehrhart E.J., Johnson Y., Jones C., Kiupel M., Labelle P., Lester S., Miller M., Moore P., Moroff S., Roccabianca P., Ramos-Vara J., Ross A., Scase T., Tvedten H. & Vernau W. 2011. Classification of canine malignant lymphomas according to the World Health Organization Criteria. Vet Pathol. 48(1):198-211. <http://dx.doi.org/10.1177/0300985810379428> <PMid:20861499>

Valli V.E., Kass P.H., San Myint M. & Scott F. 2013. Canine lymphomas: association of classification type, disease stage, tumor subtype, mitotic rate, and treatment with survival. Vet. Pathol. 50(5):738-748. <http://dx.doi.org/10.1177/0300985813478210> <PMid:23444036>

Van Stee L.L., Boston S.E., Singh A., Romanelli G., Rubio-Guzman A. & Scase T.J. 2015. Outcome and prognostic factors for canine splenic lymphoma treated by splenectomy (1995-2011). Vet Surg. 44(8):976-982. <http://dx.doi.org/10.1111/vsu.12405> <PMid:26426095>

Vezzali E., Parodi A.L., Marcato P.S. & Bettini G. 2009. Histopathologic classification of 171 cases of canine and feline non-Hodgkin lymphoma according to the WHO. Vet. Comp. Oncol. 8(1):38-49. <http://dx.doi.org/10.1111/j.1476-5829.2009.00201.x> <PMid:20230580>

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Original Article
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Effect of calcium sources in the diets of adult cats on urinary parameters and acid-base balance[1]

Dóris P. Halfen[2], Alexandre M. Kessler[3], Luciano Trevizan[3], Thiago H.A. Vendramini[4], João P.F. Santos[4], Vivian Pedrinelli[2], Marcio A. Brunetto[4]* and Aulus C. Carciofi[5]

ABSTRACT.- Halfen D.P., Kessler A.M., Trevizan L., Vendramini T.H.A., Santos J.P.F., Pedrinelli V., Brunetto M.A. & Carciofi A.C. 2018. **Effect of calcium sources in the diets of adult cats on urinary parameters and acid-base balance.** *Pesquisa Veterinária Brasileira 38(11):2133-2138.* Departamento de Nutrição e Produção Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Duque de Caxias Norte 225, Pirassununga, SP 19635-900, Brazil. E-mail: mabrunetto@usp.br

Calcium is a macroelement that is part of the mineral composition of the diet of companion animals, and is considered a cation of strong alkalizing power, increasing urinary pH. Calcium salts have different solubilities and depending on the anion to which calcium is associated with, it can be more or less absorbed, modifying the pH of the urine. The aim of this study was to evaluate the efficiency of calcium sources on alkalinization of urinary pH, as well as excretion of urinary electrolytes and acid-base balance of adult cats. An extruded diet for cats was selected, and had 160mEq/kg of calcium from the sources of either calcium carbonate ($CaCO_3$) or calcium gluconate ($C_{12}H_{22}CaO_{14}$) added. In the control treatment there was no addition of calcium sources, resulting in three treatments. Nine adult cats were used, mixed breed, in two experimental periods, with six replicates per treatment. Animal average age was 4±1.3 years old and average weight was 3.96±0.71kg. The cats remained in metabolic cages for an adaptation period of seven days, followed by six days of urine total collection, with volume, density, pH and calcium concentration (g/d) measurements. The acid-base balance was studied by blood gas analysis of venous blood. The two sources of calcium alkalinized the urine ($P<0.001$). However, calcium gluconate had less alkalinization power compared to the calcium carbonate ($P<0.05$). Urinary calcium was not affected by treatments, and represented less than 0.5% of calcium intake. The experiment showed that calcium, although an alkaline cation and considered strong influencer of the EB of the diet, cannot be evaluated individually, because depending on its associated anion it may have greater or lesser influence on cats urine pH.

INDEX TERMS: Calcium, diets, cats, urinary parameters, acid-base balance, base excess, calcium carbonate, calcium gluconate, urinary calcium, urolithiasis, clinics.

RESUMO.- [**Efeitos das fontes de cálcio na dieta de gatos adultos sobre parâmetros urinários e equilíbrio ácido-básico.**] O cálcio (Ca) é um macroelemento que faz parte da composição mineral da dieta de animais de companhia. Este macroelemento é considerado um cátion de forte capacidade alcalinizante e, de acordo com a fonte e quantidade inclusa, pode aumentar o pH urinário. Os sais de cálcio têm diferentes solubilidades e dependendo do ânion ao qual o cálcio está associado, pode ser mais ou menos absorvido e assim, alterar o pH da urina. O objetivo deste estudo foi avaliar os efeitos de duas fontes de cálcio na alcalinização do pH urinário, bem como a excreção de eletrólitos urinários

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[2] Departamento de Clínica Veterinária, Universidade de São Paulo (USP), Avenida Prof. Dr. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP 05508-270, Brazil.

[3] Departamento de Zootecnia, Universidade Federal do Rio Grande do Sul, Porto Alegre (UFRGS), Av. Bento Gonçalves 2600, Porto Alegre, 91540-500, Brazil.

[4] Departamento de Nutrição e Produção Animal, USP, Avenida Prof. Dr. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP 05508-270. *Corresponding author: mabrunetto@usp.br

[5] Departamento de Clínica e Cirurgia Veterinárias, Universidade Estadual Paulista (Unesp), Via de acesso Prof. Paulo Donato Castellane s/n, Jaboticabal, SP 14884-900, Brazil.

e o equilíbrio ácido-básico de felinos. Foi selecionada uma dieta extrusada para gatos e adicionados 160mEq/kg de cálcio das fontes carbonato de cálcio ($CaCO_3$) ou gluconato de cálcio ($C_{12}H_{22}CaO_{14}$). No tratamento controle, não houve adição de fontes de cálcio. Foram utilizados nove gatos adultos, de raças mistas, em dois períodos experimentais, com seis repetições por tratamento. Os animais apresentavam idade média de 4,0±1,3 anos e peso corporal médio de 3,96±0,71kg. Estes permaneceram em gaiolas metabólicas em período de adaptação durante sete dias, seguido de coleta total de urina durante seis dias. Nestas amostras foram aferidos o volume, densidade, pH e concentração de cálcio (g/d). O equilíbrio ácido-básico foi avaliado por hemogasometria em amostras de sangue venoso. As duas fontes de cálcio alcalinizaram a urina (P<0,001). No entanto, o gluconato de cálcio apresentou menor potencial de alcalinização em comparação ao carbonato de cálcio (P<0,05). O cálcio urinário não foi afetado pelos tratamentos e representou menos de 0,5% da ingestão de Ca. O experimento demonstrou que o cálcio, apesar de ser um cátion alcalinizante e influenciador do EB da dieta, não pode ser avaliado individualmente, porque dependendo do ânion associado, pode apresentar maior ou menor influência no pH da urina de gatos.

TERMOS DE INDEXAÇÃO: Cálcio, dieta, felinos, parâmetros urinários, equilíbrio ácido-básico, carbonato de cálcio, cálcio urinário, excesso de base, gluconato de cálcio, urolitíase, clínica.

INTRODUCTION

Nutrition, through the manipulation of the electrolytic composition modifies the base excess of dietary (BE), which is a mEq difference between cations, such as calcium, and anions, calculated with predictive equations (Jeremias et al. 2013). BE can alter urinary pH (Allen & Kruger 2000), and changes in urine pH are, in fact, due to the impact of the cations and anions balance on the acid-base state of the animals (Pizzorno et al. 2010).

Urolithiasis in dogs and cats has been much studied in recent years and management of this disorder is evolving, with nutrition being a keystone for the management of these animals (Jeremias et al. 2013, Lulich et al. 2016), since diet influences urinary pH, and it has a strong influence on certain types of urolith formation (Bartges & Callens 2015, Bartges 2016).

The cations that most influence urinary pH are calcium (Ca), magnesium (Mg), sodium (Na) and potassium (K), and the anions are phosphorus (P), sulfur (S), and chlorine (Cl) (Block 1984). Thus, calcium is part of the mineral composition of the diet and is considered a cation of strong alkalizing power, increasing urinary pH by altering BE (Dibartola 2006). Calcium carbonate ($CaCO_3$) is considered a strong dietary alkali (Kienzle et al. 1991), however other chemical forms of calcium, such as calcium sulfate, present cation-anion balance close to zero and may have lower effects on the acid-base status of animals.

Kienzle et al. (1991) and Pastoor et al. (1994a) obtained discrepant results regarding the action of calcium as a urinary alkalinizer. They observed that the addition of $CaCO_3$ raised urinary pH, unlike calcium chloride ($CaCl_2$), which acidified the pH. The acidifying effect of calcium chloride is due to the fact that calcium is less absorbed than chloride and urinary chloride excretion decreases the excretion of bicarbonate and increases ammonium, acidifying the urinary pH (Pastoor et al. 1994a).

Thus, considering that the effect of calcium on urinary pH is apparently influenced by the composition of calcium salts, and based on the need for new approaches and adequate information on the storage potential of each mineral source to make the right decision during cat food formulation, the present study evaluated the efficiency of two calcium sources on alkalinization of urinary pH, as well as excretion of urinary electrolytes and acid-base balance of adult cats.

MATERIALS AND METHODS

Ethics statement. The experiment was conducted at the Laboratory of Research on Nutrition and Nutritional Diseases of Dogs and Cats, São Paulo State University, Jaboticabal, Brazil, in compliance with procedures approved by the Ethics Research Committee for Animal Welfare of the College of Agrarian and Veterinary Sciences, São Paulo State University (protocol number 017648/11).

Animals and experimental design. Eighteen healthy adult mixed-breed cats aged 5±1.4 years and with 3.96±0.71kg of body weight. The experiment was conducted with 2 groups and each group of 9 cats received three diets in a randomized complete block design (3 cats/diet in each group), resulting in 6 cats/diet. The blocking factors were body weight (BW), body condition score (BCS) and age. About 3.21g Ca/kg (+160mEq/kg) of calcium carbonate ($CaCO_3$) or calcium gluconate ($C_{12}H_{22}CaO_{14}$) were added to a dry diet for cats with BE around -140mEq/kg. In the control diet, no calcium source was added. All diets were pelleted and their nutritional level was similar (Table 1), all being within recommendations for adult cat maintenance (AAFCO 2008). Calcium sources were added by replacing corn starch.

Cats belonged to the same laboratory colony, and their health was assessed prior to the beginning of the survey by clinical and hematologic examination, as well as by urine analysis. The animals were kept in individual stainless steel metabolic cages (90x80x90cm) equipped with a system to separate faeces and urine. Cats were fed once daily (8h00) and the metabolizable energy (ME) of diets was calculated from their chemical composition. The amount of food supplied was calculated as 130kcal ME per $kg^{0.4}$ (NRC 2006). Water was provided *ad libitum*, and food was offered *ad libitum* until the next meal. Food intake was daily measured by weighing offered food and refusal amounts.

Urine analysis. Diet adaptation occurred from days 1 to 7, and the following 6 days were used for total urine collection, in two periods. In the first period (days 8-10), urine was collected twice daily in plastic containers with 0.1g of thymol (0.1g per 100mL of urine) and kept refrigerated (4°C). Immediately after collection, urine volume was measured, as well as urine density with a refractometer (model T2-N3, ATAGO CO., LTD, Tokyo, Japan), and pH with a pH meter (model DM20, DIGIMED, São Paulo, Brazil). The urine collected in 24h was maintained frozen. The urine produced in 72h was mixed and evaluated for sodium (mmol/L) and potassium (mmol/L) using an ion-selective method (AVL unit - OMNI4, Roche, Basel, Switzerland) determined by mercuric thiocyanate methodology (Labtest, Minas Gerais, Brazil) followed by spectrophotometry.

In the second period (days 11-13), urine was collected twice daily in plastic containers with HCl 6N (2mL per 100mL of urine) and kept refrigerated (4°C). This measure has been reported to reduce the urinary pH to about 1-2, preventing the formation of crystals (Griyth & Dunn (1978). Calcium (mg/dL), phosphorus (mg/dL), magnesium (mg/dL) and sulfur (g/L) were measured in

Table 1. Ingredient and chemical composition of the experimental diets

	Control	Calcium carbonate	Calcium gluconate
Ingredients (%)			
Commercial diet for cats[a]	96.49	96.49	96.49
Corn starch	3.51	2.70	0
Calcium carbonate[b]	0	0.81	0
Calcium gluconate[c]	0	0	3.51
Chemical composition (%, dry-matter basis)			
Dry matter	91.4	93.5	92.9
Crude Protein	30.09	29.87	30.16
Fat	13.36	13.16	12.58
Crude Fibre	2.56	2.37	2.46
Ash	7.92	8.83	8.83
Calcium	1.53	1.79	1.86
Phosphorus	1.54	1.54	1.50
Magnesium	0.15	0.15	0.15
Sodium	0.54	0.50	0.51
Potassium	0.72	0.68	0.72
Chlorine	0.66	0.65	0.67
Sulphur	0.43	0.42	0.42

[a] Ingredient composition: maize, maize gluten meal, meat meal, wheat bran, soybean meal, fish meal, poultry by-products meal, poultry fat, beet pulp, sodium chloride, flavour, calcium propionate, potassium sorbate, potassium chloride, vitamin and mineral premix; [b] Labsynth produtos para laboratório Ltda, Diadema, Brazil, 98% purity; [c] Jiangxi Xinganjiang, chemical importer Delaware Ltda, Porto Alegre, Brazil, 98% purity.

urine produced in 72h. Calcium quantification was analyzed using colorimetric method (CPC - cresolphthalein, calcium Liquiform, Minas Gerais, Brazil), and magnesium by the colorimetric method (Magon sulfonate, magnesium Liquiform, Minas Gerais, Brazil).

Faecal score. Faecal score was always determined by the same researcher, according to a scale from 1 to 5, as follows: 1 = watery (liquid, can be poured from a container); 2 = soft, unshaped stool (assumes the shape of the container); 3 = soft, shaped, and moist stool; 4 = firm, shaped, dry stool (remains firm and soft); 5 = hard, dry pellets (small, hard masses) (De-Oliveira et al. 2008).

Blood gas analysis. After 13d of diet consumption, 0.5mL of venous blood was collected from the medial saphenous vein without compression, at 08:00 am (before feeding) and 02:00 pm (six hours after feeding). Before blood was collected, the syringe was washed out with a heparin solution, leaving the dead-space filled. Blood was immediately analyzed for pH, sodium, potassium, ionized calcium, partial pressure of carbon dioxide, bicarbonate, base excess and osmolality using Omini C Blood Gas Analyzer (Roche Diagnostics, Indianapolis, USA).

Chemical analysis. Diet samples were analyzed for dry matter (DM), crude protein (CP), crude fiber (CF), mineral matter (MM) and acid-hydrolyzed fat (AHF) according to AOAC (2006). The samples were ground in a cutting mill equipped with 1mm screen and analyzed for DM by submitting samples to oven drying; ash by muffle furnace incineration; and crude protein CP using the Kjeldahl method. Samples were also analyzed for AHF using Soxhlet apparatus, and CF using the Weende method (AOAC 2006). Nitrogen-free extract (NFE) was calculated by subtracting ash, AHF, CP and CF components from DM (NFE = DM-ash-AHF-CP-CF). Minerals were analyzed after nitric-perchloric digestion. Phosphorus was measured with the spectrophotometer using the vanadate-molybdate method. Calcium, potassium, magnesium, chloride, and sodium were measured by flame atomic absorption spectrophotometry.

Statistical analysis. Data were analyzed by the statistical software SAS (version 9.2, SAS Institute Inc., Cary, USA), in a randomised block design by ANOVA, three treatments in two blocks, and the averages of treatments were compared by the SNK test. All analyses were performed using GLM module from the Statigraphics plus 4.1 software. Values of $p \leq 0.05$ were considered significant.

RESULTS

Cats showed average daily food intake of 14.7±1.5g/kg DM/ BW/d, which is considered satisfactory and did not differ among groups. The group fed with added calcium gluconate diet had lower faecal score (P=0.050) and faecal dry matter (P=0.003), and higher venous Na^+ (P=0.049) and osmolality (P=0.008; Table 2).

Daily urinary volume and urine density were not significantly different. Cats fed with diets with added calcium showed increased urine pH and the addition of calcium carbonate in the diet showed greater effect than calcium gluconate on pH, (P<0.001; Table 3).

Cats fed with control diet had increased Na^+ excretion (P=0.018). The group fed with calcium carbonate diet had lower percentage of urinary phosphorus excretion (P=0.044). The addition of calcium in either form did not result in increased excretion of phosphorus in urine (P=0.233; Table 3).

The correlation between urinary pH and Na^+ excretion was negative (R^2=-0.49, P<0.05). Ca^{2+} excretion was correlated only with the Mg^{2+} excretion (R^2=0.49, P<0.05). Chloride excretion (R^2=0.79, P<0.001) was highly correlated with K^+ excretion, as well as K^+ to Na^+ (R^2=0.47, P<0.05; Table 4).

DISCUSSION

Cats fed with diet containing calcium gluconate had lower faecal score (P=0.050) compared to cats that received control diet and diet with added calcium carbonate, which indicates production of faeces with higher moisture content (Table 2). Since there was no difference in urinary volume between groups, the possible explanation for higher water loss in the stool could be the increased osmolality by osmotic active glucose from gluconate. Blood gas analysis showed slight metabolic acidosis in control and calcium gluconate treatments, with pH, HCO_3 and BE below normal values (Dibartola 2006), probably due to low BE of the control diet. However, deviations from normality were minimal and did not differ from calcium carbonate values. Ionized calcium was found below normal blood gas parameters for adult cats in all experimental diets, before and after feeding (Table 2). Hypocalcemia found in this study (reference values of iCa: 1.15 to 1.35; Dibartola 2006), may be explained by the use of heparin in the syringe for blood collection.

Na^+ and osmolality values were higher (P<0.05) in the treatment with calcium gluconate. Hypernatremia could have occurred by the presence of impermeable solutes such as glucose. Initially, the elevation of blood glucose draws intracellular water to the extracellular fluid in order to normalise osmolality, with consequent dilution of sodium and hyponatremia. Subsequently, there may be development of hypernatremia due to osmotic diuresis, which occurs as a

Table 2. Dry matter intake, faecal parameters and ion gas analysis of venous blood of cats

		Control	Calcium carbonate	Calcium gluconate	CV (%)	SEM[a]	p
Dry matter intake (g/kg BW/d)		14.7	14.8	14.7	8.10	1.63	0.991
Faeces							
Score (out of 5)		3.6[a]	3.4[a]	3.1[b]	7.90	0.38	0.050
g/d (moist matter)		10.05	11.34	10.06	11.13	1.12	0.333
g/d (dry matter)		4.06	4.39	3.44	13.59	0.72	0.088
Dry matter, %		40.3[a]	38.7[a]	34.0[b]	4.79	2.84	0.003
Ion and gas analysis of venous blood							
pH (venous)	8h[b]	7.26	7.30	7.26	0.36	0.15	0.154
	14h[c]	7.29	7.27	7.31	0.29	0.16	0.161
PCO_2 (mmHg)	8h	39.4	36.9	38.8	6.77	0.46	0.465
	14h	41.0	40.9	39.2	9.00	0.77	0.776
Na (mmol/L)	8h	159[b]	158[b]	163[a]	1.13	0.04	0.049
	14h	160	159	161	1.50	0.47	0.477
K (mmol/L)	8h	3.60	3.77	3.45	7.57	0.38	0.380
	14h	3.58	3.80	3.45	7.13	0.36	0.278
Cai (mmol/L)	8h	0.78	0.76	0.69	11.46	0.27	0.275
	14h	0.81	0.78	0.77	13.43	0.89	0.893
EB (mmol/L)	8h	-8.6	-7.6	-8.88	21.08	0.63	0.632
	14h	-6.75	-7.56	-6.16	25.29	0.56	0.565
HCO_3 (mmol/L)	8h	17.7	17.6	17.4	9.14	0.91	0.915
	14h	19.4	18.7	19.4	9.84	0.86	0.862
Osm (mOsm/kg)	8h	314[b]	312[b]	321[a]	1.10	0.00	0.008
	14h	316	314	318	1.41	0.47	0.470

Means not sharing the same superscripts are significant different between feeding groups (p<0.05); [a] Standard error of the mean (n=6), [b] before animals fed, [c] 6h after after animals fed.

Table 3. Urine pH, specific gravity, and volume and macroelements intake and excreted by urine of cats

	Control	Calcium carbonate	Calcium gluconate	CV (%)	SEM[a]	P
Urine						
Ph	5.84[c]	6.35[a]	6.18[b]	1.42	0.12	<0.001
Specific gravity, g/dl	1.059	1.051	1.056	0.91	0.01	0.611
Volume, ml/cat/day	55.9	61.9	53.6	34.03	23.26	0.809
Macroelements, mmol/kg BW/d, unless indicated otherwise						
Ca						
Intake	5.63[b]	7.05[a]	7.10[a]	7.59	00.68	0.003
Urinary excretion	0.02	0.01	0.02	22.44	0.009	0.233
% of intake excreted by urine	0.44	0.35	0.23	25.36	0.168	0.119
Mg						
Intake	0.90	0.97	0.92	8.07	0.10	0.448
Urinary excretion	0.13	0.13	0.13	23.79	0.037	0.976
% of intake excreted by urine	14.65	14.05	14.41	21.12	3.54	0.957
Na						
Intake	3.41	3.27	3.18	11.29	0.50	0.070
Urinary excretion	3.36[a]	2.05[b]	1.65[b]	28.31	0.95	0.018
% of intake excreted by urine	97.47[a]	62.34[b]	52.97[b]	25.93	25.87	0.022
K						
Intake	2.69	2.68	2.65	11.28	0.414	0.984
Urinary excretion	2.21	2.52	2.06	17.29	0.66	0.551
% of intake excreted by urine	82.53	93.43	79.61	16.37	21.63	0.596
P						
Intake	7.30	7.82	7.42	7.61	0.79	0.606
Urinary excretion	1.90	1.42	1.68	15.93	0.39	0.197
% of intake excreted by urine	26.48[a]	18.28[b]	22.66[ab]	13.56	4.54	0.044
Cl						
Intake	2.99	2.98	2.95	11.25	0.43	0.988
Urinary excretion	2.56	3.23	2.96	15.51	0.76	0.339
% of intake excreted by urine	85.87	101.1	109.15	15.98	22.44	0.226

Means not sharing the same superscripts are significant different between feeding groups (p<0.05); [a] Standard error of the mean (n=6).

way of removing excess extracellular fluid (Dibartola 2006). Cats fed the diet with calcium gluconate showed no increase in urine volume, but had faeces with higher moisture content. Osmolality, calculated from the Na^+, also increased.

Cats fed control the diet had lower calcium intake ($P<0.05$) due to lower calcium concentration in the diet, showing lower urinary pH, which is consistent with low dietary BE. Cats fed with the diet containing calcium carbonate showed higher urinary pH than cats fed with the diet with added calcium gluconate ($P<0.001$), although both groups had ingested similar amounts of calcium.

Cats that received the diet with calcium gluconate possibly absorbed more Ca^{2+}, but still there was no greater urinary Ca^{2+} excretion. Urine pH was more acidic than in cats fed the diet containing calcium carbonate. Diet containing calcium carbonate may have had less Ca^{2+} absorption, but induced higher urine alkalinization. This difference in the urine alkalization levels is similar to that observed between calcium chloride and calcium carbonate reported by Pastoor et al. (1994a). The authors observed that urine pH was always lower when calcium chloride was added to the diet, while the addition of calcium carbonate increased the urine pH. Kienzle et al. (1991) observed that the addition of calcium carbonate and calcium lactate ($C_6H_{10}CaO_6$) increased urine pH, and calcium chloride acidified urine pH.

Although calcium is absorbed by the intestinal tract, not all is retained in tissues. About 90% of daily Ca^{2+} intake is excreted in faeces, while the remaining is eliminated in urine (Guyton & Hall 1996). Despite being a high-power alkalinizing cation, its absorption is dependent on the associated anion, being more or less absorbed (Allen & Kruger 2000). Renal calcium excretion levels in healthy cats are low and seem to not be strictly correlated with dietary calcium intake (Zentek & Schulz 2004).

Pansu et al. (1993) studied the solubility and intestinal transit time limit of calcium absorption in rats. According to them, calcium carbonate has low solubility, and is preferably absorbed via transcellular in the proximal parts of the intestine, when it becomes slightly soluble in higher pH. Salts with greater solubility such as calcium gluconate will be soluble at higher pH values, which may be absorbed on other portions of the intestine, on calcium paracellular pathway, since it is considered non-saturable.

Passlack & Zentek (2013) investigated the impact of dietary calcium and phosphorus on urine composition of cats and demonstrated that increasing dietary calcium and phosphorus did not affect urinary mineral concentrations and had more influence on PTH levels. An increase in faecal calcium was observed, showing that the homeostatic regulatory process of calcium metabolism in cats seems to be related to the intestinal tract and not to renal excretion. Moreover, PTH enhances blood calcium concentration by increasing intestinal calcium absorption, reducing the renal calcium excretion (Healy et al. 2003).

Jeremias et al. (2013) observed that equations predicting base excess of foods tended to overestimate urine pH and according to the study, they could have some limitations. It is possible that not the entire acid load produced in the intermediary metabolism is related to food macroelements, because differences in absorption of certain acid and alkaline minerals are not taken into account.

Kienzle & Wilms-Eilers (1994) tested diets with ammonium chloride (NH_4Cl) and observed that it suppresses postprandial alkaline tide when combined with calcium carbonate, but this effect can be reversed when it is associated with sodium carbonate (Na_2CO_3). Calcium carbonate is not readily soluble and therefore, it is absorbed more slowly while sodium carbonate is rapidly absorbed, contributing to the postprandial alkaline tide.

Lower urinary calcium excretion was also reported by Pastoor et al. (1994a), who evaluated the balance of daily Ca^{2+} in cats fed with a diet high in Ca^{2+} (13g/kg). The percentage excreted in urine was approximately 0.59% of Ca^{2+} consumed and faecal percentage was around 98% of Ca^{2+} consumed, similar to findings in the present experiment, which found even lower urinary calcium excretion levels.

In the calcium carbonate metabolism in human patients, Ca^{2+} was minimally excreted in the urine, while carbonate contributes to the increased potential of bicarbonate buffer, demonstrating that carbonate is most likely responsible for urine alkalization (Clarkson et al. 1970).

In this study, urinary K^+ and Ca^{2+} excretions were similar to concentrations reported by Lulich et al. (2004). However, Na^+, Mg^{2+} and P excretions were higher in this experiment (Table 3), which may be related to the different amount of macrominerals in the diets.

In the control diet, high Na^+ and phosphorus excretions were observed and pH was negatively correlated with Na^+ (R^2=-0.49, $P<0.05$; Table 4). This can be explained by the acid-base balance, which in an attempt to compensate for the metabolic acidosis, increases the excretion of H^+ ions, which are buffered by HPO_4, forming H_2PO_4, which may be excreted as sodium salt (NaH_2PO_4). Phosphorus excretion in the treatment with calcium gluconate was significantly higher than in the treatment with calcium carbonate, which can also be explained by the buffering of HPO_4.

According to Pastoor et al. (1994b), increased Ca^{2+} intake decreased phosphorus and Mg^{2+} absorption due to the formation of insoluble calcium-magnesium-phosphate complexes (Brink et al. 1992). In this study, there was no difference in urinary Mg^{2+} excretion, but there was lower phosphorus excretion in cats fed with the diet containing calcium carbonate. This may have occurred because calcium carbonate, due to its lower absorption or reexcretion into the intestinal lumen, may have had more time to react with phosphorus, generating complexes, which possibly could have increased its faecal excretion, concomitantly decreasing its urinary excretion. Calcium gluconate, due to its higher and

Table 4. Correlation between the electrolytes excreted in the urine of cats

	pH	Ca	Mg	Na	K	P	Cl
pH	1	NS	NS	-0.49*	NS	NS	NS
Ca		1	0.49*	NS	NS	NS	NS
Mg			1	NS	NS	0.51*	NS
Na				1	0.47*	NS	NS
K					1	NS	0.79**
P						1	NS
Cl							1

NS = Not significant; * $p< 0.05$, ** $p<0.001$.

faster Ca^{2+} absorption, had less time for reactions and plays a different role in urine pH when compared to calcium gluconate.

This study has some limitations that should be considered: the major one was the lack of data regarding digestibility and faecal excretion of minerals, requiring further studies. In addition, Buckley et al. (2011) demonstrated that high water intake influences urinary evaluations in cats by increasing the urine volume, reducing the urine density. The amount of water ingested by cats was not measured in this study. However, no significant differences among evaluations mentioned above were observed.

CONCLUSIONS

The experiment demonstrated that calcium carbonate and gluconate of calcium alkalinized the urine. Calcium carbonate has highest potential than calcium gluconate, which demonstrates that carbonate also contributes to alkalinization, and this action is not exclusive to calcium.

The calcium ion should not be evaluated in isolation, since its presence in urine affects the urinary pH variably according to the anion forming the different Ca salts.

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

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REFERENCES

AAFCO 2008. Association of American Feed Control Officials. Association of American Feed Control Officials, Oxford. 246p.

Allen T.A. & Kruger J.M. 2000. Feline lower urinary tract disease, p.689-723. In: Hand M.S., Thatcher C.D., Remillard R.L. & Roudebush P. (Eds), Small Animal Clinical Nutrition. 4th ed. Mark Morris Institute, Topeka.

AOAC 2006. Official methods of analysis. 18th ed. Association of Official Analytical Chemist, Arlington. 1141p.

Bartges J.W. 2016. Feline calcium oxalate urolithiasis: risk factors and rational treatment approaches. J. Feline Med. Surg. 18(9):712-722. <http://dx.doi.org/10.1177/1098612X16660442> <PMid:27562981>

Bartges J.W. & Callens A.J. 2015. Urolithiasis. Vet. Clin. N. Am., Small Anim. Pract. 45(4):747-768. <http://dx.doi.org/10.1016/j.cvsm.2015.03.001>

Block E. 1984. Manipulating dietary anions and cations for prepartum dairy cows to reduce incidence of milk fever. J. Dairy Sci. 67(12):2939-2948. <http://dx.doi.org/10.3168/jds.S0022-0302(84)81657-4> <PMid:6530489>

Brink E.J., Beynen A.C., Dekker P.R., Van Beresteijn E.C.H. & Van der Meer R. 1992. Interaction of calcium and phosphate decreases ileal magnesium solubility and apparent magnesium absorption in rats. J. Nutr. 122(3):580-586. <http://dx.doi.org/10.1093/jn/122.3.580> <PMid:1542015>

Buckley C.M., Hawthorne A., Colyer A. & Stevenson A.E. 2011. Effect of dietary water intake on urinary output, specific gravity and relative supersaturation for calcium oxalate and struvite in the cat. Brit. J. Nutr. 106(Suppl.1):S128-S130. <http://dx.doi.org/10.1017/S0007114511001875> <PMid:22005408>

Clarkson E., Durrant C., Phillips M., Gower P., Jewkes R. & De Wardener H.E. 1970. The effect of a high intake of calcium and phosphate in normal subjects and patients with chronic renal failure. Clin. Sci. 39(6):693-704. <http://dx.doi.org/10.1042/cs0390693> <PMid:5493940>

De-Oliveira L.D., Carciofi A.C., Oliveira M.C.C., Vasconcellos R.S., Bazolli R.S., Pereira G.T. & Prada F. 2008. Effects of six carbohydrate sources on diet digestibility and postprandial glucose and insulin responses in cats. J. Anim. Sci. 86(9):2237-2246. <http://dx.doi.org/10.2527/jas.2007-0354> <PMid:18469063>

Dibartola S.P. 2006. Fluid Therapy in Small Animal Practice. Philadelphia, Pennsylvania. 611p.

Griyth D.P. & Dunn D. 1978. Collection and preservation of urine for biochemical analyses. Investig. Urol. 15(6):459-461. <PMid:649294>.

Guyton A.C. & Hall J.E. 1996. Tratado de Fisiologia Médica. Guanabara Koogan, Rio de Janeiro. 1176p.

Healy K.D., Zella J.B., Prahl J.M. & DeLuca H.F. 2003. Regulation of the murine renal vitamin D receptor by 1,25-dihydroxyvitamin D3 and calcium. Proc. Natl. Acad. Sci. U.S.A. 100(17):9733-9737. <http://dx.doi.org/10.1073/pnas.1633774100> <PMid:12900504>

Jeremias J.T., Nogueira S.P., Brunetto M.A., Pereira G.T., Loureiro B.A., Ferreira C.S., Gomes M.O.S. & Carciofi A.C. 2013. Predictive formulas for food base excess and urine pH estimations of cats. Anim. Feed Sci. Technol. 182(1/4):82-92. <http://dx.doi.org/10.1016/j.anifeedsci.2013.04.003>

Kienzle E. & Wilms-Eilers S.K. 1994. Struvite diets in cats: effect of ammonium chloride and carbonates on acid base balance of cats. J. Nutr. 124(Suppl.12):2652-2659. <http://dx.doi.org/10.1093/jn/124.suppl_12.2652S>

Kienzle E., Schuknecht A. & Meyer H. 1991. Influence of food composition on the urine pH in cats. J. Nutr. 121(Suppl.11):S87-S88. <http://dx.doi.org/10.1093/jn/121.suppl_11.S87> <PMid:1941250>

Lulich J.P., Osborne C.A., Lekcharoensuk C., Kirk C.A. & Bartges J.W. 2004. Effects of diet on urine composition of cats with calcium oxalate urolithiasis. J. Am. Anim. Hosp. Assoc. 40(3):185-191. <http://dx.doi.org/10.5326/0400185> <PMid:15131098>

Lulich J.P., Berent A.C., Adams L.G., Westropp J.L., Bartges J.W. & Osborne C.A. 2016. ACVIM Small Animal Consensus Recommendations on the Treatment and Prevention of Uroliths in Dogs and Cats. J. Vet. Intern. Med. 30(5):1564-1574. <http://dx.doi.org/10.1111/jvim.14559> <PMid:27611724>

NRC 2006. Nutrient requirements of dogs and cats. National Research Council, National Academy of Science Press, Washington. 424p.

Pansu D., Duflos C., Bellaton C. & Bronner F. 1993. Solubility and intestinal transit time limit calcium absorption in rats. J. Nutr. 123(8):1396-1404. <PMid:8336210>

Passlack N. & Zentek J. 2013. Urinary calcium and oxalate excretion in healthy adult cats are not affected by increasing dietary levels of bone meal in a canned diet. PloS One 8(8):e70530. <http://dx.doi.org/10.1371/journal.pone.0070530> <PMid:23940588>

Pastoor F.J., Opitz R., Van 't Klooster A.T. & Beynen A.C. 1994a. Dietary calcium chloride vs. calcium carbonate reduces urinary ph and phosphorus concentration, improves bone mineralization and depresses kidney calcium level in cats. J. Nutr. 124(11):2212-2222. <http://dx.doi.org/10.1093/jn/124.11.2212> <PMid:7965206>

Pastoor F.J.H., Van 'T Klooster A.T., Mathot J.N.J.J. & Beynen A.C. 1994b. Increasing calcium intakes lower urinary concentration of phosphorus and magnesium in adult ovariectomized cats. J. Nutr. 124(2):299-304. <http://dx.doi.org/10.1093/jn/124.2.299> <PMid:8308580>

Pizzorno J., Frassetto L.A. & Katzinger J. 2010. Diet-induced acidosis: is it real and clinically relevant? Brit. J. Nutr. 103(8):1185-1194. <PMid:20003625>

Zentek J. & Schulz A. 2004. Urinary composition of cats is affected by the source of dietary protein. J. Nutr. 134(Suppl.8):2162S-2165S. <http://dx.doi.org/10.1093/jn/134.8.2162S> <PMid:15284427>

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Original Article
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Incidence of skin tumors in dogs in Salvador, Bahia state, Brazil (2007-2016)[1]

Gessica A.C. Machado[2], Thanielle N. Fontes[2], Daniela F. Larangeira[3], Alessandra Estrela-Lima[3], Eduardo L.T. Moreira[3], Lorena S. Ribeiro[4], Marcela P.R. Pinto[5] and Tiago C. Peixoto[3]*

ABSTRACT.- Machado G.A.C., Fontes T.N., Larangeira D.F., Estrela-Lima A., Moreira E.L.T., Ribeiro L.S., Pinto M.P.R. & Peixoto T.C. 2018. **Incidence of skin tumors in dogs in Salvador, Bahia state, Brazil (2007-2016).** *Pesquisa Veterinária Brasileira 38(11):2139-2145.* 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

This study aimed to establish the incidence of skin tumors (cutaneous proliferative lesions of neoplastic or non-neoplastic nature) in dogs diagnosed by histopathological evaluation at the Veterinary Pathology Laboratory (LPV) of the Federal University of Bahia (UFBA) in a 10-year (2007-2016) historical series. Of the 1945 histopathological diagnoses made in this period, 503 were skin biopsies, and 617 dermatological problems (87 dogs, 17.3%, presented more than one positive diagnosis) were found. Of the 617 diagnoses of dermatopathy, 546 (88.49%) were tumors and 71 (11.51%) were non-tumorous alterations. The 546 conditions more profoundly studied were from 453 dogs, 468 (85.7%) neoplastic and 78 (14.3%) non-neoplastic tumors. The 468 neoplasms were classified as follows: 230 benign (49.14%), 215 malignant (45.94%), 23 borderline (epitheliomas) (4.91%), 51.92% (243/468) mesenchymal, 42.74% (200/468) epithelial, 4.91% (23/468) melanocytic, and 0.43% (2/468) metastatic (mammary gland). The most commonly diagnosed neoplastic dermatopathies were mastocytoma (14.7%) and lipoma (7.48%). Among the 78 non-neoplastic conditions (14.3%), epidermal inclusion cyst (39.74%) and trichogranuloma (15.38%) were the most frequent. Canine dermatopathies accounted for 26% of the biopsy files of the LPV-UFBA. Distinct simultaneous dermatological problems were frequently found in the dogs assessed (one in six). Considering that these conditions can present with different cellular origin and biological behavior, it is crucial that histopathological evaluation be performed in fragments from the different cutaneous lesions.

INDEX TERMS: Skin tumors, dogs, Bahia state, dermatology, dermatopathology, cutaneous neoplasms, pathology.

RESUMO.- [**Frequência de dermatopatias tumorais em cães da cidade de Salvador, Bahia (2007-2016).**] Objetivou-se com esse estudo determinar a frequência de dermatopatias tumorais (lesões proliferativas cutâneas que cursam com aumento de volume de natureza neoplásicas ou não neoplásicas) em cães, diagnosticadas por exame histopatológico no Laboratório de Patologia Veterinária (LPV) da Universidade Federal da Bahia (UFBA) na série histórica de 10 anos (2007-2016). Dos 1.945 exames histopatológicos realizados no período, 503 tratava-se de biópsias cutâneas, dentre os quais, foram diagnosticados 617 dermatopatias (87 cães, 17,3%, apresentavam mais de um diagnóstico). Dos 617 diagnósticos de dermatopatias 546 (88,49%) foram tumorais e 71 (11,51%) não tumorais. As 546 dermatopatias tumorais, estudadas com mais ênfase, foram diagnosticadas em 453 cães, 468 (85,7%) eram neoplásicas e 78 (14,3%) não neoplásicas. Das 468 dermatopatias tumorais neoplásicas encontradas 230 foram benignas (49,14%), 215 malignas

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

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

[4] Autonomous Veterinary, Salvador, BA.

[5] Resident in Veterinary Pathology, Laboratório de Patologia Veterinária (LPV), EMEVZ-UFBA, Salvador, BA 40170-110.

(45,94%), 23 *borderline*/epiteliomas (4,91%), 51,92% (243/468) de origem mesenquimal, 42,74% (200/468) epiteliais, 4,91% (23/468) melanocíticas e 0,43% (2/468) metastáticas para a pele (primárias de glândula mamária). As dermatopatias neoplásicas mais diagnosticadas foram o mastocitoma (14,7%) e o lipoma (7,48%). Dentre as 78 dermatopatias tumorais não neoplásicas (14,3%), os cistos de inclusão epidermal (39,74%) e o tricogranuloma (15,38%) foram os mais frequentes. As dermatopatias caninas representaram 26% da casuística no LPV/UFBA. A ocorrência de dermatopatias tumorais simultâneas distintas foi comum nos cães desse estudo (um a cada seis); como podem ter origens celulares e comportamentos biológicos diferentes, enfatiza-se a importância da coleta e envio para exame histopatológico de fragmentos das diferentes lesões cutâneas.

TERMOS DE INDEXAÇÃO: Dermatopatias, tumores, caninos, Salvador, Bahia, dermatologia, dermatopatologia, neoplasias cutâneas, patologia.

INTRODUCTION

Skin diseases are often diagnosed in domestic animals (Balda et al. 2004), accounting for 20-75% of the cases in routine small animal general practice (Scott et al. 2001). In dogs and cats, tumor diseases can be neoplastic or non-neoplastic in nature, such as cysts, nodular hyperplasia, and hamartomas, that is, the term skin tumor is generically used for proliferative lesions that occur with increased cutaneous volume. There is a wide variety of tumoral and non-tumoral dermatopathies with similar clinical manifestation or macroscopic appearance. This fact underscores the importance of performing a correct diagnostic investigation. Therefore, veterinarians need to know the main diseases that affect animals in their region. In some situations, histopathological examination can establish a definitive diagnosis or, at least, assist clinicians with the exclusion of differential diagnoses. In addition, close contact between pathologists and clinical dermatologists is fundamental, as it enables discussion of the cases and, when necessary, accomplishment of histochemical or immunohistochemical techniques to confirm or exclude a suspicion (Scott et al. 2001).

It is thus evident that the study of the major skin diseases that affect dogs becomes increasingly important for clinicians and pathologists who have an interest in dermatopathology. However, the few existing epidemiological surveys are, for the most part, international (Sischo et al. 1989, Scott & Paradis 1990, Hill et al. 2006) and, in Brazil, studies of this nature in dogs, which encompass the occurrence of skin tumors, have been conducted only in the states of Santa Catarina (Bellei et al. 2006), Rio Grande do Sul (Souza et al. 2006, Meirelles et al. 2010), Paraíba (Andrade et al. 2012), and Ceará (Bastos et al. 2017).

It is worth noting that no studies on this theme have been conducted in the state of Bahia to date. Furthermore, due to the great territorial extension of Brazil and its diverse epidemiological conditions, the few surveys conducted in the aforementioned states may not reflect the real situation of other Brazilian states and municipalities. These facts justified the accomplishment of this retrospective research, which aimed to determine the incidence of skin tumors in dogs diagnosed at the Laboratory of Veterinary Pathology of the Federal University of Bahia (LPV-UFBA) in a 10-year (2007-2016) historical series.

MATERIALS AND METHODS

This study was conducted at the Laboratory of Veterinary Pathology (LPV) of the Federal University of Bahia (UFBA). All skin biopsy protocols (requests and histopathological reports) of dogs with a conclusive diagnosis of neoplastic and non-neoplastic dermatopathies (inclusion criterion) archived at the LPV-UFBA from January 2007 to December 2016 were revised.

Data on breed, sex, age (pups, ≤1 year; adults, >1 year and ≤8 years; elderly, >8 years) and histopathological diagnosis were collected from the biopsy reports revised in this study. Reproductive status (sexually intact or castrated) was not included in this survey because many reports did not present this information.

Data on anatomical location of the tumor lesions were analyzed and the distribution proposed by Goldschmidt & Shofer (1992) was used. Dogs that presented more than one tumor lesion with the same microscopic diagnosis were considered as of multicentric anatomical distribution, whereas dogs that showed more than one skin tumor (multiple tumors) at histopathological diagnosis were analyzed and included in the survey individually within each category.

Cutaneous neoplasms were classified into benign, malignant, or borderline (group composed of epitheliomas) according to biological behavior, and into epithelial, mesenchymal, and melanocytic according to origin and characteristic of cells, based on the classifications by Yager & Wilcock (1994) and Gross et al. (2009). Tumor-like lesions were classified as non-neoplastic proliferative and neoplastic lesions of the primary mammary glands were classified as metastatic to the skin.

Non-neoplastic proliferations were analyzed separately and classified microscopically as cysts, nodular hyperplasia, acrocordon, fibroanal and collagenous hamartoma, organized hematoma, callus pyoderma, trichogranuloma, and nodular cutaneous amyloidosis, based on the terminology proposed by Gross et al. (2009). Because this is a 10-year retrospective study, it should be noted that changes in the nomenclature of some skin tumors have occurred during this period; therefore, in order to standardize and compile the results adequately, old nomenclatures were replaced with their current corresponding terms according to Gross et al. (2009).

The annual activity reports of the LPV-UFBA were also revised from 2007 to 2016 aiming to obtain data on the cases of histopathological biopsy examinations in animals of the canine species. This study was conducted following the norms of animal use and experimentation and was approved by the Animal Ethics Committee (CEUA), College of Veterinary Medicine and Animal Science (EMEVZ), Federal University of Bahia (UFBA), under protocol no. 06/2016.

RESULTS

From January 2007 to December 2016 (a historical series of 10 years), 1945 histopathological examinations of samples from dogs were performed by the pathology teachers who work in the diagnostic service of the LPV-UFBA. Among these cases, 503 were skin biopsies; dermatopathies in dogs accounted for 26% (approximately ¼) of all the histopathological diagnoses issued by the LPV-UFBA for this species in this period. In this period (2007-2016), the annual number of dog skin biopsies received by the LPV-UFBA ranged from 30 to 90, with a mean of 50 cases. Of the 503 cases analyzed, 617 dermatopathies were diagnosed, of which 416 (82.7%) dogs presented only one diagnosis and two or more different skin diseases were observed in the other 87 cases (17.3%), namely, 69 dogs (79.31%) had two diagnoses of dermatopathies, 12 dogs (13.79%) showed three diagnoses, four dogs (4.6%) presented

four diagnoses, one dog (1.15%) had five diagnoses, and one dog (1.15%) showed six diagnoses.

Of the 617 diagnoses of dermatopathy, 546 (88.49%) were tumoral and 71 (11.51%) were non-tumoral. Among the 546 skin tumors, 468 (85.7%) were neoplastic and 78 (14.3%) were non-neoplastic. These 546 tumoral diseases (table 1) were diagnosed in 453 dogs (table 2), which were more comprehensively assessed in this study. Of the 468 neoplasms found, 230 were benign (49.14%), 215 were malignant (45.94%), and 23 were borderline (4.91%) with respect to their biological behavior. Among these, 51.92% (243/468) were mesenchymal, 42.74% (200/468) were epithelial, 4.91% (23/468) were melanocytic, and 0.43% (2/468) were metastatic for the skin (primary mammary gland) according to their origin. Regarding the sex of the 453 animals affected by skin tumors, 0.88% (4/453) of the reports did not present

Table 1. Frequency of 546 tumoral dermatophaties diagnosed in 453 dogs at Laboratório de Patologia Veterinária, Universidade Federal da Bahia, 2007-2016

Tumoral dermatophaties	Sex			Total of cases	%
	Male	Female	NI		
Mastocytoma	40	28	1	69	11.18
Lipoma	9	26		35	5.67
Hemangioma	18	15		33	5.35
Trichoblastoma	18	15		33	5.35
Epidermal inclusion cyst	12	19		31	5.02
Hepatoide gland adenome (perianal)	20	3		23	3.73
Histiocytoma	10	11	1	22	3.57
Melanotic melanoma	9	10		19	3.08
Sebaceous epithelioma	11	7		18	2.92
Hemangiosarcoma	7	10	1	18	2.92
Adenocarcinoma of sweat gland	9	7		16	2.59
Plasmocytoma	9	7		16	2.59
Sebaceous adenocarcinoma	7	7		14	2.27
Squamous cell carcinoma	9	5		14	2.27
Adenocarcinoma of hepatocellular gland (perianal)	9	4		13	2.11
Sebaceous adenoma	7	6		13	2.11
Trichogranuloma	4	9		13	1.94
Fibrosarcoma of the type “Hemangiopericytoma”	5	6		11	1.78
Fibroma	5	5		10	1.62
Fibrosarcoma	4	6		10	1.62
Nodular hyperplasia of sebaceous gland	6	3		9	1.46
Basosquamous carcinoma	6	2		8	1.3
Pilomatricoma	5	3		8	1.3
Papilloma	4	3		7	1.13
Trichoepithelioma	6	1		7	1.13
Apocrine cystadenoma	4	1		5	0.81
Myxosarcoma	2	3		5	0.81
Transmissible venereal tumor (extragenital form)	3	2		5	0.81
Acrocordon	2	2		4	0.65
Apocrine adenoma	1	3		4	0.65
Basal cell carcinoma	1	3		4	0.65
Infundibular keratinizing acanthoma	1	2		3	0.49
Apocrine cyst	1	2		3	0.49
Hepatocellular gland epithelioma (perianal)	2	1		3	0.49
Collagenous hamartoma	2	1		3	0.49
Sebaceous cyst	2			2	0.32
Fibroexamic hamartoma		2		2	0.32
Hematoma in organization	1	1		2	0.32
Hyperplasia of hepatocellular gland (perianal)	1	1		2	0.32
Liposarcoma	2			2	0.32
Melanocytoma	1	1		2	0.32
Amelanotic melanoma	2			2	0.32
Myxoma	1	1		2	0.32
Undifferentiated sarcoma	1	1		2	0.32

NI = Non informed.

Table 1. Continued...

Tumoral dermatophaties	Sex			Total of cases	%
	Male	Female	NI		
Malignant neoplasm of the peripheral nerve sheath	2			2	0.32
Apocrine adenocarcinoma of the anal sac		1		1	0.16
Cerumen adenocarcinoma of the gland		1		1	0.16
Apocrine gland adenoma of the anal sac	1			1	0.16
Nodular cutaneous amyloidosis		1		1	0.16
Meibomian gland carcinoma	1			1	0.16
Ceratogranuloma			1	1	0.16
Follicular cyst		1		1	0.16
Proliferative matrical cyst		1		1	0.16
Basosquamous epithelioma	1			1	0.16
Meibomian gland epithelioma		1		1	0.16
Granuloma associated with leishmaniasis		1		1	0.16
Fungal granuloma	1			1	0.16
Glandular epithelial hyperplasia	1			1	0.16
Metastasis of anaplastic carcinoma of the mammary gland		1		1	0.16
Metastasis of tubular carcinoma of the mammary gland		1		1	0.16
Malignant trichoepithelioma		1		1	0.16
Round cell tumor	286	1	4	1	0.16
TOTAL		256		546	100

NI = Non informed.

Table 2. Fequency of breeds of 453 dogs with tumoral dermatopathies diagnosed at Laboratório de Patologia Veterinária, Universidade Federal da Bahia, 2007-2016

Breed	Total os cases	%
No defined breed	135	29.8
Poodle	85	18.76
American Pit Bull Terrier	30	6.62
English Cocker Spaniel	25	5.52
Siberian Husky	19	4.19
Rottweiler	18	3.97
Pinscher	17	3.75
Dachshund	12	2.65
Boxer	10	2.21
Labrador Retriever	10	2.21
Shih Tzu	10	2.21
Brazilian Terrier	9	1.99
Yorkshire Terrier	9	1.99
American Staffordshire Terrier	8	1.77
Old English Sheepdog	8	1.77
French Bulldog	6	1.32
Schnauzer	5	1.1
Beagle	4	0.88
Golden Retriever	4	0.88
Basset Hound	3	0.66
Deutsche Dogge	3	0.66
Pug	3	0.66
Weimaraner	3	0.66
Akita	2	0.44
Chihuahua	2	0.44
Chow Chow	2	0.44
Brazilian Mastiff	2	0.44
Pekingese	2	0.44
West Highland White Terrier	2	0.44
English Bulldog	1	0.22
Dogo Argentino	1	0.22
Non informed	1	0.22
German Sheperd	1	0.22
St. Bernard	1	0.22
TOTAL	453	100%

this information. Of the 449 dogs whose sex was specified, 243 (53.64%) were males and 206 (45.47%) were females, that is, a male:female ratio of approximately 1.2.

A great diversity was observed with respect to the breeds of the 453 dogs affected by skin tumors (33 different breeds), with 135 (29.8%) mongrels and 317 (69.98%) dogs of defined breed, as shown in table 2. In one case (0.22%), the breed was not informed in the biopsy report. Of the 453 dogs with skin tumors evaluated, 26 (5.74%) did not have their age reported. Of the other 427 (94.26%) dogs that had their age described in the forms, 214 (50.12%) were classified as elderly, 200 (46.84%) were adults, and 13 (3.04%) were pups.

Comparative analysis between age group and skin tumor in the 546 diagnoses of dogs showed the following results: puppies presented higher incidence of histiocytoma (33.3%, 5/15), plasmacytoma (13.3%, 2/15), and mast cell tumor (13.3%, 2/15); adults had higher incidence of hemangioma (7.14%, 17/238), lipoma (6.72%, 16/238), sebaceous epithelioma (6.72%, 16/238), and trichoblastoma (6.72%, 16/238); elderly showed higher incidence of mast cell tumor (10.11%, 26/257), lipoma (7%, 18/257), hepatocellular adenoma (6.6%, 17/257), trichoblastoma (6.22%, 16/257), melanotic melanoma (5,83%, 15/257), and epidermal inclusion cyst (5.83%, 15/257).

As for the anatomical sites where the 468 neoplasms were observed, distribution of incidence and main types of tumors were as follows: 76 (16.2%) in the head (trichoblastoma 21%, sebaceous epithelioma 10.5%, and histiocytoma 7.9%); 44 (9.4%) in the thorax (mast cell tumor 18.2% and lipoma 11.3%); 43 (9.2%) in the abdomen (mast cell tumor 23.2%, hemangiosarcoma 18.6%, and lipoma 16.3%); 42 (9%) in the pelvic limb (mast cell tumor 38.1%); 36 (7.6%) in the perineum (adenocarcinoma of hepatocellular gland 33.3% and mast cell tumor 16.6%); 30 (6.4%) in the thoracic limb (fibrosarcoma 13.3%); 21 (4.5%) in the neck (sweat gland adenocarcinoma 19%); 17 (3.6%) were multicentric (mast cell tumor 47%);

13 (2.8%) on the back (hemangioma 15.4%); 10 (2.1%) in the scrotum (mast cell tumor 50% and plasmacytoma 20%); seven (1.5%) in the tail without predominance of neoplasm.

Regarding non-neoplastic tumors, cysts accounted for 48.71% (38/78) of the total diagnoses. Among these, epidermal inclusion cysts (81.58%, 31/38) presented the highest incidence, and most of them were multicentric (25.8%, 8/31), followed by trichogranulomas (15.38%, 12/78), which occurred mainly in the neck, thoracic limb, or were multicentric with equal incidence in these sites (25% each 3/12), and nodular hyperplasia of the sebaceous gland (11.54%, 9/78), which was mostly multicentric (55.5%, 5/9).

DISCUSSION

There are several studies in the specific scientific literature that emphasize the fact that the skin is the organ most affected by diseases (Scott & Paradis 1990, Scott et al. 2001, Balda et al. 2004), in addition to being one of the main sites for the development of neoplasms (Goorman & Dobson 1995, Brønden et al. 2010). The findings of the present study corroborate the literature: among the 617 dermatopathies diagnosed, skin neoplasms presented the highest incidence (75.85%, 468/617) and were responsible for approximately ¾ of the cases in the time series investigated, which explains why this neoplasia has been one of the most studied in veterinary oncology over the years (Goldschmidt & Shofer 1992).

This high incidence of skin neoplasms is attributed, by some authors, to the fact that the skin is the largest organ of the body and constitutes an important barrier between the organism and the external environment (Jones et al. 2000) because of its exposure to oncogenic factors such as solar radiation (Medleau & Hnilica 2009), its cell renewal capacity, which increases the susceptibility of mutations, and of the increased attention and care provided by owners, which prolongs the life of domestic animals (Bonnett et al. 2005). In contrast, it is worth noting that the high incidence of neoplastic lesions found in this retrospective survey (2007-2016) may be justified by the fact that skin tumors are the main reason for skin biopsy, because other non-tumoral skin diseases are routinely diagnosed during clinical consultation or by other complementary tests.

Souza et al. (2006) conducted a national retrospective study of 40 years (1964-2003) that analyzed the diagnoses of 703 dog skin biopsy reports of the Laboratory of Veterinary Pathology of the Federal University of Santa Maria (LPV-UFSM) and found a mean of 17 skin biopsies per year, which was considered a significant number for the LPV-UFSM according to the authors. In the present study, 26% (503/1945) of the histopathological evaluations of samples from dogs were skin biopsies, that is, approximately ¼ of the cases at the LPV-UFBA in this period refers to dermatopathies, and nearly 50 histopathological examinations of dog skin were performed per year on average. This frequency of diagnoses in the LPV-UFBA routine is also quite significant, considering that most of the samples received come from the Hospital of Veterinary Medicine (Hospmev-UFBA).

With regard to the sex of dogs affected by tumor skin diseases, several studies have shown that males are more affected than females (Kaldrymidou et al. 2002, Bellei et al. 2006, Paranhos 2014); however, no statistically significant difference between the sexes of the dogs assessed was observed in this study, corroborating the findings described by Souza (2005). In contrast, the literature reports that specific types of neoplasms, such as infundibular keratinizing acanthoma (Vail & Withrow 2007) and sebaceous gland carcinoma (Goldschmidt & Hendrick 2008) are more commonly found in male dogs. In the present study, however, females presented infundibular keratinizing acanthoma more often, but this comparison could not be performed significantly because of the small number of diagnosed cases (only three: one in a male dog and two in female dogs). As for sebaceous gland carcinoma, the same number of males (seven) and females (seven) was observed in this study. Regarding hepatocellular neoplasms (39 cases), hepatocellular adenoma presented the highest incidence (59%, 23/39), mainly in males (86.95%, 20/23), with a ratio of approximately 7:1. It is worth mentioning that this type of neoplasia presents marked predisposition in male dogs because of the influence of testosterone on tumor development (Wilson & Hayes Junior 1979, Turek & Withrow 2007, Daleck et al. 2009, North & Banks 2009); however, reproductive status (sexually intact or castrated) could not be assessed in the biopsy reports of this study.

Apparently, the high incidence of dogs with undefined breed (30.56%) with skin tumors found in this retrospective study may be associated with the fact that mongrels represent the majority of the canine population in Brazil (Fonseca 1999, Hataka 2004), as well as to the fact that the population assisted at the Hospmev-UFBA is, in general, of low-income. In contrast, it is known that, currently, many families with good standard of living have made an option to adopt mongrels, which also increases the population of these dogs even in upper-class neighborhoods. Similar findings have been reported in other studies that addressed the incidence of neoplastic lesions in mongrels (Pires et al. 2003, Bellei et al. 2006, Paranhos 2014).

Some pertinent considerations on the age group of all the dogs included in this study are as follows: older dogs presented skin neoplasms more frequently compared with adult dogs, but no statistically significant difference was observed between these two age groups, with an elderly:adult ratio of only 1.07; however, the number of skin tumors in pups (15 cases) was directly influenced by the occurrence of histiocytomas (33.3%, 5/15), which corroborates the data reported by Bellei et al. (2006), considering that histiocytoma is a benign mesenchymal neoplasia more frequently found in dogs aged <2 years (Raskin & Meyer 2003, Cowell et al. 2009, Gross et al. 2009).

Among the neoplastic disorders observed (468), those with benign biological behavior were the most prevalent (230/468, 49.14%), in agreement with the findings described by Bellei et al. (2006) and Souza et al. (2006), but differing from the results found in a study conducted at the Hospital of Veterinary Clinics of the Federal University of Rio Grande do Sul (UFRGS) from January 2008 to December 2012 (Mazzocchin 2013), which described that 62.37% of the skin tumors diagnosed were of malignant biological behavior.

Malignant neoplasms presented fewer diagnoses in this study, but corresponded to 45.94% (215/468) of all neoplastic lesions. In addition, neoplasms classified as borderline accounted for 4.91% (23/468) of the cases, with prevalence of epitheliomas (Gross et al. 2009). Nevertheless, the fact that some of the benign non-neoplastic, pre-neoplastic, or neoplastic proliferative lesions may become malignant

should be considered (Cotran et al. 2000), which reinforces the importance of adoption of early therapeutic measures for each lesion. In this study as a whole, the possibility of neoplasms of benign biological behavior (230) was similar to that of malignant neoplasms (215), with a ratio between them of only 1.07. The following ratios were observed when neoplasms were analyzed separately: 1.04 for benign and malignant mesenchymal tumors, 1.42 for benign and malignant epithelial tumors, and 3.17 for malignant and borderline tumors. As for melanocytic lesions, the occurrence of malignant tumors was 10.5 fold that of benign tumors. In comparison with the findings by Souza (2005), although the incidence of malignant tumors (359) was higher than that of benign tumors (314), the ratio between them was 1.14, a result similar to that observed in the present study; however, this ratio could have been even lower if the tumors had been classified as borderline as in this survey. Conversely, in the study conducted in the municipality of Santa Maria, Rio Grande do Sul state, prevalence of mesenchymal tumors of malignant biological behavior was higher than that of benign tumors, with a ratio of 1.6, whereas in relation to melanocytic tumors, the findings were similar for the higher incidence of malignant tumors, but in Santa Maria the malignant and benign ratio was only 2.5.

Of the 546 skin tumors (88.5%, 546/617) diagnosed in the 503 dogs of this research, 468 (85.7%) were neoplastic and 78 (14.3%) were non-neoplastic, that is, neoplastic proliferative lesions were six times more common than tumor-like lesions. This finding is quite similar to those observed in other retrospective studies (Bostock 1977, Finnie & Bostock 1979, Goldschmidt & Shofer 1992, Yager & Wilcock 1994, Souza 2005).

Of all the skin neoplasms evaluated in this survey (468), those of mesenchymal origin (243) were 1.2 and 10.5 times more common than those of epithelial and melanocytic origins, respectively, and incidence of epithelial tumors was 8.7 fold that of melanocytic lesions. These results corroborate those described by other authors (Bostock 1977, Finnie & Bostock 1979, Goldschmidt & Shofer 1992, Yager & Wilcock 1994, Souza 2005). In addition, melanocytic tumors were 11.5 times more frequent than skin metastases; however, the number of cases of metastatic skin neoplasms was extremely low in this study.

It is of uttermost importance to emphasize that in 87 of the 503 biopsy reports (17.3%) revised in this study more than one concurrent skin disease was observed, with one dog presenting five different dermatopathies. Thus, an extremely varied array of possible combinations (22 ratios) was observed within the classifications used in this study. Consequently, some dogs presented tumoral (neoplastic or not) and non-tumoral lesions at the same time, which resulted in diagnoses with prognoses that varied from good to unfavorable for the same dog. This finding stresses how careful clinicians and surgeons must be when collecting samples of skin lesions (skin biopsies) for histopathological diagnosis. It is essential that, in the presence of a dog with more than one skin tumor of macroscopic appearance similar to each other or not, common sense should always be used and, therefore, a significant number of fragments of different lesions should be collected and separately identified, considering that approximately one in six dogs presented more than one type of dermatopathy in this study.

This recommendation is also reinforced by the fact that mast cell tumor was the most prevalent skin tumor (11.18%) in this survey, considering that in dermatological veterinary clinical practice this tumor has been termed by many authors as "the great imitator" or "tumor of a thousand and one faces" (Murphy 2003), because of its extremely varied macroscopic appearance that can imitate numerous tumoral or non-tumoral skin lesions. Indeed, in the present study, four dogs presented multicentric cutaneous mast cell tumors with different macroscopic aspects, which were identified at histopathological examination as mastocytomas of different histological grades of differentiation. Souza (2005) described mast cell tumors with seven macroscopic patterns, namely, papule, nodule, plaque, mass, cyst, swelling, and "undeveloped feather aspect".

A large number of cases of mast cell tumors were also reported in the research by Bellei et al. (2006). Moreover, Kiupel et al. (2004) stated that mastocytomas account for 7-21% of all skin tumors and Souza et al. (2006) concluded that they are the most prevalent skin tumor in the central region of Rio Grande do Sul state (20.9%, 158/761). In this study, mast cell tumors also presented high incidence: 1.9 fold that of lipoma - the second most frequent neoplasm. In comparison with other studies, mastocytoma was 3.8 times more frequent than the 18 cases reported by Andrade et al. (2012), 3.3 times more prevalent than the 21 cases described by Paranhos (2014), 2.22 fold the 31 cases observed by Mazzocchin (2013), and 1.7 times more frequent than the 39 cases found by De Nardi et al. (2002).

The 78 non-neoplastic tumoral skin diseases accounted for 14.3% of the diagnoses of skin tumors, which may be justified by the high incidence of cutaneous cysts (48.71%, 38/78). According to Pakhrin et al. (2007), epidermal inclusion cyst is one of the five most prevalent skin tumors in dogs in Korea. Fortunately, in spite of its high incidence, this lesion presents a favorable prognosis, and surgical excision is indicated only for esthetics or comfort reasons.

CONCLUSIONS

Skin tumors were observed in one-fourth of the dogs at the LPV-UFBA, with higher incidence of mesenchymal, epithelial and melanocytic neoplasms, in this order.

Mast cell tumor was the most frequent neoplasia in dogs, whereas epidermal inclusion cysts were the most common non-neoplastic skin tumors.

Distinct concurrent dermatological problems were frequently found in the dogs assessed in this study (one in six). Considering that these conditions can present with different cellular origin and biological behavior, it is crucial that histopathological evaluation be performed in fragments from the different cutaneous lesions.

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REFERENCES

Andrade R.L.F.S., Oliveira D.M., Dantas A.F.M., Souza A.P., Nóbrega Neto P.I. & Riet-Correa F. 2012. Tumores de cães e gatos diagnosticados no semiárido da Paraíba. Pesq. Vet. Bras. 32(10):1037-1040. <http://dx.doi.org/10.1590/S0100-736X2012001000016>

Balda A.C., Larsson C.E., Otsuka M. & Gambale W. 2004. Estudo retrospectivo de casuística das dermatofitoses em cães e gatos atendidos no Serviço de Dermatologia da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo. Acta Scient. Vet. 32(2):133-140.

Bastos R.S.C., Farias K.M., Lopes C.E.B., Pacheco A.C.L. & Viana D.A. 2017. Estudo retrospectivo de neoplasias cutâneas em cães da região metropolitana de Fortaleza. Revta Bras. Hig. San. Anim. 11(1):39-53. <http://dx.doi.org/10.5935/1981-2965.20170005>

Bellei M.H.M., Neves D.S., Gava A., Liz P.P. & Pilati C. 2006. Prevalência de neoplasias cutâneas diagnosticadas em caninos no Estado de Santa Catarina, Brasil, no período de 1998 a 2002. Revta Ciênc. Agrovet. 5(1):73-79.

Bonnett B.N., Egenvall A., Hedhammar A. & Olson P. 2005. Mortality in over insured Swedish dogs from 1995 to 2000. Acta Scient. Vet. 46(3):105-120. <http://dx.doi.org/10.1186/1751-0147-46-105> <PMid:16261924>

Bostock D.E. 1977. Neoplasia of the skin and mammary glands in dogs and cats, p.493-505. In: Kirk R.W. (Ed), Current Veterinary Therapy. W.B. Saunders, Philadelphia.

Brønden L.B., Eriksen T. & Kristensen A.T. 2010. Mast cell tumours and other skin neoplasia in Danish dogs: data from the Danish Veterinary Cancer Registry. Acta Vet. Scand. 52(1):6. <http://dx.doi.org/10.1186/1751-0147-52-6> <PMid:20096110>

Cotran R.S., Kumar V. & Collins T. 2000. Neoplasias, p.233-295. In: Ibid. (Eds), Robbins Patologia Estrutural e Funcional. 6ª ed. Guanabara Koogan, Rio de Janeiro.

Cowell R.L., Tyler R.D. & Meinkoth J.H. 2009. Lesões cutâneas e subcutâneas, p.78-111. In: Cowell R.L., Tyler R.D., Meinkoth J.H. & Denicola D.B. (Eds), Diagnóstico Citológico e Hematologia de Cães e Gatos. 3ª ed. Medvet, São Paulo.

Daleck C.R., Rodigheri S.M., De Nardi A.B. & Rodasaki S. 2009. Neoplasias perianais, p.471-479. In: Daleck C.R., De Nardi A.B. & Rodaski S. (Eds), Oncologia em Cães e Gatos. Roca, São Paulo.

De Nardi A.B., Rodaski S., Sousa R.S., Costa T.A., Macedo T.R., Rodigheri S.M., Rios A. & Piekarz C.H. 2002. Prevalência de neoplasias e modalidades de tratamentos em cães, atendidos no Hospital Veterinário da Universidade Federal do Paraná. Arch. Vet. Sci. 7(2):15-26. <http://dx.doi.org/10.5380/avs.v7i2.3977>

Finnie J.W. & Bostock D.E. 1979. Skin neoplasia in dogs. Aust. Vet. J. 55(12):602-604. <http://dx.doi.org/10.1111/j.1751-0813.1979.tb07068.x> <PMid:231964>

Fonseca C.S. 1999. Avaliação dos níveis séricos do estradiol e progesterona em cadelas portadoras de neoplasias mamárias. Dissertação de Mestrado, Faculdade de Ciências Agrárias, Universidade Estadual Paulista, Jaboticabal, SP. 65p.

Goldschmidt M.H. & Shofer F.S. 1992. Skin Tumors of the Dog and Cat. Pergamon, Oxford. 316p.

Goldschmidt M.H. & Hendrick M.J. 2008. Tumors of the skin and soft tissues, p.44-117. In: Meuten D.J. (Ed), Tumors in Domestic Animals. 4th ed. Iowa State Press, Ames. <http://dx.doi.org/10.1002/9780470376928.ch2>.

Goorman N.T. & Dobson J.M. 1995. The skin and associated tissues, p.187-200. In: White R.A.S. (Ed), Manual of Small Animal Oncology. British Small Animal, Shurdington.

Gross T.A., Ihrke P.J., Walder E.J. & Affolter V.K. 2009. Doenças de Pele do Cão e do Gato: diagnóstico clínico e histopatológico. 2ª ed. Roca, São Paulo. 904p.

Hataka A. 2004. Citologia aspirativa com agulha fina e histopatologia: valor e significado para diagnóstico e prognóstico do câncer de mama em cadelas. Tese de Doutorado, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, São Paulo. 68p.

Hill B.P., Lo A., Eden C.A., Huntley S., Morey V., Ramsey S., Richardson C., Smith D.J., Sutton C., Taylor M.D., Thorpe E., Tidmarsh R. & Williams V. 2006. Survey of the prevalence, diagnosis and treatment of dermatological conditions in small animals in general practice. Vet. Rec. 158(16):533-539. <http://dx.doi.org/10.1136/vr.158.16.533> <PMid:16632525>

Jones T.C., Hunt R.D. & King N.W. 2000. Patologia Veterinária. 6ª ed. Manole, São Paulo. 1415p.

Kaldrymidou H., Leontides L., Koutinas A.F., Saridomichelakis M.N. & Karayannopoulou M. 2002. Prevalence, distribution and factors associated with the presence and potential for malignancy of cutaneous neoplasms in 174 dogs admitted to a clinic in Northern Greece. J. Am. Vet. Med. Assoc. 49(2):87-91. <http://dx.doi.org/10.1046/j.1439-0442.2002.jv408.x> <PMid:11958472>

Kiupel M., Webster J.D., Kaneene J.B., Miller R. & Yuzbasiyan-Gurkan V. 2004. The use of Kit and Tryptase expression patterns as prognostic tools for canine cutaneous mast cell tumors. Vet. Pathol. 41(4):371-377. <http://dx.doi.org/10.1354/vp.41-4-371> <PMid:15232137>

Mazzocchin R. 2013. Neoplasias cutâneas em pele. Monografia, Universidade Federal do Rio Grande do Sul, Porto Alegre. 64p.

Medleau L. & Hnilica K.A. 2009. Dermatologia de Pequenos Animais: atlas colorido e guia terapêutico. 2ª ed. Roca, São Paulo. 512p.

Meirelles A.E.W.B., Oliveira E.C., Rodrigues B.A., Costa G.R., Sonne L., Tesser E.S. & Driemeier D. 2010. Prevalência de neoplasmas cutâneos em cães da Região Metropolitana de Porto Alegre, RS: 1.017 casos (2002-2007). Pesq. Vet. Bras. 30(11):968-973. <http://dx.doi.org/10.1590/S0100-736X2010001100011>

Murphy S. 2003. Mast cell tumors, p.161-167. In: Dobson J.M. & Lascelles B.D.X. (Eds), BSAVA Manual of Canine and Feline Oncology. 2nd ed. BSAVA, Gloucester.

North S. & Banks T. 2009. Tumors of skin and subcutaneous tissues, p.94-125. In: Ibid. (Eds), Introduction to Small Animal Oncology. Saunders Elsevier, London.

Pakhrin B., Kang M., Bae I., Park M., Jee H., You M., Kim J., Yoon B., Choi Y. & Kim D. 2007. Retrospective study of canine cutaneous tumors in Korea. J. Vet. Sci. 8(3):229-236. <http://dx.doi.org/10.4142/jvs.2007.8.3.229> <PMid:17679768>

Paranhos C.A. 2014. Neoplasias cutâneas caninas: um estudo descritivo de quatro anos. Dissertação de Mestrado, Universidade de Trás-os-Montes e Alto Douro, Vila Real. 77p.

Pires I., Oliveira J., Queiroga F. & Alves A. 2003. Doenças hístiocíticas do cão, p.14-29. In: Ibid. (Eds), Série Didáctica. Universidade de Trás-os-Montes e Alto Douro, Vila Real.

Raskin R.E. & Meyer D.J. 2003. Atlas de Citologia de Cães e Gatos. Roca, São Paulo. 354p.

Scott D.W. & Paradis M. 1990. A survey of canine and feline skin disorders seen in a university practice: Small Animal Clinic, University of Montreal, Saint-Hyacinthe, Quebec (1987-1988). Can. Vet. J. 31(12):830-835. <PMid:17423707>

Scott D.W., Miller D.H., Griffin C.E. & Muller K. 2001. Small Animal Dermatology. 6th ed. W.B. Saunders, Philadelphia. 1528p.

Sischo W.M., Ihrke P.J. & Franti C.E. 1989. Regional distribution of ten common skin diseases in dogs. J. Am. Vet. Med. Assoc. 195(6):752-756. <PMid:2676928>

Souza T.M. 2005. Estudo retrospectivo de 761tumores cutâneos em cães. Dissertação de Mestrado, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Santa Maria, RS. 296p.

Souza T.M., Fighera R.A., Irigoyen L.F. & Barros C.S.L. 2006. Estudo retrospectivo de 761 tumores cutâneos em cães. Ciência Rural 36(2):555-560. <http://dx.doi.org/10.1590/S0103-84782006000200030>

Turek M.M. & Withrow S.J. 2007. Perianal tumors. p.503-508. In: Vail D.M. & Withrow S.J. (Eds), Small Animal Clinical Oncology. 4th ed. W.B. Saunders, Missouri.

Vail D.M. & Withrow S.J. 2007. Tumors of the skin and subcutaneous tissues, p.233-260. In: Withrow S.J. & Macewen E.G. (Eds), Small Animal Clinical Oncology. 4th ed. W.B. Saunders, Missouri. <http://dx.doi.org/10.1016/B978-072160558-6.50021-6>.

Wilson G.P. & Hayes Junior H.M. 1979. Castration for treatment of perianal gland neoplasms in the dog. J. Am. Vet. Med. Assoc. 174(12):1301-1303. <http://dx.doi.org/10.2460/javma.246.12.1301> <PMid:511727>

Yager J.A. & Wilcock B.P. 1994. Color Atlas and Text of Surgical Pathology of the Dog and Cat: dermatopathology and skin tumors. Wolfe, London, p.239-313.

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Original Article
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Epidemiological and clinical aspects of ophidian bothropic accidents in dogs[1]

Laís G. Silva[2], Welden Panziera[3], Carlos A.S. Lessa[4] and David Driemeier[3]*

ABSTRACT.- Silva L.G., Panziera W., Lessa C.A.S. & Driemeier D. 2018. **Epidemiological and clinical aspects of ophidian bothropic accidents in dogs.** *Pesquisa Veterinária Brasileira 38(11):2146-2149*. Setor de Patologia Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, prédio 42505, Porto Alegre, RS 91540-000, Brazil. E-mail: davetpat@ufrgs.br

Snakebites are included in the group of emergencies for domestic animals, and these consultations demand technical knowledge as well as careful clinical evaluation of patients. Because of the importance of this theme and the higher prevalence of snakebites caused by snakes of the genus *Bothrops* in Rio Grande do Sul state, Brazil, this study aimed to address the epidemiological and clinical aspects of these accidents in dogs in the state, in addition to establishing their incidence. Clinical records of dogs, with a diagnosis of ophidian bothropic accident, assisted at the Toxicological Information Center of Rio Grande do Sul (CIT-RS) were revised from 2014 to 2016. These data were collected at the Toxicological Information Center, Statistics and Evaluation Center, State Secretary of Health. Data on the incidence of accidents and epidemiological and clinical information were obtained for each case. A total of 53 records were revised. In the records that included animal sex (n=49), 53% were female and 46.9% were male. As for animal age (n=47), the dogs ranged from one to 14 years, and most of them (46.9%) were in the 1-4-year age group. Over half of the cases occurred in the rural area (60.7%), and the head and neck were the main venom inoculation sites (76.3%). Diseases were characterized by clinical courses varying from hyperacute (<30 min to 6h), acute (6-24h), to subacute (4-5 days). Severe accidents accounted for 40% of the cases, with edema as the most frequent clinical sign (88.7%) followed by hemorrhagic manifestations (41.5%). Associations of epidemiological and clinical aspects coupled with history of exposure are important characteristics to assist with suspicion and definitive diagnosis of bothropic accidents in dogs.

INDEX TERMS: Canine diseases, ophidian accidents, *Bothrops* spp., Rio Grande do Sul, toxicoses.

RESUMO.- [**Aspectos epidemiológicos e clínicos dos acidentes ofídicos botrópicos em cães.**] Os acidentes ofídicos se enquadram no grupo de emergências para animais domésticos e esses atendimentos demandam conhecimento técnico, aliado a uma criteriosa avaliação clínica do paciente. Devido à importância do tema e a maior frequência de acidentes ofídicos provocados por serpentes do gênero *Bothrops* no Rio Grande do Sul, esse estudo teve como objetivo abordar os aspectos epidemiológicos e clínicos desses acidentes em cães nesse estado, além de estabelecer a frequência em que ocorrem. Foram revisados os protocolos de atendimentos clínicos de cães realizados pelo Centro de Informações Toxicológicas do Rio Grande do Sul (CIT-RS) com diagnóstico de acidente ofídico botrópico, entre o período de 2014 e 2016. Esses dados fazem parte do Centro de informação Toxicológica, Secretaria da Saúde, RS, Núcleo de Estatística e Avaliação. Foram obtidos dados relacionados à frequência dos acidentes e informações referentes à epidemiologia e à clínica de cada caso. Totalizaram-se 53 registros de atendimentos. Dos protocolos que informaram o sexo (n=49), 53% corresponderam a fêmeas e 46,9%, a machos. Em relação

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[2] Veterinary Medicine student, Faculdade de Veterinária (Favet), Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil. Intern, Centro de Informações Toxicológicas do Rio Grande do Sul (CIT-RS), Av. Ipiranga 5400, Jardim Botânico, Porto Alegre, RS 90610-000, Brazil.

[3] Setor de Patologia Veterinária, Departamento de Patologia Clínica Veterinária, Favet-UFRGS, Av. Bento Gonçalves 9090, prédio 42505, Porto Alegre, RS 91540-000. *Corresponding author: davetpat@ufrgs.br

[4] Veterinarian, coordinator of statistical and evaluation core, CIT-RS, Av. Ipiranga 5400, Jardim Botânico, Porto Alegre, RS 90610-000.

à idade (n=47), houve uma variação entre um e 14 anos, com maior concentração dos casos entre 1-4 anos (46,9%). Mais da metade dos atendimentos ocorreu em zona rural (60,7%) e a cabeça e o pescoço foram os principais locais de inoculação do veneno botrópico (76,3%). Observou-se uma doença com um curso que variou de hiperagudo (<30 min à 6h), agudo (6-24h) a subagudo (4-5 dias). Acidentes graves representaram 40% dos casos, no qual edema foi o sinal clínico mais frequente (88,7%), seguido por manifestações hemorrágicas (41,5%). A associação dos aspectos epidemiológicos e clínicos, aliados ao histórico de exposição, constituem características importantes que auxiliam na suspeita e no diagnóstico definitivo do acidente botrópico em animais.

TERMOS DE INDEXAÇÃO: Doenças de caninos, acidentes ofídicos, *Bothrops* spp., Rio Grande do Sul, toxicoses.

INTRODUCTION

Snakebite accidents are common in humans and domestic animals in different parts of the world, and are associated with a number of factors including climate, ecological parameters, biodiversity, distribution of venomous snakes, population density, economic activities and types of dwellings. In addition, severity of the accidents varies according to the snake species involved and the amount of venom inoculated (Otero-Patiño 2009). In Brazil, accidents caused by snakes of the genus *Bothrops* and *Crotalus* are common (Amaral et al. 1985, Tokarnia & Peixoto 2006, Nogueira 2011, Blanco & Melo 2014, Tokarnia et al. 2014) and, more seldom, of the genus *Lachesis* and *Micrurus* (Blanco & Melo 2014). In Rio Grande do Sul state, bothropic accidents are predominant, and are usually associated with the species *Bothrops jararaca* (jararaca), *Bothrops neuwiedi* (jararaca-pintada), and *Bothrops alternatus* (cruzeira) (Tokarnia & Peixoto 2006, Dallegrave & Sebben 2008, Herrera 2009). Owing to the proteolytic, coagulant and hemorrhagic action of bothropic venom, hemostatic disorders are the main clinical signs observed (Tokarnia & Peixoto 2006, Otero-Patiño 2009).

Snakebites are included in the group of emergencies for domestic animals, and these consultations demand technical knowledge as well as careful clinical evaluation of patients. Because of the importance of this theme and the higher prevalence of snakebites caused by snakes of the genus *Bothrops* in Rio Grande do Sul state, Brazil, this study aimed to address the epidemiological and clinical aspects of these accidents in dogs in the state, in addition to establishing their incidence.

MATERIALS AND METHODS

Clinical records of dogs, with a diagnosis of ophidian bothropic accident, assisted at the Toxicological Information Center of Rio Grande do Sul (CIT-RS) were revised from 2014 to 2016. These data were collected at the Toxicological Information Center, Statistics and Evaluation Center, State Secretary of Health. Data on the incidence of accidents and epidemiological and clinical information were obtained from each case. Epidemiological aspects included sex, age, year of occurrence, and area of exposure (rural or urban). Clinical aspects were based on the history described by veterinarian in the reports. In addition, severity of the accidents, most frequent site of the bites, and existence of previous clinical care were assessed.

RESULTS

From 2014 to 2016, 53 bothropic ophidian accidents (*Bothrops* spp.) involving dogs were assisted at the CIT-RS. The canids involved were from cities of different regions of Rio Grande do Sul state and, in all cases, diagnosis was based on identification of snakes of the genus *Bothrops* or presumptively on the clinical signs presented. Of the 49 records that included animal sex, 53% were female and 46.9% were male. Age of the animals was informed in 47 records, and ranged from <1 to 14 years. Age distribution of the affected dogs was as follows: 23 (46.9%), 1-4 years; 18 (36.7%), 4-9 years; three (6.3%), 10-14 years; three (6.3%), <1 year; two (4.25%), 15-19 years.

As for the annual distribution of the 53 consultations, 26 (49%) occurred in summer, 16 (30.1%) in autumn, eight (15.1%) in spring, and three (5.6%) in winter. Of the cases with information about the area of exposure (51/53), most of the accidents occurred in the rural area (60.7%), followed by the urban area (39.2%). Regarding medical assistance (50/53), 28 (56%) patients were in a clinic or hospital when they were referred to the CIT-RS, 19 (38%) received first aid in the place where they lived, two (4%) were in a veterinary's office or outpatient clinic, and one (2%) was under pre-hospital care on the way to veterinary assistance.

Of the records with information about location of the snakebite (38/53), 37 dogs showed only one venom inoculation site and one dog had more than one site (2.6%). The head and neck accounted for most of the cases (76.3%), followed by the pelvic (10.5%) and thoracic limbs (5.2%). Sites such as the chest, abdomen, and back represented one case each (5.2%).

Fifty of the 53 records provided descriptions of clinical manifestations. Disease was characterized by clinical courses varying from hyperacute (<30 min to 6h), acute (6-24h), to subacute (4-5 days). Of the 50 cases, 20 (40%) were considered severe accidents and seven of them evolved to death. Accidents were considered severe when marked edema and hemorrhage with occasional necrosis at the bite site were observed (Fig.1 and 2). Moderate accidents (moderate edema and bleeding at the bite site) accounted for 32% of the cases, whereas mild accidents (mild edema at the bite site) totaled 28%. In all cases of mild and moderate accidents, the dogs recovered completely. Edema at the bite site and



Fig.1. Ophidian bothropic accident in dog. Marked increase in submandibular and labial volume (edema) is observed. Two hemorrhagic spots corresponding to the venom inoculation site are visualized adjacent to the labial commissure.



Fig.2. Ophidian bothropic accident in dog. The dog shows marked submandibular edema. The labial commissure also presents a hemorrhagic spot corresponding to venom inoculation.

adjacent tissue was the most frequently observed clinical sign (88.7%), varying from mild to severe. Associated with edema, 22 (41.5%) dogs presented hemorrhagic manifestations such as bleeding at the bite site (68.2%), ecchymosis (18.2%), epistaxis (4.5%), gingival hemorrhage (4.5%), and hyphema (4.5%). Other clinical signs included apathy (13.2%), pain (11.3%), necrosis at the bite site (3.8%), and hyperthermia (3.8%). Hypothermia, anuria, and cardiorespiratory arrest were reported in only one case (1.9%).

DISCUSSION

Associations of epidemiological and clinical aspects coupled with history of exposure are important characteristics to assist with suspicion and definitive diagnosis of bothropic accidents in dogs (Méndez & Riet-Correa 2007). All species of domestic animals are affected by the venom of vipers of the genus *Bothrops*, but sensitivity varies between them. The domestic animal species most susceptible to this venom, in descending order, are equines, sheep, bovines, goats, canines, swine, and felines (Blanco & Melo 2014). Despite the intermediate resistance, dogs, especially young, occupy the first positions among the domestic species involved in ophidian accidents caused by snakes of the genus *Bothrops* (Méndez & Riet-Correa 2007, Nogueira 2011, Barni et al. 2012, Blanco & Melo 2014). In this study, over half of the consultations recorded were with young canids, and this can be attributed to the inquisitive nature of this age group, associated with active behavior with habitat exploration through smell and the mouth (Méndez & Riet-Correa 2007, Heckler et al. 2010, Nogueira 2011, Blanco & Melo 2014). Moreover, accidents are facilitated by the aggressive behavior of snakes of this genus, even though they present the habit of staying still and camouflaged when not disturbed (Pereira 2006, Méndez & Riet-Correa 2007).

Occurrence of ophidian accidents is also directly associated with the activity of the snakes, which increases when they are in search of food, mating, a place to spawn, and control of their body temperature. Low temperatures reduce their metabolism and, consequently, their activity (Blanco & Melo 2014). These epidemiological aspects justify the higher incidence of accidents observed in summer in the dogs of this study and corroborate with the observation of other authors, who also reported a larger number of cases in this season (Méndez & Riet-Correa 2007, Nogueira 2011, Blanco & Melo 2014).

In the present study, over 60% of the ophidian bothropic accidents occurred in rural settings, which shows a relation between the natural habitat of these vipers and the presence of food (Boff 2005, Méndez & Riet-Correa 2007, Nogueira 2011, Blanco & Melo 2014). Serpents inhabit mainly rural areas, where they prefer humid environments such as forests, areas with crops and high vegetation, and places where rodents proliferate easily, such as barns, abandoned buildings, firewood deposits, etc. Although most of the cases in this study occurred in rural areas, it is suggested that the significant number of consultations in urban areas (approximately 40%) is associated with sites with accumulation of food waste (garbage and grains), which attracts rodents that serve as prey for these snakes. In addition, the advancement of cities onto less populated areas and regions with ecological imbalance associated with the absence of predators promotes an increase in the ophidian population and, consequently, in the number of accident cases (Boff 2005, Méndez & Riet-Correa 2007).

Head and neck corresponded to the anatomical regions most affected by ophidian bothropic accidents in the dogs of this study, and they are sites that may denote stronger severity to the case. These sites are frequently reported in the literature, and are associated with the habit of dogs of trying to immobilize the snakes with the mouth, either as a jest or an attack (Méndez & Riet-Correa 2007). Unlike the dogs of the present study, lesions in cattle and horses are predominant in the limbs and abdomen (Tokarnia & Peixoto 2006, Méndez & Riet-Correa 2007). When the snakebite occurs in the head and neck region, there is formation of edema and hemorrhage, sometimes severe, in the spot and adjacent tissue, with occasional progression to the thoracic region. In these cases, the edema obstructs the upper airways, causing dyspnea and edema of glottis, which constitute serious secondary complications of these accidents, requiring an emergency tracheostomy (Nogueira 2011, Blanco & Melo 2014, Cintra et al. 2014).

Severity of the clinical condition depends on the volume of inoculated venom, the size of the injured dog, the time elapsed between the accident and appropriate treatment, and the bite site (Blanco & Melo 2014). In accordance with the literature (Azevedo-Marques et al. 2003), the accidents of the present study were classified into severe, moderate, and mild. Severe cases were more representative (40%), with seven dogs evolving to death. In contrast, dogs with mild and moderate symptoms showed improvement. The local clinical signs emerge shortly after the bite, and are characterized by intense pain, edema, and hemorrhage (Azevedo-Marques et al. 2003, Tokarnia & Peixoto 2006, Méndez & Riet-Correa 2007, Nogueira 2011, Blanco & Melo 2014, Tokarnia et al. 2014). Corroborating the literature, edema and hemorrhagic disorders were the most evident and frequent clinical manifestations observed in the dogs of this study, and they are important clinical features to assist with suspicion of bothropic poisoning. These manifestations, associated with tissue necrosis at the bite site, are due to the action of bothropic venom, which presents proteolytic or

necrotizing, anticoagulant, and vasculotoxic (hemorrhagic) properties (Amaral et al. 1985, Barbosa et al. 2002, Méndez & Riet-Correa 2007, Nogueira 2011, Blanco & Melo 2014).

Hemorrhagic changes found in approximately 40% of the cases in this study are often observed in bothropic accidents. These changes may be local or systemic, and are caused by activation of the enzymes hemorrhages that cause lesions in the basement membrane of the capillaries, associated with thrombocytopenia and coagulation disorders (Tokarnia & Peixoto 2006, Méndez & Riet-Correa 2007, Blanco & Melo 2014). The proteolytic action of bothropic venom is another important characteristic that is directly related to necrotic lesions clinically observed at the bite site. This change is due to activation of proteases, hyaluronidases, and phospholipases, as well as to release of inflammatory response mediators such as bradykinin, prostaglandins and leukotrienes. Activation of these enzymes and release of these mediators cause destruction in the bite site and adjacent tissue, and are accompanied by pain, blushing, local or regional edema, and tissue necrosis (Tokarnia & Peixoto 2006, Méndez & Riet-Correa 2007, Puzzi et al. 2008, Nogueira 2011, Blanco & Melo 2014). The deaths of the seven dogs in this study may be associated with severe systemic changes, such as hypovolemic shock, extensive bleeding, respiratory arrest, or acute renal insufficiency (Blanco & Melo 2014).

It is important to differentiate ophidian bothropic accidents from other conditions. Among these conditions, the most important are allergic and toxic (especially poisoning by ornamental plants) diseases and accidents caused by other poisonous or venomous animals such as bees, frogs, spiders, and scorpions (Méndez & Riet-Correa 2007, Nogueira 2011, Blanco & Melo 2014). Accidents caused by bees or other insects can produce severe edema and represent important differentials (Nogueira 2011). In the present study, these differential diagnoses were discarded owing to absence of epidemiological evidence.

CONCLUSIONS

Dogs aged 1-4 years are the most predisposed to ophidian bothropic accidents in Rio Grande do Sul state.

The accidents occur predominantly in rural settings during summer, the head and neck are the most common sites of venom inoculation.

Clinically, ophidian bothropic accidents occur acutely and severely, with specific clinical presentation as early as the first few hours after the bite.

Edema and hemorrhages were the main clinical manifestations observed in the canine reports, followed by apathy and pain.

The association of epidemiological and clinical findings provides good subsidies for the diagnosis of ophidian bothropic accident in dogs.

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REFERENCES

Amaral C.F.S., Silva O.A., Goody P. & Miranda D. 1985. Renal cortical necrosis following *Bothrops jararaca* and *B. jararacussu* snake bite. Toxicon 23(6):877-885. <http://dx.doi.org/10.1016/0041-0101(85)90379-4> <PMid:4095703>

Azevedo-Marques M.M., Palmira C. & Hering S.E. 2003. Acidentes por animais peçonhentos: serpentes peçonhentas. Medicina 36:480-489.

Barbosa P.S.F., Havt A., Facó P.E.G., Sousa T.M., Bezerra I.S.A.M., Fonteles M.C., Toyama M.H., Marangoni S., Novello J.C. & Monteiro H.S.A. 2002. Renal toxicity of *Bothrops moojeni* snake venom and its main myotoxins. Toxicon 40(10):1427-1435. <http://dx.doi.org/10.1016/S0041-0101(02)00156-3> <PMid:12368112>

Barni B.S., Mottin I.B., Vidor S.B., Albuquerque P.B. & Contesini E.A. 2012. Incidência e perfil dos animais atendidos devido a acidente ofídico no Hospital de Clínicas Veterinárias da Universidade Federal do Rio Grande do Sul entre os anos de 2005 e 2010. Acta Scient Vet. 40(Suppl.1):1-60.

Blanco B.S. & Melo M.M. 2014. Acidente botrópico. Cad. Tec. Vet. Zootec. 75:1-77.

Boff G.J. 2005. Envenenamento por picada de serpente, gênero *Bothrops*: revisão. Vet. Foco 2(2):121-135.

Cintra C.A., Paulino Junior D., Dias L.G.G.G., Pereira L.F. & Dias F.G.G. 2014. Acidentes ofídicos em animais domésticos. Encicl. Biosf. 10(18):58-61.

Dallegrave E. & Sebben V.C. 2008. Toxicologia clínica: aspectos teórico-práticos, p.145-210. In: González H.D. & Silva S.C. (Eds), Patologia Clínica Veterinária: texto introdutório. Universidade Federal do Rio Grande do Sul, Porto Alegre.

Heckler M.C.T., Santos F.H. & Amorim R.M. 2010. Teste para avaliação das funções cognitivas de memória e aprendizado em cães. Neurobiologia 73(4):141-149.

Herrera M.S. 2009. Acidente com serpente do gênero *Bothrops* em cão: relato de caso. Revta Cient. Eletrôn. Med. Vet. 12:1-6.

Méndez M.L. & Riet-Correa F. 2007. Envenenamento botrópico, p.31-38. In: Riet-Correa F., Schild A.L., Lemos R.A.A. & Borges J.R. (Eds), Doenças de Ruminantes e Equídeos. Vol.2. 3ª ed. Pallotti, Santa Maria. 692p.

Nogueira R.M.B. 2011. Lagartas e serpentes, p.295-315. In: Nogueira R.M.B & Andrade S.F. (Eds), Toxicologia Veterinária. Roca, São Paulo. 323p.

Otero-Patiño R. 2009. Epidemiological, clinical and therapeutic aspects of *Bothrops asper* bites. Toxicon 54(7):998-1011. <http://dx.doi.org/10.1016/j.toxicon.2009.07.001> <PMid:19591857>

Pereira M.T. 2006. Acidente botrópico em cães. Tese de Especialização em Clínica Médica e Cirúrgica em Pequenos Animais, Universidade Castelo Branco, Campo Grande. 46p.

Puzzi M.B., Vicarivento N.B., Xavier A., Polizer K.A., Neves M.F. & Sacco S.R. 2008. Acidentes ofídicos. Revta Cient. Eletrôn. Med. Vet. 6(10):1-7.

Tokarnia C.H. & Peixoto P.V. 2006. A importância dos acidentes ofídicos como causa de morte de bovinos no Brasil. Pesq. Vet. Bras. 26(2):55-68. <http://dx.doi.org/10.1590/S0100-736X2006000200001>

Tokarnia C.H., Brito M.F., Barbosa J.D. & Döbereiner J. 2014. Quadros clínico-patológicos do envenenamento ofídico por *Crotalus durissus terrificus* e *Bothrops* spp. em animais de produção. Pesq. Vet. Bras. 34(4):301-312.

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Antimicrobial susceptibility and diarrheagenic diagnosis of *Escherichia coli* and *Salmonella enterica* isolated from feral pigeons (*Columba livia*) captured in Fortaleza, Brazil [1]

Ruben V. Horn[2]*, Windleyanne G.A. Bezerra[2], Elisângela S. Lopes[2], Régis S.C. Teixeira[2], Isaac N.G. Silva[2], Mariana D. Bona[3], Alexandre Havt[3] and William M. Cardoso[2]

ABSTRACT- Horn R.V., Bezerra W.G.A., Lopes E.S., Teixeira R.S.C., Silva I.N.G., Bona M.D., Havt A. & Cardoso W.M. 2018. **Antimicrobial susceptibility and diarrheagenic diagnosis of *Escherichia coli* and *Salmonella enterica* isolated from feral pigeons (*Columba livia*) captured in Fortaleza, Brazil.** *Pesquisa Veterinária Brasileira 38(11):2150-2154*. Laboratório de Estudos Ornitológicos, Faculdade de Veterinária, Universidade Estadual do Ceará, Av. Paranjana 1700, Fortaleza, CE 60740-000, Brazil. E-mail: rubenhorn@hotmail.com

This study aimed to isolate *Escherichia coli* and *Salmonella enterica* from captured feral pigeons in Fortaleza, Brazil, and, in addition to evaluate the antimicrobial susceptibility profiles and diagnose diarrheagenic *E. coli* strains. Pigeons were captured in four public locations in Fortaleza with three techniques. Individual cloacal swab samples were collected and submitted to bacterial isolation, biochemical identification and antimicrobial susceptibility test. Disk diffusion technique was used with twelve antibiotics. *E. coli* strains were submitted to DNA extraction followed by PCR to diagnose five diarrheagenic pathotypes. A total of 124 birds were captured. One bird was positive for *Salmonella enterica* (0.81%) and 121 (97.58%) were positive for *E. coli.* Among these, 110 isolates were submitted to antimicrobial susceptibility test and 28.18% (31/110) presented resistance to at least one antibiotic. Resistance to azithromycin was the most frequent (21.82%), followed by tetracycline (10.91%) and sulfamethoxazole with trimethoprim (8.9%). Multidrug resistance, calculated as a resistance to at least 3 antimicrobial classes, was identified in 3.64% (4/110) of strains. The maximum number of antimicrobial classes to which one strain was resistant was seven. Results demonstrated nine different resistance profiles and the most frequent was tetracycline and sulfamethoxazole with trimethoprim (4 strains), followed by chloramphenicol, azithromycin, tetracycline and sulfamethoxazole with trimethoprim (3 strains). Amoxicillin with clavulanic acid and tobramycin presented lowest levels of antimicrobial resistance, to which none of the tested strains were resistant. A single strain was positive for the *eltB* gene, which is a diagnostic tool to identify the Enterotoxigenic *E. coli* (ETEC) pathotype. None of the other investigated genes (*stx1, stx2, estA, eaeA, ipaH, aatA* and *aaiC)* were identified. The single isolate of *S. enterica* was a rough strain of *Salmonella enterica* subsp. e*nterica,* but serotype identification was not possible. However, this isolate presented resistance to amoxicillin, amoxicillin with clavulanic acid, tetracycline and sulfamethoxazole with trimethoprim. Therefore, captured feral pigeons of Fortaleza presented a low prevalence of *S. enterica* and diarrheagenic *E. coli*. Considering

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[2] Post-Graduate Program in Veterinary Science, Laboratório de Estudos Ornitológicos, Faculdade de Veterinária, Universidade Estadual do Ceará (UECE), Avenida Silas Munguba 1700, Fortaleza, CE 60740-000, Brazil. *Corresponding author: rubenhorn@hotmail.com

[3] Laboratório de Toxinologia Molecular, Faculdade de Medicina, Universidade Federal do Ceará, Rua Alexandre Baraúna 949, Fortaleza, CE 60430-160.

the investigated pathogens, our results suggest a good health status and a low public health risk. However, important antimicrobial resistance profiles were identified.

INDEX TERMS: Antimicrobial susceptibility, diarrheagenic diagnosis, *Escherichia coli, Salmonella enterica,* feral pigeons, Fortaleza, Brazil, rock pigeon, multidrug resistance, wild birds.

RESUMO.- [**Isolamento, sensibilidade antimicrobiana e diagnóstico de cepas diarreiogênicas de *Escherichia coli* e *Salmonella enterica* isoladas de pombos urbanos (*Columba livia*) capturados em Fortaleza, Brasil**.] O objetivo deste estudo foi isolar cepas de *Escherichia coli* e *Salmonella enterica* de pombos urbanos capturados em Fortaleza, Brasil, e avaliar os perfis de resistência antimicrobiana dos isolados, bem como diagnosticar patotipos diarreiogênicos de *E. coli*. Pombos foram capturados em quatro locais públicos de Fortaleza utilizando três técnicas. Amostras individuais de suabes cloacais foram coletadas e submetidas a isolamento bacteriano, seguido de identificação bioquímica e teste de susceptibilidade a antimicrobianos. A técnica de disco difusão foi utilizada para avaliar resistência antimicrobiana a doze antibióticos. Cepas de *E. coli* foram submetidas à extração de DNA seguido de PCR para o diagnóstico de cinco patotipos diarreiogênicos. Um total de 124 aves foram capturadas, a partir das quais em uma houve isolamento de *Salmonella enterica* (0,81%) e em 121 (97,58%) houve isolamento de *E. coli.* Destas, 110 isolados foram submetidos a teste de suscetibilidade a antimicrobianos e 28,18% (31/110) apresentaram resistência a pelo menos um antibiótico. Resistência a azitromicina foi a mais frequente (21,82%), seguida por tetraciclina (10,91%) e sulfametoxazol com trimetoprim (8,9%). Resistência a múltiplas drogas foi identificada em 3,64% (4/110) dos isolados e o número máximo de antibióticos aos quais uma única cepa foi resistente foi sete. Resultados demonstraram nove diferentes perfis de resistência e o mais frequente foi tetraciclina e sulfametoxazol com trimetoprim (4 cepas), seguido por cloranfenicol, azitromicina, tetraciclina e sulfametoxazol com trimetoprim (3 cepas). Amoxicilina com ácido clavulânico e tobramicina foram os antibióticos com menor resistência antimicrobiana, aos quais nenhuma cepa apresentou resistência. Uma única cepa foi positiva para o gene *eltB* que é usado para diagnóstico do patotipo *E. coli* enterotoxigênica (ETEC), enquanto que os demais genes investigados (*stx1, stx2, estA, eaeA, ipaH, aatA* e *aaiC)* não foram identificados. A única cepa de *S. enterica* isolada foi identificada como uma cepa rugosa de *Salmonella enterica* subsp. *enterica* e, portanto, a identificação do sorotipo não foi possível. Entretanto, este isolado apresentou resistência a amoxicilina, amoxicilina com ácido clavulânico, tetraciclina e sulfametoxazol com trimetoprim. Portanto, pombos urbanos capturados em Fortaleza apresentaram baixa prevalência de cepas de *S. enterica* e *E. coli* diarreiogênicas. Considerando os patógenos investigados, os resultados encontrados sugerem um bom status sanitário destas aves e um baixo risco à saúde pública. Entretanto, importantes perfis de resistência antimicrobiana foram identificados.

TERMOS DE INDEXAÇÃO**:** Antimicrobiana, diagnóstico diarreiogênicas, *Escherichia coli, Salmonella enterica,* pombos urbanos, Fortaleza, Brasil, pombo das rochas, resistência a múltiplas drogas, aves selvagens.

INTRODUCTION

Feral pigeons (*Columba livia*) have adapted to the urban environment and populations are now widespread in several countries (Morabito et al. 2001, Pedersen et al. 2006, Dutta et al. 2013b), including Brazil (Silva et al. 2009). In some locations, increasing populations have been reported and control measures have been applied, such as in Chile, in which this avian species was considered a plague (González-Acuña et al. 2007). These birds can host several human pathogens and a total of 60 have already been identified, including protozoa, bacteria, viruses and fungi (Haag-Wackernagel & Moch 2004). Among these, different serotypes of the genus *Salmonella* have been isolated from these animals, raising concern for the public health in several locations.

Salmonella enterica is a species of Gram-negative bacteria, member of the Enterobacteriaceae family, and over 2,500 different serotypes have been identified. Some of these are species-specific, while many others may infect multiple hosts, including humans (Grimont & Weill 2007). Several reports show that these microorganisms are frequently isolated from feral pigeons in different countries (Refsum et al. 2002, Dovč et al. 2004, Sousa et al. 2010, Dutta et al. 2013a, Osman et al. 2013). However, there is a great variation between prevalence values in these reports and which serotypes are found. In addition, there is considerable effort employed by the scientific community to understand the dispersal of this pathogen among these hosts.

Another aspect of concern related to microorganisms isolated from feral pigeons is antimicrobial resistance. This is a matter of concern for the public health worldwide for limiting therapeutic options in infectious cases. The increase of resistance in human pathogens is frequently related to food producing animals and *Escherichia coli* is often used as an indicator in different studies (EFSA & ECDC 2016). However, free-living birds may host resistant strains and this microbial species is frequently isolated from feral pigeons with variable levels of antimicrobial resistance (Silva et al. 2009, Dutta et al. 2013b). In addition, considering that, diarrheagenic *E. coli* strains have been isolated from feral pigeons (Morabito et al. 2001, Gargiulo et al. 2014), the proximity of these birds with humans could be a risk for the public health. Therefore, this study aimed to isolate and evaluate antimicrobial resistance of *Salmonella enterica* and *Escherichia coli* strains from feral pigeons (*Columba livia*) captured in Fortaleza, Brazil.

MATERIALS AND METHODS

Ethics statement. This project was authorized by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) with the following protocol number: 47316-1. In addition, this study was submitted and approved by the local Ethics Committee for the Use of Animals of the State University of Ceará with the following protocol number: 2081925.

Capture. From November 17, 2014 to June 14, 2015, feral pigeons were captured in four locations in Fortaleza, the Capital

city of the State of Ceará - Brazil. Two locations (A: 3°43'45.5"S 38°31'35.1"W; B: 3°44'24.0"S 38°31'28.9"W) were public squares in which pigeons were found in large quantities in close contact with humans. The other two locations (C: 3°47'43.4"S 38°33'30.0"W; D: 3°46'31.4"S 38°32'49.7"W) were places in which pigeons are not used to the human presence. Three different techniques were used based on the location and behavior of the birds, which were: net throwing, tomahawk trapping and a noose carpet was built. Noose carpet trap is a variation of the Bal-Chatri technique and was prepared with nooses tied to a $1m^2$ wired frame using corn as bait in the middle. Net throwing technique was used in locations in which pigeons were accustomed to humans, allowing an approach necessary for successfully throwing the net. In these locations, early in the morning, before pedestrian activity initiated, corn was thrown as bait and when an adequate amount of birds were within a distance of at least 2m the net was thrown. Noose carpets and tomahawk traps were used in the locations in which pigeons did not tolerate well the human presence, often flying away at a minimum distance of 15m. In these locations, from 8h a.m. to 4h p.m. traps were placed with corn as bait and observed in intervals of 15min for removing trapped birds. Once captured, individual cloacal swab samples were collected and immediately submitted to microbiological procedure. Pigeons that died in traps were submitted to necropsy and representative fragment samples were collected aseptically from liver, spleen and intestines for bacteriological procedure.

Microbiological procedure. For the isolation of *Escherichia coli*, cloacal swabs and organ samples were placed in tubes containing 10mL of 0.1% buffered peptone water and incubated. Then, a loopful was collected from each tube and streaked in Eosin Methylene Blue agar plate (EMB). After incubation colonies with morphological characteristics of *E. coli* were collected and submitted to biochemical identification with the following tests: triple-sugar-iron agar (TSI), sulfide-indole-motility agar (SIM), lysine iron agar (LIA), methyl red production, Voges-Proskauer, malonate and citrate. Isolates confirmed as *Escherichia coli* were maintained in nutrient agar for antimicrobial susceptibility test. For the isolation of *Salmonella enterica*, the guidelines of the normative instruction 62 of the Brazilian Ministry of Agriculture, Livestock and Supply (Ministério da Agricultura Pecuária e Abastecimento, MAPA) were followed with some modifications. From the tubes containing buffered peptone water, after incubation aliquots of 0.1mL and 1mL were collected from each sample and transferred to the selective enrichment step. This step was performed with tubes containing the broths Rappaport-Vassiliadis and Selenite-Cystine added novobiocin 0.4µg/mL, respectively, which were then incubated. Then, a loopful from each broth was streaked in plates containing brilliant green agar added novobiocin 0.4µg/mL and Salmonella-Shigella agar. After incubation, colonies suggestive of *S. enterica* were selected and submitted to a screening with the biochemical tests: TSI, LIA, SIM and urease production. The isolates with biochemical profile suggestive of *Salmonella enterica* were confirmed with rapid slide agglutination test performed with polyvalent *Salmonella* sp. antisera. Positive samples were cultured in nutrient agar and sent to the Laboratory of Enterobacteria of the Oswaldo Cruz Institute Foundation (LABENT-FIOCRUZ) for serotype identification. All of the incubation steps were performed at 37°C for 24h in bacteriological incubator.

Antimicrobial susceptibility test. The *S. enterica* and *Escherichia coli* strains isolated in this study were submitted to antimicrobial susceptibility testing using the disk diffusion method. After incubation in Mueller-Hinton agar plates at 37°C for 24h, inhibition zones around the antimicrobial disks were measured and compared to the standards provided by CLSI (2014). Intermediate results were interpreted as resistant. Disks containing the following antimicrobials and concentrations were used: amoxicillin (10µg), amoxicillin with clavulanic acid (20/10µg), cephalexin (30µg), ceftiofur (30µg), ciprofloxacin (5µg), sulfamethoxazole with trimethoprim (25µg), polymyxin B (300I.U.), gentamycin (10µg), tobramycin (10µg), chloramphenicol (30µg), tetracycline (30µg) and azithromycin (15µg). *E. coli* ATCC 25922 strain was used as control.

Diarrheagenic *Escherichia coli*. DNA was extracted from the *E. coli* isolates and were submitted to polymerase chain reaction (PCR) to diagnose five diarrheagenic pathotypes. Briefly, isolates were inoculated in BHI broth, incubated and plated on MacConkey agar plates. Then, three colonies of each isolate were collected and submitted to a simple extraction method. Colonies were placed in tubes with 1mL of Triton X-100, boiled for 20min at 94°C, followed by centrifugation at 10.000rpm for 10min. After this procedure, the supernatant was collected, quantified by spectrophotometry and used as DNA sample for the reaction. The GoTaq Green Master Mix® kit (Promega, São Paulo, Brazil) was used for the PCR in a total volume of 20µL per reaction with the following protocol: 95°C for 5min; 40 cycles of 95°C for 30s, 57°C for 30s and 72°C for 1min. Finishing the PCR, samples were heated at 72°C for 10min. The following genes were used to diagnose the diarrheagenic pathotypes: genes *stx1* (348pb) and *stx2* (584pb) for the identification of Shiga-Toxin producing *E. coli* (STEC); *eltB* (508pb) and *estA* (147pb) for enterotoxigenic *E. coli* (ETEC); *eaeA* (881 pb) for enteropathogenic *E. coli* (EPEC); *ipaH* (483 pb) for enteroinvasive *E. coli* (EIEC); *aatA* (630 pb) and *aaiC* (215pb) for enteroaggregative *E. coli* (EAEC) (Taniuchi et al. 2012). Reference strains EAEC 042, EHEC O157:H7, EIEC O124, EPEC 2348/69 and ETEC H10407 were used as positive controls for the reactions. Amplified products were submitted to 2% agarose gel electrophoresis stained with ethidium bromide and photo documented using Chemidoc XRS System (Bio-Rad Laboratories).

RESULTS

Captured birds. In public squares, pigeons were successfully captured with the net throwing technique, while noose carpets and tomahawk traps were used in the other two locations. A total of four birds died in noose carpets, three asphyxiated between the 15min intervals of observation, and one was predated by a raptor (Southern Caracara, *Caracara plancus*) while trapped. Necropsied birds were positive for *Escherichia coli* only in samples collected from intestine, which were accounted and processed equally as the cloacal swab samples. No *E. coli* or *S. enterica* was isolated from samples collected from extra-intestinal organs and no macroscopic lesions were identified in the necropsies. Most pigeons were collected in location B (n=34), followed by C (n=31), A (n=30) and D (n=29). A total of 124 birds were captured and 121 were positive for *E. coli* (97.58%). A single strain of *S. enterica* was isolated from samples (0.81%) and was identified as a rough strain of *Salmonella enterica* subsp. *enterica*.

Antimicrobial resistance. Among the *E. coli* isolates, 110 were submitted to antimicrobial susceptibility tests. In storage, 11 strains were lost and were not submitted to further tests. The antibiotic to which strains presented the most frequent resistance was azithromycin (21.82%), followed by tetracycline (10.91%) and sulfamethoxazole with trimethoprim (8.9%) (Table 1). Considering the 110 strains that were tested, 28.18% (31/110) presented resistance to at least one antibiotic.

Multidrug resistance, calculated as a resistance to at least 3 antimicrobial classes, was identified in 3.64% (4/110) of strains. A total of 9 different resistance profiles was identified and the most frequent was tetracycline and sulfamethoxazole with trimethoprim (4 strains), followed by chloramphenicol, azithromycin, tetracycline and sulfamethoxazole with trimethoprim (3 strains). The maximum number of antibiotic classes to which a strain presented resistance was 7 (1 strain), followed by 5 (1 strain) (Table 2). Amoxicillin with clavulanic acid and tobramycin were the most effective antibiotics, to which none of the analyzed strains presented resistance. The *S. enterica* isolate was resistant to four antibiotics with the following profile: amoxicillin, amoxicillin with clavulanic acid, tetracycline and sulfamethoxazole with trimethoprim.

Table 1. Antimicrobial resistance rates obtained from 110 *Escherichia coli* strains isolated from feral pigeons captured in Fortaleza, Brazil, from November 2014 to June 2015

Antimicrobial	Resistance rates
Azithromycin	21.8% (24/110)
Tetracycline	10.9% (12/110)
Sulfamethoxazole with trimethoprim	8.1% (9/110)
Amoxicillin	3.6% (4/110)
Chloramphenicol	3.6% (4/110)
Cephalexin	2.7% (3/110)
Ceftiofur	2.7% (3/110)
Ciprofloxacin	0.9% (1/110)
Polymyxin B	0.9% (1/110)
Gentamycin	0.9% (1/110)
Tobramycin	0% (0/110)
Amoxicillin with clavulanic acid	0% (0/110)

Table 2. Antimicrobial resistance profiles of 31 *Escherichia coli* strains isolated from feral pigeons captured in Fortaleza, Brazil, from November 2014 to June 2015

No. of antimicrobial classes	Profiles	No. of isolates
1	AZI	18
2	TET+SUT	4
2	AZI+POL	1
2	AMO+TET	1
2	AZI+TET	1
2	CFE+CTF +AMO	1
4	CLO+AZI+TET+SUT	3
5	CFE+CTF+AMO+AZI+TET+SUT	1
7	CFE+CTF+AMO+GEN+CLO+TET+CIP+SUT	1
Total	9 profiles	31

CFE = Cephalexin, CTF = ceftiofur, AMO = amoxicillin, AMC = amoxicillin with clavulanic acid, TOB = tobramycin, GEN = gentamycin, CLO = chloramphenicol, AZI = azithromycin, TET = tetracycline, POL = polymyxin B, CIP = ciprofloxacin, SUT = sulfamethoxazole with trimethoprim.

Diarrheagenic *Escherichia coli*. The *eltB* gene was identified in one isolate (1/110; 0.91%), belonging to the ETEC pathotype, while the remaining genes were not detected.

DISCUSSION

Several techniques have been used over the centuries to capture wild birds, mostly for feeding and clothing. However, current interests for capturing wild birds are more diverse, often implicated as part of disease control programs, population regulation activities, wildlife management efforts, and research studies (Schemnitz 2005). In addition, animal welfare concerns implicate in a continuous search for techniques to capture wild birds without causing unnecessary suffering (FAO 2007). In this study, three techniques were used in different situations, two of which presented good results. However, the noose carpet technique led to occasional deaths and presented animal welfare implications.

A study performed in Juiz de Fora, Brazil, with one hundred fresh fecal samples collected in an urban environment revealed that 86% of samples were positive for *Escherichia coli* (Silva et al. 2009). However, this study showed a higher isolation rate (97.58%), which could be explained by the different microbiological methodology. In that report, swabs were placed in saline and directly plated, while the pre-enrichment broth used in the present study may have promoted a better recovery of bacterial cells.

In general, antimicrobial resistance rates appear to vary greatly among *E. coli* strains isolated from pigeons depending on the location (Radimersky et al. 2010, Dutta et al. 2013b). However, in Brazil, low resistance rates have been reported in a pigeon population (Silva et al. 2009), which corroborates with the results in this study. This may indicate that, in Brazil these birds are not frequently in contact with antibiotics or resistant bacteria, as we can see in other countries in which resistant bacteria were isolated from pigeons (Radimersky et al. 2010, Dutta et al. 2013b). Despite the great variation in rates observed in different studies, resistance to some specific antimicrobial groups, such as tetracycline and sulfonamides, are commonly reported (Silva et al. 2009, Radimersky et al. 2010, Dutta et al. 2013b).

Low frequencies of diarrheagenic *E. coli* have been identified in feral pigeons (Morabito et al. 2001, Pedersen et al. 2006, Gargiulo et al. 2014). However, different pathotypes have been identified recently in Brazil and ETEC prevalence was 7.1% (Silva et al. 2009). In this study, a single strain was identified as diarrheagenic, belonging to the ETEC pathotype. This result suggests that the studied population does not frequently host these microorganisms and appears to offer little risk to the public health, concerning these pathogens.

Several studies reported different isolation rates of *Salmonella* from feral pigeons, such as: 8% of pigeons in India, 13.3% in Egypt, 4.17% in Norway, 5.7% in Slovenia, and 7.94% in Brazil (Refsum et al. 2002, Dovč et al. 2004, Sousa et al. 2010, Dutta et al. 2013a, Osman et al. 2013). However, the isolation rate in this study was lower than all of the previously reported studies, which is an indication that the population of feral pigeons in Fortaleza have a low prevalence of this bacterium.

The link between poultry and salmonellosis in humans is well established and an outbreak have been reported in Brazil (De Almeida et al. 2015). In addition, there are several

studies (Refsum et al. 2002, Dovč et al. 2004, Sousa et al. 2010, Dutta et al. 2013a, Osman et al. 2013) demonstrating the isolation of *Salmonella* strains from feral pigeons in different countries, including Brazil (De Sousa et al. 2010). Despite that, the public health risk that these animals pose of transmitting this pathogen may be overestimated due to the lack of reported cases of human salmonellosis originated from feral pigeons (Haag-Wackernagel & Moch 2004). In fact, there is only a single reported case of human salmonellosis transmitted by a pigeon in recent years (Lacassin et al. 1995). Therefore, in this study public health risk may be considered low, despite the isolation of a *Salmonella* strain, which is an important human pathogen.

CONCLUSIONS

Captured feral pigeons of Fortaleza presented a low prevalence of *Salmonella enterica* and diarrheagenic *Escherichia coli*. Considering these pathogens, this study suggests a good health status and a low risk to the public health.

However, important multidrug resistance profiles were identified in the investigated isolates. In addition, the noose carpet technique did not present adequate results concerning animal welfare restrictions and it is not recommended for capturing feral pigeons.

REFERENCES

Almeida I.A.Z.C., Peresi J.T.M., Alves E.C., Marques D.F., Teixeira I.S.C., Silva S.I.L., Pigon S.R.F., Tiba M.R. & Fernandes S.A. 2015. *Salmonella* Alachua: causative agent of a foodborne disease outbreak. Braz. J. Infect. Dis. 19(3):233-238. <http://dx.doi.org/10.1016/j.bjid.2014.12.006> <PMid:25661321>

CLSI 2014. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement. CLSI document M100-S24. Clinical and Laboratory Standards Institute, Wayne, USA.

Dovč A., Zorman-Rojs O., Vergles Rataj A., Bole-Hribovšek V., Krapež U. & Dobeic M. 2004. Health status of free-living pigeons (*Columba livia domestica*) in the city of Ljubljana. Acta Vet. Hungarica 52(2):219-226. <http://dx.doi.org/10.1556/AVet.52.2004.2.10> <PMid:15168753>

Dutta P., Borah M., Sarmah R. & Gangil R. 2013a. Isolation of *Salmonella* Typhimurium from pigeons (*Columba livia*) in Greater Guwahati, its histopathological impact and antibiogram. Comp. Clin. Pathol. 22(1):147-150. <http://dx.doi.org/10.1007/s00580-012-1614-3>

Dutta P., Borah M., Sarmah R. & Gangil R. 2013b. Isolation, histopathology and antibiogram of *Escherichia coli* from pigeons (*Columba livia*). Vet. World 6(2):91-94. <http://dx.doi.org/10.5455/vetworld.2013.91-94>

EFSA & ECDC. 2016. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2014. EFSA J. 14(2):4380.

FAO 2007. Wild Birds and Avian Influenza: an introduction to applied field research and disease sampling techniques, p.13-32. In: Whitworth D., Newman S.H., Mundkur T. & Harris P. (Eds), FAO Animal Production and Health Manual, No. 5. FAO, Rome.

Gargiulo A., Russo T.P., Schettini R., Mallardo K., Calabria M., Menna L.F., Raia P., Pagnini U., Caputo V., Fioretti A. & Dipineto L. 2014. Occurrence of enteropathogenic bacteria in urban pigeons (*Columba livia*) in Italy. Vector-Borne Zoonot Dis. 14(4):251-255. <http://dx.doi.org/10.1089/vbz.2011.0943> <PMid:24661012>

González-Acuña D., Silva G F., Moreno S L., Cerda L F., Donoso E S., Cabello C J. & López M J. 2007. Detección de algunos agentes zoonóticos en la paloma doméstica (*Columba livia*) en la ciudad de Chillán, Chile. Revta Chilena Infect. 24(3):199-203. <http://dx.doi.org/10.4067/S0716-10182007000300004> <PMid:17554438>

Grimont P.A.D. & Weill F.X. 2007. Antigenic formulae of the *Salmonella* serovars, p.6-9. In: Ibid. (Eds), WHO Collaborating Centre for Reference and Research on *Salmonella*. 9th ed. OCLC ResearchWorks Terms and Conditions, Paris.

Haag-Wackernagel D. & Moch H. 2004. Health hazards posed by feral pigeons. J. Infect. 48(4):307-313. <http://dx.doi.org/10.1016/j.jinf.2003.11.001> <PMid:15066331>

Lacassin F., Mino J.C., Benoit C., Perronne C., Leport C. & Vildé J.L. 1995. A propos d'un cas de salmonellose aviaire [A case of avian salmonellosis]. Revta Med. Intern. 16(1):77-78. <http://dx.doi.org/10.1016/0248-8663(96)80671-0>

Morabito S., Dell'omo G., Agrimi U., Schmidt H., Karch H., Cheasty T. & Caprioli A. 2001. Detection and characterization of Shiga toxin-producing *Escherichia coli* in pigeons. Vet. Microbiol. 82(3):275-283. <http://dx.doi.org/10.1016/S0378-1135(01)00393-5> <PMid:11470548>

Osman K.M., Mehrez M., Erfan A.M. & Nayerah A. 2013. A. *Salmonella enterica* isolated from pigeon (*Columba livia*) in Egypt. Foodborne Pathogens Dis. 10(5):481-483. <http://dx.doi.org/10.1089/fpd.2012.1347> <PMid:23531124>

Pedersen K., Clark L., Andelt W.F. & Salman M.D. 2006. Prevalence of Shiga toxin-producing *Escherichia coli* and *Salmonella enterica* in rock pigeons captured in Fort Collins, Colorado. J. Wildl. Dis. 42(1):46-55. <http://dx.doi.org/10.7589/0090-3558-42.1.46> <PMid:16699148>

Radimersky T., Frolkova P., Janoszowska D., Dolejska M., Svec P., Roubalova E., Cikova P., Cizek A. & Literak I. 2010. Antibiotic resistance in faecal bacteria (*Escherichia coli, Enterococcus* spp.) in feral pigeons. J. Appl. Microbiol. 109(5):1687-1695. <PMid:20602656>

Refsum T., Handeland K., Baggesen D.L., Holstad G. & Kapperud G. 2002. Salmonellae in avian wildlife in Norway from 1969 to 2000. Appl. Env. Microbiol. 68(11):5595-5599. <http://dx.doi.org/10.1128/AEM.68.11.5595-5599.2002> <PMid:12406754>

Schemnitz S.D. 2005. Capturing and handling wild animals, p.239-285. In: C.E. Braun (Ed.), Techniques for Wildlife Investigations and Management. The Wildlife Society, Bethesda, USA.

Silva V.L., Nicoli J.R., Nascimento T.C. & Diniz C.G. 2009. Diarrheagenic *Escherichia coli* strains recovered from urban pigeons (*Columba livia*) in Brazil and their antimicrobial susceptibility patterns. Curr. Microbiol. 59(3):302-308. <http://dx.doi.org/10.1007/s00284-009-9434-7> <PMid:19504156>

Sousa E., Berchieri Junior A., Pinto A.A., Machado R.Z., Carrasco A.O.T., Marciano J.A. & Werther K. 2010. Prevalence of *Salmonella* spp. antibodies to *Toxoplasma gondii*, and Newcastle disease virus in feral pigeons (*Columba livia*) in the city of Jaboticabal, Brazil. J. Zoo Wildl. Med. 41(4):603-607. <http://dx.doi.org/10.1638/2008-0166.1> <PMid:21370639>

Taniuchi M., Walters C.C., Gratz J., Maro A., Kumburu H., Serichantalergs O., Sethabutr O., Bodhidatta L., Kibiki G., Toney D.M., Berkeley L., Nataro J.P. & Houpt E.R. 2012. Development of a multiplex polymerase chain reaction assay for diarrheagenic *Escherichia coli* and *Shigella* spp. and its evaluation on colonies, culture broths, and stool. Diagn. Microbiol. Infect. Dis. 73(2):121-128. <http://dx.doi.org/10.1016/j.diagmicrobio.2012.03.008> <PMid:22541788>

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Original Article
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Nephroblastoma in a black-tufted marmoset (*Callithrix penicillata*)[1]

Jair A. Ferreira Junior[2], Daniel R. Rissi[3], Mônica A. Elias[2], André S. Leonardo[2], Karla A. Nascimento[2], Juliana T.S.A. Macêdo[2] and Pedro M.O. Pedroso[2]*

ABSTRACT.- Ferreira Junior J.A., Rissi D.R., Elias M.A., Leonardo A.S., Nascimento K.A., Macêdo J.T.S.A. & Pedroso P.M.O. 2018. **Nephroblastoma in a black-tufted marmoset (*Callithrix penicillata*)**. *Pesquisa Veterinária Brasileira 38(11):2155-2158*. Laboratório de Patologia Veterinária, Hospital Veterinário, Fundação Universidade de Brasília, Via L4 Norte, Brasília, DF 70910-970, Brazil. E-mail: pedrosovet@yahoo.com.br

A renal nephroblastoma is described in a free-living black-tufted marmoset (*Callithrix penicillata*) in Central Brazil. The monkey was found dead and subjected to necropsy. Gross anatomic changes consisted of a ruptured left kidney, which was almost completely effaced by a white to yellow, partially encapsulated friable mass. The left ureter was distended due to obstruction by a red, spherical, 2mm in diameter friable mass. The urinary bladder was also distended. Histologically the renal and ureteral masses consisted of a triphasic embryonal neoplasm composed of embryonic epithelium forming glomeruli and tubules, polygonal blastemal cells, and a mesenchymal stroma. The embryonic epithelium exhibited rare nuclear immunoreactivity for WT-1, whereas blastemal cells exhibited robust cytoplasmic and rare nuclear immunoreactivity for WT-1; blastemal cells were also immunoreactive for vimentin. No immunoreactivity was detected for pan-cytokeratin (AE1/AE3), actin, and desmin. Morphological and immunohistochemical features of the present neoplasm are consistent with those described for renal nephroblastoma.

INDEX TERMS: Nephroblastoma, black-tufted marmoset, *Callithrix penicillata*, neoplasm, non-human primate, Wilms tumor, pathology.

RESUMO.- [**Nefroblastoma em um sagui-de-tufos-pretos (*Callithrix penicillata*).**] Descreve-se um caso de nefroblastoma maligno em um sagui de vida livre no Brasil Central. O macaco foi encontrado morto e encaminhado para necropsia. Na macroscopia, o rim esquerdo apresentava-se rompido e o parênquima estava substituído por um tecido neoplásico friável, parcialmente encapsulado e de superfície natural branca e de corte amarela. O ureter esquerdo apresentava-se distendido devido à obstrução por uma massa friável, vermelha, esférica, de 2mm de diâmetro. Histologicamente, as massas renal e ureteral consistiam de uma neoplasia embrionária composta por três populaçõies de células neoplásicas, composta por epitélio embrionário formando glomérulos e túbulos, células blastemais poligonais e um estroma mesenquimal. O epitélio embrionário exibiu imunorreactividade nuclear rara para WT-1, enquanto que as células blastemais exibiram imunorreactividade nuclear citoplasmática e rara para WT-1; As células blastemais também foram imunorreativas à vimentina. Nenhuma imunorreatividade foi detectada para pan-citoqueratina (AE1/AE3), actina e desmina. As características morfológicas e imuno-histoquímicas da presente neoplasia são consistentes com as descritas para o nefroblastoma renal.

TERMOS DE INDEXAÇÃO: Nefroblastoma, sagui-de-tufos-pretos, *Callithrix penicillata*, neoplasma, primata não-humano, tumor de Wilms, patologia.

INTRODUCTION

Nephroblastoma is an embryonic tumor that is commonly described in humans, especially in children (Goens et al. 2005). Nefroblastoma has been uncommonly described in non-human primates (NHP), such as cynomolgus monkeys (*Macaca fascicularis*) (Bennett et al. 1982, Zoller et al. 2008), baboons (*Papio* sp.) (Goens et al. 2005), cotton-top marmosets

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[2] Laboratório de Patologia Veterinária, Universidade de Brasília (UnB), Via L4 Norte, Brasília, DF 70910-970, Brazil. *Corresponding author: pedrosovet@yahoo.com.br
[3] Department of Pathology, Athens Veterinary Diagnostic Laboratory, University of Georgia College of Veterinary Medicine, Athens, GA, USA.

(*Saguinus oedipus*) (Jones & Casey 1981), and in a common tamarin (*Callithrix jacchus*) (Zoller et al. 2008). In NHP, renal nephroblastomas are usually an incidental finding in young individuals. They appear not only as spontaneous cases, but also in association with radiation exposure, carcinogenic chemical agents and parasites (Jones & Casey 1981, Zoller et al. 2008). In dogs, tumors typically affect the thoracolumbar spinal cord and cause clinical signs related to spinal cord compression (JPC 2017).

Nephroblastoma arises from the primitive metanephric blastema and typically exhibits epithelial and stromal differentiation in varying proportions (Meuten et al. 2004). Renal tumors can be solitary or multiple and unilateral or bilateral. Histologically, nephroblastoma is characterized by the presence of three cell populations (Meuten et al. 2004, JPC 2017). The embryonic epithelial component exhibits glomerular or tubular differentiation (Zoller et al. 2008); the blastemal component consists of densely packed, round to polygonal cells with a serpentine, diffuse, or nodular arrangement; and the mesenchymal component is arranged in streams of spindle cells that can undergo differentiation to other mesenchymal tissues such as skeletal muscle, cartilage, and/or bone (Meuten et al. 2004).

Callithrix penicillata (Geoffroy 1812) is a New World primate known as "black-tufted marmoset" or "star tamarin" due to its long, black pre-auricular hair ornaments in the shape of a brush. These NHP are insectivorous-gumivores and inhabit the Amazon Forest, the Atlantic Forest, and the Brazilian Cerrado, usually forming groups of approximately 10 individuals (Nitta et al. 2013). Primates of the genus *Callithrix* are phylogenetically close to humans and have the ability to adapt in urban or secondary vegetation environments, often acting as invasive species and as reservoirs of zoonotic diseases such as yellow fever, rabies, and dengue (Do Vale 2016). The purpose of the report is to describe a case of renal nephroblastoma in a free-living black-tufted marmoset in Central Brazil.

MATERIALS AND METHODS

A young female black-tufted marmoset (*Callithrix penicillata*) was found dead in the territory of the Federal District and subjected to necropsy. Representative samples of various organs were collected, fixed in 10% buffered formalin, routinely processed for histology, and stained with hematoxylin and eosin (HE). Immunohistochemistry (IHC) was performed using the streptavidin-biotin-peroxidase technique (LSAB+System HRP, EasyLink One, Esaypath) for anti-WT-1 (Wilms tumor factor; manufacturer's pre-dilution, Cell Marque, USA), anti-cytokeratin (AE1/AE3, 1:100 dilution, Biocare Medical, USA), and anti-vimentin (1:100 dilution, Biocare Medical, USA), anti-human smouth muscle actin (1:500 dilution, clone 1A4, DakoCytomation), and anti-desmin (1:50 dilution, Biocare Medical, USA). Immunostaining was visualized with the use of 3-3'-diaminabenzidine (DAB). Sections were stained with Harris hematoxylin. Positive and negative controls were used for each antibody.

RESULTS

Gross anatomic changes consisted of 10ml of serosanguineous fluid in the abdominal cavity and blood clots covering the omentum and the intestinal loops. The left kidney was ruptured was and almost completely effaced by a white to yellow, partially encapsulated friable mass that had a yellow cut surface. Between the affected kidney and the liver, other soft and friable white masses were present. The left ureter was distended due to obstruction by a red, spherical, 2mm in diameter friable mass. The urinary bladder was also distended (Fig.1). The hepatic capsule was moderately pale.

Microscopically a poorly demarcated, partially encapsulated, infiltrative neoplasm effaced and replaced most of the renal parenchyma. The tumor consisted of three intertwined cell populations (Fig.2): an embryonic epithelial component was composed of flat to cuboidal epithelial cells that formed primitive glomerular (Fig.3) and tubular structures; a second cell population was characterized by round to polygonal blastemal cells; and a third population of cells composed of

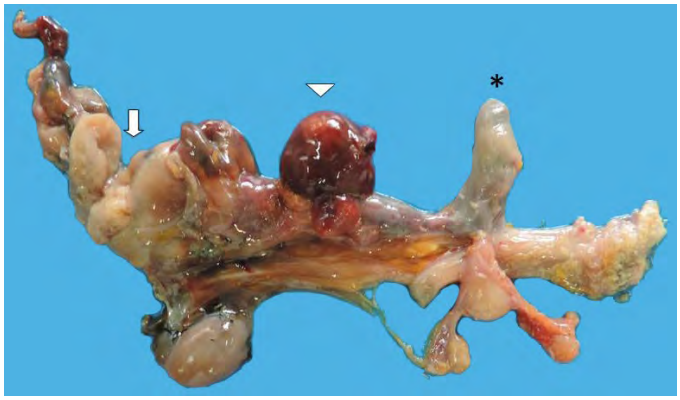


Fig.1. Renal nephroblastoma in a black-tufted marmoset (*Callithrix penicillata*), genito-urinary system. The left kidney is effaced by a friable white mass (arrow). A red mass also expands the mid-third portion of the ureter (arrowhead). The urinary bladder is shown as a reference (asterisk).

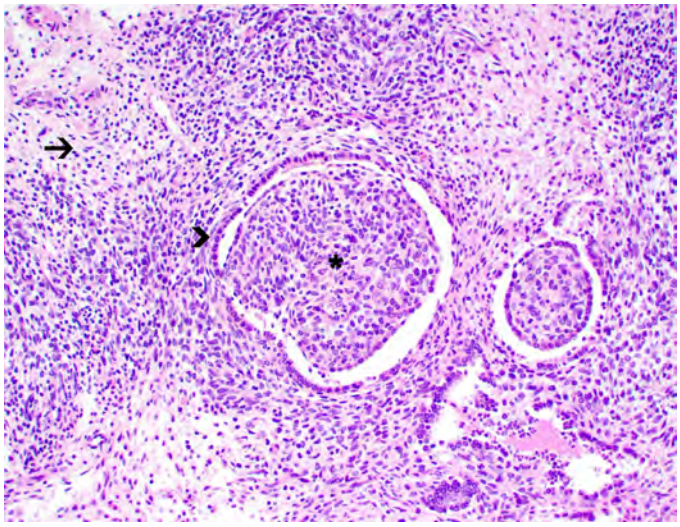


Fig.2. Renal nephroblastoma in a black-tufted marmoset (*Callithrix penicillata*). Scattered throughout the neoplasm are tubules lined by epithelial cells (arrowhead) and lined by epithelial cells and containing nests of polygonal blastemal cells (asterisks). The mesenchymal component (arrow) consists of streams of spindle cells. HE, obj.20x.

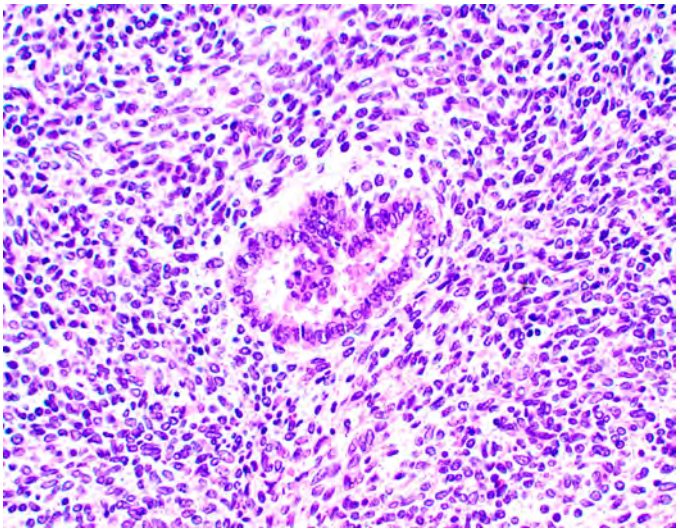


Fig.3. Renal nephroblastoma in a black-tufted marmoset (*Callithrix penicillata*). The neoplasm is composed of irregular glomerular tufts lined by epithelial cells. HE, obj.20x.

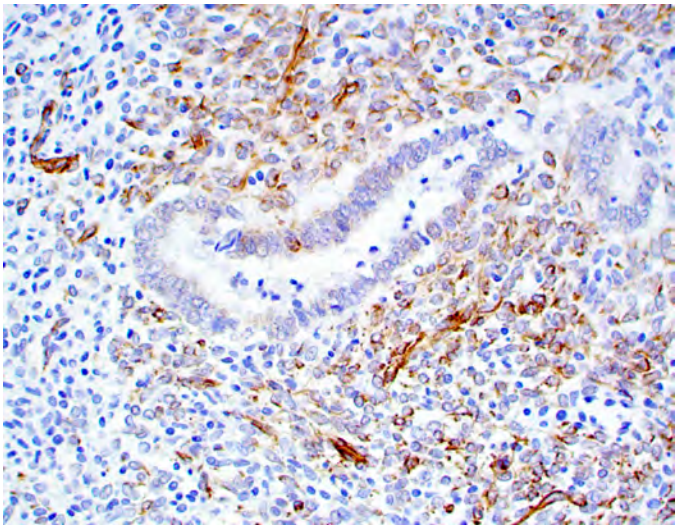


Fig.4. Renal nephroblastoma in a black-tufted marmoset (*Callithrix penicillata*). There is rare nuclear immunostaining for WT-1 within tubular cells and cytoplasmic immunostaining within blastemal cells. Immunohistochemistry, streptavidin-biotin-peroxyase method, Harris haematoxylin counterstaining, obj.20x.

thin bundles of fibroblasts, collagen, and small blood vessels (mesenchymal stroma). The epithelial cells had poorly defined and eosinophilic cytoplasm, and oval nuclei with coarse chromatin and 1 to 3 nucleoli. The blastemal cells were densely packed, had indistinct cytoplasmic borders, and round nuclei with loosely arranged chromatin and 1 to 2 nucleoli. There was moderate anisocytosis and anisokaryosis, and 5 mitoses in 10 400x fields. Adjacent renal tissue was compressed and atrophied, with multifocal infiltrates of moderate numbers of lymphocytes, plasma cells, and macrophages. The lumen of the ureter and urinary bladder contained large numbers of erythrocytes and strands of fibrin that extended into the urothelium and lamina propria. In these areas there were foci of neoplastic transformation similar to those described for the kidney.

The embryonic epithelium exhibited rare nuclear immunoreactivity for WT-1 (Fig.4), whereas blastemal cells exhibited robust cytoplasmic and rare nuclear immunoreactivity for WT-1; blastemal cells were also immunoreactive for vimentin (Fig.5). No immunoreactivity was detected for pan-cytokeratin (AE1/AE3), actin, and desmin.

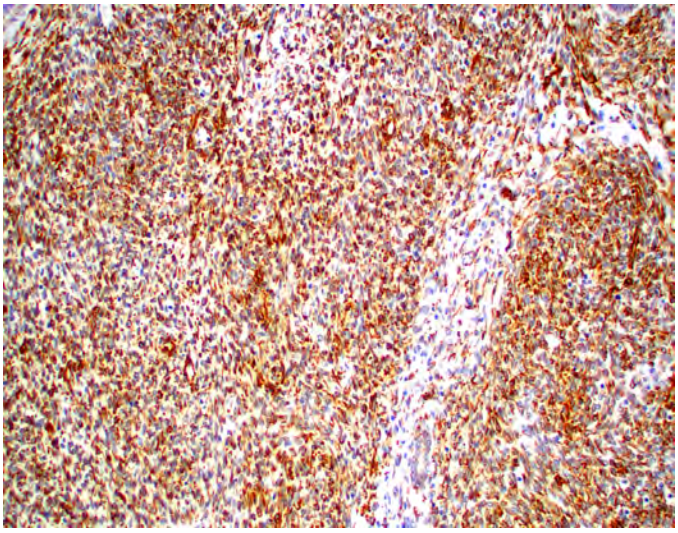


Fig.5. Renal nephroblastoma in a black-tufted marmoset (*Callithrix penicillata*). Positive immunostaining for vimentin within peritubular blastemal cells. Immunohistochemistry, streptavidin-biotin-peroxidase method, Harris haematoxylin counterstaining, obj.20x.

DISCUSSION

The diagnosis of nephroblastoma in this case was made based on the morphological and IHC findings. Nephroblastomas are poorly described in free-living tamarins in South America. In Brazil, these neoplasms are highly prevalent in pigs and poultry during slaughter (Brum et al. 2015, Serakides & Silva 2017). The primary renal tumors most commonly described in NHP are adenomas and carcinomas; nephroblastomas are rarely described (Jones & Casey 1981, Zoller et al. 2008). In humans, nephroblastomas affect mainly children, where carcinogenesis has been related to abnormalities in the Wilms tumor gene (WT-1 in chromosome 11). However, the genetics of nephroblastoma or Wilms tumor appear to be multifactorial and probably include other chromosomal abnormalities. Phylogenetic studies with two NHP with nephroblastoma (*Macaca fascicularis* and baboon) with pairs of identical chromosomes and homologous to human chromosome 11 did not reveal any chromosomal abnormalities similar to those associated with those tumors in humans (Goens et al. 2005). Primary renal neoplasms, including nephroblastoma, have also been associated with radiation, carcinogenic chemicals, and parasites in NHPs (Jones & Casey 1981, Zoller et al. 2008) and with N-ethylnitrosouria in laboratory mice (Zoller et al. 2008, Meuten & Meuten 2017). A familial form of nephroblastoma occurs in some strains of laboratory mice (Zoller et al. 2008, Meuten & Meuten 2017).

In the few reported cases in NHPs, nephroblastomas were directly related to the death of the animals (Jones & Casey 1981, Zoller et al. 2008). In the present case, the tumor likely caused death due to its invasive and destructive nature, with hemorrhage into the abdomen and hypovolemic shock. Although nephroblastomas are usually unilateral, located at one pole of the kidney, tumors can occasionally compress adjacent organs or invade the renal capsule, adhering to the abdominal wall and mesentery and, exceptionally, invading the pelvis (Meuten & Meuten 2017). Microscopically, the tumor exhibited the classic triphasic features typically described in cases of nephroblastoma in humans and other animals (Jones & Casey 1981, Goens et al. 2005, Zoller et al. 2008, Meuten & Meuten 2017). A striking feature in this case was the metastatic foci observed in the ureter, which is a feature of malignancy in these tumors (Meuten & Meuten 2017). In pigs, birds and primates, metastases are rare, but in dogs and cats, metastasis is expected in more than 50% of cases (Goens et al. 2005, Zoller et al. 2008, Meuten & Meuten 2017).

WT-1 IHC is useful for diagnostic confirmation of nephroblastoma. However, immunolabeling patterns have been mainly described for spinal cord nephroblastomas in dogs, where nuclear staining occurs within neoplastic cells forming glomerular and tubular structures and blastemal cells (Brewer et al. 2011). In the current case, the typical nuclear WT-1 immunolabeling was rare throughout the embryonic epithelium and, in addition, nuclear and cytoplasmic WT-1 immunostaining was detected within the blastemal component. While nuclear staining is considered specific for the diagnosis of nephroblastoma, studies have shown that WT-1 can be also expressed in the cytoplasm of neoplastic cells (Niksic et al. 2004) and can occur within all three tumor components (Sangkhathat et al. 2010).

CONCLUSIONS

The morphological and IHC features of the current neoplasm were typical of those described for renal nephroblastoma.

Although rare, nephroblastoma should be considered in the differential diagnosis of primary renal neoplasms in NHPs.

REFERENCES

Bennett B.T., Beluhan F.Z. & Welsh T.J. 1982. Malignant nephroblastoma in *Macaca fascicularis.* Lab. Anim. Sci. 32(4):403-404. <PMid:6292577>

Brewer D.M., Cerda-Gonzalez S., Dewey C.W., Diep A.N., Van Horne K. & McDonough S.P. 2011. Spinal cord nephroblastoma in dogs: 11 cases (1985-2007). J. Am. Vet. Med. Assoc. 238(5):618-624. <http://dx.doi.org/10.2460/javma.238.5.618> <PMid:21355804>

Brum J.S., Martins T.B., Vielmo A., Hammerschmitt M.E., Talini R., Minozzo C.D. & Barros C.S.L. 2015. Neoplasmas em suínos: 37 casos. Pesq. Vet. Bras. 35(6):541-546. <http://dx.doi.org/10.1590/S0100-736X2015000600009>

Do Vale C.A. 2016. Distribuição e potencial de invasão do sagui *Callithrix penicillata* (É. Geoffroy, 1812) no território brasileiro. Dissertação de Mestrado, Universidade Federal de Juiz de Fora, Juiz de Fora. 53p.

Goens S.D., Moore C.M., Brasky K.M., Frost P.A., Leland M.M. & Hubbard G.B. 2005. Nephroblastomatosis and nephroblastoma in nonhuman primates. J. Med. Primatol. 34(4):165-170. <http://dx.doi.org/10.1111/j.1600-0684.2005.00113.x> <PMid:16053493>

Jones S.R. & Casey H.W. 1981. Primary renal tumors in nonhuman primates. Vet. Pathol. 18(Suppl. 6):89-104. <http://dx.doi.org/10.1177/0300985881018s0610> <PMid:6283711>

JPC 2017. Case IV. 14th Wednesday Slide Conference, Joint Pathology Center Veterinary Pathology Services, Maryland. Available at <https://www.askjpc.org/wsco/wsc/wsc16/16WSC14.pdf> Access on Jun. 5, 2018.

Meuten D.J. & Meuten T.L.K. 2017. Tumors of the urinary system, p.632-688. In: Meuten D.J. (Ed.), Tumors in Dosmestic Animals. 5th ed. John Wiley and Sons, Ames.

Meuten D.J., Everitt J., Inskeep W., Jacobs R.M., Peleteiro M. & Thompson K.G. 2004. Histological Classification of Tumors of the Urinary System of Domestic Animals. Armed Forces Institute of Pathology, WHO, Washington, D.C. 70p.

Niksic M., Slight J., Sanford J.R., Caceres J.F. & Hastie N.D. 2004. The Wilms' tumour protein (WT1) shuttles between nucleus and cytoplasm and is present in functional polysomes. Hum. Mol. Genet. 13(4):463-471. <http://dx.doi.org/10.1093/hmg/ddh040> <PMid:14681305>

Nitta C.Y., Silva L.C.S., Miglino M.A., Ambrosio C.E., Bombonato P.P. & Rici R.E.G. 2013. Comparative ultrastructure of the tongue of black-tufted-ear-marmoset (*Callithrix penicillata*) and black-howler-monkey (*Alouatta caraya*) in different age groups. Pesq. Vet. Bras. 33(Suppl. 1):75-84. <http://dx.doi.org/10.1590/S0100-736X2013001300013>

Sangkhathat S., Kanngurn S., Chaiyapan W., Gridist P. & Maneechay W. 2010. Wilms' tumor 1 gene (WT1) is overexpressed and provides an oncogenic function in pediatric nephroblastomas harboring the wild-type WT1. Oncol. Lett. 1(4):615-619. <http://dx.doi.org/10.3892/ol_00000109> <PMid:22966353>

Serakides R. & Silva J.F. 2017. Sistema urinário, p.267-310. In: Santos R.L. & Alessi A.C. (Eds), Patologia Veterinária. 2ª ed. Roca, Rio de Janeiro.

Zoller M., Matz-Rensing K., Fahrion A. & Kaup F.J. 2008. Malignant nephroblastoma in a common marmoset (*Callithrix jacchus*). Vet. Pathol. 45(1):80-84. <http://dx.doi.org/10.1354/vp.45-1-80> <PMid:18192583>

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Original Article
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Morphological analysis of the elastic and collagen fibers in the ram penis[1]

Bruno Cesar Schimming[2]* and Gustavo N. Moraes[3]

ABSTRACT.- Schimming B.C. & Moraes G.N. 2018. **Morphological analysis of the elastic and collagen fibers in the ram penis.** *Pesquisa Veterinária Brasileira 38(11):2159-2165.* Departamento de Anatomia, Universidade Estadual Paulista, Cx. Postal 510, Botucatu, SP 18618-970, Brazil. E-mail: bruno.schimming@unesp.br

The penis represents the organ of the male's copulation. It is essential to know the reproductive biology and the morphology of the reproductive organs to increase animal production. In order to contribute to this knowledge and provides information on the ram reproductive morphology, the purpose of this work was to describe the distribution, based on light microscopy, of the collagen and elastic fibers in the ram penis. For that, were collected transverse fragments of the penis (root, sigmoid flexure, body and glans) of seven rams. The specimens were fixed in paraformaldehyde for 24h and destined for the histological routine. The extracellular matrix of the ram penis was composed of collagen and elastic fibers. The penis was enveloped by the tunica albuginea, consisting essentially of collagen fibers, which were arranged in two layers: an outer longitudinal and an inner circular. This tunic emitted septa that penetrated the corpus cavernosum. The elastic fibers appeared transversely and longitudinally in the corpus cavernosum, corpus spongiosum, and next to the neurovascular bundle of the penis. This structure was not different to that reported for other domestic ruminants such as cattle and buffaloes.

INDEX TERMS: Morphological analysis, collagen fibers, extracellular matrix, ram, penis, elastic fibers.

RESUMO.- [**Análise morfológica de fibras elásticas e colágenas no pênis de ovinos.**] O pênis representa o órgão da cópula do macho. É fundamental que se conheça a biologia reprodutiva e a morfologia dos órgãos reprodutores para o incremento da produção animal. Com o objetivo de contribuir para este conhecimento e fornecer informações sobre a morfologia reprodutiva de ovinos, o propósito deste trabalho foi descrever a distribuição, com base na microscopia de luz, das fibras colágenas e elásticas no pênis de ovinos. Para tanto, foram coletados fragmentos transversais do pênis (raiz, flexura sigmoide, corpo e glande) de sete ovinos. Os exemplares foram fixados em paraformaldeído por 24h e destinados à rotina histológica. A matriz extracelular do pênis de ovinos estava constituída por fibras colágenas e elásticas. O pênis estava envolvido pela túnica albugínea, formada essencialmente por fibras colágenas, que estavam arranjadas em duas camadas: longitudinal externa e circular interna. Esta túnica emitiu septos que penetraram no corpo cavernoso. As fibras elásticas apareceram de modo transversal e longitudinal nos corpos cavernoso e esponjoso e junto ao feixe vásculo-nervoso do pênis de ovinos. Esta estrutura mostrou-se similar ao encontrado em outros ruminantes domésticos como bovinos e bubalinos.

TERMOS DE INDEXAÇÃO: Análise morfológica, fibras colágenas, matriz extracelular, ovinos, pênis, fibras elásticas.

INTRODUCTION

According to the architecture of the corpus cavernosum, the penis of domestic animals is classified into two types: fibroelastic and musculocavernous penises. The fibroelastic type has small and confined blood spaces, and these spaces are divided by a large amount of fibroelastic tissue. This type of penis, when quiescent, exhibits a sigmoid flexure. The fibroelastic penis appears in ruminants and boars (Hafez & Hafez 2004, Dyce et al. 2010). Stallions, dogs and rabbits

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[2] Departamento de Anatomia, Instituto de Biociências de Botucatu, Universidade Estadual Paulista (Unesp), Distrito de Rubião Junior, Cx. Postal 510, Botucatu, SP 18618-970, Brazil. *Corresponding author: bruno.schimming@unesp.br

[3] Veterinary Medicine student, Faculdade de Medicina Veterinária e Zootecnia, Unesp, Campus de Botucatu, Distrito de Rubião Júnior, Botucatu, SP 18618-970.

have the musculocavernous type of penis. In this type of penis, blood spaces are relatively larger, and the lining and septa are more delicate and more muscular (Hafez & Hafez 2004, Dyce et al. 2010, Ribeiro et al. 2013).

The rabbit penis is covered by a capsule of connective tissue, the tunica albuginea, which projects intracavernous septa, especially in the corpus cavernosum. There are elastic fibers in the corpus cavernosum, corpus spongiosum, and in the tunica albuginea of the penis (Maia et al. 2006). The concentration of elastic fibers in different penile regions is related to age-related changes in rabbits. From one month of life, rabbits have a gradual increase in the concentration of these fibers. This trend is interrupted at two years, which may be related to initial changes due to senescence of the animals (Abidu-Figueiredo et al. 2013).

There are few reports on the distribution of elastic, collagen and muscle fibers in the penis in domestic or wild animals. Babinski et al. (2005) studied the boar penis in order to verify the distribution of smooth muscle fibers and the volumetric density of the elastic fiber system. Elastic fibers appeared in both the corpus spongiosum and corpus cavernosum of all the animals studied. The authors suggest that the large amount of elastic fibers and the distribution of smooth muscle cells between the endothelium could represent the role that these structures would exert in the process of penile erection in boars. The zebu and bubaline penis, which is a fibroelastic type, has much of its rigidity conferred by the collagen and elastic fibers present in the extracellular matrix. Many collagen fibers were found in the corpus cavernosum and spongiosum of these penises, which is a common pattern in animals with this type of penis (Ribeiro et al. 2013). The extracellular matrix of penis in intact and neutered cats was analyzed and the findings of this study showed a significant decrease in the density of the elastic fibers and a significant increase of the density of the collagen fibers in the corpus spongiosoum in neutered cats, suggesting that hormonal deprivation caused modifications in the extracellular matrix in the penises of neutered cats (Borges et al. 2017).

The extracellular matrix formed by the collagen and elastic fibers is a dynamic cellular skeleton that plays an important role in modulating the physiological function of the cavernous tissue. This regulates cell morphology, movement, growth and differentiation. In the cavernous body, the matrix consists of a fibrillar collagen network that is closely connected to smooth muscle trabeculae (Traish & Kim 2005). Type I and II collagen are the major components of the cavernous body of the penis (Luangkhot et al. 1992, Moreland et al. 1995, Raviv et al. 1997).

The system of elastic and collagen fibers is therefore an important structure for penile erection and allows adequate resistance during the return of the penis to a detumescence (Hsu et al. 1994, Sattar et al. 1994, Da Silva & Sampaio 2002, Bastos et al. 2004, Babinski et al. 2005). Therefore, it may be considered that the extracellular matrix of the corpus cavernosum is essential for normal penile erection and is involved in erectile dysfunction. The structural description and quantification of the elements of the extracellular matrix represent a good method to evaluate the morphological and functional alterations associated with pathological conditions in humans and animal models (Siracusano et al. 1996, Maia et al. 2006)

The gross anatomy of the ram penis has been studied and described in veterinary anatomy treatises such as Dyce et al. (2010), but there is no information about the elements of the penile connective tissue in these animals. Thus, the purpose of this study was to provide morphological data on the distribution of collagen and elastic fibers in the ram penis, because is essential to know the reproductive biology and the morphology of the reproductive organs to increase animal production.

MATERIALS AND METHODS

Penises of seven rams, sexually mature, were used in this study. The animals were Santa Ines, Dorper and Texel breeds. The samples were collected after the slaughter and inspection procedures of the animals, in a slaughterhouse. After collection, the penises were sectioned in regions such as root, sigmoid flexure, penile body and glans.

The tissues were fixed in 4% paraformaldehyde for 24h. After histological fixation, tissue samples were washed for 24 hours in running water and submitted for inclusion in Paraplast™ (Sigma, St Louis/MO, USA). Following inclusion, 5µm thick sections were cut and stained in Hematoxylin and Eosin (HE), Masson's Trichrome, Calleja and Picrosirius red. The slides were observed, analyzed and photographed in the Olympus BX41 optic microscope connected to a digital camera Olympus SC30, Department of Anatomy, Institute of Biosciences of Botucatu, Unesp. The blades stained in Picrosirius red were analyzed in the polarized light microscope Leica DMLB (Leica Microsystems, Mannheim, Germany) The images were captured by a digital camera Leica DFC 300FX and processed with Leica Qwin V3 software, Department of Morphology, Institute of Biosciences of Botucatu, Unesp.

RESULTS

The ram has a fibroelastic penis, with a characteristic sigmoid flexure. There were no morphological differences, by light microscopy, between of the penile regions such as root, sigmoid flexure, and penile body. The penis was covered by a dense connective tissue capsule named tunica albuginea. The tunica albuginea involved the penile structure, which was formed by the corpus cavernosum and corpus spongiosum. The corpus cavernosum was located dorsolateral, deep into the tunica albuginea, while the corpus spongiosum enveloped the urethra (Fig.1 and 2).

The histological analysis confirmed that the extracellular matrix of the ram penis was basically formed by elastic and collagen fibers. The tunica albuginea was composed essentially of collagen fibers (Fig.1-4), which were arranged in two layers: an outer longitudinal and an inner circular (Fig.1A,B, 2C,D). The inner circular layer of the tunica albuginea projects intracavernosal septa in the corpus cavernosum. These septa consisted mainly of collagen fibers. The collagen fibers were the most abundant component of the tunica albuginea, septa, and corpus cavernosum (Fig.2 and 4).

An irregular system of elastic fibers was observed in the studied penises. Elastic fibers were intercalated between the collagen fiber bundles of the tunica albuginea and its septa. These fibers were abundant in the neurovascular bundles, around the blood vessels and nerves. There was a predominance of elastic fibers in the cavernous structure of the corpus spongiosum of the ram penis and in the neurovascular bundles

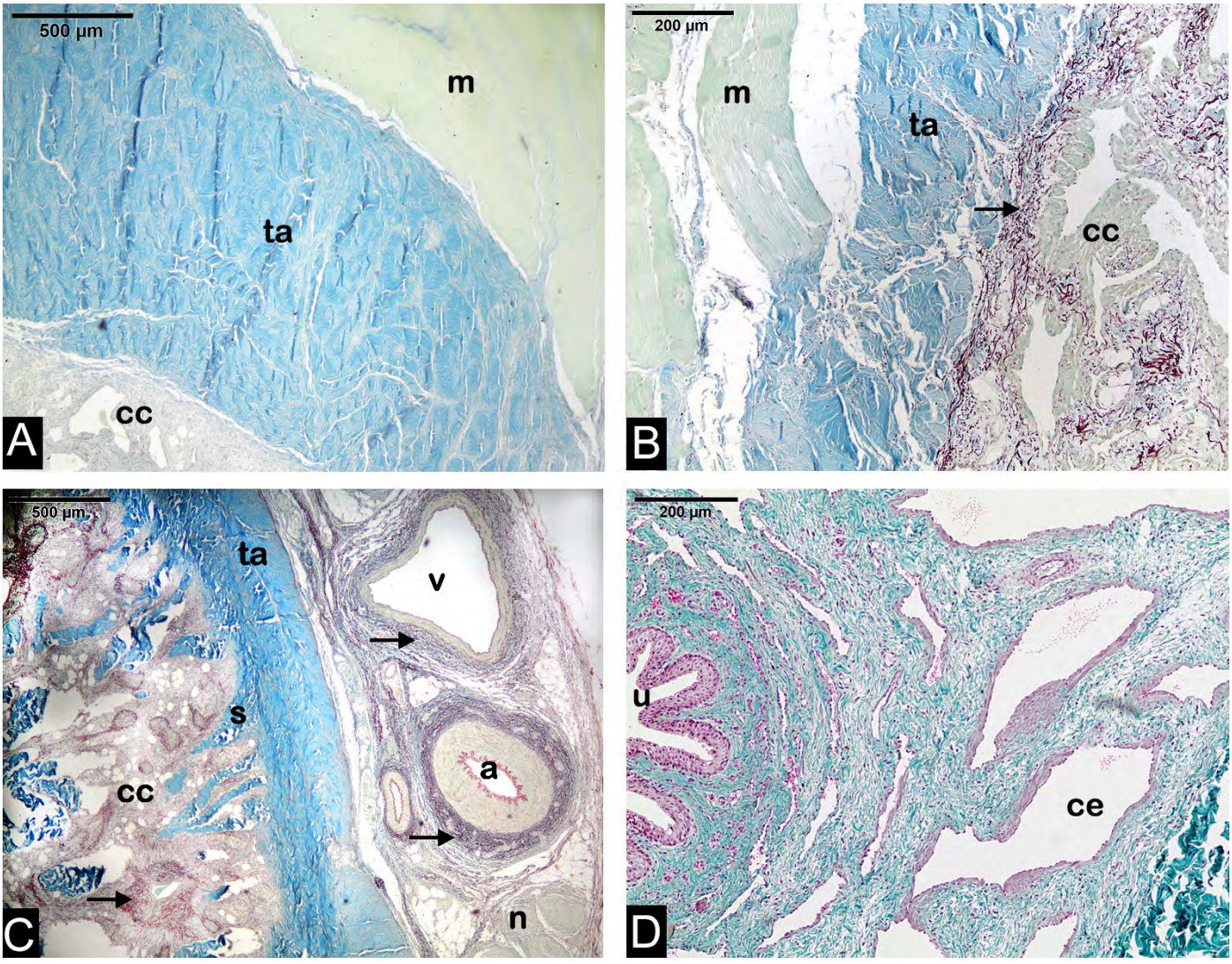


Fig.1. (**A**,**B**) Regions of the root and (**C**,**D**) the sigmoid flexure in the ram penis. Ischiocavernous muscle (m), tunica albuginea (ta), septa of tunica albuginea (s), corpus cavernosum (cc), corpus spongiosum (ce), elastic fibers (arrows), urethra (u), artery (a), vein (v) and nerve (n) that comprised the neurovascular bundle of the penis. Note the collagen fibers in blue in Calleja stain indicating that tunica albuginea consists mainly of collagen fibers. (**A**,**C**) Calleja, bar = 500µm. (**B**) Calleja, bar = 200µm. (**D**) Masson's trichrome, bar = 200µm.

(Fig.1-3). This system of elastic fibers was composed of elastic fibers that were appeared in several ways, longitudinally and transversely bundles (Fig.3). The region of the ram penile glans was formed only by a corpus spongiosum, which was formed by the system of elastic fibers (Fig.2E,F).

DISCUSSION

The ram penis contains two erectile structures, the supero-lateral corpus cavernosum and the ventral corpus spongiosum that surrounds the urethra. The wild boar penis has a similar structure with a tunica albuginea covering the corpus cavernosum and spongiosum (Babinski et al. 2005). In this study, the extracellular matrix of the ram penis was described and is formed by collagen and elastic fibers. Ribeiro et al. (2013) claimed that the fibroelastic penis of cattle and water buffalo is an organ of great interest in animal breeding, due to the presence of extracellular matrix which is involved in penile erection. Trabeculae of the corpus cavernosum represent the major morphological structure involved in penile erection. They are composed of endothelial cells, smooth muscle cells and extracellular matrix (Sattar et al. 1994, Sampaio et al. 1998, Ribeiro et al. 2013). The integrity of the corpus spongiosum is also important for the erection mechanism since it acts synergistically with the tunica albuginea and the corpus cavernosum (Conti et al. 1988, Ribeiro et al. 2013).

Penile erection occurs by the transformation of erectile tissue and vasculature from a state of minimally perfused sagging to one of engorgement with blood. This process is mediated by vascular, neuronal, hormonal, and psychological components. There is relaxation of the smooth muscles present in the blood vessels and trabeculae of the corpus cavernosum, which leads to an increase in blood flow to the penis, which compresses the venules that lie just below the

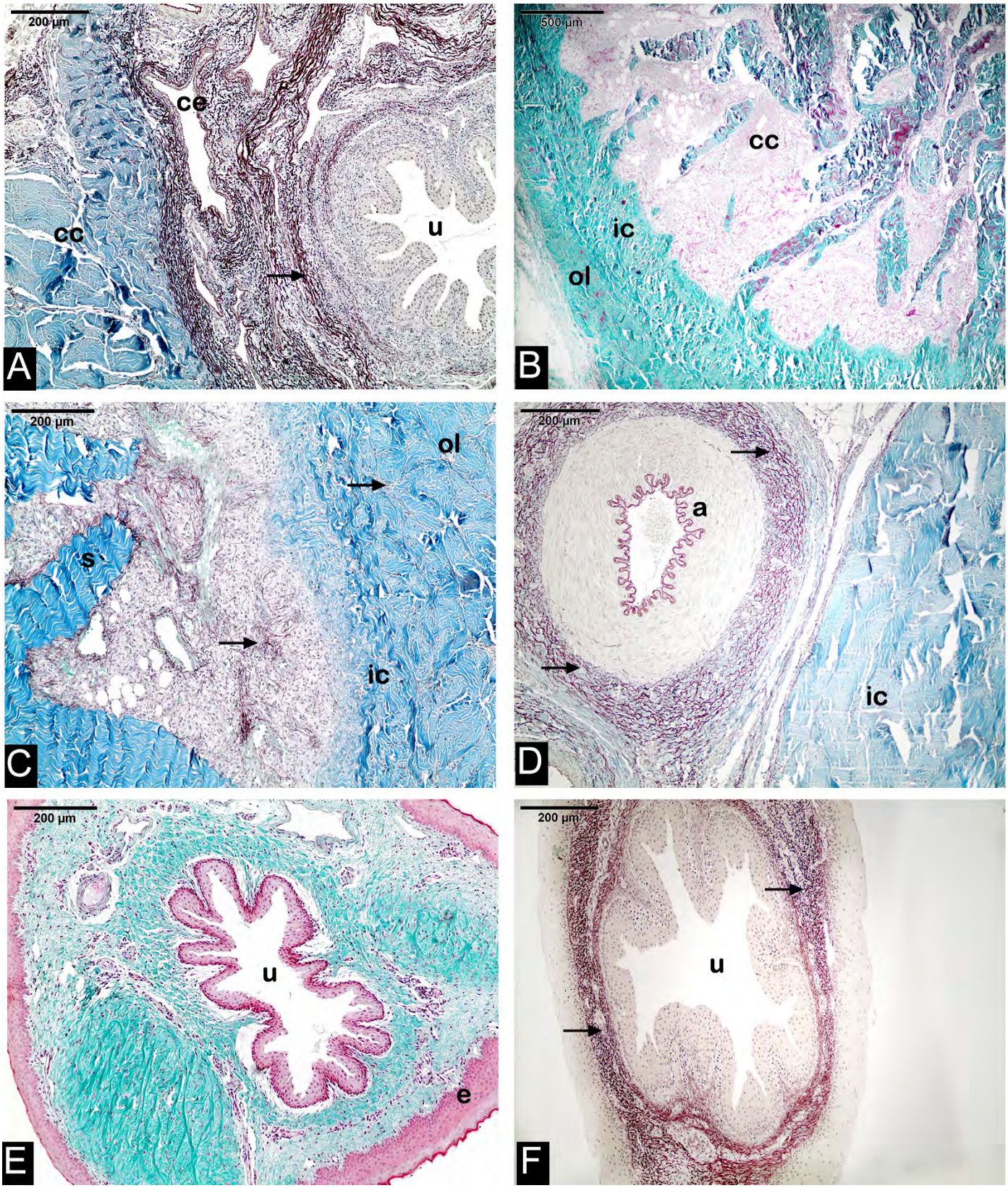


Fig.2. (**A**-**D**) Ram penile body and (**E**,**F**) glans. Corpus cavernosum (cc), corpus spongiosum (ce), elastic fibers (arrows), urethra (u), artery (a), and lining epithelium of the glans (e). The tunica albuginea are arranged in two layers: an outer longitudinal (ol) and an inner circular (ic). These two layers are comprised basically of collagen fibers (in blue in Calleja stain). (**A**,**C**,**D**,**F**) Calleja, bar = 200µm. (**B**) Masson's Trichrome, bar = 500µm. (**E**) Masson's Trichrome, bar = 200µm..

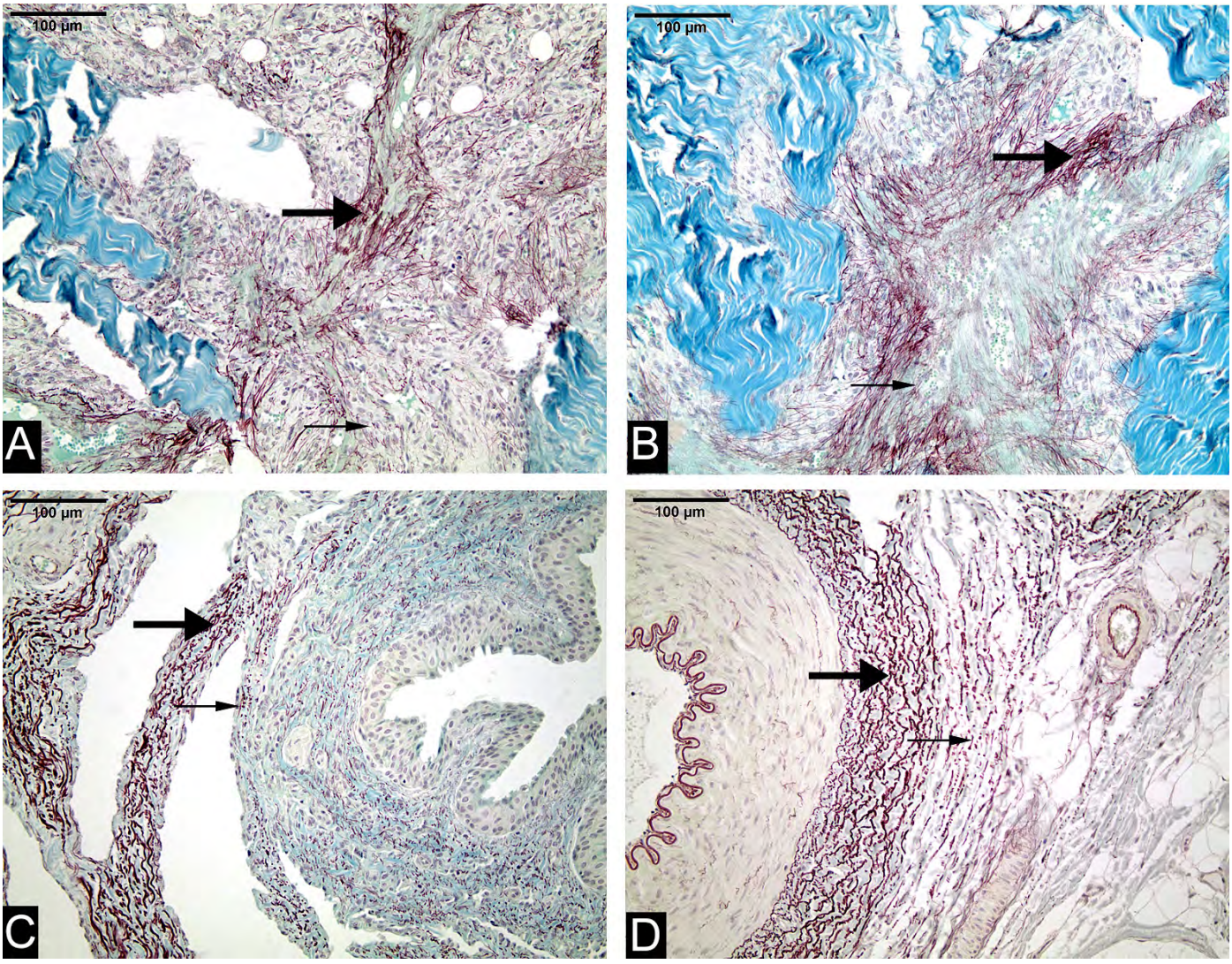


Fig.3. (**A-D**) Elastic fibers in the region of the body of the ram penis. Elastic fibers arranged transversely (thick arrows) and longitudinally (thin arrows). Note the collagen fibers in blue. Thus, the extracellular matrix of the ram penis is formed by elastic and collagen fibers. Calleja, bar = 100µm.

tunica albuginea against it, leading to drainage occlusion (Mostafa et al. 2013).

The ram tunica albuginea was divided into two layers: an outer longitudinal and an inner circular, which is in agreement with that reported for bovine and buffalo penises (Ribeiro et al. 2013). The collagen fibers appear mainly in the two layers of the tunica albuginea and in their septa, which is also similar to that described for the tunica albuginea of the corpus cavernosum of cattle and buffalo (Ribeiro et al. 2013). The organization of collagen fibers in the tunica albuginea of ram are in agreement with reports on the cattle and water buffalo penis (Ribeiro et al. 2013). The tunica albuginea, a connective tissue sheath which surrounds the cavernous body, also plays an important role in erection, and is essential for penile compliance, venous occlusion, flexibility, extensibility, and rigidity (Brock et al. 1997).

Pinheiro et al. (2000) have been studied the structure of the corpus cavernosum of the rat penis, and reported that the collagen fibers represented the main component of the trabeculae of the corpus cavernosum of the mouse penis. These authors claimed that the extracellular matrix of the corpus cavernosum is essential for the normal erection of the penis and seems to be related to several erectile dysfunctions.

The ram penis is a fibroelastic penis. The elastic fiber system appeared in the corpus spongiosum and corpus cavernosum. The boar penis had a well developed elastic fiber system in the corpus spongiosum and cavernosum, but there was a higher volume density of these fibers in the corpus spongiosum when compared to the corpus cavernosum (Babinski et al. 2005). In this study, the elastic fibers, besides constituting the extracellular matrix of the cavernous tissue, are also abundant near the blood vessels and nerves. Several studies reported that tissues, normally submitted to stretching, present a large amount of elastic fibers (Cotta-Pereira et al. 1976, Kreis & Vale 1993, Ribeiro et al. 2013), and this could explain the presence of abundant elastic fibers in the cavernosus structure of the ram penis.

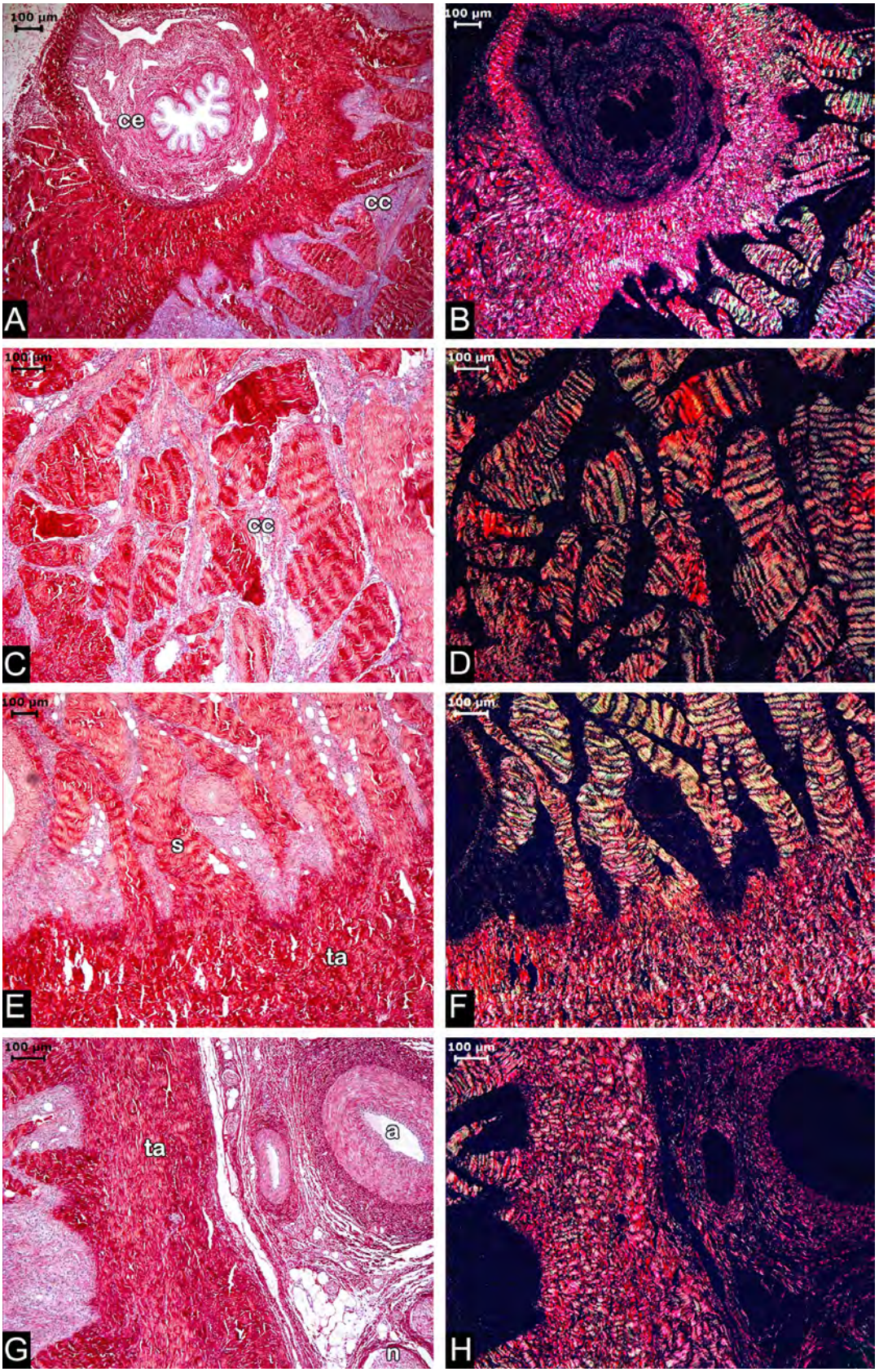


Fig.4. (**A**-**H**) Corpus spongiosum (ce), corpus cavernosum (cc) and tunica albuginea (ta) of ram penis. Note the great amount of collagen fibers in the corpus cavernosum (cc), and in the septa (s) of the tunica albuginea (ta). Neurovascular bundles with artery (a) and nerve (n). (**A**,**C**,**E**,**G**) Picrosirius Red under conventional light and (**B**,**D**,**F**,**H**) under polarized light, bar = 100µm.

CONCLUSION

The arrangement of the elastic and collagen fibers in the ram penis suggests that this animal shows a typical extracellular matrix corresponding to the fibroelastic penis, similar to other mammals such as the boar, bull and buffalo.

Like these fibers play an important role in penile erection and detumescence, this morphological knowledge can contribute to the reproduction routine in veterinary medicine.

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REFERENCES

Abidu-Figueiredo M., Costa W.S., Chagas M.A., Sampaio F.J.B. & Cardoso L.E.M. 2013. Age-related changes in the concentration of elastic fibers in different regions of the rabbit penis. Acta Cir. Bras. 28(5):378-384. <http://dx.doi.org/10.1590/S0102-86502013000500010> <PMid:23702941>

Babinski M.A., Brito-Gitirana L., Chagas M.A., Abidu-Figueiredo M., Costa W.S. & Sampaio F.J.B. 2005. Immunohistochemical analysis of smooth muscle cells and volumetric density of the elastic system fibers of wild boar (*Sus scrofa*) penis. Anim. Reprod. Sci. 86(3/4):317-328. <http://dx.doi.org/10.1016/j.anireprosci.2004.08.002> <PMid:15766809>

Bastos A.L., Silva E.A., Costa W.S. & Sampaio F.J.B. 2004. Concentration of elastic fibers in the male urethra during human fetal development. BJU Int. 94(4):620-623. <http://dx.doi.org/10.1111/j.1464-410X.2004.05012.x> <PMid:15329125>

Borges N.C.S., Pereira-Sampaio M.A., Pereira V.A., Abidu-Figueiredo M. & Chagas M.A. 2017. Effects of castration on penile extracellular matrix morphology in domestic cats. J. Feline Med. Surg. 19(12):1261-1266. <http://dx.doi.org/10.1177/1098612X16689405> <PMid:28176530>

Brock G., Hsu G.L., Nunes L., Von Heyden B. & Lue T.F. 1997. The anatomy of the tunica albuginea in the normal penis and Peyronie's disease. J. Urol. 157(1):276-281. <http://dx.doi.org/10.1016/S0022-5347(01)65359-X> <PMid:8976279>

Conti G., Virag R. & von Niederhäusern W. 1988. The morphological basis for the polster theory of penile vascular regulation. Acta Anat. 133(3):209-212. <http://dx.doi.org/10.1159/000146641> <PMid:3227779>

Cotta-Pereira G., Rodrigo G. & Bittencourt-Sampaio S. 1976. Oxylatan, elaunin and elastic fibers in the human skin. J. Investigative Dermatol. 66(3):143-148. <http://dx.doi.org/10.1111/1523-1747.ep12481882> <PMid:1249442>

Dyce K.M., Sack W.O. & Wensing C.J.G. 2010. Tratado de Anatomia Veterinária. 4ª ed. Elsevier, Rio de Janeiro, p.193-195.

Hafez E.S.E. & Hafez B. 2004. Reprodução Animal. 7ª ed. Manole, São Paulo, p.3-10.

Hsu G.-L., Brock G., Heyden B.V.O.N., Nunes L., Lue T.F. & Tanagho E.A. 1994. The distribution of elastic fibrous elements within the human penis. BJU Int. 73(5):566-571. <http://dx.doi.org/10.1111/j.1464-410X.1994.tb07645.x> <PMid:8012781>

Kreis T. & Vale R. 1993. Guidebook to the Extracellular Matrix and Adhesion Proteins. Oxford University Press, Oxford, p.40-51.

Luangkhot R., Rutchik S., Agarwal V., Puglia K., Bhargava G. & Melman A. 1992. Collagen alterations in the corpus cavernosum of men with sexual dysfunction. J. Urol. 148(2 Pt 1):467-471. <http://dx.doi.org/10.1016/S0022-5347(17)36630-2> <PMid:1635159>

Maia R.S., Babinski M.A., Figueiredo M.A., Chagas M.A., Costa W.S. & Sampaio F.J. 2006. Concentration of elastic system fibers in the corpus cavernosum, corpus spongiosum, and tunica albugínea in the rabbit penis. Int. J. Impot. Res. 18(2):121-125. <http://dx.doi.org/10.1038/sj.ijir.3901404> <PMid:16224492>

Moreland R.B., Traish A., McMillin M.A., Smith B., Goldstein I. & Saenz de Tejada I. 1995. PGE1 supresses the induction of collagen synthesis by transforming growth factor-beta 1 in human corpus cavernosum smooth muscle. J. Urol. 153(3 Pt 1):826-834. <PMid:7861547>

Mostafa M.E., Senbel A.M. & Mostafa T. 2013. Effect of chronic low-dose Tadalafil on penile cavernosous tissues in diabetic rats. Urology 81(6):1253-1259. <http://dx.doi.org/10.1016/j.urology.2012.12.068> <PMid:23726447>

Pinheiro A.C.A.D., Costa W.S., Cardoso L.E.M. & Sampaio F.J.B. 2000. Organization and relative contento f smooth muscle cells and extracellular matrix components in the corpus cavernosum of the rat penis. J. Urol. 164:1802-1806. <http://dx.doi.org/10.1016/S0022-5347(05)67110-8> <PMid:11025773>

Raviv G., Kiss R., Vanegas J.P., Petein M., Danguy A., Schulman C. & Wespes E. 1997. Objective measurement of the different collagen types in the corpus cavernosum of potent and impotent men: an immunohistochemical staining with computerized-image analysis. World J. Urol. 15(1):50-55. <http://dx.doi.org/10.1007/BF01275157> <PMid:9066095>

Ribeiro I.C.A., Abidu-Figueiredo M., Costa F.B., Pereira-Sampaio M.A. & Chagas M.A. 2013. Stereological study of the elastic fiber and smooth muscle cell system in the bovine and buffalo penis. Pesq. Vet. Bras. 33(Suppl. 1):107-112. <http://dx.doi.org/10.1590/S0100-736X2013001300017>

Sampaio F.B., Pinto J.F., Silva-Costa W. & Pereira M.S. 1998. Quantitative analysis of smooth muscle fibers in corpus cavernosum of human fetuses. J. Urol. 159(6):2226-2228. <http://dx.doi.org/10.1016/S0022-5347(01)63310-X> <PMid:9598574>

Sattar A.A., Wespes E. & Schulman C.C. 1994. Computerized measurement of penile elastic fibers in potent and impotent men. Eur. Urol. 25(2):142-144. <http://dx.doi.org/10.1159/000475269> <PMid:8137855>

Silva E.A. & Sampaio F.J.B. 2002. Uretral extensibility applied to reconstructive surgery. J. Urol. 167(5):2042-2045. <http://dx.doi.org/10.1016/S0022-5347(05)65079-3> <PMid:11956434>

Siracusano S., Bosincu L., Marras V., Pisano I., Deriu M., Marceddu S., Trignano M. & Belgrano E. 1996. Preliminary reports on morphological and ultrastructural changes in the corpora cavernosa of the rat after chronic arterial obstruction. Arch. Esp. Urol. 49(2):191-195. <PMid:8702334>

Traish A.M. & Kim N.N. 2005. The physiological role of androgens in penile erection: Regulation of corpus cavernosum structure and function. J. Sexual Med. 2(6):759-770. <http://dx.doi.org/10.1111/j.1743-6109.2005.00094.x> <PMid:16422801>

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Histological characterization of reproductive tract and fetal annexes of the West Indian Manatee (*Trichechus manatus*) from Brazil[1]

Arielle R. Bezerra[2], Carminda S.B. Salmito-Vanderley[2], Paulo R.O. Bersano[2], Vitor L. Carvalho[3], Ana C.O. Meirelles[3], Fernanda L.N. Attademo[4], Fábia O. Luna[4] and Lúcia D.M. Silva[2]*

ABSTRACT.- Bezerra A.R., Salmito-Vanderley C.S.B., Bersano P.R.O., Carvalho V.L., Meirelles A.C.O., Attademo F.L.N., Luna F.O. & Silva L.D.M. 2018. **Histological characterization of reproductive tract and fetal annexes of the West Indian Manatee (*Trichechus manatus*) from Brazil.** *Pesquisa Veterinária Brasileira 38(11):2166-2174*. Universidade Estadual do Ceará, Avenida Dr. Silas Munguba 1700, Campus do Itaperi, Fortaleza, CE 60714-903, Brazil. E-mail: lucia.daniel.machado@hotmail.com

The West Indian manatee (*Trichechus manatus*) is one of the most threatened aquatic mammals in Brazil, and is currently classified as "endangered" (MMA). The objective of this study was to characterize histologically the reproductive tract and fetal annexes of stranded manatees in northeastern Brazil. Tissue samples were collected from the reproductive tract of 23 manatees, which were fixed in 10% buffered formalin, processed using standard histological protocols and stained with hematoxylin eosin. We qualitatively described the histological and histomorphometric characteristics of each structure. Six ovaries were analyzed. In four ovaries, we found a large number of primordial and primary follicles. Two ovaries were different from the others: one had inflammatory infiltration and the other had a thickening in the cortex and absence of follicles. We also analyzed seven uteri (of which four were in the proliferative phase, two in the secretory phase, and one in the recovery phase), four placentas, one vagina, six testes (four were in the immature phase, one in the pubertal phase, and one in the mature phase), two epididymides, two penises, and one umbilical cord. The histological and morphometric findings in our work will support future analyses of the reproductive tract of *T. manatus* from Brazil.

INDEX TERMS: Histology, reproductive tract, fetal annexes, West Indian Manatee, *Trichechus manatus*, Brazil, sirenian, reproductive biology, embryonic annexes.

RESUMO.- [**Caracterização histológica do trato reprodutor e anexos fetais do peixe-boi marinho (*Trichechus manatus*) do Brasil.**] O peixe-boi marinho (*Trichechus manatus*) é um dos mamíferos aquáticos mais ameaçados do Brasil e, atualmente é classificado como "em perigo" (MMA). O objetivo deste estudo foi caracterizar histologicamente o trato reprodutor e os anexos fetais de peixes-bois marinhos encalhados no Nordeste do Brasil. Foram coletadas amostras de tecidos do trato reprodutor de 23 peixes-bois marinhos (*T. manatus*), que foram fixadas em formol tamponado a 10%, processados usando protocolos histológicos padrão e corados com hematoxilina eosina. Foi realizada a descrição qualitativa das características histológicas e histomorfométricas de cada estrutura. Foram analisados seis ovários. Em quatro ovários, foi encontrado um grande número de folículos primordiais e primários. Dois ovários eram diferentes dos outros: um tinha infiltração inflamatória e o outro tinha um espessamento no córtex e ausência de folículos. Também foram analisadas sete amostras uterinas (das quais quatro estavam na fase proliferativa, duas na fase secretória e uma

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[2] Post-Graduate Program in Veterinary Science, Universidade Estadual do Ceará, Avenida Dr. Silas Munguba 1700, Campus do Itaperi, Fortaleza, CE 60714-903, Brazil. *Corresponding author: lucia.daniel.machado@hotmail.com

[3] Associação de Pesquisa e Preservação de Ecossistemas Aquáticos (Aquasis), Av. José Alencar 150, Praia de Iparana, Caucaia, CE 61627-210, Brazil.

[4] Centro Nacional de Pesquisa e Conservação de Mamíferos Aquáticos (CMA-ICMBio), Estrada do Forte Orange s/n, Pilar, Ilha de Itamaracá, PE 53900-000, Brazil.

na fase de recuperação), quatro placentas, uma vagina, seis testículos (quatro na fase imatura, um na fase puberal e um na fase madura), dois epidídimos, dois pênis e um cordão umbilical. Os achados histológicos e morfométricos em nosso trabalho apoiarão futuras análises do trato reprodutivo de *T. manatus* do Brasil.

TERMOS DE INDEXAÇÃO: Histologia, trato reprodutor, anexos fetais, peixe-boi marinho, *Trichechus manatus*, Brasil, sirênio, biologia reprodutiva, anexos embrionários.

INTRODUCTION

The population of manatees (*Trichechus manatus*) in the Brazilian Northeast has been estimated to range from 485 to 2,221 individuals, based on overflight measurements, which indicates that there are few manatees in this region (Alves et al. 2016). This species represents one of the most endangered aquatic mammals in Brazil, and is classified as "endangered" in the Official National List of Endangered Species (Brasil 2014).

Some issues faced by the manatee population of the Brazilian Northeast include the rapid growth of anthropogenic activities in the coastal zones, habitat destruction owing to the loss of mangroves, estuarine sedimentation (leading females to have their offspring in more agitated waters, risking the cubs to be carried away by tides and stranded on beaches), water pollution, and debris ingestion; because manatees have coastal habits and this area has a higher concentration of litter (Parente et al. 2004, Meirelles 2008, Lima et al. 2011, Attademo et al. 2015).

To develop effective conservation strategies for endangered species, such as the manatee, it is crucial to understand their life history and reproductive biology (Marsh et al. 2011). Studies on manatees from the United States, where a large number of dead animals were evaluated, resulted in an analysis of the reproductive tracts of males and females (Hernandez et al. 1995, Marmontel 1995). According to these authors, male manatees present a vascular penis that retracts when not engorged, do not have a crotch, have intra-abdominal testicles (with a ventrolateral position to the kidneys), and bilateral seminal vesicles, which are in a dorsal position in relation to the urinary bladder. However, the prostate is composed of erectile muscle tissue (Reynolds III et al. 2002).

Regarding the females, their reproductive system is composed of ovaries, uterine tubes, uterus, vagina, vaginal vestibule, and clitoris. The mammary glands also contribute to the reproductive process. Female manatees have a bicornuate uterus, and with a zonary (anatomical classification) and endotheliochorial (histological classification) placenta (Rodrigues et al. 2008).

Although this species has been studied for more than three decades, research on its reproductive system has not yet been performed in Brazil. The histological evaluation of the reproductive system has been carried out by the analysis of samples collected from stranded dead animals. However, access to these types of samples has been limited in Brazil because as the small size of the population, the high frequency of newborn stranding, the stage of decomposition of dead stranded individuals and the storage of samples in the collections (Meirelles & Carvalho 2016).

Thus, the objective of this study was to characterize the reproductive tract and fetal annexes of male and female manatees stranded in northeastern Brazil to support future studies on the age of sexual maturation in these animals.

MATERIALS AND METHODS

Ethical aspects. This experiment was approved by the Ethics Committee for Animal Use of the State University of Ceará (number 0971110/2016) and the Biodiversity Authorization and Information System (SISBIO)/Chico Mendes Institute for Biodiversity Conservation (ICMBio, license number 51456-3).

Animals. We analyzed tissue samples from the reproductive tracts of 23 manatees (*Trichechus manatus* Linnaeus, 1758), including 14 females and 9 males of different age groups. These animals were stranded, died in rehabilitation, or gave birth in captivity from July 1995 to October 2016 (Tables 1 and 2). Manatees were necropsied according to conventional protocols (Geraci et al. 2005). Samples were included in the biological collections of the Association for Research and Preservation of Aquatic Ecosystems (Aquasis) in Caucaia, Ceará and the National Center for Research and Conservation of Aquatic Mammals (CMA/ICMBio) in Itamaracá, Pernambuco.

Histological processing. Tissues from the reproductive tracts were fixed in 10% buffered formalin. We collected tissue samples approximately 2cm-thick and processed them using standard histological protocol before histological staining with hematoxylin eosin (HE). Slides containing the processed samples were analyzed using light microscopy. During the histological processing, samples were dehydrated through successive washing in alcohol solutions with gradually increased concentrations (70, 80, 95, and 100%). Samples were washed for 50 min with each solution and then maintained in an alcohol/xylol solution for 24h. Thereafter, samples were diaphanized, where they were successively placed in two xylol solutions for 50 min each. They were then subjected to an inclusion process, in which samples were impregnated with two sets of paraffin for 50 min at 60°C. We then embedded the biological material in histological paraffin.

The embedded samples were cut into 5µm-thick sections using a histological resin microtome (Leica RM 2235). Sections were then immersed in a histological water bath at a mean temperature of 45°C and collected on slides pre-prepared with albumin. The slides containing the sections were maintained in an oven (MD 1.2) for sterilization and drying. Next, the slides were histologically stained with hematoxylin eosin. Fragments of testis, penis, umbilical cord, and placenta tissue were later stained with Masson's trichrome.

After this procedure, slides were analyzed using light microscopy and were photomicrographed (Nikon Eclipse 80i) using a digital photomicrographic system (Nikon NIS-Elements Advanced Research). During the analysis, we qualitatively described the histological and histomorphometric characteristics of each structure: a) ovaries (cortex thickness, largest and smallest diameter of the follicles with an observable oocyte); b) uterus (thickness of mucosa and endometrium); c) vagina (thickness of the vaginal epithelium), and d) testicles (major and minor diameter of the seminiferous tubules). The analysis was performed using ImageJ software, with an average of ten measurements for each structure.

Statistical analysis. Subjective data were presented in descriptions, whereas objective data were expressed as mean ± standard deviation.

Table 1. Information on the female manatees (*Trichechus manatus*) analyzed

Register number	Date of stranding or death	Place of stranding	Total length (meters)	Age group	Samples
02S0112/31	14/11/2005	Fontaínhas, Aracati/CE	3.15	Adult	Uterus
02S0112/36	26/09/2006	Between Redonda and Peroba, Icapuí/CE	3.07	Adult	Ovary
02S0112/38	25/02/2007	Retiro Grande, Icapuí/CE	3.18	Adult	Uterus
02S0112/57	19/10/2012	Canto Verde, Beberibe/CE	1.20	Calf	Uterus
02S0112/60	21/09/2013	Retirinho, Aracati/CE	2.41	Adult	Ovary
02S0112/77	17/10/2016	Ponta Grossa, Icapuí/CE	3.18	Adult	Ovary
08S0112/20	24/03/2015	Alagamar, Grossos/RN	1.20	Calf	Ovary and uterus
01S0112/02	21/01/2008	Barro Preto/CE	3.07	Adult	Placenta
01S0112/07	08/06/2007	Ponta de Pedra/PE	2.90	Adult	Placenta
01S0112/08	30/07/2008	Barra de Sucatinga, Beberibe/CE	2.89	Adult	Placenta
01S0112/12	28/07/1995	Jacarecica, Maceió/AL	1.08	Calf	Ovary, uterus and vagina
01S0112/25	11/04/2008	Born in captivity, 10/4/97, PE	2.59	Adult	Placenta
01S0112/30	10/11/1999	Barra de Camaratuba/PB	1.36	Calf	Uterus
01S0112/31	02/03/1998	Pitimbi/PB	-	-	Ovary and uterus

The age group was defined according to Marmontel (1995).

Table 2. Information on the male manatees (*Trichechus manatus*) analyzed

Register number	Date of stranding or death	Place of stranding	Total length (meters)	Age group	Samples
02S0111/42	13/02/2008	Redonda, Icapuí/CE	1.19	Calf	Testicles
02S0111/45	10/10/2008	Parajuru, Beberibe/CE	1.15	Calf	Testicles
02S0111/55	23/09/2011	Ponta Grossa, Icapuí/CE	2.01	Juvenile	Testicles
02S0111/59	23/03/2013	Manibu, Icapuí/CE	1.08	Calf	Testicles and epididymis
02S0111/71	05/03/2015	Ariós, Beberibe/CE	1.35	Calf	Epididymis
02S0111/73	03/10/2015	Ponta Grossa, Icapuí/CE	3.01	Adult	Testicles and penis
01S0111/123	04/11/2002	Born in captivity/PE	1.06	Calf	Testicles
01S0111/180	24/12/2004	Barretas, Nisia Floresta/RN	2.72	Adult	Penis
01S0111/304	16/11/2011	Born in captivity/PE	0.79	Calf	Umbilical cord

The age group was defined according to Marmontel (1995).

RESULTS

We analyzed six ovaries, two of which were different from the others. We also analyzed seven uteri, four placentas, one vagina, six testicles, two epididymides, two penises, and one umbilical cord. These tissues were collected from manatees of different sizes and age groups (Table 1 and 2).

Ovaries

The ovary was coated with a serum layer consisting mainly of simple cubic epithelium, which, in some cases, had features of pseudostratified epithelium or small areas of columnar epithelium (Fig.1A).

The ovarian cortex consisted of a narrow band (Table 3) with the presence of follicles surrounded by supporting tissue. In manatees 08S0112/20, 01S0112/12, and 01S0112/31, there was a predominance of primordial follicles, but some primary follicles were also observed (Fig.1B). A secondary and a tertiary follicle were also observed in manatee 01S0112/31. In the ovary fragment of manatee 02S0112/60, we detected primordial, primary, secondary, tertiary, and Graafian follicles (Fig.1C).

This organ contained a large area of bone marrow (approximately 80% of the ovary), which consisted of loose connective tissue with many adipocytes and was richly and diffusely vascularized with vessels of different calibers (Fig.1D).

At the time of the necropsy of manatee 02S0112/36, the ovaries were not found. However, we observed the presence of tissue in the left antimer, with discrete signals indicative of possible ovarian tissue. We did not find similar tissue or ovarian tissue in the right antimer. In the ovary of manatee 02S0112/36, the cortex presented a thicker region (403,882μm) than in the ovaries of the other analyzed manatees, containing almost no follicle. In addition, this ovary presented considerable connective tissue in the supporting tissue of the cortex, where we observed a band with an intense increase in the cellularity composed of dense connective tissue and another band with less cellularity composed of loose connective tissue (Fig.1E). The ovarian marrow in manatee 02S0112/36 was similar to that observed in the ovaries of the other analyzed manatees.

In the ovary of manatee 02S0112/77 (supposedly an adult female), we found a large number of blood vessels, histiocytic inflammatory infiltrate (predominance of macrophages),

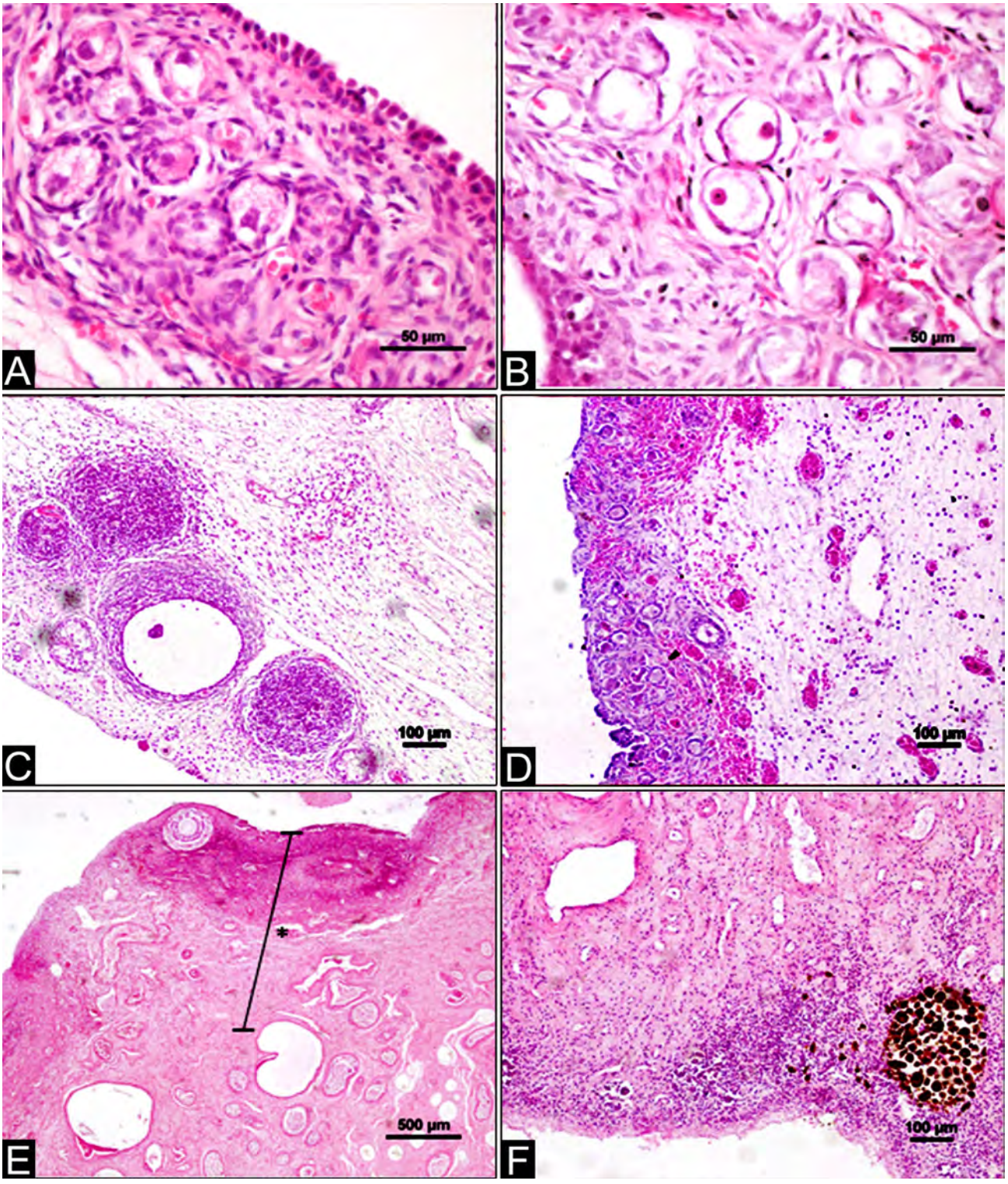


Fig.1. Ovary of *Trichechus manatus*. (**A**) Narrow cortex and germinal epithelium. HE, obj.40x. (**B**) Primary follicles. HE, obj.40x. (**C**) Tertiary follicle. HE, obj.10x. (**D**) Narrow cortex and large marrow area, highly vascularized. HE, obj.10x. (**E**) Altered ovary of manatee 02S0112/36 with thickened cortex. HE, obj.40x. (**F**) Ovary of manatee 02S0112/77 with an inflammatory infiltrate. HE, obj.10x.

multifocal necrosis, coalescent and congested vessels, congestion areas in the periphery, and the presence of some follicles (primary and secondary), which were in an advanced degeneration state (Fig.1F).

Uterus

The uterine mucosa was lined with simple cylindrical epithelium followed by endometrial glands with the same coating, in a perpendicular position to the mucosa. The submucosa

Table 3. Mean ± standard deviation (SD) of the epithelium, cortex, primordial follicle major diameter (PFMa), primordial follicle minor diameter (PFMi), primary follicle major diameter (PaFMa), primary follicle minor diameter (PaFMi), secondary follicle major diameter (SFMa), and secondary follicle minor diameter (SFMi) measured in µm in female manatees (*Trichechus manatus*)

Ovary	Epithelium	Cortex	PFMa	PFMi	PaFMa	PaFMi	SFMa	SFMi
01S0112/12	18.262	192.438	43.149	38.162	42.127	39.927	ND	ND
08S0112/20	12.592	197.413	42.583	34.218	51.648	45.825	ND	ND
01S0112/31	9.983	200.277	43.274	35.250	55.656	41.880	ND	ND
02S0112/60	12.346	291.739	45.260	37.087	51.195	47.580	124.960	95.270
02S0112/36	16.364	403.882	ND	ND	ND	ND	ND	ND
02S0112/77	10.544	200.050	ND	ND	ND	ND	ND	ND
Mean ± SD	13.348 ± 3.287	247.633 ± 85.363	43.567 ± 1.126	36.179 ± 1.776	50.156 ± 5.716	43.803 ± 3.516	124.96 ± 0	95.27 ± 0

ND = no data.

was composed of abundant fibrocellular and fibrovascular tissue (Table 4). The muscular layer was composed of three layers, longitudinal, circular, and longitudinal. The serosa was composed of mesothelium-lined connective tissue. The uteri of the manatees 08S0112/20, 01S0112/12, 01S0112/31, and 02S0112/31 were in the proliferative phase (Fig.2A). The uterus of the manatees 01S0112/30 and 02S0112/57 were in the secretory phase (Fig.2B), with glands opening into the lumen of the organ. The uterus of the manatee 02S0112/38 was in the recovery phase (Fig.2C).

Table 4. Mean ± SD of mucosal and endometrium thickness in female manatees (*Trichechus manatus*)

Uterus	Mucosal (µm)	Endometrium (µm)
01S0112/12	22.40	1338.34
08S0112/20	19.03	1282.34
01S0112/30	13.91	1221.82
01S0112/31	14.27	1141.44
02S0112/38	18.04	1282.11
02S0112/57	23.86	ND
Mean ± SD	18.585 ± 4.083	1253.21 ± 74.848

ND = no data.

Placenta

In the placenta, we detected the presence of an artery in the fetal chorion, and a vein. We also observed a well-developed villus region (lacunar region with the presence of a syncytial region, syncytiotrophoblast), and the presence of maternal tissue cells with chorionic villi (conjunctival and mesoderm). We also observed connective tissue filled with blood and maternal tissue (Fig.2D).

Vagina

The vagina presented a mucosa consisting of keratinized squamous epithelium (hyperkeratosis) with an average length of 267.1115µm. The submucosa was composed of very thick and vascularized connective tissue, and areas containing longitudinal muscular glands that were also vascularized (Fig.2E).

Umbilical cord

This structure presented three vessels (two arteries, one vein) supported by loose mucous connective tissue, or Wharton jelly. These two arteries had a histological structure similar to that of other mammals, but with little definition in the middle layer. All were coated by common endothelium and exhibited an adventitious layer that was continuous with the surrounding tissue (Fig.2F).

Testicles

Manatees 02S0111/42, 02S0111/59, and 01S0111/123 were characterized as newborns owing to their body size (1.19, 1.08, and 1.06m, respectively). Their testes had a structure separated by connective tissue septa, which was richly vascularized with small seminiferous tubules and had sparse cell content, namely spermatogonia and Sertoli cells. The intralobular connective tissue was rich in spindle cells that sometimes formed concentric blades around the tubules (Fig.3A).

Manatee 02S0111/45 was also classified as a newborn (1.15m), and the general structure of its testis was the same as that of the previously described animals, with a similar general organization of the seminiferous tubules, which were only different in size (Table 5). The intralobular stroma cells did not appear to be predominantly spindle-shaped and had a globular aspect (Fig.3B).

Manatee 02S0111/55 was classified as a juvenile manatee according to the size (2.01m), and the general structure of the testis was also similar to the previous ones. However, there was a clear change in the structure of the lobe, as the seminiferous tubules were similar in size to those of manatee 02S0111/45, but with an evidently increased cell population and presence of spermatogonia, spermatocytes I and II, being characterized as an immature animal. The intralobular stroma had the appearance of mature connective tissue with observable clusters of Leydig cells that were not previously seen in the other animals (Fig.3C).

Manatee 02S0111/73 was considered an adult (3.01m) and the structure of his testis resembled that of other adult mammals. The seminiferous tubules presented a mature and organized production of spermatogonia, spermatocytes I and II, and spermatids with evident spermiogenesis, as confirmed by the presence of spermatozoa in the lumen of the seminiferous tubule. The intralobular stroma was loose and exhibited small clusters of mature Leydig cells (with vacuolized cytoplasm, Fig.3D).

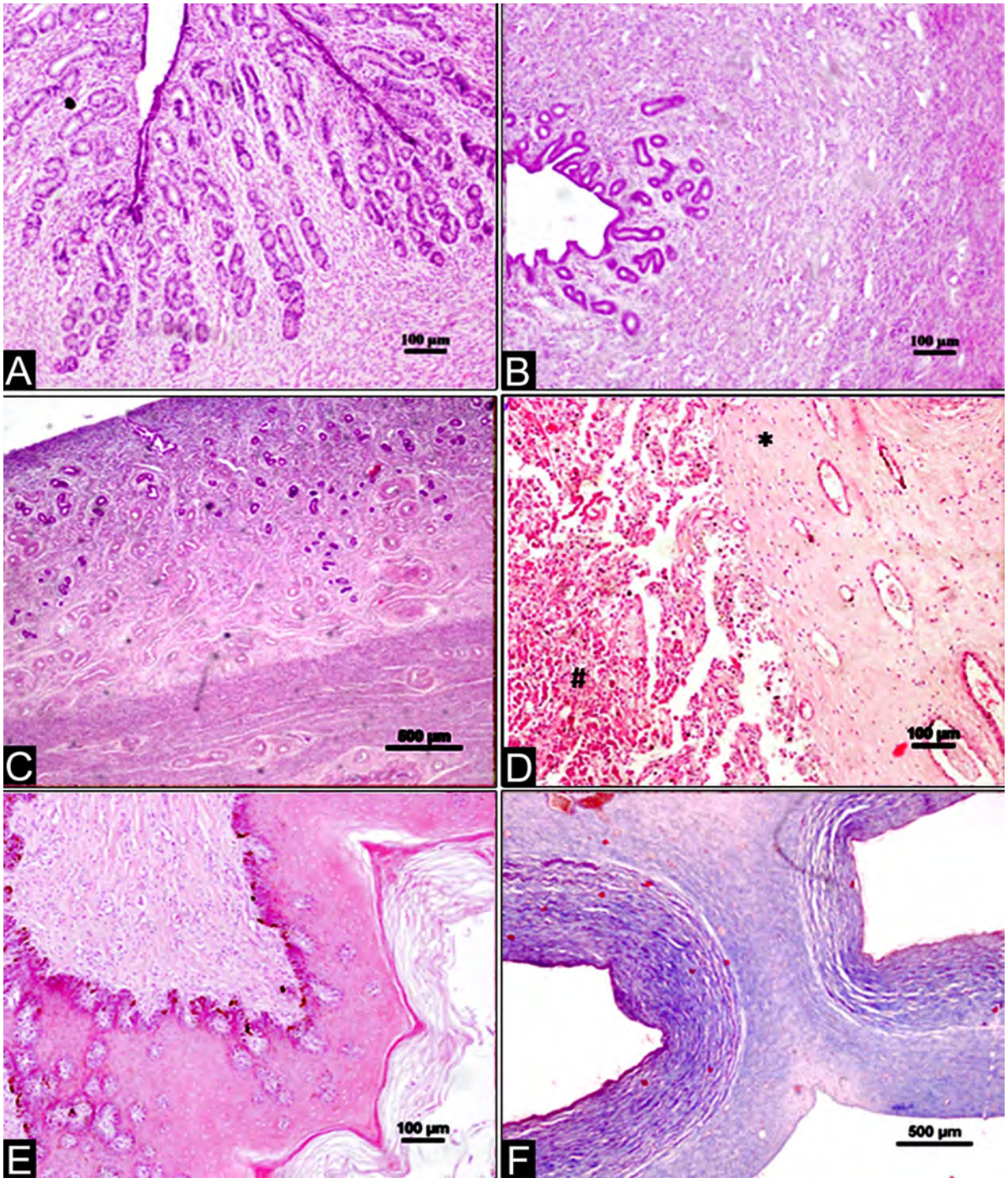


Fig.2. Uteri, placenta, vagina and umbilical cord of *Trichechus manatus*. (**A**) Uterus in the proliferative phase, with many endometrial glands. HE, obj.40x. (**B**) Uterus in the secretory phase, with large endometrial glands opening to the light. HE, obj.10x. (**C**) Uterus in the recovery phase. HE, obj.40x. (**D**) Placenta with chorionic villi (#) and syncytiotrophoblast (*). HE, obj.10x. (**E**) Vagina with a keratinized squamous epithelium. HE, obj.10x. (**F**) Umbilical cord showing the two arteries. Masson's trichrome, obj.40x.

Epididymis

In manatees 02S0111/59 and 02S0111/71, we found multiple tubular structures composed of multiple channels with variable dilation light, coated with pseudo-stratified epithelium and stereocilia, and some portions contained intraepithelial cysts. The wall was composed of abundant fibromuscular tissue, but the stroma contained loose fibromuscular tissue (Fig.3E). Manatee 02S0111/59 had a less obvious fibromuscular structure.

Penis

Manatees 02S0111/73 and 01S0111/123 presented multiple endothelium-coated vascular channels with variable dilation, filled with blood, in abundant fibromuscular support tissue

Table 5. Mean values of the major diameter (STMa) and minor diameter (STMi) of the seminiferous tubules and mean diameter of the spermatogonia (DS) in male manatees (*Trichechus manatus*)

Testicles	STMa	STMi	DS
02S0111/42	32.692	26.758	3.642
02S0111/45	46.546	39.854	4.363
02S0111/55	56.437	42.041	4.624
02S0111/59	39.778	32.692	4.036
02S0111/73	172.345	134.394	6.162
01S0111/123	35.7680	28.032	ND
Mean ± SD	63.928 ± 53.786	50.629 ± 41.494	4.565 ± 0.965

ND: no data.

with longitudinal, transverse, and oblique fibers. The urethral canal was lined with simple cylindrical epithelium (Fig.3F).

DISCUSSION

This is the first study providing a description of the histological characteristics of the female and male reproductive tracts of manatees (*Trichechus manatus*) from Brazil. Despite the publication of studies on this species in Florida, detailed descriptions of the histological characteristics of the reproductive tract are limited.

We compared the histology of the organs described in this work with that of elephants, which phylogenetically are the

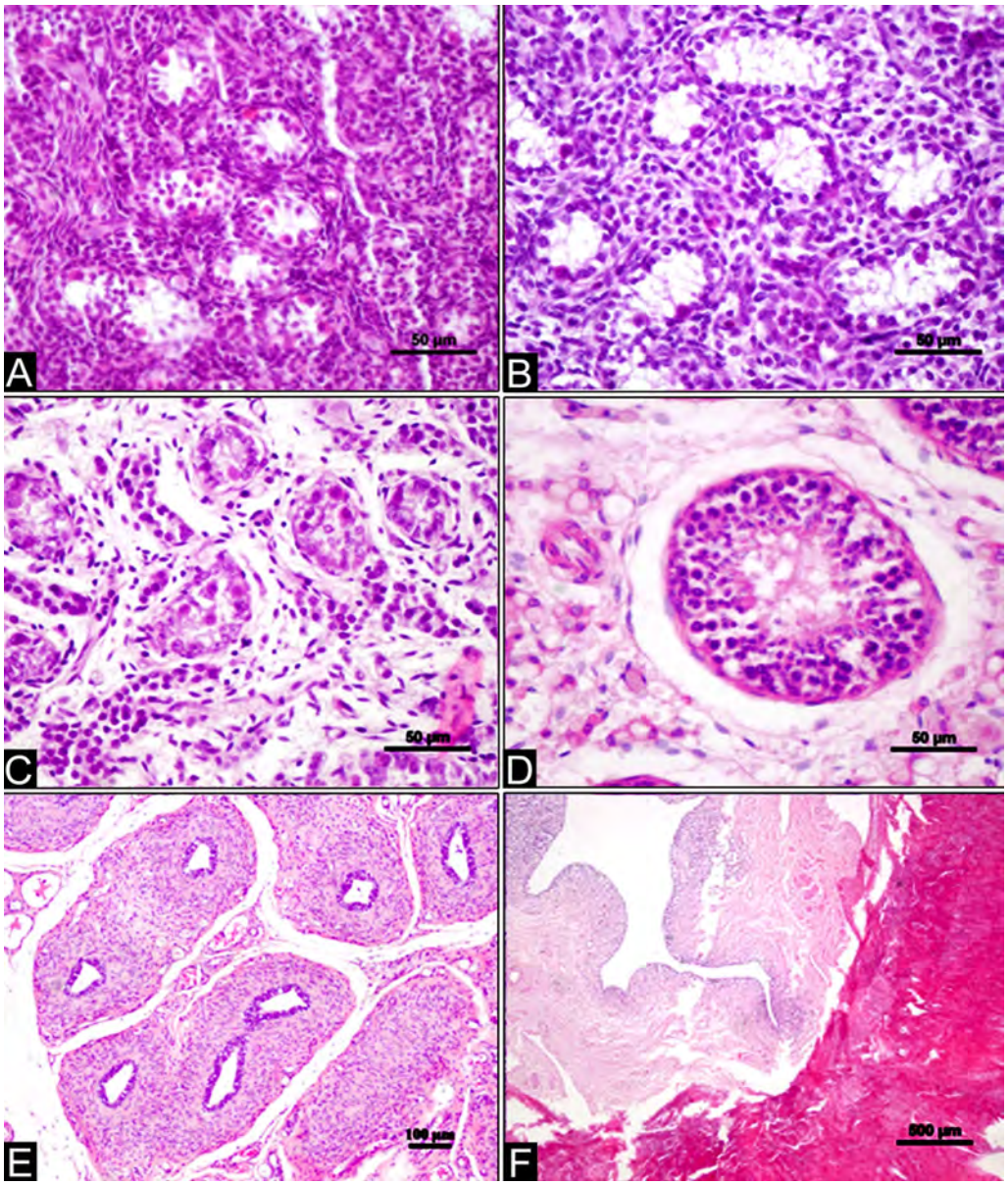


Fig.3. Testis, epididymis and penis of *Trichechus manatus*. (**A**) Testis showing the spindle cells around the seminiferous tubules. HE, obj.40x. (**B**) Testis showing the rounded cells around the seminiferous tubules. HE, obj.40x. (**C**) Testis showing spermatogonia and primary and secondary spermatocytes within the seminiferous tubules. HE, obj.40x. (**D**) Testis showing complete spermiogenesis within the seminiferous tubules. HE, obj.40x. (**E**) Epididymis with presence of intraepithelial cysts. HE, obj.10x. (**F**) Penis in which the cavernous space and the tunica albuginea can be observed. HE, obj.10x.

most closely related group to sirenians, and cetaceans, as a matter of evolutionary convergence.

Female reproductive tract

At the time of necropsy of manatee 02S0112/36, we noticed the absence of ovaries and the presence of tissue in the left antimer, with discrete signs of possible ovarian tissue. Marsh & Kasuya (1984) observed an altered ovary cortex in the short-finned pilot whale (*Globicephala macrorhynchus*), which presented no follicles and had reduced cortex thickness and a large amount of fibrous tissue. This female was lactating and was probably in the puerperal period. The manatee 02S0112/36 also presented a large amount of fibrous tissue in the cortex and almost no follicle, but unlike that of the short-finned pilot whale, the cortex was thicker and, during necropsy, the mammary glands had no increase in volume in the axillary region, or milk.

The uteri of manatees 08S0112/20 and 01S0112/12 were in the proliferative phase, but these animals were neonates, so this characteristic was probably owing to the action of maternal hormones. The uteri of manatees 01S0112/31 and 02S0112/31 were in a proliferative phase, presenting a well-developed epithelium, increased endometrial glands, increased density near the myometrium, and similar features to those of non-pregnant adult female elephants (Hanks & Short 1972).

The uterus of manatee 02S0112/38 was in the recovery phase and belonged to a lactating female, according to the characterization at the time of the necropsy. Histologically, the uterus of this female manatee resembled the uterus of an African elephant female (*Loxodonta africana*) in lactational anestrous, presenting small endometrial glands and low density near the myometrium (Hanks & Short 1972). Coincidentally, one week after the registration of this female, a live newborn was found stranded in the same place. Genetic studies confirmed the relationship between the two animals (Silva 2015).

The placenta was similar to that of Amazonian manatees with a well-developed villus region, syncytiotrophoblast, and the presence of maternal tissue cells and chorionic villi. The umbilical cord was similar to that of *T. inunguis* (Carter et al. 2008).

Male reproductive tract

The general histological appearance of the male reproductive tract in *T. manatus* was similar to that of the African elephant (*L. africana*) and the common dolphin (*Delphinus delphis*). The histological characteristics of the testis were similar to those described for testes of mature and immature animals in Florida manatees (Hernandez et al. 1995).

Murphy et al. (2005) described four stages of testis development in *D. delphis*, and in our work three similar phases were observed. We noticed the immature phase, marked by abundant interstitial tissue and the presence of few spermatogonia; the pubertal stage, in which there is a reduction of the interstitial tissue and a small increase in the diameter of the seminiferous tubules with spermatogonia and spermatocyte; the mature young phase (not observed in this work), which is characterized by the increase of the diameter of the seminiferous tubules and the presence of a central and small lumen; and the sexually mature phase, marked by the presence of spermatozoa, thus confirming complete spermatogenesis.

According to the classification mentioned above, it was possible to observe that cubs 02S0111/42, 02S0111/45, 02S0111/59, and 01S0111/123 were in the immature phase. The juvenile manatee 02S0111/55 was in the pubertal stage and the adult manatee 02S0111/73 was in the mature phase.

We observed that the mean diameter of the seminiferous tubules varied with the age and size of the manatee, and that smaller and younger animals had smaller, larger tubule diameters, whereas older animals showed larger tubule diameters, similar to what is observed in cetaceans and elephants (Short et al. 1967, Murphy et al. 2005, Westgate & Read 2007).

We also found similarities between the epididymis of the samples studied in this study and the efferent tubules in *L. africana*, which were composed of a ciliary pseudo-stratified epithelium. However, there were no spermatozoids observed in the epididymides, because they belonged to young animals (Short et al. 1967).

CONCLUSIONS

The systematic study of the different components of the reproductive tract, at different stages of growth and sexual development, is necessary to provide a complete profile of the histological characteristics of the reproductive tracts of male and female manatees.

It should be noted that this work appears to be the first to describe the histological characteristics of the tissues of the reproductive tract, placenta, and umbilical cord in this species, which present similar features to those of other aquatic mammals.

The present study seems to contribute significantly to current knowledge of the reproductive histology of the manatee.

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REFERENCES

Alves M.D., Kinas P.G., Marmontel M., Borges J.C.G., Costa A.F., Schiel N. & Araújo M.E. 2016. First abundance estimate of the Antillean manatee (*Trichechus manatus manatus*) in Brazil by aerial survey. J. Mar. Biol. Assoc. U.K. 96(04):955-966. <http://dx.doi.org/10.1017/S0025315415000855>

Attademo F.L.N., Balensiefer D.C., Bôaviagem Freire A.C., Sousa G.P., Cunha F.A.G.C. & Luna F.O. 2015. Debris ingestion by the Antillean Manatee (*Trichechus manatus manatus*). Mar. Pollut. Bull. 101(1):284-287. <http://dx.doi.org/10.1016/j.marpolbul.2015.09.040> <PMid:26431561>

Brasil 2014. Portaria MMA nº 444, de 17 de dezembro de 2014. Available at <http://www.icmbio.gov.br/portal/images/stories/biodiversidade/faunabrasileira/avaliacao-do-risco/PORTARIA_N%C2%BA_444_de_17_de_dezembro_2014.pdf> Accessed on Dec. 7, 2017.

Carter A.M., Miglino M.A., Ambrósio C.E., Santos T.C.D., Rosas F.C.W., d'Afonseca Neto J.A., Lazzarini S.M., Carvalho A.F. & Silva V.M.F. 2008. Placentation in the Amazonian manatee (*Trichechus inunguis*). Reprod. Fertil. Develop. 20(4):537-545. <http://dx.doi.org/10.1071/RD08009> <PMid:18462617>

Geraci J.R., Lounsbury V.J. & Nathan J. 2005. Marine Mammals Ashore: a field guide for strandings. 2nd ed. College Station, Texas A&M Sea Grant Publication, Texas. 382p.

Hanks J. & Short R.V. 1972. The formation and function of the corpus luteum in the African elephant, *Loxodonta africana.* J. Reprod. Fertil. 29(1):79-89. <http://dx.doi.org/10.1530/jrf.0.0290079> <PMid:5017016>

Hernandez P., Reynolds 3rd J.E., Marsh H. & Marmontel M. 1995. Age and seasonality in spermatogenesis of Florida manatees, p.84-97. In: O'Shea T.J., Ackerman B.B. & Percival H.F. (Eds), Population Biology of the Florida Manatee. National Biological Service, Fort Collins.

Lima R.P., Paludo D., Soavinski R.J., Silva K.G. & Oliveira E.M.A. 2011. Levantamento da distribuição, ocorrência e status de conservação do Peixe-Boi Marinho (*Trichechus manatus,* Linnaeus, 1758) no litoral nordeste do Brasil. Nat. Resour. 1:41-57.

Marmontel M. 1995. Age and reproduction in female Florida manatees, p.98-119. In: O'Shea T.J., Ackerman B.B. & Percival H.F. (Eds), Population Biology of the Florida Manatee. National Biological Service, Information and Technology Report, Fort Collins.

Marsh H. & Kasuya T. 1984. Changes in the ovaries of the short-finned pilot Whale, *Globicephala macrorhynchus*, with age and reproductive activity. Rep. Int. Whal. Commn. 6(Special issue):311-335.

Marsh H., O'Shea T.J. & Reynolds 3rd J.E. 2011. Ecology and Conservation of the Sirenia: dugongs and manatees. Cambridge University Press, Cambridge. 521p. <http://dx.doi.org/10.1017/CBO9781139013277>.

Meirelles A.C.O. 2008. Mortality of the Antillean manatee, *Trichechus manatus manatus*, in Ceará State, north-eastern Brazil. J. Mar. Biol. Assoc. U.K. 88(06):1133-1137. <http://dx.doi.org/10.1017/S0025315408000817>

Meirelles A.C.O. & Carvalho V.L. 2016. Peixe-boi marinho: biologia e conservação no Brasil. Aquasis, Bambu Editora e Artes Gráficas, São Paulo. 176p.

Murphy S., Collet A. & Rogan E. 2005. Mating strategy in the male common dolphin (*Delphinus delphis*): what gonadal analysis tells us. J. Mammal. 86(6):1247-1258. <http://dx.doi.org/10.1644/1545-1542(2005)86[1247:MSITMC]2.0.CO;2>

Parente C.L., Vergara-Parente J.E. & Lima R.P. 2004. Strandings of Antillean manatees, *Trichechus manatus manatus*, in northeastern Brazil. Lat. Am. J. Aquat. Mamm. 3(1):69-75. <http://dx.doi.org/10.5597/lajam00050>

Reynolds III J.E., Rommel S.A. & Bolen M.E. 2002 Anatomical dissection: thorax and abdomen, p.21-30. In: Perrin W.F., Wursig B. & Thewissen J.G.M. (Eds), Encyclopedia of Marine Mammals. Academic Press, Massachussets.

Rodrigues F.R., Silva V.M.F., Barcellos J.F.M. & Lazzarini S.M. 2008. Reproductive anatomy of the female Amazonian manatee *Trichechus inunguis* Natterer, 1883 (Mammalia: Sirenia). Anat. Rec. 291(5):557-564. <http://dx.doi.org/10.1002/ar.20688> <PMid:18383272>

Short R.V., Mann T. & Hay M.F. 1967. Male reproductive organs of the African elephant, *Loxodonta africana.* J. Reprod. Fertil. 13(3):517-536, NP. <http://dx.doi.org/10.1530/jrf.0.0130517> <PMid:6029179>

Silva S.M. 2015. Caracterização populacional e estimativa de parentesco entre peixes-boi marinhos *Trichechus manatus* (Linnaeus, 1758) usando marcadores microssatélites. Dissertação de Mestrado em Biologia Ambiental, Universidade Federal do Pará, Pará.

Westgate A.J. & Read A.J. 2007. Reproduction in short-beaked common dolphins (*Delphinus delphis*) from the western North Atlantic. Mar. Biol. 150(5):1011-1024. <http://dx.doi.org/10.1007/s00227-006-0394-1>

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Original Article
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Subplacental development in *Galea spixii*[1]

Ferdinando V.F. Bezerra[2]*, Phelipe O. Favaron[3], Andrea M. Mess[3], Hélio N. Araújo Júnior[2], Gleidson B. Oliveira[2], Alexsandra F. Pereira[2], Maria A. Miglino[3] and Moacir F. Oliveira[2]

ABSTRACT.- Bezerra F.V.F., Favaron P.O., Mess A.M., Araújo Júnior H.N., Oliveira G.B., Pereira A.F., Miglino M.A. & Oliveira M.F. 2018. **Subplacental development in *Galea spixii*.** *Pesquisa Veterinária Brasileira 38(11):2175-2182.* Universidade Federal Rural do Semi-Árido, Av. Costa e Silva s/n, Mossoró, RN 59625-900, Brazil. E-mail: ferdinando_vinicios@hotmail.com

Animal models are essential to understand healthy human placentation. Guinea pig related rodents became on focus for such purposes. In particular, processes of trophoblast invasion are similar. The latter is associated with a specialized area, the subplacenta. Since previous results showed differences between the guinea pig and its close relative *Galea spixii*, we aimed to study subplacental development with more detail. We investigated 16 pregnant females of 14 to 55 days of gestation by means of histology, morphometrics, immunohistochemistry and electron microscopy. The overlap between the fetomaternal blood systems resulted as intimate, suggesting some exchange processes. Proliferation was revealed by three independent methods, being most active in early and mid-gestation, which was in accordance to former results. Though degeneration of tissues took place, the subplacenta was maintained towards term with access to the fetal vascularization, supporting a hypothesis about the release of substances to the fetal unit in advanced gestation. In contrast to other species, the extraplacental trophoblast showed a shift from syncytial streamers to giant cells during mid-gestation. Views on placentation in caviomorphs were influenced by the guinea pig, but our data supported recent studies that the subplacenta had a much greater placidity. In regard to subplacental grow, degeneration and likely also exchange processes, *Galea* and other species showed a more basal pattern of caviomorphs than the guinea pig. Such differences should be considered, when choosing most adequate animal models for special purposes in comparison to human placentation.

INDEX TERMS: Placenta, cytotrophoblast, syncytiotrophoblast, trophoblast invasion, guinea pig, *Galea spixii*, animal model.

RESUMO.- [**Desenvolvimento subplacentário do preá (*Galea spixii*).**] Modelos animais são essenciais para entender a placenta humana sadia. Neste sentido os roedores relacionados ao porquinho da índia tornaram-se foco para tal entendimento. Em particular, os processos de invasão trofoblástica são semelhantes. O último está associado a uma área especializada, a subplacenta. Uma vez que os resultados anteriores mostraram diferenças entre o porquinho da índia e seu relativo o preá, buscamos estudar o desenvolvimento subplacentário com mais detalhes. Pesquisamos 16 fêmeas gestantes de 14 a 55 dias de gestação por meio de histologia, morfometria, imuno-histoquímica e microscopia eletrônica. A sobreposição entre os sistemas sanguíneos materno e fetal apresentou-se com íntima relação, sugerindo alguns processos de troca. A proliferação foi revelada por três métodos independentes, sendo mais ativos no início e metade da gestação, o que corroborou com os resultados anteriores. Embora a degeneração dos tecidos tenha ocorrido, a subplacenta foi mantida até o termo gestacional com acesso à vascularização fetal, apoiando uma hipótese sobre a liberação de substâncias para a unidade fetal em gestação avançada. Em contraste com outras espécies, o trofoblasto extraplacentário mostrou uma mudança de flâmulas sinciciais para células gigantes durante a metade da gestação. As visualizações sobre a placentação em caviomorfos foram influenciadas pelo porquinho da

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[2] Universidade Federal Rural do Semi-Árido (UFERSA), Av. Costa e Silva s/n, Mossoró, RN 59625-900, Brazil.*Corresponding author: ferdinando_vinicios@hotmail.com

[3] Departamento de Cirurgia, Faculdade de Medicina Veterinária, Universidade de São Paulo (USP), Avenida Prof. Dr. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP 05508-000, Brazil.

índia, mas nossos dados apoiaram estudos recentes de que a subplacenta apresentava uma plasticidade muito maior. Em relação ao crescimento subplacentário, a degeneração e provavelmente também os processos de troca, o preá e outras espécies apresentaram um padrão mais basal de caviomorfos do que o porquinho da índia. Tais diferenças devem ser consideradas, ao escolher os modelos animais mais adequados para fins especiais em comparação com a placentação humana.

TERMOS DE INDEXAÇÃO: Placenta, citotrofoblasto, sinciciotrofoblasto, invasão trofoblástica, preá, *Galea spixii*, modelo animal.

INTRODUCTION

In regard to better understanding healthy pregnancy as well as potential problems in human beings, animal models on placentation are essential (Carter 2007, Carter & Mess 2014, Mess 2014). Recently the guinea pig and caviomorph rodents in general have been regarded as suitable models for placentation. They share more similarities of placental fine structure and trophoblast invasion with humans than other mammalian taxa such as murids including the mice (Mess et al. 2007, Mess 2014). Moreover, they possess relatively long gestation periods and give birth to well-developed offspring that allows modeling conditions of advanced human pregnancy (Carter & Mess 2014).

In the last decade placentation was investigated in several caviomorph species (Davies et al. 1961a, 1961b, Kaufmann & Davidoff 1977, Wolfer & Kaufmann 1980, Miglino et al. 2002, 2004, Kaufmann 2004, Bonatelli et al. 2005, Oliveira et al. 2006, 2008, Rodrigues et al. 2006, Bosco et al. 2007, Mess 2003, 2007a, 2007b, Mess & Carter 2007, Mess et al. 2007, Kanashiro et al. 2009, Flamini et al. 2011, Oliveira et al. 2012a, 2012b) revealing a quite stable pattern of evolution that is largely independent from size diameters or reproductive specializations (Kanashiro et al. 2009, Flamini et al. 2011, Mess 2011, 2014). In particular, trophoblast invasion in caviomorphs is associated with a specialized area within the placental disk, the so-called subplacenta (Kaufmann 2004, Rodrigues et al. 2006, Mess et al. 2007, Mess 2007a, 2011, 2014). It represents an area of folded layers of cellular and syncytial trophoblast, formed at the central excavation in association to the maternal blood system early in gestation, but that is supplied by fetal vessels only in later phases (Fig.1).

It serves as origin and proliferation center for extraplacental trophoblast that migrates into the decidua to destroy and remodel the maternal arteries (Kaufmann 2004, Mess et al. 2007, Mess 2007a, 2011). However, there are some differences within the group, especially when comparing the guinea pig with one of its closest relatives, the prea *Galea spixii* that exhibits the semiarid *caatinga* vegetation of northeast Brazil (Moojen 1952, Pinheiro et al. 1989, Bonvicino et al. 2008, Oliveira et al. 2008). In the latter, illustrations of subplacental structures focus on early ontogeny until day 12, whereas older stages are only superficially described (Oliveira et al. 2008). Moreover, the length of gestation in the prea is variable and recent data indicated that it is about 55 to 60 days in animals from our breeding facilities (Vale 2011) and not 48 days as was assumed for former placental studies (Oliveira et al. 2008, 2012b). Thus, real near term conditions of the subplacenta are absent in the literature (Oliveira et al. 2008). To better understand subplacental development throughout gestation in *Galea* and to compare with already established animal models for human placentation, we investigated placentas of days 14 to 55 by means of histology, morphometric analysis, immunohistochemistry and electron microscopy.

MATERIALS AND METHODS

Animals and ethics statement. Sixteen females of gestational days 14, 15, 23, 25, 30, 33, 45, 50, 52, 53 and 55 were investigated, obtained from the Multiplication Center Wild Animals (CEMAS/UFERSA), Mossoro (Register #1478912 by IBAMA), authorized by the System of Authorization of Biodiversity Information in Brazil (SISBIO #35231-1). The project was approved by the Ethical Committee (CEUA/UFERSA) (Protocol #68/2012). For breeding, four groups with four adult females and one male, respectively, have been established. These animals are small rodents, with small ears, devoid of tail and with average weight of 300g and that have dorsal gray coloration, with brunáceo tone and white belly and hind legs with three digits. They inhabit the capoeiras, between stones, barriers of rivers and reeds, feeding on grasses and roots and were housed in a $5m^2$ area and fed rations for rodents, fruits, soybeans, maize and water ad libitum, so that each group had the same food management. Pregnancy was detected by daily vaginal cytology tests, with the presence of sperm indicating day zero.

Anesthetic surgical procedure. Once the pregnancy was identified, the animals were separated and the day before the collection were subjected to a solid twelve hour fast and a six hour liquid. At the time of collection, the animals were weighed and submitted to anesthetic procedure. For this, the combination of xylazine hydrochloride (Xilazin® 2% at the dose of $1mg.kg^{-1}$, manufacturer Syntec) and hydrochloride of ketamine (10%) was administered intramuscularly, after waiting time (10min) the thiopental anesthetic 1g (Thiopental sodium 1g at the dose of $25mg.kg^{-1}$, manufacturer Richmond, Division Veterinaria SA) by intravenous route as an anesthetic-inducing drug. The anesthetic plan was followed by administration of Potassium Chloride (19.1% potassium chloride at a dose of $2.56mEq.kg^{-1}$, manufacturer Halexistar) also by intravenous route, confirming the death of the animal after cardiorespiratory arrest.

Histology. Tissues for histology were fixed by immersion in buffered 4% paraformaldehyde after female euthanasia, transferred to decreasing concentrations of alcohol and xylene, embedded in paraffin, cut and then stained with hematoxylin and eosin (HE), Masson's trichrome and the periodic acid-Schiff reaction (PAS) (Oliveira et al. 2008, 2012a, 2012b). Slides were analyzed by a Leica ICC50 HD microscope.

Qualitative proliferation. Was revealed in main stages. In slides photographed at 100x magnification, a total of 1000 cells were counted and the mitotic index was obtained by the relative number of cells with positive signals of mitosis compared to the total number of cells. Also, Argyrophilic Nucleolar Organizer Regions (AgNOR) were calculated in silver-stained slides (Santos et al. 2012). Sections were deparafinized, post-fixed in absolute ethanol and acetic acid (3:1), rehydrated and stained by a 2:1 mixture of (a) 2% unflavored gelatin in aqueous 1% formic acid and (b) 50% silver nitrate in water, protected from light. Slides were post-fixed with 5% sodium thiosulfate, dehydrated, mounted and AgNORs were counted 200 nuclei in each slide and were used 4 slides, randomly chosen, per animal totaling 800 nuclei per animal.

Immunohistochemistry. Was performed for vimentin, cytokeratin and PCNA (Oliveira et al. 2008, 2012b). The material was rehydrated in ethanol and submitted to endogenous peroxidase blockage in 3% hydrogen peroxide. The sections were treated in 0.1M citrate

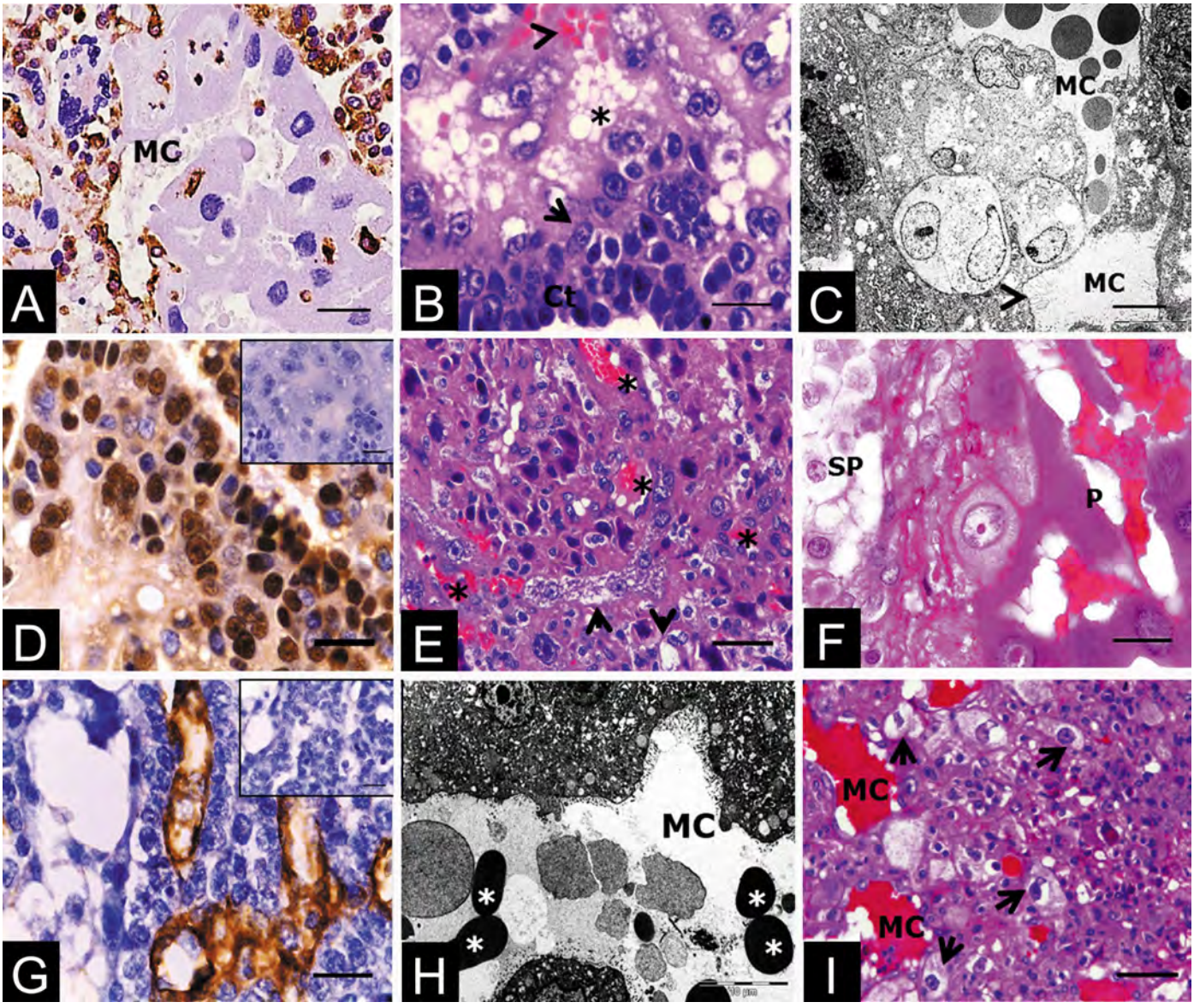


Fig.1. (**A-E**) Day 15 and (**F-I**) day 23. (**A**) The subplacenta was supplied by the maternal arterial system that partly possessed a vimentin-positive endothelium. Vimentin reaction, bar = 20µm. (**B**) An area of cytotrophoblast surrounded by syncytiotrophoblast with lots of vacuoles (*) and associated with maternal blood channels (arrowheads). HE, bar = 20µm. (**C**) Maternal blood channels lined by syncytiotrophoblast. Giant cells occurred in between. TEM, bar = 10µm. (**D**) Cellular trophoblast and giant cells inside the syncytial layer were proliferative. PCNA with negative control, bar = 20µm. (**E**) Syncytial streamers were frequent between the subplacenta and maternal blood vessels of the decidua. HE, bar = 50µm. (**F**) The subplacenta was separated from the main placenta by connective tissue with giant cells situated in-between. HE, bar = 20µm. (**G**) Positive reaction showed fetal vessels that supplied the organ, associated to remnants of maternal blood channels that were lined by vimentin-negative trophoblast. Vimentin and negative control, bar = 20µm. (**H**) Functional maternal blood channel. TEM, bar = 10µm. (**I**) Extraplacental trophoblast included mainly giant cells. HE, bar = 50µm.

buffer at pH 7.4, irradiated in a microwave (700MHz), washed in 0.1M sodium phosphate buffer at pH 7.4 and nonspecific binding was blocked by Dako Protein Block. Then, incubation with the primary antibodies, i.e. polyclonal goat anti-vimentin (SC-1226, Santa Cruz Biotchnology, USA) in dilution 1:400, anti-cytokeratin rabbit polyclonal (PU071-UP, Biogenex) in dilution 1:300 and anti-PCNA monoclonal mice (107 904, Novocastra, UK) in dilution 1:300 too, was done for 12h in a humid chamber at 4°C. Finally, incubation with the peroxidase-conjugated secondary antibody was performed and slides were counterstained with Harris hematoxylin and mounted in Resin-Entellan (Merck, Darmstadt, Germany). Negative controls were performed using goat anti-mouse IgG as the primary antibody solution.

Transmission electron microscopy (TEM). Samples were fixed in buffered 2.5% glutaraldehyde, post-fixed in osmium tetroxide, embedded in Spurr's Resin and then cut with 0.07µm thickness in an automatic ultramicrotome (Ultracut R, Leica, Germany). Sections were contrasted with 2% uranyl acetate and 0.5% lead citrate (Oliveira et al. 2008, 2012a, 2012b) and analyzed in a Morgagni 268D transmission electron microscope (FEI Company, The Netherlands) with a Mega View III camera (Soft Imaging, Germany).

RESULTS

Days 14 and 15

The subplacenta was confluent with the main placenta and had access to the maternal arterial system that in parts still possessed its endothelial lining (Fig.1A). Attached to the folded basal membrane facing the central excavation, cytotrophoblast composed of cubic, mostly multilayered, but partly pseudo-stratified cells occurred (Fig.1B). The cytoplasm was basophil and the nuclei were round with chromatin and scattered clumps (Fig.1B). Above was an extended syncytiotrophoblast with eosinophilic cytoplasm and vacuoles (Fig.1B) that lined the maternal blood channels (Fig.1C). It was positive to PAS. In between, giant cells occurred (Fig.1C). The cellular trophoblast and these giant cells were actively proliferating (Fig.1D). Extraplacental trophoblast was frequent, consisting predominately of syncytial streamers intermingled with some giant cells (Fig.1E).

Days 23 and 25

The lobulated, highly proliferative subplacenta was confluent to the decidua (Fig.1), but in large parts separated from the main placenta by a thin layer of connective tissue with giant cells being frequent at the border (Fig.1F). Fetal vessels and maternal blood channels were present (Fig.1G,H), but some of the channels were filled with amorphic material. Extraplacental trophoblast primarily included mononucleated giant cells instead of syncytial streamers (Fig.1I).

Days 30 and 33

The prominent subplacenta is mainly, but not exclusively separated from the main placenta, showing some lateral cell clusters continuing towards the interlobium (Fig.2A). Inside, fetal vessels could be followed in-between the cellular and syncytial areas of the folds (Fig.2B,C). Also, several functional maternal blood channels were present, in parts associated with only thin syncytiotrophoblast (Fig.2D). The proliferation activity of subplacental cells was high (Fig.2E). The extraplacental trophoblast was decreased to only some trophoblast giant cell clusters.

Days 41 and 45

Syncytiotrophoblast was most prominent, characterized by plenty, very large vacuoles and positive reactions to PAS, indicating a continued accumulation of glycogen or related carbohydrates (Fig.2F,G). Remnants of the maternal blood channels were found with small internal projections to the inside (Fig.2G). The cellular trophoblast was in parts discontinuous and thin (Fig.3H). Mitotic figures were rare and proliferation low (Fig.2I). Signs of degeneration occurred such as the presence of pyknotic nuclei in the syncytiotrophoblast (Fig.2F).

Days 50, 52, 53 and 55

Though the relative extension of the subplacenta was strongly decreased, the organ was present and supplied by fetal vessels (Fig.3A). However, extended areas of apomorphic structure occurred inside (Fig.3B), in addition to large amounts of connective tissue in-between the cellular trophoblast that also possessed vacuoles now (Fig.3C,D). Nuclei were often disorganized (Fig.3E). The syncytiotrophoblast was PAS-positive and contained positive amorphous material; it was still found near some rests of the former maternal blood system. Severe signs of degeneration and apoptosis occurred. The proliferation activity was very low (Fig.3F), in contrast to areas inside the placental disk (data not shown).

Qualitative data

The mitotic index revealed an abrupt decrease between days 30 to 45, revealing that up to 30 days of gestation the proliferative activity of the subplacenta is high and that in the sequence decreases, until becoming minimal at the end of gestation, whereas counting AgNORs suggested a more graduate decrease throughout gestation (Fig.4A,B), considering that the Agnor´s technique portrays not only the proliferation but also the metabolism of the cells, revealing that even with the abrupt decrease of ploriferative activity the organ remains functional.

DISCUSSION

Our findings agree with Conceição et al. (2008) that report the central positioning of the subplacenta in relation to the main placenta and basal decidua in paca and agouti and showed a relatively close overlap between the fetomaternal blood systems inside the subplacenta, continuation of the organ and its supplement by the fetal vasculature towards term and more detailed views in regard to proliferation and extraplacental trophoblast.

The subplacenta historically was regarded as an organ without fetomaternal exchange, mainly based on studies of the guinea pig where the maternal blood system was stopped by coagulation from day 20 on and fully disappeared at least at day 27, in contrast to the fetal system that started around day 26 to 32 (Kaufmann & Davidoff 1977, Wolfer & Kaufmann 1980, Kaufmann 2004). However, recent studies showed a short-term overlap in the prea, the degu *Octodon degus*, the punare *Thrichomys aperoides*, the capybara *Hydrochaeris hydrochaeris* as well as paca *Cunniculus paca* and agouti *Dasyprocta leporina* (Miglino et al. 2002, 2004, Bonatelli et al. 2005, Bosco et al. 2007, Mess 2007a, Oliveira et al. 2008, 2012a). Here, we argued for an even more intimate overlap, On the contrary, a hemotricorial barrier is observed in Sigmodontinae (Favaron et al. 2011). Maternal blood spaces in *Galea* were functional throughout mid-gestation until near term, i.e. found largely in parallel with the fetal vasculature. Only near term, they became filled with amorphous material, indicating a non-functional state. Also, the syncytiotrophoblast was partly very thin and fetal vessels had access towards this layer. Since data suggested a relatively long-lasting and close interaction between the two systems, at least some exchange of substances supporting the effective fetomaternal exchange units in the placental disk should no longer be ruled out.

Our findings in the prea were in accordance to former studies that the subplacenta was active and proliferative in early and mid-gestation (Davies et al. 1961a, 1961b, Kaufmann & Davidoff 1977, Wolfer & Kaufmann 1980, Kaufmann 2004, Miglino et al. 2002, 2004, Bonatelli et al. 2005, Oliveira et al. 2006, Rodrigues et al. 2006, Bosco et al. 2007, Mess 2003, 2007a, 2007b, 2011, Mess et al. 2007, Mess & Carter 2007, Oliveira et al. 2008, 2012a, 2012b, Kanashiro et al. 2009, Flamini et al. 2011). Immunostaining for the proliferation marker PCNA and revealing the mitotic index by morphology both resulted in high proliferation early on, followed by an

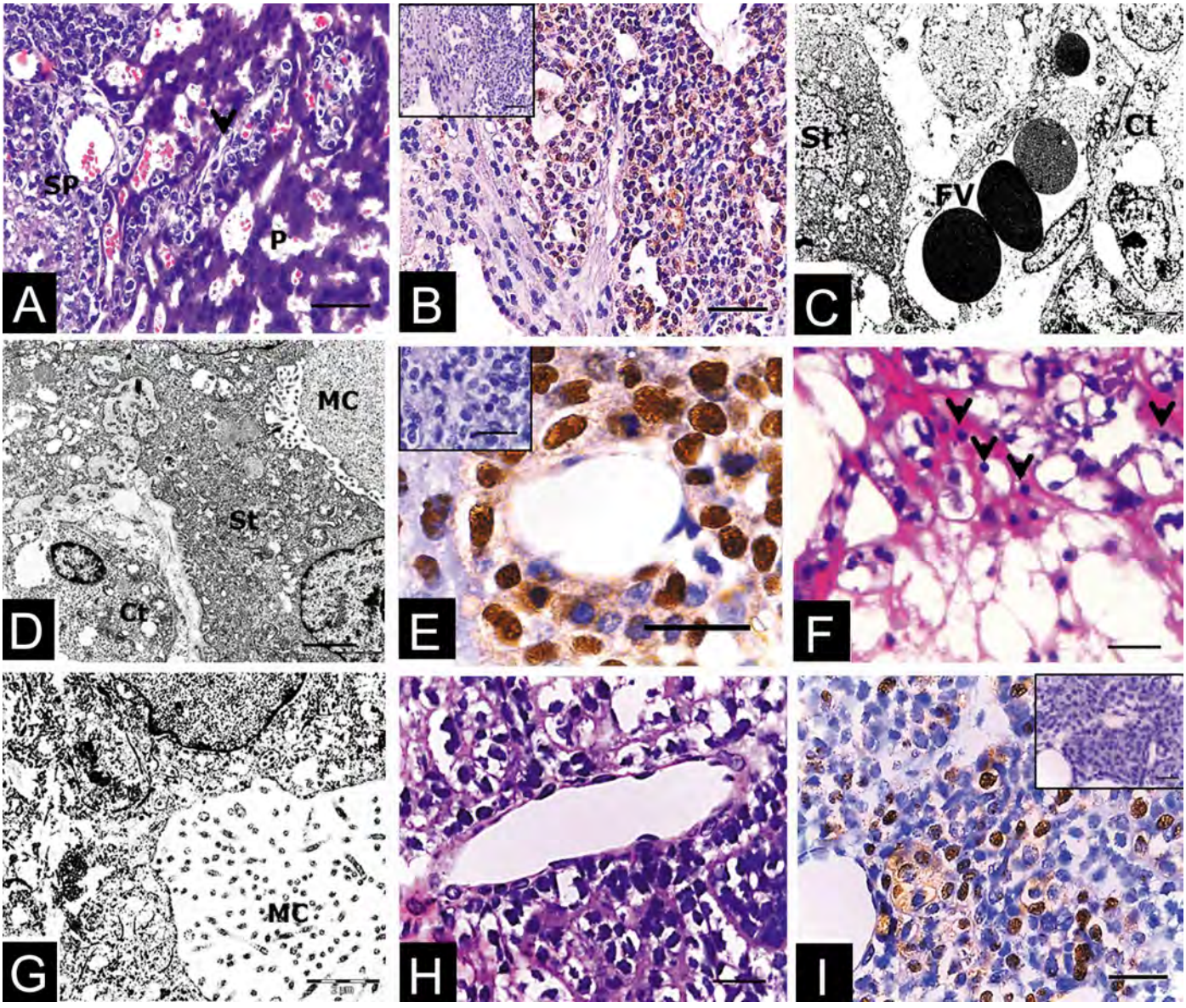


Fig.2. (**A-E**) Day 30 and (**F-I**) day 45. (**A**) Clusters of subplacental trophoblast cells could be followed towards the placental disk. HE, bar = 50µm. (**B**) The endothelium of a fetal vessel was negative, in contrast to (especially cellular) trophoblast. Cytokeration, bar = 50µm. (**C**) Fetal capillary in-between the cellular and syncytial trophoblast. TEM, bar = 5µm. (**D**) In parts, syncytiotrophoblast was very thin near remnants of maternal blood channels. TEM, bar = 5µm. (**E**) Subplacental trophoblast was still very active in proliferation. PCNA, bar = 20µm. (**F**) Syncytiotrophoblast with plenty of large vacuoles and positive reaction. Pyknotic nuclei as signs of degeneration were frequent. PAS, bar = 20µm. (**G**) Rest of a maternal blood channel. TEM, bar = 2µm. (**H**) Cellular trophoblast partly discontinuous and thin. HE, bar = 20µm. (**I**) Rate of proliferation rate was low. PCNA and negative control, bar = 20µm.

abrupt decrease around day 45 and further decrease towards term. Calculation of AgNORs focusing on metabolic active nuclei fit into the frame. As to be expected, data showed a more graduate decrease, caused by the inclusion of both pre-mitotic and mitotic stages that lead to a relatively higher number of positive results. For the first time, proliferation processes inside the subplacenta were revealed by three independent methods, supporting the former results. During advanced gestation, the subplacenta generally degenerated (Davies et al. 1961a, 1961b, Kaufmann & Davidoff 1977, Wolfer & Kaufmann 1980, Miglino et al. 2002, 2004, Mess 2003, 2007a, 2007b, 2011, Kaufmann 2004, Bonatelli et al. 2005, Oliveira et al. 2006, 2008, 2012a, 2012b, Rodrigues et al. 2006, Bosco et al. 2007, Mess et al. 2007, Mess & Carter 2007, Kanashiro et al. 2009, Flamini et al. 2011). In addition, these results corroborate with those described in the capybara (Kanashiro et al. 2009) and in the punaré (Oliveira et al. 2012a), however the final phases are not approached in this regard. In the same way, as described by Oliveira et al. (2008), the cytotrophoblast is highly proliferative in the middle third of gestation, which agrees with Davies et al. (1961a, 1961b) who affirm that this is the phase of greater activity, however, it was observed that in prea the phase of greater proliferative and metabolic activity is the initial phase and that from the middle of

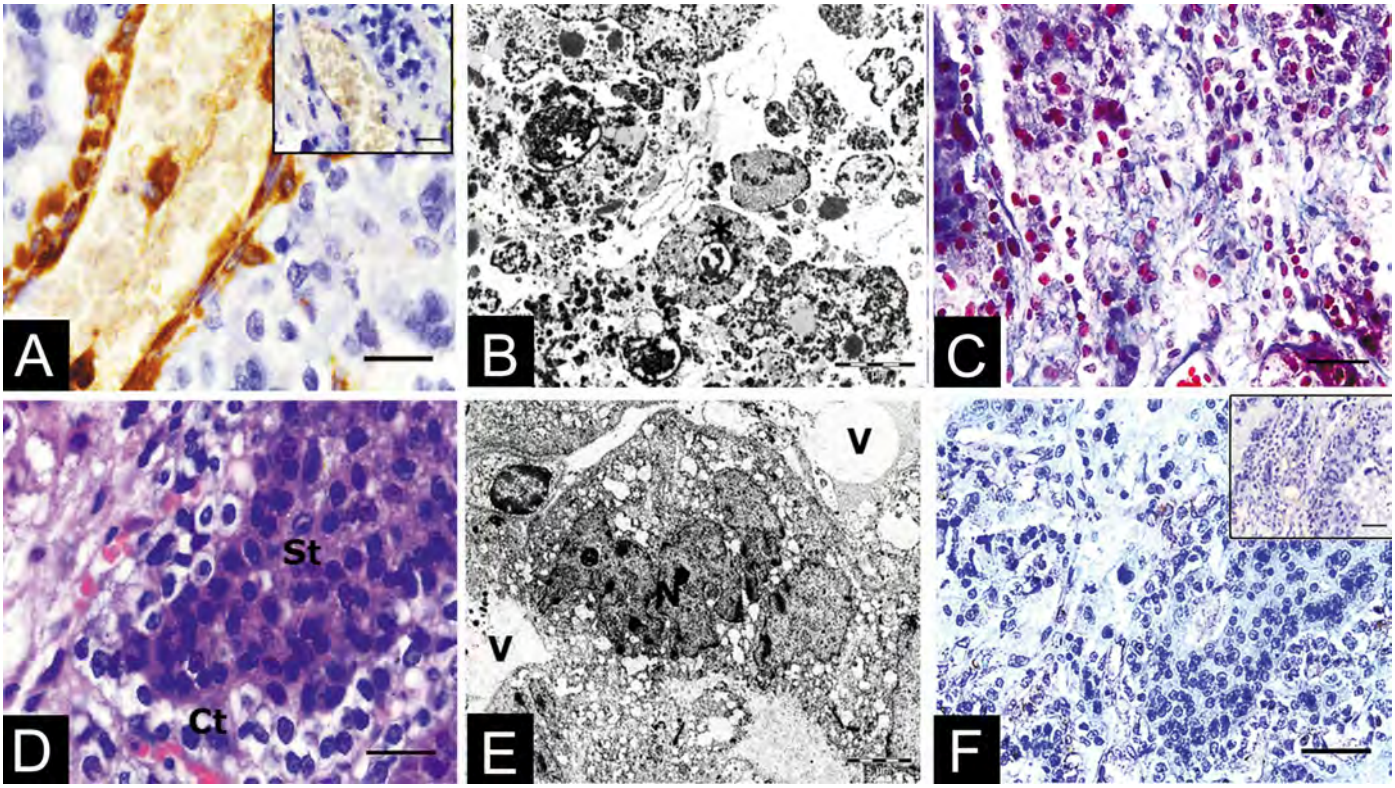


Fig.3. (**A**) Day 53 to 55. Main fetal vessels supplying the organ. Vimentin and negative control, bar = 20µm. (**B**) Large amorphic areas were present. TEM, bar = 5µm. (**C**) High amounts of connective tissue in-between the cytotrophoblast. Gomori, bar = 50µm. (**D**) Also, cellular trophoblast possessed vacuoles now. HE, bar = 20µm. (**E**) Disorganized nuclei of syncytiotrophoblast. TEM, bar = 5µm. (**F**) Proliferation activity had been stopped. PCNA and negative control, bar = 50µm.

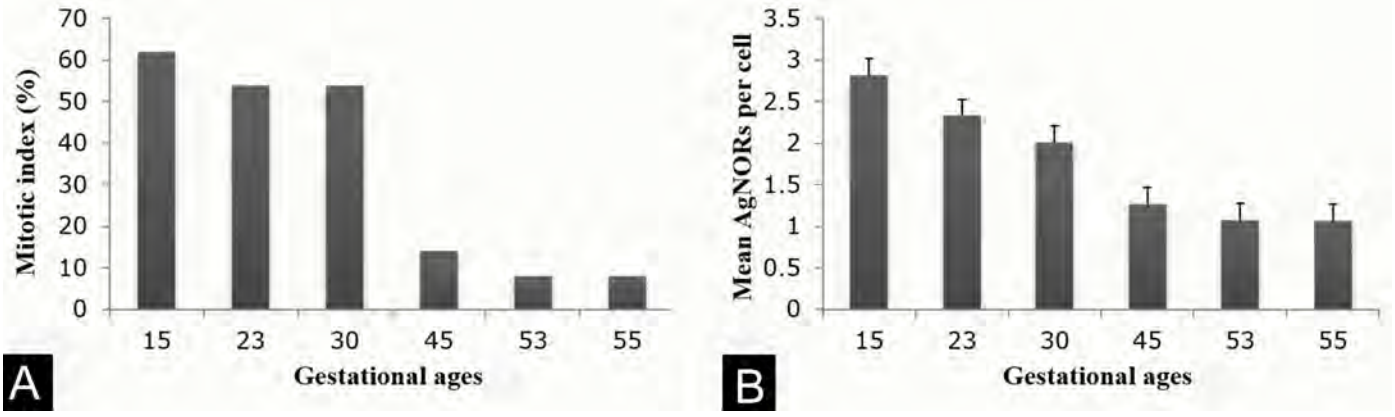


Fig.4. Qualitative proliferative activity. (**A**) Mitotic index, (**B**) mean of AgNORs per cell.

gestation both decay. *Galea* showed the normal pattern with degeneration starting in the syncytiotrophoblast and then affecting the cellular trophoblast. However, a functional state was maintained much longer than in the guinea pig, where the organ was fully resorbed near term (Davies et al. 1961a, 1961b, Kaufmann & Davidoff 1977, Kaufmann 2004, Mess 2007a, 2011, Mess et al. 2007, Flamini et al. 2011, Carter & Mess 2014). In contrast, the subplacenta was reduced and filled with amorphous material, but still present and supplied by fetal vessels in the degu, punare, paca, agouti, capybara, and the rock cavy *Kerodon rupestris* (Miglino et al. 2002, 2004, Bonatelli et al. 2005, Oliveira et al. 2006, 2008, 2012a, 2012b, Rodrigues et al. 2006, Mess 2007a, 2011, Mess et al. 2007, Kanashiro et al. 2009). The here investigated near term stages indicated that *Galea* belonged to the second group. The positive PAS reaction of the syncytiotrophoblast and the material inside its lacunae, indicating active glycolysation processes associated with secretion, supported a former hypothesis (Rodrigues et al. 2006) that the release of hormones, growth factors or other substances to the fetal unit may be of special

importance and may also be involved in processes of forthcoming birth. Thus, *Galea* and other species represented a more basal pattern of caviomorphs than the guinea pig itself, which is also observed in embryos as concluded by Franciolli et al. (2011) studying South American histricomorphic embryos and this variations need to be considered for comparative studies with human placentation as concluded by Miglino et al. (2008).

Finally, a greater placidity in regard to trophoblast invasion was indicated. Whereas in most species both syncytial streamers and giant cells were involved throughout gestation (Kaufmann 2004, Mess 2007a, 2011, Mess et al. 2007, Kanashiro et al. 2009, Flamini et al. 2011), our data suggested that in *Galea* a shift from syncytial streamers to mononucleated giant cells took place in mid gestation. Further studies are necessary to more fully understand the functional significance and differences of the two types of extraplacental trophoblast.

However, the subplacenta had access to partly intact maternal arteries in early ontogeny and seemed to be involved with the destruction and reorganization of the maternal vessel walls, likewise to what was known from other species of caviomorphs (Kaufmann 2004, Bosco et al. 2007, Mess 2007a, 2011, 2014, Mess et al. 2007, Kanashiro et al. 2009, Flamini et al. 2011, Oliveira et al. 2012a, Carter & Mess 2014).

CONCLUSIONS

The subplacental development in the studied species is initially confluent with a main placenta, being initially irrigated by maternal circulation and later replaced by fetal circulation, where both as circulations showed great intimacy without the middle third of gestation.

Its proliferative and metabolic activity are higher in the initial periods of gestation and decay from the middle of gestation, being the most pronounced fall in relation to the proliferation.

Thus, when we observe a subplaced as the center of origin for the trophoblastic invasion and in particular for the intimate relation between the fetal and maternal blood systems during the middle third of gestation, and bearing in mind that there is no ideal model for the human placentation, these are more important in choosing the most suitable model for specifications cases.

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Conflict of interest.- The authors declare that they have no competing interests.

REFERENCES

Bonatelli M., Carter A.M., Machado M.R., Oliveira M.F., Lima M.C. & Miglino M.A. 2005. Placentation in the paca (*Agouti paca* L.). Reprod. Biol. Endocrinol. 3(1):1-12. <http://dx.doi.org/10.1186/1477-7827-3-9> <PMid:15737234>

Bonvicino C.R., Oliveira J.D. & D'Andrea P.S. 2008. Guia dos Roedores do Brasil, com Chaves para Gêneros Baseadas em Caracteres Externos. Centro Pan-Americano de Febre Aftosa, Rio de Janeiro. 120p.

Bosco C., Buffet C., Bello M.A., Rodrigo R., Gutierrez M. & García G. 2007. Placentation in the degu (Octodon degus): Analogies with extrasubplacental trophoblast and human extravillous trophoblast. Comp. Biochem. Physiol. A, Mol. Integr. Physiol. 146(4):475-485. <http://dx.doi.org/10.1016/j.cbpa.2005.12.013> <PMid:16448832>

Carter A.M. 2007. Animal models of human placentation: a review. Placenta 28(Suppl.A):S41-S47. <http://dx.doi.org/10.1016/j.placenta.2006.11.002> <PMid:17196252>

Carter A.M. & Mess A. 2014. Mammalian placentation: implications for animal models, p.2423-2442. In: McManus L.M. & Mitchell R.N. (Eds), Pathobiology of Human Disease: a dynamic encyclopedia of disease mechanisms. Elsevier, Berlin, Germany.

Conceição R.A., Ambrósio C.E., Martins D.S., Carvalho A.F., Franciolli A.L.R., Machado M.R.F., Oliveira M.F. & Miglino M.A. 2008. Aspectos morfológicos do saco vitelino em roedores da subordem Hystricomorpha: paca (*Agouti paca*) e cutia (*Dasyprocta agouti*). Pesq. Vet. Bras. 28(5):253-259. <http://dx.doi.org/10.1590/S0100-736X2008000500005>

Davies J., Dempsey E.W. & Amoroso E.C. 1961a. The subplacenta of the guinea pig: an electron microscopic study. J. Anat. 95:311-324. <PMid:17105123>

Davies J., Dempsey E.W. & Amoroso E.C. 1961b. The subplacenta of the guinea-pig: development, histology and histochemistry. J. Anat. 95:457-473. <PMid:13883829>

Favaron P.O., Carter A.M., Ambrósio C.E., Morini A.C., Mess A.M., Oliveira M.F. & Miglino M.A. 2011. Placentation in Sigmondontinae: a rodent táxon native to South America. Reprod. Biol. Endocrinol. 9(1):55. <http://dx.doi.org/10.1186/1477-7827-9-55> <PMid:21518439>

Flamini M.A., Portiansky E.L., Favaron P.O., Martins D.S., Ambrósio C.E., Mess A.M., Miglino M.A. & Barbeito C.G. 2011. Chorioallantoic and yolk sac placentation in the plains viscacha (*Lagostomus maximus*) a caviomorph rodent with natural polyovulation. Placenta 32(12):963-968. <http://dx.doi.org/10.1016/j.placenta.2011.09.002> <PMid:21982335>

Franciolli A.L.R., Ambrósio C.E., Oliveira M.F., Morini A.C., Favaron P.O., Machado M.R.F. & Miglino M.A. 2011. Os histricomorfos sul-americanos: uma análise comparativa do desenvolvimento embriológico. Pesq. Vet. Bras. 31(5):441-446. <http://dx.doi.org/10.1590/S0100-736X2011000500012>

Kanashiro C., Santos T.C., Miglino M.A., Mess A. & Carter A.M. 2009. Growth and development of the placenta in the capybara (*Hydrochaeris hydrochaeris*). Reprod. Biol. Endocrinol. 7(1):1-13. <http://dx.doi.org/10.1186/1477-7827-7-57> <PMid:19493333>

Kaufmann P. 2004. Capybara: *Hydrochaeris hydrochaeris*. In: Benirschke K. (Ed.), Comparative Placentation. Available at <http://placentation.ucsd.edu/capy.htm> Accessed on June 20, 2017.

Kaufmann P. & Davidoff M. 1977. The guinea pig placenta. Adv. Anat. Embriol. Cell. Biol. 53(2):5-91. <PMid:331890>

Mess A. 2003. Evolutionary transformations of chorioallantoic placental characters in Rodentia with special reference to hystricognath species. J. Exp. Zool. A, Comp. Exp. Biol. 299(1):78-98. <http://dx.doi.org/10.1002/jez.a.10292> <PMid:12950036>

Mess A. 2007a. The subplacenta in *Octodon degus* and *Petromus typicus*, two hystricognath rodents without significant placental lobulation. J. Exp. Zool. B, Mol. Dev. Evol. 308B(2):172-188. <http://dx.doi.org/10.1002/jez.b.21126> <PMid:17016808>

Mess A. 2007b. The guinea pig placenta: model of placental growth dynamics. Placenta 28(8/9):812-815. <http://dx.doi.org/10.1016/j.placenta.2007.02.005> <PMid:17382996>

Mess A. 2011. Character transformations and their functional significance as a key to the evolution of hystricognath Rodentia. Pesq. Vet. Bras. 31(12):1108-1115. <http://dx.doi.org/10.1590/S0100-736X2011001200012>

Mess A. 2014. Placental evolution within the supraordinal clades of eutheria with the perspective of alternative animal models for human placentation. Adv. Biol. 2014:1-21. <http://dx.doi.org/10.1155/2014/639274>

Mess A. & Carter A.M. 2007. Evolution of the placenta during the early radiation of placental mammals. Comp. Biochem. Physiol. A, Mol. Integr. Physiol. 148(4):769-779. <http://dx.doi.org/10.1016/j.cbpa.2007.01.029> <PMid:17347003>

Mess A., Zaki N., Kadyrov M., Korr H. & Kaufmann P. 2007. Caviomorph placentation as a model for trophoblast invasion. Placenta 28(11/12):1234-1238. <http://dx.doi.org/10.1016/j.placenta.2007.08.003> <PMid:17915313>

Miglino M.A., Carter A.M., Ferraz R.H.S. & Machado M.R.F. 2002. Placentation in the capybara (Hydrochaerus hydrochaeris), agouti (*Dasyprocta aguti*) and paca (*Agouti paca*). Placenta 23(5):416-428. <http://dx.doi.org/10.1053/plac.2002.0806> <PMid:12061858>

Miglino M.A., Franciolli A.L., Oliveira M.F., Ambrósio C.E., Bonatelli M., Machado M.R. & Mess A. 2008. Development of the inverted visceral yolk sac in three species of caviids (Rodentia, Caviomorpha, Caviidae). Placenta 29(8):748-752. <http://dx.doi.org/10.1016/j.placenta.2008.05.007> <PMid:18586321>

Miglino M.A., Carter A.M., Ambrósio C.E., Bonatelli M., Oliveira M.F., Ferraz R.H.S., Rodrigues R.F. & Santos T.C. 2004. Vascular organization of the hystricomorph placenta: a comparative study in the agouti, capybara, guinea pig, paca and rock cavy. Placenta 25(5):438-448. <http://dx.doi.org/10.1016/j.placenta.2003.11.002> <PMid:15081638>

Moojen J. 1952. Os Roedores do Brasil. Instituto Nacional do Livro, Ministério da Educação e Saúde, Rio de Janeiro. 214p.

Oliveira M.F., Carter A.M., Bonatelli M., Ambrósio C.E. & Miglino M.A. 2006. Placentation in the rock cavy, *Kerodon rupestris* Wied. Placenta 27(1):87-97. <http://dx.doi.org/10.1016/j.placenta.2004.11.012> <PMid:16310042>

Oliveira M.F., Mess A., Ambrósio C.E., Dantas C.A., Favaron P.O. & Miglino M.A. 2008. Chorioallantoic placentation in *Galea spixii* (Rodentia, Caviomorpha, Caviidae). Reprod. Biol. Endocrinol. 6(1):39. <http://dx.doi.org/10.1186/1477-7827-6-39> <PMid:18771596>

Oliveira M.F., Favaron P.O., Ambrósio C.E., Miglino M.A. & Mess A. 2012a. Chorioallantoic and yolk sac placentation in Thrichomys laurentius (Echimyidae) and the evolution of hystricognath rodents. J. Exp. Zool. B, Mol. Dev. Evol. 318:13-25.

Oliveira M.F., Vale A.M., Favaron P.O., Vasconcelos B.G., Oliveira G.B., Miglino M.A. & Mess A. 2012b. Development of yolk sac inversion in *Galea spixii* and *Cavia porcellus* (Rodentia, Caviidae). Placenta 33(10):878-881. <http://dx.doi.org/10.1016/j.placenta.2012.06.019> <PMid:22809674>.

Pinheiro M.J.P., Andrade S.A. & Cunha J.N. 1989. Preservação e exploração de animais silvestres: preá, cutia e mocó. Revta Caatinga 6:28-49.

Rodrigues R.F., Carter A.M., Ambrósio C.E., Santos T.C. & Miglino M.A. 2006. The subplacenta of the red-rumped agouti (*Dasyprocta leporina* L.). Reprod. Biol. Endocrinol. 4(1):31. <http://dx.doi.org/10.1186/1477-7827-4-31> <PMid:16740154>

Santos T.C., Tonarelli C., Teixeira F.A., Oliveira M.F., Maria D., Reginato P., Kfoury Junior J.R., Oliveira C.A.L., Lourenço D.A.L. & Miglino M.A. 2012. Histomorphometrical and proliferative aspects of placenta and uterus of the collared peccary (*Tayassu tajacu*). Histol. Histopathol. 27(6):793-806. <PMid:22473699>

Vale A.M. 2011. Dinâmica da inversão do saco vitelino em preás (*Galea spixii* Wagler, 1831). Dissertação de Mestrado, Universidade Federal Rural do Semi-Árido, Mossoró, RN. 115p.

Wolfer J. & Kaufmann P. 1980. Die ultrastruktur der meerschweinchen-subplazenta. Anat. Histol. Embryol. 9(1):29-43. <http://dx.doi.org/10.1111/j.1439-0264.1980.tb00838.x> <PMid:6445695>

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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 garante o contato com o Conselho Editorial da PVB. Asteriscos de chamadas para o rodapé devem ser mais uma vez elevados (sobrescritos), para aparecerem maiores e mais nítidos.

c) O **Cabeçalho do ABSTRACT** deve conter, além dos nomes dos autores abreviados invertidos, 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. Devem-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; de conveniência quando seguida de letra alta (35 kg ou 35kg, 4 h ou 4h). 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 usem 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.

3. **Apresentação das Figuras:**

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

b) Numerar cada figura separadamente (1, 2, ...).

c) Figuras com assuntos similares (subfiguras) devem ser agrupadas em pranchas com espaço entre elas de aprox. 1mm. Identifique cada imagem com uma letra maiúscula (A, B, ...) colocada no canto inferior esquerdo, de preferência fonte Arial 14, branca, em um quadro preto sem bordas.

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

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

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:

Pavarini S.P., Soares M.P., Bandarra P.M., Gomes D.C., Bandinelli M.B., Cruz C.E.F. & Driemeier D. 2011. Mortes súbitas causadas por *Amorimia exotropica* (Malpighiaceae) no Rio Grande do Sul. Pesq. Vet. Bras. 31(4):291-296.

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.

(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 "ponto-vírgulas" (;) 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:

Silva R.M.M. 2016. Prevalência, identificação e distribuição das lesões abscedativas em caprinos e ovinos abatidos em um matadouro frigorífico no Estado da Bahia. Dissertação de Mestrado, Universidade Federal do Recôncavo da Bahia, Cruz das Almas. 56p.

Sant´Ana V.A.C. 2004. Proteinograma do leite de vacas: padrões e variabilidade. Tese de Doutorado, Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP. 161p.

(Notem: (1) Deve-se evitar se referir a Dissertações ou Teses em vez de aos 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!)

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.

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.

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, 15h; but 35 kg or 35kg, 5 h or 5h as convenient).

The abbreviation for number is "nº" and not "n°"; for degree Celsius "°C" 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.

3. **Figure apresentation:**

a) Save images at 300 dpi, TIF files.

b) Number each Figure separately (1, 2, …).

c) Similar subjects as images should be grouped into a composition divided with spaces of about 1mm width. Label each subimage with a letter (A, B, ...) at the lower left corner, preferably with white, 14-point Arial font inside a black board with no border.

d) 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.

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

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:

Silva R.M.M. 2016. Prevalência, identificação e distribuição das lesões abscedativas em caprinos e ovinos abatidos em um matadouro frigorífico no Estado da Bahia. Dissertação de Mestrado, Universidade Federal do Recôncavo da Bahia, Cruz das Almas. 56p.

(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)

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

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

(Note: Consult entire papers instead of only Abstracts)

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