ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)
Volume 40 Number 10
October 2020

PESQUISA VETERINÁRIA BRASILEIRA

Brazilian Journal of Veterinary Research



Colégio Brasileiro de Patologia Animal
www.pvb.com.br

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

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

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

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

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

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

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

Founding General Editor, 1981-2018
Jürgen Döbereiner

Editorial Board

Chief-Editor: Claudio S.L. Barros (UFMS), *Campo Grande, MS* (cbarros@pvb.com.br).
Managing Editor: Daniel G. Ubiali (UFRRJ), *Seropédica, RJ* (daniel.ubiali@pvb.com.br).
Image-Editor: Asheley H.B. Pereira (UFRRJ), *Seropédica, RJ* (imagens@pvb.com.br).
Publisher: Grillo Tecnologia & Colégio Brasileiro de Patologia Animal (CBPA)

Livestock Diseases

Editor: Claudio S.L. Barros (UFMS), *Campo Grande, MS* (cbarros@pvb.com.br).
Associate Editors: Ana Lucia Schild (UFPel), *Pelotas, RS* (alschild@terra.com.br); David Driemeier (UFRGS), *Porto Alegre, RS* (davetpat@ufrgs.br); Francisco A. Uzal, (UCDavis), *San Bernardino, CA, USA* (fuzal@cahfs.ucdavis.edu); Franklin Riet-Correa (Instituto Nacional de Investigación Agropecuaria), *La Estanzuela*, *Uruguay* (frcorrea@inia.org.uy); Leticia Trevisan Gressler (IF Farroupilha), Frederico Westphalen, RS (letrevi@gmail.com); Mateus M. Costa (Univasf), *Petrolina, PE* (mmatiuzzi@hotmail.com); Raquel Rech (Texas A&M University), *College Station, TX, USA* (rrech@cvm.tamu.edu); Ricardo A.A. Lemos (UFMS), *Campo Grande, MS* (ricardo.lemos@ufms.br); Roselene Ecco (UFMG), *Belo Horizonte, MG* (eccoro.ufmg@gmail.com).

Small Animal Diseases

Editor: Claudio S.L. Barros (UFMS), *Campo Grande, MS* (cbarros@pvb.com.br).
Associate Editors: Alexandre Mazzanti (UFSM), *Santa Maria, RS* (alexamazza@yahoo.com.br); Corrie Brown (University of Georgia), *Athens, GA, USA* (corbrown@uga.edu); Daniel R. Rissi (Athens Vet. Diagn. Lab.), *Athens, GA, USA* (danielricardorissi@yahoo.com.br); Ingeborg M. Langohr (Louisiana State University), *Baton Rouge, USA* (langohri@dcpah.msu.edu); Leticia Trevisan Gressler (IF Farroupilha), Frederico Westphalen, RS (letrevi@gmail.com); Luciana Sonne (UFRGS), *Porto Alegre, RS* (lusonne@yahoo.com.br); Saulo P. Pavarini (UFRGS), *Porto Alegre, RS* (sauloppvet@yahoo.com.br); Ticiana N. França (UFRRJ), *Seropédica, RJ* (ticianafranca19@gmail.com).

Wildlife Medicine

Editor: Pedro M.O. Pedroso (UnB), *Brasília, DF* (pedrosovet@yahoo.com.br).
Associate Editors: Juliana T.S.A. Macêdo (UnB), *Brasília, DF* (jtsam_targino@yahoo.com.br); Mauro Pereira Soares (UFPel), *Pelotas, RS* (gmpsoares@gmail.com); Ricardo B. Lucena (UFPB), *Areia, PB* (lucena.rb@gmail.com).

Animal Morphophysiology

Editor: Carlos Eduardo Ambrósio (USP), *FZEA-Pirassununga, SP* (ceambrosio@usp.br).
Associate Editors: Antônio C. Assis Neto (USP), *São Paulo, SP* (antonioassis@usp.br); Daniele S. Martins (USP), *FZEA-Pirassununga, SP* (daniele@usp.br); Maria Angélica Miglino (USP), *São Paulo, SP* (miglino@usp.br); Tatiana C. Santos (UEM), *Maringá, PR* (tcsantos@uem.br).

Apoio:

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

Advisory Board

Adivaldo H. Fonseca (UFRRJ), Seropédica, RJ
Aldo Gava (UDESC), Lages, SC
Alessandra E.S. Lima (UFBA), Salvador, BA
Alexandre Mazzanti (UFSM), Santa Maria, RS
Alexandre S. Borges (Unesp), Botucatu, SP
Aline M. Viott (UFPR), Palotina, PR
Aline R. Hoffmann, Diplomada ACVP (Texas A&M University), College Station, TX, USA
Amauri A. Alfieri (UEL), Londrina, PR
Ana Lucia Schild (UFPel), Pelotas, RS
Angélica T.B. Wouters (UFLA), Lavras, MG
Aníbal G. Armién, Diplomado ACVP (Univeristy of Minnesota), St. Paul, USA
Antônio F.M. Dantas (UFCG), Patos, PB
Axel Colling (CSIRO), Gueelong, Australia
Bruno L. Anjos (Unipampa), Uruguaiana, RS
Carlos L. Massard (UFRRJ), Seropédica, RJ
Caroline A. Pescador (UFMT), Cuiabá, MT
Cláudio E.F. Cruz (UFRGS), Porto Alegre, RS
Claudio S.L. Barros, Membro honorário ACVP (UFMS), Campo Grande, MS
Claudio W. Canal (UFRGS), Porto Alegre, RS
Corrie Brown, Diplomada ACVP (University of Georgia), Athens, GA, USA
Daniel A. Balthazar (UFRRJ), Seropédica, RJ
Daniel C.L. Linhares (Iowa State University), Ames, USA
Daniel G. Ubiali (UFRRJ), Seropédica, RJ
Daniel R. Rissi, Diplomado ACVP (University of Georgia), Athens, GA, USA
Daniela I.B. Pereira (UFPel), Pelotas, RS
Danilo C. Gomes (UFMS), Campo Grande, MS
David Driemeier (UFRGS), Porto Alegre, RS
David E.S.N. Barcellos (UFRGS), Porto Alegre, RS
Djeison L. Raymundo (UFLA), Lavras, MG
Eduardo F. Flores (UFSM), Santa Maria, RS
Edson M. Colodel (UFMT), Cuiabá, MT
Fabiana M. Boabaid (Univ. La Republica), Tacuarembo, Uruguay
Fabiano J.F. Sant'Ana (UnB), Brasília, DF
Fábio S. Mendonça (UFRPE), Recife, PE
Felício G. Júnior (UFCG), Patos, PB
Felipe Pierezan, Diplomado ACVP (UFMG), Belo Horizonte, MG
Félix Gonzáles (UFRGS), Porto Alegre, RS
Fernando H. Furlan (UFMT), Cuiabá, MT
Fernando R. Spilki (Univ. Feevale), Novo Hamburgo, RS
Flademir Wouters (UFLA), Lavras, MG
Francisco A. Uzal, Diplomado ACVP (UCDavis), San Bernardino, CA, USA
Franklin Riet-Correa (Instituto Nacional de Investigación Agropecuaria), La Estanzuela, Uruguay
Glaucia D. Kommers (UFSM), Santa Maria, RS
Glauco J.N. Galiza (UFCG), Patos, PB
Guilherme G. Verocai (Texas A&M University), College Station, TX, USA
Gustavo S. Silva (Iowa State University), Ames, USA
Huarrison A. Santos (UFRRJ), Seropédica, RJ
Ingeborg M. Langohr, Diplomada ACVP (Louisiana State University), Baton Rouge, LA, USA
Iveraldo S. Dutra (Unesp), Araçatuba, SP
Janildo L. Reis Júnior, Diplomado ACVP (UFJF), Juiz de Fora, MG
Jean Carlos R. Silva (UFRPE), Recife, PE
Jeann Leal de Araujo (Texas A&M University), College Station, TX, USA
John Edwards, Diplomado ACVP (Texas A&M University), College Station, TX, USA
José Diomedes Barbosa (UFPA), Castanhal, PA
José Luiz Catão-Diaz (USP), São Paulo, SP
José M.V. Garcia (Univ. La Republica), Montevideo, Uruguay
José R.J. Borges (UnB), Brasília, DF
Josué Díaz-Delgado, Diplomado ACVP (USP), São Paulo, SP
Juliana S. Brum (UFPR), Curitiba, PR
Juliana T.S.A. Macêdo (UnB), Brasília, DF
Luciana Sonne (UFRGS), Porto Alegre, RS
Luciano A. Pimentel (UFRB), Cruz das Almas, BA
Luciano Nakazato (UFMT), Cuiabá, MT
Luciano S. Alonso (UFRRJ), Seropédica, RJ
Luis Fernando Pita Gondim, (UFBA), Salvador, BA
Marcelo B. Labruna (USP), São Paulo, SP
Mauro Pereira Soares (UFPel), Pelotas, RS
Mateus M. Costa (Univasf), Petrolina, PE
Paula R. Giaretta, Diplomada ACVP (Texas A&M University), College Station, TX, USA
Paulo César Maiorka (USP), São Paulo, SP
Paulo M. Roehe (UFRGS), Porto Alegre, RS
Paulo V. Peixoto (UFRRJ), Seropédica, RJ
Pedro M.O. Pedroso (UnB), Brasília, DF
Pedro Malafaia (UFRRJ), Seropédica, RJ
Pedro S. Bezerra Junior (UFPA), Castanhal, PA
Peres R. Badial (Mississipi State University), Starkville, MS, USA
Raquel R. Rech, Diplomada ACVP (Texas A&M University), College Station, TX, USA
Renata A. Casagrande (UDESC), Lages, SC
Ricardo A.A. Lemos (UFMS), Campo Grande, MS
Ricardo B. Lucena (UFPB), Areia, PB
Roberto M.C. Guedes (UFMG), Belo Horizonte, MG
Roselene Ecco (UFMG), Belo Horizonte, MG
Rudi Weiblen (UFMS), Santa Maria, RS
Saulo A. Caldas (UFRRJ), Seropédica, RJ
Saulo P. Pavarini (UFRGS), Porto Alegre, RS
Tatiane T.N. Watanabe, Diplomada ACVP (North Carolina State University), Raleigh, NC, USA
Tessie B. Martins (UFMS), Campo Grande, MS
Tiago C. Peixoto (UFBA), Salvador, BA
Ticiana N. França (UFRRJ), Seropédica, RJ
Valéria Dutra (UFMT), Cuiabá, MT
Welden Panziera (UFRGS), Porto Alegre, RS

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

il.; 28 cm

Mensal.

Resumos em inglês e português.

ISSN 0100-736X (*Print*).

ISSN 1678-5150 (*Online*).

Título traduzido: *Brazilian Journal of Veterinary Research*.

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

I. Colégio Brasileiro de Patologia Animal.

Tiragem: 100 exemplares.

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

PESQUISA VETERINÁRIA BRASILEIRA

Brazilian Journal of Veterinary Research

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

Colégio Brasileiro de Patologia Animal

www.pvb.com.br

Cover illustration: Diffuse fibrinous pericarditis in swine (Piva M.M. et al., pag. 763).

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(10):739-749, October 2020
DOI: 10.1590/1678-5150-PVB-6700

Original Article
Livestock Diseases
BY-NC

Research and extension action for parasitic control in pig breeding families located in Tanguá, Rio de Janeiro[1]

Camila S.C. Class[2], Renato L. Silveira[3], João Pedro S. Palmer[2], Priscila A. Fialho[2], Lucas F. Lobão[2], Laís V. Dib[2], Claudia Maria A. Uchôa[2] and Alynne S. Barbosa[2,4]*

ABSTRACT.- Class C.S.C., Silveira R.L., Palmer J.P.S., Fialho P.A., Lobão L.F., Dib L.V., Uchôa C.M.A. & Barbosa A.S. 2020. **Research and extension action for parasitic control in pig breeding families located in Tanguá, Rio de Janeiro**. *Pesquisa Veterinária Brasileira 40(10):739-749*. Laboratório de Bioagentes Ambientais, Departamento de Microbiologia e Parasitologia, Instituto Biomédico, Universidade Federal Fluminense, Niterói, RJ 24210-130, Brazil. E-mail: alynnedsb@gmail.com

This study aimed to analyze the parasites infesting pigs and their producers on family farms in Tanguá, RJ, Brazil, and to conduct extension activities between 2018 and 2019. Fecal samples were collected from 132 pigs, as well as, skin scrapings from the ears of 125 animals. In addition, 36 stool specimens from farmers and their families were analyzed. The collected material was processed by direct examination, sedimentation and flotation techniques. Gastrointestinal parasites were detected in 88.6% of the pigs, especially coccidia (71.8%), *Balantioides coli* (55%), strongyles (40.2%) and *Strongyloides ransomi* (31.6%) which showed statistically significance ($p<0.05$). Ectoparasites were identified in 16% of the pigs, mostly *Sarcoptes scabiei* var. *suis* (76.2%). Parasites were detected in 19.4% of the humans, including, *Entamoeba coli* (19.4%), *Ascaris lumbricoides* (8.3%) and *Trichuris trichiura* (5.5%). The frequency of *B. coli* and *S. scabiei* var. *suis* ($p<0.05$) were statistically significant, when analyzing the age group of the animals, having been more detected in the older years, fatterners phase. The pig farmers participated in these activities extension, which included lecture and "field day", aimed at imparting information about parasites and their prophylaxis. The situation in Tanguá pig farms reinforces the need to carry out programs that provide technical support to these farmers, whose livelihood depends on pig farming.

INDEX TERMS: Parasitic control, pig breeding, Rio de Janeiro, pigs, gastrointestinal parasites, scabies, rural extension.

RESUMO.- [Pesquisa e ação de extensão para controle das parasitoses em propriedades familiares de criação de suínos localizadas em Tanguá, Rio de Janeiro.] Este estudo objetivou analisar os parasitos que infectam suínos e seus produtores em propriedades familiares em Tanguá, RJ, Brasil, realizando atividades extensionistas entre 2018 e 2019. Amostras fecais foram coletadas de 132 suínos, bem como raspados da pele da orelha de 125 animais. Além disso, 36 amostras fecais dos produtores e seus familiares foram analisadas. O material coletado foi processado pelo exame direto e por técnicas de sedimentação e flutuação. Parasitos gastrointestinais foram detectados em 88,6% dos suínos, principalmente coccídios (71,8%), *Balantioides coli* (55%), estrôngilos (40,2%) e *Strongyloides ransomi* (31,6%) que apresentaram significância estatística ($p<0,05$). Ectoparasitos foram identificados em 16% dos suínos, principalmente *Sarcoptes scabiei* var. *suis* (76,2%). Parasitos foram detectados em 19,4% das amostras fecais humanas, incluindo *Entamoeba coli* (19,4%), *Ascaris lumbricoides* (8,3%) e *Trichuris trichiura* (5,5%). A frequência de *B. coli* e *S. scabiei* var. *suis* foi estatisticamente significativa ($p<0,05$), quando se analisou os animais por faixa etária, tendo sido principalmente detectado nos animais mais velhos; como na fase de terminação. Os produtores de suínos participaram

[1] Received on May 9, 2020.
Accepted for publication on July 11, 2020.
[2] Laboratório de Bioagentes Ambientais, Departamento de Microbiologia e Parasitologia, Instituto Biomédico, Universidade Federal Fluminense (UFF), Rua Professor Hernani Melo, 101, Niterói, RJ 24210-130, Brazil.
[3] Departamento de Morfologia, Instituto Biomédico, Universidade Federal Fluminense (UFF), Rua Professor Hernani Melo 101, Niterói, RJ 24210-130, Brazil.
[4] Laboratório de Toxoplasmose e Outras Protozooses, Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, RJ 21040-360, Brazil. *Corresponding author: alynnedsb@gmail.com

ativamente das atividades de extensão, incluído a palestra e a atividade "dia de campo", que tinham como objetivo mediar informações sobre parasitos e suas medidas profiláticas. A situação nas granjas familiares de Tanguá demonstra a necessidade de realização de programas que apoiem tecnicamente esses produtores, que utilizam a suinocultura como fonte de subsistência.

TERMOS DE INDEXAÇÃO: Controle de parasitoses, criação de suínos, Rio de Janeiro, suínos, parasitos gastrointestinais, sarna, extensão rural.

INTRODUCTION

Brazil stands out worldwide in pig farming, mainly in industrial animal agriculture, where pigs are raised in total confinement in intensive systems under highly sanitary conditions. Today, research and investments in pig farming have placed Brazil in fourth place in the ranking of the world largest pork producers and exporters (Embrapa 2018).

Notwithstanding the excellence of industrial farming in Brazil, a large part of pig production is concentrated on small farms, which generally raise these animals as a source of primary or secondary income and/or of subsistence. Generally, this type of pig farming involves simple intensive or extensive systems, with little financial and technological investment. This activity takes place at approximately 46.5% of the 5.8 million farms in Brazil, which typically employ family labor and constitute an important source of income (Silva Filha et al. 2011).

In national pig herds, health problems can be attributed to a variety of biological agents, including gastrointestinal parasites and ectoparasites, which are one of the limiting factors in raising these animals (Carreiro et al. 2016). The main negative effects of parasitic diseases in pigs are economic losses for producers, such as reduced feed conversion, reduced fertility, low number of piglets born and weaned, low weight at birth and at weaning, in addition to losses resulting from high discard rates in slaughterhouses (Roepstorff et al. 1998, Nansen & Roepstorff 1999, Barbosa et al. 2015a). Moreover, pigs are considered the main reservoirs of *Balantioides coli*, *Entamoeba polecki* and also *Blastocystis* sp. a protozoan with zoonotic potential that can infect humans, especially farmers that raise these animals (Solaymani-Mohammadi & Petri Jr 2006).

Not only endoparasites but also arthropods represent a major problem in pig farming, especially *Sarcoptes scabiei* variety *suis*, which is considered the most important ectoparasitic mite in pig farming. In pigs, this ectoparasite can cause sarcoptic mange, i.e., scabies, manifested in the form of intense itching, causing extreme discomfort and favoring infestation by other secondary pathogenic organisms. Thus, scabies can cause economic losses for the producer through reduced growth rates of the animals, low reproductive efficiency, and increased carcass disposal rates in slaughterhouses (Sobestiansky et al. 2005).

The national scientific literature contains several studies analyzing pig parasites recovered from fecal samples, especially studies conducted on family farms. These studies have reported high positivity rates for gastrointestinal parasites, i.e., about 90% of pigs raised on family farms in the states of Sergipe, Bahia, Distrito Federal and Rio de Janeiro (Pinto et al. 2007, Aguiar 2009, Brito et al. 2012, Barbosa et al. 2015a). With regard to ectoparasites, all the studies conducted in Brazil focused on industrial farms, but none of them involved family farms. In the biological samples collected from pigs on industrial farms, positivity for *Sarcoptes scabiei* variety *suis* was reported, ranging from 0.09% to 16.6% in biological samples collected from pigs raised on farms in southern Brazil and 12.1% and 43.5% on farms in the north (Silva et al. 2002, Pedroso-de-Paiva et al. 2003, Sobestiansky et al. 2005, Oliveira et al. 2006).

In view of the above, it is important to carry out studies on family pig farms to ascertain the parasite infection rates among these animals and their producers, and to conduct qualitative research to learn about these farmers' conditions of management and socioeconomic health, associating them with extension activities aimed at mediating information about parasites. It should be noted that studies on this topic have not yet been carried out in Brazil. In the state of Rio de Janeiro, these small family farmers are concentrated in the interior of the state, as is the case of the Tanguá city and neighboring cities, which still have areas with little urbanization. Thus, this study aimed to analyze the frequency of parasites detected in pigs, relating their positivity rates to the sex and age of the animals, and to perform the same analysis on human fecal samples from pig farmers on family farms, associating the information garnered in this research with extension activities.

MATERIALS AND METHODS

Ethical consideration

This study was approved by the Ethics Committee on Animal Use of the Universidade Federal Fluminense (CEUA/UFF) under Protocol no. 1048, and by the Human Research Ethics Committee of UFF under Protocol no. 2686336.

Study location

This study was carried out from June 2018 to August 2019 on family owned pig farms located in Tanguá city (22°43′48″ S, 42°42′50″ W), which covers an area of 146.62km^2, with tropical climate and is part of the metropolitan region of the state of Rio de Janeiro. Tanguá is surrounded by Itaboraí, Maricá, Rio Bonito and Saquarema cities. Tanguá consists of 10 neighborhoods, has a population of about 30,732, is rich in natural resources, and has small farms run by nuclear families (Brasil 2015) (Fig.1).

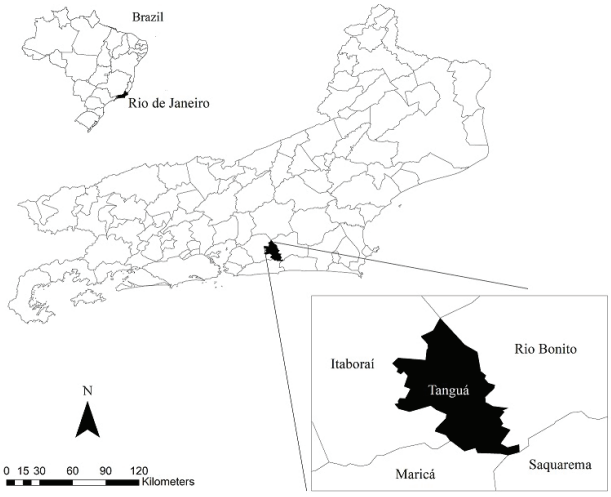


Fig.1. Location of Tanguá city in the state of Rio de Janeiro, Brazil.

Study design, application of forms and collection of biological samples

This study involved seven technical visits to each farm for a variety of objectives. The visits occurred at intervals of approximately 10 days. During the period of this study, a leaflet was delivered to the neighborhood community outpatient health clinic to inform the local doctor about the study, in case anyone included in it sought medical treatment.

First visit. Consisted of presenting the study in a simple conversation with farm owners and other family members and obtaining their signature on the informed consent forms required by the Ethics Committees. Since the city does not have a register of these small family farms, they were identified based on information provided by other farmers living in the city. The farmers who agreed to participate in the study filled out two semi-structured forms, one containing questions about the pigs and pig-raising management, which was answered only by the producer, and the other, which was answered individually by each family member, and included sanitation-related questions. After the forms were filled out, the research team walked around the farm in order to familiarize themselves with the property and the pig raising operation.

Second visit. Fecal samples from these animals were collected directly from the rectal ampulla using a rectal palpation glove lubricated with glycerin, and were duly tagged. Material from the outer ears was collected using a stainless steel spatula soaked in glycerin and was deposited in sterile plastic Petri dishes, which were then tagged and sealed with tape. Piglets younger than one month and pregnant sows were not included in the sampling procedures. In addition, during this visit, stool collection kits were delivered to farmers and their family members for their own use. These kits contained two labeled 80mL stool specimen containers without chemical preservative, two wooden spatulas and a leaflet describing the proper collection procedure. Each person was also told that the samples should be collected on two different days and stored in a refrigerator door. The stool samples from each person were picked up by the study team at a prearranged time.

Third visit. Included the delivery of the parasitology test results of humans and pigs. The test results of the pigs were explained to each farmer, while the human test results were explained individually to clear up any doubts. At the end of this visit, a presentation called "Parasites and the importance of their control" was made on a structure resembling a large book made of canvas posters. Most of the information in this material was visual with figures.

Fourth visit. Consisted of a "field day," accompanying the farmer in his daily routine activities of cleaning pig pens and sanitizing the animal feed and water troughs. During this visit, the team members held discussions with the farmers about the correct and incorrect animal handling procedures they had adopted and suggested steps to correct them. Also during this visit, antiparasitic drugs containing fenbendazole as active ingredient were mixed into the pig feed. At the end of this visit, the producer was given two posters. One of them was entitled "Ten important steps for the producer: hygiene in pig farming," which repeated the information about good pig raising practices. The other poster was a "Health Calendar," whose purpose was schedule medications and to remind them of the dates and drugs given to their pigs. The two posters were posted on the farm at specific locations selected jointly by the farmers and researchers.

Fifth, sixth and seventh visits. These visits involved an activity called "homework checking," to determine whether the animal handling information and advice was being carried out on the farm. These visits also provided opportunities for questions to be asked and doubts cleared up about the subjects previously discussed.

Laboratory processing of fecal samples and skin scrapes from the ear

A part of the human and pig fecal samples was immediately processed by direct examination. Another part of the sample was homogenized and the filtrate was aliquoted and placed in 15ml conical bottom centrifuge tubes and processed by the centrifugal sedimentation method proposed by Ritchie (1948) modified by Young et al. (1979), to the centrifugal flotation technique of Faust et al. (1938); and to the centrifugal flotation method of Sheather (1923) modified by Huber et al. (2003). Part of the filtered material was allowed to settle for 24 hours in conical bottom flasks to perform the spontaneous sedimentation technique (Lutz 1919).

In the Parasitology laboratory, the samples collected from the pigs' ear pavilion were submitted to the protocol described by Ferreira (2010) with some modifications. The Petri dishes containing the samples collected from the ear were incubated at 30ºC in a bacteriological incubator for 30 min, and then examined in an Olympus® CKX41 inverted microscope under 100x magnification. The Petri dishes were then covered with a lactophenol solution, sealed again, and incubated at room temperature for one month. After this period, the samples were transferred to 15ml centrifuge tubes, with 1ml of sediment in each tube, which were completed with distilled water to a volume of 15ml, and then centrifuged at 2500rpm for five min. After centrifugation, a microscope slide of the tube sediment was prepared, covered with a 24 x 32mm coverslip, and examined under an optical microscope.

After examining the slide, the remaining pellet in the tube was suspended with sucrose solution at a density of 1,300g/ml until a volume of 15mL. This solution was then centrifuged at 1500rpm for 10 min. After this procedure, the tubes were placed on shelves and the sucrose solution was added until a meniscus was formed over which were placed 24 x 32mm coverslips, which were left to rest on the meniscus for 20 min in this flotation stage.

An Olympus BX 41 optical microscope was used to examine the slides obtained in each technique, initially under 50x and 100x magnification, and then under 400x for confirmation, when necessary. An ocular micrometer was used for morphometry of evolutionary forms of the identified parasites.

Data analysis

All the qualitative information retrieved from the forms was tabulated and presented descriptively, by means of a percentage, while the most widely reported information was presented in tables. Data on the animals' sex and age, in months, were also retrieved and tabulated. Thus, the pigs were classified in stages, as follows: Initial stage - from one to two months of age to weaners, Growing stage - from two to four months old, and Fattening stage - four months and older.

A biological material was considered positive when at least one parasite was found (trophozoites, eggs, larvae, cysts or oocysts). The frequency was determined by dividing the number of positive samples by the total number of samples collected, and these data were presented in percentages. Fisher's exact test with a 5% confidence level was applied, using Graph Pad version 6 software, to analyze the parasite positivity rate according to the taxonomic classification of parasites and also to the categories of age and sex of the pigs.

RESULTS

This study involved a total of 14 family farms, which were identified by the letters A to N. The number of pigs on these farms varied 1 to 56. Of the 132 pigs included in this study, 88.6% of them had gastrointestinal parasites, with protozoa

detected more than helminths. In fact, the parasite positivity rate among the animals on these farms exceeded 70%. In general, non-sporulated coccidia oocysts were the most frequently detected parasites, followed by *Balantioides coli*, strongyles and *Strongyloides ransomi*, whose presence was statistically significant (p<0.05). Other parasites were also detected, such as amoebic cysts, eggs of *Trichuris suis* and *Ascaris suum*, cysts of *Blastocystis* spp. and eggs of *Capillaria* spp. However, the frequency of these parasites was not statistically significant (p>0.05). Among the 125 biological samples collected from the pigs' ears, forms similar to ectoparasites were diagnosed in 20 (16%) samples, and adult forms of the mite *Sarcoptes scabiei* variety *suis* was the most frequently detected, showing a statistically significant frequency (p<0.05) (Table 1).

Of the 132 pigs included in the study, 62 were females and 70 were males, with 90.3% and 87.1%, respectively, testing positive for parasites. Protozoa and helminths were detected in the feces of both females and males. However, no significant statistical relevance was found in the diagnosis of parasite taxa when the frequency was compared as a function of sex (p>0.05). As for age groups, the older pigs, i.e., those in the fattening stage, showed a higher positivity rate than the other age groups. *B. coli* and *S. scabiei* var. *suis* were the parasites that showed a significant difference in a comparison of frequency rates as a function of age groups (p<0.05) (Table 2).

Based on information obtained from the forms answered by the farmers about their pig management procedures and on the observations of team members, it can be stated that more than 78.6% of the farmers raised their pigs in the backyard of their own homes, where they also grew fruit trees, vegetables, and herbs. All the farms (100%) raised crossbred pigs i.e., mixed breeds, and also kept other farm animals such as cows, horses, chickens, ducks, dogs, cats, birds, and even rodents such as guinea pigs. All the farmers stated that they raised pigs for the family's own consumption, although there was also one farm that sold or exchanged the animals (Table 3).

About 92.9% of the farms had pig pens with cement floors, and 64.3% of them were covered with fiber cement roof tiles. Some farmers also allowed their pigs to roam freely around the houses during part of the day. As for animal handling, 100% of the farmers reported providing drinking water for their pigs, wheat bran in their feed, and human food leftovers that were previously stored in plastic drums. The farmers also stated that they did not adopt the practice of sanitary

Table 1. Parasites detected in fecal and ear skin scraping samples from pigs raised on different family farms located in Tanguá city, state of Rio de Janeiro, Brazil

Gastrointestinal parasites	Family pig farming properties															
	A (n=56)	B (n=5)	C (n=4)	D (n=2)	E (n=1)	F (n=2)	G (n=4)	H (n=1)	I (n=4)	J (n=8)	K (n=21)	L (n=13)	M (n=1)	N (n=10)	Total (n=132)	p value
Balantioides coli	12 (27.3%)	1 (20%)	2 (66.7%)	2 (100%)	1 (100%)	2 (100%)	3 (75%)	1 (100%)	4 (100%)	5 (62.5%)	11 (52.4%)	10 (91%)	1 (100%)	9 (90%)	64 (55%)	<0.0001*
Amoebids	5 (11.4%)	3 (60%)	0	0	0	1 (50%)	1 (25%)	0	2 (50%)	2 (25%)	0	0	0	8 (80%)	22 (18.8%)	0.0744
Coccidia oocysts	33 (75%)	4 (80%)	0	1 (50%)	1 (100%)	0	3 (75%)	1 (100%)	2 (50%)	5 (62.5%)	20 (95.2%)	7 (63.6%)	0	7 (70%)	84 (71.8%)	<0.0001*
Blastocystis sp.	0	0	0	0	1 (100%)	1 (50%)	0	0	0	0	0	0	0	1 (10%)	3 (2.6%)	1
Ascaris suum	3 (6.8%)	3 (60%)	2 (66.7%)	0	0	0	0	0	0	1 (12.5%)	2 (9.5%)	1 (9.1%)	0	0	12 (10.2%)	0.3591
Nematode larvae	2 (4.5%)	0	0	0	0	0	1 (25%)	0	0	0	3 (14.3%)	0	0	4 (40%)	10 (8.5%)	0.6084
Strongyloides ransomi	7 (15.9%)	3 (60%)	0	0	0	0	2 (50%)	0	0	0	17 (80.9%)	0	0	8 (80%)	37 (31.6%)	0.0112*
Strongyles	14 (31.8%)	1 (20%)	0	2 (100%)	0	0	2 (50%)	1 (100%)	4 (100%)	3 (37.5%)	4 (19%)	5 (45.4%)	1 (100%)	8 (80%)	47 (40.2%)	0.0006*
Trichuris suis	3 (6.8%)	2 (40%)	0	0	0	0	0	0	0	0	10 (47.6%)	2 (18.2%)	0	0	17 (14.5%)	0.2159
Capillaria sp.	0	0	0	0	0	0	0	0	0	0	1 (4.8%)	0	0	0	1 (0.8%)	1
Subtotal	44 (78.6%)	5 (100%)	3 (75%)	2 (100%)	1 (100%)	2 (100%)	4 (100%)	1 (100%)	4 (100%)	8 (100%)	21 (100%)	11 (85%)	1 (100%)	10 (100%)	117 (88.6%)	
Ectoparasites	A (n=54)	B (n=4)	C (n=4)	D (n=2)	E (n=1)	F (n=0)	G (n=4)	H (n=1)	I (n=4)	J (n=7)	K (n=21)	L (n=12)	M (n=1)	N (n=10)	Total (n=125)	p value
Sarcoptes scabiei var. *suis*	4 (66.6%)	1 (50%)	1 (100%)	0	0	0	0	1 (100%)	1 (100%)	1 (100%)	4 (80%)	0	0	3 (100%)	16 (76.2%)	<0.0001*
Demodex phylloides	0	1 (50%)	0	0	0	0	0	0	0	0	1 (20%)	0	0	0	2 (12.5%)	0.0266*
Arthropod egg	2 (33.3%)	0	0	0	0	0	0	0	1 (100%)	0	0	0	0	0	3 (13.6%)	0.0036*
Subtotal	6 (11.1%)	2 (50%)	1 (25%)	0	0	0	0	1 (100%)	1 (25%)	1 (14.2%)	5 (23.8%)	0	0	3 (30%)	20 (16%)	

* p value <0.05.

breaks, and not use a flamethrower to disinfect the pig pens. Most of the farmers, i.e., 85.7%, reported pouring water over the animals to cool them (Table 3).

Based on the forms, the technical visits, and especially the "field day" activity, it was found that more than 75% of the farmers fed and watered the pigs directly in cement troughs built on the floor of the pens. During the field activities, the team members noticed accumulated excreta in many of the pig pens. Most of the farmers also reported that they had already seen pigs scratching themselves, had never found blood in the pigs' feces, and vaccinated and wormed their animals. They reported that they did not treat their pigs with anti-ectoparasitic drugs, and that they had seen rats on the farm. They reported that they washed the pens with water, and used brooms and shovels only to muck out the enclosures. Half of the farmers stated that they wore the clothes the entire day, and did not wear them solely when handling the pigs (Table 3).

Of the 42 participants in this study, i.e., the farmers and their family members, 36 delivered stool samples. Seven of these 36 stool samples, 7/36 (19.4%), contained evolutionary forms of parasites, including *Entamoeba coli* cysts in 4/36 (19.4%), which were identified in stool samples from four individuals from farms A, B, I and N. In addition, *Ascaris lumbricoides* eggs were found in 3/36 (8.3%) stool samples from two people on farm I, and from one person on farm J, as well as *Trichuris trichiura* eggs in the stool of two individuals, 2/36 (5.5%), one from farm B and the other from farm J.

The information provided by the farmers and their families who delivered the fecal samples revealed that more than 80% of them had never noticed fragments of helminths in their stool or blood, and had already had stool tests and taken antiparasitic medicine. About 61.1% of the participants stated they felt no intestinal pain and 69.4% had not suffered from diarrhea in the previous six months. The majority (58.3%) stated that they wash roots and leafy vegetables in water and vinegar, and 55.5% stated they use tap water from the public water supply system and artesian well for drinking and cooking. About half of the farms who delivered the stool samples (52.3%) use filters to treat their drinking water (Table 4).

All the farmers participated in the extension activities carried out on the farms. The 3rd technical visit was particularly relevant, since this was when the lab test results of the pigs and humans were delivered and the interactive lecture "Parasites and the importance of their control" was given. The participants demonstrated their interest by asking numerous questions about the lab test results, parasite transmission routes, and possible treatments and preventive measures.

On the 4th visit, all the farmers participated in the proposed "field day" activities with the team. The team interacted with them as they cleaned the pig pens, suggesting changes in some routine practices that might be harmful to the animals' health. The following suggestions stand out: remove dry feces from the pens (mucking out), especially on rainy and cold days, and avoid cleaning your home with the same cleaning supplies used in pig pens. Another important point is to remember to wash out the feed and water troughs regularly and provide fresh clean water *ad libitum*. Provide straw bedding for farrowing sows close to parturition and newborn piglets. Try to use natural resources available on the farm to feed the pigs, such as frugivorous plants and leaves such as banana, Eucalyptus and kingwood (*Astronium fraxinifolium*).

During the 5th, 6th and 7th visits to each farm, the researchers observed that the farmers and their families were adopting strategies the team had suggested, such as

Table 2. Frequency of parasites detected in fecal and ear skin scraping samples from pigs, according to sex and age group, raised on family farms in Tanguá city, state of Rio de Janeiro, Brazil

Gastrointestinal parasites	Sex			Age range			
	Female (n=62)	Male (n=70)	p value	Iniciation (n=58)	Growing (n=48)	Fatteners (n=26)	p value
Amoebids	11 (19.6%)	11 (18%)	0.81	4 (8.3%)	9 (20.9%)	9 (34.6%)	0.11
Balantioides coli	32 (57.1%)	32 (52.5%)	0.6	13 (27.1%)	30 (69.8%)	21 (80.8%)	< 0.0001*
Blastocystis sp.	2 (3.6%)	1 (1.6%)	0.6	1 (2.1%)	1 (2.3%)	1 (3.8%)	0.99
Coccidia oocysts	35 (62.5%)	49 (80.3%)	0.14	38 (79.2%)	33 (76.7%)	13 (50%)	0.84
Subtotal of samples with protozoa (n=109)	51 (91.1%)	58 (95.1%)	-	43 (89.6%)	41 (95.3%)	25 (96.2%)	-
Ascaris suum	5 (8.9%)	7 (11.5%)	0.2	7 (14.6%)	2 (4.7%)	3 (11.5%)	0.89
Strongyles	25 (44.6%)	22 (36.1%)	0.85	13 (27.1%)	22 (51.2%)	12 (46.2%)	0.24
Nematode larvae	4 (7.1%)	6 (9.8%)	0.74	3 (6.3%)	7 (16.3%)	0	0.42
Strongyloides ransomi	11 (19.6%)	26 (42.6%)	0.01	17 (35.4%)	20 (46.5%)	0	0.02*
Trichuris suis	11 (19.6%)	6 (9.8%)	0.12	7 (14.6%)	9 (20.9%)	1 (3.8%)	0.75
Capillaria sp.	0	1 (1.6%)	1	0	1 (2.3%)	0	0.94
Subtotal of samples with helminths (n=74)	34 (60.7%)	40 (65.6%)	-	28 (58.3%)	32 (74.4%)	14 (53.8%)	-
Total positive samples for gastrointestinal parasites (n=117)	56 (90.3%)	61 (87.1%)	-	48 (82.8%)	43 (89.6%)	26 (100%)	-
Ectoparasites	Female (n=59)	Male (n=66)	p value	Iniciation (n=58)	Growing (n= 48)	Fatteners (n=19)	p value
Sarcoptes scabiei var. *suis*	5 (71.4%)	11 (84.6%)	0.59	4 (50%)	8 (100%)	4 (100%)	0.002*
Demodex phylloides	1 (14.2%)	1 (7.7%)	0.99	2 (25%)	0	0	0.88
Arthropod egg	1 (14.2%)	2 (15.3%)	0.97	2 (25%)	0	1 (25%)	0.9

* p value <0.05.

Table 3. Social, economic and pig management information garnered from the questionnaires pertaining to the 14 family pig farms, located in Tanguá city, state of Rio de Janeiro, Brazil

Information	Responses in order of frequency					
	1st place	Farms (%)	2nd place	Farms (%)	3rd place	Farms (%)
Property type	Backyard of the homes	78.6	Little farm	21.4	-	0
Grew fruit trees, vegetables, and herbs	Yes	92.9	No	7.1	-	0
Breed of the pig	Mixed breeds	100	-	0	-	0
Purpose of breeding	Consumption and sale	57.1	Consumption and exchange	28.6	Consumption	14.3
Pig reproduction	No	64.3	Hand Mating	35.7	-	0
Care of piglets born on your property	Teeth cutting. iron shot and anti-parasitic medicine	50	Doesn´t do anytihing	35.7	Teeth cutting. iron shot e breast-feeding after born	14.3
Type of housing for pigs	Pen	78.6	Pen and free pigs in the vicinity of the house	21.4	-	0
Facilities type	Pen with mansory walls	85.7	Pen with wood fence wall	14.3	-	0
Floor pen	Cemented	92.9	Dirt floor	7.1	-	0
Roof pen	Fully covered with fiber cement roof tile	64.3	Partially covered with fiber cement roof tile or french roof tile	28.6	Without roof	7.1
Water to cool the pig	Play on the animal's body with a hose or bucket	85.7	Water slide on the floor like a little pool	14.3	-	0
Supply of drinking water	Once or twice a day	71.4	Three times or more a day	28.6	-	0
Type of drinking fountains	Cement container attached to the floor	78.6	Cement sink attached on the floor	21.4	-	0
Food provided to animals	Wheat bran, cornmeal, food remnants from producers and commercial establishments	57.1	Wheat bran and food remnants of the producers	28.6	Wheat bran. granulated wheat, food remnants from producers and commercial establishments	14.3
Feeder type	Cement container attached to the floor	92.9	Wooden container	7.1	-	0
Food storage location	Plastic drum type	85.7	Bathtub with lid or does not store	14.3	-	0
Other animals in the farms	Yes	100	No	0	-	0
Observed rats on the property	Yes	64.3	No	35.7	-	0
Observed the pigs itch	Yes	50	No	50	-	0
Noted blood on the pig feces	No	85.7	Yes	14.3	-	0
Vaccinated animals	Yes. association with any injection	78.6	No	21.4	-	0
Anti - parasitc medicine	Yes. worm medicine	85.7	Yes. medicine made with plant or iron shot	14.3	-	0
Annti - ectoparasitic medicine	No	71.4	Yes. cresol-based disinfectant	28.6	-	0
Accumulation of excreta in the pig enclosure (team observation)	Yes	64.3	No	35.7	-	0
Cleaning the enclosures	Removal of feces manually dry and with water, brooms and shovels	57.1	Removal of feces with water	42.9	-	0
Sanitary break	No	100	-	-	-	0
Use of the flamethrower like to fire broom	No	100	-	-	-	0
Cleaning utensils intended only for cleaning the pig facility	Yes	85.7	No	14.3	-	0
Specific clothing only for handling pigs	Yes	50	-	0	-	0
	No	50	-	0	-	0

Table 4 . Social and health information garnered from the questionnaires answered by the 36 study participants, pig farmers and their families, in Tanguá city, Rio de Janeiro, Brazil

Information	Responses in order of frequency							
	1st palce	Delivered the stool (%)	2nd place	Delivered the stool (%)	3rd place	Delivered the stool (%)	4th place	Delivered the stool (%)
Performed stool examination	Yes	88.8	No	11.1	-	0	-	0
Anti - parasitic medicine	Yes	83.3	No	16.7	-	0	-	0
Intestinal pain	No	61.1	Yes	38.8	-	0	-	0
Diarrhea in the last six months	No	69.4	Yes	30.5	-	0	-	0
Pieces of helminth or whole helminth in their feces	No	88.9	Yes	11.1	-	0	-	0
Observed blood in his stool	No	97.2	Yes	2.7	-	0	-	0
Residence water supply	Public piped water and artesian well	55.5	Artesian well	19.4	Public piped water	19.4	Spring water	5.5
Water treatment	Filtered	52.3	Untreated	47.6	-	0	-	0
Hygiene of roots and leafy vegetables	Washing with water and vinegar	58.3	Washing with water	22.2	Washing with water. vinegar and lemon	5.5	Washing vinegar and bleach water	5.5
	-	-	-	-	Washing bleach water	8.3		-

providing mothers and newborn piglets with straw bedding, mucking out the pens before washing them down, and even improving the animals' facilities, such as building roofs and walls, as well as expanding these facilities.

DISCUSSION

The voluntary participation of family farmers in Tanguá/RJ and parasitology research enabled the detection of gastrointestinal parasites in 88.6% of the pig fecal samples, with parasite positivity rates ranging from 75% to 100%. Positivity rates lower than those in this study were reported in pigs on 66.3% of family farms located in Fálcon, Venezuela (Perfetti et al. 2013). However, parasite positivity rates higher than those found in this study were reported at family farms located in different municipalities in the state of Rio de Janeiro, such as Itaboraí, Rio Bonito, Silva Jardim, Saquarema, Maricá, Araruama and Casimiro de Abreu, where an overall positivity rate of 93.1% was recorded, while the Federal District showed a positivity rate of 96.1% and Sergipe a rate of 90% (Aguiar 2009, Brito et al. 2012, Barbosa et al. 2015a).

The high parasite positivity rates detected in the feces of pigs on family farms in Tanguá was already expected. Although most of the farmers raised pigs in a confined system, they clearly invested very little in sanitary management. The team's technical visits enabled them to observe heaps of organic matter, mainly excreta, in several pens, which may have favored the maintenance of evolutionary forms of parasites in the environment, and hence, infections. The lack of investments in health management by pig farmers was also revealed in their answers on the questionnaire. None of the family farms reported using a sanitary break, nor sanitizing the pig pens with a flamethrower. In fact, the farmers were unfamiliar with these terms, which had to be explained to them by the team members. D'Alencar et al. (2011) reported that pig pens on small family pig farms in Pernambuco were cleaned once a day using only water jets. This was a practice also found on the family farms in Tanguá. Most farmers stated they washed the pens, as well as feed and water troughs, only with water, without using any chemical product facilitate the removal of organic matter. This practice was also observed during the "field day" extension activity on the 4th technical visit.

In this study, evolutionary forms of protozoa were more commonly identified than those of helminths. A similar situation was reported at family farms in the Federal District and in the state of Rio de Janeiro (Aguiar 2009, Barbosa et al. 2015a). Although most farmers had reportedly already dewormed their animals, these drugs are known to be effective in the elimination of helminths, but ineffective in the control of protozoa, which may have favored the higher incidence of the latter group of parasites. Family farmers in Pernambuco interviewed by D'Alencar et al. (2011) also reported deworming their pigs with anthelmintics, and helminth positivity rates in Pernambuco ranged from 1.7% to 13.8%, i.e., much lower than those detected in pigs in Tanguá. Although the farmers in this study stated that they treated their animals with anthelmintics, they were not asked how often this treatment was applied. However, it should be noted that although the farmers reported having dewormed their animals, they could not recall the brand name of the drug, the color of the packaging, or the active ingredient it contained. Infrequent deworming, as well as the active ingredient in the antiparasitic drug, may have contributed the high parasite positivity rate, especially of helminths, found in the fecal samples of pigs in Tanguá.

Non-sporulated oocysts of coccidia compatible with the genus *Eimeria* and with *Cystoisospora suis* were the most frequently detected evolutionary forms in the animals' feces. Although the presence this parasite was not statistically

significant as a function of age group, oocysts were mainly found in the fecal matter of the youngest piglets, i.e., in the weaning stage, followed by the growing and fattening stages. On a subsistence farm located in the municipality of Pinheiral, state of Rio de Janeiro, non-sporulated coccidium oocysts were also the parasites most frequently detected in pig feces (Carreiro et al. 2016). The high frequency of coccidia detected in the pigs of this study can be directly attributed to the high resistance of the oocyst in the environment, since it can resist different temperatures and disinfectants, and also due to the large numbers of oocysts released in the feces of infected piglets, i.e., between 1,000 and 400,000 oocysts per gram of feces (Sobestiansky et al. 1999). These factors favor the reinfection of subsequent litters housed on these pig pens, which are not properly sanitized after the piglets are weaned, as evidenced at the family farms of this study.

The second most frequently detected parasite in pig feces was *Balantioides coli*. This protozoan was found in both female and male pigs, and in all the age groups, but more frequently in the growing and fattening stages. This protozoan is widely reported in parasitology research on pig, including the traditional farms in China studied by Lai et al. (2011), who reported a lower frequency than that found in this study (22.7%), on family farms analyzed in Venezuela (45.3%), and among pigs raised in confined and semi-confined systems on the outskirts of Salvador, Bahia, Brazil (46%) (Pinto et al. 2007, Perfetti et al. 2013). *B. coli* was the most frequently detected parasite on family farms in Sergipe and in various municipalities in Rio de Janeiro, showing higher positivity rates than that of the present study, i.e., 78% and 71.6%, respectively (Brito et al. 2012, Barbosa et al. 2015a).

Pigs are considered the main reservoirs of this parasite, which has a potential for zoonotic transmission (Zaman 1978). It should be noted that the farmers in Tanguá fed wheat bran and leftovers from commercial establishments to their pigs. Wheat is known to be highly rich in carbohydrates, and this nutrient, according to Zaman (1978), Schuster & Ramirez-Ávila (2008), and Barbosa et al. (2015b, 2018), is the main source of energy for *B. coli*, and is essential for its development, even in vitro. Thus, the wheat-based feed that was supplied to the pigs, especially in the fattening stage, may have favored the high positivity rates of this protozoan, particularly in the growing and fattening age groups.

Despite evidence of high positivity rates of *B. coli* in pigs, the evolutionary forms of this protozoan have not been detected in humans. High levels of *B. coli* in fecal samples from pigs and negative findings in their producers have also been reported on family farms in Rio Janeiro, as well as in rural communities in Venezuela (Guzmán et al. 2013, Barbosa et al. 2016). Unlike this study, in rural areas of Bolivia, *B. coli* was detected in stool samples from children and adolescents and in fecal samples from pigs roaming freely in the region (Esteban et al. 1998). It is worth mentioning that none of the farmers and their family members participating in this study reported they had ever detected blood in their stool. Blood is usually a visible element in symptomatic infections by *B. coli*, with dysentery and mucous and bloody diarrhea stools one of the most commonly reported symptoms. The marked presence of evolutionary forms of *B. coli* in the fecal samples from the animals in this study, and their absence in the stool tests of their owners and family members, may be directly related to the personal hygiene and sanitary habits of these individuals, which reduced their exposure to the infectious form of the parasite. It is possible that the low prevalence of balantidiasis in humans could also be explained by antigenic differences, i.e., humans would naturally be resistant to infection by *B. coli*, even when living in close proximity to parasitized pigs (Walzer et al. 1973). However, this theory has never been proven.

Amoeboids and *Blastocystis* sp. were also detected in the pigs' feces. The evolutionary forms of these parasites were not studied in depth using parasitological staining techniques. However, it was found that amoeboid cysts generally had only one nucleus and their size was comparable to that of *Entamoeba polecki* and *Entamoeba suis*, with a length of $\bar{x} = 13.74\mu m$ (±1.8μm) and a width of $\bar{x} = 13.21\mu m$ (±1.9μm). Despite the relevant difference between *E. polecki* and *E. suis*, since only the former species has so far been reported infecting humans, they could not be differentiated in this study because no molecular techniques were used (Stensvold et al. 2010). In addition to these amoeboids, uninucleated cysts were also detected in five pig fecal samples, showing a morphology compatible with immature cysts of the *Entamoeba histolytica*/*Entamoeba dispar*/*Entamoeba moshkovskii* complex, $\bar{x} = 22.72\mu m$ long (±4.3) and $\bar{x} = 21.14\mu m$ (±2.6) wide. Like the findings of this study, uninucleated amoeboid cysts were also found in pig feces on family farms located in other municipalities in the state of Rio de Janeiro, albeit with a higher frequency (44%) than that of this study (Barbosa et al. 2015a). It should be noted that *E. polecki*, the *E. histolytica*/*E. dispar*/*E. moshkovskii* complex, and *Blastocystis* spp. are protozoa with zoonotic potential and are therefore transmissible to humans. Furthermore, with the exception of *E. polecki*, it is not known whether these protozoa can produce symptomatic infections in pigs, which underscores the need for further study with of protozoa. Despite their zoonotic potential, such evolutionary forms were not identified in the stool samples from the human participants of this study, in which only amoeboid cysts with more than five nuclei compatible with *E. coli* were identified.

As for helminths, only nematodes were detected, the most prevalent of which were strongyles, whose eggs were found in all the animals' age groups. Strongyle eggs were considered nematode eggs possibly belonging to the superfamilies Strongyloidea and Trichostrongyloidea. The eggs of these superfamilies are identical; hence, differentiating the species taxonomically requires fecal cultures or molecular biology techniques, which were not used in this study. Strongyles eggs were considered those with thin shells, containing an embryonic mass and having an average length of 102.4μm (±12.4) and average width of 59.5μm (±7.5). Lower frequencies than those of this study, i.e., 23.6% and 10.6%, were reported on family farms in the municipality of Pinheiral/RJ and on traditional farms in China, respectively (Lai et al. 2011, Carreiro et al. 2016). Barbosa et al. (2015a), who analyzed gastrointestinal parasitosis in swine on family farms located in different municipalities of the state of Rio de Janeiro, reported a positivity rate similar to that found in this study (46.6%). According to Roepstorff & Nansen (1996), it should be emphasized that strongyles, particularly *Hyostrongylus rubidus* and *Oesophagostomum* spp., are highly relevant in pig farming, since they can cause "thin sow syndrome." This syndrome develops when 4th stage larvae of the parasite in the stomach and intestinal mucosa leave their hypobiotic

state in response to the hormonal stimulation of parturition in the host and to climate conditions, developing into adult and pathogenic evolutionary forms precisely in the period when sows are suckling their offspring, i.e., when these animals have higher energy needs.

Nematode larvae and larvae of thin-shelled nematode larvae, $\bar{x} = 75\mu m$ (±5) long and $\bar{x} = 47\mu m$ (±4.8) wide, similar to *Strongyloides ransomi*, were identified in the feces of pigs in the initial and growing stages. The average size of the eggs resembling those of *S. ransomi* was slightly larger than that described in the literature for the Rhabditoidea family. Unfortunately, a search of the literature failed to identify any scientific articles that measure the size of this parasite, and only a very few that discuss the biology of *Strongyloides* spp. in animals. Even so, the eggs detected here were typical of the species that infects pigs. Note the fecal material was processed in the laboratory immediately after collection, thus minimizing the possibility that it belongs to a strongyle species. Although the fecal samples were processed in the laboratory as quickly as possible on the same day they were collected, the fecal samples from pigs that contained eggs typical of *S. ransomi* also contained nematode larvae, which may have hatched from *S. ransomi* eggs. Strongyloidiasis is a parasitic disease that affects mainly young piglets, and may cause them to suffer from severe catarrhal enteritis. It should be noted that sows are the main source of infection for their offspring, and that the parasite is transmitted via percutaneous, oral and transmammary routes (Nansen & Roepstorff 1999, Sobestiansky et al. 1999). The presence of evolutionary forms of this parasite in piglet feces, especially in the initial stage, on family farms in Tanguá, confirms the parasite is transmitted by infected sows.

Evolutionary forms of *Ascaris suum* and *Trichuris suis* were identified in pig feces in all the age groups and in both sexes. These nematodes are extremely important in pig farming because they reduce weight gains, particularly in the fattening stage. In the case of the family farms in Tanguá, would be inserted in the period of growth and fatteners, since the prepatent period of these nematodes varies by about 8 weeks, according to Roepstorff & Nansen (1996). Moreover, slaughterhouses may discard pig livers due to milk spots, a pathology characteristic of larval migrations in the liver tissue (Bordin 1987). However, pig livers are rarely discarded on family farms, where most of these animals are slaughtered and inspections are not carried out.

Like the family farms in Tanguá, evolutionary forms of *A. suum*, *T. suis* and *S. ransomi* have also been identified in Venezuela, *A. suum* and *T. suis* on family farms in Rio de Janeiro, Sergipe, and China, and *S. ransomi* on industrial farms in Rio de Janeiro (Lai et al. 2011, Brito et al. 2012, Perfetti et al. 2013, Barbosa et al. 2015a, Carreiro et al. 2016). The helminths identified in this study as strongyles, *A. suum*, *T. suis* and *S. ransomi*, are geohelminths, i.e., helminths that are transmitted primarily through contaminated soil, where they develop the infective structure, which may be 3rd stage nematode larvae (L3) or embryonated eggs. The floors of pigpens on the family farms in Tanguá/RJ, are made of a very thin layer of cement, and most of them were cracked and worn, thus facilitating the accumulation of organic matter. In addition, it was found that most of the water and feed troughs were made of cement built into the floor. This type of trough easily becomes contaminated the animal excreta. Hence, the type of construction of the animals' enclosures on the family farms in Tanguá seems to have favored the development and persistence of the infective stages of parasites, especially of geohelminths.

In addition to these parasites, the identification of eggs typical of *Capillaria* spp. in pig feces on farm K was a significant finding, since this nematode does not usually infect pigs. The pigs on this farm may have been ingested *Capillaria* spp. eggs from the feces of other animals or from the predation of synanthropic animals such as rodents, indicating a possible case of pseudoparasitism. The family farmers in Tanguá reported the sporadic appearance of rats on their farms. It should be kept in mind that pigs are omnivores and may prey on these rodents if they enter their pens. Synanthropic rodents such as rats may be carriers of a variety of infectious agents that are harmful to animal health. This sanitary problem was discussed with the farmers during the fieldwork. The presence of rodents on pig farms does not seem to be a problem solely of family farms in Brazil, since it also occurs in intensive pig farming in France, according to Beloeil et al. (2003).

The presence of rodents in herds in the municipality of Tanguá may be attributed to the practice of storing pig feed, as the farmers themselves reported. The practice of feeding leftovers to pigs was also reported by Rocha et al. (2016), who analyzed 67 farms in Senador Canedo, Goiás based on socioeconomic, zootechnical and environmental questionnaires. Another important point is the fact that the financial status of most of the pig farmers in this study is precarious. This means that commercial pig feed is too expensive for most farmers, who feed their pigs leftovers of human food, knowing that pigs readily consume leftovers and are omnivorous, like humans.

Sixteen percent of the ear skin scrapings from pigs on the family farms of Tanguá showed evolutionary forms of ectoparasites compatible with *Sarcoptes scabiei* variety *suis*, arthropod eggs and *Demodex phylloides*. *S. scabiei* var. *suis* infestation rates in pigs lower than and similar to that of this study were reported in industrial farms in Paraná (0.09%) and in Goiás (12.1%), respectively (Oliveira et al. 2006, Silva et al. 2002). Unlike the case records in Tanguá, Sobestiansky et al. (2005) reported a high infestation rate (43%) on an industrial farm in Goiás, which already had a previous history of scabies.

Apart from detecting these mites by parasitological techniques, on several occasions during their technical visits the researchers saw pigs rubbing their bodies against the walls of pig pens, trees and against other individuals in the pens, a behavior suggestive of itching caused by scabies. Some of the farmers also described this behavior in their answers to the questionnaire. The infestation rate of evolutionary forms of *S. scabiei* var. *suis* might have been even higher if the skin of the ear had been scraped to a greater intradermal depth, where most mites burrow, or if biological material had also been collected from other parts of the pigs' bodies. According to Sobestiansky et al. (2005), scabies can pose problems in pig farming by lowering production rates because mite infested pigs reduce their feed intake, thus slowing down their growth and weight gains. Other evolutionary forms of arthropods were detected in this study, such as eggs characteristic of arthropods that could be *S. scabiei* var. *suis*, in addition to *D. phylloides*, a mite considered a commensal on the skin of pigs and unharmful to their health.

Evolutionary forms of parasites, mostly of *E. coli*, were also detected in the stool samples of the farmers and their family members that participated in this study. This protozoan is not pathogenic, but it can be considered a bioindicator of fecal contamination. In addition to *E. coli*, the geohelminths *Ascaris lumbricoides* and *Trichuris trichiura* were also found in the stool of these participants. This finding was expected, since most of the people on these farms worked on the land, cultivating fruit trees, vegetables, and herbs for their own consumption and for sale. The researchers noticed that the children on the farms went barefoot, roaming around all over the place. An important fact that the participants reported at various times during the fieldwork was the lack of basic sanitation in the municipality. They reported that sewage was not treated and that they suspected the public water supply, which was used occasionally, was also untreated. For this reason, the farms sought alternative water sources that are not subject to sanitary monitoring, such as wells and natural springs. In addition to possible infections transmitted through contaminated soil, environment and water, the way in which the participants washed their vegetables and fruits may also have contributed to their ingestion of parasites, since most of them stated they used vinegar incorrectly for this purpose.

In addition to the parasitological evaluation of biological samples from pigs and human participants, extension activities were carried out to transmit information and provide technical support to these small farmers. Unfortunately, extension activities are still undervalued and rarely carried out in Brazil. Therefore, no articles about this theme in national pig farming were found in the literature, indicating that this is a pioneering study.

Even though this information was imparted using simple language in informal conversations, the researchers found that people sometimes found it difficult to understand. Although the participants' educational level was not included in the questionnaire in order to avoid embarrassment, many participants proved to be illiterate when it came time to sign the informed consent form. This may have be why they found it difficult to understand some of the information and/or questions on the forms. During the interactive lecture, the use of visual images clearly facilitated the transmission of information. Moreover, the intervention activity carried out on the field day was also apparently more effective in transmitting information, as it was imparted dynamically while the farmer was mucking out the pig pens. To remind the participants of the most relevant points explained during the technical visits, a poster visually illustrating this information, as well as a calendar, were posted on each farm to encourage and remind the farmers to deworm their animals according to schedule.

In the remaining technical visits to the farms, the researchers checked to ascertain if their instructions were being followed. They found that this was being done properly, such as mucking out the pens regularly and building roofs over pens lacking protection from weather. Over the course of the various visits, a relationship of mutual trust was established between the farmers and their families and the research team. This relationship was further reinforced through the continuing contact of the farmers with the researchers to ask for technical support, even after the study was concluded. The primary focus of this study was parasitological research allied to extension activities carried out in seven technical visits to impart information for improved sanitary management in raising pigs, thereby improving the economic status of small farmers. However, it is impossible to predict whether or not the information imparted to these farmers will be lost over time. In view of this real possibility, it would be ideal for small farmers to be given permanent technical support.

CONCLUSIONS

The high parasite positivity rates found in the fecal samples from pigs in the municipality of Tanguá appear to be directly attributable to the ineffective health management these farmers give their animals, as was revealed in the forms and observed by the researchers during their technical visits to the farms. In addition, evolutionary forms of parasites were also detected in the stool samples of farmers and their family members. This situation may be the result of poor sanitation in the municipality, according to reports by the participants of this study, as well as inadequate hygiene habits, according to information retrieved from the questionnaires.

The overall situation underscores the need for publicly sponsored programs to provide technical and financial aid for these small farmers. In the case of the family farms in the municipality of Tanguá, these farmers raise pigs as a means of subsistence, since they all eat pork and some of them reported that the sale and/or exchange of pigs for other products was a second source of family income. Silva Filha et al. (2011) stated that for these breeders, owning these animals represents not only a source of income but in many cases is also a family tradition. However, this study clearly revealed the farmers' precarious financial status and inadequate technical conditions to raise their animals properly, as well as their lack of access to and/or understanding of what constitutes proper hygienic habits.

Acknowledgements.- The authors would like to thank for project support: Proex (Dean's Office of extension of "Universidade Federal Fluminense") and to family producers in Tanguá, Rio de Janeiro, who participated in this study.

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

REFERENCES

Aguiar P.C. 2009. Aspectos epidemiológicos das parasitoses gastrintestinais de suínos naturalizados de criações familiares do Distrito Federal. Doctoral Dissertation, Universidade de Brasília, Brasília.

Barbosa A.S., Bastos O.M.P., Dib L.V., Siqueira M.P., Cardozo M.L., Ferreira L.C., Chavez W.T., Fonseca A.B.M., Uchôa C.M.A. & Amendoeira M.R.R. 2015a. Gastrointestinal parasites of swine raised in different management systems in the State of Rio de Janeiro, Brazil. Pesq. Vet. Bras. 35(12):941-946. <https://dx.doi.org/101590/S0100-736X2015001200001>

Barbosa A.S., Bastos O.M.P., Uchôa C.M.A., Dib L.V. & Amendoeira M.R.R. 2016. Avaliação da frequência de *Balantidium coli* em suínos, tratadores de suínos e primatas não humanos no estado do Rio de Janeiro. Revta Patol. Trop. 45(3):285-293. <https://dx.doi.org/10.5216/rpt.v45i3.43505>

Barbosa A.S., Bastos O.M.P., Uchôa C.M.A., Pissinatti A., Ferreira Filho P.R., Dib L.V., Azevedo E.P., de Siqueira M.P., Cardozo M.L. & Amendoeira M.R. 2015b. Isolation and maintenance of *Balantidium coli* (Malmsteim, 1857) cultured from fecal samples of pigs and non-human primates. Vet. Parasitol. 210(3/4):240-245. <https://dx.doi.org/10.1016/j.vetpar.2015.03.030> <Pmid:25920329>

Barbosa A.S., Cardozo M.L., Dib L.V., Fonseca A.B.M., Uchôa C.M.A., Bastos O.M.P. & Amendoeira MRR. 2018. Comparative study of three xenic media

culture for cultivation of *Balantidium coli* strains. Revta Bras. Parasitol. Vet. 27(1):19-25. <https://dx.doi.org/10 1590/s1984-29612017075>

Beloeil P.A., Chauvin C., Fablet C., Jolly J.P., Eveno E., Madec F. & Reperant J.M. 2003. Helminth control practices and infections in growing pigs in France. Livest. Product. Sci. 81(1):99-104. <https://dx.doi.org/10.1016/S0301-6226(02)00192-6>

Bordin E.L. 1987. Relações entre infecções por parasitas internos de suínos e o custo de alimentação: uma revisão. Hora Vet. 7(39):21-27.

Brasil 2015. Lei nº 0971 de 24 de junho de 2015 - Aprova o plano municipal de educação para o decênio 2015-2025 e dá outras providências. Avalaible at <https://www mprj mp br/documents/20184/203908/Tangua5 pdf> Accessed on Mar. 30, 2019.

Brito G.G., Santos T.B., Melo C.M., Jeraldo V.L.S. 2012. Ocorrência de enteroparasitas em amostras fecais de suínos do município de Simão Dias, SE. Ciênc. Biol. Saúde 1(15):11-18.

Carreiro C., Coelho C.D., Jorge J.L.B.P. & Costa N.O.G., Paiva R.V., Filho Teixeira W.L., Rosa A.G. & Jesus V.L.T. 2016. Parasitos intestinais em suínos confinados em uma criação no município de Pinheiral, RJ. Revta Bras. Med. Vet. 38(supl.2):117-122.

D'Alencar A.S., Farias M.P.O., Rosas E.O., Lima M.M., Alves L.C. & Faustino M.A.G. 2011. Influência do manejo higiênico-sanitário na infecção por helmintos gastrintestinais em suínos de granjas tecnificadas e de subsistência abatidos na Região Metropolitana de Recife e Zona da Mata do Estado de Pernambuco, Brasil. Arqs Inst. Biológico, São Paulo, 78(2):207-215.

Embrapa 2018. Qualidade da carne suína. Avalaible at <https://www.embrapa.br/qualidade-da-carne/carne-suina>. Accessed on Oct. 30, 2018.

Esteban J.G., Aguirre C., Angles R., Ash L.R. & Mas-Coma S. 1998. Balantidiasis in Aymara children from northern Bolivian altiplano. Am. J. Trop. Med. Hyg. 59(6):922-927. <https://dx.doi.org/10.4269/ajtmh.1998.59.922> <PMid:9886201>

Faust E.C., D'Antoni J.S., Odon V., Miller M.J., Perez C., Sawitz W., Thomen L.F., Tobie J.E. & Walker J.H. 1938. A critical study of clinical laboratory technics for the diagnosis of protozoan cysts and helminth eggs in feces I Preliminary communication. Am. J. Trop. Med. 18(4):169-183. <https://dx.doi.org/10.4269/ajtmh.1938.s1-18.169>

Ferreira S.C.T. 2010. Contribuição para o estudo de sarna sarcóptica em suínos abatidos para consumo. Master's Thesis, Universidade de Trás-os-Montes e Alto Douro, Vila Real.

Guzmán C.R., Nessi A.P., Gonzáles O.H., Hernández M.O. & Galindo M. 2013. *Balantidium* spp. en cerdos y sus criadores: prevalencia em comunidades de dos Estados de Venezuela. Vitae 54:1-10.

Huber F., Bomfim T.C. & Gomes R.S. 2003. Comparação da eficiência da técnica de sedimentação pelo formaldeído-éter e da técnica de centrífugo flutuação modificada na detecção de cistos de *Giardia* sp. e oocistos de *Cryptosporidium* sp. em amostras fecais de bezerros. Revta Bras. Parasitol. Vet. 12(2):135-137.

Lai M., Zhou R.Q., Huang H.C. & Hu S.J. 2011. Prevalence and risk factors associated with intestinal parasites in pigs in Chongqing, China. Res. Vet. Sci. 91(3):121-124. <https://dx.doi.org/10.1016/j.rvsc.2011.01.025> <PMid:21349561>

Lutz A.O. 1919. *Schistosomum mansoni* e a Schistosomatose segundo observações, feitas no Brazil. Mem. Inst. Oswaldo Cruz 11(1):121-155. <https://dx.doi.org/10.1590/S0074-02761919000100006>

Nansen P. & Roepstorff A. 1999. Parasitic helminths of the pig: factors influencing transmission and infection levels. Int. J. Parasitol. 29(6):877-891. <https://dx.doi.org/10.1016/S0020-7519(99)00048-X>

Oliveira A.B., Biondo A.W., Alberton G.C., Santis A.P.T., Vianna G.N.O., Teixeira M.A. & Pieper M. 2006. Prevalência de *Sarcoptes scabiei* var. *suis* em granjas de reprodutores suídeos certificadas do estado do Paraná, no período de 2002 a 2004. Arch. Vet. Sci. 11(2):61-65. <https://dx.doi.org/10.5380/avs.v11i2.6801>

Pedroso-de-Paiva D., Móres N., Barioni Júnior W., Costa O.A.D., Sobestianski J. & Amaral A.L. 2003. Fatores de risco associados à ocorrência de sarna sarcóptica e prevalência em suínos nas fases de crescimento e terminação, na região sul do Brasil. Ciência Rural 33(4):731-736. <https://dx.doi.org/10.1590/S0103-84782003000400023>

Perfetti D.J.C., Quintero M.E.A., Low J.L.T. & Moreno P.M. 2013. Prevalencia de enteroparásitos porcinos en una comunidad rural de la península de Paraguaná, Estado Falcón, Venezuela. Revta Cient. Facultad Ciencias Veterinarias, Zulia, 23(1):19-25. <https://www researchgate net/publication/288575568>

Pinto J.M.S., Costa J.O. & Souza J.C.A. 2007. Ocorrência de endoparasitos em suínos criados em Itabuna, Bahia, Brasil. Ciênc. Vet. Trop. 10(2/3):79-85.

Ritchie L.S. 1948. An ether sedimentation technique for routine stool examinations. Bull. US Army Med. Depart. 8(4):326. <PMid:18911509>

Rocha L.O., Oliveira R.M., Hellmeister Filho P., Gomes N.A., Carneiro M.F., Silva O.M & Fernandes L.C. 2016. Diagnóstico Participativo/Rural aplicado à criação de aves e suínos caipiras em regiões periurbanas no município de Senador Canedo (GO). Fronteiras, J. Social Technol. Environ. Sci. 5(2):135-152. <https://dx.doi.org/10.21664/2238-8869.2016v5i2.p135-152>

Roepstorff A. & Nansen P. 1996. FAO Animal Health Manual: epidemiology, diagnosis and control of helminth parasites of swine. Food and Agricultural Organization of United States Press, Rome.

Roepstorff A., Nilsson O., Oksanen A., Gjerde B., Richter S.H., Örtenberg E., Christensson D., Martinsson K.B., Bartlett P.C., Nansen P., Eriksen L., Helle O., Nikander S. & Larsen K. 1998. Intestinal parasites in swine in the Nordie countries: prevalence and geographical distribution. Vet. Parasitol. 76(4):305-319. <https://dx.doi.org/10.1016/s0304-4017(97)00223-9> <PMid:9650867>

Schuster F.L. & Ramirez-Ávila L. 2008. Current world status of *Balantidium coli*. Clin. Microbiol. Rev. 21(4):626-638. <https://dx.doi.org/10.1128/CMR.00021-08> <PMid:18854484>

Sheather A.T. 1923. The detection of intestinal protozoa and mange parasites by a floatation technique. J. Comp. Pathol. 36:266-275. <https://dx.doi.org/10.1016/S0368-1742(23)80052-2>

Silva E.V., Sobestiansky J., Linhares G.F.C., Vieira R.C., Oliveira J.P.O. & Vieira M.C.M. 2002. Prevalência de sarna sarcóptica em suínos mantidos em criações intensivas na microrregião de Goiânia, GO, Brasil. Revta Patol. Trop. 31(2):225-232. <https://dx.doi.org/10.5216/rpt.v31i2.14567>

Silva Filha O.L., Barbosa E.J.R., Lima A.D., Melo A.G.P., Melo Filho A.J. & Sá MS. 2011. Os produtores de suínos no Município de Floresta, Estado de Pernambuco, Brasil. AICA 1(2011):416-418.

Sobestiansky J., Barcelos J., Mores N., Carvalho L. & Oliveira S. 1999. Clínica e Patologia Suína. Gráfica Art3, Goiânia. 464p.

Sobestiansky J., Linhares G.F.C., Silva E.V. & Linhares D. 2005. Aspectos clínicos e epidemiológicos de um foco de sarna sarcóptica em um sistema intensivo de produção de suínos localizado no município de Teresópolis-GO, Brasil. Revta Patol. Trop. 34(1):61-67. <https://dx.doi.org/10.5216/rpt v34i1 2138>

Solaymani-Mohammadi S. & Petri Jr W.A. 2006. Zoonotic implications of the swine-transmitted protozoal infections. Vet. Parasitol. 140(3/4):189-203. <https://dx.doi.org/10.1016/j.vetpar.2006.05.012> <PMid:16828229>

Stensvold C.R., Lebbad M. & Clark C.G. 2010. Genetic characterization of uninucleated cyst producing *Entamoeba* spp. from ruminants. Int. J. Parasitol. 40(7):775-778. <https://dx.doi.org/10.1016/j.ijpara.2010.03.003> <PMid:20359482>

Walzer P.D., Judson F.N., Murphy K.B., Healy G.R., English D.K. & Schultz M.G. 1973. Balantidiasis outbreak in Truk. Am. J. Trop. Med. Hyg. 22(1):33-41. <https://dx.doi.org/10.4269/ajtmh.1973.22.33> <PMid:4684887>

Young K.H., Bullock S.L., Melvin D.M. & Spruill C.L. 1979. Ethyl acetate as a substitute for diethyl ether in the formalin-ether sedimentation technique. J. Clin. Microbiol. 10(6):852-853. <https://dx.doi.org/10.1128/JCM.10.6.852-853.1979> <PMid:574877>

Zaman V. 1978. *Balantidium coli*, p.633-653. In: Kreier J.P. (Ed.), Parasitic Protozoa. Academic Press, New York.

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(10):750-757, October 2020
DOI: 10.1590/1678-5150-PVB-6595

Original Article
Livestock Diseases
BY-NC

Embryonic loss, abortion, and malformations in ewes caused by the ingestion of hay leaves of *Cenostigma pyramidale*[1]

Suélen D.S. Reis[2], Juliana T.S.A. Macêdo[3], Ariana L. Pereira[3], Rubens S. Jesus[5], Luciano A. Pimentel[4], Ivo Pivato[6], Tiago C. Peixoto[2,7] and Pedro M.O. Pedroso[2,3]*

ABSTRACT.- Reis S.D.S., Macêdo J.T.S.A., Pereira A.L., Jesus R.S., Pimentel L.A., Pivato I., Peixoto T.C. & Pedroso P.M.O. 2020. **Embryonic loss, abortion, and malformations in ewes caused by the ingestion of hay leaves of *Cenostigma pyramidale***. *Pesquisa Veterinária Brasileira 40(10):750-757*. Universidade Federal da Bahia, Av. Adhemar de Barros 500, Ondina, Salvador, BA 40170-110, Brazil. E-mail: pedrosovet@yahoo.com.br

Determining if reproductive failures in ewes at the semiarid region in the state of Bahia are related to the consumption of the species *Cenostigma pyramidale* (Tul.) Gagnon & G.P. Lewis, and this study was developed using pregnant ewes divided into six groups: G1, G2, G3, G4 with six animals each, G5 and G6 with ten animals. Each group received fence leaves in the proportion of 1%, 2%, 0.5%, and 0.25% of live weight (LW) respectively; G5 and G6, with ten animals each, receiving 0.25% and 0.5% of the LW, respectively, and the Control Group, comprising 16 ewes, were grass feeding (*Cynodon dactylon*). Ewes from G1 to G4 were the same, except for two, and started ingestion of the plant four days after ending of natural mating on the 80th day of gestation, while those regarding from G5 to G6 groups started ingestion on the 26th day of gestation ending on the 98 day. The ultrasonographic test was performed weekly. In G1 ewes (1%), there was an embryonic loss on the 32nd and 39th days of gestation and abortion on the 46th day. In G2 (2%), the embryo loss was earlier (on the 26th day of gestation), and abortion on the 46th day of gestation. In G3 group (0.5%), there was an embryonic loss around the 40th day of gestation. In G4 group (0.25%), it was observed the occurrence of one death lamb with bone malformations. In G6 (0.5%), abortion occurred later (108 days), followed by retained placenta. This was also verified in G5 group (0.25%). The presence of fetal malformation was found in death lambs born in G4 group, born alive from G5 and G6 groups, and one aborted from G6. In G5 and G6 groups, there were also genetic alterations on surviving lambs. In addition to these results, recurrent estrus was observed without gestation in G1, G2, G3, and G4 ewes. From the Control Group, 13 normal lambs were born without genetic alterations; furthermore, concerning a quadruple birth, three lambs were born dead. The results infer that species of *C. pyramidale* in low doses causes reproductive losses in pregnant ewes, therefore it is not recommended for sheep diet over the first 60 days of gestation.

INDEX TERMS: Embryonic loss, abortion, malformation, ewes, *Cenostigma pyramidale*, hay, reproductive losses, teratogenic plant, sheep, catingueira.

[1] Received on April 13, 2020.
Accepted for publication on April 27, 2020.
[2] Graduate Program in Animal Science in the Tropics (PPGCAT), Universidade Federal da Bahia (UFBA), Av. Adhemar de Barros 500, Ondina, Salvador, BA 40170-110, Brazil.
[3] 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
[4] Setor de Patologia Veterinária, Hospital Universitário de Medicina Veterinária, Universidade Federal do Recôncavo da Bahia (UFRB), Rua Rui Barbosa 710, Campus Universitário, Cruz das Almas, Bahia, BA 44380-000, Brazil.
[5] Setor de Clínica e Cirurgia de Grandes Animais, Hospital Universitário de Medicina Veterinária, Universidade Federal do Recôncavo da Bahia (UFRB), Rua Rui Barbosa 710, Campus Universitário, Cruz das Almas, Bahia, BA 44.380-000, Brazil.
[6] Laboratório de Reprodução Animal, Fundação Universidade de Brasília (UnB), Campus Universitário Darcy Ribeiro, Via L4 Norte s\n, Brasília, DF 70910-970, Brazil.
[7] Laboratório de Patologia Veterinária, Universidade Federal da Bahia (UFBA), Av. Adhemar de Barros 500, Ondina, Salvador, BA 40170-110, Brazil.

RESUMO.- [Perda embrionária, aborto e malformações em ovinos por ingestão de *Cenostigma pyramidale* na forma fenada.] Para determinar se falhas reprodutivas em ovelhas na região semiárida da Bahia estão relacionadas ao consumo de *Cenostigma pyramidale* (Tul.) Gagnon & G.P. Lewis, foi realizado um estudo utilizando-se ovelhas prenhes divididas em seis grupos e dois Grupos Controle. Os grupos G1, G2, G3 e G4 com seis animais cada. Cada grupo recebeu folhas fenadas na proporção de 1%, 2%, 0,5% e 0,25% do peso vivo (PV) respectivamente; G5 e G6, com 10 animais cada, que receberam 0,25% e 0,5% do PV respectivamente. Os Grupos Controle foram alimentados com ração e capim (*Cynodon dactylon*). Ovelhas dos grupos 1 a 4 iniciaram ingestão da planta quatro dias após monta natural com término aos 80 dias de gestação, enquanto as dos grupos 5 a 6 iniciaram ingestão no 26º dia de gestação com término aos 98 dias. Avaliação ultrassonográfica foi realizada semanalmente. Nos animais do G1 (1%), verificou-se perda embrionária aos 32 e 39 dias de gestação, e aborto aos 46 dias. Nos do G2 (2%) a perda embrionária foi mais precoce (26 dias), e aborto aos 46 dias. No G3 (0,5%), houve perda embrionária em torno dos 40 dias. No G4 (0,25%), verificou-se ocorrência de natimorto com malformações aos 150 dias de gestação. No G6 (0,5%) o aborto ocorreu mais tardiamente (108 dias), seguido de retenção de placenta. Essa ocorrência também foi verificada no G5 (0,25%). A presença de malformação fetal foi encontrada em fetos natimorto do G4, nascidos vivos do G5 e G6, e um abortado do G6. No G5 e G6 também foram observadas alterações de aprumos em cordeiros sobreviventes. Do Grupo Controle nasceram 13 borregos normais, porém uma ovelha apresentou gestação quádrupla com três natimortos. Os resultados inferem que *C. pyramidale* fenada em baixas doses causa perdas reprodutivas em ovelhas gestantes, não sendo por isso recomendada para a dieta de ovelhas durante os primeiros 60 dias de gestação.

TERMOS DE INDEXAÇÃO: Perda embrionária, aborto, malformações, ovinos, *Cenostigma pyramidale*, feno, perdas reprodutivas, planta teratogênica, ovelhas, catingueira.

INTRODUCTION

The association between the consumption of toxic plants and the occurrence of reproductive changes in farm animals is known in Brazil and other countries (Panter et al. 1990, Pimentel et al. 2007, Welch et al. 2009). The reported toxic effects result in miscarriages, embryonic loss, embryonic resorption and teratogeny.

Concerning the Northeast region of Brazil, where goat breeding is an important economic activity and source of income, the abortive and teratogenic effects of *Mimosa tenuiflora*, also known as "jurema-preta", have been proven to be harmful both in ruminants and in rats (Nóbrega Júnior et al. 2005, Pimentel et al. 2007, Medeiros et al. 2008, Dantas et al. 2010, Santos et al., 2012). Additionally, *Cenostigma pyramidale* (*Poincyanella pyramidalis*) (Tul.) Gagnon & G.P. Lewis is teratogenic for goats (Reis et al. 2016), rats (Câmara et al. 2017), and sheep (Lopes et al. 2017).

Based on reports of malformation outbreaks and abortions in small ruminants in the state of Bahia, an experiment was carried out to verify whether hay leaves of *C. pyramidale* (hay) induces reproductive failures in pregnant ewes at different gestational periods.

MATERIALS AND METHODS

Stages. The experiment was divided into three Stages. Stages 1 and 2 were performed at the "Universidade Federal do Recôncavo da Bahia" (UFRB), from March to December 2016. Stage 3 was conducted at the "Universidade de Brasília" (UnB), from September 2016 to January 2017. In each of the three Stages, two treatment groups (G) were formed. In Stage 1, groups G1 and G2 were formed, while stage 2 was composed of G3 and G4 groups, and Stage 3, by G5 and G6 groups. The Animal Use Ethics Committee approved this study of the UFRB (protocol number 23007.013398 / 2012-21).

Animals. To conduct the experiment, 50 Santa Inês, Bergamácia, and crossbred ewes, aged from one to five years, were chosen. Of these animals, 14 underwent treatment in Stages 1 and 2, 20 were distributed in stage 3, and 16 comprised the Control Group. Semen was collected from four sheep aged from two to three years (three Santa Inês and one Bergamácia) for artificial insemination and natural breeding.

Treatment and Control Groups. Six treatment groups were randomly set up (G1, G2, G3, G4, G5, and G6), in addition to two Control Groups. Groups 1-4 were made up of six ewes each, and groups 5-6 were made up of 10 ewes each. Groups 1, 2, 3, 4, 5, and 6 received daily doses of *Cenostigma pyramidale* (Tul.) Gagnon & G.P. Lewis (hay leaves) of concentrations of 1%, 2%, 0.5%, 0.25%, 0.25% and 0.5%. The females used were examined clinically and submitted to ultrasound evaluation to rule out pregnancy and changes in the reproductive system before starting treatment. Males were subjected to andrological evaluation before semen collection and natural breeding (NB). Leaves of *C. pyramidale* were harvested in the rural area of the municipalities of Tupiaçu and Geolândia in the region of Recôncavo Bahiano. After harvesting, leaves were stored to dry in the shade for 10 to 15 days. After desiccated, the leaves were crushed and bagged according to the animal's weight (g/kg of live weight (LV)). A sample of the specimen plant was authenticated and deposited at the Herbarium of the "Universidade Estadual de Feira de Santana" (UEFS) under number (HUEFS 240996).

The negative pregnancy was confirmed by ultrasonography (USG) through the rectum (SonoScape device, 7.0MHz); a short synchronization protocol was performed. On the day 0, intravaginal sponges impregnated with 60mg of medroxyprogesterone acetate (MAP) (Progespon®, Laboratório Syntex S.A.) were inserted. Equine chorionic gonadotropin (eCG PMSG-Novormon® 5000UI, Zoetis-Pfizer, 2.0IU/animal) prostaglandin (Sincrocio®, OuroFino, 0.6ml/animal) were administered intramuscularly on the 5th day. On the 6th day, the sponges were removed. The NB occurred on the 7th, 9th, and 10th days, and on the 8th day, Fixed-Time Artificial Insemination (FTAI) with fresh semen was performed (Fig.1). The ewes were placed in individual stalls, and after four days, that is, counted from the last natural mount, a daily supply of dried and ground leaves of *C. pyramidale* was initiated for ewes in Stages 1 and 2 (G1-G4) until the 80th day of gestation. Ewes in groups 5-6 could ingeste the hay leaves from the 26th day of gestation until the 98th day of gestation (Fig.2).

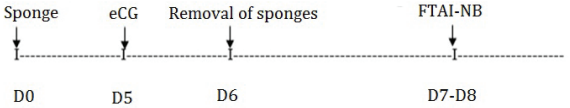


Fig.1. Short protocol regarding the synchronization and artificial insemination in a fixed time period. Fixed-Time Artificial Insemination (FTAI), natural breeding (NB).

Animals that were not pregnant, that is, suffered embryonic loss or abortion remained in the treatment groups, being together with the others. Concentrate and mineral salt was added to the dried and ground leaves of *C. pyramidale* to adjust the acceptability. Any residues found in the troughs were collected and weighed. After the total consumption of dry and ground leaves, the ewes received grass or silage until the end of the afternoon.

The USG was performed weekly to monitor the gestational evolution. In addition, weights were checked weekly to update the quantities of dried leaves and leaves on the ground to be administered. Ewes, that have more than one estrus or "heat", were again subjected to NB. In case of abortion or stillbirth lambs, necropsy and, when possible, radiological examinations were performed.

RESULTS

Regarding Stage 1, in G1 group (1% LW of hay leaves), there was embryonic loss in three ewes on the 32nd day of gestation and also on the 39th days, while in G2 (2% LW of hay leaves), two ewes suffered embryonic loss on the 32nd and 26th days, respectively. Abortion occurred in one ewe in G1 and one in G2 group on the 46th days of gestation (Table 1).

Pregnancy confirmation was performed by USG on the 20th day after FTAI, as well as confirming evidence of embryonic loss and abortion (Table 1 and Fig.3). One G1 and two G2 animals returned to estrus 18 days after coating covering, again submitted to NB, remaining non-pregnant. The ewes, that suffered embryonic loss or abortion after ingesting hay

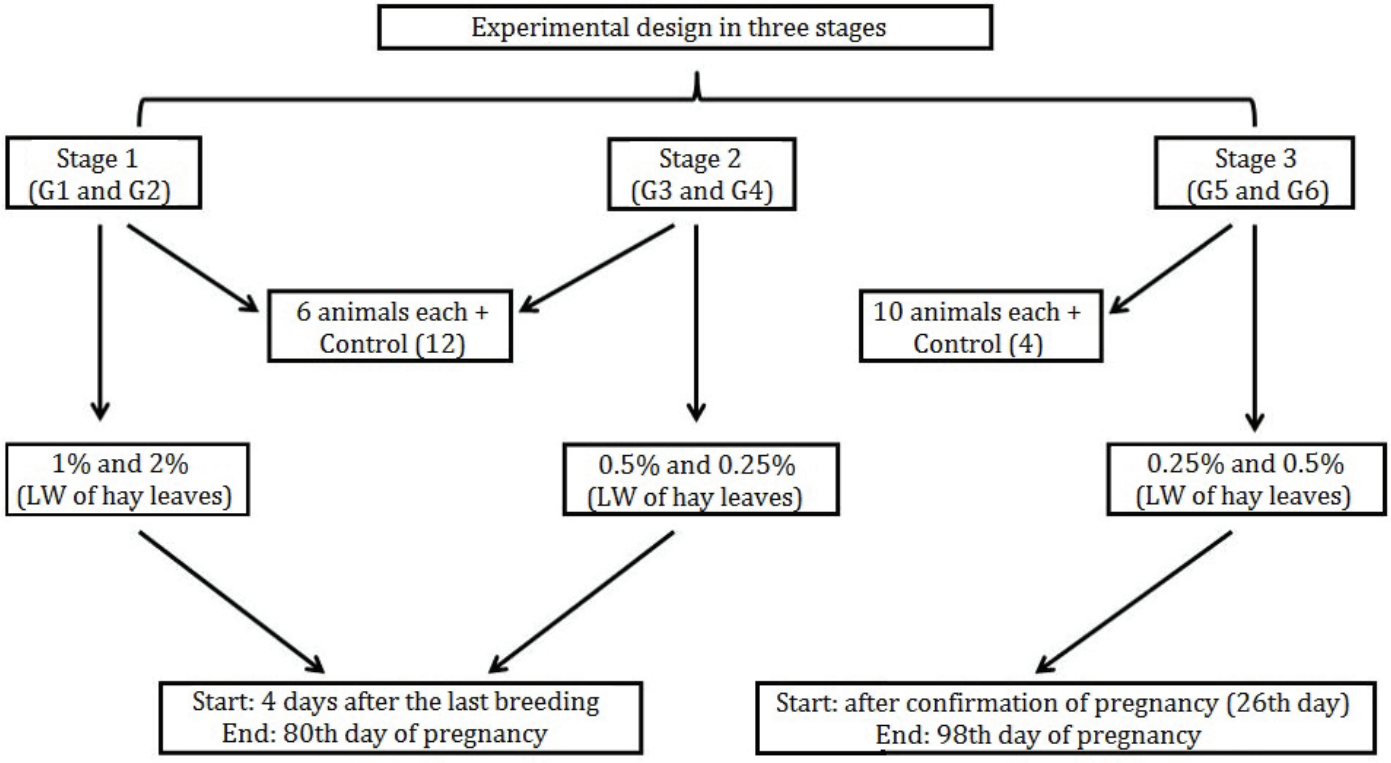


Fig.2. Flowchart of the experimental design in three stages. Live weight (LW).

Table 1. Main data on experimental design and outcome of Stage 1

Ewe	Dosage[a]	Consumption		Weight (kg)			Outcome	
		Total (g)	Average (g/kg/dia)	Initial	Final	Average	Diagnosis	Gestation (days)
0	1	13767.5	9.51	50	50	49.9	EL[b]	32
349	1	14021.5	9.09	55	52	53.2	EL	32
344	1	15252.5	8.77	63	54	60	EL	32
310	1	17925.5	9.03	56.5	54	55.17	Abortion	39
193	1	29191	7.16	48	39	43.42	NP[c]	NP
355	1	62458	6.27	47	45	46.43	Abortion	46
319	2	22469.33	16.38	50	45.5	47.3	EL	32
322	2	22458.67	16.51	49	42.5	46.9	EL	32
346	2	1383.33	23.46	49	37	40.78	NP	NP
365	2	16322.67	14.63	50	44	48.5	EL	26
100	2	17860	31.28	67	67	66.29	Abortion	46
187	2	80800.66	22.91	52	40	44.08	NP	NP

[a] % of live weight (LW), [b] EL = embryonic loss, [c] NP= non-pregnant.

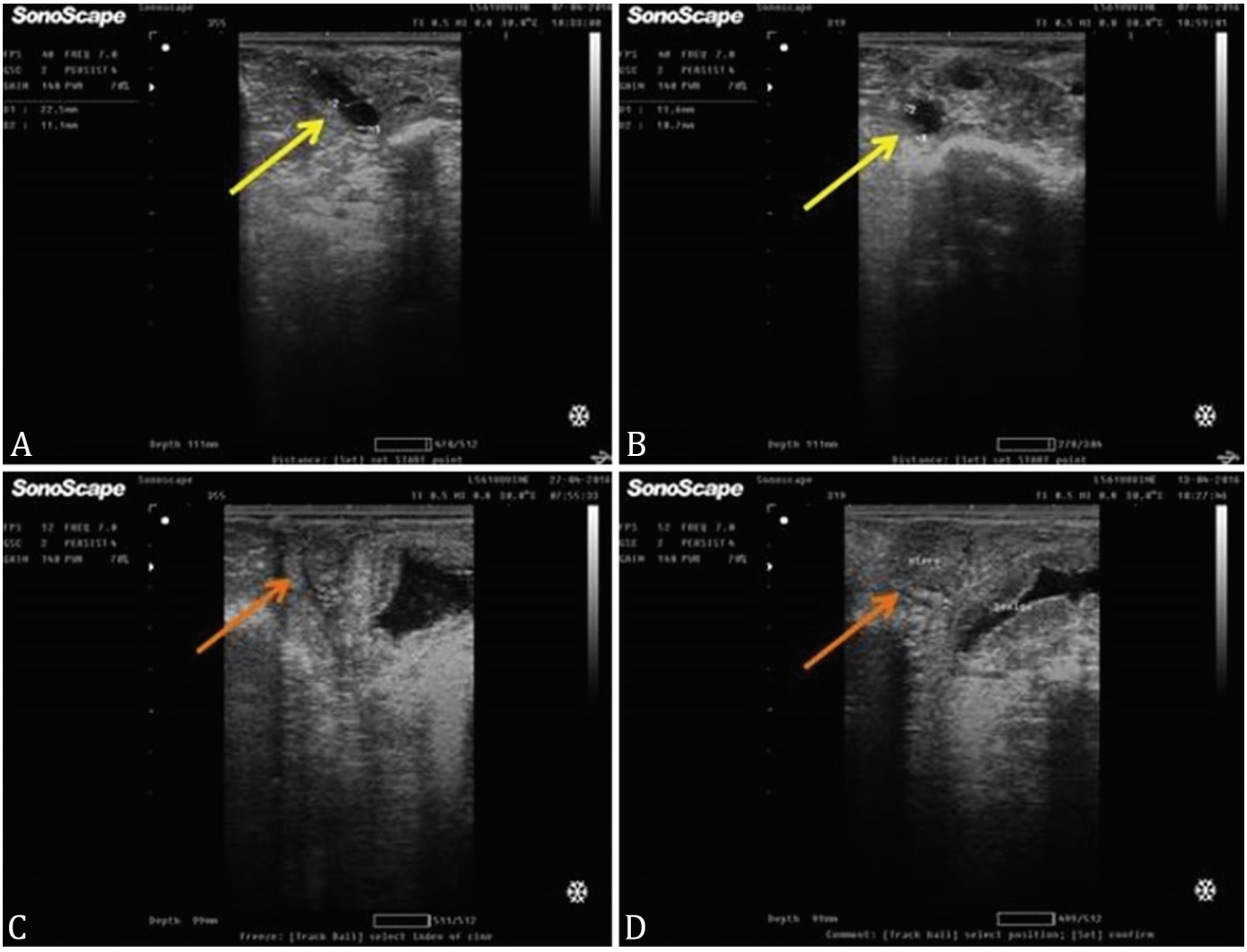


Fig.3. (**A**,**B**) Confirmation of pregnancy in animals in Groups 1 and 2, respectively (embryonic vesicle is indicated at the tip of the yellow arrows). (**C**,**D**) Confirmation of abortion (on the 46th day of gestation, ewe 355) and embryonic loss (on the 32nd day of gestation, ewe 319) in the previously mentioned animals, which received hay leaves of *Cenostigma pyramidale* in doses of 1% and 2% of live weight (LW), respectively (non-pregnant uterus indicated at the tip of the orange arrows).

leaves of *Cenostigma pyramidale*, could return to estrus; however, they were not pregnant after new NB.

In Stage 2, only two ewes (16.67%) were pregnant on the 23rd day after the last NB, that is, after being confirmed by USG. The other ewe returned to estrus; however, pregnancy was not found after new NB. From G3 group (0.5% LW of hay leaves), one ewe suffered embryonic loss at around the 40th day of gestation. A G4 ewe (0.25% LW of hay leaves) gave birth to a stillborn fetus on the 150th day of gestation (Table 2). The stillborn fetus was necropsied, with malformations as brachygnathia inferior (underbite), scoliosis (from the cervical spine to the 13th thoracic vertebra), arthrogryposis of the four limbs and arthrogryposis of the tibial tarsal joint and fibular tarsal joint, in addition to a rudimentary tongue (Fig.4A-C). The same animal was radiographed, confirming the presence of scoliosis (Fig.5).

In Stage 3, comprising the 20 synchronized ewes, 100% of pregnancies were found by USG around the 20th day of gestation. Placental retention was observed in 15% of the tested ewes, two from G6 group (corresponding to 20% of the group) and one from G5 (10%). One ewe (10%) of the G6 group suffered an abortion at approximately 108 days of gestation, accompanied by placental retention.

Malformations were observed in five lambs (45.46%) from G6 group, of which two presented slight tortuosity of the proximal phalanges of the thoracic limbs, two presented malformations of the frontal plane of thoracic limb alignment (Fig.6A,B) and deviation of the animal tail, and one, which was aborted, presented brachygnathia superior in addition to anasarca (Fig.7).

A lamb from G5 group showed slight deformities of the four limbs. In total, 23 full-term lambs were born, 12 from G5 group and 11 from G6 group. In eight (66.67%) lambs from G5 group, there were no changes, but in three of them (25%), there were slight changes in an upright posture (front posture). In five (45.46%) lambs from G6 group, no changes were observed, and one (9.1%) showed a slight change in an upright posture (open of the front part). There were no changes in the Control Groups. The results and the experimental design of this stage are shown in Table 3.

Table 2. Main data on experimental design and outcome of Stage 2

Ewe	Dosage[a]	Consumption		Weight (kg)			Outcome	
		Total (g)	Average (g/kg/dia)	Initial	Final	Average	Diagnosis	Gestation (days)
344	0.5	16353	3.64	61	50	56.08	NP[c]	NP
310	0.5	16511	3.7	58	52	55.71	NP	NP
355	0.5	6563.75[b]	3.54	47	41	46.33	EL[d]	40
322	0.5	14321	3.77	47.5	47	47.46	NP	NP
0	0.5	15647	3.73	52	51	52.37	NP	NP
100	0.5	19687	3.73	66.5	67	66.04	NP	NP
346	0.25	5700	1.57	46	47	45.5	NP	NP
90	0.25	5204.8	1.6	42	41	40.67	NP	NP
599	0.25	4296.2	1.58	35.4	34	33.94	NP	NP
349	0.25	6731.97[b]	1.58	54	54	53.38	Malformed stillborn	150
365	0.25	6264.6	1.61	49	50	48.54	NP	NP
193	0.25	6207.5	1.58	50	48	49.21	NP	NP

[a] % of live weight (LW), [b] total consumed until outcome, [c] NP = non-pregnant, [d] EL = embryonic loss.



Fig.4. Stillborn of ewe 349 that ingested 0.25% of the live weight (LW) from hay leaves of *Cenostigma pyramidale* for 80 days. (**A**) Evidence of deviation of the cervical region and arthrogriposis of the four limbs. (**B**) Brachygnathia inferior. (**C**) Rudimentary tongue (arrow).



Fig.5. Dorsoventral radiography showing lateral deviation of the cervical spine, compatible with scoliosis in stillborn lamb, from ewe 349, which received a daily dose of 0.25% of the live weight (LW) from hay leaves of *Cenostigma pyramidale* for 80 days.



Fig.6. Lambs from mothers that ingested leaves of *Cenostigma pyramidale* at 0.5% of live weight (LW) for 73 days. Note tortuosity of the thoracic limbs and alteration of hock joint in pelvic limbs ("jarretes cambaios").



Fig.7. An aborted fetus at approximately the 108th day of gestation, whose matrix ingested daily hay leaves of *Cenostigma pyramidale* at 0.5% of live weight (LW) from the 26th day of gestation. Note anasarca and brachygnathia superior (arrow).

DISCUSSION

The experimental ingestion of dry and ground leaves of *Cenostigma pyramidale* resulted in reproductive failures corresponding to abortion and embryonic loss, as well as malformations in ewes. These results infer that spontaneous cases of malformations and other reproductive losses in sheep and goats in the northeastern semiarid region are associated with the consumption of this species. The low acceptability of this plant, observed in experiments where it was necessary to add chemical concentrate, reinforces that poisoning is facilitated in periods of forage scarcity, remaining as the only or almost only alternative for feeding small ruminants, as reported in poisonings by other species (Tokarnia et al. 2012). On the other hand, the lesions observed are similar to those induced in small ruminants that consumed *Mimosa tenuiflora* ("jurema-preta") in its natural or experimental reproductive period (Pimentel et al. 2007, Dantas et al. 2010, Santos et al. 2012). Studies with *M. tenuiflora* and *C. pyramidale* in rats also demonstrated similar changes regarding the reproductive period (Medeiros et al. 2008, Câmara et al. 2017). As demonstrated in goats, that ingested fresh leaves of *C. pyramidale* (Reis et al. 2016), the primary malformations were also related to the limbs. The same findings were mentioned by Lopes et al. (2017), who tested doses of 10, 20, and 40% (within 2% of the doses supplied by roughage of the animals) of dry leaves of *C. pyramidale* for three groups of four ewes from the 18th day of gestation. Of the animals that ingested a 20% dose, three animals (75%) had a preterm birth over 120 days of gestation, and one (25%) gave birth to a lamb with bilateral arthrogryposis of the thoracic limbs. Concerning the group that received a 40% dose, one ewe (25%) gave birth to a normal fetus, another one (25%) lost the embryo on the 25th day of gestation, one (25%) had a miscarriage associated with arthrogryposis and prognathism over 140 days of the gestation period, and the last one (25%) had a miscarriage of a normal fetus on the 103rd day of pregnancy. The high rate of embryonic loss and abortion with less than 50 days of gestation seen in Stage 1 of the present study, in comparison to the aforementioned work, suggests that it results from the time of consumption of the plant (four days after the last natural harvest). According to Sinowatz (2010), when there is an action of agents that cause disturbances to the embryo in the first three weeks of life, there may be associated with embryonic loss or death, except in cases where the fetus may invoke compensatory mechanisms.

In addition to malformations, abortion, and embryonic loss, it was observed that ewes that ingested the dried leaves and leaves on the ground of *C. pyramidale* returned to estrus normally. However, when subjected to the breeding season, these ewes did

Table 3. Main data on experimental design and the outcome of Stage 3

Ewe	Dosage[a]	Consumption		Weight (kg)			Outcome	
		Total[b] (g)	Average (g/kg/dia)	Initial	Final	Average	Diagnosis	Gestation (days)
109	0.25	7369	2.48	35	43.8	35.83	Open of the front part	149
101	0.25	9930.5	2.47	47	58.4	48.43	Open of the front part	150
122	0.25	8941.8	2.44	44	47	44.08	No genetic alterations	150
14	0.25	10090	2.46	50	60	49.48	Open of the front part	150
99	0.25	8835	2.47	50	53.4	48.95	Subtle deformities of the four limbs	150
11	0.25	11376	2.48	65	72.4	62.83	No genetic alterations	150
5	0.25	10377	2.47	57	65.4	57.65	No genetic alterations	150
3	0.25	9300.5	2.48	50	60	51.28	Placental retention	150
15	0.25	9639.5	2.48	55	61.6	53.23	No genetic alterations	150
12	0.25	10340	2.5	54	61.8	56.68	No genetic alterations	150
108	0.5	15369	4.98	39.6	46.2	42.28	Partial/discrete placental retention tortuosity of the phalanges of the thoracic limbs	150
1	0.5	15577	4.97	41	50	42.98	Slight tortuosity of the phalanges	150
105	0.5	15534	4.96	42	51.2	42.93	Tortuous thoracic limbs	150
114	0.5	17861	4.95	49	53.2	49.45	Abortion/placental retention/fetus with anasarca and superior bragnatism	≈108
76	0.5	15109	4.95	40	50.4	41.83	Malformation of the four limbs and deviation of the animal tail	150
115	0.5	15159	4.94	43	52	42.08	No genetic alterations	150
126	0.5	14538	4.92	39.6	52	40.48	Hock joint injury	150
102	0.5	17447	5	47	53.6	47.83	No genetic alterations	150
103	0.5	14923	4.95	43	50	41.34	No genetic alterations	150
10	0.5	14831	4.77	44	54.4	42.58	No genetic alterations No genetic alterations	150

[a] % of live weight (LW), [b] total consumed until outcome.

not become pregnant. The return of estrus without pregnancy was later observed in ewe that ingested *M. tenuiflora* at the beginning of the reproductive period (Santos et al. 2012). Even after six months without ingesting *C. pyramiale*, it could be noted that pregnancy was not observed in 80% of the ewe submitted to the experiment in Stage 2, which suggests the possibility of a residual chemical effect of the plant. This result indicates the possibility of genetic alterations in the matrices for each offspring that ingested *C. pyramidale*, since studies with this species are in the initial stages and data have not yet been investigated. Likewise, there is no information regarding males, either for studies with *C. pyramidale* or with *M. tenuiflor*a.

The difference at the beginning of the ingestion of *C. pyramidale* did not prevent reproductive failures. As seen in Stage 3, despite the low doses and the start of consumption occurring on the 26th day of gestation, there were essential losses such as abortion and placental retention. The latter was not mentioned in similar studies in the northeastern semiarid region (Dantas et al. 2010, Santos et al. 2012, Lopes et al. 2017) and had not been recorded in the herd of ewes where the study was carried out. As the ewes in Stage 3 came from the Brazilian Midwest, where there is no occurrence of *C. pyramidale* and *M. tenuiflora*, it is suggested that the lack of contact of these animals with these plants has led to a greater predisposition to this alteration.

The geographical distribution of *C. pyramidale* and *M. tenuiflora* occurs in the northeastern semiarid region. It can be suspected that natural cases previously attributed to *M. tenuiflora* may have resulted from the poisoning by *C. pyramidale*. However, the possibility of synergistic action between species should not be ruled out since both can coexist in the same region. There is a possibility that the toxic principle is similar to that of *M. tenuiflora*, or that there is a potentization when the two species are ingested concomitantly.

CONCLUSIONS

Dry leaves and leaves on the ground of *Cenostigma pyramidale* maintain their toxicity, causing malformations, embryonic loss, and abortion in ewes.

Based on the observed results, it is recommended that pregnant ewe, preferably during the first 60th day of gestation, do not ingest the plant.

Acknowledgments.- The authors would like to thank "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES), Brazil, for the doctoral scholarship of the first author as well as all interns and employees who collaborated with the research project. The work was supported by the "Fundação de Apoio à Pesquisa do Estado da Bahia" (Proc. FAPESB PET 0006/2015), "Instituto Nacional de Ciência e Tecnologia" (INCT), and "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq) regarding the "Controle das Intoxicações por Plantas" (Proc. no. 573534/2008-0) and Universal Project 2014/CNPq (Proc. no. 460979 / 2014-0).

Conflict of interest statement.- There are no conflicts of interest.

REFERENCES

Câmara A.C.L., Gadelha I.C.N., Castro M.B., Medeiros R.M.T., Riet-Correa F. & Soto-Blanco B. 2017. Embryotoxic effects of *Poincianella* (*Caesalpinia*) *pyramidalis* leaves on pregnant rats. J. Vet. Diagn. Invest. 29(2):137-142. <https://dx.doi.org/10.1177/1040638716682564> <PMid:28064563>

Dantas A.F.M., Riet-Correa F., Medeiros R.M.T., Galiza G.J.N., Pimentel L.A., Anjos B.L. & Mora R.A. 2010. Malformações congênitas em ruminantes no semiárido do Nordeste Brasileiro. Pesq. Vet. Bras. 30(10):807-815. <https://dx.doi.org/10.1590/S0100-736X2010001000002>

Lopes J.R.G., Santos J.R.S., Medeiros M.A., Campos E.M., Riet-Correa F. & Medeiros R.M.T. 2017. Reproductive losses caused by the ingestion of *Poincianella pyramidalis* in sheep. Toxicon 138:98-101. <https://dx.doi.org/10.1016/j.toxicon.2017.08.020> <PMid:28843661>

Medeiros R.M.T., Figueiredo A.P.M., Benício T.M.A., Dantas F.P.M. & Riet-Correa F. 2008. Teratogenicity of *Mimosa tenuiflora* seeds to pregnant rats. Toxicon 51(2):316-319. <https://dx.doi.org/10.1016/j.toxicon.2007.06.012> <PMid:18078971>

Nóbrega Júnior J.E., Riet-Correa F., Nóbrega R.S., Medeiros J.M., Vasconcelos J.S., Simões S.V. & Tabosa I.M. 2005. Mortalidade perinatal de cordeiros no semi-árido da Paraíba. Pesq. Vet. Bras. 25(3):171-178. <https://dx.doi.org/10.1590/S0100-736X2005000300008>

Panter K.E., Keeler R.F., Bunch T.D. & Callan R.J. 1990. Congenital skeletal malformations and cleft palate induced in goats by ingestion of *Lupinus*, *Conium* and *Nicotiana* species. Toxicon 28(12):1377-1385. <https://dx.doi.org/10.1016/0041-0101(90)90154-Y>

Pimentel L.A., Riet-Correa F., Gardner D., Panter K.E., Dantas A.F.M., Medeiros R.M.T., Mota R.A. & Araújo J.A.S. 2007. *Mimosa tenuiflora* as a cause of malformations in ruminants in the Northeastern Brazilian semiarid rangelands. Vet. Pathol. 44(6):928-931. <https://dx.doi.org/10.1354/vp.44-6-928> <PMid:18039908>

Reis S.D.S., Oliveira R.S., Marcelino S.A.C., Macêdo J.T.S.A., Riet-Correa F. & Pedroso P.M.O. 2016. Congenital malformations and other reproductive losses in goats due to poisoning by *Poincianella pyramidalis* (Tul.) L.P. Queiroz (*Caesalpinia pyramidalis* Tul.). Toxicon 118:91-94. <https://dx.doi.org/10.1016/j.toxicon.2016.04.043> <PMid:27125469>

Santos J.R.S., Dantas A. F. M. & Riet-Correa F. 2012. Malformações, aborto e mortalidade embrionária causada pela ingestão de *Mimosa tenuiflora* (Leguminosae). Pesq. Vet. Bras. 32(11):1103-1106. <https://dx.doi.org/10.1590/S0100-736X2012001100005>

Sinowatz F. 2010. Teratology, p.339-382. In: Hyttel P., Sinowatz F. & Vejlsted M. (Eds), Essential of Domestic Animal Embriology. Elsevier, China.

Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Döbereiner J. 2012. Plantas Tóxicas do Brasil. 2ª ed. Helianthus, Rio de Janeiro. 586p.

Welch K.D., Panter K.E., Lee S.T., Gardner D.R., Stegelmeier B.L. & Cook D. 2009. Cyclopamine-induced synophthalmia in sheep: defining a critical window and toxicokinetic evaluation. J. Appl. Toxicol. 29(5):414-421. <https://dx.doi.org/10.1002/jat.1427> <PMid:19301244>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(10):758-775, October 2020
DOI: 10.1590/1678-5150-PVB-6708

Original Article
Livestock Diseases
BY-NC

Causes of death in growing-finishing pigs in two technified farms in southern Brazil[1]

Manoela M. Piva[2], Claiton I. Schwertz[2], Ronaldo M. Bianchi[2], Regina T. Kemper[2], Luan Cleber Henker[2], Ricardo Y. Nagae[3,4], Tais Regina M. Cê[3], David Emilio S.N. Barcellos[4], David Driemeier[2] and Saulo P. Pavarini[2]*

ABSTRACT.- Piva M.M., Schwertz C.I., Bianchi R.M., Kemper R.T., Henker L.C., Nagae R.Y., Cê T.R.M., Barcellos D.E.S.N., Driemeier D. & Pavarini S.P. 2020. **Causes of death in growing-finishing pigs in two technified farms in southern Brazil.** *Pesquisa Veterinária Brasileira 40(10):758-775.* 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

The aim of this study was to investigate the main causes of death in growing-finishing pigs in southern Brazil. During a one-year period (from 2018 to 2019), two industrial pig herds (18 and 20 thousand pigs each farm) in southern Brazil were monitored along the four seasons of the year (12 days per season on each farm), in order to perform necropsies of all pigs that died in that period. The two farms had an average monthly mortality rate ranging from 0.94 to 3.93% in the evaluated months. At necropsy, tissues were collected, fixed in 10% formalin solution and processed routinely for histopathological examination. When necessary, samples were sent for bacterial culture and PCR to identify etiologic agents. A total of 601 necropsies were performed, with 94.9% of conclusive diagnoses. Infectious diseases corresponded to 64.4% of conclusive diagnosis and non-infectious diseases to 35.6%. The most prevalent causes of death were: pneumonia (33%), gastric ulcers (15.4%), circovirosis (9.9%), systemic bacterial embolism (5.4%), polyserositis (4.4%), dilated cardiomyopathy and torsion of abdominal organs (4.3% each), and bacterial pericarditis (3.4%). Regarding pneumonias (199/601), the main agents identified in these cases were *Pasteurella multocida, Influenza A* virus and *Mycoplasma hyopneumoniae*, mainly in associations.

INDEX TERMS: Growing-finishing pigs, farms, Brazil, swine pathology, mortality, pneumonia, gastric ulcer, circovirus.

RESUMO.- [**Causas de morte em suínos de crescimento e terminação em duas granjas tecnificadas no Sul do Brasil.**] O objetivo do presente trabalho foi investigar as principais causas de morte de suínos em fase de crescimento e terminação no Sul do Brasil. Durante o período de um ano (entre 2018 e 2019), duas granjas tecnificadas de suínos no Sul do Brasil foram acompanhadas nas quatro estações (12 dias por estação em cada granja), para realização de necropsias dos suínos que morreram nesse período. As duas propriedades apresentavam mortalidade mensal média entre 0,94 e 3,93% nos meses avaliados. Na necropsia, amostras de órgãos foram colhidas, fixadas em formol 10% e processadas rotineiramente para o exame histopatológico. Quando necessário, amostras foram enviadas para o cultivo bacteriano e PCR para identificação de agentes etiológicos. Foram realizadas um total de 601 necropsias, com 94,9% de diagnósticos conclusivos. As doenças infecciosas corresponderam a 64,4% dos diagnósticos conclusivos e as não infecciosas a 35,6%. As principais causas de morte foram: pneumonias (33%), úlcera gástrica (15,4%), circovirose (9,9%), embolia bacteriana sistêmica (5,4%), polisserosite (4,4%), cardiomiopatia dilatada e torção de órgãos abdominais (4,3% cada) e pericardite bacteriana (3,4%). Com relação às pneumonias (199/601), os principais agentes associadas as lesões foram *Pasteurella*

[1] Received on May 20, 2020.
Accepted for publication on June 3, 2020.

[2] Setor de Patologia Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil. *Corresponding author: saulo.pavarini@ufrgs.br

[3] Empresa Seara Alimentos, Laboratório de Sanidade Animal, Avenida Paludo 155, Bairro Industrial, Seara, SC 89770-000, Brazil.

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

multocida, vírus da Influenza A e *Mycoplasma hyopneumoniae*, principalmente associados entre si.

TERMOS DE INDEXAÇÃO: Suínos de crescimento e terminação, granjas, Brasil, patologia suína, mortalidade, pneumonia, úlcera gástrica, circovirose.

INTRODUCTION

To investigate and define the patterns of diseases that affect pigs is an efficient way to identify specific problems of the farms, whether they are sanitary, nutritional, structural or related to management practices. Recognizing the causes of mortality in swine farms may represent an important tool to assess the general health status of the herd. Therefore, determining the causes of death may be important to assess the efficiency of programs of disease prevention and control (Mondal et al. 2012). The mortality rate considered acceptable for growing-finishing pig herds varies from 2.5 to 3.3%. The diseases that occur more frequently in this phase are generally multifactorial, and the severity of their occurrence does not depend only on the virulence of the agents involved, but mainly on the risk factors present in the system (Sobestiansky et al. 2012).

Some studies have been conducted to investigate the aspects of swine mortality in pre-weaning piglets and weaned pigs (Straw et al. 1983, Tubbs et al. 1993, Dutta & Rahman 2006, Mondal et al. 2012). However, there are few studies that broadly assess the causes of death in pigs (Straw et al. 1983, Wilson 1970, Mondal et al. 2012), since there is a greater focus on the evaluation of epidemiological aspects (Losinger 1998, Dutta & Rahman 2006) and risk factors (Maes et al. 2004, Jensen & Toft 2009). Thus, the aim of this study was to determine the main diseases that cause mortality in pig herds from two commercial farms in southern Brazil, through necropsy and histopathological analysis, as well as to identify the etiology of the diseases and evaluate their distribution throughout the year and during the growing-finishing period.

MATERIALS AND METHODS

Two farms (Farms A and B) located in the western region of the state of Santa Catarina, were assessed in this study. Farm A contained 14 pig-sheds with total capacity to house 20,000 pigs and had mortality rates ranging from 0.94 to 3.93% during the months of data gathering. Farm B had eight pig-sheds with capacity to house 18,000 pigs, and mortality rates ranged from 0.90 to 1.54% during the period. Each of the farms housed piglets from a single origin, with an average age of 70 days. The two farms were GRSC (Granjas de Reprodutores Suídeos Certificadas - Certified Suidae Breeder Farms) and used to transfer the selected gilts with approximately 140 days of age. The males and the gilts not selected were usually sent to slaughter at 175 days of age, with an average weight of 125kg. In both farms, automatic feeding systems were used, with *ad libitum* supply of ration. In both farms flooring consisted of solid concrete and slat concrete. The pigs received commercial vaccines for swine circovirus type 2 and *Mycoplasma hyopneumoniae* (days 21 and 42 of age), in addition to autogenous vaccination against *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica,* and *Pasteurella multocida* (days 42 and 63 of age). Farm A implemented autogenous vaccination during the visits, while Farm B underwent updating of vaccine strains for these agents.

Each farm was visited in four moments, which covered the four seasons, with a total of eight visits (Table 1). Each of the visits lasted 12 days, during which all pigs that died spontaneously or were euthanized by farm employees (usually fallen pigs which could no longer be fed without aid) were submitted for necropsy.

During the necropsies, organ fragments were collected, fixed in 10% formalin solution and subsequently routinely processed for the preparation of histological slides, which were stained by the hematoxylin and eosin (HE) technique. Data from individual pigs, including age, sex, clinical history, and treatments performed by the employees were recorded. When infectious agents were suspected to be involved in the lesions, fragments of organs and body fluids were collected, kept refrigerated and sent for bacteriological and biochemical examinations within 24 hours.

The diagnoses were concluded through the association of clinical history, macroscopic and microscopic lesions, as well as complementary tests, when necessary. Based on this, specific criteria were established for the completion of the diagnosis of each condition, as described below. For the diagnosis of pneumonia as the cause of death, pigs with severe macroscopic and microscopic lesions in the lungs (more than 30% of the pulmonary parenchyma affected), associated with the absence of significant lesions in other vital organs were considered. All lungs were evaluated according to the Pneumonia Severity Index (PSI), in order to quantify the pulmonary lesions through a validated methodology described elsewhere (Piffer et al. 1991). Due to the complex etiological character of respiratory diseases in pigs (Rech et al. 2018), different techniques were used to detect etiological agents and/or characteristic lesions, including bacteriology, histology and PCR. In all cases of pneumonia, lung fragments were inoculated in blood agar plates (BA) and MacConkey agar (MC) and incubated at 36°C+/-1°C for 18-48 hours in aerobiosis atmosphere. A colony of *Staphylococcus hyicus* supplemented with nicotinamide adenine dinucleotide (NAD) was added to the blood agar plates and these were incubated in microaerophilia at 36°C+/-1°C for 24-48h, enabling the growth of *Glaesserella (Haemophilus) parasuis* and *A. pleuropneumoniae.* Biochemical characterization of

Table 1. Temporal distribution of the collections, number of necropsied pigs and monthly mortality rate in each of the farms visited

Visit	Farm	Month/Year	Number of necropsies	Monthly mortality rate (%)
01	A	July/2018	64	3.93
02	B	August/2018	69	1.12
03	B	November/2018	90	1.54
04	A	November/2018	61	0.94
05	B	February/2019	88	1.39
06	A	February /2019	59	2.05
07	B	April/2019	85	0.90
08	A	May/2019	94	2.84

the colonies was performed according to Quinn et al. (2011) for the identification of bacteria. For the capsular type of *Pasteurella multocida,* the hyaluronidase test described by Carter & Rundell (1975) was performed to identify type A strains. The neutral acryflavine test (Carter & Subronto 1973) was used to characterize type D strains.

Diagnoses of bacterial pneumonia included cases with marked cranioventral consolidation (Fig.1A), which sometimes extended to the caudal lobes, often associated with pleuritis (Fig.1B). These areas of consolidation were dark red, firm, and the cut surface revealed yellowish-white areas that sometimes coalesced, as well as abscesses (Fig.1C and 1D) (López & Martinson 2017). The pattern of histological lesions in these cases consisted of marked suppurative bronchopneumonia with fibrin deposition, in bronchi, bronchioles and alveoli, associated or not with areas of coagulative necrosis, hemorrhage and sometimes abscess formation.

The participation of influenza virus in the etiology of pneumonia was considered through the observation of the macroscopic and histological lesions characteristic of the disease. Polymerase chain reaction techniques (PCR) and immunohistochemistry (IHC) were not used for the detection of this virus due to the rapid period of elimination of infection, as well as the subacute and chronic nature of the lesions observed in many cases. The lungs of pigs with diagnosis compatible with influenza virus pneumonia did not collapse after the opening of the thoracic cavity and showed multifocal areas of consolidation and/or atelectasis, interspersed with areas of normal appearance ("checkerboard appearance") (Fig.1E). Microscopic findings observed in these cases included necrotizing and obliterative bronchiolitis (Fig.1F), associated with bronchointerstitial pneumonia (Schaefer et al. 2013), which are highly suggestive of influenza infection in pigs in Brazil, since other diseases that could cause similar lesions such as PRRS do not occur in the country (Rech et al. 2018). Frequently, concomitant occurrence of the two described patterns was observed. Furthermore, to prove the concomitant involvement of *M. hyopneumoniae* in lung lesions, 80 samples of pig lungs (10 samples from each visit) with final diagnosis of pneumonia were randomly chosen and submitted to PCR for amplification of the 16S rRNA m gene of *M. hyopneumoniae* (Yamaguti et al. 2008), added to ROX as a reference dye. The extraction was performed with a commercial kit (MagMAX CORE Nucleic Acid Purifications, Thermo Fisher Scientific) according to the manufacturer's instructions, and the samples were stored at -20°C.

The diagnosis of gastric ulcer was established through the observation of ulceration of the *pars oesophagea* of the stomach (Fig.2A and 2B), associated with signs of blood loss from the lesion, such as the presence of clots in the gastric lumen (Fig.2C), pallor of the carcass (Fig.2D) and organs, as well as dark and pasty fecal content inside the intestinal lumen (melena) (Fig.2E). Gastric ulcers were graded according to Sobestiansky et al. (2012) and only grade IV ulcers, when in association with signs of hemorrhage, were considered as the cause of death of the animal. Pigs with peritonitis due to rupture of gastric ulcers were also allocated in this group (Fig.2F).

In the diagnosis of circovirosis, pigs with lesions of porcine dermatitis and nephropathy syndrome (PDNS) and post-weaning multisystemic wasting syndrome (PMWS) were included. All pigs with PDNS presented fibrinosuppurative glomerulonephritis (Fig.3A and 3B), granulomatous lymphadenitis, systemic vasculitis and, in most cases, characteristic skin lesions (Fig.3C). All pigs with PMWS presented emaciation, systemic granulomatous lymphadenitis with lymphoid depletion, mononuclear interstitial pneumonia and nephritis, besides vasculitis (Caswell& Willians 2016, Cianciolo & Mohr 2016) (Fig.3D-F). Pool samples of lymph nodes from 21 pigs were submitted to PCR for the detection of swine circovirus type 2 (PCV2) following the methodology of Xiangdong et al. 2018.

The diagnosis of systemic bacterial embolism comprised pigs with multifocal suppurative and abscedative lesions of hematogenous origin, such as embolic pneumonia, arthritis, osteomyelitis and endocarditis, often associated with a primary lesion, usually tail lesions (Fig.4). Fragments and swabs of the lesions were inoculated on BA and MC agar and incubated at 36°C+/-1°C for 18-48 hours in aerobic atmosphere.

The diagnosis of bacterial polyserositis was attributed to pigs with large amounts of fluid and fibrin exudation in both the abdominal and thoracic cavities (Fig.5A and 5B), with concomitant involvement of other sites, including joints, pericardial sac, meninges and lungs. For the diagnoses of bacterial pericarditis, the presence of a large amount of exudate inside the pericardial sac was used as a criterion (Fig.5C-E), associated with extracardiac lesions of congestive heart failure, such as ascites and mottled liver ("nutmeg liver") (Fig.5F). In both cases, swabs and fluids were collected and kept refrigerated for microbiological examination using methodology similar to that used for cases of bacterial pneumonia.

Pigs with dilated cardiomyopathy presented cardiomegaly (Fig.6A), with marked dilation of the cardiac chambers (Fig.6B), especially the ventricles (Cruz et al. 2019). These pigs also had extracardiac lesions of congestive heart failure, such as ascites (Fig.6C), pulmonary edema and "nutmeg liver" (Fig.6D). The group of cardiorespiratory failure associated with fights included pigs with a history of recent fight (up to 12 hours), with abrasions and hyperemia of the skin, in addition to marked pulmonary edema and congestion.

Pigs presenting displacement of viscera were classified into a group of torsion of abdominal cavity organs. In this group, the macroscopic visualization of mesenteric torsion (Fig.7A), hepatic lobe torsion (Fig.7B) and spleen torsion, together with histological lesions supported the diagnosis. Similar criteria were used to reach the diagnosis of pigs with hemoperitoneum due to liver rupture. In this case, a large amount of free blood was observed in the abdominal cavity and ruptures of the capsule and hepatic parenchyma were seen (Fig.7C and 7D).

For the diagnosis of meningoencephalomyelitis, four distinct histopathological patterns were considered: suppurative meningoencephalitis, of bacterial origin (Fig.8A); non-suppurative meningoencephalitis of probable viral origin; ascending myelitis of bacterial origin (Fig.8B); and eosinophilic meningoencephalitis, compatible with salt poisoning or water deprivation. In cases of meningoencephalitis, fragments of the central nervous system and/or liquor were submitted to bacteriological examination. These were inoculated in BA and MC, maintained in similar conditions of samples from cases of bacterial pneumonia in microaerobiosis, adding *a Staphylococcus hyicus* streak to the plate culture.

Pigs that presented bone fractures in various places, and who died because of this injury, comprised a group called "bone fracture". Acute bleeding associated with this lesion was often observed (Fig.8C). Some animals with umbilical or inguinal hernias presented intestinal incarceration or rupture within the hernia. This diagnosis was established through the macroscopic observation of the lesions (Fig.8D-F).

For the diagnosis of bacterial enterocolitis, affected pigs presented acute and chronic lesions in the intestines, associated with the absence of alterations in other vital organs. In the diagnosis of salmonellosis, cases with characteristic fibrinonecrotic lesions in the intestinal mucosa were included (Fig.9A and 9B). Fragments of intestines, spleen and bile were submitted to bacterial isolation in BA

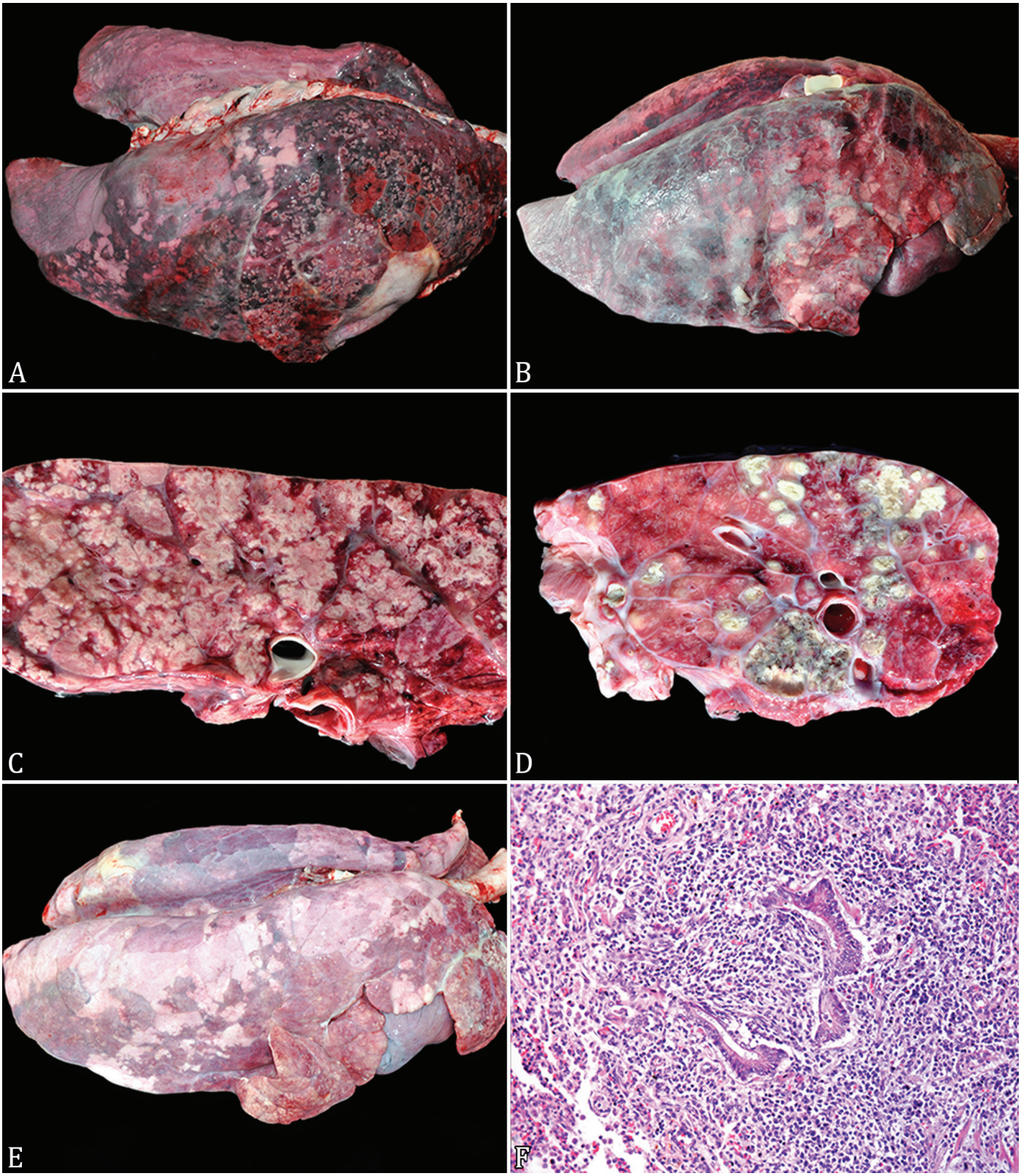


Fig.1. Pneumonias in growing-finishing pigs. (**A**) Bacterial pneumonia. Non-collapsed lungs, with marked consolidation affecting predominantly the cranioventral area. (**B**) Bacterial pleuropneumonia. Non-collapsed lungs, with marked consolidation affecting the cranial, middle and caudal lobes, and intense fibrin deposition on the pleura. (**C**) Bacterial pneumonia (cut surface). Multifocal to coalescent whitish and friable areas (necrosis), surrounded by reddish areas. (**D**) Bacterial pneumonia (cut surface). Areas of necrosis and multifocal abscesses are seen in the pulmonary parenchyma. (**E**) Bronchointerstitial pneumonia suggestive of influenza and secondary bacterial infection. Non-collapsed lungs, with "checkerboard aspect", as well as marked cranioventral consolidation. (**F**). Swine influenza. Obliterative bronchiolitis with marked peribronchiolar infiltrate of lymphocytes. HE, obj.20x.

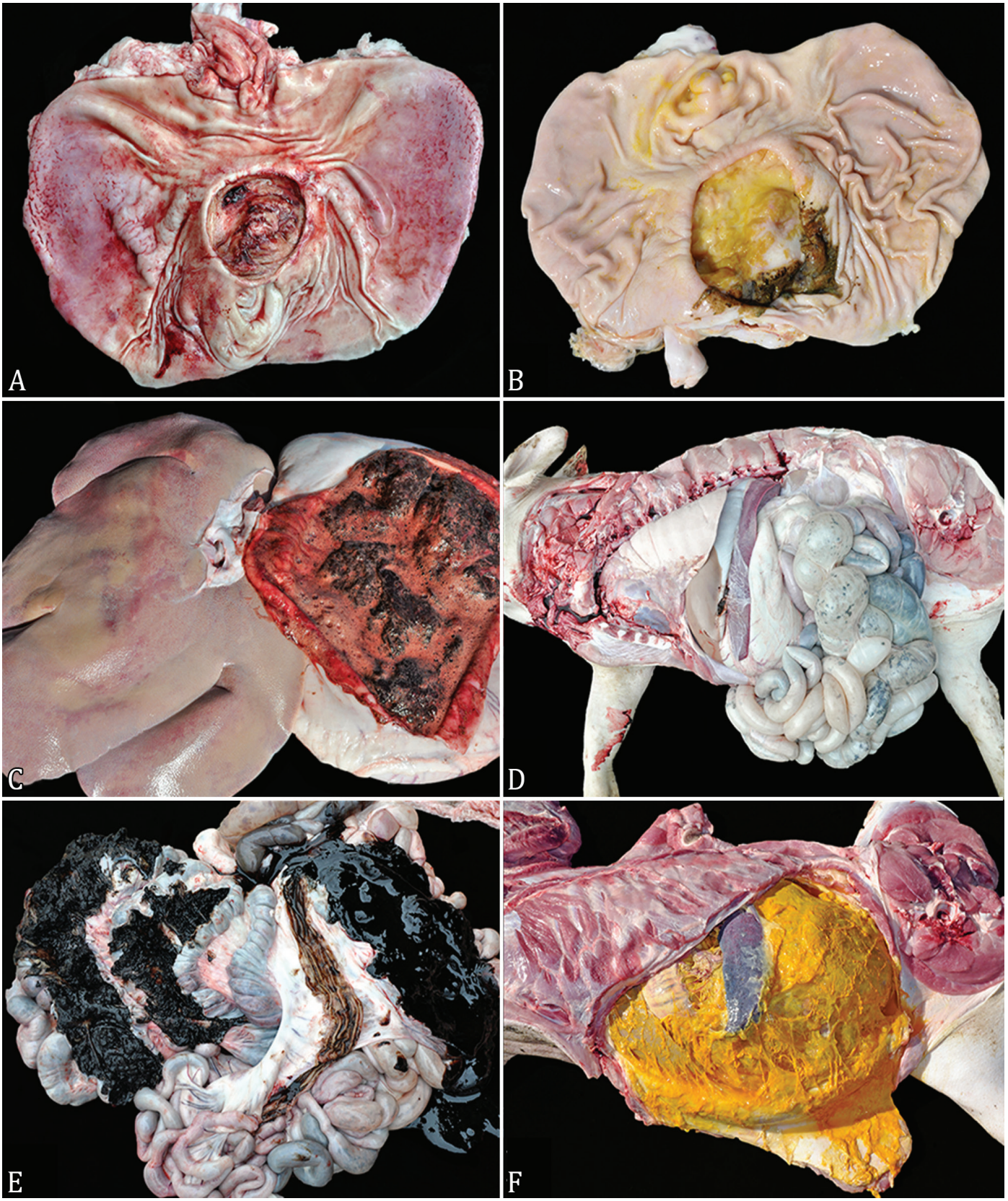


Fig.2. Gastric ulcer in growing-finishing pigs. (**A**) Stomach with focally extensive ulceration affecting the *pars oesophagea*. (**B**) Stomach with marked ulceration of the *pars oesophagea*, which presents elevated edges and yellowish coloration (fibrin deposition). (**C**) Stomach filled with blood clots, and diffusely pale liver. (**D**) Diffuse pallor of the carcass and organs in a pig with gastric ulcer. (**E**) Intestines. In the lumen of large gut there is a great amount of pasty and blackened feces. (**F**). Fibrinous peritonitis in a pig with perforated gastric ulcer. Abdomen filled with fibrillar and intensely yellowish material (bile discoloration).

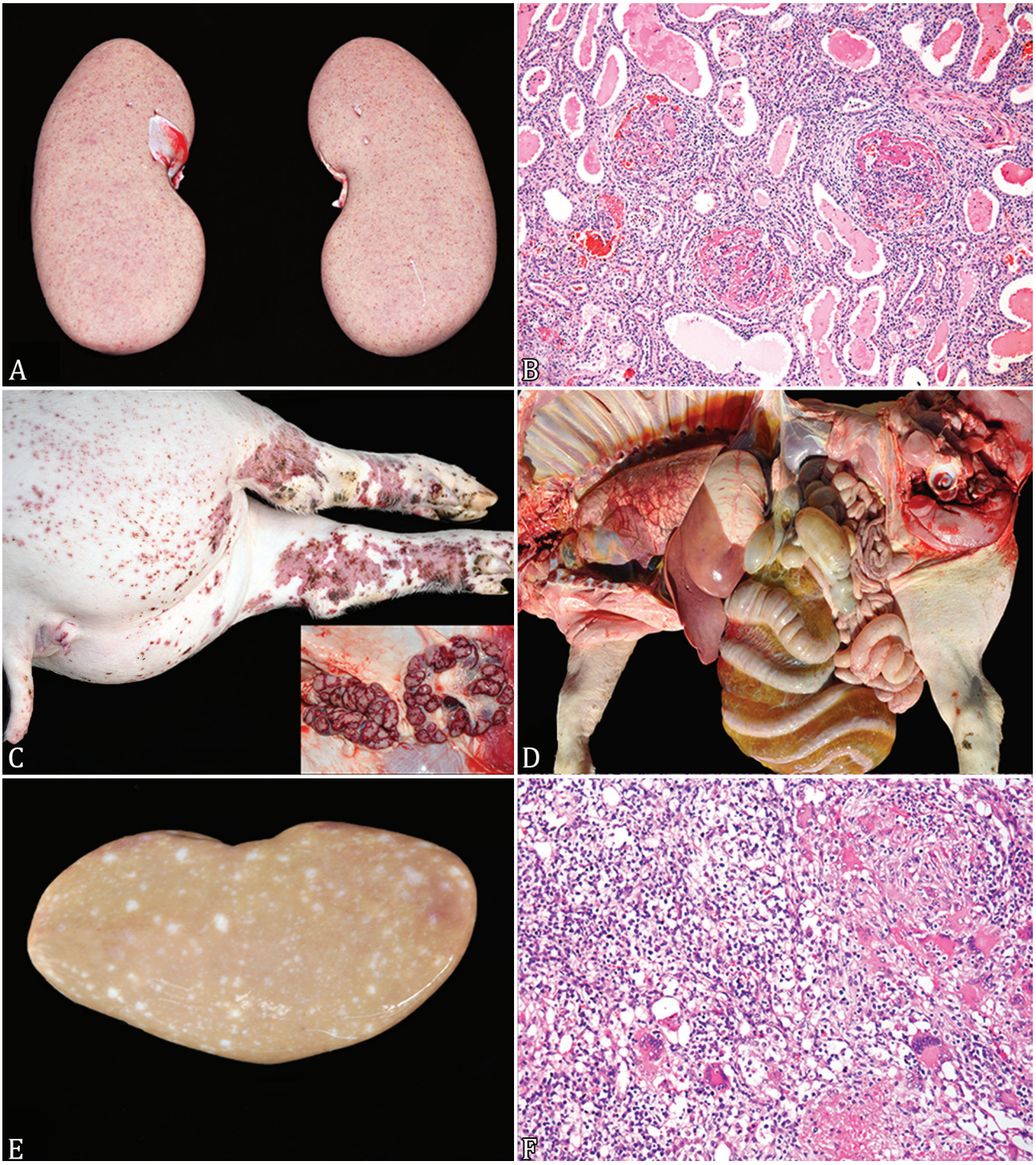


Fig.3. Circovirosis in growing-finishing pigs. (**A**) Porcine dermatitis and nephropathy syndrome (PDNS). Enlarged kidneys with reddish multifocal pinpoint areas throughout the cortical surface. (**B**) PDNS. Fibrinosuppurative glomerulonephritis. Enlarged glomeruli, with intense deposition of densely fibrillar and eosinophilic material in the urinary spaces (fibrin), in addition to infiltrate of neutrophils, lymphocytes, plasma cells, and macrophages that extends to the interstitium. The urinary tubules are dilated and filled with proteinaceous material (proteinuria). HE, obj.20x. (**C**) PDNS. Skin of the perineal region and pelvic limbs with multifocal irregular areas of reddish discoloration, sometimes covered by crusts (necrotic dermatitis). Inset: enlarged inguinal lymph nodes are observed, which present reddish peripheral areas (hemorrhage). (**D**) Post-weaning multisystemic wasting syndrome (PMWS) in pigs. At the opening of the carcass, marked edema of the mesocolon is observed, in addition to mild deposition of clear effusion in the abdominal and thoracic cavities (ascites and hydrothorax, respectively). The lung is not collapsed and shows interlobular septa demarcated by intense edema (interlobular edema). (**E**) PMWS. Kidney with whitish multifocal areas throughout the cortical surface (interstitial nephritis). (**F**) PMWS. Lymph node with lymphoid necrosis and depletion, inflammatory infiltrate and fibrin deposition, as well as infiltrate of Langhans-type multinucleated giant cells (granulomatous lymphadenitis). HE, obj.20x.

and MC agar, incubated for 24-48 hours at 36°C+1°C in aerobiosis. In some cases of chronic *Salmonella* sp. infection, pigs presented marked stenosis of the final portion of the colon or rectum, with secondary megacolon (Fig.9C and 9D). In cases of swine proliferative enteropathy, marked thickening of the ileum wall was observed, which often extended to the jejunum and cecum, and resulted in the "ceribriform aspect" of the mucosa associated with a large amount of blood and necrotic debris in the lumen (Fig.9E). For these cases, the Warthin-Starry histochemical technique (WS) of silver impregnation was used to evidence the agent inside enterocytes (Fig.9F). Diseases with a frequency equal to or less than three cases were grouped as others.

RESULTS

In this study, 601 necropsies of growing-finishing pigs were performed, and in 94.9% (570/601) of the cases, the diagnosis was conclusive. The frequency of diagnosis of the main conditions, as well as the age and sex of the affected pigs are shown in Table 2. Figure 10 shows the seasonal distribution of the main diagnosed diseases and figure 11 highlights the main diseases diagnosed according to the age of the pigs, as well as the distribution of deaths in each age group. Figure 11 highlights that there was an increase in the number of deaths due to pneumonia in older pigs, and a similar pattern occurred for deaths due to gastric ulcer, producing greater mortality between 151 and 175 days. The occurrence of systemic bacterial embolism was higher in pigs after 111 days of life, and this condition was especially common in animals at the end of the finishing phase (151 to 175 days). Circovirosis, bacterial polyserositis and dilated cardiomyopathy were diagnosed at all ages, as well as pericarditis, but these presented a higher frequency of cases between 70-90 days of life. The mortality of growing-finishing pigs increased along with the time of housing, meaning that the mortality rate among older pigs was higher when compared with recently housed pigs.

Infectious diseases represented the majority of conclusive diagnosis, accounting for 64.4% of these (367/570), while non-infectious diseases accounted for 35.6% (203/570) of the cases. Pneumonia comprised the most frequent diagnosis of

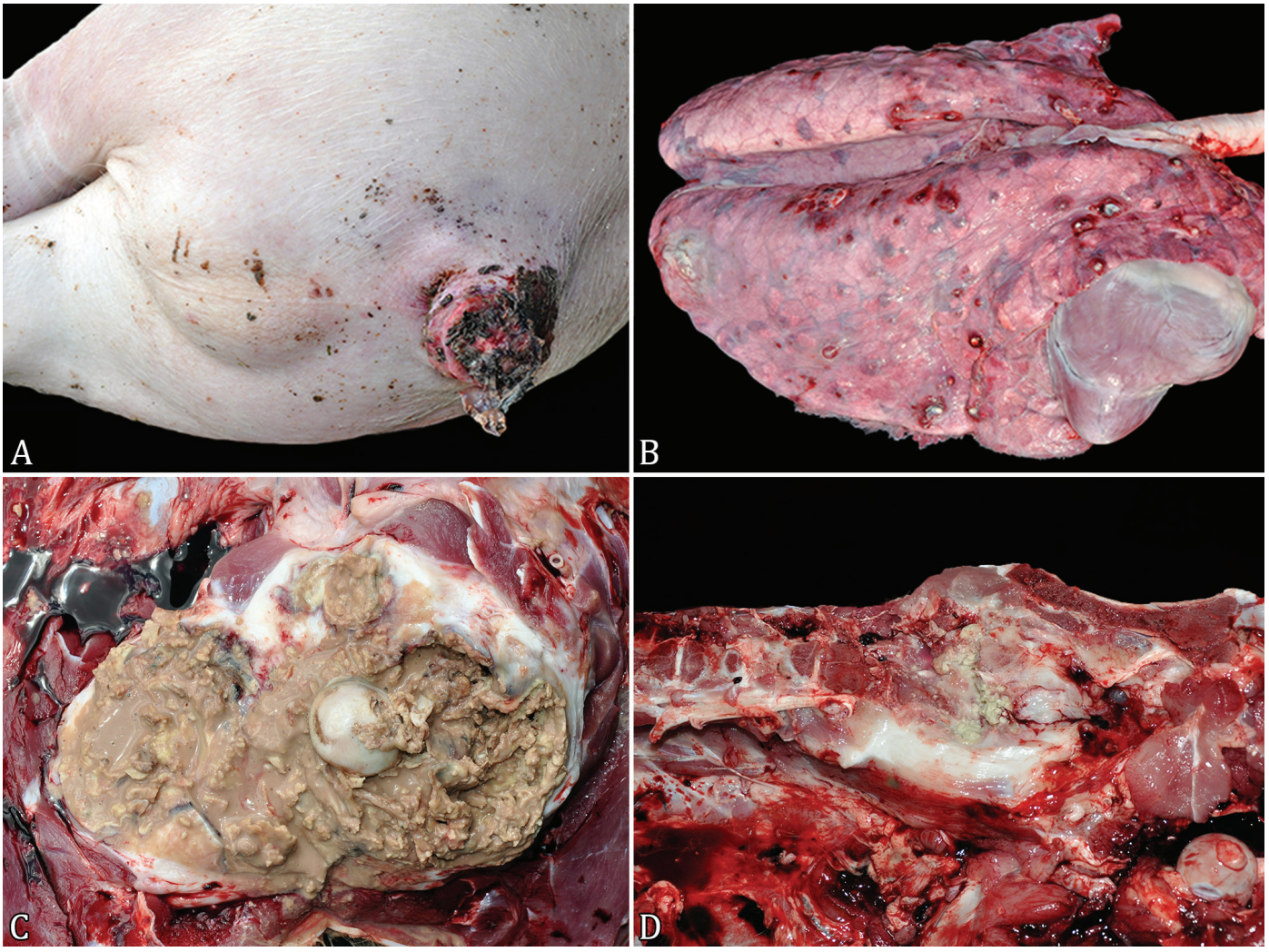


Fig.4. Systemic bacterial embolism in growing-finishing pigs. (**A**) Tail biting lesion. Tail with tissue laceration, ulcerated surface and crust formations. (**B**) Embolic pneumonia. Lungs with nodular and firm multifocal areas, yellowish and shrouded by a reddish halo (abscesses). (**C**) Suppurative arthritis. Coxofemoral joint with a marked amount of purulent content, surrounded by a thick fibrous capsule. (**D**) Abscedative spondylitis. The sacral region is enlarged and contains friable and purulent material in the center, surrounded by whitish and firm areas.

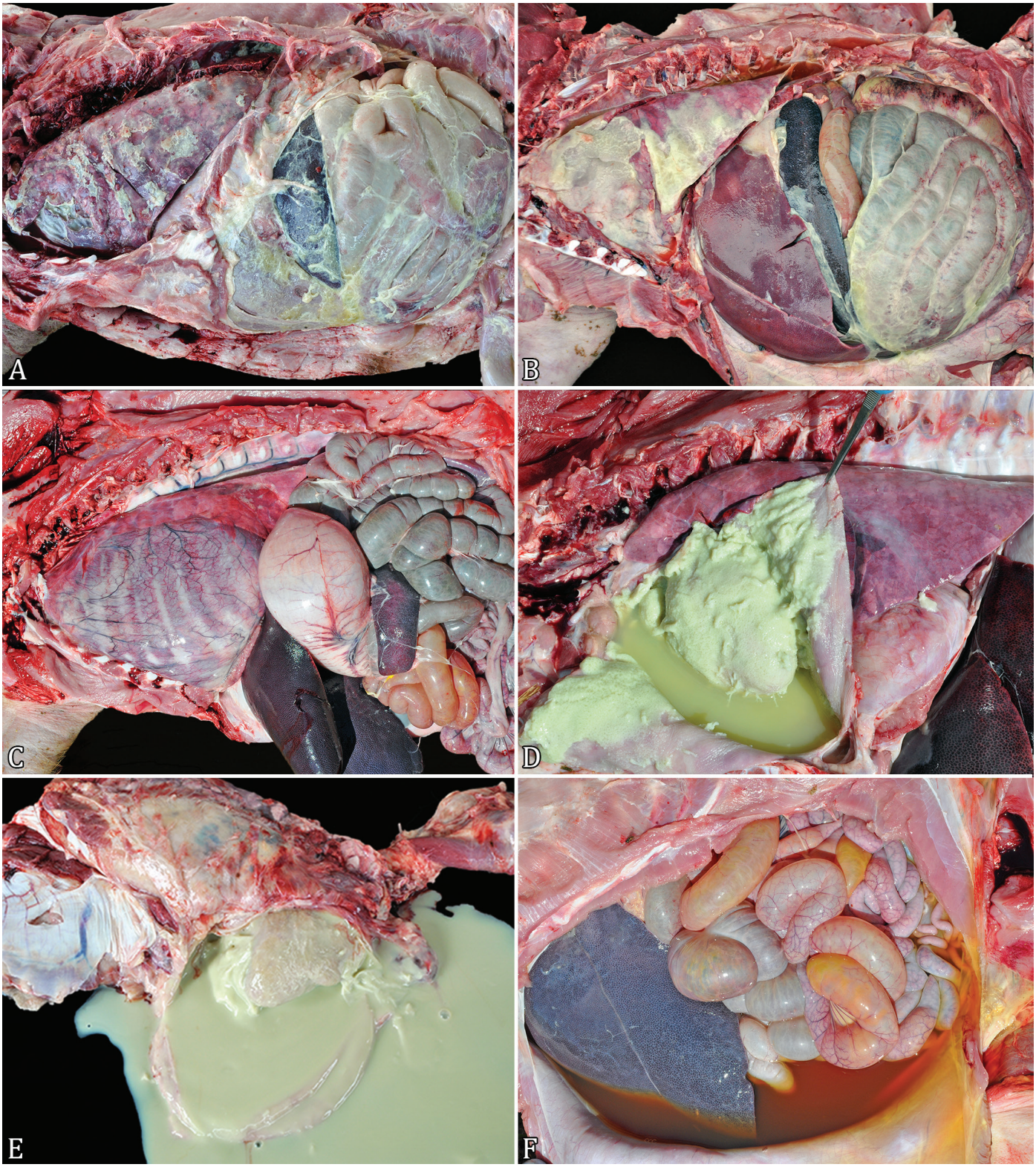


Fig.5. Bacterial polyserositis and pericarditis in growing-finishing pigs. (**A**) Pneumonia and polyserositis by *Pasteurella multocida.* Intense fibrin deposition in the abdominal and thoracic cavities. Not collapsed and markedly consolidated lungs. (**B**) Polyserositis by *Glaesserella (Haemophilus) parasuis*. Intense fibrin deposition in the abdominal and thoracic cavities. (**C**) Bacterial pericarditis. Pericardial sac severely distended and occupying nearly the entire thoracic cavity. Lung severely compressed and reduced in size (compressive atelectasis). (**D**) Bacterial pericarditis. In the opening of the pericardial sac there is a large amount of yellowish liquid and marked deposition of dense fibrin filaments on the surface of the epicardium. (**E**) Bacterial pericarditis. Pericardial sac with a large amount of purulent material, with adhesion of fibrin filaments in the pericardium. (**F**). Congestive heart failure in cases of bacterial pericarditis. Large amount of light orange fluid in the abdominal cavity (ascites) and severe liver enlargement (congestion).

this study, with 33% of the cases (199/601) and, from these, by the evaluation of PSI, the mean pulmonary involvement was 57.8%. Bacterial pneumonia corresponded to 93.9% of cases of pneumonia (187/199), and 79.1% had histological lesions suggestive of concomitant influenza infection (148/187). Bacterial isolation was possible in 61.30% of cases (122/199). The most commonly isolated bacterial agent was *Pasteurella multocida*, with a total of 101 isolations (50.7% of pneumonia diagnoses). Of these, 49 were capsular type A, 40 were capsular type D and 12 were non-typable. Other bacterial agents isolated were *Streptococcus suis* (10 cases), *Actinobacillus pleuropneumoniae* (five cases), *Trueperella pyogenes* (five cases), and *Actinobacillus suis* (one case). Viral pneumonia with changes compatible with primary swine influenza was diagnosed in 12 cases. Fibrinonecrotic tracheitis was also observed in seven cases of pneumonia, and in six of these, severe lesions suggestive of influenza were noted. In 75% of the lungs tested by PCR (60/80), the involvement of *Mycoplasma hyopneumoniae* was identified. It is also worth mentioning that in 72.88% (293/402) of the cases, the pigs died from other diseases also presented different degrees of pneumonia.

Gastric ulcers accounted for 15.4% of conclusive diagnoses (93/570), and this condition represented the second most frequent diagnosis in this study. In only three cases the gastric ulcer ruptured with secondary peritonitis was observed.

Of the 60 pigs diagnosed with circovirosis, 34 were classified as PDNS and 26 as PMWS. Ages ranged from 90 to 181 days old for PDNS and 80 to 190 for PMWS, with a median of 108 days old for both. In cases of PMWS, concomitant lesions caused by other infectious agents were identified in 23 cases and these were: valvular bacterial endocarditis (8/26), bacterial pneumonia (7/26), suppurative meningoencephalitis, and fibrinous pericarditis (3/26 each), fibrinonecrotic enterocolitis (salmonellosis), and bacterial arthritis (1/26 each). In cases of PDNS, the main concomitant conditions were bacterial pneumonia (5/34), and swine proliferative enteropathy, salmonellosis, polyserosites, and rectal prolapse at one opportunity each.

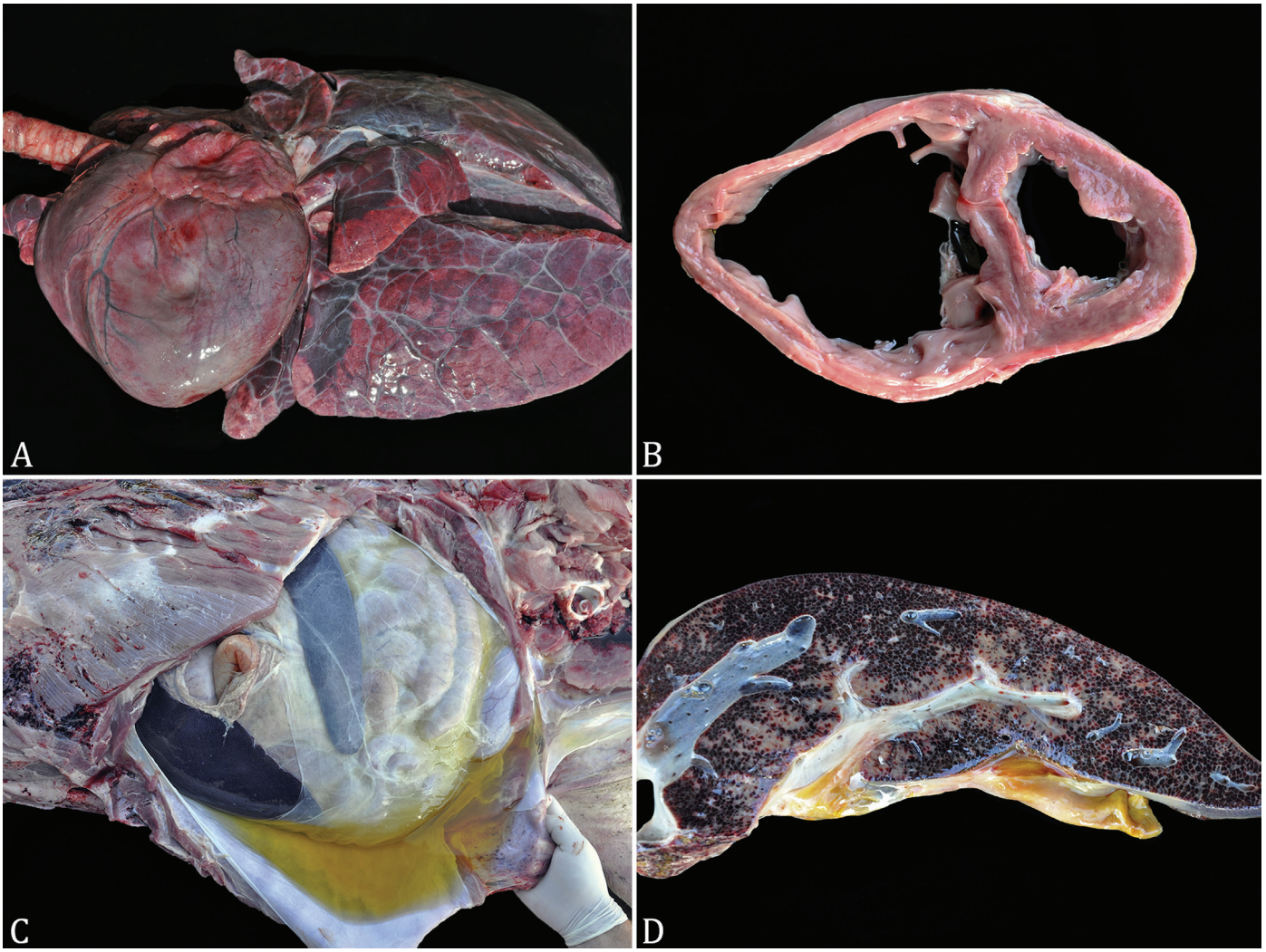


Fig.6. Dilated cardiomyopathy in growing-finishing pigs. (**A**) Heart enlarged (cardiomegaly) with globose aspect. Non-collapsed lungs, with diffuse reddish discoloration (congestion and edema). (**B**) Heart with marked dilation of the ventricular chambers and thinning of the myocardial wall, more evident in the right ventricle. (**C**) Large amount of citrine fluid in the abdominal cavity associated with moderate deposition of fibrin filaments (ascites). (**D**) Cut surface of the liver, dark red, with intense dilation of vascular spaces and marked enhancement of the lobular pattern ("nutmeg liver").

Of the 60 cases of circovirus, 21 were submitted to RT-PCR and all were positive for PCV-2 in samples of lymph nodes. In the remaining cases, the diagnosis was made based solely on the typical macroscopic and histopathological lesions.

Systemic bacterial embolism predominantly affected males (24/33), and the primary lesion of the embolic process was identified in 75.7% of the cases (25/33). In 92% of these (23/25), the laceration of the tail and perineal tissues due to biting was assumed to be the primary lesion, and in two cases, podal lesions (pododermatitis of unspecific cause) were incriminated. The pathological picture was characterized by combinations of suppurative and abscedative lesions, mainly arthritis (25/33), embolic pneumonia (21/33), osteomyelitis, especially in the spine (12/33), endocarditis (2/33), splenitis (2/33), and less frequently, serositis and regional lymphadenitis and (1/33 each).

From the 27 cases of bacterial polyserositis, in 20 (74%) it was possible to determine the etiology. Of these, in 17 pigs *P. multocida* was cultured (11 cases type D; three cases type A; and three cases non-typable); and in three cases, *Glaesserella* (*Haemophilus*) *parasuis* was cultured. These bacteria were often isolated in more than one site (e.g. exudates from the pericardium, abdomen and thorax, liquor and synovial fluid). In cases with isolation of *P. multocida* the median age of the animals was 100 days, while for *Glaesserella* (*Haemophilus*) *parasuis* it was 91 days old. In the 17 cases with isolation of *P. multocida*, 14 had suppurative pneumonia.

In the group of bacterial pericarditis, in 10 of the 21 cases, *P. multocida* was cultured, and it was the only agent identified in this condition. In these pigs with diagnosis of pericarditis, in addition to the lesions of chronic congestive heart failure, fibrinous pleuritis (5 cases), and pleuropneumonia (3 cases) were observed.

Of the 26 cases grouped into "torsion of abdominal cavity organs", 22 were mesenteric torsions (mean of 121 days of age), followed by two liver lobe torsions (mean of 175 days), and single cases of splenic torsion (160 days), and gastric torsion (125 days).

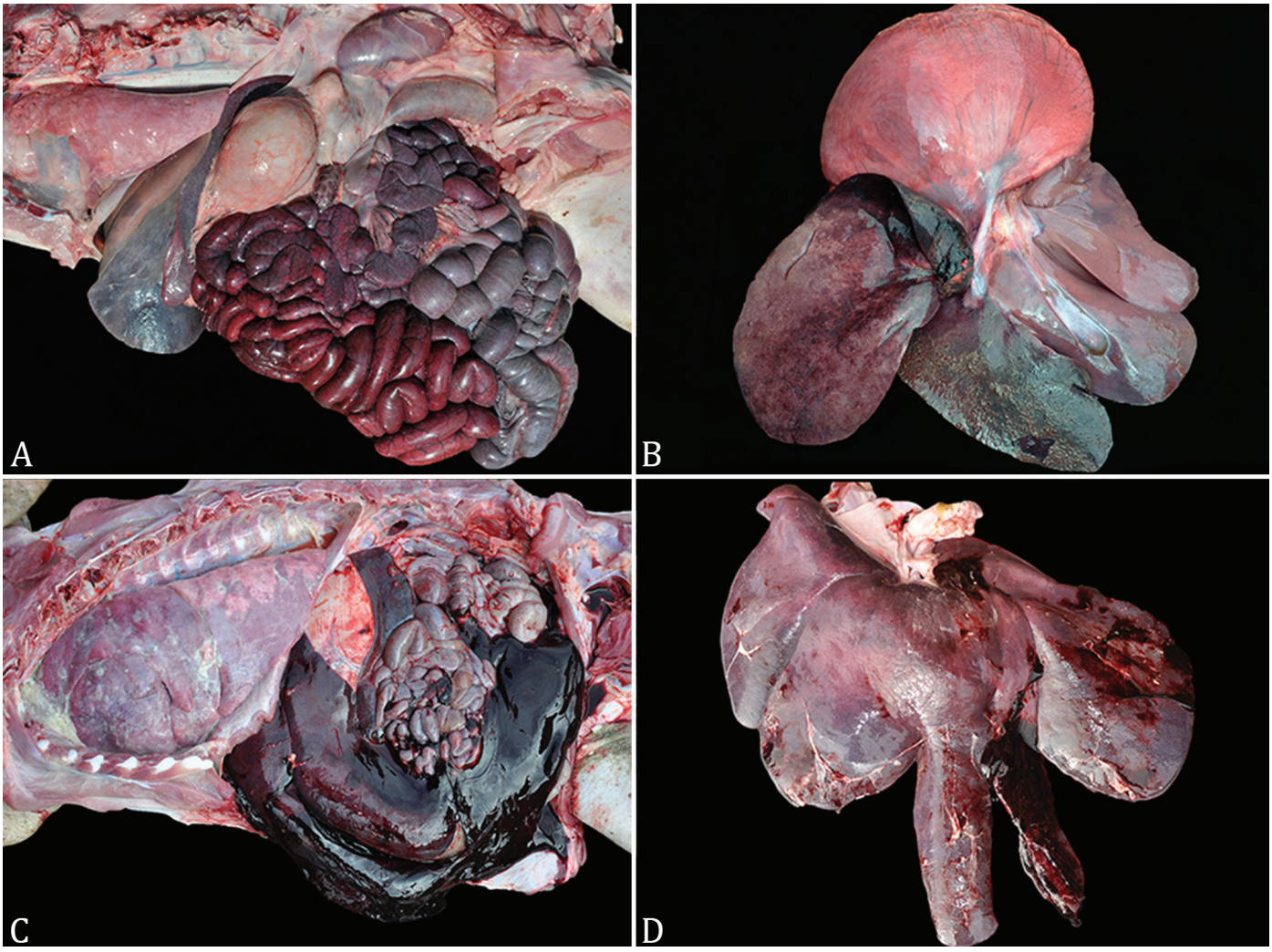


Fig.7. Torsions and ruptures of abdominal organs in growing-finishing pigs. (**A**) Mesentery torsion. Dark red intestinal wall (congestion), with 180° bowel displacement counterclockwise. (**B**)Liver lobe torsion. The left lateral lobe has a 360° twist counterclockwise. The twisted lobe is markedly enlarged and dark red in color. (**C**) Hemoperitoneum due to liver rupture. Abdominal cavity with large amount of blood clots. (**D**) Liver rupture. Left medial lobe with a focal, linear rupture of the capsule and parenchyma, with adhesion of blood clots and fibrin filaments on the capsule.

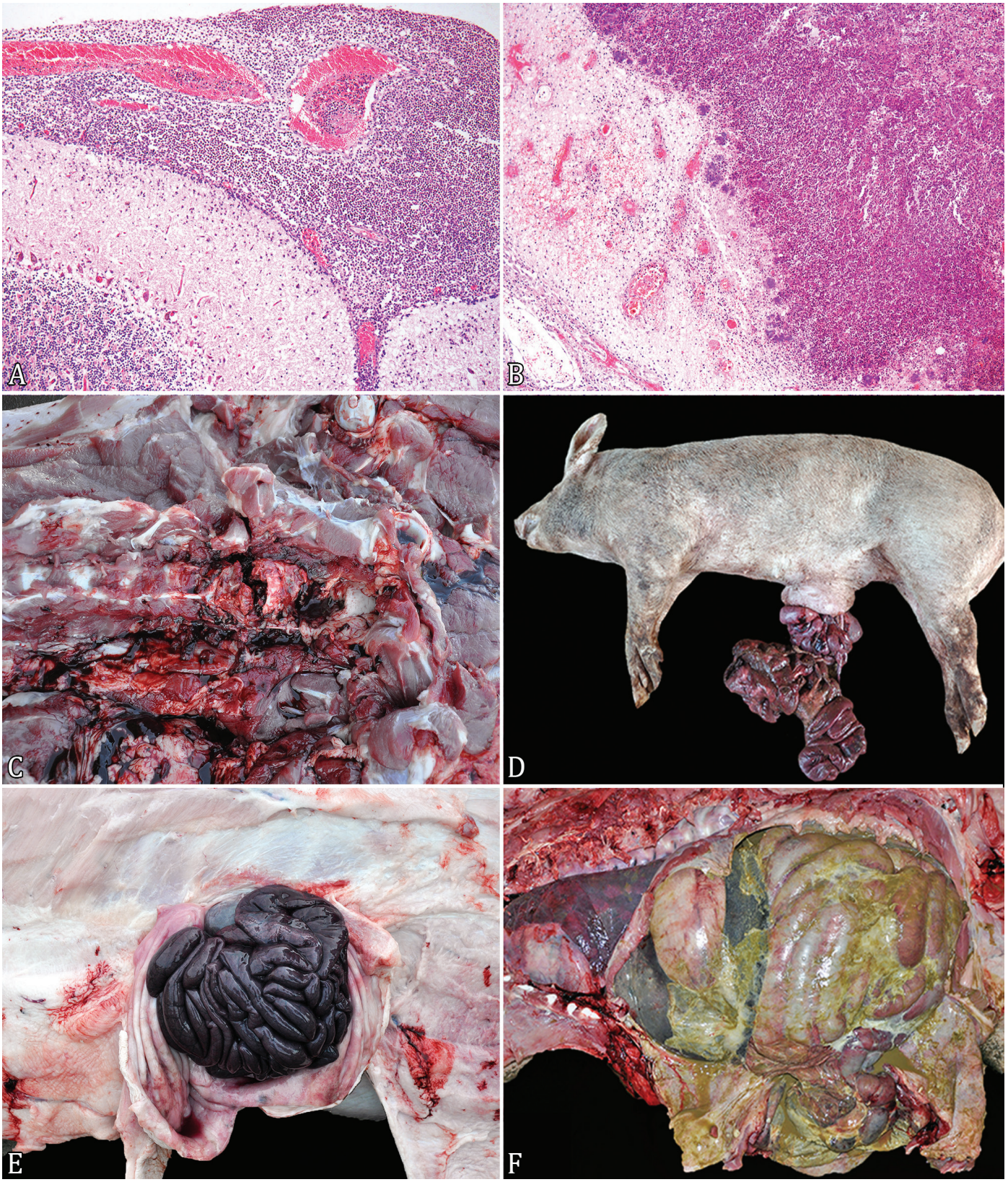


Fig.8. Causes of death in growing-finishing pigs. (**A**) Bacterial meningitis. Intense inflammatory infiltrate predominantly of neutrophils in leptomeninges. HE, obj.20x. (**B**) Ascending bacterial myelitis due to tail biting injury. Sacral spinal cord segment, with an area of central necrosis, with deposition of cellular debris, fibrin, neutrophil infiltrate, and bacterial aggregates. HE, obj.20x. (**C**) Vertebral fracture. Lumbar vertebra with total vertebral body fracture, associated with acute hemorrhage. (**D**) Hernia evisceration. Intestines exposed by umbilical hernia evisceration. (**E**) Umbilical hernia incarceration. Incarcerated intestine with dark red-colored wall. (**F**). Peritonitis secondary to incarceration and rupture of umbilical hernia. Fibrin deposition and intestinal contents spilled from the area of rupture.



Fig.9. Enteric diseases in growing-finishing pigs. (**A**) Salmonellosis. Small intestine with thickened, reddish wall and whitish nodular areas. **(B)** Salmonellosis. Mucosa markedly reddened, with multifocal areas of deposition of yellowish material forming small nodules (button ulcers). (**C**) Megacolon secondary to rectal stenosis due to chronic salmonellosis. Severe dilation of the large intestine, more evident in the colon, which is filled with feces. (**D**) Rectal stenosis associated with chronic salmonellosis. Rectum with a focal area of stenosis, with thickening and dilation of the anterior portion of the intestinal wall, in addition to a focally extensive area with fibrin deposition on the mucosa. (**E**) Swine proliferative enteropathy. Marked and diffuse thickening of the mucosa, with blood in the lumen. (**F**) Swine proliferative enteropathy. Ileus with intestinal crypt hyperplasia and evidence of bacteria compatible with *Lawsonia intracellularis* in the apical cytoplasm of epithelial cells. WS, obj.40x.

Cases of meningoencephalomyelitis were divided into four groups according to the morphological pattern of microscopic changes observed; non-suppurative meningoencephalomyelitis (six cases, affecting pigs between 81 and 170 days of age with a median of 120 days); meningitis or bacterial meningoencephalitis (five cases, affecting pigs between 70 and 80 days of age, with a median of 77 days), from which it was possible to isolate the etiological agent (*S. suis)* in only one case. The third pattern was ascending myelitis of bacterial origin, totaling three cases, which were associated with tail biting. And finally, one animal presented eosinophilic meningoencephalitis, compatible with salt poisoning or water deprivation.

Pigs with hemoperitoneum due to hepatic rupture presented focal or multifocal ruptures, mainly in the left and right medial lobes. This condition often showed comorbidities, being pneumonia (10/14), PDNS and bacterial pericarditis (2/14 each) the most frequently observed. In addition to the areas of rupture of the capsule and parenchyma, neither macroscopic nor microscopic lesions were observed in the liver of these pigs.

In the group of bone fractures, six pigs presented fracture of the spine (mean of 152 days of age), three exhibited epiphysiolisis (159 days), and one, a humerus fracture. No histological lesions of osteometabolic disease were observed in these cases. Enterocolitis comprised four cases of acute *Salmonella* sp. infection (4/9); three cases of rectum stenosis due to chronic salmonellosis (3/9); and two cases of proliferative enteropathy by *L. intracellularis* (2/9). The culture of *Salmonella* sp. was possible in only one case. Intestinal samples of both pigs with proliferative enteropathy were positive in Warthin-Starry technique.

The group "others" was composed by less frequent diagnoses (19/601). They were fibrocartilaginous thromboembolism, diaphragmatic hernia, eosinophilic granulomatous myocarditis, rectum prolapse, vaccine reaction, mediastinal lymphoma, penile urethra necrosis, bacterial peritonitis and congenital heart defects.

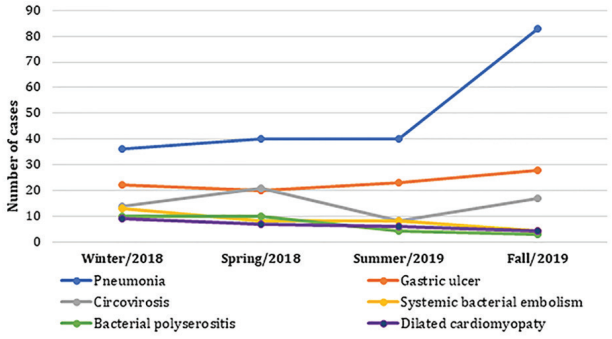


Fig.10. Distribution of the main causes of death of growing-finishing pigs diagnosed throughout the four seasons.

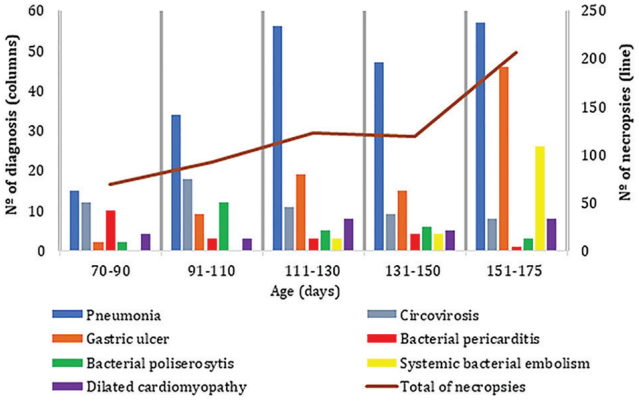


Fig.11. Distribution of the main causes of death of growing-finishing pigs diagnosed according to age and number of necropsies performed at each age.

DISCUSSION

The technified production of pigs is characterized by the confinement of large groups of animals, and this intensification generates an environment favorable to the transmission of infectious agents that clinically affect pigs (Maes et al. 2001). In addition, changes of the epidemiological profile of pig diseases can be observed today (Thrusfield 2013). Several studies show that infectious diseases are the most frequent in the growth and finishing phases, and these can represent up to 75% of the diagnoses (Wilson 1970, Lippke et al. 2007, Sobestiansky et al. 2012, Brum et al. 2013, Konradt et al. 2020). Similarly, in the present study, infectious diseases were the most frequent. However, the large number of non-infectious diseases stood out (35.6% of conclusive diagnoses). Performing necropsies *in loco* provided an adequate sampling, essential to reach the final diagnosis, and allowed the observation of the gross appearance of non-infectious conditions that are frequently and easily diagnosed, such as gastric ulcer and torsions. These are usually sporadic diseases that do not justify the submission of samples for laboratorial diagnosis, making them underrepresented in the laboratories routine, and consequently in retrospective studies.

The most frequent diseases of this study did not present apparent seasonality, however a significant increase in the number of cases of pneumonia was observed in the fall. This increase was justified by a failure to include antibiotics in the ration of Farm A in the month of visit. Regarding the mortality rate, it was evident that the number of deaths increased throughout the life of pigs, peaking in the final weeks, near the time of slaughter, as also highlighted in a previous work (Straw et al. 1983).

Pneumonias represented the group of greatest importance in this work. The term "swine respiratory disease complex" is used to refer to the interaction of many infectious agents with risk factors for the development of pneumonia in pigs (Fonseca et al. 2015). In the present study, this interaction was evident and was considered for the establishment of diagnoses. In Brazil, swine influenza became important after the entry of the pandemic influenza A H1N1 virus in 2009 (Schaefer et al. 2011). After 2013, the virus became endemic in the swine population, causing mild and less lethal lesions (Konradt et al. 2020). Although there are few studies about influenza virus infection dynamics, Nirmala et al. (2019) demonstrated that influenza A viruses isolated from the same batch at different times were genetically different, with multiple genotypes co-circulating throughout winter and summer. This suggests that Influenza virus has the potential to reinfect animals, causing year-round disease in pigs of different ages. Further studies

infer that there is cross-immunity only for homologous strains of the virus, which allows the pig to become constantly infected by heterologous strains (Everett et al. 2019). In the present study, 80.4% of the pigs that died of pneumonia presented histological lesions suggestive of influenza, demonstrating the great importance of this pathogen in finishing herds, in which mortality is influenced by its presence. The results of the present study also demonstrated that in 75% of the lungs tested (60/80) there was involvement of *Mycoplasma hyopneumoniae* in the lesions. Thus, the importance of influenza A virus and *M. hyopneumoniae* as the main primary respiratory agents of pigs in the growth and finishing phases is emphasized, in agreement with other studies (Morés et al. 2015, Rech et al. 2018).

In association with Influenza and *M. hyopneumoniae*, *Pasteurella multocida* was also an important respiratory pathogen for pigs in this study, which is in accordance with other studies on pneumonia in pigs (Choi et al. 2003, Oliveira Filho et al. 2015, Morés et al. 2015). Previously, *P. multocida* was highlighted as a secondary and opportunistic bacterium of the respiratory tract of pigs, mainly related to co-infection with *M. hyopneumoniae* (Pijoan & Fuentes 1987, Hansen et al. 2010). Currently, *P. multocida* has been also associated with primary lesions of pneumonia and septicemia in pigs (Cappuccio et al. 2004, Kich et al. 2007, Pors et al. 2011, Oliveira Filho et al. 2015, Paladino et al. 2017), and it is known to produce suppurative, necrosuppurative and necrohemorrhagic bronchopneumonias, as well as pleurites, especially when there is the involvement of strains of high pathogenicity (Paladino et al. 2017, Oliveira Filho et al. 2018). In this study, bacterial isolation was possible in 61.30% of bacterial pneumonia cases. The absence of growth or presence of contaminant microorganisms in the remaining samples may have occurred due to some factors, such as frequent use of antibiotics in water or feed, parenteral treatment of sick animals, chronicity of lesions, varying degree of autolysis, as well as the logistics of shipping samples to the laboratory.

Gastric ulcer was the second most frequent diagnosis in this study, and this condition presents great importance in mortality and decreased performance of pigs. The prevalence of death from gastric ulcer ranges from 2 to 27% in confined herds (Deen 1993, Melnichouk 2002), and its observation in slaughterhouses can reach frequencies close to 20% (Gottardo et al. 2017). It is a condition with multifactorial etiology, associated with factors such as management practices, environment, feed granulometry, fasting, genetics and stress, which may be associated (Almeida et al. 2006, Thomson & Friendship 2012). In the present study, deaths from gastric ulcers were more frequent at the end of the finishing phase, possibly related to the exacerbation of predisposing factors, such as overcrowding. Usually, these triggering factors are related, and it is difficult to determine the main cause of ulceration, especially in large herds (Gottardo et al. 2017).

Among viral diseases, circovirosis is one of the most important diseases diagnosed in pigs in Brazil (Brum et al. 2013, Konradt et al. 2020), which corroborates with our results, in which it was the third leading cause of death. Detected in the country since 1999, the porcine circovirus type 2 (PCV2) has become endemic, and it is controlled with the use of vaccines, which provide a significant reduction in the losses caused by the disease (Guedes 2012). In the present study, circovirosis was of great importance, especially in the clinical form of PDNS. This was also observed in another study, in which many cases of PDNS were noted, but without the observation of PMWS (Thomson et al. 2002). As demonstrated by Wellenberg et al. (2004), extremely high titers of antibodies against PCV-2 could contribute to the development of the syndrome. In another study, the authors argue that viral load is not a decisive factor in the development of PDNS (Olvera et al. 2004). In this work, it was not possible to determine the origin of the trigger of this condition, however, the advanced age of involvement of these pigs may be a predisposing factor, since finishing pigs are antigenically exposed for a longer period of time, which may favor the more frequent appearance of PDNS.

Although most herds are vaccinated and PCV2 is endemic, the disease has reemerged in recent years, mainly due to vaccine management failures (Konradt et al. 2018), in addition to the interference of other concomitant diseases

Table 2. Main causes of death of growing-finishing pigs from two farms in Southern Brazil

Diagnosis	Total (%)	Sex (male/female)	Age range (days)	Mean age (median)
Pneumonia	199 (33)	112/87	75-190	135 (140)
Gastric ulcer	93 (15.4)	62/31	75-190	149 (152)
Circovirosis	60 (9.9)	24/36	80-190	117 (108)
Systemic bacterial embolism	33 (5.4)	24/9	120-190	171 (180)
Bacterial polyserositis	27 (4.4)	17/11	91-150	116 (100)
Dilated cardiomyopathy	26 (4.3)	12/14	80-190	124 (117)
Torsion of organs of the abdominal cavity	26 (4.3)	11/15	78-175	102 (125)
Bacterial pericarditis	21 (3.4)	9/12	75-150	98 (93)
Meningoencephalomyelitis	15 (2.4)	9/6	70-170	112 (100)
Hemoperitoneum due to liver rupture	14 (2.2)	8/6	75-150	128 (135)
Bone fracture	10 (1.5)	5/5	90-190	159 (170)
Intestinal incarceration/rupture associated with hernia	10 (1.5)	7/3	75-181	157 (145)
Bacterial enterocolitis	9 (1.4)	4/5	80-190	142 (155)
Cardiorespiratory insufficiency associated with fighting	8 (1.2)	3/5	90-190	156 (160)
Others	19 (3.1)	7/12	75-190	148 (155)
Inconclusive	31 (5.0)	13/18	76-190	128(130)
TOTAL	601			

in the vaccination phase; use of vaccines outside the ideal age; and possible insufficiency of vaccine immunity in the protection against viral variants (Segalés 2015). Because it is an immunosuppressive disease, especially when it comes to PMWS, circovirosis makes pigs more vulnerable to secondary bacterial infections (Morés et al. 2015). Valvular endocarditis, pneumonia and bacterial meningoencephalitis were the most frequent concomitant lesions in pigs with circovirosis in the present study.

Systemic bacterial embolisms are caused by opportunistic bacterial infections, which spread hematogenously from a primary lesion and can cause suppurative and abscedative lesions in several organs. The main association of primary lesion in this study was tail biting lesions, as previously observed in pigs with embolic lesions during the slaughter process (Braga et al. 2006, Marques et al. 2012). Males were more frequently affected by this condition, which may indicate a predisposition to caudophagy (Walker & Bilkei 2006, Kritas & Morrison 2007). Other factors that may predispose to this condition are related to stress, such as overcrowding, competition for food, nutritional factors, humidity, temperature, gas level (CO2 or NH3), age and weight gain (Schroder-Petersen & Simonsen 2001, Moinard et al. 2003, Sobestiansky & Zanella 2007). In the farms evaluated, the females selected for reproductive replacement left the farm earlier than the males. Males also tended to be housed in overstocked stalls when compared with females, and these two factors may have contributed to the higher proportion of males with tail biting lesions and systemic bacterial embolism in this study. Cannibalism is also a concern in pig production due to the condemnation of carcasses affected by secondary embolic lesions (Braga et al. 2006, Kritas & Morrison et al. 2007, Marques et al. 2012). In addition to its importance as a cause of carcass condemnation in the slaughterhouse, the present study highlights the group of embolic diseases as an important cause of death of growing and finishing pigs, especially in older animals.

Bacterial polyserositis was the fifth most prevalent diagnosis in this study and was mainly associated with *P. multocida* infection. As already addressed, highly pathogenic strains of *P. multocida* have potential to produce primary lesions of necrohemorrhagic pneumonia, pleuritis, and fibrinous pericarditis (Cappuccio et al. 2004, Oliveira Filho et al. 2015). In the present study, polyserositis lesions with isolation of *P. multocida* were observed in 17 cases (62.9% of polyserositis), sometimes also associated with pneumonia (Paladino et al. 2017, Oliveira Filho et al, 2018). Thus, it is suggested that strains of high pathogenicity of *P. multocida* may be circulating in Brazilian herds, causing mortality and possibly chronic injuries, which can lead to condemnations in slaughterhouses. Therefore, studies on pathogenicity and virulence factors of super pathogenic strains of *P. multocida* become important.

In this research, cardiopathies represented one of the main diagnoses of causes of death of non-infectious origin in pigs, and the most frequent presentation was dilated cardiomyopathy. This condition is a myocardial disorder characterized by reduced contractility and ventricular dilation (Miller & Gal 2017) that may progress to chronic congestive heart failure and death. In pigs there are reports of this disease associated with gossypol and fumonisin poisoning, besides cardiomyopathies of undefined cause (Loynachan 2012, Sobestiansky 2012, Collins et al. 2015), as found in this study. Recently, this disease has been described in Brazil and experimentally reproduced in nursery pigs; in the referred study a nutritional etiology was suggested (Cruz et al. 2019). In the present study, although macroscopic and histopathological lesions were similar to those observed by Cruz et al. (2019), the disease affected pigs sporadically during the growth and finishing phases, in two farms with distinct genetics and nutritional practices, and not as outbreaks, what does not allow us to associate these cases with any epidemiological factor.

Torsions of abdominal organs are common in pigs, especially in the finishing phase (Thomson 2007). The prevalence of mesentery torsion in finishing pig herds can range from 8 to 12% (Melnichouk 2002) and can reach up to 19.3% (Desrosiers 2008). The etiology is not fully elucidated, but it is believed that feed and eating habits are risk factors (Buddle & Twomey 2002, Hollis 2006, Martineau 2008). When compared to younger pigs, finishing pigs eat fewer times a day and consume a larger amount of feed per meal, which may predispose to excessive intestinal gas production, consequently predisposing to twisting at a fixed point (Buddle & Twomey 2002, Thomson 2007, Martineau 2008).

Liver lobe torsion is considered uncommon in production animals (Hamir 1980, Schwartz et al. 2006). However, Schwertz et al. (2018) found that this change was an important cause of death of sows. Generally, twists tend to affect the left lateral lobe, because it is the largest hepatic lobe in the swine species. This lobe is connected to the left medial lobe by a narrow band of tissue, making it predisposed to twists (Hamir 1980, Schwartz et al. 2006). In addition to the displacement of abdominal organs, hepatic rupture with secondary hemoperitoneum was observed in 14 pigs in this study. However, it was not possible to determine the primary cause of the rupture, since no microscopic lesions were found in the ruptured livers, as well as no evidence of blunt trauma.

Bacterial pericarditis represented a small proportion of diagnoses in this study. They usually result from the hematogenous spread of bacterial agents from inflammatory processes in adjacent tissues, such as the lung (Robinson& Robinson 2016). In the present study, pleuritis and pleuropneumonias associated with cases of fibrinous pericarditis were found, and bacterial culture of *P. multocida* occurred in 10 samples. Pulmonary and pleural lesions associated with isolation of *P. multocida* from the pericardial sac were observed by Coelho et al. (2014) in a study on bacterial pericarditis in a slaughterhouse. As the lesions are strongly related to pneumonia and pleuritis, pulmonary agents of greater importance, such as *P. multocida* and *S. suis,* also affect the pericardium more frequently. In general, clinical detection of cases of pericarditis in pigs is difficult in the final stages of the finishing phase, because they are chronic or subclinical conditions, and chronic lesions are usually identified in the slaughterhouse (Sorensen et al. 2006). The chronicity of these lesions also contributes to the difficulty of bacterial isolation from these samples. Another important factor that indicated the chronic course of the disease in the cases diagnosed here was the development of congestive heart failure, in addition to thickening of the pericardium due to intense proliferation of fibrous connective tissue (fibrosis).

Fights are an important stressful factor for pigs. On these moments, when the stress response is exacerbated, there is

activation of the autonomic sympathetic system and simultaneous release of cortisol and catecholamines, especially adrenaline and norepinephrine by the adrenal glands (Maria et al. 2013). This hormonal discharge rapidly increases the heart and respiratory rates, as well as promotes systemic vasoconstriction, redirecting blood flow to the brain, heart and skeletal muscle (Pereira & Ribeiro 2012). This defense mechanism is known as "fight or flight", described by Cannon (1927). Deaths of dogs after manipulation in veterinary establishments and, consequently, reaction to stressful situations, are described by Maria et al. (2013), where 93.8% of the dogs evaluated exhibited edema and marked pulmonary congestion, similar to those described in the pigs of this study. Another fact that contributes to the clinical evolution is the inefficiency of the swine species of losing heat. In these cases of thermal stress, peripheral vasodilation and increased cardiac output occur. When these compensatory mechanisms are overloaded, cardiorespiratory failure and death occur (Krum & Osborne 1977, Johnson 1982). Pigs also present, in general, low heart weight and cardiac volume when compared to other domestic species, and this promotes greater sensitivity of the myocardium to hypoxia. In stressful situations, such as fights, farrowing, loading, and transportation (D'Allaire et al. 1991, Drolet et al. 1992) circulatory system overload and acute heart failure may occur (Thielscher 1987). These mechanisms triggered by stress justify the pulmonary edema and congestion observed in pigs with history of fight in this work.

Enteric diseases represented a small portion of the causes of death of pigs in the farms evaluated. According to Thomson & Friendship (2012), in the growing-finishing phase the main enteric diseases are swine dysentery, swine proliferative enteropathy, salmonellosis, and spirochaetal colitis. However, in the present study, enteritis was sporadic and found in both herds, represented by salmonellosis and the hemorrhagic form of swine proliferative enteropathy. In cases of salmonellosis, bacterial growth was possible in only one case, isolated in bile and spleen. This may be associated with the intermittent excretion of the bacteria through the intestine (Van Winsen et al. 2001), as well as the use of antibiotics in the treatment of affected animals.

CONCLUSIONS

Infectious diseases were the most common cause of death in growing-finishing pigs, especially pneumonia and circovirosis. Non-infectious diseases accounted for 35.6% of diagnoses, with emphasis on gastric ulcer, dilated cardiomyopathy and torsion of abdominal organs. There was an increase in pig mortality in older pigs compared with recently housed pigs.

The most frequent disease was pneumonia, with emphasis on the association among influenza A, *Mycoplasma hyopneumoniae,* and *Pasteurella multocida* as the main etiological agents involved. It was also observed that in addition to being frequently associated with other agents as a cause of pneumonia, *P. multocida* can also cause atypical lesions such as polyserositis.

Circovirosis, even in vaccinated herds, presented a high frequency, especially in the clinical form of porcine dermatitis and nephropathy syndrome. Tail biting was considered an important cause of bacterial thromboembolism in the two farms evaluated, mainly in males. Enteric diseases were not very common causes of death of pigs in this study.

A wide variety of conditions were diagnosed as the cause of death of growing and finishing pigs, most of which were easily diagnosed through necropsy, histopathological examination, and complementary bacteriological and molecular examinations.

Acknowledgments.-The work was developed and financed together with the support of the "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq) and the company Seara Alimentos.

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

REFERENCES

Almeida M.N., Vearick G., Lippke R.T., Lagemann F. L., Correa A.M.R. & Barcellos D.E.S.N. 2006. Úlceras gástricas em suínos. Hora Vet. 26(153):62-66.

Braga D.P., Del'Arco A.E. & Dias R.C. 2006. Condenação de carcaças suínas por caudofagia em frigorífico sob Inspeção Federal no município de Concórdia, Santa Catarina. Acta Sci. Vet. 34(3):319-323. <https://dx.doi.org/10.22456/1679-9216.15553>

Brum J.S., Konradt G., Bazzi T., Fighera R.A., Kommers G.D, Irigoyen L.F. & Barros C.S.L. 2013. Características e frequência das doenças de suínos na Região Central do Rio Grande do Sul. Pesq. Vet. Bras. 33(10):1208-1214. <https://dx.doi.org/10.1590/S0100-736X2013001000006>

Buddle J.R. & Twomey D.E. 2002. The "porcine intestinal distension syndrome". Pig J. 50:68-82.

Cannon WB. 1927. Bodily Changes in Pain, Hunger, Fear, and Rage. D. Appleton and Company, Boston. 328p.

Cappuccio J., Leotta G.A., Vigo G., Moredo F., Wolcott M.J. & Perfumo C.J. 2004. Phenotypic characterization of *Pasteurella multocida* strains isolated from pigs with broncho and pleuropneumonia. Proc. 18th Congress of the International Pig Veterinary Society, Hamburgo, p.205.

Carter G.R. & Rundell D.W. 1975. Identification of type A strains of *Pasteurella multocida* using staphylococcal hyaluronidase. Vet. Rec. 87(15):343. <https://dx.doi.org/10.1136/vr.96.15.343> <PMid:1125106>

Carter G.R. & Subronto P. 1973. Identification of type D strains of *Pasteurella multocida* with acriflavine. Am. J. Vet. Res. 34(2):293-294. <PMid:4685553>

Caswell J.L. & Willians K.J. 2016. Respiratory system, 465-591. In: Maxie M.G. (Ed.), Jubb, Kennedy and Palmer's Pathology of Domestic Animals. Vol.2. 6th ed. Elsevier, St. Louis.

Choi Y.K., Goyal S.M. & Joo H.S. 2003. Retrospective analysis of etiologic agents associated with respiratory diseases in pigs. Can. J. Vet. Res. 44(9):735-737. <PMid:14524628>

Cianciolo R.E. & Mohr F.C. 2016. Urinary system, p.376-464. In: Maxie M.G. (Ed.), Jubb, Kennedy and Palmer's Pathology of Domestic Animals. Vol.2. 6th ed. Elsevier, St. Louis.

Coelho C.F., Zlotowski P., Andrade C.P., Borowski S.M., Gaggini T.S., Almeida L.L., Driemeier D. & Barcellos D.E.S.N. 2014. Pericardite em suínos ao abate no Rio Grande do Sul: avaliação de agentes bacterianos e lesões associadas. Pesq. Vet. Bras. 34(7):643-648. <https://dx.doi.org/10.1590/S0100-736X2014000700006>

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

Cruz R.A.S., Bassuino D.M., Reis M.O., Laisse C.J.M., Pavarini S.P., Sonne L., Kessler A.M. & Driemeier D. 2019. Outbreaks of nutritional cardiomyopathy in pigs in Brazil. Pesq. Vet. Bras. 39(8):573-579. <https://dx.doi.org/10.1590/1678-5150-pvb-6248>

D'Allaire S., Drolet R. & Chagnon M. 1991. The causes of sow mortality: a retrospective study. Can. Vet. J. 32(4):241-243. <PMid:17423771>

Deen J. 1993. The problem of gastroesophageal ulcers: a field description. Proc. Leman Swine Conf. 137-138.

Desrosiers R. 2008. More on intestinal torsions/HBS/PIDS - part 2. Int. Pigletter 28(9b):1-2.

Drolet R., D'Allaire S. & Chagnon M. 1992. Some observations on cardiac failure in sows. Can. Vet. J. 33(5):325-329. <PMid:17424001>

Dutta B. & Rahman T. 2006. Epidemiological studies on the preweaning mortality of piglets in the organized farms of Assam. Indian Vet. J. 83(4):376-378.

Everett H.E., Aramouni M., Coward V., Ramsay A., Kelly M., Morgan S., Tchilian E., Canini L., Woolhouse M.E.J., Gilbert S., Charleston B., Brown I.H. & Brookes S.M. 2019. Vaccine-mediated protection of pigs against infection with pandemic H1N1 2009 swine influenza A virus requires a close antigenic match between the vaccine antigen and challenge virus. Vaccine 37(17):2288-2293. <https://dx.doi.org/10.1016/j.vaccine.2019.02.078> <PMid:30914224>

Fonseca J.A.A., Nonaka C.K.V., Guedes E.O., Lobato Z.I.P., Dias A.S., Nascimento J.A.F.B., Klein C.S., Reis J.K.P. & Heinemann M.B. 2015. Detecção de agentes associados com doenças respiratórias de suínos por PCR em tempo real. Revta Bras. Saúde Prod. Anim. 16(2):300-307. <https://dx.doi.org/10.1590/S1519-99402015000200005>

Gottardo F., Scollo A., Contiero B., Bottacini M., Mazzoni C. & Edwards S.A. 2017. Prevalence and risk factors for gastric ulceration in pigs slaughtered at 170kg. Animal 11(11):2010-2018. <https://dx.doi.org/10.1017/S1751731117000799> <PMid:28436350>

Guedes R.M.C. 2012. Como monitorar infecções subclínicas causando perdas de desempenho na recria e terminação. Soc. Med. Vet. Uruguay 48(supl.1):49-51.

Hamir A.N. 1980. Torsion of the liver in sow. Vet. Rec. 106(16):362-363. <https://dx.doi.org/10.1136/vr.106.16.362> <PMid:7376394>

Hansen M.S., Pors S.E., Jensen H.E., Bille-Hansen V., Bisgaard M., Flachs E.M. & Nielsen O.L. 2010. An investigation of the pathology and pathogens associated with porcine respiratory disease complex in Denmark. J. Comp. Pathol. 143(2/3):120-131. <https://dx.doi.org/10.1016/j.jcpa.2010.01.012> <PMid:20181357>

Hollis, W.L. 2006. Will nutrition and management stop hemorrhagic bowel syndrome? A veterinarian´s perspective. Swine Nutrition Conference, Indiana, p.38-41.

Jensen T.B. & Toft N. 2009. Causes of and predisposing risk factors for leg disorders in growing-finishing pigs. CAB Rev., Perspectives Agricult. Vet. Sci. Nutr. Nat. Resources 4(010):1-8. <https://dx.doi.org/10.1079/PAVSNNR20094010>

Johnson K.E. 1982. Pathophysiology of heatstroke. Compend. Contin. Educ. Pract. Vet. 4(2):141-144.

Kich J.D., Morés N., Triques N.J., Nogueira M.G., Locatelli C., Klein C.S. & Felicio R.P. 2007. *P. multocida* tipo A atuaria como agente primário nos processos pneumônicos dos suínos? Comunicado Técnico 469, Embrapa Suínos e Aves, Concórdia. Available at <www.cnpsa.embrapa.br/sgc/sgc_publicacoes/publicacao_x8g42b6i.pdf> Accessed on Dec. 11, 2019.

Konradt G., Bassuino D.M., Siqueira L.C., Bianchi M.V., Sonne L., Driemeier D. & Pavarini S.P. 2020. Infectious diseases dynamics in growing/finishing pigs in southern Brazil (2005-2016). Pesq. Vet. Bras. 40(4):254-260 <https://dx.doi.org/10.1590/1678-5150-pvb-6510>

Konradt G., Cruz R.A., Bassuino D.M., Bianchi M.V., de Andrade C.P., da Silva F.S., Driemeier D. & Pavarini S.P. 2018. Granulomatous necrotizing myositis in swine affected by porcine circovirus disease. Vet. Pathol. 55(2):268-272. <https://dx.doi.org/10.1177/0300985817736114> <PMid:29050543>

Kritas SK. & Morrison R.B. 2007. Relationships between tail biting in pigs and disease lesions and condemnations at slaughter. Vet. Rec. 160(5):149-152. <https://dx.doi.org/10.1136/vr.160.5.149> <PMid:17277296>

Krum S.H. & Osborne C.A. 1977. Heatstroke in the dog: a polysystemic disorder. J. Am. Vet. Med. Assoc. 170(5):531-535. <PMid:845081>

Lippke R.T., Heck A., Michelon A., Afonso J.A.B. & Barcellos D.E.S.N. 2007. Principais causas associadas a mortalidade de suínos nas fases de recria e terminação. 13º Congresso Brasileiro de Veterinários Especialistas em Suínos (ABRAVES), Florianópolis.

López A. & Martinson S.A. 2017. Sistema respiratório, mediastino e pleura, p.471-560. In: Zachary J.F. (Ed.), Bases da Patologia Veterinária. 6ª ed. Elsevier, St. Louis.

Losinger W.C., Bush E.J., Smith M.A. & Corso B.A. 1998. An analysis of mortality in the grower/finisher phase of swine production in the Unites States. Prev. Vet. Med. 33(1/4):121-145. <https://dx.doi.org/10.1016/s0167-5877(97)00052-4> <PMid:9500169>

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

Maes D.G., Duchateau L., Larriestra A., Deen J., Morrison R.B. & de Kruif A. 2004. Risk factors for mortality in grow-finishing pigs in Belgium. J. Vet. Med. B 51(7):321-326. <https://dx.doi.org/10.1111/j.1439-0450.2004.00780.x> <PMid:15525358>

Maes D., Larriestra A., Deen J. & Morrison R.B. 2001. A retrospective study of mortality in grow-finish pigs in a multi-site production system. J. Swine Health Prod. 9(6):267-273.

Maria A.C.B.E., Rego A.A.M.S. & Maiorka P.C. 2013. Necropsy findings in dogs that died during grooming or other pet service procedures. J. Foren. Sci. 58(5):1189-1192. <https://dx.doi.org/10.1111/1556-4029.12236> <PMid:23879553>

Marques B.M.F.P.P., Bernardi M.L., Coelho C.F., Almeida M., Morales O.E., Mores T.J., Borowski S.M. & Barcellos D.E.S.N. 2012. Influence of tail biting on weight gain, lesions and condemnations at slaughter of finishing pigs. Pesq. Vet. Bras. 32(10):967-974. <https://dx.doi.org/10.1590/S0100-736X2012001000003>

Martineau G. 2008. The BB dead pig: part 1. Int. Pigletter 27(12c):1-2.

Melnichouk S.I. 2002. Mortality associated with gastric ulceration in swine. Can. Vet. J. 43(3):223-225. <PMid:11901598>

Miller L.M. & Gal A. 2017. Cardiovascular system and lymphatic vessels, p.561-616. In: Zachary J.F. (Ed.), Pathologic Basis of Veterinary Disease. 6th ed. Mosby Elsevier, St Louis.

Moinard C., Mendla M., Nicola C.J. & Greenb L.E. 2003. A case control study of on-farm risk factors for tail biting in pigs. Appl. Anim. Behav. Sci. 81(4):333-355. <https://dx.doi.org/10.1016/S0168-1591(02)00276-9>

Mondal S.K., De U.K., Das G.K., Powde A.M. & Verma A.K. 2012. Pattern of mortality of crossbred pigs in an organized swine production farm. J. Livest. Sci. 3:37-44.

Morés M.A.Z., Oliveira Filho J.X., Rebelatto R., Klein C.S., Barcellos D.E.N., Coldebella A. & Morés N. 2015. Aspectos patológicos e microbiológicos das doenças respiratórias em suínos de terminação no Brasil. Pesq. Vet. Bras. 35(8):725-733. <https://dx.doi.org/10.1590/S0100-736X2015000800004>

Nirmala J., Bender J.B., Lynfield R., Yang M., Rene C. M., Nelson M. I., Sreevatsan S. & Torremorell M. 2019. Genetic diversity of influenza A viruses circulating in pigs between winter and summer in a Minnesota live animal market. Zoonoses Publ. Health. 67(3):243-250. <https://dx.doi.org/10.1111/zph.12679> <PMid:31868300>

Oliveira Filho J.X., Morés M.A.Z., Rebelatto R., Agnol A.M.D., Plieski C.L.A., Klein C.S., Barcellos D.E.N. & Morés N. 2015. *Pasteurella multocida* type A as the primary agent of pneumonia and septicaemia in pigs. Pesq. Vet. Bras. 35(8):716-724. <https://dx.doi.org/10.1590/S0100-736X2015000800003>

Oliveira Filho J.X., Morés M.A.Z., Rebellato R., Kich J.D., Cantão M.E., Klein C.S., Guedes R.M.C., Coldebella A., Barcellos D.E.S.N. & Morés N. 2018. Pathogenic variability among *Pasteurella multocida* type A isolates from

Brazilian pig farms. BMC Vet. Res. 14(1):244. <https://dx.doi.org/10.1186/s12917-018-1565-2> <PMid:30134904>

Olvera A., Sibila M., Calsamiglia M., Segalés J. & Domingo M. 2004. Comparison of porcine circovirus type 2 load in serum quantified by a real time PCR in post-weaning multisystemic wasting syndrome and porcine dermatitis and nephropathy syndrome naturally affected pigs. J. Virol. Methods 117(1):75-80. <https://dx.doi.org/10.1016/j.jviromet.2003.12.007> <PMid:15019262>

Paladino E.S., Gabardo M.P., Lunardi P.N., Morés N. & Guedes R.M.C. 2017. Anatomopathological pneumonic aspects associated with highly pathogenic *Pasteurella multocida* in finishing pigs. Pesq. Vet. Bras. 37(10):1091-1100. <https://dx.doi.org/10.1590/s0100-736x2017001000009>

Pereira M.C. & Ribeiro L. 2012. Stresse, catecolaminas e risco cardiovascular. Arq. Med. 26(6):245-253.

Piffer I.A. & Brito J.R.F. 1991. Descrição de um modelo para avaliação e quantificação de lesões pulmonares de suínos e formulação de um índice para classificação de rebanhos. Embrapa/CNPSA, Concórdia, p.16.

Pijoan C. & Fuentes M. 1987. Severe pleuritis associated with certain strains of *Pasteurella multocida* in swine. J. Am. Vet. Med. Assoc. 191(1):823-826. <PMid: 3679971>

Pors S.E., Hansen M.S., Bisgaard M. & Jensen H.E. 2011. Ocurrence and associated lesions of *Pasteurella multocida* in porcine bronchopneumonia. Vet. Microbiol. 150(1/2):160-166. <https://dx.doi.org/10.1016/j.vetmic.2011.01.005> <PMid:21296510>

Quinn P.J., Markey B.K., Leonard F.C., Fitzpatrick E.S., Fanning S. & Hartigen P.J. 2011. *Pasteurella* species, *Mannheimia haemolytica* and *Bibersteinia trehalosi*, p.300-308. In: Quinn P.J., Markey B.K., Leonard F.C., Fitzpatrick E.S., Fanning S. & Hartigen P.J. (Eds), Veterinary Microbiology and Microbial Disease. 2nd ed. Wiley-Blackwell, Ames, Iowa.

Rech R.R., Gava D., Silva M.C., Fernandes L.T., Haach V., Ciacci-Zanella J.R. & Schaefer R. 2018. Porcine respiratory disease complex after the introduction of H1N1/2009 influenza virus in Brazil. Zoonoses Publ. Health 65(1):155-161. <https://dx.doi.org/10.1111/zph.12424> <PMid:29139241>

Robinson W.F. & Robinson N.A. 2016. Cardiovascular system, p.2-99. In: Maxie M.G. (Ed.), Jubb, Kennedy and Palmer's Pathology of Domestic Animals. Vol.3. 6th ed. Elsevier, St. Louis.

Schaefer R., Rech R.R., Silva M.C., Gava D. & Ciacci-zanella J.R. 2013. Orientações para o diagnóstico de influenza em suínos. Pesq. Vet. Bras. 33(1):61-73. <https://dx.doi.org/10.1590/S0100-736X2013000100012>

Schaefer R., Zanella J.R.C., Brentano L., Vincent A.L., Ritterbusch G.A., Silveira S., Caron L. & Mores N. 2011. Isolation and characterization of a pandemic H1N1 influenza virus in pigs in Brazil. Pesq. Vet. Bras. 31(9):761-767. <https://dx.doi.org/10.1590/S0100-736X2011000900007>

Schroder-Petersen D.L. & Simonsen H.B. 2001. Tail biting in pigs. Vet. J. 162(3):196-210. <https://dx.doi.org/10.1053/tvjl.2001.0605> <PMid:11681870>

Schwartz S.G.H., Mitchell S.L., Keating J.H. & Chan D.L. 2006. Liver lobe torsion in dogs: 13 cases (1995-2004). J. Am. Vet. Med. Assoc. 228(2):242-247. <https://dx.doi.org/10.2460/javma.228.2.242> <PMid:16426198>

Schwertz C.I., Driemeier D., Bianchi R. M., Cecco B.S., Henker L.C., Lorenzett1 M.P. & Pavarini S.P. 2018. Causas de mortalidade de matrizes suínas. In: Anais do XI SINSUI-Simpósio Internacional de Suinocultura. Avanços em sanidade, produção e reprodução de suínos. Porto Alegre, Rio Grande do Sul, Brasil. p. 171-183.

Segalés J. 2015. Best practice and future challenges for vaccination against porcine circovirus type 2. Expert Rev. Vaccines 14(3):473-487. <https://dx.doi.org/10.1586/14760584.2015.983084> <PMid:25400216>

Sobestiansky J. & Zanella E. 2007. Formas anormais de comportamento, p.159-169. In: Sobestiansky J. & Barcellos D.E.S.N. (Eds), Doenças dos Suínos. Cânone Editora, Goiânia.

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

Sobestiansky J., Morés N., Weiblen R., Reis R. & Barcellos D.E.N. 2012. Classificação das doenças, p.14-20. In: Sobestianksy, J. & Barcellos D.E.N. 2012. Doenças dos suínos. 2ª ed. Cânone, Goiânia.

Sorensen V., Jorsal S.E. & Mousing J. 2006. Diseases of the respiratory system, p.149-177. In: Straw B.E., Zimmerman J.J., D´Allaire S. & Taylor D.J. (Eds), Diseases of Swine. 9th ed. Blackwell Publishing, Iowa.

Straw B., Neubauer G.D. & Leman A.D. 1983. Factors affecting mortality in finishing pigs. J. Am. Vet. Med. Assoc. 183(4):452-455. <PMid:6618972>

Thielscher H.H. 1987. The pig's heart - a problem of pathophysiology. Pro Vet. 3:12.

Thomson J.R. & Friendship R.M. 2012. Digestive system, p.199-226. In: Zimmerman J.J., Karriker L.A., Ramirez A., Schwartz K.J. & Stevenson G.W. (Eds), Diseases of Swine. 10th ed. Blackwell Publishing, Iowa.

Thomson J.R. 2007. Pressure-related abdominal changes in pigs with 'whey bloat' - a case report. Pig J. 59:152-159. <https://dx.doi.org/10.1186/s40813-017-0074-1> <PMid:29270311>

Thomson J.R., Higgins R.J., Smith W.J. & Done S.H. 2002. Porcine dermatitis and nephropathy syndrome: clinical and pathological features of cases in the United Kingdom (1993-1998). J. Vet. Med. A 49(8):430-437. <https://dx.doi.org/10.1046/j.1439-0442.2002.00475.x> <PMid:12450193>

Thrusfield M. 2013. Veterinary Epidemiology. 3rd ed. J. Wiley, New York. 624p.

Tubbs R., Hurd H., Dargatz D. & Hill G. 1993. Pre-weaning morbidity and mortality in the United States swine herds. J. Swine Health Prod. 1(1):21-27.

Van Winsen R.L., Van Nes A., Keuzenkamp D., Urlings H.A., Limpman L.J., Biesterveld S., Snijders J.M., Verheijden J.H. & Van Knapen F. 2001. Monitoring of transmission of *Salmonella enterica* serovars in pigs using bacteriological and serological detection methods. Vet. Microbiol. 80(3):267-274. <https://dx.doi.org/10.1016/s0378-1135(01)00313-3> <PMid:11337142>

Walker P.K. & Bilkei G. 2006. Tail-biting in outdoor pig production. Vet. J. 171(2):367-369. <https://dx.doi.org/10.1016/j.tvjl.2004.10.011>

Wellenberg G.J., Stockhofe-Zurwieden N., Jong M.F., Boersma W.J. & Elbers A.R. 2004. Excessive porcine circovirus type 2 antibody titers may trigger the development of porcine dermatitis and nephropathy syndrome: a case-control study. Vet. Microbiol. 99(3/4):203-214. <https://dx.doi.org/10.1016/j.vetmic.2004.01.001> <PMid:15066723>

Wilson M.R. 1970. Sudden death in pigs. Can. Vet. J. 11(9):178-180. <PMid:5466737>

Xiangdong L., Mingming Q., Sun M. & Kegong T. 2018. A duplex real time PCR assay for the simultaneous detection of porcine circovirus 2 and circovirus 3. Virol. Sinica 33(2):181-186. <https://dx.doi.org/10.1007/s12250-018-0025-2>

Yamaguti M., Muller E.E., Piffer A.I., Kich J.D., Klein C.S. & Kuchiishi S.S. 2008. Detection of *Mycoplasma hyopneumoniae* by polymerase chain reaction in swine presenting respiratory problems. Braz. J. Microbiol. 39(3):471-476. <https://dx.doi.org/10.1590/S1517-83822008000300011>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(10):776-780, October 2020
DOI: 10.1590/1678-5150-PVB-6677

Original Article
Livestock Diseases
BY-NC

Clostridium perfringens α and β recombinant toxoids in equine immunization[1]

Nayra F.Q.R. Freitas[2], José D. Barbosa[2], Denis Y. Otaka[2], Marcos Roberto A. Ferreira[3], Rafael R. Rodrigues[3], Clóvis Moreira Jr[3], Fabricio R. Conceição[3] and Felipe M. Salvarani[2]*

ABSTRACT.- Freitas N.F.Q.R., Barbosa J.D., Otaka D.Y., Ferreira M.R.A., Rodrigues R.R., Moreira Jr C., Conceição F.R. & Salvarani F.M. 2020. ***Clostridium perfringens* α and β recombinant toxoids in equine immunization**. *Pesquisa Veterinária Brasileira 40(10):776-780*. Instituto de Medicina Veterinária, Universidade Federal do Pará, BR-316 Km 61, Saudade II, Cristo Redentor, Castanhal, PA 68740-910, Brazil. E-mail: felipems@ufpa.br

Clostridium perfringens is considered one of the main causative agents of superacute enterocolitis, usually fatal in the equine species, due to the action of the β toxin, and is responsible for causing severe myonecrosis, by the action of the α toxin. The great importance of this agent in the equine economy is due to high mortality and lack of vaccines, which are the main form of prevention, which guarantee the immunization of this animal species. The aim of this study was to evaluate three different concentrations (100, 200 and 400μg) of *C. perfringens* α and β recombinant toxoids in equine immunization and to compare with a group vaccinated with a commercial toxoid. The commercial vaccine was not able to stimulate an immune response and the recombinant vaccine was able to induce satisfactory humoral immune response in vaccinated horses, proving to be an alternative prophylactic for *C. perfringens* infection.

INDEX TERMS: *Clostridium perfringens*, alpha toxin, beta toxin, recombinant toxoids, equine, immunization, vaccination, myonecrosis, enterocolitis, horses.

RESUMO.- [**Toxóides recombinantes α e β de *Clostridium perfringens* na imunização de equinos.**] *Clostridium perfringens* é considerado um dos principais agentes causadores de enterocolites superagudas, geralmente fatais na espécie equina, devido à ação da toxina β, além de ser responsável por causar quadros graves de mionecrose, pela ação da toxina α. A grande importância desses agentes na equinocultura, deve-se a elevada mortalidade e a inexistência de vacinas, principal forma de prevenção, que garantam a imunização dessa espécie animal. O objetivo deste trabalho foi avaliar três diferentes concentrações (100, 200 e 400μg) dos toxóides recombinantes α e β de *C. perfringens* na imunização de equinos, bem como comparar com um grupo vacinado com um toxóide comercial. A vacina comercial não se mostrou capaz de estimular uma resposta imune e a vacina recombinante foi capaz de induzir resposta imune humoral satisfatória em equinos vacinados, provando ser uma alternativa profilática para infecção por *C. Perfringens*.

TERMOS DE INDEXAÇÃO: *Clostridium perfringens,* toxina alfa, toxina beta,toxóides recombinantes, equinos, imunização, vacinação, mionecroses, entecolites.

INTRODUCTION

Clostridium perfringens is an anaerobic Gram-positive bacterium (Zaragoza et al. 2019), considered to be na important gastrointestinal pathogen in neonate foals of up to 10 days old (East et al. 1998). Types A and C are considered the most epidemiologically important for equine specie (Choi et al. 2003, Diab et al. 2012, Gohari et al. 2014, Olivo et al. 2016).

In equine species, *C. perfringens* type C is associated with enterocolitis and diarrhea, with or without hemorrhage, and acute progression that manifests as colic, abdominal distension, fever, dehydration, tachycardia, tachypnea and circulatory shock, for which the β toxin is responsible (Sayeed et al. 2008, Gohari et al. 2014). The death of animals results mainly from the high absorption of this toxin by the gut (Songer 1996, Songer & Uzal 2005, Uzal & Songer 2008). In a study conducted by Weese et al. (2001), *C. perfringens* type

[1] Received on July 15, 2020.
Accepted for publication on July 30, 2020.
[2] Instituto de Medicina Veterinária, Universidade Federal do Pará (UFPA), BR-316 Km 61, Saudade II, Cristo Redentor, Castanhal, PA 68740-910, Brazil. *Corresponding author: felipems@ufpa.br
[3] Centro de Desenvolvimento Tecnológico, Núcleo de Biotecnologia, Universidade Federal de Pelotas (UFPel), Pelotas, RS 96160-000, Brazil.

A was isolated in 19% of fecal samples from adult horses with diarrhea, demonstrating the association of this agent with enteric problems in adult animals also.

Myonecrosis in horses is mainly caused by *C. perfringens* type A through the action of the alpha toxin (Choi et al. 2003). The disease is characterized by rapid progression, with muscle liquefaction necrosis and gas formation, and may be associated with signs of toxemia. Most cases of myonecrosis are associated with the presence of solution of continuity of the skin, however *C. perfringens* is also able to penetrate healthy skeletal muscle (Peek et al. 2003).

The sporulation capacity of the genus *Clostridium*, and the fact that it may be a commensal microorganism of the intestinal microbiota of healthy animals, favors its presence in the soil for long periods, making eradication difficult (Freedman et al. 2016). The clinical conditions caused by the bacteria are superacute in character, and associated with, the absence of commercial vaccines that guarantee the immunization and prevention of these clostridioses in the equine species. The actions of these agents consequently lead to high mortality (Vengust et al. 2002), demonstrating the extreme importance of *C. perfringens* types A and C for the equine economy. Therefore, the objective of this study was to evaluate the humoral immune response of horses vaccinated with different concentrations of *C. perfringens* α and β recombinant toxoids.

MATERIALS AND METHODS

Ethics statement. The study was conducted in accordance with the "Conselho Nacional de Controle de Experimentação Animal" (CONCEA). It was submitted to the Animal Use Ethics Committee of the "Unviersidade Federal do Pará" (CEUA-UFPA) and approved under license number 7310201016.

Commercial vaccine. We used the commercial VISION 10® vaccine which contained the α and β *Clostridium perfringens* toxoids. The manufacturer did not specify the concentrations of either antigen. This commercial vaccine is not specific to horses, as there are no species-specific vaccines available. However, this does not make their use unfeasible, since the antigens are the same as those contained in the recombinant vaccine. The manufacturer's recommendations for use in bovine species were followed.

Recombinant vaccine. The production of recombinant toxins α and β was performed according to the methodology previously described by Milach et al. (2012) and Salvarani et al. (2013).

Recombinant toxoids at concentrations of 100, 200 and 400μg were dissolved into aluminum hydroxide suspension (2.5-3.5% $Al(OH)_3$), from Omega Produtos Ltda, in a final volume of 2mL per dose, and stirred at room temperature for 24h.

In order to verify sterility, 0.5mL of each of the vaccine formulations were sown in four tubes containing 20mL of thioglycolate broth, and four tubes containing 20mL of Sabouraud broth. Two thioglycolate broth tubes were incubated under anaerobic conditions, and the other Sabouraud and thioglycolate broth tubes were incubated under aerobic conditions. All tubes were incubated at 37°C for 21 days with daily readings (Brasil 1997).

For the safety test (European Pharmacopoeia 1998) four horses were inoculated, two with the commercial vaccine and two with the recombinant vaccine, containing twice the highest concentration to be tested (800μg), by subcutaneous and intramuscular routes. The animals were observed for seven days for the occurrence of local or systemic reactions.

Immunization of animals and titration of neutralizing antibodies. Fifty male and female Mangalarga Marchador animals, aged between one and 12 years, with no history of vaccination against *C. perfringens* were used in this study. The animals were randomly divided into five groups: 100μg Recombinant Vaccine Group (G1), 200μg Recombinant Vaccine Group (G2), 400μg Recombinant Vaccine Group (G3), Commercial Vaccine Group (G4) and Negative Control Group (G5), which received the administration of sterile saline (NaCl 0.9%). All animals received two doses (2mL), intramuscularly, on the neck board, on days 0 and 28 after the first dose.

On day 56 after the first vaccine dose, moment in which the vaccine efficiency is evaluated, through the potency test in the target species according to the literature (Moreira et al. 2016, 2018, Otaka et al. 2017), blood samples were collected by jugular venipuncture. After clot formation, centrifugation was performed at 3000g for 5 minutes to obtain serum, which was stored at -20°C until further use.

Serum titration was performed using the mouse neutralization technique; for β toxin the methodology described by the European Pharmacopoeia (1998), and for alpha toxin the methodology according to the USDA (2002). Serum dilutions were mixed with standard α and β toxins at 37°C for 30 minutes and 0.2mL of each dilution was intravenously inoculated into two Swiss Webster strain mice weighing between 18 and 22g. The animals were observed for 72 hours for death or survival, with results recorded every 24 hours. The neutralization titer of each dilution was calculated by the method of Reed and Muench (1938) and expressed in international units per milliliter (IU/mL).

Statistical analysis. We performed analysis of variance and Tukey tests to identify statistically significant differences in antibody titers between groups, using Statview statistical software version 5.0.0.0, adopting a statistical significance interval set at 95% ($p<0.05$).

RESULTS

No microbiological growth was observed in the sterility tests of the recombinant vaccine. In the innocuity test, the animals that were inoculated subcutaneously with the commercial vaccine showed intense local reaction, characterized by pain on palpation and edema, which disappeared after seven days. The animals did not show local or systemic alterations after inoculation using intramuscular administration routes for both vaccines.

The horses inoculated with Saline Group G5 did not develop antibodies against α and β toxins. The neutralizing antibody titers after 56 days of inoculation with Recombinant Vaccine Groups G1, G2, G3, and Commercial Vaccine Group G4 were obtained by the seroneutralization technique and are shown in Table 1.

When evaluating mean anti-α and anti-β (Fig.1) neutralizing antibody titers, no significant differences ($p<0.05$) were found between groups inoculated with recombinant vaccines at a concentration of 100μg (G1) and commercial (G4). No significant differences were found between the animals vaccinated with 200μg (G2) and 400μg (G3) concentrations of the recombinant vaccine. However, there was a significant difference between groups G2 and G3 compared to G4.

As the concentration of recombinant proteins increased, there was a linear increase in the humoral immune response in horses for both anti-α and anti-β antibodies. Regression analysis was performed, showing significant results ($p<0.001$), and is shown in Figure 2.

Table 1. Anti-α and anti-β neutralizing antibody titers, means and standard deviations (SD) obtained at 56 days after the first vaccination of horses with the recombinant vaccines 100 (G1), 200(G2) and 400µg(G3) and commercial vaccine (G4)

Vaccines	G1		G2		G3		G4	
Samples	Anti-α	Anti-β	Anti-α	Anti-β	Anti-α	Anti-β	Anti-α	Anti-β
1	0	10	5.7	10	5.7	14.4	0	0
2	4	0	6.9	12	4	14.4	0	10
3	0	10	5.7	14.4	4.8	17.3	0	10
4	4	10	4.8	14.4	5.7	14.4	4	10
5	4	0	4	10	6.9	12	0	10
6	0	10	6.9	14.4	6.9	12	4	0
7	0	0	6.9	14.4	6.9	12	4	10
8	4	0	4	12	5.7	10	0	10
9	4	10	4.8	12	6.9	14.4	0	0
10	0	0	5.7	17.3	8	17.3	0	10
Seroconversion rate	50%	50%	100%	100%	100%	100%	30%	70%
Means ± SD	2±2.11	5±5.27	5.54±1.13	13.09±2.30	6.15±1.19	13.82±2.40	1.2±1.93	7±4.83

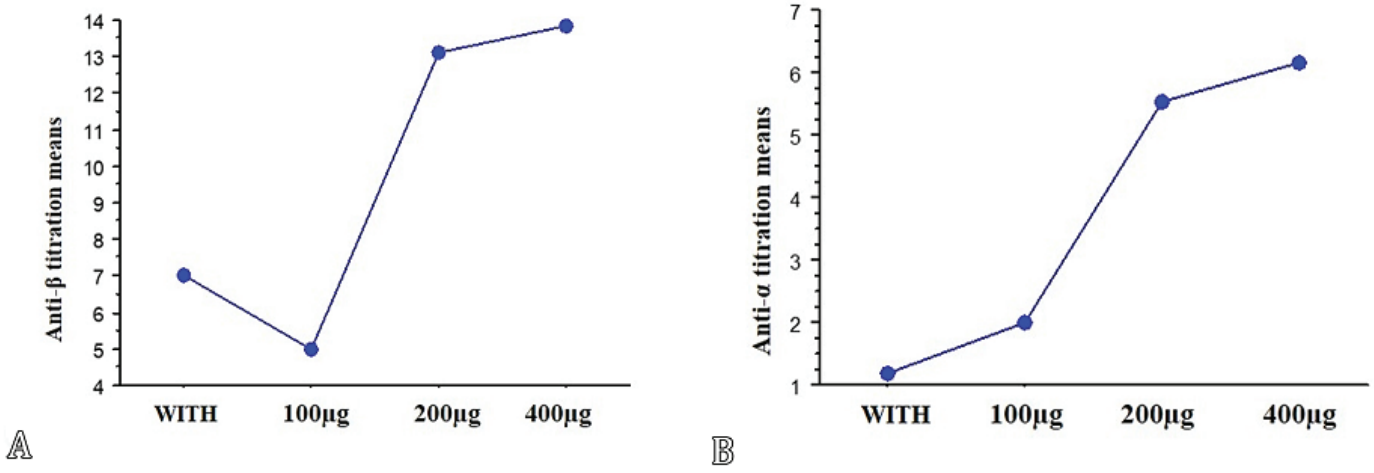


Fig.1. (**A**) Mean titers of anti-α neutralizing antibodies induced by the commercial vaccine (COM) and the different concentrations of the recombinant vaccine. (**B**) Mean titers of neutralizing anti-β antibodies induced by the commercial (COM) and the different concentrations of the recombinant vaccine.

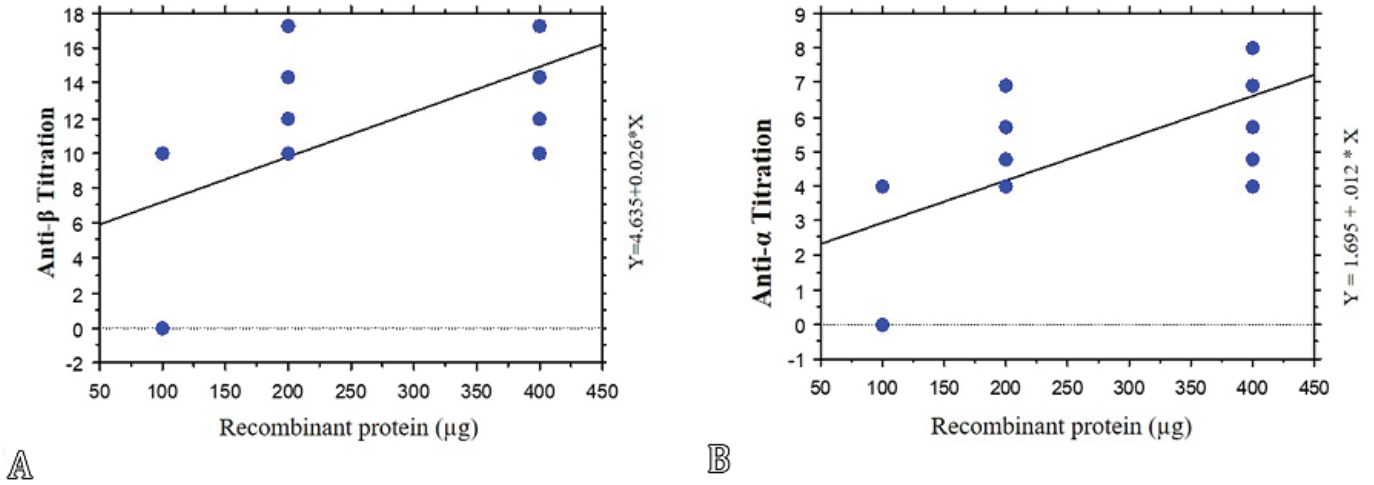


Fig.2. (**A**) Regression equation for anti-α titrations comparing different concentrations of recombinant proteins. (**B**) Regression equation for anti-β titrations comparing different concentrations of recombinant proteins.

DISCUSSION

Enterocolitis is an important cause of death in adult and neonate horses, as it usually presents as superacute changes in toxemia, which result in acute diarrhea, colic, dehydration, tachypnea, cardiovascular collapse and intestinal necrosis (Diab et al. 2012), due to the action of *Clostridium perfringens* β toxin, which is present in 40% of the intestinal content samples of horses with enterocolitis (Gohari et al. 2014). *C. perfringens* type A is also associated with severe myositis in the equine species, being the most prevalent agent in this type of infection (Peek et al. 2003), which is characterised by the presence of acute malignant edema, fever, depression, cutaneous emphysema, muscle liquefaction and toxemia, and other changes related to α toxin action (Horner 1982). The present study establishes a possible alternative to be used as prophylaxis against these superacute toxi-infections caused by the action of *C. perfringens* α and β toxins.

There is no vaccine against *C. perfringens* infections in the equine species currently available. The commercial vaccine available in Brazil, besides not being species-specific, was also not able to stimulate a humoral response with minimum antibody levels required for α and β toxins, in all inoculated animals of this study. Our work is the first to develop and test recombinant vaccines in the immunization of horses against *C. perfringens* toxins α and β.

The use of recombinant vaccines in animal immunization has been studied, and has shown excellent seroconversion results in swine (Salvarani et al. 2013), bovine, ovine, caprine (Moreira et al. 2016) and bubaline species (Otaka et al. 2017). This study tested different concentrations of α and β toxin recombinant vaccines at 100, 200, and 400μg, however neutralizing antibody levels above 4IU/mL are the USDA (2002) guidelines' minimum recommendation for toxin α, and 10IU/mL, for β toxin is required by Brazilian legislation, which is based on the European Pharmacopoeia (1998). These values were obtained only in groups immunised with formulations containing concentrations starting at 200μg, with no significant difference between the titers obtained from groups G2 and G3. None of the inoculated animals developed clinical signs of toxicity or death, demonstrating the safety of the studied recombinants.

There was a linear increase in the immune response between the amount of antigen per vaccine dose and the titers of neutralizing antibodies to α and β toxins. This correlation was similar to that found by Otaka et al. (2017) when testing concentrations of 100, 200 and 400μg of recombinant *Clostridium botulinum* neurotoxins C and D in buffalo, and also by Moreira et al. (2018) in bovine species.

As in our study, the mean titers obtained from swines (Salvarani et al. 2013) from cattles, goats and sheeps (Moreira et al. 2016) immunised with recombinant α and β vaccines, at a concentration of 200μg of each antigen, were capable of stimulating the immune response with levels above internationally required levels (USDA 2002, Otaka et al. 2017). Aside from verifying the results of studies conducted in these species, the structural characteristics of the α and β epitopes were maintained because antibodies generated in horses inoculated with the recombinant vaccine were able to recognise the standard *C. perfringens* toxins used in the seroneutralization tests.

The concentration of 200μg of recombinant toxoids was the lowest antigen concentration capable of inducing seroconversion in 100% of the inoculated horses. This concentration of α and β toxins was the same as used by Moreira et al. (2016) in ruminants and Salvarani et al. (2013) in swines, which was also effective in inducing neutralizing antibody levels higher than those required by international standards.

In neonatal foals there are an increased susceptibility of β toxin action in the first days of life due to the low level of intestinal trypsin present in colostrum (Lawrence 1997). Additional studies should be performed to check the level of circulating antibodies in newborns, which were acquired through passive immunity from mares previously immunised with the recombinant vaccine, as has been performed in pigs (Salvarani et al. 2013).

CONCLUSIONS

The present study demonstrated that the recombinant vaccine was able to stimulate satisfactory humoral immune responses in vaccinated horses, and may prove to be a prophylactic alternative in *Clostridium perfringens* type A and C infections in horses, which generally present as superacute diseases with a high mortality rate.

Future studies into the dynamic of the immune response in horses immunised with recombinant vaccines will further add to the currently scant data on this topic.

Acknowledgments.- This study was financed in part by the "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq), "Fundação Amazônia de Amparo a Estudos e Pesquisas do Estado do Pará" (FAPESPA), "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES - Finance Code 001), "Pró-Reitora de Pesquisa" and "Pós-Graduação da Universidade Federal do Pará" (PROPESP-UFPA), "Programa de Pós-Graduação em Saúde Animal na Amazônia" of the "Universidade Federal do Pará" (PPGSAAM-UFPA), "Programa de Pós-Graduação em Biotecnologia" of the "Universidade Federal de Pelotas" (PPGBIOTEC-UFPel). We also would like to thank LANAGRO-MG for their contribution to perform this study and for kindly provide the inputs.

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

REFERENCES

Brasil 1997. Instrução Normativa nº 45. Diário Oficial da União, Ministério da Agricultura Pecuária e Abastecimento, Brasília, DF.

Choi Y.K., Kang M.S., Yoo H.S., Lee D.Y., Lee H.C. & Kim D.Y. 2003. *Clostridium perfringens* type A myonecrosis in a horse in Korea. J. Vet. Med. Sci. 65(11):1245-1247. <https://dx.doi.org/10.1292/jvms.65.1245> <PMid:14665756>

Diab S.S., Kinde H., Moore J., Shahriar M.F., Odani J., Anthenill L., Songer G. & Uzal F.A. 2012. Pathology of *Clostridium perfringens* type C enterotoxemia in horses. Vet. Pathol. 49(2):255-263. <https://dx.doi.org/10.1177/0300985811404710> <PMid:21502373>

East L.M., Savage C.J., Traub-Dargatz J.L., Dickinson C.E. & Ellis R.P. 1998. Enterocolitis associated with *Clostridium perfringens* infection in neonatal foals: 54 cases (1988-1997). J. Am. Vet. Med. Assoc. 212(11):1751-1756. <PMid:9621884>

European Pharmacopoeia 1998. European Pharmacopoeia. 3rd ed. Maisonneuve S.A., Sainte-Ruffine, p.561.

Freedman J.C., Shrestha A. & McClane B.A. 2016. *Clostridium perfringens* enterotoxin: action, genetics, and translational applications. Toxins 8(3):73. <https://dx.doi.org/10.3390/toxins8030073> <PMid:26999202>

Gohari I.M., Arroyo L., Macinnes J.I., Timoney J.F., Parreira V.R. & Prescott J.F. 2014. Characterization of *Clostridium perfringens* in the feces of adult horses and foals with acute enterocolitis. Can. J. Vet. Res.78(1):1-7. <PMid:24396174>

Horner R.F. 1982. Malignant oedema caused by *Clostridium perfringens* type A in a horse. J. S. Afr. Vet. Assoc. 53(2):122-123. <PMid:7120271>

Lawrence G.W. 1997. The pathogenesis of enteritis necroticans, p.197-207. In: Rood J.I., McClane B.A., Songer J.G. & Titball R.W. (Eds), The Clostridia: molecular biology and pathogenesis. Academic Press, San Diego.

Milach A., de los Santos J.R., Turnes C.G., Moreira A.N., de Assis R.A., Salvarani F.M., Lobato F.C. & Conceição F.R. 2012. Production and characterization of *Clostridium perfringens* recombinant β toxoid. Anaerobe 18(3):363-365. <https://dx.doi.org/10.1016/j.anaerobe.2012.01.004> <PMid:22306065>

Moreira C., Ferreira M.R.A., da Cunha C.E.P., Donassolo R.A., Finger P.F., Moreira G.M.S.G., Otaka D.Y., de Sousa L.A., Barbosa J.D., Moreira Â.N., Salvarani F.M. & Conceição F.R. 2018. Immunogenicity of a bivalent non-purified recombinant vaccine against botulism in cattle. Toxins 10(10):381. <https://dx.doi.org/10.3390/toxins10100381> <PMid:30241350>

Moreira G.M.S.G., Salvarani F.M., da Cunha C.E.P., Mendonça M., Moreira Â.N., Gonçalves L.A., Pires P.S., Lobato F.C.F. & Conceição, F.R. 2016. Immunogenicity of a trivalent recombinant vaccine against *Clostridium perfringens* alpha, beta, and epsilon toxins in farm ruminants. Scient. Reports 6:22816. <https://dx.doi.org/10.1038/srep22816> <PMid:27004612>

Olivo G., Lucas T.M., Borges A.S., Silva R.O.S., Lobato F.C.F, Siqueira A.K., da Silva Leite D., Brandão P.E., Gregori F., de Oliveira-Filho J.P., Takai S. & Ribeiro M.G. 2016. Enteric pathogens and coinfections in foals with and without diarrhea. BioMed Res. Intern. 2016:1512690. <https://dx.doi.org/10.1155/2016/1512690>

Otaka D.Y., Barbosa J.D., Moreira C., Ferreira M.R.A., Cunha C.E.P., Brito A.R.S., Donassolo R.A., Moreira Â.N., Conceição F.R. & Salvarani F.M. 2017. Humoral response of buffaloes to a recombinant vaccine against botulism serotypes C and D. Toxins 9(10):297. <https://dx.doi.org/10.3390/toxins9100297> <PMid:28937601>

Peek S.F., Semrad S.D. & Perkins G.A. 2003. Clostridial myonecrosis in horses (37 cases 1985-2000). Equine Vet. J. 35(1):86-92. <https://dx.doi.org/10.2746/042516403775467513> <PMid:12553469>

Reed L.J. & Muench H. 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27(3):493-497. <https://dx.doi.org/10.1093/oxfordjournals.aje.a118408>

Salvarani F.M., Conceição F.R., Cunha C.E.P., Moreira G.M.S.G., Pires P.S., Silva R.O.S., Alves G.G. & Lobato F.C.F. 2013. Vaccination with recombinant *Clostridium perfringens* toxoids α and β promotes elevated antepartum and passive humoral immunity in swine. Vaccine 31(38):4152-4155. <https://dx.doi.org/10.1016/j.vaccine.2013.06.094>

Sayeed S., Uzal F.A., Fisher D.J., Saputo J., Vidal J.E., Chen Y., Gupta P., Rood J.I. & McClane B.A. 2008. Beta toxin is essential for the intestinal virulence of *Clostridium perfringens* type C disease isolate CN3685 in a rabbit ileal loop model. Mol. Microbiol. 67(1):15-30. <https://dx.doi.org/10.1111/j.1365-2958.2007.06007.x> <PMid:18078439>

Songer J.G. & Uzal F.A. 2005. Clostridial enteric infections in pigs. J. Vet. Diagn. Invest. 17(6):528-536. <https://dx.doi.org/10.1177/104063870501700602> <PMid:16475510>

Songer J.G. 1996. Clostridial enteric disease of domestic animals. Clin. Microbiol. Rev. 9(2):216-234. <https://dx.doi.org/10.1128/CMR.9.2.216-234.1996> <PMid:8964036>

USDA 2002. Conditional licenses for products containing *Clostridium perfringens* type A. No.02-25. Center for Veterinary Biologics Notice, United States Department of Agriculture.

Uzal F.A. & Songer J.G. 2008. Diagnosis of Clostridium perfringens intestinal infections in sheep and goats. J. Vet. Diagn. Invest. 20(3):253-265. <https://dx.doi.org/10.1177/104063870802000301> <PMid:18460610>

Vengust M., Arroyo L.G., Weese J.S., Staempfli H.R. & Baird J.D. 2002. Clostridial myositis: evaluation of normal equine skeletal muscle for the presence of clostridial spores. Proc. Am. Ass. Equine Practitioners 48:134-135.

Weese J.S., Staempfli H.R. & Prescott J.F. 2001. A prospective study of the roles of *Clostridium difficile* and enterotoxigenic *Clostridium perfringens* in equine diarrhoea. Equine Vet. J. 33(4):403-409. <https://dx.doi.org/10.2746/042516401776249534> <PMid:11469775>

Zaragoza N.E., Orellana C. A., Moonen G.A., Moutafis G. & Marcellin E. 2019. Vaccine production to protect animals against pathogenic clostridia. Toxins, Basel, 11(9):525. <https://dx.doi.org/10.3390/toxins11090525> <PMid:31514424>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(10):781-790, October 2020
DOI: 10.1590/1678-5150-PVB-6628

Original Article
Livestock Diseases
BY-NC

Investigation of *Listeria monocytogenes*, *Salmonella enterica* and *Yersinia enterocolitica* in pig carcasses in Southern Brazil[1]

Jalusa D. Kich[2]*, Agnes I.A. Souza[3], Júlia Montes[2], Mariana Meneguzzi[2], Eduardo F. Costa[4], Arlei Coldebella[2], Luis Gustavo Corbellini[4] and Marisa Cardoso[4]

ABSTRACT.- Kich J.D., Souza A.I.A., Montes J., Meneguzzi M., Costa E.F., Coldebella A., Corbellini L.G. & Cardoso M. 2020. **Investigation of *Listeria monocytogenes*, *Salmonella enterica* and *Yersinia enterocolitica* in pig carcasses in Southern Brazil.** *Pesquisa Veterinária Brasileira 40(10):781-790*. Embrapa Suínos e Aves, BR-153 Km 110, Distrito de Tamanduá, Concórdia, SC 89715-899, Brazil. E-mail: jalusa.kich@embrapa.br

The intensification of pig production and advances in the sanitary control of herds profoundly changed the profile of risk attributed to pork consumption. In the actual scenario, most microorganisms related to macroscopic lesions observed in the *post mortem* inspection are not transmitted by food, while foodborne bacteria of importance to consumer health do not cause macroscopic lesions. In Brazil, the "*Ministério da Agricultura, Pecuária e Abastecimento*" requested a scientific opinion on the prioritizing of pathogens potentially transmitted by unprocessed pork. After conducting a qualitative risk assessment, only *Salmonella enterica* was classified as of high risk to consumers. The present study was part of the validation step of the risk assessment and aimed to investigate the frequency of *S. enterica*, *Yersinia enterocolitica* and *Listeria monocytogenes* and hygienic-sanitary indicators in pig carcasses of pigs rose under intensive production and slaughtered under the Federal Inspection System in three slaughterhouses located in Southern Brazil. Additionally, the antimicrobial resistance profile of the isolated pathogens was also investigated. A total of 378 carcasses were sampled by superficial sponges before the chilling step in three slaughterhouses. Samples were investigated for the presence of the three aforementioned pathogens and subjected to enumeration of Colony Formation Units (log CFU.cm^{-1}) of total aerobic mesophiles (TAM) and Enterobacteriaceae. *Salmonella* strains were tested by disc diffusion test for resistance to eleven antimicrobials. There were significantly statistical differences ($p<0.0001$) on the median counts of both indicators between the slaughterhouses. The median of TAM was very close for Slaughterhouses A and B: 1.573 log CFU.cm^{-1} and 1.6014 log CFU.cm^{-1}, respectively. While in Slaughterhouse C, a higher TAM median was detected (2.216 log CFU.cm^{-1}). A similar profile was observed regarding to Enterobacteriaceae, and medians were calculated as follow: -0.426 log CFU.cm^{-1} in Slaughterhouse A; 0.2163 log CFU.cm^{-1} in B; and 0.633 log CFU.cm^{-1} in C. Regarding the pathogens investigated, *L. monocytogenes* was not detected and only one carcass from Slaughterhouse C was positive for *Y. enterocolitica*. Thus, the results suggest a very low prevalence of *L. monocytogenes* and *Y. enterocolitica* in the sampled population. A total of 65 (17.2%) carcasses were positive for S. *enterica*, with a difference in frequencies between slaughterhouses and slaughter days. The prevalence of *Salmonella* positive carcasses was higher in the Slaughterhouse C (25.4%; CI 95% 19-32%) in comparison with A (9.5%; CI

[1] Received on May 25, 2020.
Accepted for publication on July 4, 2020.

[2] Embrapa Suínos e Aves, BR-153 Km 110, Distrito de Tamanduá, Concórdia, SC 89715-899, Brazil. E-mails: juliahmontes@gmail.com, mariana.meneguzzi@hotmail.com, arlei.coldebella@embrapa.br; *Corresponding author: jalusa.kich@embrapa.br

[3] Graduate Program in Veterinary Science, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil. E-mail: agnes_isadora@hotmail.com

[4] Departamento de Medicina Veterinária Preventiva, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9090, Porto Alegre, RS 90540-000, Brazil. E-mails: eduardo.costa@ufrgs.br, luis.corbellini@ufrgs.br, mcardoso@ufrgs.br

95% 9-14%) and B (18.3%; CI 95% 12-24%). There was no significantly statistical association between Enterobacteriaceae counts and *Salmonella* isolation on carcass surface (p=0.69). The slaughtering day, nested within the slaughterhouse, explains 31.3% of *Salmonella* prevalence variability. *S.* Typhimurium (38.1%) was the most prevalent, followed by *S.* Infantis (30.1%). Among the 61 *Salmonella* strains tested for resistance to antimicrobials, 18 (31.6%) were full-susceptible. No strain displayed resistance to azithromycin, ceftazidime, cefotaxime and meropenem. The highest resistance frequency was displayed to tetracycline (54.1%), followed by ampicillin (50.82%), nalidixic acid (42.62%) and chloramphenicol (42.62). Multi-resistance was detected in 52.54% of the, strains. In conclusion, *S. enterica* is more prevalent in pre-chill pig carcasses than *Y. enterocolitica* and *L. monocytogenes* and thus should be prioritized in monitoring and control programs at slaughter. *Salmonella* serovars varied among slaughterhouses and present significant differences in their resistance to antimicrobials. Slaughterhouses that present higher medians of TAM or Enterobacteriaceae in a monitoring period may have higher *S. enterica* prevalences as well. However, there is a high variation of *S. enterica* prevalence among slaughter days, which cannot be always related to the hygienic indicators counts observed on a given day.

INDEX TERMS: *Listeria monocytogenes*, *Salmonella enterica*, *Yersinia enterocolitica*, pig carcasses, pork, pigs, antimicrobial resistance, Brazil.

RESUMO.- [**Pesquisa de *Listeria monocytogenes*, *Salmonella enterica* and *Yersinia enterocolitica* em carcaças de suínos no sul do Brasil.**] A intensificação da produção de suínos e os avanços no controle sanitário dos rebanhos alterou de forma importante o perfil de risco do consumo de carne suína. No cenário atual, a maioria dos microrganismos causadores de lesões macroscópicas detectáveis na inspeção *post mortem* não são transmissíveis por alimentos, enquanto bactérias de importância como causadoras de doenças transmitidas por alimentos não causam lesões macroscópicas. No Brasil, o Ministério da Agricultura, Pecuária e Abastecimento solicitou uma opinião científica sobre a priorização de patógenos potencialmente transmitidos pela carne suína in natura. Após conduzir uma avaliação de risco qualitativa, apenas *Salmonella enterica* foi classificada como de alto risco para o consumidor. O presente estudo foi parte da etapa de validação da avaliação de risco e objetivou: investigar a frequência de *S. enterica*, *Yersinia enterocolitica* e *Listeria. monocytogenes*; e enumerar indicadores higiênico-sanitários em carcaças de suínos abatidos sob inspeção federal em frigoríficos dedicados ao abate de suínos sob sistema intensivo de criação no sul do Brasil. Além disso, o perfil de resistência a antimicrobianos dos patógenos isolados foi investigado. A superfície de um total de 378 carcaças foi amostrada por esponjas, na etapa de pré-resfriamento em três matadouros frigoríficos (A, B, C). As amostras foram investigadas quanto à presença dos três patógenos acima mencionados e quanto à enumeração de Unidades Formadoras de Colônia (log UFC.cm^{-1}) de mesófilos aeróbios totais (MAT) e Enterobacteriaceae. As cepas isoladas de *Salmonella* foram testadas quanto à resistência a onze antimicrobianos pela técnica de disco difusão. As medianas de contagem de ambos os indicadores apresentaram diferença significativa (p<0,0001) entre matadouros-frigoríficos. A mediana de MAT foi bastante próxima para A e B (1,573 log UFC.cm^{-1} e 1,6014 log UFC.cm^{-1}, respectivamente), enquanto em C uma mediana de MAT mais elevada foi determinada (2,216 log CFU.cm^{-1}). Um perfil semelhante foi observado em relação a Enterobacteriaceae, sendo as medianas calculadas para A, B e C, respectivamente: -0,426 log CFU.cm^{-1}; 0,2163 log UFC.cm^{-1}; e 0,633 log UFC.cm^{-1}. Em relação aos patógenos investigados, *L. monocytogenes* não foi detectada e apenas uma carcaça, do Matadouro C, foi positiva para *Y. enterocolitica.* Portanto, os resultados sugerem uma prevalência muito baixa desses patógenos na população amostrada. Em um total de 65 (17,2%) carcaças houve isolamento de *S. enterica*, com diferença nas frequências observadas entre matadouros e dias de abate. A prevalência de carcaças positivas para *S. enterica* foi maior no Matadouro C (25,4%; IC95% 19-32%) em comparação com A (9,5%; IC95% 9-14%) e B (18,3%; IC95% 12-24%). Não houve associação estatística entre o número de Enterobacteriaceae e o isolamento de *S. enterica* na superfície das carcaças (p=0,69). O dia de abate agrupado por frigorífico explica 31,3% da variação na prevalência de *Salmonella.* O sorovar mais frequente de *S. enterica* foi Typhimurium (38,1%) seguido de *S.* Infantis (30,1%). Entre as 61 cepas de *S. enterica* testadas quanto à resistência a antimicrobianos, 18 (31,6%) foram totalmente suscetíveis aos antimicrobianos testados. Nenhuma cepa apresentou resistência a azitromicina, ceftazidima, cefotaxima e meropenem. As maiores frequências de resistência foram demonstradas contra tetraciclina (54,1%), ampicilina (50,8%), ácido nalidíxico (42,62%) e cloranfenicol (42,62%). Em 52,54% das cepas foi detectada multi-resistência. Em conclusão, *S. enterica* é mais prevalente em carcaças suínas no pré-resfriamento do que *Y. enterocolitica* e *L. monocytogenes*. Portanto, *S. enterica* deve ser priorizada em programas de monitoramento e controle ao abate. Os sorovares de *Salmonella* variam entre matadouros e apresentam diferenças significativas na resistência a antimicrobianos. Matadouros de suínos que apresentam medianas de MAT e Enterobacteriaceae num período de monitoramento podem apresentar também prevalências mais de altas de presença de *S. enterica*. Entretanto, há uma alta variabilidade na frequência de S. *enterica* entre dias de abate, e nem sempre há relação entre essa frequência e a contagem de indicadores higiênico-sanitários determinados num determinado dia.

TERMOS DE INDEXAÇÃO: Carne suína, carcaças de suínos, *Salmonella enterica*, *Listeria monocytogenes*, *Yersinia enterocolitica*, resistência a antimicrobianos, suínos, Brasil.

INTRODUCTION

Meat inspection aims to protect the consumer's health by detecting and preventing hazards, which may be transmitted by meat (FAO 2000). The identification of meat unsuitable for human consumption has traditionally been performed by *post mortem* inspection of carcasses and viscera, which are subjected to visual examination, palpation and incisions for detecting lesions. The intensification of pig production and advances in the sanitary control of herds, however, profoundly changed the profile of risk attributed to pork consumption. While in the past parasitic diseases were the most important hazards transmitted by pork, nowadays parasites are very well controlled in the animal production phase with rare evidence of lesions. In the actual scenario, most microorganisms related to macroscopic lesions observed in the *post mortem* inspection are not transmitted by food; most of them cause animal diseases or are related to occupational exposure (EFSA 2011). Data collected from 2012 to 2014 in all Brazilian slaughterhouses under the Federal Inspection System demonstrated that cysticercosis, which had been an important zoonosis in the past, was the cause of only 9.2 condemnations per million pigs slaughtered in this period. Moreover, 74.5% of the detections occurred in only one slaughterhouse, indicating an epidemiological profile found in a restricted geographical area (Kich et al. 2019).

Foodborne bacteria of importance to consumer health, in turn, do not cause lesions and thus are not detectable by the technics adopted in the *post mortem* inspection (EFSA 2011). In addition, traditional inspection system practices, such as palpation and incision, can lead to the transfer of these bacteria between carcasses, the environment and employees (Buncic et al. 2014). As long as supported by scientific evidences, several countries have changed some traditional inspection practices, and adopted a risk-based meat inspection (EFSA 2011). In Brazil, the "*Ministério da Agricultura*, *Pecuária e Abastecimento*" (MAPA) requested from "Embrapa Suínos e Aves" a scientific opinion on this topic; in order to achieve this goal a multidisciplinary scientific project was conducted. The study aimed ultimately at generating scientific evidences to support decisions to be taken by the risk manager regarding changes in the meat inspection system. The first step of the project was a qualitative risk assessment for the prioritization of biological hazards transmissible to humans by pork (Costa et al. 2020), in which only *Salmonella enterica* was classified as of high risk to consumers. Other bacteria, such as *Yersinia enterocolitica* and *Listeria monocytogenes*, which are reported as possible causes of foodborne diseases transmitted by pork, were not prioritized in the risk assessment. However, *Y. enterocolitica* is often carried in slaughtered pig tonsils and can contaminate carcasses during processing (Drummond et al. 2012), while ready-to-eat pork products may be the vehicle of *L. monocytogenes* in foodborne cases (WHO 2018a). The presence of bacterial hazards in carcasses in turn is greatly influenced by the quality of the process and self-control programs implemented at slaughterhouses (Pearce et al. 2004, Arguello et al. 2013). The process hygiene has been monitored by the enumeration of hygienic-sanitary indicators, such as mesophilic aerobes and Enterobacteriaceae, since these bacteria can be eliminated by proper hygiene and sanitation procedures during slaughtering (Ghafir et al. 2008). Therefore, the investigation of these three pathogens and two hygienic-sanitary indicators (mesophilic aerobes and Enterobacteriaceae) was kept in the present study, which will be a part of the validation step that follows the qualitative risk assessment.

In addition to the risk posed by their presence in pork, pathogenic bacteria carrying antimicrobial resistance genes have been a growing concern to consumer's health. The hazard of multi-resistant strains selection by the antimicrobial use in animals has been stressed worldwide (WHO 2018b). Resistance to antimicrobials used for treatment of human diseases has been reported in foodborne pathogens, such as *S. enterica* isolated from swine (Lopes et al. 2015, McDermott et al. 2016, Cameron-Veas et al. 2018, Wang et al. 2019), highlighting the importance of monitoring the resistance of this pathogen. In this sense, gathering information of antimicrobial resistance profile of bacteria isolated from animals and animal products belongs to the goals of the antimicrobial resistance monitoring and control program launched in Brazil (Brasil 2018).

Therefore, the present study aimed to investigate the frequency of *S. enterica*, *Y. enterocolitica* and *L. monocytogenes* and hygienic-sanitary indicators in pig carcasses slaughtered under the Federal Inspection System in three slaughterhouses in Southern Brazil. This investigation was one of the steps for the development of the scientific opinion on the adoption of a risk-based inspection of pork in the scope of the Federal Meat Inspection System. Additionally, the antimicrobial resistance profile of the isolated pathogens was also investigated.

MATERIALS AND METHODS

Study design. The sample size calculation considered the minimum number of carcass samples needed to determine the prevalence of bacterial pathogens listed as important in pig carcasses. The parameters used to calculate a simple random sample were: infinite population, 95% confidence level, 6% absolute precision and 50% prevalence. A previous study (Corbellini et al. 2016) showed an important effect of the day of sampling on the variation of *Salmonella* prevalence in pig carcasses. The design effect (Deff) of sampling day was estimated in 1.42 in this study (Corbellini et al. 2016). Thus, this inflation factor was applied to correct the number of samples, resulting in a total of 378 carcasses to be analyzed. The Brazilian swine production system includes producers integrated into large agro-industries, producers who participate in a cooperative system and independent farmers who deliver finished animals to slaughterhouses. In order to achieve representativeness of the sample, the total number of carcasses was divided equally (n=126) among three slaughterhouses (A, B, C), which represent the aforementioned three modes of swine production. Slaughterhouse A was part of a cooperative system; approximately 4,600 pigs were slaughtered daily. In Slaughterhouse B, a similar number of pigs (4,200/day) were slaughtered, which were delivered by farmers engaged in an integration system with the company. The Slaughterhouse C received pigs from independent farmers, and slaughtered 900 pigs daily. In general, the moderate variation between clusters (sampling days) implies that the addition of sample units on a given day will not add much to the sample's representativeness, being more important to distribute the sample units in as many days as possible. Thus, six weekly sampling events were carried out in each of the slaughterhouses, with 21 carcasses sampled per event. Sampling was always conducted in the morning shift, with the first carcass sampled being that corresponding to the first slaughtered animal. The other samples were collected systematically in an interval of 35 carcasses.

Sample collection and analysis. Carcasses were sampled at the pre-chill step using individual sterile abrasive sponges (NASCO®) rubbed in an area of 100cm^2 from each: loin, jowl, ham and belly (Brasil 2007). The four sponges were pooled in sterile plastic bags and constituted the carcass sample, which was kept refrigerated until processing. To each carcass sample, 100mL of 1% Buffered Peptone Water (BPW 1%) were added; the suspension was then homogenized and used for the enumeration of hygienic indicators: total aerobic mesophiles (TAM) and Enterobacteriaceae; and detection of pathogens: *Salmonella enterica*, *Yersinia enterocolitica* and *Listeria monocytogenes*.

The enumeration of TAM and Enterobacteriaceae was performed in duplicate, in Petrifilm™ Aerobic Count Plates (3M Company) and Petrifilm™ Enterobacteriaceae Count Plates (3M Company), respectively, following the manufacturer's recommendations. After incubation at 37ºC for 48h, typical colonies were counted and the result was multiplied by 0.25 to achieve the number of colony forming units (CFU) per cm^2 of carcass surface.

S. enterica was investigated following the ISO 6579:2002 Amendment 1:2007 protocol (ISO 2007), using Xylose-Lysine-Deoxycholate agar (Merck) and Brilliant Green Phenol Red Lactose Sucrose agar (Merck) for the selective differential isolation step. Typical colonies were confirmed by their biochemical profile and agglutination with somatic polyvalent serum (Probac). The isolates confirmed as *S. enterica* were sent for serotyping at the "Fundação Instituto Oswaldo Cruz" (Fiocruz).

The detection of *Y. enterocolitica* was performed according to the methodology of the American Public Health Association (Weagant & Feng 2001). Briefly, 1mL of the sample suspension was added to 9mL of Peptone Sorbitol Bile broth (PSB, Himedia) and incubated for 10 days at 10°C. After this period, 10µL of the culture was transferred to 100µL of either 0.5% potassium hydroxide (KOH) or NaCl 0.5% solutions. After homogenization for five seconds, an aliquot was immediately transferred to Cefsulodin-Irgasan-Novobiocin agar (Fluka) and to MacConkey agar (Oxoid) plates, and incubated at 30°C for 24h. Typical colonies were subjected to identification by Matrix-assisted Laser Desorption Ionization Time-of-flight (MALDI-TOFF).

L. monocytogenes was investigated according to the IN62 (Brasil 2003) protocol. An aliquot of 1mL of the sample suspension was added to 9mL of Enrichment Broth for Listeria (UVM). After incubation at 30±1°C for 24h, an aliquot (100µL) was transferred to 9.9mL of Fraser broth (Merck) and incubated at 30±1°C for 24h. Afterwards, aliquots were transferred to Tryptose agar with Nalidixic Acid, Palcam agar (Oxoid) and Chromocult© Listeria Selective Agar acc. to Agosti and Otttaviani (ALOA, Merck). After incubation at 30°C±1°C for 48h, typical colonies were identified by biochemical tests and CAMP test (Silva et al. 2010).

Antimicrobial susceptibility testing. *S. enterica* isolates were tested for antimicrobial susceptibility against eleven different antimicrobials. The agar disc diffusion method was performed and evaluated according to the specifications of the Clinical and Laboratory Standards Institute (CLSI) documents VET01-S3 and M100-S26 (CLSI 2015, 2016). The following discs (Oxoid) were used: ampicillin (10µg); azithromycin (15µg); cefotaxime (30µg); ceftazidime (30µg); chloramphenicol (30µg); ciprofloxacin (5µg); gentamicin (10µg), meropenem (10µg); nalidixic acid (30µg); tetracycline (30µg); trimethoprim (5µg). *Escherichia coli* ATCC© 25922 was used as a reference strain for quality control purposes. Antimicrobial multi-resistance was defined as resistance to three or more classes of antimicrobials (CLSI 2016).

Data analysis. The proportions of slaughterhouses in the quantiles of log CFU of mesophilic and Enterobacteriaceae counts, box-plot with the distribution of these indicators and Kruskal-Wallis test to assess if there are differences in the median counts of these indicators between slaughterhouses were made with the function *Desc* of the package *DescTools* (Signorell 2020) in R environment. The prevalence of *Salmonella* in each slaughterhouse was estimated using the package *survey* (Lumley 2020) in R environment.

An intercept-only multilevel logistic model that predicts the prevalence conditional to slaughterhouse and day of sampling was made in which the random effect was the day nested within slaughterhouse. The predicted conditional prevalence for each day of sampling within slaughterhouse was extracted from the model estimates. The model dimension has 18 subjects (i.e. the day of sampling, six in each selected slaughterhouse) with 21 observations per subject (21 carcasses in each day). The intra cluster correlation (ICC) was given by:

$$ICC = \frac{\sigma_0^2}{\sigma_0^2 + \frac{\pi^2}{3}}$$

Where σ_0^2 is the between-cluster variance (i.e., the variance between days nested within slaughterhouse) and $\left(\sigma_0^2 + \frac{\pi^2}{3}\right)$ is the total variance.

The same model structure was used to check the effect of Enterobacteriaceae counts (log CFU.cm^{-1}) on the *Salmonella* isolation, and the ICC was calculated using the same formula described above. This model estimates the average effect of Enterobacteriaceae (fixed-effect) as well as its effect conditional to the day of sampling and slaughterhouse, i.e., the model generates 18 estimates for each day of sampling nested within slaughterhouse (random-effects).

The multilevel logistic models were analyzed with SAS Studio using the procedure PROC GLIMMIX. A significance level of 0.05 was considered for all statistical tests.

The antimicrobial susceptibility testing was analyzed using Fisher's exact test to identify its association with serotype and slaughterhouse. The procedure PROC FREQ from SAS 9.3 (SAS 2012) was used to do this evaluation.

RESULTS

The distributions of log CFU of TAM and Enterobacteriaceae counts and the proportion of slaughterhouse in the quantiles of log counts are demonstrated in Figure 1 and 2. There were significantly statistical differences ($p<0.0001$) on the median counts of both indicators between the slaughterhouses. The median of TAM was very close for Slaughterhouses A and B: 1.573 log CFU.cm^{-1} and 1.6014 log CFU.cm^{-1}, respectively. While in Slaughterhouse C, a higher TAM median was detected (2.216 log CFU.cm^{-1}). A similar profile was observed regarding to Enterobacteriaceae, and medians were calculated as follow: -0.426 log CFU.cm^{-1} in Slaughterhouse A; 0.2163 log CFU.cm^{-1} in B; and 0.633 log CFU.cm^{-1} in C. For both hygienic indicators, in Slaughterhouse C the collected data were concentrated in the fourth and fifth quantiles, while in A and B there was a concentration in the first and second quantiles.

Regarding the pathogens investigated, *Listeria monocytogenes* was not detected and only one carcass from Slaughterhouse C was positive for *Yersinia enterocolitica*. A total of 65 (17.2%) carcasses were positive for *Salmonella enterica*,

with a difference in frequencies between slaughterhouses and slaughter days. The prevalence of *Salmonella* positive carcasses was higher in the Slaughterhouse C (25.4%; CI 95% 19-32%) in comparison with A (9.5%; CI 95% 9-14%) and B (18.3%; CI 95% 12-24%). Only in Slaughterhouse C was *S. enterica* detected in all slaughter days.

The *Salmonella* prevalence conditional to the day of sampling and slaughterhouse estimated by the model was 17.7% and the between-cluster variance estimates was 1.67. The resulted ICC was 33.6% [1.67/(1.67+9.9/3)], which means the between-cluster (day of slaughtering nested within the slaughterhouse) variability in the prevalence of *Salmonella* on the carcasses. Table 1 depicts the predicted and observed prevalence in each day of sampling and model estimates. It is possible to observe a marked variation of the prevalence among days.

There was no significantly statistical association between Enterobacteriaceae counts and *Salmonella* isolation on carcass surface (p=0.69, Table 2). The between-cluster variance estimates was 1.50, resulting in an ICC value of 31.3% [1.50/(1.50+9.9/3)], which demonstrate the between-cluster (day of sampling nested within slaughterhouse) variability in the effect of Enterobacteriaceae counts on the prevalence of *Salmonella*. There were three statistically significant days nested within slaughterhouses (random intercepts), which are demonstrated in Table 2. These results mean that in these days the probability of isolation of *Salmonella* was significantly different (higher) than the average probability of *Salmonella* isolation given the Enterobacteriaceae count.

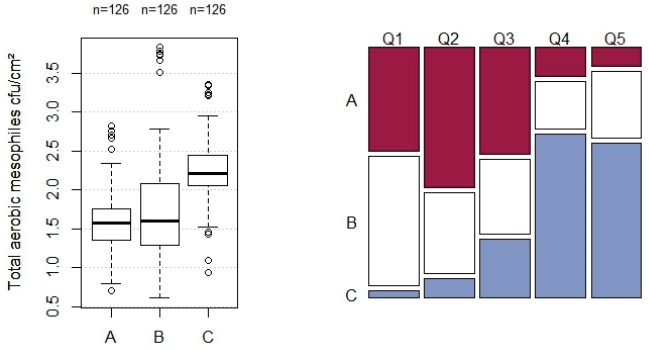


Fig.1. Box plot and quantile distribution (Q1-Q5) of total aerobic mesophiles counts on pig pre-chill carcasses in three slaughterhouses (A, B, C).

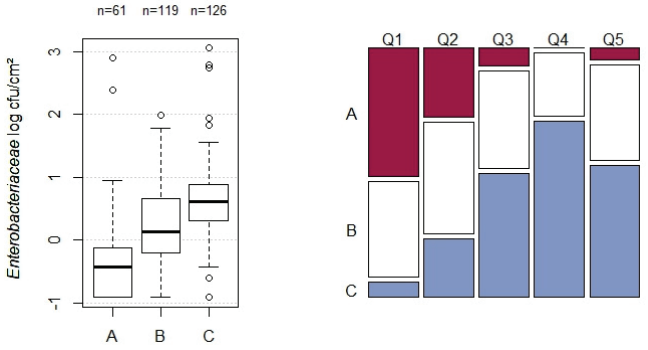


Fig.2. Box plot and quantile distribution (Q1-Q5) of Enterobacteriaceae (II) counts on pig pre-chill carcasses in three slaughterhouses (A, B, C).

Table 1. Observed *Salmonella* prevalence and predicted prevalence estimated by the model on pig pre-chill carcasses in each day of sampling conducted in three slaughterhouses in southern Brazil

Model effect	Slaughterhouse	Day of sampling	Model estimates			Observed data	
			Estimate	SE	Prevalence	No. *Salmonella* positive (n=21/day)	Prevalence
Fixed effect intercept	.	.	-1.89	0.35	-	-	-
Random effect intercept	A	1	-1.34	0.88	3.8%	0	0.0%
	A	2	-1.34	0.88	3.8%	0	0.0%
	A	3	-0.27	0.68	10.3%	2	9.5%
	A	4	-0.27	0.68	10.3%	2	9.5%
	A	5	-0.27	0.68	10.3%	2	9.5%
	A	6	0.85	0.56	26.1%	6	28.6%
	B	1	-1.34	0.88	3.8%	0	0.0%
	B	2	-1.34	0.88	3.8%	0	0.0%
	B	3	0.37	0.60	18.0%	4	19.0%
	B	4	-0.27	0.68	10.3%	2	9.5%
	B	5	0.37	0.60	18.0%	4	19.0%
	B	6	2.12	0.52	55.8%	13	61.9%
	C	1	2.12	0.52	55.8%	13	61.9%
	C	2	-0.72	0.76	6.8%	1	4.8%
	C	3	-0.27	0.68	10.3%	2	9.5%
	C	4	0.08	0.63	14.1%	3	14.3%
	C	5	1.78	0.52	47.3%	11	52.4%
	C	6	-0.27	0.68	10.3%	2	9.5%
Average prevalence					17.7%		17.7%

SE = Standard error.

Among the 63 isolates of *S. enterica* subjected to serotyping, *S.* Typhimurium (38.1%) was the most prevalent, followed by *S.* Infantis (30.1%). The profile of serovars found varied between slaughterhouses, with *S.* Typhimurium being significantly more prevalent (P<0.0001) in Slaughterhouses A and B than in C (Table 3). In Slaughterhouse C, *S.* Infantis was the most prevalent serovar.

Among the 61 *Salmonella* strains tested for resistance to antimicrobials, 18 (31.6%) were full-susceptible (Table 4). No strain displayed resistance to azithromycin, ceftazidime, cefotaxime and meropenem. The highest resistance frequency was displayed to tetracycline (54.1%), followed by ampicillin (50.82%), nalidixic acid (42.62%) and chloramphenicol (42.62). Multi-resistance was detected in 52.54% of the strains; in most cases (62.5%) the MDR profile included tetracycline, ampicillin and chloramphenicol. The association between the serovars with the resistance frequency demonstrated that *S.* Typhimurium presented significantly more resistance (P<0.05) to almost all antimicrobials than the other serovars. Therefore, the greater antimicrobial resistance frequencies were observed in slaughterhouses, in which *S*. Typhimurium was isolated.

DISCUSSION

Human salmonellosis is mainly attributed to food transmission (Scallan et al. 2011), reinforcing the need for control in food animals and their products. The high frequency of *Salmonella enterica* in pre-chill carcasses evidenced here supports the prioritizing of this hazard among those transmitted to human by unprocessed pork, which was pointed out in the risk assessment conducted by Costa et al. (2020). On the contrary and also corroborating the risk assessment results, the other two investigated bacterial hazards proved to have a very low frequency.

Table 2. Association between Enterobacteriaceae counts and *Salmonella* isolation on pig pre-chill carcasses in three slaughterhouses in southern Brazil

Model structure			Model results for Enterobacteriaceae count			Observed data			
Effects	Subjects[a]		Solutions			*Salmonella*	Enterobacteriaceae		
	Abattoir	Day of sample	Estimate	SE	*p*-value	Prevalence	Mean	Min	Max
Enterobacteriaceae count	All	All	0.10	0.25	0.69	17.7%[b]	0.28[b]	-0.9[b]	3.06[b]
Random intercepts	B	6	1.86	0.52	0.0004	61.9%	0.47	-0.90	1.13
	C	1	1.96	0.51	0.0002	61.9%	0.25	-0.90	1.94
	C	5	1.57	0.53	0.0032	52.4%	0.79	-0.43	1.54

[a] There are 18 subjects (sampling event), only three were statistically demonstrated; [b] prevalence, mean, and min-max of all data points in three slaughterhouses; SE = standard error.

Table 3. Distribution of *Salmonella enterica* serovars isolated from pig carcasses in three southern Brazilian slaughterhouses

Serovar	Slaughterhouses			Total (%)
	A	B	C	
Typhimurium	8	16	-	24 (38.1%)
Infantis	-	-	19	19 (30.1%)
Mbandaka	-	-	7	7 (11.1%)
Panama	3	1	-	4 (6.35%)
Hadar	-	-	4	4 (6.35%)
London	1	1	-	2 (3.2%)
Saintpaul	-	-	1	1 (1.6%)
S. O:4,5:-:1,2	-	-	1	1 (1.6%)
Rugose morphotype	-	-	1	1 (1.6%)
TOTAL	12	18	33	63

Table 4. Percentage distribution of antimicrobial resistance to different antimicrobials in *Salmonella enterica* serovars isolated from pig carcasses in three slaughterhouses of southern Brazil

Antimicrobials	*S.* Infantis (n=17)	*S.* Typhimurium (n=24)	Other serovars (n=20)	Fisher's probability (P)	Total (n=61)
Ampicillin	47.1[a]	83.3[b]	15.0[a]	<0.0001	54.4
Ciprofloxacin	0[a]	4.2[b]	0[a]	0.0065	1.75
Chloramphenicol	35.3[a]	75.0[b]	10.0[a]	<0.0001	45.6
Gentamicin	0[a]	54.2[b]	15.0[a]	<0.0001	28.1
Nalidixic acid	17.6[a]	83.3[b]	15.0[a]	0.0010	45.6
Tetracycline	35.3[a]	83.3[b]	35.0[a]	0.0010	57.9
Trimethoprim	35.3	12.5	20.0	0.2557	22.8
Full susceptible	41.2[a]	4.2[b]	55.0[a]	0.0003	31.2
Multi-drug resistant	41.2[b]	83.3[a]	25.0[b]	0.0003	52.5

[a,b] = Different letters in a same line differ significantly by Fisher's exact test (P≤0.05).

Regarding *Listeria monocytogenes* isolation, despite none carcass has being positive, the study design used here allow us only to conclude that the prevalence in carcasses is low, i.e. below the detection power. Although frequencies above 30% have already been reported in pig carcasses in Brazil (Ferronato et al. 2012, Pissetti et al. 2012), in both studies, the high frequency of isolation was concentrated in just one slaughterhouse. The high adaptation of *L. monocytogenes* to moist and low temperature, which characterize the slaughterhouse environment, corroborate with the hypothesis that high frequencies in pork may be associated with poor hygiene and biofilm formation on surfaces in contact with the carcasses. Moreover, *L. monocytogenes* outbreaks are more often vehiculated by ready-to-eat products than by unprocessed pork, which is usually subjected to heat treatment before consumption (Valk et al. 2001, Awofisayo-Okuyelu et al. 2016). Therefore, monitoring programs of *L. monocytogenes* targeted to processed pork products are usually the strategy adopted by the sanitary authorities (Brasil 2009, EFSA 2018) followed by a thorough investigation of the production process in cases of detection of contaminated products.

Although *Yersinia enterocolitica* has been recognized as cause of foodborne disease cases in humans, particularly associated with eating raw or undercooked pork in European countries (Huovinen et al. 2010, Rosner et al. 2011), only one carcass resulted positive in this study. Pigs have shown to be a major reservoir of pathogenic *Y. enterocolitica*, particularly strains of bioserotype 4/O:3 (Fredriksson-Ahomaa et al. 2007). A similar scenario was reported in Brazil, and *Y. enterocolitica* 4/O:3 was found in 30% of tongues and tonsils from pigs at slaughter, while none of 192 samples of pork resulted positive (Paixão et al. 2012). These results corroborate to the low priority of investigation of this pathogen in a monitoring program of pig carcasses in Brazil. Similarly, *Y. enterocolitica* was not classified as of high risk in the scientific opinion on biological hazards transmitted by pork published in the European Union (EFSA 2011). The high frequency reported in tongue and tonsils highlights the hazard represented by the tissues associated with the head regarding the cross contamination during the pluck set removal (Borch et al. 1996). Moreover, edible tissues in the head area, such as tongue and cheek meat, are often commercialized or included in processed products. In this sense, post-fabrication interventions to be applied in the trimmings may be considered and further evaluated in future studies.

The frequency of *S. enterica* positive carcasses corroborates the prevalence in previous studies conducted in Brazil, which varied from 9.8 to 24% (Kich et al. 2011, Silva et al. 2012, Corbellini et al. 2016, Brasileiro et al. 2017). As previously reported (Corbellini et al. 2016), there is a high variation in the frequencies among slaughterhouses and slaughter days. The model constructed considering these variations estimates prevalence values between 3.8% and 55.8%, with an average prevalence similar to the observed. The day of slaughter nested within the slaughterhouse represents 31.3% of the whole variation in the *Salmonella* prevalence, indicating that several factors in a slaughter day may influence the number of positive carcasses. The *Salmonella* prevalence variation may be associated to failures in the slaughter process, which should be monitored by the profile of hygienic indicators over a time period. Actually, in our study, the median of TAM and Enterobacteriaceae was significantly higher in Slaughterhouse C, which presented also the highest *Salmonella* prevalence in the pre-chill carcasses. Therefore, data of monitoring of hygienic indicators may be of outmost importance in auto-control quality programs as well as for official auditing of slaughterhouses. Deviations in the hygienic indicators should be interpreted as an enhancement of the hazard of carcass contamination with pathogens, which may be present in the slaughtered pig or in the environment. However, there is no strict correlation between the hygienic indicator counts in a given carcass or in a given slaughter day with the isolation of *Salmonella* in pre-chill carcasses. Although, in a few cases the *Salmonella* prevalence was associated with higher Enterobacteriaceae counts in our study (Table 2), in general this association was not supported by the statistical analysis. This fact highlights that, even in slaughterhouses with a controlled hygienic process, *Salmonella* can present a higher prevalence in pre-chill carcasses in some days. Therefore, the monitoring programs of *Salmonella* in carcasses cannot be discontinued in auto-control programs and official audits, even in slaughterhouses with a good hygienic process. Another important aspect to be considered is the delivery of slaughter batches with high number of *Salmonella* carrier pigs or the presence of high shedders among the slaughtered pigs, since these factors have been pointed out as leading to enhancement of carcass contamination probability (Duggan et al. 2010, Silva et al. 2012, Kerouanton et al. 2019, Paim et al. 2019). In this case, other approaches targeted to the pre-harvest stage are needed, in order to diminish the pressure in the slaughtering process represented by a high number of *Salmonella* carrier pigs.

The characterization of *S. enterica* isolated in the three slaughterhouses demonstrated a significant difference among the serovars found. While *S.* Typhimurium predominated in Slaughterhouses A and B, in C this serovar was not found and most strains were identified as *S.* Infantis. The prevalence of *Salmonella* serovars often varies among production systems and over time (Denis et al. 2013, Colello et al. 2018). This fact reflects the multiple sources of *Salmonella,* which includes, associated or not, carrier animals, contaminated feed and environment (Funk & Gebreyes 2004, Kich et al. 2005). Still, *S.* Typhimurium is often reported as the most prevalent serovar in pig and pork (Kich et al. 2011, Silva et al. 2012, Campos et al. 2019, Paim et al. 2019). Moreover, this serovar is also frequently reported in human cases and outbreaks associated with pork consumption (Campos et al. 2019). While *S*. Infantis does not figure among the most prevalent serovars identified in swine, human cases present frequently serious symptoms indicating that this serovar may be highly virulent (Almeida et al. 2013). Therefore, the serovars identified in our study highlights the need of *Salmonella* control for the consumer's health.

An additional growing concern in public health has been the antimicrobial resistance presented by pathogenic or non-pathogenic bacteria colonizing food animals. The hazard of transmission of these resistant bacteria to human has been considered as a factor that may contribute to failures of antibiotic treatments in human patients. Foodborne pathogens are of special relevance, since antimicrobial resistance represents an additional hazard for patients with low immunity, which will need antibiotic therapy. In this sense, a marked difference

was observed in the resistance level of *S*. Typhimurium compared to the other serovars. Considering the first choice for antibiotic treatment of salmonellosis in humans, resistance to ciprofloxacin was displayed by 4.2% of the *S*. Typhimurium strains, while all strains of other serovars were susceptible to this drug. Moreover, 83.3% of the *S*. Typhimurium strains were resistant to nalidixic acid, which is often the first step for full resistance to fluorquinolones (Oteo et al. 2000). These results corroborate the recommendation of phasing out the use of antimicrobials highly important to human medicine in animal production (WHO 2018b). The resistance profile in general demonstrated that *S*. Typhimurium was significantly more resistant to most tested antimicrobials and presented more MDR strains in comparison to the other serovars. *S*. Typhimurium is reported as a serovar which often presents multiresistance and, therefore, besides its pathogenic potential may also carry a plethora of resistance genes (Almeida et al. 2018, McMillan et al. 2019). In recent years, genes codifying extended spectrum beta-lactamases (ESBL) or carbapenemases as well as resistance to macrolides have been emerged as a particular concern (WHO 2018b). In this regard, all tested strains were susceptible to these antimicrobial groups in agreement with other reports of very low frequency or absence of resistance to these drugs in *Salmonella* originated from the Brazilian swine production chain (Bersot et al. 2019, Monte et al. 2019, Viana et al. 2019). On the contrary, antimicrobials such as ampicillin, tetracycline and nalidixic acid have been often reported among the group of highest levels of resistance in food borne pathogens (Lopes et al. 2015, Almeida et al. 2018, Bersot et al. 2019, Monte et al. 2019, Viana et al. 2019). Those drugs belong to antimicrobial classes broadly used in pig production in Brazil (Dutra 2017), which justify the high level of resistance found.

CONCLUSIONS

Salmonella enterica is more prevalent in pre-chill pig carcasses than *Yersinia enterocolitica* and *Listeria monocytogenes* and thus should be prioritized in monitoring and control programs at slaughter.

Salmonella serovars varied among slaughterhouses and present significant differences in their resistance to antimicrobials.

Slaughterhouses that present higher medians of total aerobic mesophiles or Enterobacteriaceae in a monitoring period may have higher *S. enterica* prevalences as well. However, there is a high variation of *S. enterica* prevalence among slaughter days, which cannot be always related to the hygienic indicators counts observed on a given day.

Acknowledgements.- This study was carried out with the support of "Sistema Embrapa de Gestão" (SEG), "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES), Brazil, Financing Code 001 (PNPD scholarship for E.F. Costa; MSc scholarship for A.I.A. Souza), and the "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq), Brazil, (MSc scholarship for D. Paim; Undergraduate scholarship for M. Meneguzzi).

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

REFERENCES

Almeida F., Pitondo-Silva A., Oliveira M.A. & Falcão J. P. 2013. Molecular epidemiology and virulence markers of *Salmonella* Infantis isolated over 25 years in São Paulo State, Brazil. Infec. Gen. Evol. 19:145-151. <https://dx.doi.org/10.1016/j.meegid.2013.07.004>

Almeida F., Seribelli A.A., Medeiros M.I.C., Rodrigues D.P., Varani A.M., Luo Y., Allard M.W. & Falcão J.P. 2018. Phylogenetic and antimicrobial resistance gene analysis of Salmonella Typhimurium strains isolated in Brazil by whole genome sequencing. PLos One 13(8):e0201882. <https://dx.doi.org/10.1371/journal.pone.0201882> <PMid:30102733>

Arguello H., Carvajal A., Naharro G., Rodicio M.R., Martin M.C. & Rubio P. 2013. Sero- and genotyping of Salmonella in slaughter pigs, from farm to cutting plant, with a focus on the slaughter process. Int. J. Food Microbiol. 161(1):44-52. <https://dx.doi.org/10.1016/j.ijfoodmicro.2012.11.013> <PMid:23261811>

Awofisayo-Okuyelu A., Arunachalam N., Dallman T., Grant K.A., Aird H., McLauchlin J., Painset A. & Amar C. 2016. An outbreak of human listeriosis in England between 2010 and 2012 associated with the consumption of pork pies. J. Food Protect. 79(5):732-740. <https://dx.doi.org/10.4315/0362-028X.JFP-15-456> <PMid:27296419>

Bersot L.S., Cavicchioli V.Q., Viana C., Burin R.C.K., Camargo A.C., Pinto J.P.A.N., Nero L.A. & Destro M.T. 2019. Prevalence, antimicrobial resistance, and diversity of Salmonella along the pig production chain in Southern Brazil. Pathogens 8(4):204. <https://dx.doi.org/10.3390/pathogens8040204> <PMid:31652972>

Borch E., Nesbakken T. & Christensen H. 1996. Hazard identification in swine slaughter with respect to foodborne bacteria. Int. J. Food Microbiol. 30(1/2):9-25. <https://dx.doi.org/10.1016/0168-1605(96)00988-9>

Brasil 2003. Métodos analíticos oficiais para análises microbiológicas para controle de produtos de origem animal e água. Instrução Normativa nº 62/2003, Diário Oficial da União de 18/09/2003, Seção 1, p.14, Brasília, DF. Available at <http:// www.agricultura.gov.br> Accessed on Jan., 2012.

Brasil 2007. Exportações de carne suína para os estados-membros da União Europeia. Circular nº 130/2007/CGPE/DIPOA, Ministério da Agricultura, Pecuária e Abastecimento (MAPA), Brasília, DF. Available at <http://www.agricultura.gov.br/sislegis> Accessed on Dec., 2015.

Brasil 2009. Programa de controle de Listeria monocytogenes em produtos de origem animal prontos para consumo. Instrução Normativa n.9/2009, Diário Oficial da União de 8/09/2009, Seção 1, Brasília, DF. Available at <http://www.agricultura.gov.br/assuntos/inspecao/produtos-animal/controle-de-patogenos/arquivos-controle-de-patogenos/in_09-_de_8_de_abril_de_2009.pdf> Accessed on May 5, 2020.

Brasil 2018. Plano de ação nacional de prevenção e controle da resistência aos antimicrobianos, no âmbito da agropecuária. Ministério da Agricultura, Pecuária e Abastecimento (MAPA), Brasília, DF. Available at <http://www.agricultura.gov.br/assuntos/insumos-agropecuarios/insumos-pecuarios/programas-especiais/resistencia-antimicrobianos/arquivos/copy2_of_publ_panagro_web.pdf> Accessed on May 5, 2020.

Brasileiro A.C.M., Santos M.A.S., Sá C.V.G.C., Rodrigues C.S. & Haddad J.P.A. 2017. National prevalence of Salmonella spp. in pork slaughterhouses under Federal Inspection in Brazil, 2014/2015. 12th International Symposium on the Epidemiology and Control of Biological, Chemical and Physical Hazards in Pigs and Pork, Foz do Iguaçu, PR, p.55-58.

Buncic S., Nychas G.J., Lee R.F., Koutsoumanis K., Hebraud M., Desvaux M., Chorianopoulos N., Bolton D., Blagojevic B. & Antic D. 2014. Microbial pathogen control in the beef chain: recent research advances. Meat Sci. 97(3):288-297. <https://dx.doi.org/10.1016/j.meatsci.2013.04.040> <PMid:23688797>

Cameron-Veas K., Fraile L., Napp S., Garrido V., Grilló M.J. & Migura-Garcia L. 2018. Multidrug resistant Salmonella enterica isolated from conventional pig farms using antimicrobial agents in preventative medicine programmes. Vet. J. 234:36-42. <https://dx.doi.org/10.1016/j.tvjl.2018.02.002> <PMid:29680391>

Campos J., Mourão J., Peixe L. & Antunes P. 2019. Non-typhoidal Salmonella in the pig production chain: a comprehensive analysis of its impact

on human health. Pathogens 8(1):19. <https://dx.doi.org/10.3390/pathogens8010019> <PMid:30700039>

CLSI 2015. Performance standards for antimicrobial disk and dilution susceptibility test for bacteria isolated from animals. CLSI document VET01-S3, Second Informational Supplement, Clinical and Laboratory Standards Institute, Wayne. 128p.

CLSI 2016. Performance standards for antimicrobial susceptibility testing. CLSI document M100-S26, Clinical and Laboratory Standards Institute, Wayne. 256p.

Colello R., Ruiz M.J., Padín V.M., Rogé A.D., Leotta G., Padola N.L. & Etcheverria A.I. 2018. Detercion and charcaterization of Salmonella serotypes in the production chain of two pig farms in Buenos Aires province, Argentina. Front. Microbiol. 9:1370. <https://dx.doi.org/10.3389/fmicb.2018.01370> <PMid:30002649>

Corbellini L.G., Bianco Júnior A., Costa E.F., Duarte A.S.R., Albuquerque E.R., Kich J.D., Cardoso M. & Nauta M. 2016. Effect of slaughterhouse and day of sample on the probability of a pig carcass being Salmonella-positive according to the Enterobacteriaceae count in the largest Brazilian pork production region. Int. J. Food Microbiol. 228:58-66. <http://dx.doi.org/10.1016/j.ijfoodmicro.2016.03.030> <PMid:27107299>

Costa E.F., Cardoso M., Kich J.D. & Corbellini L.G. 2020. A qualitative risk assessment approach to microbial foodborne hazards in Brazilian intensive pork production: a step towards risk prioritization. Microbial Risk Anal. 15(20):100105. <https://dx.doi.org/10.1016/j.mran.2020.100105>

Denis M., Houard E., Fablet A., Rouxel S. & Salvat G. 2013. Distribution of serotypes and genotypes of Salmonella enterica species in French pig production. Vet. Rec. 173(15):370. <https://dx.doi.org/10.1136/vr.101901> <PMid:24106251>

Drummond N., Murphy B.P., Ringwood T., Prentice M.B., Buckley J.F. & Fanning S. 2012. Yersinia enterocolitica: a brief review of the issues relating to the zoonotic pathogen, public health challenges, and the pork production chain. Foodborne Pathog. Dis. 9(3):179-189. <https://dx.doi.org/10.1089/fpd.2011.0938> <PMid:22217012>

Duggan S.J., Mannion C., Predergast D.M., Leonard M., Fanning S., Gonzales-Barron U., Egan J., Butler F. & Duffy G. 2010. Tracking Salmonellla status of pigs and pork from lairage through the slaughter process in the Republic of Ireland. J. Food Protect. 73(12):2148-2160. <https://dx.doi.org/10.4315/0362-028x-73.12.2148> <PMid:21219731>

Dutra M. 2017. Uso de antimicrobianos em suinocultura no Brasil: análise crítica e impacto sobre marcadores epidemiológicos de resistência. Doctoral Dissertation, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo. Available at <https://teses.usp.br/teses/disponiveis/10/10134/tde-31012018-121740/pt-br.php> Accessed on Apr. 16, 2020.

EFSA 2011. Scientific opinion on the public health hazards to be covered by inspection of meat (swine). EFSA J. 9(10):2351. <https://dx.doi.org/10.2903/j.efsa.2011.2351>

EFSA 2018. Listeria monocytogenes contamination of ready-to-eat foods and the risk for human health in EU. EFSA J. 16(1):5134. <https://dx.doi.org/10.2903/j.efsa.2018.5134>

FAO 2000. Manual on meat inspection for developing countries. FAO Animal Production and Health Paper, Food and Agriculture Organization of the United Nations, Rome.

Ferronato A.I., Pellegrini D.C.P., Guerra P. & Cardoso M. 2012. Distribuição de grupos clonais de Listeria monocytogenes em carcaças e no ambiente de matadouros frigoríficos de suínos. Arch. Vet. Sci. 17(3):42-49. <https://dx.doi.org/10.5380/avs.v17i3.24940>

Fredriksson-Ahomaa M., Stolle A. & Stephan R. 2007. Prevalence of pathogenic Yersinia enterocolitica in pigs slaughtered at a Swiss abattoir. Int. J. Food Microbiol. 119(3):207-212. <https://dx.doi.org/10.1016/j.ijfoodmicro.2007.07.050>

Funk J. & Gebreyes W. A. 2004. Risk factors associated with Salmonella prevalence in swine farms. J. Swine Health Prod. 12(5):246-251.

Ghafir Y., China B., De Zutter L. & Daube G. 2008. Hygiene indicator microorganisms for selected pathogens on beef, pork, and poultry meats in Belgium. J. Food. Prot. 71(1):35-45. <https://dx.doi.org/10.4315/0362-028x-71.1.35> <PMid:18236660>

Huovinen E., Sihvonen L.M., Virtanen M.J., Haukka K., Siitonen A. & Kuusi M. 2010. Symptoms and sources of Yersinia enterocolitica-infection: a case-control study. BMC Infect. Dis. 10:122. <https://dx.doi.org/10.1186/1471-2334-10-122> <PMid:20487529>

ISO 2007. Microbiology of food and animal feeding stuffs: horizontal method for the detection of Salmonella spp. ISO6579:2002/Amd. 1:2007, International Standard Organization, Geneva. 9p.

Kerouanton A., Souchaud F., Houdayer C., Houard E., Nagard B., Guionnet J.M., Fougeroux A., Paboeuf F. & Denis M. 2019. Pigs infected experimentally with the same dose of monophasic variant of SalmonellaTyphimurium exhibit different shedding levels. 13th International Symposium on the Epidemiology and Control of Biological, Chemical and Physical Hazards in Pigs and Pork, Berlin, p.157-159.

Kich J.D., Coldebella A., Albuquerque E.R., Cardoso M., Corbellini L.G. & Costa E.F. 2019. Modernização da inspeção sanitária em abatedouros de suínos: inspeção baseada em risco: opinião científica. Documentos 204, Embrapa Suínos e Aves, Concórdia, SC.

Kich J.D., Coldebella A., Mores N., Nogueira M.G., Cardoso M., Fratamico P.M., Call J.E., Fedorka-Cray P. & Luchansky J.B. 2011. Prevalence, distribution, and molecular characterization of Salmonella recovered from swine finishing herds and a slaughter facility in Santa Catarina, Brazil. Int. J. Food Microbiol. 151:307-313. <https://dx.doi.org/10.1016/j.ijfoodmicro.2011.09.024>

Kich J.D., Mores N., Piffer I.A., Coldebella A., Amaral A., Ramminger L. & Cardoso M. 2005. Fatores associados à soroprevalência de Salmonella em rebanhos comerciais suínos. Ciência Rural 35(2):398-405. <https://dx.doi.org/10.1590/S0103-84782005000200024>

Lopes G.V., Pissetti C., Pellegrini D.C.P., Silva L.E. & Cardoso M. 2015. Resistance phenotypes and genotypes of Salmonella enterica subsp. enterica isolates from feed, pigs annd carcasses in Brazil. J. Food Protect. 78(2):407-413. <https://dx.doi.org/10.4315/0362-028X.JFP-14-274> <PMid:25710159>

Lumley T. 2020. Package 'survey'. Version 3.37. Available at <https://cran.r-project.org/web/packages/survey/survey.pdf> Accessed on Mar. 10, 2020.

McDermott P.F., Tyson G.H., Kabera C., Chen Y., Li C., Folster J.P., Lam C., Tate H.P. & Zhao S. 2016. Whole-genome sequencing for detecting antimicrobial resistance in nontyphoidal Salmonella. Antimicrob. Agents Chemother. 60(9):5515-5520. <https://dx.doi.org/10.1128/AAC.01030-16> <PMid:27381390>

McMillan E.A., Gupta S.K., Williams L.E., Jové T., Hiott L.M., Woodley T.A., Barrett J.B., Jackson C.R., Wasileko J.L., Simmons M., Tillman G.E., McClelland M. & Frye J.G. 2019. Antimicrobial resistance genes, cassettes, and plasmids present in Salmonella enterica associated with United States food animals. Front. Microbiol. 10:832. <https://dx.doi.org/10.3389/fmicb.2019.00832> <PMid:31057528>

Monte D.F., Lincopan N., Berman H., Cerdeira L., Keelara S., Thakur S., Fedorka-Cray P.J. & Landgraf M. 2019. Genomic features of high-priority Salmonella enterica servoars circulating in the food production chain, Brazil 2000-2016. Sci. Reports 9:11058. <https://dx.doi.org/10.1038/s41598-019-45838-0>

Oteo J., Aracil B., Alós J.I. & Gómez-Garcés J.L. 2000. High rate of resistance to nalidixic acid in Salmonella enterica: its role as a marker of resistance to fluoroquinolones. Clin. Microbiol. Infect. 6(5):273-276. <https://dx.doi.org/10.1046/j.1469-0691.2000.00058-3.x> <PMid:11168127>

Paim D.S., Pissetti C., Vieira T.R., Werlang G.O., Costa E.F., Kich J.D. & Cardoso M. 2019. Enumeration, antimicrobial resistance and typing of Salmonella entrica: profile of strains carried in the intestinal contentes of pigs at

slaughter in Southern Brazil. Acta Sci. Vet. 47:1636. <https://dx.doi.org/10.22456/1679-9216.89668>

Paixão R., De Gobbi D.D.S., Raimundo D.C., Hofer E., Matte M.H. & Moreno A.M. 2012. Characterization of Yersinia enterocolitica 4/O:3 isolated from slaughterhouses and pork in the State of São Paulo, Brazil, and clinical cases in humans. In: De Almeida A. & Leal N. (Eds), Advances in Yersinia Research - Advances in Experimental Medicine and Biology. Vol. 954. Springer, New York.

Pearce R.A., Bolton D.J., Sheridan J.J., McDowell D.A., Blair I.S. & Harrington D. 2004. Studies to determine the critical control points in pork slaughter hazard analysis and critical control points systems. Int. J. Food Microbiol. 90(3):331-339. <https://dx.doi.org/10.1016/s0168-1605(03)00333-7> <PMid:14751688>

Pissetti C., Werlang G.O., Biesus L.L., Kich J.D. & Cardoso M. 2012. Detecção de Salmonella enterica e Listeria monocytogenes em carcaças suínas na etapa de pré-resfriamento. Acta Scient. Vet. 40(4):1071.

Rosner B.M., Stark K., Höhle M. & Werber D. 2011. Risk factors for sporadic Yersinia enterocolitica infections, Germany 2009-2010. Epidemiol. Infect. 140(10):1738-1747. <https://dx.doi.org/10.1017/S0950268811002664> <PMid:22313798>

SAS 2012. Package 9.3. SAS Institute Inc., Cary, NC.

Scallan E., Hoekstra R.M., Angulo F.J., Tauxe R.V., Widdowson M., Roy S.L., Jones J.L. & Griffin P.M. 2011. Foodborne illness acquired in the United States-major pathogens. Emerg. Infect. Dis. 17(1):7-15. <https://dx.doi.org/10.3201/eid1701.P11101> <PMid:21192848>

Signorell A. 2020. Package 'DescTools'. Version 0.99.32. Available at <https://cran.r-project.org/web/packages/DescTools/DescTools.pdf> Accessed on Mar. 10, 2020.

Silva L.E., Dias V., Ferronatto A., Guerra P., Berno L., Triches N., Kich J.D., Corbellini L.G. & Cardoso M. 2012. Longitudinal dissemination of Salmonella enterica clonal groups through the slaughter process of Salmonella-positive pig batches. J. Food Protect. 75(9):1580-1588. <https://dx.doi.org/10.4315/0362-028X.JFP-11-515> <PMid:22947464>

Silva N., Junqueira V.C.A., Silveira N.F.A., Taniwaki M.H., Santos R.F.S. & Gomes R.AQ.R. 2010. Manual de Métodos de Análise Microbiológica de Alimentos e Água. 4ª ed. São Paulo, Varela.

Valk H., Vaillant V., Jacquet C., Rocourt J., Le Querrec F., Stainer F., Quelquejeu N., Pierre O., Pierre V., Desenclos J.C. & Goullet V. 2001. Two consecutive nationwide outbreaks of listeriosis in France, October 1999 - February 2000. Am J. Epidemiol. 154(10):944-950. <https://dx.doi.org/10.1093/aje/154.10.944> <PMid:11700249>

Viana C., Sereno M.J., Pegoraro K., Yamatogi R.S., Call D.R., Bersot L.S. & Nero L.A. 2019. Distribution, diversity, virulence genotypes and antibiotic resitance for Salmonella isolated from a Brazilian pork production chain. Int. J. Food Microbiol. 310:108310. <https://dx.doi.org/10.1016/j.ijfoodmicro.2019.108310>

Wang X., Biswas S., Paudyal N., Pan H., Li X., Fang W. & Yue M. 2019. Antibiotic resistance in Salmonella Typhimurium isolates recovered from the food chain through National Antimicrobial Resistance Monitoring System between 1996 and 2016. Front. Microbiol. 10:985. <https://dx.doi.org/10.3389/fmicb.2019.00985> <PMid:31134024>

Weagant S.D. & Feng P. 2001. Yersinia, p.421-428. In: Downes F.P. & Ito K. (Eds), Compendium of Methods for Microbiological Examination of Foods. 4th ed. American Public Health Association, Washington D.C.

WHO 2018a. Listeriosis. World Health Organisation. Available at <https://www.who.int/news-room/fact-sheets/detail/listeriosis> Accessed on Dec. 7, 2019.

WHO 2018b. Antimicrobial resistance. World Health Organisation. Available at <https:// https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance> Accessed on Dec. 7, 2019.

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(10):791-797, October 2020
DOI: 10.1590/1678-5150-PVB-6340

Original Article
Small Animal Diseases
BY-NC

Mast cells in the pathophysiology of Duchenne muscular dystrophy in Golden Retriever dogs[1]

Isabela M. Martins[2], Lygia M.M. Malvestio[2,3], Jair R. Engracia-Filho[4], Gustavo S. Claudiano[2,5], Flávio R. Moraes[2] and Julieta R.E. Moraes[2]*

ABSTRACT.- Martins I.M., Malvestio L.M.M., Engracia-Filho J.R., Claudiano G.S., Moraes F.R. & Moraes J.R.E. 2020. **The role of mast cells in the pathogenesis of Duchenne muscular dystrophy in Golden Retriever dogs.** *Pesquisa Veterinária Brasileira 40(10):791-797*. Departamento de Patologia Veterinária, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista "Júlio de Mesquita Filho", Via Prof. Paulo Donato Castellane Km 5, Jaboticabal, SP 14884-900, Brazil. E-mail: julietaengracia@gmail.com

The Golden Retriever muscular dystrophy (GRMD) is one of the best models of Duchenne muscular dystrophy (DMD), with similar genotypic and phenotypic manifestations. Progressive proliferation of connective tissue in the endomysium of the muscle fibers occurs in parallel with the clinical course of the disease in GRMD animals. Previous studies suggest a relationship between mast cells and the deposition of fibrous tissue due to the release of mediators that recruit fibroblasts. The aim of this study was to evaluate the presence of mast cells and their relationship with muscle injury and fibrosis in GRMD dogs of different ages. Samples of muscle groups from six GRMD and four control dogs, aged 2 to 8 months, were collected and analyzed. The samples were processed and stained with HE, toluidine blue, and Azan trichrome. Our results showed that there was a significant increase in infiltration of mast cells in all muscle groups of GRMD dogs compared to the control group. The average number of mast cells, as well as the deposition of fibrous tissue, decreased with age in GRMD dogs. In the control group, all muscle types showed a significant increase in the amount of collagenous tissue. This suggests increased mast cell degranulation occurred in younger GRMD dogs, resulting in increased interstitial space and fibrous tissue in muscle, which then gradually decreased over time as the dogs aged. However, further studies are needed to clarify the role of mast cells in the pathogenesis of fibrosis.

INDEX TERMS: Mast cells, pathogenesis, Duchenne muscular dystrophy, Golden Retriever dogs, canine, dogs, fibrosis, mast cells, muscular dystrophy.

RESUMO.- [**O papel dos mastócitos na patogênese da distrofia muscular de Duchenne em cães Golden Retriever.**] O cão Golden Retriever distrófico (Golden Retriever muscular dystrophy - GRMD) é um dos melhores modelos da distrofia muscular de Duchenne (DMD), com manifestações genotípicas e fenotípicas similares. A proliferação progressiva de tecido conjuntivo no endomísio das fibras musculares ocorre paralelamente ao curso clínico da doença em animais GRMD. Estudos anteriores sugerem uma relação entre os mastócitos e a deposição de tecido fibroso devido à liberação de mediadores que recrutam fibroblastos. O objetivo deste estudo foi avaliar a presença de mastócitos e sua relação com a lesão muscular e fibrose em cães GRMD de diferentes idades. Amostras de grupos musculares de seis GRMD e quatro controles, com idade entre 2 a 8 meses, foram coletadas e analisadas. As amostras foram processadas e coradas com HE, azul de toluidina e tricrômico de Azan. Nossos resultados mostraram que houve um aumento significativo na infiltração de mastócitos em todos os grupos musculares de cães GRMD em comparação com o grupo controle. O número médio de mastócitos, assim

[1] Received on March 12, 2020.
Accepted for publication on April 23, 2020.

[2] Departamento de Patologia Veterinária, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista "Júlio de Mesquita Filho" (Unesp), Via Prof. Paulo Donato Castellane Km 5, Jaboticabal, SP 14884-900, Brazil. *Corresponding author: julietaengracia@gmail.com

[3] Escola de Medicina Veterinária, IMED, Rua Gen. Prestes Guimarães 304, Vila Rodrigues, Passo Fundo, RS 99070-220, Brazil.

[4] Graduate Program in Animal Science, Escola de Ciências da Vida, Pontifícia Universidade Católica (PUC), Rua Imaculada Conceição 1155, Prado Velho, Curitiba, PR 80215-901, Brazil.

[5] Instituto de Biodiversidade e Florestas, Universidade Federal do Oeste do Pará (UFOPA), Campus Santarém, Av. Vera Paz s/n, Santarém, PA 68035-110, Brazil.

como a deposição de tecido fibroso, diminuiu com a idade em cães GRMD. No grupo controle, todos os tipos musculares mostraram um aumento significativo na quantidade de tecido colágeno. Isto sugere o aumento da degranulação de mastócitos em cães GRMD mais jovens, resultando em aumento do espaço intersticial e tecido fibroso no músculo, que então gradualmente diminuiu com o tempo à medida que os cães envelheceram. No entanto, mais estudos são necessários para esclarecer o papel dos mastócitos na patogênese da fibrose.

TERMOS DE INDEXAÇÃO: Mastócitos, patogênese, distrofia muscular de Duchenne, cães Golden Retriever, caninos, fibrose, mastócitos.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a degenerative disease in humans caused by a mutation in the dystrophin gene, affecting one in every 3,500 male live births. DMD is X-linked recessive genetic disorder located on the short arm of the X chromosome (Xp21) (Hoffman et al. 1987, Woolf et al. 2006). The first stage of the disease is characterized by focal groups of necrotic muscle fibers and muscle hypertrophy. Later, repeated cycles of degeneration and regeneration exhaust the regenerative ability statellite cells. Mechanisms of fibrosis then induces the gradual replacement of contractile tissue by connective and fatty tissue (Collins & Morgan 2003, Deconinck & Dan 2007, Miyazato et al. 2011b, Malvestio et al. 2015). Patients affected by DMD display symptoms of muscle weakness. Rapid progression of DMD begins to occur in affected boys younger than five years of age, affecting muscles of the pelvic girdle and shoulder girdle. Patients also exhibit muscular pseudo-hypertrophy of the calf, kyphosis, scoliosis, and difficulty to ambulate during early childhood. Patients are typically confined to a wheelchair by 14 years of age. By 20 to 30 years of age, patients typically exhibit respiratory and/or cardiac complication, resulting in death (Eagle et al. 2002, Gulati et al. 2005, Van Bockel 2009).

Golden Retriever muscular dystrophy (GRMD) affected dogs are one of the best-known animal models of DMD, with genotypic and phenotypic manifestations similar to the human disease (Cooper et al. 1988, Collins & Morgan 2003, Moraes et al. 2017). GRMD dogs are the only animals that display early widespread development of secondary lesions due to deficiencies in dystrophin at two months of age. Disease progression in GRMD dogs is comparable to that of DMD affected boys (Nguyen et al. 2002, Banks & Chamberlain 2008, Lessa et al. 2014a).

Under normal conditions, damage to skeletal muscle due to contractile forces is followed by an inflammatory response, which then subsides after a few days. This transient inflammatory response is a normal homeostatic response to injury that induces muscle repair (Abdel-Salam et al. 2009). Although mutations in dystrophin represent the main cause of DMD, disease progression is exacerbated by secondary processes that are putative for the chronic inflammation. This includes repeated cycles of degeneration and regeneration that deplete the regenerative capacity of satellite cells, and mechanisms that induce the gradual replacement of the contractile muscle tissue by connective and fatty tissue (Collins & Morgan 2003, Deconinck & Dan 2007, Miyazato et al. 2011b, Moraes et al. 2015, Moraes et al. 2017). Movement becomes progressively limited, and dogs show premature signs of fatigue (Gaiad et al. 2011).

Mast cells are activated by a series of chemical and physical signals that induces the cytoplasmic release of various factors in a process known as degranulation. These factors act on various sites to increase epithelium and endothelium permeability by stimulating angiogenesis, inducing contraction of smooth muscle, recruiting neutrophils and macrophages, and stimulating fibroblasts to produce collagen (Gorospe et al. 1994, Abbas et al. 2008, Abdel-Salam et al. 2009, Frenzel & Hermine 2013).

Myocytes lacking dystrophin are more vulnerable to mechanical stress, resulting in the loss of sarcolemmal integrity. Subsequent leakage of cytoplasmic contents stimulates the recruitment of mast cells. DMD affected boys display the first clinical manifestation of dystrophin deficiency at 3 to 5 years of age. In this same period, there is a significant increase in the number of mast cells in the affected muscles, suggesting a causal relationship of mast cells in the pathogenesis of DMD (Gorospe et al. 1994).

It has been suggested that accumulation and subsequent activation of mast cells can result in progression of necrosis and fibrosis, thus resulting in deficient muscle regeneration (Gorospe et al. 1994, Nahirney et al. 1997). In a previous study, mdx mice that received intraperitoneal injections of the mast cell stabilizer cromolyn sodium for 15 days showed reduced myonecrosis in the sternocleidomastoid, anterior tibialis, and diaphragm muscles (Marques et al. 2008).

Therefore, the aim of this study was to evaluate the causal relationship between mast cells and histopathological lesions and the deposition of collagen tissue in four skeletal muscle groups (masseter, diaphragm, brachial, and biceps femoris) of 2 to 8-month-old Golden Retriever dogs affected by muscular dystrophy, in order to establish an inter-relationship of these inflammatory cells in the pathogenesis of fibrosis.

MATERIALS AND METHODS

Animals. The study was performed using six male GRMD dogs aged 2 to 8 months. Four healthy dogs aged 2 to 8 months were used as controls. These animals were obtained from the GRMD-Brazil Kennel at the "Universidade Estadual Paulista" (Unesp). Elevation of serum creatine kinase (CK) was used initially to identify the dogs with dystrophy. Subsequent PCR genotyping was used to confirm the phenotypes (Miyazato et al. 2011b). Respiratory and/or cardiac complications resulted in death in all GRMD dogs. This research was approved by the Ethical Principles of Animal Experimentation adopted by the "Ethics in the use of animals" at the Unesp (protocol no. 01465/15).

Histopathological and morphometric analysis. For analysis study the samples of skeletal muscles masseter, diaphragm, brachial triceps and biceps femoris of the GRMD and of the controls dogs with aged 2, 3, 5 and 8 months were collected, fixed in 10% phosphate buffered formalin, embedded in paraffin, and sectioned at 3μm. Sections were stained with hematoxylin and eosin (HE; histopathology), toluidine blue (identification of mast cells) and Azan trichrome (evaluation of fibrosis) by using standard procedures (Miyazato et al. 2011b).

Morphometric analysis in the number of mast cells was obtained from 50 random fields of the samples (Nahirney et al. 1997) and quantified by automatic sequential numerator KW-TRIO® in a Zeiss Primo Star light microscope (40x objective).

Quantitation of the collagen fibers was performed in 10 random fields of muscle fragments according to Prado et al. (2012). Microscopy was performed on the Leica® DMR light microscope (DFC camera 290) and analyzed with the Leica® Qwin V3 software.

Statistical analysis. Data were analyzed using GraphPad Prism 5 (GraphPad Software Inc., San Diego/CA). The Kolmogorov-Smirnov test was used to examine normality. Multiple comparisons were performed by analysis of variance (ANOVA) and Tukey's test at the significance level of 5% and a correlation between collagen-mast cells of the muscles of dogs was analyzed by the method *Pearson's (5%).*

RESULTS

Histopathology

Histological analysis of muscle samples from control dogs displayed normal muscle patterns, as demonstrated by uniformly distributed muscle fibers with normal diameter, and polyhedral shape surrounded by connective tissue and peripheral cores (Fig.1A and 2A).

However, histological analysis of muscle samples from GRMD dogs revealed degenerative alterations and variations in fiber diameter, infiltration of interfascicular fat cells, and interstitial fibrosis. Necrosis was more frequent in the diaphragm, brachial triceps, and biceps femoris, while less frequent in the masseter. Hyalinization was the most frequent alteration in the masseter, diaphragm, and triceps, while less frequent in the biceps femoris muscle. Inflammatory infiltration of mononuclear cells, particularly giant cells in the interstitium or around the fascicles was more pronounced in the triceps, biceps femoris, and masseter, in contrast to the diaphragm.

Analysis of muscle samples from GRMD dogs revealed mast cells predominantly in fibrotic areas, including the arterioles, venules, and endomysium (Fig.1B and 1C). Azan trichrome staining revealed deposition of collagenous tissue (blue) in the perimysium and endomysium in GRMD dogs (Fig.2B and 2C).

Morphometric analysis of fibro-collagenous and tissue mastocytes

Quantification in the number of mast cells was performed in 40 samples. The highest average count in mast cells was observed in triceps, followed by femoral biceps and masseter muscle ($p<0.05$). The lowest count was observed in diaphragm ($p<0.05$). The concentration of mast cells in the muscle groups of affected animals varied in relation to age group. In all muscle groups, the number of mast cells increased preferentially in the endomysium of younger GRMD dogs. In GRMD dogs, a significant increase in the amount of the infiltrating mast cells was observed, as compared to the control group ($p<0.05$) (Fig.3).

Significant deposition of fibro-collagenous tissue was observed in all GRMD dog muscle samples. The triceps had

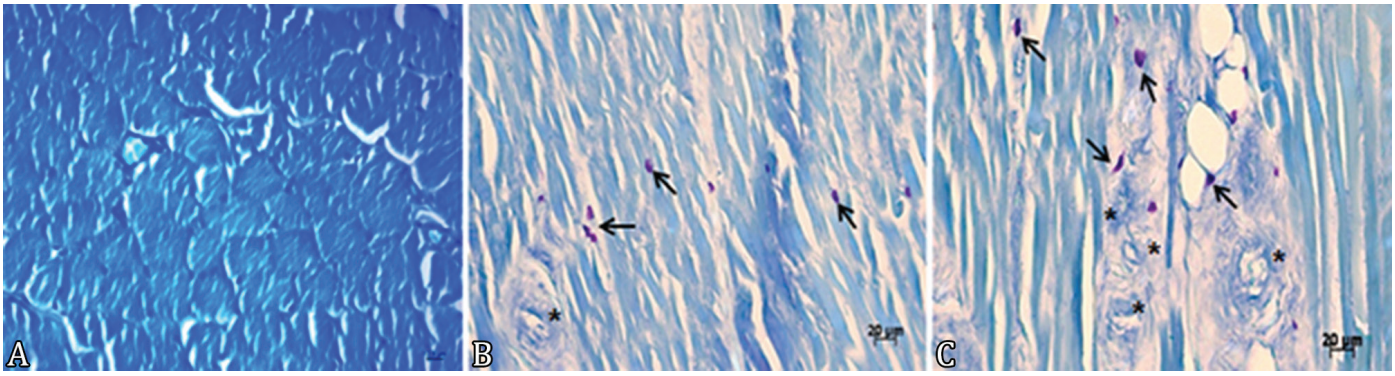


Fig.1. Mast cells in skeletal muscle samples from 2-month-old GRMD dogs. (**A**) Diaphragm. Animal control, muscle fibers distributed with regular diameter. (**B**) Brachial triceps. Presence of mast cells (arrow) in areas of fibrosis and near a venule (*). (**C**) Biceps femoris. Presence of mast cells (arrow) in areas of fibrosis, fatty infiltration, arterioles and venules (*). Toluidine blue, bar = 20µm.

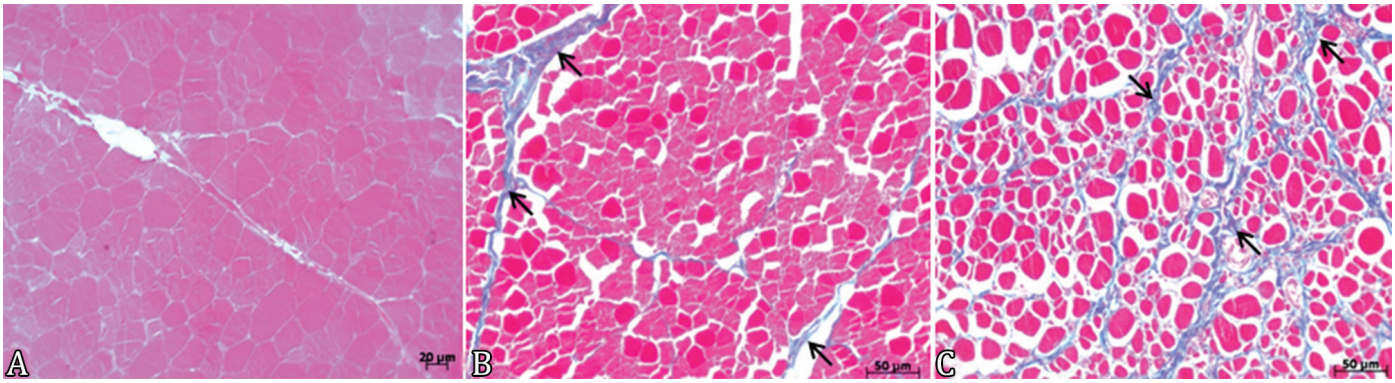


Fig.2. Fibro-collagenous tissue in skeletal muscle samples from GRMD dogs. (**A**) Masseter. Animal control, muscle fibers distributed with regular diameter. Azan trichrome, bar = 20µm. (**B**) Masseter. Mild deposition of fibro-collagenous tissue (arrow) in epimysium and endomysium in an 8-month-old dog. Azan trichrome, bar = 50µm. (**C**) Biceps Femoris. Intense deposition of fibro-collagenous tissue (arrow) in the endomysium in a 2-month-old dog. Azan trichrome, bar = 50µm.

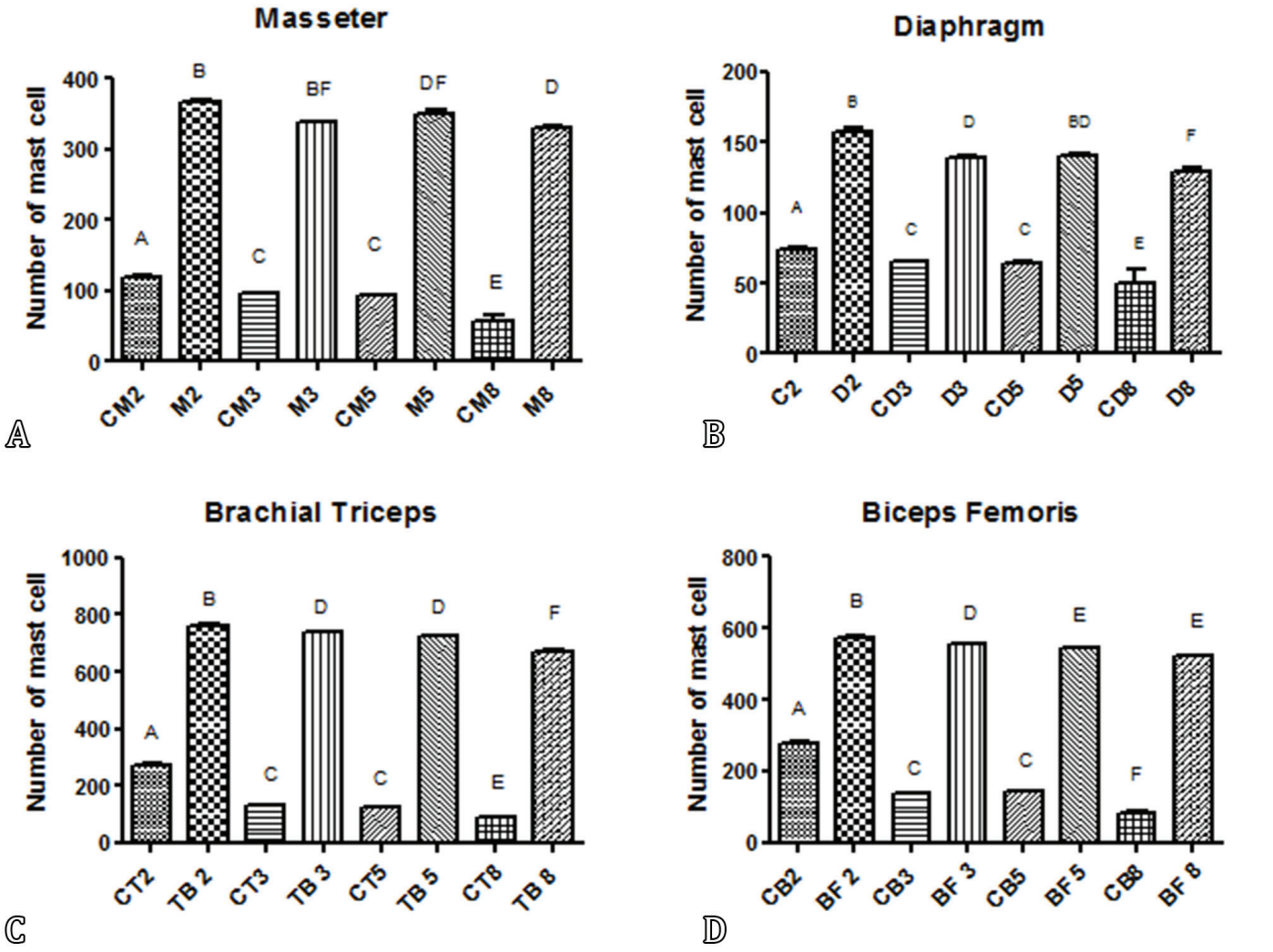


Fig.3. Number of mast cells in (**A**) Masseter (M) and Masseter control group (CM), (**B**) diaphragm (D) and diaphragm control group (CD), (**C**) brachial triceps (TB) and brachial triceps control group (CT), (**D**) biceps femoris (BF) and biceps femoris control group (CF) from GRMD dogs aged 2, 3, 5 and 8 months respectively. Vertical columns represent means of each group in the sampling times. Columns with letters in common do not differ by Tukey's test ($p<0.05$).

the highest amounts of this tissue, followed by biceps femoris, diaphragm, and masseter. The amount of fibro-collagenous tissue varied in relation to the muscle group and age group in all muscle groups studied. When compared to the control group, GRMD dogs had a significant increase in the amount of collagenous tissue in all muscle groups ($p<0.05$), as measured in percentage of tissue per field (Fig.4). It was observed a correlation ($p<0.05$) between collagen and mast cells in the muscles of these dogs, it was verified one stronger linear correlation in triceps with dystrophy in animals of 2 and 3 months of age, both r=0.99 and coefficient of determination (r^2) in 0.98 and 0.99, respectively (Table 1). In biceps femoris resembled (r=0.95, r^2=0.90) (Table 1).

DISCUSSION

In this study, GRMD dogs from 2 to 8 months in age have been characterized by variations in muscle fiber diameter, necrosis, hyalinization, mononuclear cells infiltration, fatty infiltration, and interstitial fibrosis in four muscle groups: the masseter, diaphragm, triceps, and biceps femoris. These observations are consistent with previously published studies of GRMD dogs of different ages (Beretta et al. 2014, Miyazato et al. 2011a, 2011b).

Our results suggest the masseter muscle presented a lower incidence of injuries among the four muscles evaluated. Minimal degeneration of the masseter may be due to smaller muscle fiber diameters and minimal exposure to mechanical stress as compared to muscles located in the fore and hind limbs (Lee et al. 2006).

We observed an increase in the number of mast cells in dystrophic animals compared to control group animals. This is consistent with a previous study of DMD patients with a mean age of 41 months, where an increase in the number of mast cells was observed compared to control group. These mast cells were immuno-positive for neural growth factor (NGF), while it was negative for other inflammatory cells such as lymphocytes and macrophages (Toti et al. 2003). Quantification of mast cells revealed a higher number of mast cells in the triceps, biceps

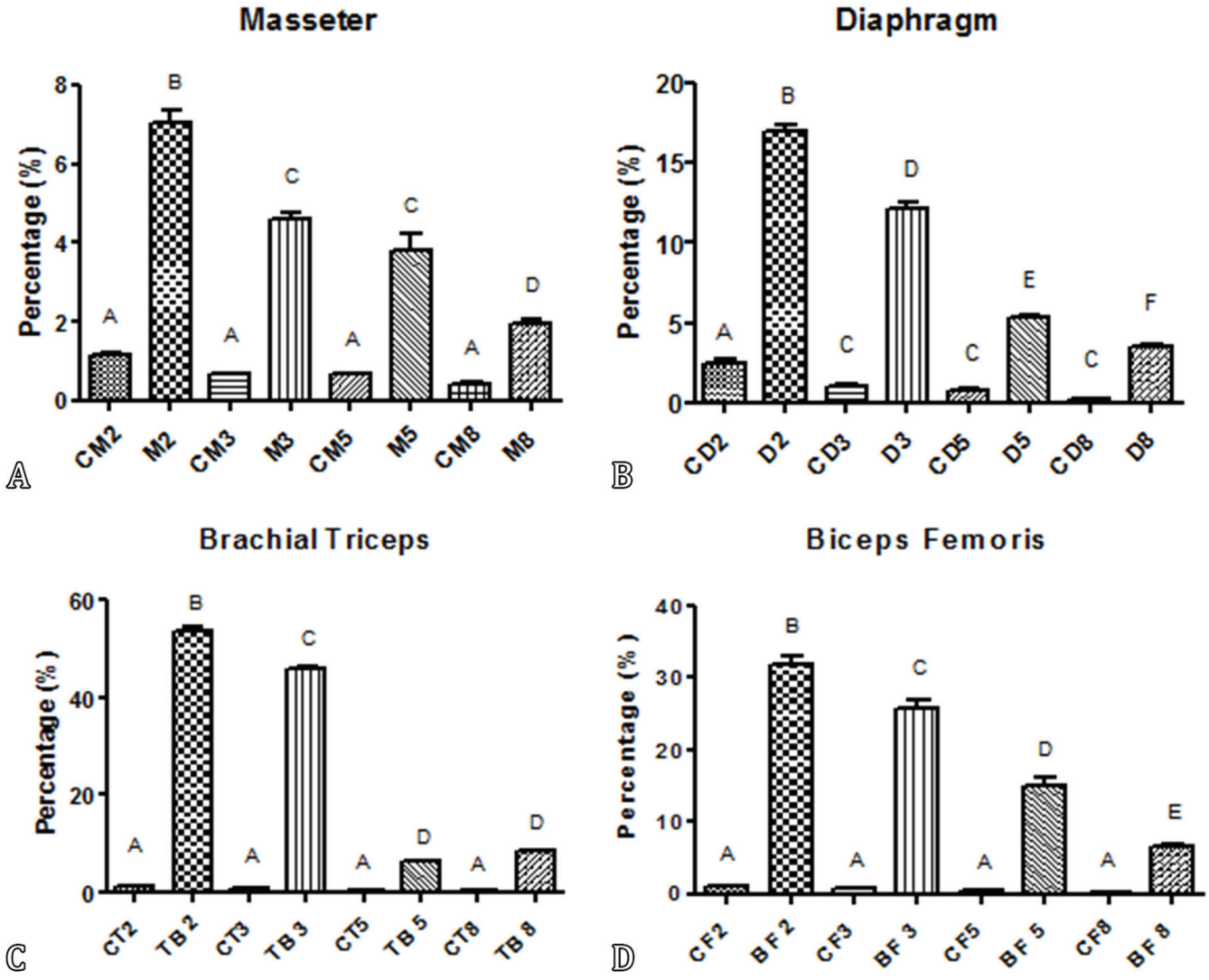


Fig.4. Deposition of fibro-collagenous tissue in (**A**) Masseter (M) and Masseter control group (CM), (**B**) diaphragm (D) and diaphragm control group (CD), (**C**) brachial triceps (TB) and brachial triceps control group (CT), (**D**) biceps femoris (BF) and biceps femoris control group (CF) from GRMD dogs aged 2, 3, 5 and 8 months respectively. Vertical columns represent means of each group in the sampling times. Columns with letters in common do not differ by Tukey's test ($p<0.05$).

Table 1. Ratio fibro-collagenous-mast cells in skeletal muscle samples from golden retriever muscular dystrophy dogs of four times and control

Treatments		Age of animals							
		2 months		3 months		5 months		8 months	
		D	C	D	C	D	C	D	C
Masseter	r	0.36	- 1.0 -	0.39	0.6 -	0.79 -	0.14	0.50	- 0.33 -
	r^2	0.13	0.01	0,15	0.4	0.63	0.02	0.50	0.11
	p-value	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05
Diaphragm	r	- 0.57 -	- 0.50 -	0.92 -	0.31	0.90	- 0.08 -	0.96	0.98
	r^2	0.32	0.30	0.86	0.09	0.81	0.007	0.92	0.97
	p-value	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05
Brachial triceps	r	0.99 -	0.70 -	0.99	0.57	0.90 -	0.8	- 0.096 -	0.2
	r^2	0.98	0.50	0.99	0.33	0.94	0.7	0.092	0.05
	p-value	p<0.05	p>0.05	p<0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05
Biceps femoris	r	0.95 -	0.60 -	0.70	0.99 -	- 0.64 -	- 0.7 -	0.79	0.82 -
	r^2	0.90	0.30	0.50	0.99	0.41	0.51	0.63	0.6
	p-value	p>0.05	p>0.05	p>0.05	p<0.05	p>0.05	p>0.05	p>0.05	p>0.05

D = Animals with dystrophy, C = control; r = Pearson's correlation coefficient with significance, r2 = coefficient of determination.

femoris, and masseter of 2-month-old dogs. These muscles are more severely affected by the inflammatory process in younger animals due to high usage and activity during the post-natal period (Valentine & Cooper 1991, Nguyen et al. 2002, Miyazato 2010), thereby suggesting a direct relationship between muscle degeneration and the number of mast cells.

We also observed a decrease in the average number of mast cells and percentage of deposition of collagenous tissue the diaphragm, indicating a lower degree of lesion in this muscle. This is consistent with a previous study, in which eleven muscles from GRMD dogs of varying ages revealed that the diaphragm was the least affected muscle, while showing a greater regenerative capacity similar to the masseter muscle (Miyazato 2010). However, the diaphragm of human DMD patients is the most affected muscle in adulthood. These structural deformities likely result from confinement to a wheelchair (Lessa et al. 2014b). Therefore, the degree of lesion in the diaphragm of DMD patients could be associated with age. In GRMD dogs, this occurs in younger animals, in contrast to DMD patients of older age.

Furthermore, another study showed that 3-month-old GRMD dogs displayed a higher number of mast cells in the peroneus longus muscle as compared to 6-month-old GRMD dogs. Comparatively, 6-month-old GRMD dogs correspond to 11-year-old patients with DMD (Gorospe et al. 1994), a period for which a wheelchair is typically needed (Gorospe et al. 1994). The degree of lesions may be related to inflammatory response and muscle activity, since patients in wheelchairs had limited movement (Gorospe et al. 1994).

Another study showed, the 12-week physical therapy protocol consisting of active exercises 3 times a week for 40 minutes with rest intervals demonstrated negative effects on walking speed and increased the amount of type I collagen in 5-months-old dystrophic dogs (Gaiad et al. 2014). The degree of injury may be related to inflammatory response, as well as muscle activity, since patients in wheelchairs reduce the movement of members.

The interrelationship of mast cells in the pathogenesis of fibrosis in GRMD dogs is poorly understood. In this study, we observed fibrosis in animals as early as 2 months of age. Previous studies with GRMD dogs of varying ages displayed significant increases in the deposition of fibrous tissue in the quadriceps and tibialis anterior muscles (Cozzi et al. 2001). However, no increase of fibrous tissue deposition in relation to age was observed. The dystrophic process, including replacement of muscle by fibrotic tissue, was evident in 15 days old animals, well before the completion of the muscle maturation (Cozzi et al. 2001). This is in contrast to that observed in DMD patients, in which fibrosis deposition is correlated with age (Cozzi et al. 2001). Decreased fibrosis with the increase in age may be due to depletion of muscle potential, followed by repeated cycles of degeneration and regeneration (Cozzi et al. 2001). The extent of proliferation of the connective tissue is also variable in DMD patients, despite the fact that fibrosis generally increases with the progression of the disease.

The first stage after muscle injury is the formation of hematoma between damaged fibers that is rapidly invaded by inflammatory cells and cytokines. In addition to stimulating the functions of satellite cells, these molecules also induce massive proliferation and activation of fibroblasts. Fibrosis is the aberrant deposition of extracellular matrix (ECM) during the healing, DMD and some other muscular pathologies, it might ultimately lead to substitution of skeletal and cardiac muscle by fibrous tissue (Serrano & Muñoz-Cánoves 2010, Serrano et al. 2011). An alternative explanation this study could be that higher mast cells track with overall inflammation and edema, which could potentially expand the extracellular space, including the interstitium (endomysium and perimysium). However, in older dogs, you would no longer expect to see this degree of active disease.

Current pharmacological study evaluated the bortezomib an inhibitor of the ubiquitin-proteasome pathway associated with degradation of the dystrophin-glycoprotein complex (DGC). Study has shown a decrease in the deposition of fibrous tissue and inflammatory cells in the skeletal muscle of 4-months-old GRMD dogs treated for 4 weeks with bortezomib. However, in this study CK levels presented altered indicating that the disease progressed despite treatment (Araujo et al. 2013).

Our findings confirm the hypothesis that mast cells are more numerous muscles containing defective dystrophin, as well as participate in the histopathologic characterization of lesions and deposition of collagenous tissue in the muscle fibers. The femoral biceps, triceps, and masseter displayed a higher average number of mast cells and minor lesions, thus suggesting a direct relationship between the intensity of the injury and the number of mast cells.

CONCLUSIONS

The results demonstrate a direct relationship between the histopathological lesions and the number of mast cells and collagen fibers in skeletal muscle of four groups of dogs GRMD from 2 to 8 months of age. The age group of 2 months presented the highest average number of mast cells and collagenous tissue deposition and in contrast the age of 8 months presented the lowest average number and lowest deposition of fibrous tissue.

Nevertheless, more studies are needed to clarify the role of mast cells in the pathogenesis of fibrosis in order to contribute to the development of new therapeutic approaches and improve the life expectancy of humans and dogs affected by muscular dystrophy.

Acknowledgments.- The authors are grateful for the support of the "Fundação de Amparo à Pesquisa do Estado de São Paulo" (FAPESP), grant number 13/25957-6 and "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES).

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

REFERENCES

Abbas A.K., Lichtman A.H. & Pillai S. 2008. Hipersensibilidade imediata, p.442-461. In: Abbas A.K. (Ed.), Imunologia Celular e Molecular. 6ª ed. Elsevier, Rio de Janeiro.

Abdel-Salam E., Abdel-Meguid I. & Korraal S.S. 2009. Markers of degeneration and regeneratio in Duchenne muscular dystrophy. Acta Myol. 28(3):94-100. <PMid:20476668>

Araujo K.P.C., Bonucelli G., Duarte C.N., Gaiad T.P., Moreira D.F., Feder D., Belizario J.E., Miglino M.A., Lisanti M.P. & Ambrosio C.E. 2013. Bortezomid (PS-341) treatment decreases inflammation and partially rescues the expression of the dystrophin-glycoprotein complex in GRMD dogs. Plos One 8(4):e61367. <https://dx.doi.org/10.1371/journal.pone.0061367> <PMid:23579193>

Banks G.B. & Chamberlain J.S. 2008. The value of mammalian models for duchenne muscular dystrophy in developing therapeutic strategies. Curr. Top. Dev. Biol. 84:431-453. <https://dx.doi.org/10.1016/S0070-2153(08)00609-1> <PMid:19186250>

Beretta D.C., Moraes J.R.E., Engracia-Filho J.R., Malvestio L.M.M. & Moraes F.R. 2014. Immunohistochemical and morphometric evaluation of gastrocnemius muscle and myotendinous junction of Golden Retriever dogs with muscular dystrophy. Braz. J. Vet. Pathol. 7(2):70-81.

Collins C.A. & Morgan J.E. 2003. Duchenne's muscular dystrophy: animal models used to investigate pathogenesis and develop therapeutic strategies. Int. J. Exp. Pathol. 84(4):165-172. <https://dx.doi.org/10.1046/j.1365-2613.2003.00354.x> <PMid:14632630>

Cooper B.J., Winand N.J. & Stedman H., Valentine B.A., Hoffman E.P., Kunkel L.M., Marion-Oronzi S., Fischbeck K.H., Kornegay J.N., Avery R.J., Williams J.R., Schmickel R.D. & Sylvester J.E. 1988. The homologue of the Duchenne locus is defective in X-linked muscular dystrophy of dogs. Nature 334:154-156. <https://dx.doi.org/10.1038/334154A0>

Cozzi F., Cerletti M., Luvoni G.C., Lombardo R., Brambilla P.G., Faverzani S., Blasevich F., Cornelio F., Pozza O. & Mora M. 2001. Development of muscle pathology in canine X-linked muscular dystrophy. II. Quantitative characterization of histopathological progression during postnatal skeletal muscle development. Acta Neuropathol. 101(5):469-478. <https://dx.doi.org/10.1007/s004010000308> <PMid:11484818>

Deconinck N. & Dan B. 2007. Pathophysiology of Duchenne muscular dystrophy: current hypotheses. Pediatr. Neurol. 36(1):1-7. <https://dx.doi.org/10.1016/j.pediatrneurol.2006.09.016> <PMid:17162189>

Eagle M., Baudouin S.V., Chandler C., Giddings D.R., Bullock R. & Bushby K. 2002. Survival in Duchenne muscular dystrophy: Improvements in life expectancy since 1967 and the impact of home nocturnal ventilation. Neuromuscul. Disord. 12(10):926-929. <https://dx.doi.org/10.1016/s0960-8966(02)00140-2> <PMid:12467747>

Frenzel L. & Hermine O. 2013. Mast cells and inflammation. Joint Bone Spine 80(2):141-145. <https://dx.doi.org/10.1016/j.jbspin.2012.08.013> <PMid:23116710>

Gaiad T.P., Araujo K.P.C., Serrão J.C., Miglino M.A. & Ambrosio C.E. 2014. Motor physical therapy affects muscle collagen type I and decreases gait speed in dystrophin-deficient dogs. Plos One 9(4):1-9. <https://dx.doi.org/10.1371/journal.pone.0093500> <PMid:24713872>

Gaiad T.P., Silva M.B., Silva G.C.A., Caromano F.A., Miglino M.A. & Ambrósio C.E. 2011. Physical therapy assessment tools to evaluate disease progression and phenotype variability in Golden Retriever muscular dystrophy. Res. Vet. Sci. 91(2):188-193. <https://dx.doi.org/10.1016/j.rvsc.2011.01.007> <PMid:21315399>

Gorospe J.R.M., Tharp M.D., Hinckley J., Kornegay J.N. & Hoffman E.P. 1994. The role for mast cells in the progression of Duchenne muscular dystrophy? Correlations in dystrophin-deficient humans, dogs, and mice**.** J. Neurol. Sci. 122(1):44-56. <https://dx.doi.org/10.1016/0022-510X(94)90050-7>

Gulati S., Saxena A., Kumar V. & Kalra V. 2005. Duchenne muscular dystrophy: prevalence and patterns of cardiac involvement. Indian J. Pediatr. 72(5):389-393. <https://dx.doi.org/10.1007/BF02731732> <PMid:15973020>

Hoffman E.P., Brown-Junior R.H. & Kunkel LM. 1987. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 51(6):919-928. <https://dx.doi.org/10.1016/0092-8674(87)90579-4> <PMid:3319190>

Lee W.H., Abe S., Kim H.J., Usami A., Honda A., Sakiyama K. & Ide Y. 2006. Characteristics of muscle fibers reconstituted in the regeneration process of masseter muscle in an mdx mouse model of muscular dystrophy. J. Muscle Res. Cell Motil. 27(3/4):235-240. <https://dx.doi.org/10.1007/s10974-006-9066-5> <PMid:16752197>

Lessa T.B., Abreu D.K., Rodrigues M.N., Brólio M.P., Miglino M.A. & Ambrósio C.E. 2014a. Morphological and ultrastructural evaluation of the Golden Retriever muscular dystrophy trachea, lungs, and diaphragm muscle. Microsc. Res. Tech. 77(11):857-861. <https://dx.doi.org/10.1002/jemt.22408> <PMid:25081087>

Lessa T.B., Carvalho R.C., Spagnolo J.D., Silva L.C., Cortopassi S.R. & Ambrósio C.E. 2014b. Laparoscopic guided local injection in the X-linked muscular dystrophy mouse (mdx) diaphragm. An advance in experimental therapies for Duchenne muscular dystrophy. Acta Cir. Bras. 29(11):715-720. <https://dx.doi.org/10.1590/S0102-86502014001800004>

Malvestio L.M.M., Martins I.M., Moares F.R. & Moraes J.R.E. 2015. Histopathologic evolution of cardiomyopathy in a canine model of Duchenne muscular dystrophy. J. Advanc. Vet. Res. 5(3):121-126.

Marques M.J., Machado R.V., Minatel E. & Santo-Neto H. 2008. Disodium cromoglycate protects dystrophin-deficient muscle fibers from leakiness. Muscle Nerve 37(1):61-67 <https://dx.doi.org/10.1002/mus.20892> <PMid:17724738>

Miyazato L.G., Beretta D.C., Engracia-Filho J.R., Moraes F.R. & Moraes J.R.E. 2011a. Evaluation of intracellular calcium in Golden Retriever muscular dystrophy. Braz. J. Vet. Pathol. 4(2):95-102.

Miyazato L.G., Beretta D.C., Engracia-Filho J.R., Moraes F.R. & Moraes J.R.E. 2011b. Involvement of organic systems in golden retriever X-linked muscular dystrophy. Braz. J. Vet. Pathol. 4(2):87-94.

Miyazato L.G. 2010. Histopatologia e imunoistoquímica na Distrofia Muscular do Golden Retriever. Doctoral Dissertation, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Jaboticabal. 89p.

Moraes J.R.E., Malvestio L.M.M., Martins I.M. & Moraes F.R. 2015. The value of dog for knowledge of human Duchenne muscular dystrophy: New findings in pathogenesis and therapeutic advances, p.141-153. In: Honório S.A.A. (Ed.), Duchenne Muscular Dystrophy: symptoms, management and prognosis. Nova Science Publishers, New York.

Moraes J.R.E., Malvestio L.M.M., Martins I.M., Mosko P.R.E, Engracia-Filho J.R. & Moraes F.R. 2017. Relevant aspects of Golden retriever muscular dystrophy for the study of Duchenne muscular dystrophy in humans. Ciência Rural 47(10):1-11. <https://dx.doi.org/10.1590/0103-8478cr20160470>

Nahirney P.C., Dow P.R. & Ovalle W.K. 1997. Quantitative morphology of mast cells in skeletal muscle of normal and genetically dystrophic mice. Anat. Rec. 247(3):341-349. <https://dx.doi.org/10.1002/(SICI)1097-0185(199703)247:3<341::AID-AR5>3.0.CO;2-X> <PMid:9066911>

Nguyen F., Cherel Y., Guiand L., Goubault-Leroux I. & Wyers M. 2002. Muscle lesions associated with dystrophin deficiency in neonatal golden retriever puppies. J. Comp. Pathol. 126(2/3):100-108. <https://dx.doi.org/10.1053/jcpa.2001.0526> <PMid:11944998>

Prado C.M., Celes M.R.N., Malvestio L.M., Campos E.C., Silva J.S., Jelicks L.A., Tanowitz H.B. & Rossi M.A. 2012. Early dystrophin disruption in the pathogenesis of experimental chronic chagas cardiomyopathy. Microbes Infect. 14(1):59-68. <https://dx.doi.org/10.1016/j.micinf.2011.08.010>

Serrano A.L. & Muñoz-Cánoves P. 2010. Regulation and dysregulation of fibrosis in skeletal muscle. Exp. Cell Res. 316(18):3050-3058. <https://dx.doi.org/10.1016/j.yexcr.2010.05.035> <PMid:20570674>

Serrano A.L., Mann C.J., Vidal B., Ardite E., Perdiguero E. & Muñoz-Cánoves P. 2011. Cellular and molecular mechanisms regulating fibrosis in skeletal muscle repair and disease. Curr. Top. Dev. Biol. 96:167-282. <https://dx.doi.org/10.1016/B978-0-12-385940-2.00007-3> <PMid:21621071>

Toti P., Villanova M., Vatti R., Schuerfeld K., Stumpo M., Barbagli L., Malandrini A. & Costantini M. 2003. Nerve growth fator expression in human dystrophic muscles. Muscle Nerve 27(3):370-373. <https://dx.doi.org/10.1002/mus.10332> <PMid:12635125>

Valentine B.A. & Cooper B.J. 1991. Canine X-linked muscular dystrophy: selective involvement of muscles in neonatal dogs. Neuromuscul. Disord. 1(1):31-38. <https://dx.doi.org/10.1016/0960-8966(91)90040-Y>

Van Bockel E.A., Lind J.S., Zijlstra J.G., Wijkstra P.J., Meijer P.M., Van den Berg M.P., Slart R.H., Aarts L.P. & Tulleken J.E. 2009. Cardiac assessment of patients with late stage Duchenne muscular dystrophy. Neth. Heart J. 17(6):232-237. <https://dx.doi.org/10.1007/BF03086253> <PMid:19789685>

Woolf P.J., Lu S., Cornford-Nairn R., Watson M., Xiao X.H., Holroyd S.M., Brown L. & Hoey A.J. 2006. Alterations in dihydropyridine receptors in dystrophin-deficient cardiac muscle. Am. J. Physiol. Heart Circ. Physiol. 290(6):2439-2445. <https://dx.doi.org/10.1152/ajpheart.00844.2005> <PMid:16415078>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(10):798-803, October 2020
DOI: 10.1590/1678-5150-PVB-6709

Original Article
Small Animal Diseases
BY-NC

Use of bone marrow for detection of toxic chemicals for the elucidation of poisoning in forensic veterinary medicine[1]

Sóstenes A.C. Marcelino[2], Rogéria Serakides[2], Viviane N. Castro-Silva[2], Maria L. Ramos[2], Natália M. Ocarino[2] and Marília M. Melo[2]*

ABSTRACT.- Marcelino S.A.C., Serakides R., Castro-Silva V.N., Ramos M.L., Ocarino N.M. & Melo M.M. 2020. **Use of bone marrow for detection of toxic chemicals for the elucidation of poisoning in forensic veterinary pathology**. *Pesquisa Veterinária Brasileira 40(10):798-803*. Escola de Veterinária, Universidade Federal de Minas Gerais, Av. Presidente Antônio Carlos 6627, Belo Horizonte, MG 31275-013, Brazil. E-mail marilia.melo@pq.cnpq.br

In forensic toxicology, the detection of toxic chemicals from human bone marrow is often used in cases with an extended *post mortem* interval; however, in veterinary medicine, this practice is not used. Therefore, this study was performed to investigate the suitability of bone marrow for toxicological analysis in dogs and cats. Six animals with suspected poisoning were selected; the carcasses were sent for necropsy, and the organs were collected and preserved in buffered formalin and processed routinely for histological examination. In addition, bone marrow samples from the femur, humerus, and tibia were collected for toxicological analysis by liquid chromatography coupled to mass spectrometry detection (LC-MS). This analysis confirmed the presence of aldicarb, aldicarb sulfone, asulam, carbendazim, chlorpyrifos, dichlorvos, thifensulfuron methyl and trifloxysulfuron-sodium and associated with clinical symptoms and anatomo-histopathological alterations it was recognized the poisonings. It is expected that this study will promote the toxicological investigation of bone marrow and open avenues for the use of this tissue as an option for the detection of toxic chemicals in cases of forensic pathology.

INDEX TERMS: Bone marrow, toxic chemicals, poisoning, forensic toxicology, aldicarb, dichlorvos, chlorpyrifos, pesticides, herbicides, crimes.

RESUMO.- **[Uso de medula óssea para detecção de produtos químicos tóxicos para a elucidação de intoxicação em patologia veterinária forense].** Na toxicologia forense, a detecção de substâncias químicas tóxicas provenientes da medula óssea humana é frequentemente usada em casos com intervalo *post mortem* prolongado; no entanto, na medicina veterinária, essa prática não é utilizada. Portanto, este estudo foi realizado para investigar a utilização da medula óssea nas análises toxicológicas em cães e gatos. Seis animais com suspeita de intoxicação foram selecionados; as carcaças foram enviadas para necropsia e os órgãos foram coletados e preservados em formalina tamponada e processados rotineiramente para exame histológico. Amostras de medula óssea de fêmur, úmero e tíbia foram coletadas para análise toxicológica por cromatografia líquida acoplada à espectrometria de massa-massa (LC-MS). A análise por LC-MS confirmou a presença dos agrotóxicos aldicarbe, aldicarbe sulfona, asulam, carbendazim, clorpirifós, diclorvós, tifensulfuron metil e trifloxisulfuron-sódico, e em associação com sinais clínicos e achados anatomo-histopatológicos comprovou-se as intoxicações. Espera-se que este estudo promova a utilização da medula óssea como uma opção na investigação toxicológica para a detecção de produtos químicos tóxicos em casos de patologia forense.

TERMOS DE INDEXAÇÃO: Medula óssea, produtos químicos tóxicos, intoxicação, toxicologia forense, aldicarbe, diclorvos, clorpirifós, agrotóxicos, herbicidas, crimes.

INTRODUCTION

Intentional exogenous poisoning is one of the main causes of crimes against animals leading to death. Carbamates and organophosphates are the most common active compounds used in criminal poisoning (Siqueira et al. 2015). Regardless of the toxic chemicals suspected, samples of the stomach contents, kidney, liver and adipose tissues are collected for

[1] Received on August 6, 2020.
Accepted for publication on August 20, 2020.
[2] Escola de Veterinária, Universidade Federal de Minas Gerais (UFMG), Av. Presidente Antônio Carlos 6627, Belo Horizonte, MG 31275-013, Brazil.
*Corresponding author: marilia.melo@pq.cnpq.br

routine toxicological examination. However, carcasses in an advanced state of decomposition or skeletonization can compromise *post mortem* evaluation and make it difficult to process samples for toxicological analysis because of inadequate soft tissue content (Fukushima et al. 2018).

In such cases, bones have emerged as vital for macroscopic and toxicological evaluations. In forensic science, toxicological examination of human bones, particularly bone marrow, is used for the detection of psychoactive illicit drugs, and some pesticides, involved in cases of suicide, overdose, and poisoning among professionals working with these substances (McIntyre et al. 2000, Drummer 2008, Cartiser et al. 2011). As the bone marrow is located inside the medullary canal, it is protected from external contaminations and the heterolytic actions of putrefying bacteria. Thus, its structure is preserved for a longer period than other tissues (Cartiser et al. 2011) and it was showed that the histological preservation of bone marrow for up to 3 months after death (Grellner & Glenewinkel 1997).

In addition to its greater resistance to putrefaction, the bone marrow is highly vascular with a high lipid content that favors the accumulation of lipophilic drugs. Owing to these characteristics, bone marrow can be considered as a potential tissue for the toxicological analysis of skeletal bodies or cadavers with extended *post mortem* intervals (Cartiser et al. 2011).

The first use of bone marrow in forensic toxicology was used to study the kinetics of alcohol (Casier et al. 1943). In the 1980s, further studies examined the correlation between toxic chemicals concentrations in the bone marrow and in the blood of poisoned animal models and humans (Winek & Esposito 1981, Winek & Luhanik 1981, Winek & Janssen 1982, Winek et al. 1982, 1983, 1985). Bone marrow from skeletal remains has only recently been studied to investigate the use of xenobiotics and correlate their use with mortality (Cartiser et al. 2011).

In the study of bone marrow, different collection sites may be used, such as the femur (Winek & Jones 1980), ribs (Schloegl et al. 2006) or vertebral bodies (Maeda et al. 2006). Nevertheless, a standard sampling procedure has not yet been established in the scientific community. Depending on the lipophilicity of the drug, the concentration may depend on the amount of yellow or red bone marrow present (Cartiser et al. 2011). Many substances such as, ethanol, bromoisoval, diazepam, flurazepam, triazolam, amitriptyline, nortriptyline, desipramine, doxepin, sertraline, moclobemide, cocaine, methamphetamine, amphetamine, morphine and paraquat could be detected in bone marrow (Drummer 2008). However, in veterinary medicine, there are no studies using bone marrow as a site for the detection of toxic chemicals. Thus, the aim of the present study was to investigate toxic chemicals in the bone marrow of dogs and cats with suspected exogenous intoxication.

MATERIALS AND METHODS

In this study, six animals (three dogs and three cats) were necropsied, and fragments of abdominal and thoracic organs were collected in 10% buffered formalin for histological analysis. The organs were processed by a routine paraffin inclusion technique, and histological sections of 3–5µm thickness were stained with hematoxylin and eosin (HE).

The bone marrow of the femur, tibia and humerus was collected of each animal, weighed and a pool was used to toxicological analysis. Chromatographic analyses were performed using an ultra-fast liquid chromatographic (UFLC) system (Shimadzu LC20) equipped with a binary pump (Shimadzu LC20ADXR), an autosampler (Shimadzu SIL20ACXR), and a column oven (Shimadzu CTO20AC).

Each bone marrow pool was extracted by modified QuEChERS method (Anastassiades et al. 2003). The sample (5.0g) was transferred to a polypropylene tube (50mL), following the addition 5.0mL of ultrapure water and 10.0mL of acetonitrile/0.1% acetic acid and ethyl acetate (70:30, v/v), and the mixture was vortexed for 1 minute. Magnesium sulfate (4.0g) and sodium acetate (1.0g) were added, and the mixture was vortexed for another 1 minute and centrifuged at 4,000 rpm for 11 minutes. Subsequently, the mixture was kept at −20°C overnight (12 hours) in order to break emulsions and then centrifuged at 4,000 rpm, −5°C, for 5 minutes. The organic layer (1.0mL) was transferred to a 2.0mL microtube containing 150mg of anhydrous magnesium sulfate, 30mg of primary and secondary amine (PSA), and 30mg of dispersive C18. Tubes were shaken for 1 minute and centrifuged at 4,000 rpm for 12 minutes. The extracts were used for chromatographic analyses.

A total of 171 analytes from different classes was used to study the full scope of the method. All pesticides were evaluated by LC-MS using a multiresidue analysis technique and this analysis was carried out using a 5500 Triple Quad mass spectrometer (Applied Biosystems, MDS SCIEX, Ontario, Canada). The instrument was operated using electrospray ionization source (ESI) in positive and negative ion modes. Instrument settings, data acquisition and data processing were controlled by the Analyst software (Version 1.5.1, Applied Biosystems). Source parameters were optimized as follows: ion spray voltage, 5.5 kV for ESI (+) and 4.5 kV for ESI (-); curtain gas, 20psi; collision gas, 8psi; nebulizer gas, 30psi; auxiliary gas, 30psi; ion source temperature, 500°C.

Separations were achieved using a Shimadzu Shim-pack XR-ODSII column (2.0 × 100mm, 2.2µm particle size) and a Phenomenex Synergi Fusion-RP column (2.0 × 50mm, 2.5µm particle size). The column temperature was set to 60°C.

The samples were injected into the LC-MS/MS system under the following conditions: flow rate, 0.5mL/minute; injection volume, 5µL; column furnace temperature, 60°C; automatic sampler temperature; 20°C; and total run time, 13 minutes. These conditions were established internally by the laboratory and were used after validation in studies (Oliveira et al. 2018).

For the mobile phase, used throughout the experiment, phase A was the aqueous fraction composed of a mixture of aqueous ammonium acetate solution 10mmol/L acidified with 0.1% formic acid, and phase B was an organic fraction, comprising methanol.

Calibration curves were obtained using acetonitrile as a solvent to standardize the results of recovery and simplify the experiment. The following concentrations were used for calibration: 5.0, 7.5, 10.0, 25.0, 50.0, 75.0 and 100µg.kg^{-1}. All solutions were prepared independently. For the purpose of simultaneous quantification and identification, two or three multiple reaction monitoring (MRM) transitions were used for each analyte to avoid false negatives at trace pesticide levels. The peaks were evenly distributed along the chromatographic window and were resolved symmetrically. Data were analyzed using the Analyst program (Version 1.5.1, Applied Biosystems). The regression curve model for each compound was selected using a homoscedasticity test. The quality of fit and significance of the employed regression model were evaluated using the lack-of-fit test. The limit of detection (LOD) and the limit of quantification (LOQ) for all tested pesticides were determined to be 5.0 and 10.0ppb, respectively.

All analytical reagents used had a purity of >98% and were purchased from Riedel-de Haën grade PESTANAL (Seelze, Germany) or Sigma-Aldrich (Saint Louis, USA).

RESULTS

A summary of the case and animal identification, the anatomopathological conclusions from the necropsy reports, microscopic examination and toxicological results are enumerated in Table 1.

All the cadavers were in excellent conditions because they were kept in a cold room. Within this study, we detected 8 different pesticides and their metabolites in bone marrow (Table 1). Measured concentrations of all compounds were higher than the LOD (5.0 ppb), but lower than the LOQ (10.0 ppb).

DISCUSSION

The main purpose of this study was not to propose that the toxicological examination of the viscera is substituted by that of bone marrow, but to demonstrate that the bone marrow may be a good choice as a sampling site for toxicological screening. The bone marrow is a highly vascularized tissue, well

Table 1. Identification of animals, clinical signs, anatomopathological conclusions and results of toxicological analyses by LC-MS

Animal	Specie	Sex/Age	Clinical signs	Anatomopathological results	Microscopic examination	Toxicological analyses
1	Feline/ Mixed-breed	Male/11 months	This cat was found dead without any clinical signs or prior disease	Macroscopically, the animal had cyanotic external mucous membranes, epistaxis, hyperemic and edematous lungs and multifocal hemorrhages. The liver, spleen and kidneys were moderately hyperemic. The esophageal and stomach content revealed numerous rounded, blackened and firm millimetric structures with an appearance suggestive of aldicarb pesticide. The anatomopathological conclusion was acute respiratory insufficiency with a generalized circulatory disorder.	Lungs, liver, spleen and kidneys were hyperemic.	Aldicarb Aldicarb sulfone Asulam Carbendazim
2	Feline/ Mixed-breed	Male/6 months	This cat was taken to emergency veterinary care due to the sudden appearance of respiratory and neurological symptoms	The main findings were pulmonary hyperemia, edema and multifocal hemorrhages. Hyperemia of the spleen, liver, kidneys, and leptomeninges was observed. The anatomopathological conclusion was generalized circulatory disorder.	Edema and multifocal hemorrhages in lungs. Spleen, liver, kidneys, and leptomeninges were hyperemic.	Aldicarb Carbendazim Trifloxysulfuron
3	Feline/ Mixed-breed	Female/1 year	This cat presented with prostration, sialorrhea, vomiting and tremors, and was taken into emergency veterinary care	Macroscopically, the animal displayed cyanotic external mucous membranes, epistaxis, hyperemic and edematous lungs and multifocal hemorrhages. The liver, spleen and kidneys were intensely hyperemic. The anatomopathological conclusion was acute respiratory insufficiency with a generalized circulatory disorder.	The main findings were pulmonary hyperemia, edema with multifocal hemorrhage, and intense hyperemia of the liver, kidneys and leptomeninges.	Dichlorvos
4	Canine/ Mixed-breed	Male/ Unknown	Unknown medical history	The animal had hyperemic external mucous membranes, and the lungs showed moderate hyperemia and edema. The liver, spleen, kidneys, and leptomeninges were intensely hyperemic. The anatomopathological conclusion of necropsy was generalized circulatory disorder.	The lungs showed moderate hyperemia and edema. The liver, spleen, kidneys, and leptomeninges were intensely hyperemic.	Aldicarb Chlorpyrifos Thifensulfuron-methyl
5	Canine/ Border collie	Female/1 year	The animal was found dead with no clinical signs	Hyperemia of the external mucous membranes, lymph nodes, musculature, liver, kidneys and leptomeninges. The lungs showed moderate hyperemia and edema, in addition to multifocal hemorrhages. In the heart, there was an area of hemorrhage in the epicardium. The anatomopathological conclusion of necropsy was generalized circulatory disorder.	Pulmonary hyperemia, edema with multifocal hemorrhage, and intense hyperemia in the liver, kidneys, and heart.	Aldicarb
6	Canine/ Golden Retriever	Male/5 years	The animal presented with prostration, vomiting and diarrhea and was taken to an emergency veterinary care	Cyanotic external mucous membranes, epistaxis and hyperemic and edematous lungs. The liver, kidneys, and muscles were intensely hyperemic and the heart showed hemorrhages in the endocardium and epicardium. The anatomopathological conclusion was neutrophilic and fibrinous myocarditis with generalized hyperemia.	Neutrophilic and fibrinous myocarditis in the heart with focally extensive hemorrhage, hyperemia in the stomach and kidneys, pulmonary hyperemia, and edema were observed.	Not detected

LC-MS = Liquid chromatography-mass spectrometry.

protected by bone, and its lipid matrix may act as a repository for chemical agents. This may be particularly important when the carcass is in an advanced stage of decomposition or skeletonization.

One of the greatest difficulties during sample collection was the small amount of bone marrow present in each bone. Therefore, the collection of bone marrow from various bones (femur, humerus, and tibia) to generate a pool of bone marrow for each animal was necessary. In this study, the pool was used to provide the minimum amount of sample needed for extraction (5.0g) as per the QueChERS methodology and the assay. These bones were selected because they had a relatively larger medullary canal and, consequently, a greater amount of marrow in relation to the vertebrae and ribs. The LC-MS assay was able to detect the toxic chemicals in the bone marrow of five animals (Table 1). In addition, it was identified a greater number of toxicants (aldicarb, aldicarb sulfone, asulam, carbendazim, trifloxysulfuron, dichlorvos, chlorpyrifos, and thifensulfuron-methyl).

Aldicarb is a carbamate and is one of the most commonly chemicals used for criminal poisoning of animals (Xavier at al. 2007a, Siqueira et al. 2012). Aldicarb was detected in bone marrow of animals 1, 2, 4 and 5 and its metabolite, aldicarb sulfone (Harper et al. 1998) just in Feline 1. That is, it was found in 80% (4/5) of the poisoned animals. Aldicarb is extremely toxic to mammals and its toxicity is mediated through inhibition of acetylcholine esterase, a respiratory enzyme. All of these animals showed generalized circulatory disorders compatible with poisoning picture. The pesticide aldicarb known as "chumbinho", is one of the most toxic of registered pesticides, and has its use restricted to agriculture in Brazil. In spite of it, aldicarb is being very often involved in severe poisoning in animals (Xavier et al. 2007b). It is controlled in Brazil and is allowed only for agricultural uses upon prescription from a responsible technician. However, aldicarb is sold illegally for the extermination of domestic animals and is erroneously indicated as rat poison. As the marketing of aldicarb is illegal, to avoid detection during inspection, it is routinely mixed with other toxic substances in an unlabeled manner. Its use is justified by its high toxicity, ease of acquisition in the illegal trade, and inefficiency of the monitoring mechanisms (Xavier et al. 2017b). Our results agree with other reports of fatal cases of carbamate poisoning in dogs and cats in Brazil (Medeiros et al. 2009, Siqueira et al. 2015).

Asulam, a systemic herbicide highly effective against bracken, also was detected in Feline 1, and belongs to the group of carbamates - sulfanilylcarbamate. When administered orally or intravenously, it is excreted in the urine in 24 hours (Heijbroek et al. 1984). Kaufmann & Kaenzig (2004) found sulfanilamide residues in 15 Swiss honeys out of some 350 samples. Bees occasionally collect nectar from meadows treated with the herbicide asulam. Such honey is not only contaminated by asulam, but also by its degradation product sulfanilamide. Carbamates pesticides can be divided into carbamate insecticides, mostly monomethyl carbamates, fungicidal dithiocarbamates and carbamates herbicides and fungicides. The toxicity of carbamate herbicides and fungicides is very heterogenous and its rather related to the aromatic moiety (herbicides) and the benzimidazole moiety (fungicides) than to the carbamate rest. Acute toxicity of carbamates herbicides and fungicides is generally low compared to the carbamate insecticides. However, as previously reported, Aldicarb is sold illegally, and many other substances could be added to this product, like the asulam.

The animals 3 and 4, showed other pesticides, from the organophosphorus group, also with action anti-acetylcholinesterase: dichlorvos (Feline 3) and chlorpyrifos (Canine 4). Dichlorvos is classified as highly toxic and it is used an agricultural insecticide on crops and stored products, besides as an antihelminthic for dogs, swine and horses, and to kill fly larvae. It is poisonous if swallowed, inhaled, or absorbed through the skin. The high dose of dichlorvos produces a detrimental effect on acetylcholinesterase activity (Verma et al. 2008).

Chlorpyrifos is an organophosphorothionate insecticide with nonsystemic anticholinesterase effects reported following exposure by skin contact, ingestion, or inhalation. It is one of the most widely used organophosphorus insecticides in the world. Chlorpyrifos has been widely used in agriculture, horticulture, viticulture and forestry on a wide range of crops, as well as in residential and nonresidential applications to control cockroaches, fleas, ticks on cattle and pests in animal dwellings (Sultatos 1994). The Feline 3 presented prostration and tremors, and the nervous system is the primary target because acetylcholinesterase and/or butlycholinesterase catabolyze the neurotransmitter acetylcholine, thereby terminating its synaptic function. The sialorrhea and vomiting also was presented by this animal are typical muscarinic signals showed in the anticholinesterase poisoning. It is well-established that severe poisoning by organophosphorus compounds strongly affects the cardiorespiratory system (Cunha et al. 2018).

Owing to its greater sensitivity, the LC-MS technique can be used to detect other toxin residues, including carbendazim, a fungicidal compound from the benzimidazole group; trifloxysulfuron-sodium, an herbicidal compound of the sulfonylurea class and thifensulfuron-methyl, a sulfonylurea herbicide. Our results confirmed the deposition of carbendazim within the bone marrow of Felines 1 and 2 (Table 1). The carbendazim is a highly effective, broad-spectrum, systemic fungicide used to protect fruits and other products from spoiling. and, it is relatively stable and persistent in the environment (Thomas et al. 1996). It is important to note that the presence of more than one type of chemical substance in the marrow bone has been detected in some animals (Feline 1, Feline 2 and Canine 4). The Feline 1 showed four substances in bone marrow, and this animal was found dead without any clinical signs or prior disease. Macroscopically, the esophageal and stomach content revealed numerous rounded, blackened, and firm millimetric structures with an appearance suggestive of aldicarb pesticide.

Besides the aldicarb and carbendazim, trifloxysulfuron-sodium was found just in Feline 2. This cat was taken to emergency veterinary care due to the sudden appearance of respiratory and neurological symptoms. But, the clinical picture worsened, and this animal died. Trifloxysulfuron-sodium is a post-emergence herbicide without European Union regulatory approval for use. It has a high aqueous solubility, is relatively volatile and, based on its chemical properties, is mobile and may leach to groundwater in some situations. It is moderately persistent in soil systems but is generally non-persistent in aquatic systems. It has a low mammalian toxicity and has a high potential to bioaccumulate (PPDB 2020).

Finally, the thifensulfuron-methyl was found in Canine 4. Thifensulfuron-methyl is a post-emergence herbicide for the control of grass and broad-leaved weeds and it is unlikely to presente an acute hazard. This herbicide has relatively long persistence in water and its partitioning into sediments is minimal. The sulfonylurea linkage in this compound is resistant to hydrolysis in weakly alkaline waters, and the microbial and photolytic degradation in dugout waters are slow (Cessna et al. 2006). Lazartigues et al. (2011) used the same extraction method (QuEChERS) and LC-MS to detect thifensulfuron-methyl on three fish species (in their muscles), naturally or orally exposed: roach (*Rutilus rutilus*), perch (*Perca fluviatilis*) and carp (*Cyprinus carpio*).

Brazil is one of the greatest consumers of pesticides in the world. Thus, the comparison of toxicological results with the clinical signs and anatomopathological findings is important to confirm whether the toxic chemicals found were related to the death of the animal. The success in the detection of toxic chemicals in human bone marrow was essentially dependent on the analytical method used and the methodology used in the pretreatment and extraction of the toxic chemicals from the sample, especially the adipose tissue matrix (Cartiser et al. 2011). In the present study, the QuEChERS extraction technique and the analytical method (LC-MS) were effective for the detection of pesticides in the bone marrow of cats and dogs. The degree of contact of drugs to the bone structures depends on the anatomical location of the bone and the local blood supply. The long bones (i.e., femur) receive the most blood supply while the short, flat, or irregular bones receive more superficial supply through the periosteum (Drummer 2008). Given this and the our results, the choice of femur, humerus and tibia bones was adequate.

In addition to the lack of studies in veterinary medicine that have investigated the detection of toxic chemicals in the bone marrow, there have been no studies in human forensic toxicology on the use of bone marrow for the detection of pesticides. Ackan et al. (2009) treated six rabbits with endosulfan and six with diazinon and collected bone marrow and analyzed by chromatography gas (GC-MS), and found these isomers and its metabolites in this sample. The authors concluded that cause of death can be determined as acute pesticide poisoning by toxicological analysis of samples from bone marrow.

Finally, the anatomopathological examination is very important and should always be accompanied by a toxicological examination in cases of suspected poisoning and may reveal pre-existing diseases that may not have been diagnosed antemortem. Canine 6 was the only one that did not show any pesticides in the bone marrow. The anatomopathological conclusion was neutrophilic and fibrinous myocarditis with generalized hyperemia.

Forensic toxicological analyses have traditionally focused the use blood, body fluids, and certain organs in examination of deaths due to poisoning. This study is preliminary, but showed that bone marrow might be used as an alternative specimen since it's a potential depot for pesticides.

CONCLUSION

Based on the results of this case study, we have concluded that bone marrow can be used for the detection of toxic chemicals to investigate poisoning in forensic veterinary medicine. Aldicarb, aldicarb sulfone, asulam, carbendazim, chlorpyrifos, dichlorvos, thifensulfuron methyl and trifloxysulfuron were deposited in the bone marrow of cats and dogs and were detected by LC-MS analysis.

Acknowledgments.- We thank the "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq) for the financial support (Process 305617/201/-6).

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

REFERENCES

Ackan R., Hila A., Daglioglu N., Cekin N. & Gulmen M.K. 2009. Determination of pesticides in postmortem blood and bone marrow of pesticide treated rabbits. Forensic Sci. Int. 189(1/3):82-87. <https://dx.doi.org/10.1016/j.forsciint.2009.04.017> <PMid:19446970>

Anastassiades M., Lehotay S.J., Stajnbaher D. & Schenck F.J. 2003. Fast and easy multiresidue method employing acetonitrile extraction/ partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. J. AOAC Int. 86(2):412-431. <PMid:12723926>

Cartiser N., Bévalot F., Fanton L., Gaillard Y. & Guiltton J. 2011. State-of-the-art of bone marrow analysis in forensic toxicology: a review. Int. J. Legal Med. 125(2):181-198. <https://dx.doi.org/10.1007/s00414-010-0525-6> <PMid:21061013>

Casier H., Thomas F. & Delanouis A.L. 1943. La répartition de l'alcool chez l'homme et les animaux au cours de l'intoxication éthylique. Arch. Int. Pharmacodyn. 69:186-204.

Cessna A.J., David B Donald D.B., Bailey J., Waiser M. & Headley J.V. 2006. Persistence of the sulfonylurea herbicides thifensulfuron-methyl, ethametsulfuron-methyl, and metsulfuron-methyl in farm dugouts (ponds). J. Environ. Qual. 35(6):2395-2401. <https://dx.doi.org/10.2134/jeq2005.0462> <PMid:17071910>

Cunha A.F., Felippe I.S.A., Ferreira-Junior N.C., Resstel L.B.M., Guimarães D.A.M., Beijamini V., Paton J.F.R. & Sampaio K.N. 2018. Neuroreflex control of cardiovascular function is impaired after acute poisoning with chlorpyrifos, an organophosphorus insecticide: possible short and long term clinical implications. Toxicology 398/399:13-22. <https://dx.doi.org/10.1016/j.tox.2018.02.005> <PMid:29471072>

Drummer O.H. 2008. Drugs in bone and bone marrow, p.131-137. In: Jenkins A.J. (Ed.), Drug Testing in Alternate Biological Specimens. Humana Press, New Jersey.

Fukushima A.R., Gonzalez M.V.D., Florio J.C., Salomone G.M., De-Siqueira A., Carvalho V.M., Zaccarelli-Magalhaes J., Anazawa T.A., Leoni L.A.B., Nicoletti M.A., Maiorka P.C. & Spinosa H.S. 2018. Study of aldicarb commercial granule using dissolution profile: a contribution for identification of metabolites. Int. J. Curr. Innov. Res. 4(4):1173-1176.

Grellner W. & Glenewinkel F. 1997. Exhumations: synopsis of morphological and toxicological findings in relation to the postmortem interval Survey on a 20-year period and review of the literature. Forensic Sci. Int. 90(1/2):139-159. <https://dx.doi.org/10.1016/S0379-0738(97)00154-0> <PMid:9438373>

Harper F.D., Weisskopf C.P. & Cobb G.P. 1998. Extraction of aldicarb and its metabolites from excreta and gastrointestinal tissue. Anal. Chem. 70(15):3329-3332. https://dx.doi.org/10.1021/ac971268k> <PMid:11013730>

Heijbroek W.M.H., Muggleton D.F. & Parke D.V. 1984. Metabolism of the carbamate herbicide, asulam, in the rat. Xenobiotica 14(3):235-247. <https://dx.doi.org/10.3109/00498258409151408> <PMid:6711013>

Kaufmann A. & Kaenzig A. 2004. Contamination of honey by the herbicide asulam and its antibacterial active metabolite sulfanilamide. Food Add. Cont. 21(6):564-571. https://dx.doi.org/10.1080/02652030410001677790> <PMid:15204534>

Lazartigues A., Wiest L., Baudot R., Thomas M., Feidt C. & Crein-Olivé C. 2011. Multiresidue method to quantify pesticides in fish muscle by QuEChERS-based extraction and LC-MS/MS. Anal. Bioanal. Chem. 400(7):2185-2193. <https://doi.org/10.1007/s00216-011-4945-z>

Maeda H., Zhu B.L., Ishikawa T., Oritani S., Michiue T., Li D.R., Zhao D. & Ogawa M. 2006. Evaluation of post-mortem ethanol concentrations in pericardial fluid and bone marrow aspirate. Forensic Sci. Int. 161(2/3):141-143. <https://doi.org/10.1016/j.forsciint.2006.01.016> <PMid:16842951>

McIntyre I.M., King C.V., Boratto M. & Drummer O.H. 2000. Post-mortem drug analyses in bone and bone marrow. Ther. Drug Monit. 22(1):79-83. <https://doi.org/10.1097/00007691-200002000-00017> <PMid:10688265>

Medeiros R.J., Monteiro F.O., Silva G.C. & Nascimento Junior A. 2009. Casos de intoxicações exógenas em cães e gatos atendidos na Faculdade de Veterinária da Universidade Federal Fluminense durante o período de 2002 a 2008. Ciência Rural 39(7):2105-2110. <https://dx.doi.org/10.1590/S0103-84782009005000151>

Oliveira F.A.S., Pereira E.N.C, Gobbi J.M., Soto-Blanco B. & Melo M.M. 2018. Multiresidue method for detection of pesticides in beef meat using liquid chromatography coupled to mass spectrometry detection (LC-MS) after QuEChERS extraction. Food Add. Cont., A 35(1):94-109. <https://doi.org/10.1080/19440049.2017.1395519> <PMid:29058520>

PPDB 2020. Pesticides Propetiers DataBase, University of Hertfordshire, Hertfordshire. Available at <https://sitem.herts.ac.uk/aeru/ppdb/en/Reports/1151.htm> Accessed on May 18, 2020.

Schloegl H., Rost T., Schmidt W., Wurst F.M. & Weinmann W. 2006. Distribution of ethyl glucuronide in rib bone marrow, other tissues and body liquids as proof of alcohol consumption before death. Forensic Sci. Int. 156(2/3):213-218. <https://dx.doi.org/10.1016/j.forsciint.2005.03.024> <PMid:15946814>

Siqueira A., Cassiano F.C., Landi M.F.A. Marlet E.F. & Maiorka P.C. 2012. Non-accidental injuries found in necropsies of domestic cats: a review of 191 cases. J. Feline Med. Surg. 14(10):723-728. <https://dx.doi.org/10.1177/1098612X12451374> <PMid:22694958>

Siqueira A., Salvagni F.A, Yoshida A.S., Gonçalves-Junior V., Calefi A.S., Fukushima A.R., Spinosa H.S. & Maiorka P.C. 2015. Poisoning of cats and dogs by the carbamate pesticides aldicarb and carbofuran. Res. Vet. Sci. 102:142-149. <https://dx.doi.org/10.1016/j.rvsc.2015.08.006> <PMid:26412534>

Sultatos L.G. 1994. Mammalian toxicology of organophosphorus pesticides. J. Toxicol. Environ. Health 43(3):271-289. <https://dx.doi.org/10.1080/15287399409531921> <PMid:7966438>

Thomas D.H., Loez-Ávila V., Betowsk L.D. & Emom J.V. 1996. Determination of carbendazim in water by high-performance immunoaffinity chromatography on-line with high-performance liquid chromatography with diode-array or mass spectrometric detection. J. Chromatogr. A 724(1/2):207-217.

Verma S.K., Raheja G. & Gill K.D. 2008. Role of muscarinic signal transduction and CREB phosphorylation in dichlorvos-induced memory deficits in rats: An acetylcholine independent mechanism. Toxicology 256(3):175-182. <https://dx.doi.org/10.1016/j.tox.2008.11.017>

Winek C.L. & Esposito F.M. 1981. Comparative study of ethanol levels in blood versus bone marrow, vitreous humor, bile and urine. Forensic Sci. Int. 17(1):27-36. <https://dx.doi.org/10.1016/0379-0738(81)90185-7> <PMid:7216079>

Winek C.L. & Janssen J.K. 1982. Blood versus bone marrow isopropanol concentrations in rabbits. Forensic Sci. Int. 20(1):11-20. <https://dx.doi.org/10.1016/0379-0738(82)90100-1>

Winek C.L. & Jones T. 1980. Blood versus bone marrow ethanol concentrations in rabbits and humans. Forensic Sci. Int. 16(2):101-109. <https://dx.doi.org/10.1016/0379-0738(80)90162-0> <PMid:7429375>

Winek C.L. & Luhanik J.M. 1981. A storage study of ethanol in rabbit and human bone marrow. Forensic Sci. Int. 17(3):191-196. <https://dx.doi.org/10.1016/0379-0738(81)90166-3> <PMid:7239369>

Winek C.L., Costantino A.G., Wahba W.W. & Collom W.D. 1985. Blood versus bone marrow pentobarbital concentrations. Forensic Sci. Int. 27(1):15-24. <https://dx.doi.org/10.1016/0379-0738(85)90100-8> <PMid:3988192>

Winek C.L., Matejczyk R.J. & Buddie E.G. 1983. Blood, bone marrow and eye fluid ethanol concentrations in putrefied rabbits. Forensic Sci. Int. 22(2/3):151-159. <https://dx.doi.org/10.1016/0379-0738(83)90008-7> <PMid:6642341>

Winek C.L., Pluskota M. & Wahba W.W. 1982. Plasma versus bone marrow flurazepam concentration in rabbits. Forensic Sci. Int. 19(2):155-163. <https://dx.doi.org/10.1016/0379-0738(82)90041-X>

Xavier F.G., Righi D.A. & Spinosa H.S. 2007a. Fatal poisoning in dogs and cats: a 6-year report in a veterinary pathology servisse. Braz. J. Vet. Res. Anim. Sci. 44(4):304-309. <https://dx.doi.org/10.11606/issn.1678-4456.bjvras.2007.26632>

Xavier F.G., Righi D.A. & Spinosa H.S. 2007b. Toxicology of the pesticide aldicarb ("chumbinho"): general, clinical and therapeutical features in dogs and cats. Ciência Rural 37(4):1206-1211. <https://dx.doi.org/10.1590/S0103-84782007000400051>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(10):804-813, October 2020
DOI: 10.1590/1678-5150-PVB-6420

Original Article
Small Animal Diseases
BY-NC

Antimicrobial resistance evaluation of bacteria isolated from infections in small animals in the Umuarama region, Paraná[1]

Marilia M. Souza[2], Jéssica T. Bordin[3], Ana Cláudia L. Pavan[3], Raquel G.A. Rodrigues[2], Ricardo A.P. Sfaciotte[4], Vanessa K.C. Vignoto[5], Marcos Ferrante[6] and Sheila R. Wosiacki[5]*

ABSTRACT.- Souza M.M., Bordin J.T., Pavan A.C.L., Rodrigues R.G.A., Sfaciotte R.A.P., Vignoto V.K.C., Ferrante M. & Wosiacki S.R. 2020. **Antimicrobial resistance evaluation of bacteria isolated from infections in small animals in the Umuarama region, Paraná**. *Pesquisa Veterinária Brasileira 40(10):804-813*. Departamento de Medicina Veterinária, Universidade Estadual de Maringá, Estrada da Paca s/n, Jardim São Cristóvão, Umuarama, PR 87507-190, Brazil. E-mail: srwosiacki@uem.br

Bacterial resistance is shown to be an inevitable side effect due to the excessive use of antibiotics, becoming a significant concern worldwide. Knowledge of regional bacterial resistance profiles enables the development of site-specific infection control practices, making conscious and moderate use of commercially available antibiotics. The aim of this study was the retrospective evaluation of the antimicrobial resistance profile of bacteria isolated from companion animal infections in the region of Umuarama/PR, from 2013 to 2017. This research was performed by analyzing the database belonging to the "Laboratório de Microbiologia Animal" at the "Universidade Estadual de Maringá" (UEM). *Staphylococcus* spp. represented 45.53% of the bacteria isolated from clinical infections in small animals in the period and place evaluated, followed by enterobacteria (34.04%), non-fermenting Gram-negative bacilli (NFGNB, 11.06%) and *Streptococcus*/*Enterococcus* (9.36%). A high number of antimicrobial resistance to antibiotics used in veterinary medicine was found. The lowest resistances associated with the best impact factor values were found for aminoglycosides, especially amikacin, chloramphenicol, and fluoroquinolones (norfloxacin and ciprofloxacin). Intermediate results were found for sulbactam-associated ampicillin, ceftriaxone, amoxicillin-clavulanic acid, and enrofloxacin. According to the number of resistant antimicrobial drugs, 64.26% (151/235) of the isolates were classified as multidrug-resistant, being 15.32% extensively resistant. Considering the resistance to antimicrobial classes, 68.94% (162/235) of the isolates were classified as multiresistant, being 19.15% extensively resistant. No bacterial strains were characterized as pan-resistant, but ten bacteria were resistant to all classes tested, with isolated susceptibility to certain drugs. Through the evaluation of resistance profiles found in the period and place studied and relevant literature, it is clear that there is a growing increase in the number of multiresistant bacteria among domestic animals which characterizes a serious risk to public health. The therapeutic arsenal is becoming increasingly diminished, and there is more difficulty in empirical drug selection, making antimicrobial susceptibility testing essential for more specific selection in antimicrobial therapy. Educational

[1] Received on August 9, 2019.
Accepted for publication on July 11, 2020.

[2] Graduate Program in Sustainable Production and Animal Health, Universidade Estadual de Maringá (UEM), Estrada da Paca s/n, Bairro São Cristóvão, Umuarama, PR 87507-190, Brazil.

[3] Residence in Infectious and Parasitic Diseases, Universidade Estadual de Maringá (UEM), Estrada da Paca s/n, Bairro São Cristóvão, Umuarama, PR 87507-190, Brazil.

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

[5] Departamento de Medicina Veterinária, Universidade Estadual de Maringá (UEM), Estrada da Paca s/n, Bairro São Cristóvão, Umuarama, PR 87507-190, Brazil. *Corresponding author: srwosiacki@uem.br

[6] Departamento de Medicina Veterinária, Universidade Federal de Lavras (UFLA), Campus Universitário, Cx. Postal 3037, Lavras, MG 37200-000, Brazil.

measures on the conscious use of antibiotics, infection control, and prevention of local specific zoonoses need to be instituted for the knowledge of health professionals and general access of the population.

INDEX TERMS: Antimicrobial resistance, bacteria, infection, small animals, Brazil, antibiotics, multiresistance, dogs, cats.

RESUMO.- [**Avaliação da resistência antimicrobiana de bactérias isoladas de infecções em pequenos animais na região de Umuarama, Paraná.**] A resistência bacteriana, mostra-se como um efeito colateral inevitável pelo excessivo uso de antibióticos, tornando-se alvo de grande preocupação mundial. O conhecimento dos perfis de resistência bacteriana regionais possibilita o desenvolvimento de práticas de controle de infecções específicas para cada localidade, fazendo uso consciente e moderado dos antibióticos disponíveis no mercado. O objetivo deste estudo foi a avaliação retrospectiva do perfil de resistência antimicrobiana de bactérias isoladas de infecções de animais de companhia na região de Umuarama/PR, no período de 2013 a 2017. Esta pesquisa foi realizada por meio da análise do banco de dados pertencente ao Laboratório de Microbiologia Animal da Universidade Estadual de Maringá (UEM). Os *Staphylococcus* spp. representaram 45,53% das bactérias isoladas de infecções clínicas em pequenos animais no período e local avaliado, seguido por enterobactérias (34,04%), bacilos Gram-negativos não fermentados (BGNNF, 11,06%) e *Streptococcus*/*Enterococcus* (9,36%). Um número elevado de resistência antimicrobiana frente aos antibióticos utilizados na medicina veterinária foi encontrado. As menores resistências associadas aos melhores valores do fator de impacto foram encontrados para aminoglicosídeos, em especial amicacina, cloranfenicol, fluoroquinolonas (norfloxacina e ciprofloxacina). Já resultados intermediários foram encontrados para ampicilina associada a sulbactam, ceftriaxona, amoxacilina com ácido clavulônico e enrofloxacina. Conforme o número de drogas antimicrobianas resistentes, foram classificados como multirresistentes 64,26% (151/235) dos isolados, sendo 15.32% extensivamente resistentes. Já considerando a resistência a classes de antimicrobianos, 68,94% (162/235) dos isolados foram classificados como multirresistentes, sendo 19.15% extensivamente resistentes. Nenhum isolado bacteriano foi caracterizado como pan-resistente, porém 10 bactérias foram resistentes a todas as classes testadas, com susceptibilidade isolada a determinadas drogas. Por meio da avaliação dos perfis de resistência encontrados no período e local estudados e de literatura pertinente, percebe-se que há um aumento crescente no número de bactérias multirresistentes entre os animais domésticos o que caracteriza um grave risco à saúde pública. O arsenal terapêutico está se tornando cada vez mais diminuto e há mais dificuldade na seleção empírica de drogas, tornando essencial a realização de testes de susceptibilidade antimicrobiana para uma seleção mais específica na terapêutica antimicrobiana. Medidas educativas sobre o uso consciente dos antibióticos, controle de infecções e prevenção de zoonoses específicas para as localidades precisam ser instituídas para conhecimento dos profissionais do setor da saúde e acesso geral da população.

TERMOS DE INDEXAÇÃO: Resistência antimicrobiana, bactérias, infecção, pequenos animais, Brazil, antibióticos, multirresistência, cães, gatos.

INTRODUCTION

Antimicrobials include any natural substance of semi-synthetic or synthetic origin that kills or inhibits the growth of a microorganism, causing little or no damage to the host. Among these, the most widely used are antibiotics that were defined by Giguère (2013) as a low molecular weight substance produced by a microorganism that inhibits or kills other organisms at low concentrations. Since its discovery in 1928, when Alexandre Flemming noted that staphylococci colonies were lysed on a Penicillium-contaminated plaque (Prescot 2013), they are widely used for the treatment or prevention of bacterial infections and are among the most important advances in modern medicine (Murphy et al. 2012).

As soon as penicillin was discovered, the presence of *in vitro* resistance was observed and it was said, in the early years of its application, that there was a need to be aware of the precautions to be taken when administering the drug due to possible undesirable reactions and even of antibiotic resistance (Pereira & Pita 2005).

Although numerous studies have provided substantial evidence of the spread of multi-resistant bacteria from animals to humans, current research indicates that humans can also transmit animal-resistant pathogens in a reverse zoonotic event called zooanthroponosis (Messenger et al. 2014). Pathogens acquired in the hospital environment by owners, for example, can be transmitted directly to the animal or indirectly through the domestic environment, becoming a critical route of transmission between them (Fernandes et al. 2018).

Resistant pathogen transfer rates at the human-animal interface as well as antibiotic resistance were increased in both companion animals (Aidara-Kane 2012) and humans. This increase was accompanied by the more frequent use of these broad-spectrum drugs in the treatment of patients without real certainty of this need as bacterial agent identification, and susceptibility tests are not performed most of the time (Guardabassi et al. 2010).

Antimicrobial resistance is defined as the condition in which a microorganism can survive when exposed to an antimicrobial to which it was initially susceptible (Barie 2012, WHO 2019) or organisms capable of multiplying in the presence of therapeutic or higher antimicrobial concentrations (Sfaciotte et al. 2017). It is considered a complex phenomenon, especially in veterinary medicine, due to the number of species and the diversity of environments in which animals are reared, the differences in the bacteria involved, the range of pathogenicity mechanisms and the intricate epidemiology (Acar et al. 2012). Antimicrobial resistance is an inevitable side effect due to the excessive use of these drugs (Guardabassi et al. 2010) and is a subject of intense worldwide concern (Ventola 2015).

Antimicrobial susceptibility testing is considered to be one of the most important factors governing drug selection for veterinary clinical use and is used in the elaboration of local therapeutic guides (ANVISA 2017). Joseph & Rodvold (2008) described the "4 D's of Ideal Antimicrobial Therapy": right Drug, right Dose, De-escalated to pathogen-directed therapy, and right Duration of therapy. De-escalating is referred to by Molina et al. (2012) as an intervention aimed at improving the

use of antimicrobials and implying their discontinuation when there is no infection or restricting the spectrum of antimicrobial coverage according to clinical response and bacterial cultures.

The aim of this study was to retrospectively evaluate the antimicrobial resistance profile found in bacteria isolated from infections in small animals in the Umuarama/PR region.

MATERIALS AND METHODS

This research was conducted through the analysis of the database belonging to the "Laboratório de Microbiologia Animal" of the "Universidade Estadual de Maringá" (UEM), located at the "Campus Regional de Umuarama" (CAU), Fazenda, from 2013 to 2017.

The Laboratory database refers to the exam files containing animal identification items (name, ID number, species, race, sex, age, material collected and clinical diagnosis), identification of the bacteria (s) isolated (s) and results of the diameter of the bacterial growth inhibition halos obtained by disc diffusion or antibiogram technique.

The information obtained from the exam files was inserted into spreadsheets using Excel software, where it was separated by year of isolation, bacterial type, and place of infection. A total of 32 antibiotics in 11 classes were used. The interpretation of antimicrobial resistance/susceptibility was performed by CLSI (2008, 2013, 2018) and BrCAST (2019) standards, as shown in Table 1.

Following the guidance of BrCAST (2019), members of the old family Enterobacteriaceae were referred to as members of the new order Enterobacterales. The order Enterobacterales currently includes the families (Adeolu et al. 2016). Enterobacteriaceae, Erwiniaceae, Pectobacteriaceae, Yersiniaceae, Hafniaceae, Morganellaceae, and Budviciaceae

Table 1. Antimicrobial susceptibility interpretation standard, in millimeters of growth inhibition halos, for bacteria of animal origin

		Staphylococcus spp.	*Enterococcus* spp.	*Streptococcus* spp.	Enterobacterales	*Pseudomonas* spp.	Acinetobacter baumannii
Penicillin G	10U	≥ 29 [a]	≥ 15 [a]	≥ 24 [a]	-	- [a]	
Ampicillin	10 µg		≥ 17 [a]	≥ 24 [a]	≥ 17 [a]	- [a]	
Amoxacillin	10 µg			amp [a]	amp [a]	- [a]	
Ampicillin + sulbactam	20 µg	pen e oxa [a]	pen e oxa [a]	- [a]	≥ 14 [b]	- [a]	
Amoxacillin + clavulonate	30 µg			- [a]	≥ 19 [a]	- [a]	
Oxacillin	1 µg	≥ 18 [a]	- [a]	≥ 20	- [a]	- [a]	
Cefoxitin	30 µg	≥ 22 [a]	IR [a]		≥ 19 [b]	- [a]	
Cephalothin/ Cephalexin	30 µg	pen e oxa [a]	IR [a]		≥ 14 [b]		
Ceftazidime	30 µg		IR [a]		≥ 22 [b]	≥ 17 [b]	
Cefotaxime	30 µg		IR [a]			≥ 23 [c]	
Ceftriaxone	30 µg	pen e oxa [a]	IR [a]	≥ 24 [a]	≥ 25 [a]	≥ 21 [c]	
Cefepime	30 µg		IR [a]	≥ 24 [a]	≥ 27 [b]	≥ 21 [b]	
Aztreonam	30 µg	IR [a,b]	IR [a]		≥ 26 [b]	≥ 16 [b]	
Meropenem	10 µg	pen e oxa [a]	- [a]		≥ 22 [a]	≥ 24 [b]	≥ 21 [b]
Imipenem	10 µg		≥ 21 [b]		≥ 23 [a]	≥ 19 [a]	≥ 23 [b]
Vancomycin	30 µg	≥ 18 [d]	≥ 17 [a]	≥ 17 [a]	IR [a]		
Teicoplamine	30 µg	van [a]	- [a] ≥ 16 [b]	≥ 17 [b]	IR [a]		
Linezolid	30 µg	≥ 21 [b]	- [a] ≥ 19 [b]	≥ 19 [a]	IR [a]		
Amikacin	30 µg	≥ 18 [a]	IR [a]	- [a]	≥ 17 [a]	≥ 17 [a]	≥ 19 [b]
Gentamicin	10 µg	≥ 15 [a]	IR [a] ≥ 8 [b]	- [a]	≥ 16 [a]	≥ 16 [a]	≥ 17 [b]
Streptomycin	10 µg	-	IR [a] ≥ 14 [b]	- [a]	≥ 15 [a]		≥ 17 [b]
Tobramycin	10 µg	≥ 18 [b]	IR [a]	- [a]	≥ 17 [a]	≥ 16 [b]	
Erythromycin	15 µg	≥ 23 [a]	≥ 23 [a]	≥ 21 [a]	IR [a]		
Azithromyx	15 µg			≥ 18 [a]	IR [a]		
Clindamycin	2 µg	≥ 21 [a]	IR [a]	≥ 17 [a]	IR [a]		
Rifampicin	5 µg	≥ 20 [a]	≥ 20 [a]	≥ 21 [a]	- [a]		
Chloramphenicol	30 µg	≥ 18 [a]	≥ 18 [a]	≥ 19 [a]	≥ 18 [a]		
Tetracycline	30 µg	≥ 23 [a]	≥ 19 [a]	≥ 23 [a]	≥ 15 [a]		
Doxycycline	30 µg	≥ 25 [a]	≥ 16 [a]	≥ 28 [a]	≥ 14 [a]		
Sulfamethoxazole + trimethoprim	23.7/1.25 µg	≥ 16 [a]	IR [a]	≥ 19 [a]	≥ 16 [a]		
Enrofloxacin	10 µg	≥ 23 [a]	- [a]	≥ 23 [a]	≥ 23 [a]	≥ 23 [a]	
Norfloxacin	10 µg	≥ 17 [b]	≥ 12 [a]	≥ 12 [a]	≥ 22 [a]	≥ 17 [c]	
Ciprofloxacin	5 µg	≥ 21 [b]	- [a]		- [a] ≥ 26 [b]	≥ 26 [b]	≥ 21 [b]
Levofloxacin	5 µg	≥ 22 [b]	- [a]	≥ 17 [a]	- [a] ≥ 23 [b]	≥ 22 [b]	≥ 23 [b]

[a] CLSI (2018), [b] BrCAST (2019), [c] CLSI (2013), [d] CLSI (2008); IR = intrinsic resistance.

The categories were organized into:

I) Gram-positive bacteria: *Staphylococcus* spp., *Enterococcus* spp., *Streptococcus* spp., *Enterococcus*/*Streptococcus* (when it was not possible to differentiate gender);

II) Gram-negative bacteria: order Enterobacterales and NFGNB (non-fermenting Gram-negative bacilli). Clinically significant NFGNB associated with severe nosocomial infections such as *Pseudomonas* spp. and *Acinetobacter baumannii* were reported separately from the other NFGNB, which were included in the "other" category.

Absolute and relative frequencies of antimicrobial resistance were determined using descriptive statistical analysis. The graphics were made by Excel software.

The impact factor of each antimicrobial drug used to help choose empirical antimicrobial therapy was calculated according to Rampacci et al. (2018) and referred to the results of the prevalence study and antimicrobial susceptibility test (antibiogram) with their frequency expressed as a percentage, according to the formula:

$$F = \left[\frac{\%P(_{St}) \times \%S(_{St})}{100}\right] + \left[\frac{\%P(_{E/S}) \times \%S(_{E/S})}{100}\right] + \left[\frac{\%P(_{E}) \times \%S(_{E})}{100}\right] + \left[\frac{\%P(_{N}) \times \%S(_{S})}{100}\right]$$

Where F = impact factor of the evaluated antibiotic, % P = prevalence of bacteria isolated from each bacterial group, % S = percentage of antibiotic susceptibility of each bacterial group, ($_{St}$) = *Staphylococcus*, ($_{E/S}$) = *Enterococcus*/*Streptococcus*, ($_{E}$) = Enterobacteriales, ($_{N}$) = NFGNB.

The Multiple Antibiotic Resistance Indexing (MAR), according to Krumperman (1983), was calculated by the number of antibiotics to which the isolate had resistance (including intermediate resistance) divided by the total number of antibiotics tested. Bacteria with an index equal to or greater than 0.2 (20% of the tested antibiotics) were considered multidrug-resistant.

Multidrug-Resistant Bacteria (MDR), according to Magiorakos et al. (2012), were detected by the calculation, through an index, considering the number of resistant classes in relation to the total number of tested classes. The class was considered resistant when one or more drugs were resistant. Values equal to or greater than 0.3 were considered multidrug-resistant, values equal to or greater than 0.8 as extensively drug-resistant, and values of 1.0 with resistance to all drugs tested were considered pandrug-resistant.

RESULTS AND DISCUSSION

During the study period, 235 bacterial strains collected from infections in small animals were isolated, 207 from dogs and 28 from cats. The records were evaluated regardless of age and gender, and all animals were from the Umuarama region and surrounding areas in the state of Paraná, Brazil.

Given the analysis of the data obtained, the most common microorganisms (54.89%, 129/235) in dogs and cats were Gram-positive bacteria. Of these, 82.17% (106/129) were identified as *Staphylococcus* spp., representing 45.10% of the total bacterial isolates studied (for further analysis *Micrococcus* spp. was included with *Staphylococcus* spp. for similarity). The second most prevalent genus of Gram-positive bacteria was *Enterococcus* (8.53%, 11/129), followed by *Streptococcus* (4.65%, 6/129). Five isolates were not differentiated between *Enterococcus* and *Streptococcus* genera. Of the 106 (45.1%) Gram-negative bacteria, Enterobacterales were identified in 75.47% (80/106) of the isolates, their largest representative was *Escherichia coli* identified in 13.75% (11/80), while NFGNB were identified in 26 isolates, of which 73.08% (19/26) comprised *Pseudomonas* spp. Despite the low frequency, the identification of *Acinetobacter baumannii* and *Burkholderia pseudomallei* (Table 2) is noteworthy.

The highest prevalence of Gram-positive bacteria, especially *Staphylococcus* spp., is found in several studies (Pereira et al. 2009, Ishii et al. 2011, Sfaciotte et al. 2014, Kohl et al. 2016) of infections in dogs and cats. Sfaciotte et al. (2014) and Kohl et al. (2016) also identified *Escherichia coli* as the second most prevalent bacteria. Ishii et al. (2011) detected a high prevalence of *Pseudomonas* spp. in samples from wounds, followed by *Escherichia coli*. The prevalence of other bacteria was similar in studies conducted by Ishii et al. (2011), Kohl et al. (2016) and in the present work. Ishii et al. (2011) was also detected *Acinetobacter* spp.

Most cases of infections evaluated occurred in the integumentary system (98/235, 41.70%); followed by infections in the sensory system (83/235, 35.32%), divided into otological infections (69/235, 29.36%), ophthalmic and nasal infections (7/235, 2.98% each); in the urinary system (32/235; 13.62%); in the female reproductive system collected from vaginal secretions (10/235, 4.26%); and other systems, with body fluids by CSF collection or abdominal aspiration from peritonitis (4/235, 1.70%). The origin of the materials was not identified in the eight-sample examination sheet (3.40%) (Table 3).

With a higher frequency of clinical infections, and more straightforward diagnosis and collection, samples of integumentary infections, urinary and otological infections, are often more sent to microbiology laboratories by clinicians, as also reported by Pereira et al. (2009), Ishii et al. (2011) and Kohl et al. (2016).

A high number of antimicrobial resistance to antibiotics used in veterinary medicine was found (Fig.1). In *Staphylococcus* spp. resistance/susceptibility to beta-lactam drugs is predicted by penicillin, oxacillin, and cefoxitin (CLSI 2018, BrCAST 2019). Penicillin resistance predicts resistance to penicillins and aminopenicillins, and these strains are considered susceptible to the association of aminopenicillins with beta-lactamase inhibitors (clavulanic acid and sulbactam). Oxacillin/cefoxitin resistance predicts resistance to all beta-lactam drugs, including penicillins, aminopenicillins, whether or not associated with inhibitors, cephalosporins, except the 5th generation, untested, and carbapenems. In this study, 24.30% (26/107) of *Staphylococcus* spp. were susceptible to all beta-lactams drugs, 37.38% (40/107) were resistant to penicillins and aminopenicillins but sensitive to associations with beta-lactamase inhibitors, cephalosporins, and carbapenem, and 38.32% (41/107) were resistant to all beta-lactams.

Staphylococcus spp. resistant to oxacillin/cefoxitin are reported as methicillin-resistant *Staphylococcus* spp. (MRS), often being studied for their great clinical and epidemiological importance. These strains are often associated with resistance to several other antimicrobial drugs (Bardiau et al. 2013, Haenni et al. 2014).

Bardiau et al. (2013) detected high resistance to penicillin, gentamicin, amikacin, erythromycin, tetracycline, ciprofloxacin, clindamycin, sulfa, and chloramphenicol in methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) from dogs and cats from Belgium. All isolates were resistant to at least five of the fifteen tested antibiotics. One isolate was resistant to all tested antibiotics.

Haenni et al. (2014) in their study also with canine origin MRSP found high resistance to penicillin, tetracycline, erythromycin, enrofloxacin, amikacin, tobramycin and

gentamicin, only chloramphenicol had a lower resistance (22%). In MSSP strains, low resistance was found for all drugs, especially enrofloxacin (9.4%), tobramycin and gentamicin (5.4% each).

The Enterobacteriales order has intrinsic resistance to penicillin, but aminopenicillins, considered broad-spectrum penicillins, also show adequate Gram-negative action. Resistance to these drugs was found in 68.75% (55/80) of

Table 2. Identification of isolated bacteria in small animal infections in the Umuarama region, Paraná

Identification			n	%
Gram-positive		*Staphylococcus* spp.	106	45.10
		Micrococcus spp.	1	0.43
		Enterococcus spp.	11	4.68
		Streptococcus spp.	6	2.55
		Streptococus/Enterococcus	5	2.13
	Total		129	54.89
Gram-negative	Enterobacteriales	*Escherichia coli*	11	4.68
		Escherichia fergusoni	6	2.55
		Salmonella spp.	4	1.70
		Citrobacter spp.	3	1.28
		Citrobacter braakii	2	0.85
		Citrobacter freundii	1	0.43
		Citrobacter youngae	1	0.43
		Proteus mirabilis	5	2.13
		Proteus spp.	2	0.85
		Proteus vulgaris	1	0.43
		Klebsiella pneumoniae	3	1.28
		Klebsiella oxytoca	1	0.43
		Klebsiella ozaenae	1	0.43
		Morganella morganii	4	1.70
		Enterobacter amnogenus 2	2	0.85
		Enterobacter sakazakii	1	0.43
		Enterobacter cloacae	2	0.85
		Enterobacter cancerogenus	3	1.28
		Enterobacter spp.	1	0.43
		Hafnia alvei	3	1.28
		Serratia spp.	2	0.85
		Serratia liquefaciens	1	0.43
		Providencia spp.	3	1.28
		Plesiomonas shigelloides	1	0.43
		Yersinia frederiksenii	1	0.43
		Yersinia enterocolitica	1	0.43
		Yernisia pseudotuberculosis	1	0.43
		Yersinia intermedius	1	0.43
		Yersinia spp.	1	0.43
		Shigela boydii sorogrupo C	1	0.43
		Leclercia adecarboxylata	2	0.85
		Ralstonia picketti va2	1	0.43
		Kluyvera ascorbata	1	0.43
		Not identified	6	2.55
	Total		80	34.04
	Non-fermenting Gram-negative bacilli	*Pseudomonas aeruginosa*	19	8.09
		Acinetobacter baumannii	2	0.85
		Burkholderia pseudomallei	1	0.43
		Ochrobactrum anthropi	1	0.43
		Chromobacterium violaceum	1	0.43
		Brevundimonas vesicularis	1	0.43
		Not identified	1	0.43
	Total		26	11.06
TOTAL			235	100%

the studied enterobacterial isolates. In the combination of amoxicillin with clavulanic acid, resistance was identified in 52.50% (42/80) of the isolates, whereas in the combination of ampicillin with sulbactam the resistance was only 28.95% (22/76). Resistance to cephalosporins was detected in 55.70% (44/79) of enterobacteria for first generation (cephalothin), 48.68% and 55.00% for the third generation (ceftriaxone and ceftazidime, respectively), and 56.06% for fourth generation (cefepime). Carbapenems, hospital drugs indicated for the treatment of severe infections by multiresistant enterobacteria, presented a resistance percentage of 7.50% (6/80) and 18.42% (14/76) for meropenem and imipenem, respectively.

Carvalho et al. (2016), studying *E. coli* from dog feces in Rio de Janeiro, Brazil, found a resistance percentage of 85.7% to ampicillin, 35.7% to amoxicillin with clavulanic acid, 33.3% to cefalexin, 16.6% to ceftazidime, 19.4% to cefotaxime, 21.4% to ceftriaxone and 9.5% to cefepime.

Pseudomonas are susceptible only to third and fourth generations of cephalosporins and carbapenems. There were found 66.67% of resistance to ceftriaxone and cefotaxime and 16.67% to cefepime. Carbapenem resistance was found in 21.05% of isolates for meropenem and 16.67% for imipenem.

Acinetobacter baumannii and *Enterococcus* spp. are intrinsically resistant to beta-lactams except for carbapenems. Both isolates of *Acinetobacter baumannii* were susceptible to meropenem, and one was resistant to imipenem, and of the three *Enterococcus* tested for imipenem, one was resistant.

The second broad class broad-spectrum drug, fluoroquinolones, tested on all bacterial isolates, showed general resistance in the 30-40% range for the second generation, represented by enrofloxacin (42.56%), ciprofloxacin (36.45%) and norfloxacin (32.59%). The third generation represented by levofloxacin presented resistance in 29.71% of the isolates tested. Resistance values were similar between *Staphylococcus* spp. and enterobacteria, in pseudomonas the resistance to enrofloxacin was 40% while the other fluoroquinolones had resistance in 26 and 29% in the isolates. The two *Acinetobacter* isolates were resistant to ciprofloxacin and levofloxacin, as well as all *Streptococcus* spp. Fifty percent of *Enterococcus* spp. were resistant to norfloxacin.

Stefanetti et al. (2017) detected resistance to fluoroquinolones in 76% of isolates to enrofloxacin; Silva et al. (2014) by 20.1% for enrofloxacin and 18.8% for ciprofloxacin, representing 22.3% of fluoroquinolone-resistant bacterial isolates; Ishii et al. (2011) found resistance to enrofloxacin in 53.8% of isolates and norfloxacin in 41.3%. Scherer et al. (2018) detected 38.6% of resistance to enrofloxacin; Kohl et al. (2016) detected 30% of resistance to enrofloxacin in Gram-negative bacteria and 13.33% in Gram-positive bacteria; and Carvalho et al. (2016), 11.9% to ciprofloxacin.

The third primary class, aminoglycosides, showed resistance in 10.78% of the isolates to amikacin, 22.01% to gentamicin

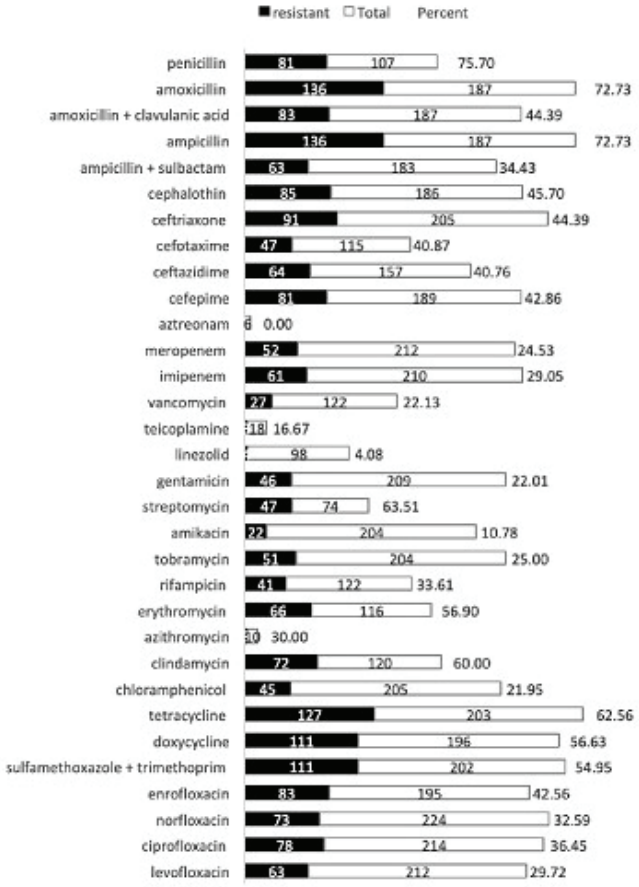


Fig.1. General profile of antimicrobial resistance found in bacteria isolated from infections in small animals in the region of Umuarama/PR, Brazil.

Table 3. Distribution of bacteria found according to the origin of infection in small animals in Umuarama region, Paraná

System	Infection site	*Staphylococcus* spp.		*Enterococcus*/ *Streptococcus* spp.		Enterobacterales		NFGNB		Total	
		n	%	n	%	n	%	n	%	n	%
Integumentary	Skin / Attachments	47	47.96	10	10.20	32	32.65	9	9.18	98	41.70
	Otological	40	57.97	4	5.8	15	21.74	10	14.49	69	83.13
Sensory	Ophthalmic	4	57.14	0	-	1	14.29	2	28.50	7	8.43
	Nasal	3	42.86	0	-	4	57.14	0	-	7	8.43
Urinary	Urine	10	31.25	6	18.75	14	43.75	2	6.25	32	13.60
Female breeder	Vaginal	1	10.00	0	-	7	70.00	2	20.00	10	4.26
Others	Body fluids	2	50.00	1	25.00	1	25.00	0	-	4	33.33
	Not identified	0	-	1	12.50	6	75.00	1	12.50	8	66.67
TOTAL		107	45.53	22	9.36	80	34.04	26	11.06	235	100.00

NFGNB = Non-fermenting Gram-negative bacilli.

and 25% to tobramycin, maintaining similar values between *Staphylococcus* and enterobacteria. *Pseudomonas* showed 5.55% of resistance to amikacin and 17.65% of resistance to tobramycin. Both *Acinetobacter* isolates were susceptible to amikacin, and one was resistant to gentamicin. *Streptococcus* and *Enterococcus* are intrinsically resistant to aminoglycosides.

Resistance rates vary according to the type of microorganism studied and location. The lowest resistances found in this study for conventional antimicrobial drugs in veterinary medicine were for amikacin, gentamicin, and tobramycin. Unlike Stefanetti et al. (2017) in Italy, with resistance in 43% of *S. pseudintermedius* isolates for amikacin and 61% for gentamicin. Scherer et al. (2018) studying *S. pseudintermedius* in dogs with otitis external in Minas Gerais, Brazil, found 27.3% of resistance to gentamicin. Kohl et al. (2016) in the state of Santa Catarina, Brazil, detected gentamicin resistance in 33.33% of Gram-negative and 12% of Gram-positive. Ishii et al. (2011) detected resistance to amikacin in 32.6%, gentamicin in 36.6% and tobramycin in 33.3% of bacterial isolates. Carvalho et al. (2016), studying *Escherichia coli* from dog feces in Rio de Janeiro, Brazil, detected resistance of 30.9% to gentamicin. Yadav et al. (2018) found 100% of isolates of *S. aureus* of canine origin susceptible to gentamicin and amikacin.

The resistance to tetracycline and doxycycline was observed in 62.56% and 56.63%, respectively, remaining in these proportions in staphylococci and enterobacteria. In enterococci and streptococci, the resistance to tetracycline was 85% and 73.68%, respectively. Potentiated sulfa resistance was found in 54.95% of the bacterial isolates tested, where the same patterns of tetracyclines were found in staphylococci and enterobacteria, increasing in enterococci and streptococci (66.67%). The resistance to phenicols, represented by chloramphenicol was 21.95%, being 16.04% in staphylococci, 30.77% in enterobacteria and 19.05% in enterococci and streptococci. Intrinsic resistance for these three classes is observed in NFGNB, including pseudomonas and acinetobacter.

Resistance to tetracyclines and potentiated sulfonamides was also found by Stefanetti et al. (2017) with 73% of resistance to doxycycline and 80% to sulfamethoxazole-trimethoprim; Scherer et al. (2018) with 61.4% to tetracycline and 63.6% to sulfamethoxazole + trimethoprim; and Ishii et al. (2011) with 75.6% to sulfamethoxazole + trimethoprim. While Silva et al. (2014) detected 4.5% of resistance to doxycycline and 29.9% to sulfamethoxazole + trimethoprim and Carvalho et al. (2016), 50% to tetracycline, 33.3% to doxycycline and 30.9% to sulfamethoxazole + trimethoprim. Carvalho et al. (2016), in *E. coli*, found chloramphenicol resistance in 23.8% of dog isolates.

Drugs considered to be shorter spectrum also showed high resistance, observed in 53.0% of staphylococci and 90.91% of enterococci and streptococci to clindamycin; 54.74% of staphylococci to erythromycin; 30% of enterococci and streptococci to azithromycin; 25% of staphylococci and 83.33% of enterococci and streptococci to rifampicin; and 63.51% of enterobacteria to streptomycin. The shortest and most modern drugs presented resistance in 25.49% of staphylococci and 5% of enterococci and streptococci to vancomycin; 16.67% of enterococci and streptococci to teicoplanin; and 3.75% of staphylococci and 5.56% of enterococci and streptococci to linezolid. Aztreonam, known as the anti-pseudomonas drug, had a 100% susceptibility in the evaluated pseudomonas.

Because pseudomonas has high intrinsic resistance, a small number of antimicrobials are tested (CLSI 2018). A critical resistance found in these microorganisms is resistance to fluoroquinolones, found in 7 of 19 isolates (36.84%). Lin et al. (2012) in their study with Pseudomonas isolated from dogs in China found only 14.8% of resistance to fluoroquinolones, as well as resistance to amikacin (11.1%), gentamicin and tobramycin (14.8%), ceftazidime (3.7%) and imipenem (0 %), unlike this study which did not detect resistance to ceftazidime, but 16.67% of resistance to cefepime and imipenem was detected. Low resistance was also found in the present study in pseudomonas for amikacin (5.55%), gentamicin (22.22%) and tobramycin (17.65%).

High intrinsic resistance is also observed in Acinetobacter (BrCAST, 2019), where both isolates were resistant to all fluoroquinolones tested, one to imipenem and gentamicin and none to amikacin.

According to Jackson et al. (2009), in their study with enterococci isolated from dogs, high resistance was found to chloramphenicol, ciprofloxacin, and gentamicin, unlike the present study which detected high resistance to all cephalosporins, azithromycin, clindamycin, and tetracyclines.

Multidrug resistance, evaluated by the MAR index (Krumperman 1983), showed 64.25% of bacterial isolates as multidrug-resistant, according to Magiorakos et al. (2012), 68.94%. When classified, 48.94% were considered multiresistant (between 0.2 and 0.8) and 15.32% as extensively resistant (above 0.8) according to MAR index, according to Magiorakos et al. (2012), 49.79% were considered multiresistant (between 0.3 and 0.8) and 19.15% extensively resistant (above 0.8). No bacterial isolates were characterized as pan-resistant (all classes and drugs tested resistant), although ten isolates were resistant to all classes, isolated drugs were considered susceptible in these (Table 4).

Table 4. Evaluation of multiresistance in bacteria isolated from infections in small animals in the region of Umuarama/PR, Brazil

Resistance	*Staphylococcus* spp.		*Enterococcus*/ *Streptococcus* spp.		Enterobacterales		NFGNB		Total	
	MDR	MAR	MDR	MAR	MDR	MAR	MDR	MAR	MDR	MAR
>0.3/>0.2	70	64	18	17	61	58	13	12	162	151
>0.8	21	22	2	5	19	9	3	0	45	36
1	6	0	1	0	3	0	0	0	10	0
MDR	49	42	16	12	42	49	10	12	117	115
XDR	21	22	2	5	19	9	3	0	45	36
Non-Resistant	37	43	4	5	19	22	13	14	73	84
TOTAL	107	107	22	22	80	80	26	26	235	235

NFGNB = Non-fermenting Gram-negative bacilli; MDR = multidrug-resistant bacteria, MAR = multiple antibiotic resistance indexing, XDR = extensively drug-resistance bacteria.

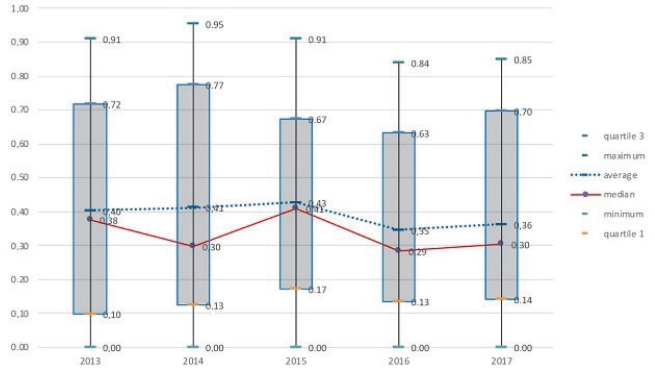


Fig. 2. Boxplot containing the multiple antibiotic resistance indexing (MAR), regardless of the isolated agent, in the respective years studied.

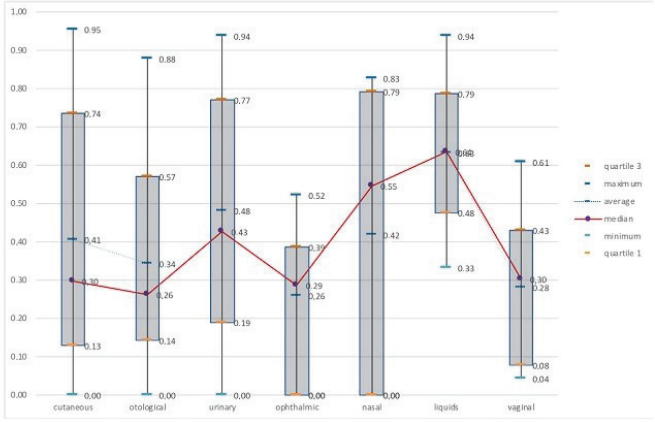


Fig.3. Boxplot containing the multiple antibiotic resistance indexing (MAR), independent of the isolated agent, in the respective infection sites.

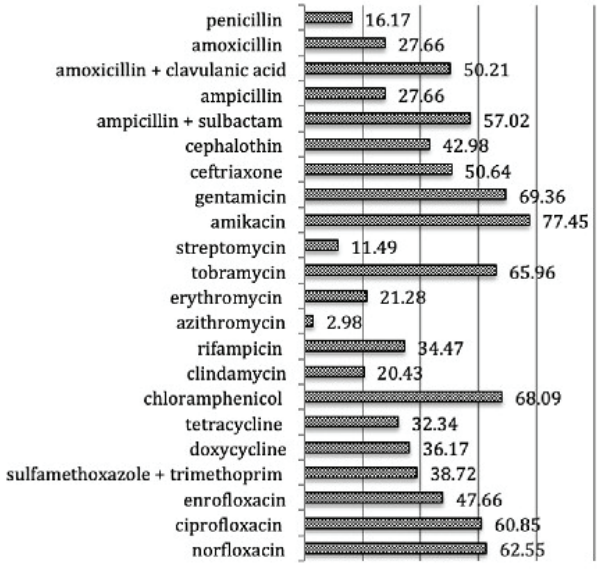


Fig.4. Impact factor of antibiotics used in veterinary medicine of bacteria isolated from infections in small animals in the region of Umuarama/PR, Brazil.

Arias et al. (2013) report a gradual increase in multidrug resistance of antimicrobial agents in veterinary medicine. High rates of multiresistant bacteria were found by Arias et al. (2008) in bacteria isolated from infected and contaminated traumatic animal wounds, with a MAR Index ≥0.2 representing 95% of isolated bacteria, with a mean of 0.7. Three isolates presented MAR = 1.0. Sfaciotte et al. (2014) found similar values, where 89.4% of isolates were considered multiresistant, with a mean MAR index of 0.65 and a maximum of 1.0. Kohl et al. (2016) detected multiresistant strains in 33.3 to 100% of the groups evaluated per period, between Gram-positive and -negative bacteria, with MAR averages between 0.2 and 0.52. Corroborating the study by Arias et al. (2013), who at the Veterinary Hospital of Londrina-Paraná, found high multidrug resistance in all isolates evaluated. All three studies found higher MAR values in Gram-negative bacteria.

Verifying the distribution of the MAR index and evaluating the resistance profile in the period studied (Fig.2), the average curve remains constant from 2013 to 2015, with a low for the following two years. It is also observed that both the average and the median presented values above 0.2 in all evaluated years.

The distribution of the MAR index according to the site of infection (Fig.3) shows superior position measurements for urinary tract infections, respiratory infections represented by nasal samples and body fluids, although the latter two, by low sampling, may not represent the real importance of this resistance.

One of the pillars of prudent antimicrobial therapy is the use of evidence-based antimicrobials (Guardabassi & Prescott 2015). The prevalence of each microorganism associated with its susceptibility underlies the calculation of the impact factor of each antimicrobial, according to Hall et al. (2013) and Rampacci et al. (2018), considered the most accurate indicator of antimicrobial efficacy, according to which the higher the impact factor, the greater the predicted clinical efficacy. The drugs used in antimicrobial therapy in animals with the highest impact factor (Fig.4) were amikacin, followed by gentamicin, chloramphenicol, tobramycin, for topical use only, norfloxacin and ciprofloxacin. Intermediate results for sulbactam-associated ampicillin, ceftriaxone, clavulanic acid amoxicillin, and enrofloxacin. The worst results were found for azithromycin, streptomycin, and penicillin, where we can mention the short spectrum of these antibiotics.

Ishii et al. (2011) point out the difficulty of the empirical selection of antimicrobial drugs with the emergence of antimicrobial resistance. Knowledge of bacterial resistance profiles in the region is essential so that antibiotic prescriptions are not based on resistance patterns described in the literature that may not reflect the reality of the site, as observed in this study. According to Guardabassi & Prescott (2015), these studies increase the possibilities of developing infection control practices, specific to each location, making conscious and moderate use of available antibiotics.

CONCLUSIONS

Staphylococcus spp. represented 45.53% of bacteria isolated from clinical infections in small animals in the period and placed evaluated, followed by enterobacteria (34.04%), non-fermenting Gram-negative bacilli (11.06%) and *Streptococcus*/*Enterococcus* (9.36%).

According to the number of resistant antimicrobial drugs, 64.26% (151/235) of the isolates were classified as multidrug-resistant, being 15.32% extensively resistant. Considering the resistance to antimicrobial classes, 68.94% (162/235) of the isolates were considered multiresistant, being 19.15% extensively resistant. No bacterial isolates were characterized as pan-resistant, but ten bacteria were resistant to all classes tested, with isolated susceptibility to certain drugs.

Resistance to all antimicrobial drugs used in veterinary medicine was found. The lowest resistances associated with the best impact factor values were found for amikacin, gentamicin, chloramphenicol, tobramycin, norfloxacin, and ciprofloxacin. Intermediate results were found for sulbactam-associated ampicillin, ceftriaxone, clavulanic acid amoxicillin, and enrofloxacin.

By evaluating the antimicrobial resistance profiles found at the time and place studied, it can be concluded that there is an increasing in the number of multidrug-resistant agents among domestic animals, which becomes a serious public health risk for all those come into direct or indirect contact with them - including through the environment - especially their tutors and healthcare professionals such as the veterinarian.

It can also be seen that the therapeutic arsenal is becoming smaller and smaller, and there is more difficulty in the empirical selection of drugs to be instituted in clinical treatment. Making it essential to perform bacterial identification tests and their awareness of their sensitivity to a more specific selection of drugs to be used, and educational measures on the conscious use of antibiotics, infection control, and prevention of site-specific zoonoses need to be instituted for knowledge of health professionals and general access of the population.

Acknowledgements.- The authors are grateful to the "Fundação Araucária de Apoio ao Desenvolvimento Científico e Tecnológico do Estado do Paraná" (001/2016); "Laboratório de Microrganismos de Referência" of "Fundação Oswaldo Cruz" (Fiocruz) for assigning ATCC strains; and Graduate Program in Sustainable Production and Animal Health, "Universidade Estadual de Maringá" (UEM).

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

REFERENCES

Acar J.F., Moulin G., Page S.W. & Pastoret P.P. 2012. Antimicrobial resistance in animal and public health: introduction and classification of antimicrobial agents. Rev. Sci. Tech. Off. Int. Epiz. 31(1):15-21. <https://dx.doi.org/10.20506/rst.31.1.2093> <PMid:22849264>

Adeolu M., Alnajar S., Naushad S. & Gupta R.S. 2016. Genome-based phylogeny and taxonomy of the 'Enterobacterales': proposal for Enterobacterales ord. nov. divided into the families Enterobacteriaceae, Erwiniaceae fam. nov., Pectobacteriaceae fam. nov., Yersiniaceae fam. nov., Hafniaceae fam. nov., Morganellaceae fam. nov., and Budviciaceae fam. nov. Int. J. Syst. Evol. Microbiol. 66:5575-5599. <https://dx.doi.org/10.1099/ijsem.0.001485> <PMid:27620848>

Aidara-Kane A. 2012. Containment of antimicrobial resistance due to use of antimicrobial agents in animals intended for food: WHO perspective. Rev. Sci. Tech. Off. Int. Epiz. 31(1):277-287. <https://dx.doi.org/10.20506/rst.31.1.2115> <PMid:22849282>

Anvisa 2017. Diretriz nacional para elaboração de programa de gerenciamento do uso de antimicrobianos em serviços de saúde. Agência Nacional de Vigilância Sanitária, Brasília, DF. 90p.

Arias M.V.B., Aiello G., Battaglia L.A. & Freitas J.C. 2013. Estudo da ocorrência de infecção hospitalar em cães e gatos em um centro cirúrgico veterinário universitário. Pesq. Vet. Bras. 33(6):771-779. <https://dx.doi.org/10.1590/S0100-736X2013000600014>

Arias M.V.B., Battaglia L.A., Aiello G., Carvalho T.T. & Freitas J.C. 2008. Identificação da suscetibilidade antimicrobiana de bactérias isoladas de cães e gatos com feridas traumáticas contaminadas e infectadas. Semina, Ciênc. Agrárias 29(4):861-874. <https://dx.doi.org/10.5433/1679-0359.2008v29n4p861>

Bardiau M., Yamazaki K., Ote I., Misawa N. & Mainil J.G. 2013. Characterization of methicillin-resistant *Staphylococcus pseudintermedius* isolated from dogs and cats. Microbiol. Immunol. 57(7):496-501. <https://dx.doi.org/10.1111/1348-0421.12059> <PMid:23607810>

Barie P.S. 2012. Multidrug-resistant organisms and antibiotic management. Surg. Clin. N. Am. 92(2):345-391. <https://dx.doi.org/10.1016/j.suc.2012.01.015> <PMid:22414417>

BrCAST 2019. Tabela de pontos de corte para interpretação de CIMs e diâmetros de halos. Brazilian Commitee on Antimicrobial Suscetibilitty Testing, s.l. 70p.

Carvalho A.C., Barbosa A.V., Arais L.R., Ribeiro P.F., Carneiro V.C. & Cerqueira A.M.F. 2016. Resistance patterns, ESBL genes, and genetic relatedness of Escherichia coli from dogs and owners. Braz. J. Microbiol. 47(1):150-158. <https://dx.doi.org/10.1016/j.bjm.2015.11.005>

CLSI 2008. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals. CLSI document M31-A3, Approved Standard, 3rd ed., Clinical and Laboratory Standards Institute, Wayne, PA. 103p.

CLSI 2013. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals. CLSI document VET01-A4, Approved Standard, 4th ed., Clinical and Laboratory Standards Institute, Wayne, PA. 81p.

CLSI 2018. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals. CLSI standard VET01, 5th ed., Clinical and Laboratory Standards Institute, Wayne, PA. 156p.

Fernandes M.R., Sellera F.P., Moura Q., Carvalho M.P.N., Rosato P.N., Cerdeira L. & Lincopan N. 2018. Zooanthroponotic transmission of drug-resistant *Pseudomonas aeruginosa*, Brazil. Emerg. Infect. Dis. 24(6):1160-1162. <https://dx.doi.org/ 10.3201/eid2406.180335> <PMid:29774849>

Giguère S. 2013. Antimicrobial drug action and interaction: an introduction, p.3-7. In Giguère S., Prescott J.F. & Dowling P.M. (Eds), Antimicrobial Therapy in Veterinary Medicine. 5th ed. John Wiley and Sons, Hoboken, New Jersey.

Guardabassi L. & Prescott J.F. 2015. Antimicrobial stewardship in small animal veterinary practice. Vet. Clin. N. Am., Small Anim. Pract. 45(2):361-376. <https://dx.doi.org/10.1016/j.cvsm.2014.11.005> <PMid:25721619>

Guardabassi L., Jensen L.B. & Kruse H. 2010. Guia de Antimicrobianos em Veterinária. Artmed, Porto Alegre. 268p.

Haenni M., De Moraes N.A., Châtre P., Médaille C., Moodley A. & Madec J.Y. 2014. Characterisation of clinical canine meticillin-resistant and meticillin-susceptible *Staphylococcus pseudintermedius* in France. J. Glob. Antimicrob. Resist. 2(2):119-123. <https://dx.doi.org/10.1016/j.jgar.2014.02.002> <PMid:27873590>

Hall J.L., Holmes M.A. & Baines S.J. 2013. Prevalence and antimicrobial resistance of canine urinary tract pathogens. Vet. Rec. 173(22):549. <https://dx.doi.org/10.1136/vr.101482> <PMid:24158327>

Ishii J.B., Freitas J.C. & Arias M.V.B. 2011. Resistência de bactérias isoladas de cães e gatos no Hospital Veterinário da Universidade Estadual de Londrina (2008-2009). Pesq. Vet. Bras. 31(6):533-537. <https://dx.doi.org/10.1590/S0100-736X2011000600013>

Jackson C.R., Fedorka-Cray P.J., Davis J.A., Barrett J.B. & Frye J.G. 2009. Prevalence, species distribution and antimicrobial resistance of enterococci isolated from dogs and cats in the United States. J. Appl. Microbiol. 107(4):1269-1278. <https://dx.doi.org/10.1111/j.1365-2672.2009.04310.x> <PMid:19486402>

Joseph J. & Rodvold K.A. 2008. The role of carbapenems in the treatment of severe nosocomial respiratory tract infections. Expert Opin. Pharmacother. 9(4):561-575. <https://dx.doi.org/10.1517/14656566.9.4.561> <PMid:18312158>

Kohl T., Pontarolo G.H. & Pedrassani D. 2016. Resistência antimicrobiana de bactérias isoladas de amostras de animais atendidos em hospital veterinário. Saúde Meio Ambiente, Revta Interdisciplinar 5(2):115-127. <https://dx.doi.org/10.24302/sma.v5i2.1197>

Krumperman P.H. 1983. Multiple antibiotic resistance indexing of Escherichia coli to identify high-risk source of fecal contamination of foods. Appl. Environ. Microbiol. 46(1):165-170. <https://dx.doi.org/10.1128/AEM.46.1.165-170.1983> <PMid:6351743>

Lin D., Foley S.L., Qi Y., Han J., Ji C., Li R, Wu C., Shen J. & Wang Y. 2012. Characterization of antimicrobial resistance of Pseudomonas aeruginosa isolated from canine infections. J. Appl. Microbiol. 113(1):16-23. <https://dx.doi.org/10.1111/j.1365-2672.2012.05304.x> <PMid:22487022>

Magiorakos A.P., Srinivasan A., Carey R.B., Carmeli Y., Falagas M.E., Giske C.G., Harbarth S., Hindler J.F., Kahlmeter G., Olsson-Liljequist B., Paterson D.L., rice L.B., Stelling J., Struelens M.J., Vatopoulos A., Weber J.T. & Monnet D.L. 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 18(3):268-281. <https://dx.doi.org/10.1111/j.1469-0691.2011.03570.x> <PMid:21793988>

Messenger A.M., Barnes A.N. & Gray G.C. 2014. Reverse zoonotic disease transmission (zooanthroponosis): a systematic review of seldom-documented human biological threats to animals. PLoS One 9(2):e89055. <https://dx.doi.org/10.1371/journal.pone.0089055> <PMid:24586500>

Molina F., Alí A., Beltrán N., Camargo R., Castro L., Fonseca N., Gil B., Gómez U., Mercado M., Solarte M., Oñate J., Osorio J., Pinillos O., Urbina Z., Valderrama S. & Villarreal A. 2012. DE-ESCALAR: una estrategia terapéutica en la Unidad de Cuidado Intensivo. Acta Colomb. Cuid. Intens. 12(3):163-169.

Murphy P., Reid-Smith R.J., Boerlin P., Weese J.S., Prescott J.F., Janecko N. & Mcewen S.A. 2012. Out-patient antimicrobial drug use in dogs and cats for new disease events from community companion animal practices in Ontario. Can. Vet. J. 53(3):291-298. <PMid:22942447>

Pereira A.L. & Pita J.L. 2005. Alexander Fleming (1881-1955): da descoberta da penicilina (1928) ao Prémio Nobel (1945). Revta Facul. Letras, Hist., Porto 6:129-151.

Pereira I.A., Soares L.C., Coelho S.M.O., Pribul B.R. & Souza M.M.S. 2009. Suscetibilidade à azitromicina de isolados bacterianos de processos infecciosos em cães e gatos. Pesq. Vet. Bras. 29(2):153-156. <https://dx.doi.org/10.1590/S0100-736X2009000200012>

Prescot J.T. 2013. Beta-lactam antibiotics: penam penicillins. p.135-152. In Giguère S., Prescott J.F. & Dowling P.M. (Eds), Antimicrobial Therapy in Veterinary Medicine. 5th ed. John Wiley and Sons, Hoboken, New Jersey.

Rampacci E., Bottinelli M., Stefanetti V., Hyatt D.R., Sgariglia E., Coletti M. & Passamonti F. 2018. Antimicrobial susceptibility survey on bacterial agentes of canine and feline urinary tract infections: weight of the empirical treatment. J. Glob. Antimicrob. Resist. 13:192-196. <https://dx.doi.org/10.1016/j.jgar.2018.01.011> <PMid:29408407>

Scherer C.B., Botoni L.S., Coura F.M., Silva R.O., Santos R.D., Heinemann M.B. & Costa-Val A.P. 2018. Frequency and antimicrobial susceptibility of *Staphylococcus pseudintermedius* in dogs with otitis externa. Ciência Rural 48(4):e20170738. <https://dx.doi.org/10.1590/0103-8478cr20170738>

Sfaciotte R.A.P., Coronel L.G., Snak A., Bordin J.T., Wildemann P., Melo F.D., Vignoto V.K.C., Ferraz S.M., Wosiacki S.R. & Osaki S.C. 2017. Antimicrobial resistance phenotypic profile of isolates from clinical infections in dogs. Acta Scient. Vet. 45:1485. <https://dx.doi.org/10.22456/1679-9216.80501>

Sfaciotte R.A.P., Vignoto V.K.C. & Wosiacki S.R. 2014. Perfil de resistência antimicrobiana de isolados bacterianos de afecções clínicas do Hospital Veterinário da Universidade Estadual de Maringá. Revta Ciênc. Vet. Saúde Públ. 1(1):29-38. <https://dx.doi.org/10.4025/revcivet.v1i1.23281>

Silva A.P., Schmidt C., Vargas A.C., Maboni G., Rampelotto C., Schwab M.L., Escobar T.P. & Amaral A.S. 2014. Suscetibilidade antimicrobiana de Staphylococcus spp. isolados de cães com pioderma superficial. Pesq. Vet. Bras. 34(4):355-361. <https://dx.doi.org/10.1590/S0100-736X2014000400010>

Stefanetti V., Bietta A., Pascucci L., Marenzoni M.L., Coletti M., Franciosini M.P., Passamonti F. & Proietti P.C. 2017. Indagine su antibiotico-resistenza e formazione di biofilm in ceppi di *Staphylococcus pseudintermedius* isolati da piodermiti canine. Vet. Ital. 53(4):289-296. <https://dx.doi.org/10.12834/VetIt.465.2275.6>

Ventola C.L. 2015. The antibiotic resistance crisis: part 1: causes and threat. PT 40(4):277-283. <PMid:25859123>

WHO 2019. Critically important antimicrobials for human medicine. 6th ed. World Health Organization, Geneva. 45p.

Yadav R., Kumar A., Singh V.K., Jayshree & Yadav S.K. 2018. Prevalence and antibiotyping of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) in domestic animals in India. J. Glob. Antimicrob. Resist. 15:222-225. <https://dx.doi.org/10.1016/j.jgar.2018.08.001> <PMid:30092364>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(10):814-817, October 2020
DOI: 10.1590/1678-5150-PVB-6494

Original Article
Wildlife Medicine
BY-NC

Rickets in white-eared opossums (*Didelphis albiventris*)[1]

Jair A. Ferreira Júnior[2], Marina F. Landi[3], Ariane O. Passos[4], Karla A. Nascimento[2], João Paulo M.V.B Azevedo[2], Juliana T.S.A. Macêdo[2] and Pedro M.O. Pedroso[2]*

ABSTRACT.- Ferreira Júnior J.A., Landi M.F., Passos A.O., Nascimento K.A., Azevedo J.P.M.B.V., Macêdo J.T.S.A. & Pedroso P.M.O. 2020. **Rickets in white-eared opossums (*Didelphis albiventris*).** *Pesquisa Veterinária Brasileira 40(10):814-817*. Laboratório de Patologia Veterinária, Universidade de Brasília, Campus Universitário Darcy Ribeiro, Via L4 Norte s\n, Brasília, DF 70910-970, Brazil. E-mail: pedrosovet@yahoo.com.br

Two young opossums were necropsied and diagnosed with rickets. This study aims to describe the clinical-pathological aspects of rickets in *Didelphis albiventris*. Macroscopically, the opossums presented kyphosis and scoliosis, lateral deviation of the limbs in *varus*, locomotion difficulty, and enlargement with softening of costochondral junctions (rickety rosary). Samples of bones and joints were processed for hematoxylin and eosin staining and Masson's trichrome. Microscopically, we observed thickening of the epiphyseal plate, characterized by irregular and multifocal proliferation of serialized and hypertrophic cartilage zones, which formed circular groups of large, dysplastic chondrocytes towards the spongy zone, often surrounded by non-mineralized osteoid tissue. In the cortical bone, there were pale eosinophilic zones around the Havers channels consistent with non-mineralized osteoid. The staining of Masson's trichrome evidenced the accumulation of osteoid tissue in cortical and trabecular bones. It is possible that a mixed cause of absorption deficiency of vitamin D3 associated with an unbalanced Ca:P diet based on lactose-free milk and fruits may have triggered the disease.

INDEX TERMS: Rickets, white-eared opossum, *Didelphis albiventris*, opossum, metabolic bone disease, pathology, vitamin D, calcium.

RESUMO.- [Raquitismo em gambás-de-orelha-branca (*Didelphis albiventris*).] Dois gambás jovens foram necropsiados e diagnosticados com raquitismo. O objetivo do trabalho é descrever os aspectos clínico-patológicos de raquitismo em *Didelphis albiventris*. Macroscopicamente os gambás apresentaram cifose e escoliose, desvio lateral dos membros em *varus*, dificuldade de locomoção e alargamento com amolecimento das junções costocondrais (rosário raquítico). Amostras dos ossos e articulações foram processadas para coloração de hematoxilina e eosina e Tricrômico de Masson. Microscopicamente havia espessamento da placa epifisária, caracterizada pela proliferação irregular e multifocal das zonas de cartilagem seriada e hipertrófica, que formavam grupos circulares de condrócitos grandes, displásicos em direção a zona esponjosa frequentemente cercados por tecido osteoide não mineralizado. No osso cortical haviam zonas eosinofílicas pálidas ao redor dos canais de Havers consistentes com osteoide não mineralizado. A coloração de Tricrômico de Masson evidenciou o acúmulo de tecido osteoide no nosso cortical e trabecular. Acredita-se que uma causa mista de déficit de absorção de vitamina D3 associada a uma dieta desbalanceada em Ca:P a base de leite sem lactose e frutas tenha desencadeado a doença.

TERMOS DE INDEXAÇÃO: Raquitismo, gambá-de-orelha-branca, *Didelphis albiventris*, gambá, doença óssea metabólica, patologia, vitamina D, cálcio.

INTRODUCTION

Rickets is a metabolic disease that occurs in humans and animals. It is characterized by a deficiency in the mineralization of the physical and epiphyseal cartilage during the endochondral ossification of the newly formed osteoid in young animals

[1] Received on February 4, 2020.
Accepted for publication on May 16, 2020.
[2] Laboratório de Patologia Veterinária, Universidade de Brasília (UnB), Via L4 Norte, Brasília, DF 70910-970, Brazil. *Corresponding author: pedrosovet@yahoo.com.br
[3] Faculdade de Medicina Veterinária e Zootecnia (FMVZ), Universidade de São Paulo (USP), Av. Prof. Dr. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP 05508-000, Brazil.
[4] Setor de Animais Silvestres, Hospital Veterinário, Universidade de Brasília (UnB), Via L4 Norte, Brasília, DF 70910-970, Brazil.

before the end of growth. It affects bone tissue and growth cartilages, and occurs due to calcium and/or phosphorus deficiency, which can be primary, dietary deficiency, or secondary (Sekarides 2016). Metabolic bone disease (MBD) is one of the terms used to treat diseases that cause bone morphology and functional changes in captive Virginia opossums (*Didelphis virginiana*). In these animals, the syndrome is usually caused by inadequate diet, Ca deficiency, or Ca:P imbalance and less likely by vitamin D deficiency (McRuer & Jones 2009). Vitamin D can be of dietary origins, such as D2 or D3, or the isomerization of 7-dehydrocholesterol (7-DHC) in the skin from ultraviolet light. Herbivores would typically perform this conversion, while carnivores are more dependent on eating other animal's fat. Vitamin D acts on the intestines, bones, kidneys, and parathyroid glands, maintaining the balance between calcium and phosphorus concentrations (Dittmer & Thompson 2011). In the disease, there is an increase in the volume of bony epiphyses and costochondral junctions, with an accumulation of non-eroded and osteoid cartilage; irregular epiphyseal plate, with delay and alteration of endochondral growth characterized by irregular invasion of the vessels in the growth cartilages, resulting in arching and reduction in bone length; and decreased bone strength, with spontaneous fractures and loss of radiopacity (Craig et al. 2016, Sekarides 2016). Altered skeletal integrity can manifest itself as retarded growth, fractured bones, and/or disturbed calcium and phosphorus levels in the blood (Madson et al. 2012). This study's objective was to describe ricket's clinical-pathological aspects in two free-living opossums (*Didelphis albiventris*) kept in the hospital.

MATERIALS AND METHODS

Two male, white-eared, young opossums (*Didelphis albiventris*) ("Saruê" no. 1 and 2) were referred to the "Laboratório de Patologia Veterinária" of the "Universidade de Brasília" for necropsy. During the necropsy, fragments of various organs and bones of the pelvic, thoracic, and ribs were collected and fixed in 10% buffered formaldehyde. The fragments were then sent for routine histological processing, embedded in paraffin, cut to 5µm thick, and stained with hematoxylin and eosin (HE). The special Masson's trichrome staining was also performed to show the non-mineralized osteoid matrix and connective tissue proliferation. Additional information was obtained from the requesting veterinarian.

RESULTS

The white-eared opossums ("saruês") were referred to as puppies for clinical care and, during the first month of life. Lactose-free milk and vitamin D3 supplementation were provided, in addition to fruits supplemented with calcium carbonate. The animals were kept in galvanized cages and without access to sunlight for the same period. In the third month, kyphosis (1/2) and scoliosis (2/2) were observed, in addition to limbs curved in varus in both animals, which promoted locomotor difficulties. Scoliosis and locomotion difficulty of the Saruê 1 made him unable to return to nature, and he died in transport to the Veterinary Hospital. Saruê 2 started to present dyspnea and nasal discharge. Laboratory tests showed an increase in metarrubycites (nucleated red cells), lymphopenia, a marked increase in total alkaline phosphatase 1,119 U/L (mean 11.9 U/L for the species (standard deviation 4.2) (Malta & Luppi 2007) and a slight reduction in creatinine (0.3 mg/dl). Treatment with antibiotics was carried out, but there was no success, and due to the worsening of the clinical condition, euthanasia was chosen.

During necropsy, we observed marked curvature of the pelvic and thoracic limbs in varus (Fig.1A), enlargement of the joints, enlargement of the costochondral junctions (rachitic rosary), and spinal deformities (kyphosis and scoliosis). The long bones showed elasticity and softening and, when cut, regular thickening of the epiphyseal plates.

Microscopically, we observed the femur and ribs' metaphysis at the costochondral joint with thickening of the epiphyseal plate, characterized by the irregular and multifocal proliferation of the zones of serial and hypertrophic cartilage, which formed circular groups of large, dysplastic or anucleate (necrotic) chondrocytes. (Fig.2A) towards the spongy area, often surrounded by non-mineralized osteoid tissue. The cortical bone presented pale eosinophilic zones around the Haversian channels consistent with non-mineralized osteoid. The Masson's trichrome stain showed osteoids non-mineralization, staining in blue instead of red compared to a compact, healthy opossum bone (Figs.2C and 2D). Also, the first opossum, in addition to bone alterations, presented a marked multifocal pulmonary lesion, characterized by macrophage infiltration into alveolar spaces and edema.

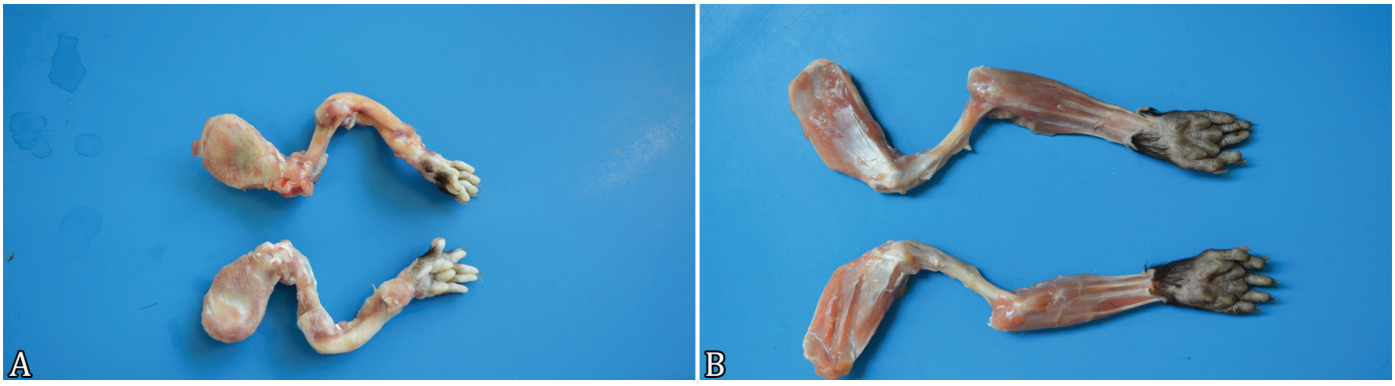


Fig.1. Rickets in white-eared opossums (*Didelphis albiventris*). (**A**) Opossum with lateral varus deviation in the thoracic limbs. (**B**) Normal opossum without curvature of the thoracic limbs.

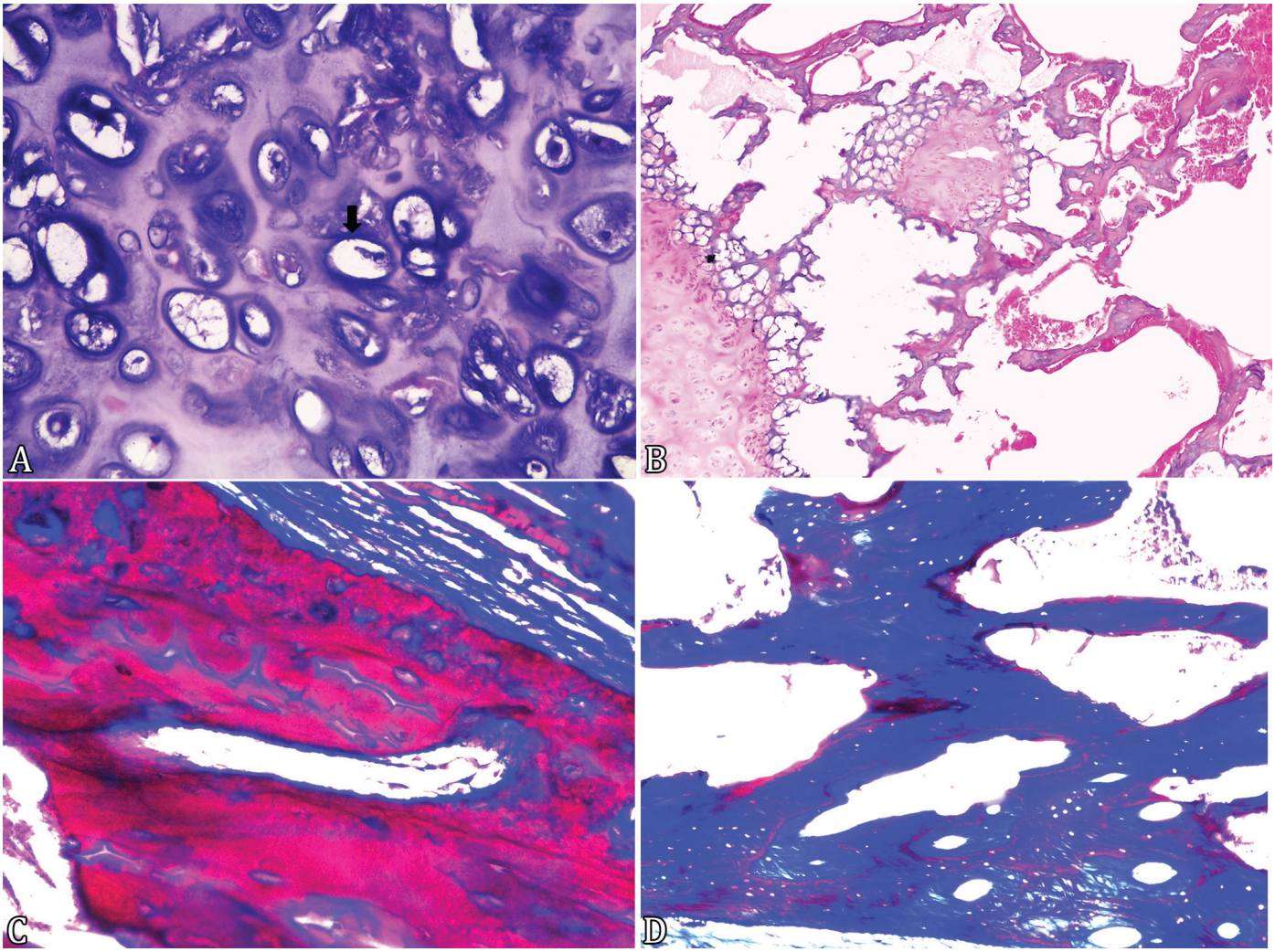


Fig.2. Rickets in white-eared opossums (*Didelphis albiventris*). (**A**) Rib metaphysis. Disorganized, large, dysplastic, and sometimes anucleate chondrocytes are noted (arrow). HE, obj.40x. (**B**) Femur metaphysis with irregular and multifocal proliferation of the serial and hypertrophic cartilage zones. HE, obj.10x. (**C**) Cortical bone of the femur. A red coloring is noted, representing mineralized bone tissue in a normal opossum of the same age. Masson's trichrome, obj.10x. (**D**) Cortical bone of the rib. Bone's trabeculae are mostly stained in blue representing non-mineralized and fibrous bone tissue. Masson's trichrome, obj.10x.

DISCUSSION AND CONCLUSION

The diagnosis of rickets in white-eared opossums ("saruês", *Didelphis albiventris*) was based on clinical-pathological and epidemiological aspects. MBD that occurs in captive *Didelphis virginiana* is treated as an entity involving a group of metabolic bone diseases such as osteoporosis, osteomalacia, rickets, imperfect osteogenesis, cage palsy, bone atrophy, juvenile osteoporosis, bone disease, fibrous osteodystrophy, and cystic fibrous osteitis, which have osteopenia and may or may not occur at the same time (McRuer & Jones 2009). Due to conditions of captivity and the absence of commercial formulations in the opossum's diet, we believe that the imbalance of the Ca:P ratio, with a cholecalciferol bioavailability deficit were determinant for the development of the lesions. The metabolic bone disease supported this hypothesis in opossums currently associated with food that does not reproduce the conditions of nature, mostly related to a calcium deficiency or an inadequate Ca:P ratio in the diet (McRuer & Jones 2009). However, vitamin D deficiency due to low sun exposure alone would be a less likely cause. Unlike humans, herbivores, pigs, and rats (How et al. 1994, Dittmer & Thompson 2011), nocturnal mammals such as *D. albiventris* and other twilight mammals are highly efficient at producing vitamin D3 (cholecalciferol) on the skin with less exposure to sunlight (McRuer & Jones 2009).

In opossums, the pathogenesis of rickets seems to be similar to what occurs in infants. Studies show some rickets cases attributed to deficient dietary calcium intake in the presence of adequate vitamin D intake. Also, in humans, a low calcium diet is known to exacerbate rickets' development due to vitamin D deficiency in children (Olson & Carlson 2017).

In addition to the dietary and environmental factors, other unknown causes could be raised for the development of rickets in opossums: hereditary factors or genetic defects of calcium or vitamin D malabsorption. Hereditary cases of rickets resulting from genetic defects have been described in several species, including humans, sheep, pigs (Madson et al. 2012), and domestic cats (Dittmer & Thompson 2011, Uhl 2018). These genetic mutations have primarily resulted in

defects in vitamin D synthesis or abnormalities in phosphorus metabolism, supporting the central role of vitamin D and phosphorus in rickets' pathogenesis (Uhl 2018). Although uncommon, a possible phosphorus's primary difficulty was not ruled out in the two opossums since the offered diet's mineral composition is unknown, not supplemented with phosphate. In addition, thickening of the growth plates can directly affect phosphorus deficiency because average serum phosphorus concentrations are necessary for apoptosis of hypertrophic chondrocytes (Craig et al. 2016).

The main clinical sign in both opossums was locomotion difficulty due to bone deformities, which justified euthanasia or prevented the animals from returning to nature. MBD is considered one of the main problems in Didelphidae's orphaned puppies, and clinical signs include locomotion difficulty, inability to climb, bone fragility, fractures, and bone deformities (McRuer & Jones 2009, Nascimento & Horta 2014). As the syndrome progresses, systemic changes, such as polyuria, polydipsia, hyposthenuria, and folding fractures in the fingers, long bones, and spine, may occur. Long bones become painful when touched, and the opossum develops a squat posture (McRuer & Jones 2009). In both cases, bone deformities in the epiphyses were the most evident and justified by the irregular epiphyseal plate with irregular endochondral growth, resulting in arching and reduced bone length (Sekarides 2016). Serum increase in alkaline phosphatase was observed and empirically related to bone disease marker since concomitant liver pathology was not observed (Saraiva & Lazaretti-Castro 2002).

The anatomopathological findings were similar to those observed in domestic animals. Clusters of hypertrophic chondrocytes in endochondral ossification sites, both in the physis and below the articular cartilage, are histopathological findings characteristic of rickets (Craig et al. 2016). Dysplastic hypertrophic chondrocytes were a finding similar to that described in chondrodysplasias in dogs (Craig et al. 2016) and autosomal recessive hypophosphatemic rickets in Corriedale sheep (JPC 2015). Despite not being pathognomonic, osteoid buildup in the cortical bone was observed histologically and in sheep with rickets (Craig et al. 2016). The animals evaluated presented marked curvature of the pelvic and thoracic limbs and widening of the costochondral junctions. Lesions are typically more severe in the bones that grow faster, including the radius, tibia and metacarpals, and metatarsals (Dittmer & Thompson 2011). Enlargement of costochondral junctions, the so-called "rickety rosary" is a classic rickets lesion in most domestic species and in children that can be seen on radiographic or *post-mortem* exams (Craig et al. 2016, Dittmer & Thompson 2011, Jaffe & Metabolic 1972, Olson & Carlson 2017). Masson's trichrome staining proved to be useful to evidence mineralization deficiency. However, like Goldner's trichrome, it should not be used as a diagnostic method alone (Craig et al. 2016).

Rickets in any species must be distinguished from metabolic diseases that develop osteopenia, in addition to arthritis, osteochondrosis, hereditary chondrodysplasias, vitamin A poisoning, or secondary renal hyperparathyroidism. In general, these diseases have ossification defects but not cortical bone mineralization. There are growth deformities and premature closure of growth plates in vitamin A poisoning due to necrosis of chondrocytes and osteoblasts, which leads to reduction in bone mass and non-mineralization (Olson & Carlson 2017).

Finally, according to the macro and microscopic findings, we conclude that both animals developed rickets, which caused locomotor difficulties and consequent depletion, in addition to secondary diseases.

Acknowledgments.- The authors would like to thank the "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES), Brazil, for granting the scholarship to carry out this work.

Conflict of interest statement.- The author(s) declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

REFERENCES

Craig L.E., Dittmer K.E. & Thompson K.G. 2016. Bones and joints. p.16-163. In: Maxie M.G. (Ed.), Jubb, Kennedy, and Palmer's Pathology of Domestic Animals. Vol. 1. 6th ed. Vol. 1. Elsevier, St Louis, Missouri.

Dittmer K.E. & Thompson K.G. 2011. Vitamin D metabolism and rickets in domestic animals: A review. Vet. Pathol. 48(2):389-407. <https://dx.doi.org/10.1177/0300985810375240> <PMid:20634407>

How K.L., Hazewinkel H.A. & Mol J.A. 1994. Dietary vitamin D dependence of cat and dog due to inadequate cutaneous synthesis of vitamin D. Gen. Comp. Endocrinol. 96(1):12-18. <https://dx.doi.org/10.1006/gcen.1994.1154> <PMid:7843559>

Jaffe H.L. 1972. Metabolic, Degenerative, and Inflammatory Diseases of Bones and Joints. Lea & Febiger, Philadelphia, p.381-448.

JPC 2015. Case I. 9th Wednesday Slide Conference, Joint Pathology Center Veterinary Pathology Service, Maryland. Available at <https://www.askjpc.org/wsco/wsc_showcase2.php?id=Ukg0VXhXQ2djTHJwQjl2a2kvM29CQT09> Accessed on Abr. 02, 2019.

Madson D.M., Ensley S.M., Gauger P.C., Schwartz K.J., Stevenson G.W., Cooper V.L., Janke B.H., Burrough E.R., Goff J.P. & Horst R.L. 2012. Rickets: case series and diagnostic review of hypovitaminosis D in swine. J Vet Diagn Invest 24(6):1137-1144. <https://dx.doi.org/10.1177/1040638712461487> <PMid:22991390>

Malta M.C.C. & Luppi M.M. 2007. Marsupialia - Didelmorphia (Gambá, Cuíca). p.340-357. In: Cubas Z.S. (Org.) Tratado de Animais Selvagens: Medicina Veterinária. Vol.1. 1ºed. Roca, São Paulo, SP.

McRuer D.L. & Jones K.D. 2009. Behavioral and nutritional aspects of the Virginian opossum (*Didelphis virginiana*). Vet Clin North Am Exot Anim Pract. 12(2):217-236. <https://dx.doi.org/10.1016/j.cvex.2009.01.007> <PMid:19341950>

Nascimento C.C. & Horta M.C. 2014. Didelmorphia (Gambá, Cuíca). p. 682-707. In: Cubas Z.S. (Org). Tratado de Animais Selvagens: Medicina Veterinária. Vol.1. 2th ed. Roca, São Paulo, SP.

Olson EJ & Carlson C.S. 2017. Bones, Joints, Tendons and Ligaments. p. 954-1008. In: Pathologic Basis of Veterinary Disease. 6th ed. Elsevier, St. Louis, Missouri.

Saraiva G.L. & Lazaretti-Castro M. 2002. Marcadores bioquímicos da remodelação óssea na prática clínica. Arq bras endocrinol metab. 46(1):72-78. <https://dx.doi.org/10.1590/S0004-27302002000100010>

Sekarides R. 2016. Ossos e Articulações. p. 619-663. In: Santos R.L. & Alessi A.C. (Org). Patologia Veterinária. 2th ed. Roca, Rio de Janeiro, RJ.

Uhl E.W. 2018. The pathology of vitamin D deficiency in domesticated animals: An evolutionary and comparative overview. Int J Paleopathol. 23:100-109. <https://dx.doi.org/10.1016/j.ijpp.2018.03.001> <PMid:29544996>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(10):818-823, October 2020
DOI: 10.1590/1678-5150-PVB-6580

Original Article
Wildlife Medicine
BY-NC

Avipoxvirus detected in tumor-like lesions in a white-faced whistling duck (*Dendrocygna viduata*)[1]

Juliana F.V. Braga[2]*, Rodrigo M. Couto[3], Marcelo C. Rodrigues[4] and Roselene Ecco[3]

ABSTRACT.- Braga J.F.V., Couto R.M., Rodrigues M.C. & Ecco R. 2020. ***Avipoxvirus* detected in tumor-like lesions in a white-faced whistling duck (*Dendrocygna viduata*)**. *Pesquisa Veterinária Brasileira 40(10):818-823.* Laboratório de Patologia Animal, Universidade Federal do Piauí, Campus Ministro Petrônio Portella, Bairro Ininga, Teresina, PI 64049-550, Brazil. E-mail: juliana.braga@ufersa.edu.br

Avipoxvirus is the etiological agent of the avian pox, a well-known disease of captive and wild birds, and it has been associated with tumor-like lesions in some avian species. A white-faced whistling duck (*Dendrocygna viduata*) raised in captivity was referred to a Veterinary Teaching Hospital in Northeast due to cutaneous nodules present in both wings. A few days after the clinical examination, the animal died naturally. Once submitted to necropsy, histopathological evaluation of the lesions revealed clusters of proliferating epithelial cells expanding toward the dermis. Some of these cells had round, well-defined, intracytoplasmic eosinophilic material suggestive of poxvirus inclusion (Bollinger bodies). PCR performed on the DNA extracted from tissue samples amplified a fragment of the 4b core protein gene (*fpv* 167), which was purified and sequenced. This fragment of *Avipoxvirus* DNA present in these tumor-like lesions showed high genetic homology (100.0%) with other poxviruses detected in different avian species in several countries, but none of them were related to tumor-like lesions or squamous cell carcinoma. This is the first report of *Avipoxvirus* detected in tumor-like lesions of a white-faced whistling duck with phylogenetic analysis of the virus.

INDEX TERMS: *Avipoxvirus*, tumor-like lesions, white-faced whistling duck, *Dendrocygna viduata*, skin diseases, viral diseases, phylogenetic analysis, Anatidae.

RESUMO.- [***Avipoxvirus* detectado em lesões tipo tumorais em uma marreca-irerê (*Dendrocygna viduata*).**] *Avipoxvirus* é o agente etiológico da varíola (bouba) aviária, uma doença bem descrita em aves de cativeiro e selvagens, tendo sido associada a lesões semelhantes a tumores em algumas dessas espécies. Uma marreca piadeira (*Dendrocygna viduata*), criada em cativeiro, foi atendida em um Hospital Veterinário na região nordeste devido à presença de nódulos cutâneos em ambas as asas. Alguns dias após o exame clínico, o animal veio a óbito naturalmente. A ave foi submetida à necropsia e coletados fragmentos das lesões para análise histopatológica, que revelou proliferação de células epiteliais expandindo para a derme. Algumas dessas células possuíam material eosinofílico intracitoplasmático e bem definido, sugestivo de inclusão de poxvírus (corpúsculos de Bollinger). A PCR realizada a partir do DNA extraído de amostras das lesões amplificou um fragmento do gene da proteína do núcleo 4b (*fpv* 167), que foi purificado e sequenciado. Esse fragmento de DNA de *Avipoxvirus* presente nas lesões relevou alta homologia genética (100,0%) com outros poxvírus detectados em diferentes espécies de aves em vários países, mas nenhum deles estava relacionado a lesões tumorais ou carcinoma espinocelular. Este é o primeiro relato de *Avipoxvirus* detectado em lesões semelhantes a tumores em uma marreca piadeira com caracterização molecular do vírus.

TERMOS DE INDEXAÇÃO: *Avipoxvirus,* lesões tumorais, marreca-irerê, *Dendrocygna viduata*, dermatopatias, doenças virais, análise filogenética, Anatidae.

[1] Received on May 23, 2020.
Accepted for publication on July 15, 2020.

[2] Departamento de Ciências Animais, Centro de Ciências Agrárias, Universidade Federal Rural do Semi-Árido (UFERSA), Av. Francisco Mota 572, Bairro Costa e Silva, Mossoró, RS 59625-900, Brazil. *Corresponding author: juliana.braga@ufersa.edu.br

[3] Setor de Patologia, Departamento de Clínica e Cirurgia Veterinárias, Escola de Veterinária, Universidade Federal de Minas Gerais (UFMG), Av. Antônio Carlos 6627, Pampulha, Belo Horizonte, MG 30161-970, Brazil.

[4] Departamento de Clínica e Cirurgia Veterinária, Centro de Ciências Agrárias, Universidade Federal do Piauí (UFPI), Campus Ministro Petrônio Portella, Ininga, Teresina, PI 64049-550, Brazil.

INTRODUCTION

Members of the family Poxviridae are double-stranded DNA viruses that infect and cause disease in many vertebrates. The genus *Avipoxvirus* is responsible for causing Avian pox, a well-known disease of captive and wild birds that affects a wide range of species worldwide (McFerran & McNulty 1993, Friend & Franson 1999, van Riper & Forrester 2007). Many species of domestic, pet and free-living birds can present various disease manifestations. The clinical signs in naturally infected birds vary depending on the virulence of the virus, susceptibility of the host, distribution and type of lesions and other complicating factors (Tripathy & Reed 2008). The most common forms of the disease are cutaneous, characterized by nodular lesions on feather-free regions of the body (Bolte et al. 1999), and/or diphtheritic form, with lesions in the digestive and upper-respiratory tracts mucosa (Gerlach 1994).

Poxviridae members have oncogenic properties characterized by the expression of factors that are homologous to growth factors. These molecules can disturb the cell cycle and induce mitoses (Schafer 1998, Bolte et al. 1999) and tumor-like lesions, which have been reported in domestic and wild birds (Arai et al. 1991, Ritchie & Carter 1995, Tsai et al. 1997, Bolte et al. 1999, Mete et al. 2001, Fallavena et al. 2002, Pesaro et al. 2009). Although *Avipoxvirus* has also been associated with a dermal squamous cell carcinoma (SCC), (Fallavena et al. 1993, Back et al. 1995, Fallavena et al., 1997, Hafner & Goodwin 1997), the etiology of naturally occurring cases of dermal SCC is still unknown.

In birds, the neoplastic changes induced by poxviruses are seen mainly in the skin of Passeriformes and Columbiformes (Gerlach 1994, Ritchie & Carter 1995). Tumor-like lesions were also reported in the skin of young Chilean flamingos (Arai et al. 1991), in the cornea, bursa of Fabricius, in the lungs of Galapagos doves (Mete et al. 2001) and canaries (Ritchie & Carter 1995), and head bones of rosy-faced lovebirds (Tsai et al. 1997). In Anseriformes, poxvirus infection was reported to be associated (Pereira et al. 2014) and not associated (Morton & Dieterich 1979) with cutaneous tumor-like lesions.

Although, there are a few reports of tumor-like lesions and their possible association with *Avipoxvirus*, there is no description of the molecular characteristics of the viruses detected in these lesions. This paper describes the first report of tumor-like lesion in white-faced whistling duck (*Dendrocygna viduata*) and the phylogenetic analysis of *Avipoxvirus* present in the lesion of this bird.

MATERIALS AND METHODS

Case history. A white-faced whistling duck (*Dendrocygna viduata*, family Anatidae, order Anseriformes), male, 15 years-old, 1.15kg, raised in captivity was admitted to Veterinary Teaching Hospital in Northeast Brazil, due to the presence of cutaneous nodules in the humerus-radio-ulnar joint region of both wings. These nodules were painful and the bird was restless and reluctant to allow physical examination. During the clinical examination, the bird presented lethargy and anorexia, but the cloacal temperature was normal (41°C).

The bird was sedated with tiletamine-zolazepam (18mg/kg, IM), and referred for radiographic examination, in dorsoventral and laterolateral positions. The radiographic exam showed areas of radiolucency in the femuro-tibiotarsal joint suggestive of neoplasia. No treatment was recommended and a few days after the consultation the animal died naturally and necropsy was performed.

Necropsy and histopathology. After death, the bird was necropsied and samples from the cutaneous nodules, lungs, liver, kidney, gastrointestinal tract and brain were fixed in a 10% formalin-buffered solution for 48 hours. Then, the tissue samples were dehydrated in increasing ethanol concentrations, diaphanised in xylene and embedded in paraffin wax to obtain 4µm thick serial sections, which were stained with haematoxylin-eosin (HE) for analysis under a light microscope (Luna 1968).

DNA extraction and Polymerase Chain Reaction (PCR). DNA extraction was performed from formalin-fixed and paraffin-embedded (FFPE) tumor-like lesion samples using a commercial DNA extraction kit (QIAamp DNA FFPE Tissue Kit, Qiagen), according to recommendations of the manufacturer with some adaptations. Briefly, sections (5µm) were cut from each paraffin block and placed in a sterile microcentrifuge tube. The paraffin was removed by extraction with 1.0mL of xylene and, after centrifugation, residual xylene was removed from the tissue pellet by using 1.0mL of absolute ethanol twice. The sample was centrifuged, the ethanol was carefully removed and the DNA was eluted. The quantity and purity of the DNA extracted was assessed using Nanodrop spectrophotometer (Thermo Scientific®), and the DNA was stored at -20°C.

The DNA extracted from the tissue samples were submitted to PCR to amplify a 500 bp fragment of the 4b core protein gene (*fpv* 167) using previously published primers (P1: 5'-CAGCAGGTGCTAAACAACAA-3'; and P2: 5'-CGGTAGCTTAACGCCGAATA-3') (Lee & Lee 1997). PCR reactions were performed in a final volume of 25µL (PCR Master Mix Promega®), with 10µmol of each primer and using 250ng of DNA sample. The amplification program was performed according to Lawson et al. (2012), as follows: initial denaturation at 94°C for 5 min, followed by 45 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and then a final extension at 72°C for 7 min. The PCR products were subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized using a UV transilluminator. The LowRanger 100 bp DNA Ladder (Norgen Biotek Corp.) was used to estimate the molecular size of the PCR products.

Phylogenetic analysis. The *Avipoxvirus* DNA amplified from tumor-like lesions by PCR was purified and sequenced by the Sanger method using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City/CA) on ABI 3130 XL Genetic Analyzer automated sequencer (Applied Biosystems) and the sequence obtained was deposited in the GenBank database (NCBI) under the accession number MH286510. The sequence was aligned with sequences of reference strains from the GenBank database in BioEdit using CLUSTAL W (Thompson et al. 1994). Then, the phylogenetic tree was generated using the neighbor-joining distance method (Saitou & Nei 1987) coupled with the Kimura 2-parameter model (Kimura 1980) with bootstrap analysis of 1000 replicates in Mega6 (Tamura et al. 2013).

RESULTS

Gross and histopathology

In the skin of the left wing, on the medial surface, there was a 4.5 x 3.0 x 2.0cm neoformation. The nodule was round to oval, and firm, with an irregular and locally extensive ulcerated surface (Fig.1A). On the skin of the medial surface of the right wing there was also a 2.5 x 1.5 x 1.0cm red nodule with a dry surface. Both nodules were diffusively pinkish and soft on the cut surface. In addition to this, the lungs had diffuse hyperemia and multifocal areas of hemorrhage.

Histopathological examination of the cutaneous nodules revealed marked thickening of epidermis due to proliferating clusters of epithelial cells arranged in islands and expanding toward the dermis. These cells had abundant cytoplasm with eosinophilic fibrillar material (keratin), which occasionally accumulated in the center of the cell clusters forming keratin "pearl"-like structures (Fig.1B and 1C). In both nodules, some cells had round, well-defined, intracytoplasmic eosinophilic material compatible with poxvirus inclusion (Bollinger bodies) (Fig.1D). The superficial skin had ulceration with heterophils infiltration into the epidermis and dermis. At the histopathological examination of the lung, kidney, gastrointestinal tract and brain, no relevant lesions were found.

Molecular characterization of *Avipoxvirus*

The phylogenetic analysis revealed that the *Avipoxvirus* DNA (4b core protein gene, *fpv* 167) present in the tumor-like lesions of the white-faced whistling duck showed high genetic homology (100.0%) with other poxvirus detected in different avian species in several countries (Fig.2), which were grouped in Cluster A. The viruses belonging to Cluster A were found to be mainly present in domestic avian species and none of them were previously associated with tumor-like lesions. The sequences grouped in Cluster B shared 95.0% of genetic homology with Cluster A. Major genetic differences were observed between our strain and those obtained from some pigeon species, eagle own, great bustard and a Harris's hawk which grouped in Cluster B.

DISCUSSION AND CONCLUSION

This is the first report of *Avipoxvirus* detected in tumor-like lesions in white-faced whistling duck (*Dendrocygna viduata*). The site, gross and histological features revealed tumor-like lesions. Moreover, histopathology revealed microscopic evidence of poxvirus infection in some hyperplastic epithelial cells, which was represented by eosinophilic intracytoplasmic inclusion bodies in these cells. These inclusion bodies in the

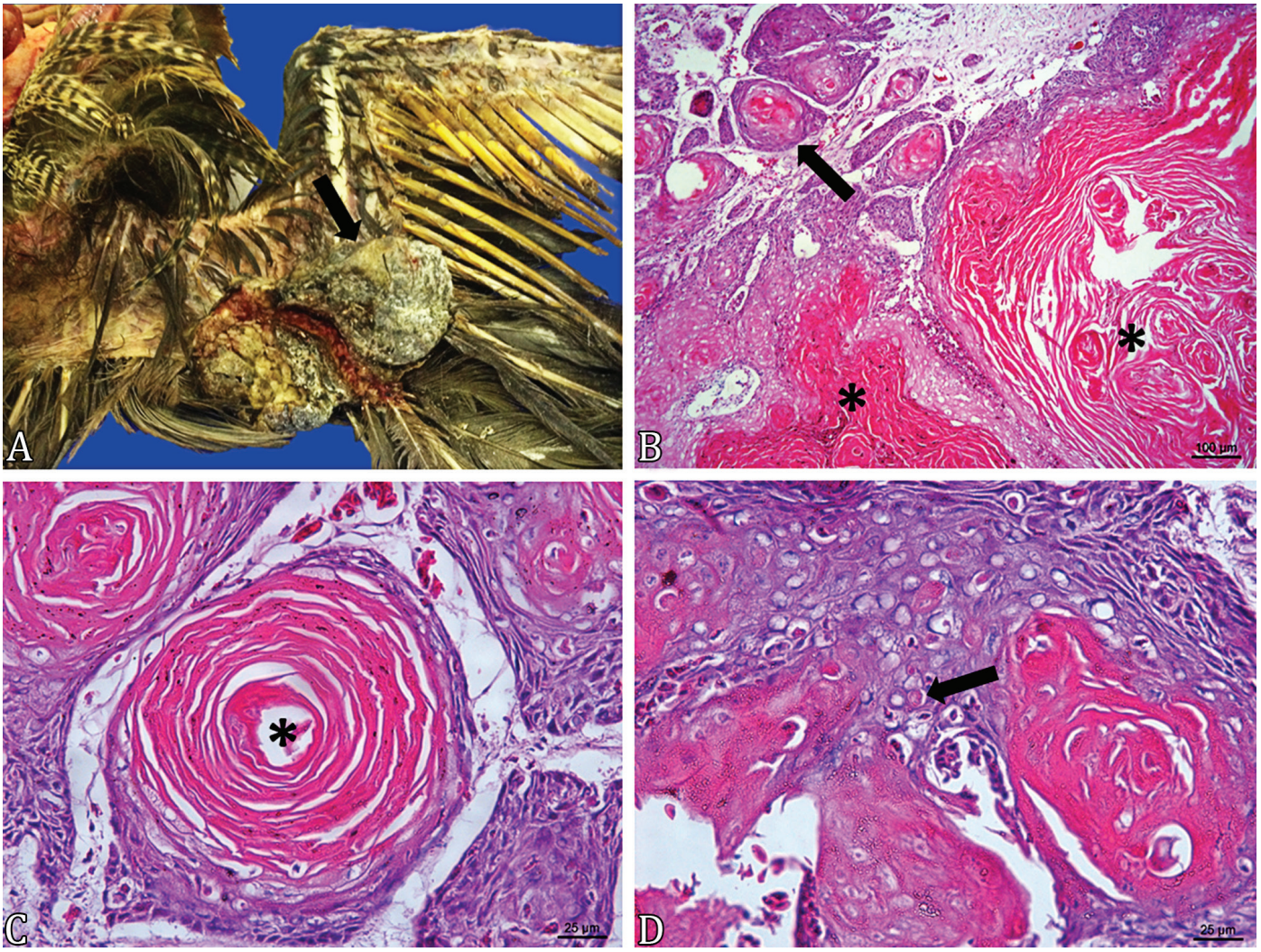


Fig.1. Tumor-like lesion in a white-faced whistling duck (*Dendrocygna viduata*). (**A**) Ulcerated nodule (→) in the skin of humerus-radio-ulnar joint region on the medial surface of the left wing. (**B**-**D**) Histopathology of the tumor-like lesions. (**B**) Proliferating cells arranged in islands (arrow) with keratin production and accumulation (*), (**C**) occasionally forming keratin "pearl"-like structures (*). (**B**) HE, bar = 100µm. (**C**) HE, bar = 25µm. (**D**) Intracytoplasmic eosinophilic inclusion bodies compatible with poxvirus inclusion (Bollinger bodies) in epithelial cell (arrow). HE, bar = 25µm.

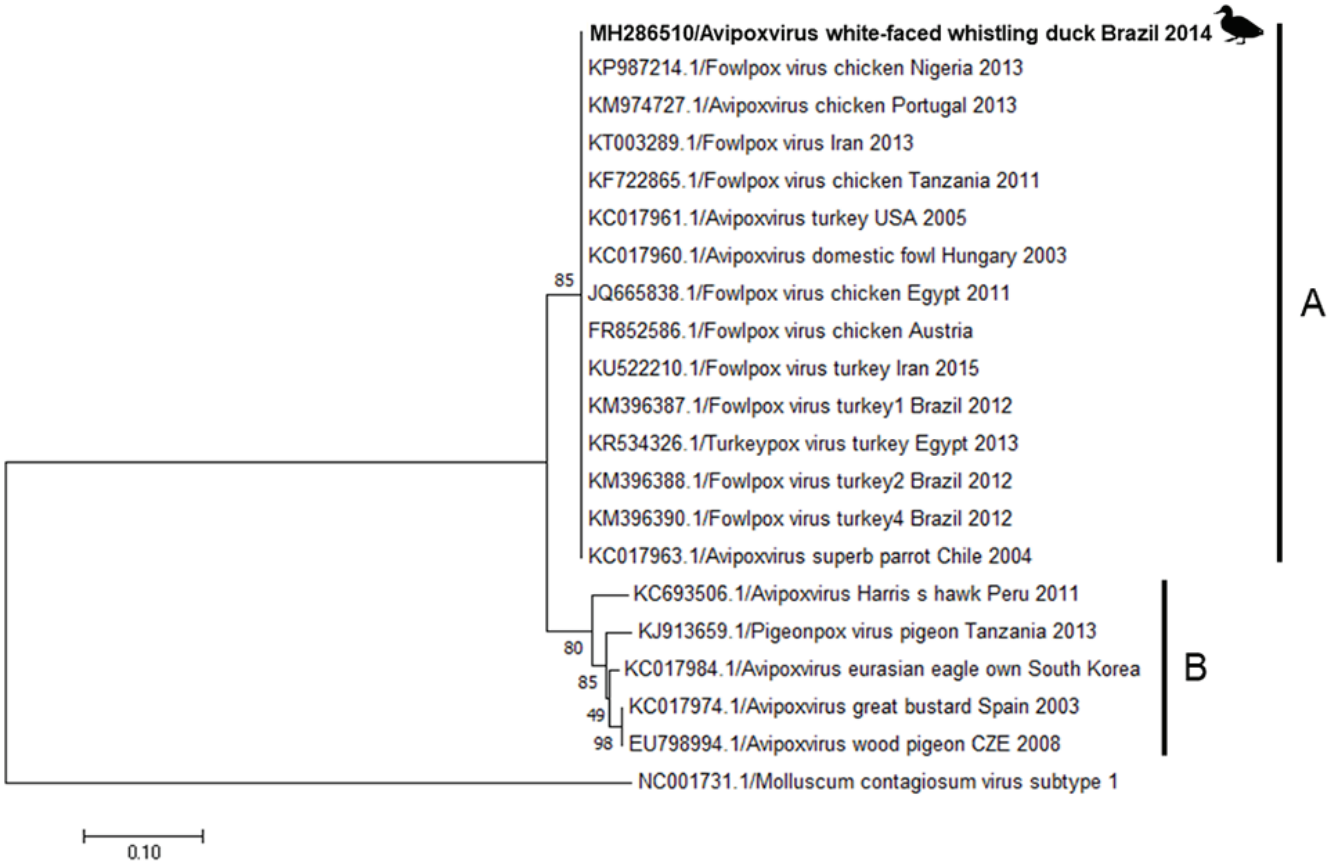


Fig.2. Evolutionary relationships among the sequences of *Avipoxvirus* detected in tumor-like lesions in white-faced whistling duck (*Dendrocygna viduata*) (MH286510) and other avian poxvirus sequences available in the GenBank database (NCBI). The sequences accession numbers, avian poxviruses, avian hosts, countries and years of viral detection are shown. Construction of Neighbour-joining tree was performed using Kimura 2-parameter with bootstrap values of 1000 replicates.

cytoplasm of infected cells consist of an aggregation of virus particles and makes histological examination one of the most effective tools for the diagnosis of fowlpox, since they can be detected in smears or tissue sections stained with Wright's, Giemsa or other stains (Eaves & Flewett 1955).

Atypical fowlpox lesions and dermal SCC begin and progress in the feather follicle epithelium (Langheinrich 1991, Fallavena et al. 1993), and this cell transformation, induced by viruses, is accompanied by the persistence of at least a part of the viral genome (Cann 1993). In cases of avian dermal SCC, the pathogenesis may be related to an error that may occur during the G1 or S phases of the cell cycle in the feather follicular epithelium, which could result in dysplastic changes able to trigger dermal SCC lesions (Langheinrich 1991).

The PCR confirmed the presence of the 4b core protein gene (p4b) of *Avipoxvirus* in the tumor-like lesions. This gene is usually chosen for comparative genetic analysis and its amplification by PCR has been used as a molecular tool for the detection of avian poxviruses (Manarolla et al. 2010), being one of the most sensitive techniques for the etiologic diagnosis (Nayeri Fasaei et al. 2014).

Some members of Poxviridae family have already been consistently associated with tumors in other animal species, such as rabbit fibroma virus (RFV) and Myxoma virus (MYXV), which have been associated with fibroma and myxoma. In nonpermissive cells, viral DNA is mostly integrated into the different sites of cell chromosomes, encoding binding proteins and inactivating cell growth, regulating proteins such as p53. This results in cell transformation due to the expression of proteins that control viral and cellular DNA synthesis (Şevik 2012). Although this is known to be the case for the previously mentioned Poxviridae family members, there is still a lack of studies regarding *Avipoxvirus.*

Sequencing of the *Avipoxvirus* DNA detected in tumor-like lesions was performed to study the genetic relationships among this strain and other avian poxviruses detected in lesions from diverse avian species. The phylogenetic analysis showed that our strain was genetically similar to other *Avipoxvirus* involved in cutaneous and/or diphtheritic forms of the disease in domestic and wild avian species, but not in tumor-like lesions. Molecular analysis also showed that the *Avipoxvirus* strain detected in the tumor-like lesions of this case was more genetically related to domestic bird strains than to wild bird strains.

This report provides the first molecular characterization of *Avipoxvirus* detected in tumor-like lesions in avian species. While previous reports have described simultaneous occurrences of SCC or tumor-like lesions and poxvirus detection in lesions in black-bellied whistling duck (*Dendrocygna autumnalis*) (Pereira et al. 2014) and in broilers in Brazil (Fallavena et

al. 2002), and pink-backed pelican in Italy (Pesaro et al. 2009), there is a lack of molecular characterization of these viruses. Poxvirus involved in the neoplasm of the white-faced whistling duck (*D. viduata*) exhibited high genetic similarity to other avian poxviruses detected mainly in domestic fowl, such as chickens and turkeys (Cluster A). Among these highly similar strains are Brazilian fowlpox virus detected in non-neoplastic skin lesions in commercial turkeys (Kunert-Filho et al. 2016). The phylogenetic analysis also showed major genetic differences between our strain and those obtained from non-neoplastic lesions in some pigeon species, eagle own, great bustard and Harris's hawk.

There is a lack of information regarding diseases and their frequency in white-faced whistling ducks (*D. viduata*). A retrospective study over a period of 22 years (1974-1996), in Brazil, described only one case of clinical care for this species and the bird had an undifferentiated carcinoma in the coelomic cavity (Werner et al. 1998).

Molecular characterization showed the genetic similarity of the *Avipoxvirus* detected in tumor-like lesions of this white-faced whistling duck (*D. viduata*) to other avian poxviruses detected mainly in domestic chicken and turkeys worldwide. As the information about the association between poxvirus and the development of neoplasm is scarce, our understanding is still limited. Therefore, experimental studies are required to elucidate the possible association between the *Avipoxvirus* and tumor-like lesions in avian species.

Acknowledgements.- We would like to thank MSc. Bruna C. Ferreira for kindly provide the *Avipoxvirus* DNA for Polymerase Chain Reaction (PCR) positive control.

Conflict of interest statement.- The authors reported no potential conflict of interest.

REFERENCES

Arai S., Arai C., Fujimaki M., Iwamoto Y., Kawarada M., Saito Y., Nomura Y. & Suzuki T. 1991. Cutaneous tumour-like lesions due to poxvirus infection in Chilean flamingos. J. Comp. Pathol. 104(4):439-441. <https://dx.doi.org/10.1016/S0021-9975(08)80154-5>

Back A., Soncini R.A., Ruthes O., Madureira Jr. S. & Flores R. 1995. An atypical fowl pox outbreak in broilers in southern Brazil. Avian Dis. 39(4):902-906. <https://dx.doi.org/10.2307/1592431>

Bolte A.L., Meurer J. & Kaleta E.F. 1999. Avian host spectrum of avipoxviruses. Avian Pathol. 28(5):415-432. <https://dx.doi.org/10.1080/03079459994434> <PMid:26911595>

Cann A.J. 1993. Infection, p.173-219. In: Cann A.J. (Eds), Principles of Molecular Virology. 6ª ed. Academic Press, London.

Eaves G. & Flewett T. 1955. The structure of fowl-pox inclusions (Bollinger bodies). Epidemiol. Infect. 53(1):102-105. <https://dx.doi.org/10.1017/s0022172400000541> <PMid:14367806>

Fallavena L.C., Canal C.W., Salle C.T., Moraes H.L., Rocha S.L., Pereira R.A. & Silva A.B. 2002. Presence of avipoxvirus DNA in avian dermal squamous cell carcinoma. Avian Pathol. 31(3):241-246. <https://dx.doi.org/10.1080/03079450220136558> <PMid:12396347>

Fallavena L.C., Rodrigues N.C., Moraes H.L., Salle C.T., Silva A.B., Nascimento V.P. & Rodrigues O. 1997. Squamous cell carcinoma-like and pox lesions occurring simultaneously in chorioallantoic membranes of chicken embryos inoculated with materials from squamous cell carcinoma and pox lesions in broiler chickens. Avian Dis. 41(2):469-471. <PMid:9201417>

Fallavena L.C., Rodrigues N.C., Scheufler W., Martins N.R., Braga A.C., Salle C.T. & Moraes H.L. 1993. Atypical fowlpox in broiler chickens in southern Brazil. Vet. Rec. 132(25):635. <https://dx.doi.org/10.1136/vr.132.25.635> <PMid:8394610>

Friend M. & Franson J.C. 1999. Avian pox, p.163-170. In: Friend M. & Franson J.C. (Eds), Field Manual of Wildlife Diseases: General Field Procedures and Diseases of Birds. Biological Resources Division, Madison.

Gerlach H. 1994. Viruses, p.862-948. In Ritchie B.W., Harrison G.J. & Harrison L.R. (Eds), Avian Medicine: principles and application. Wingers publishing Lake Worth, Florida.

Hafner S. & Goodwin M.A. 1997. Dermal squamous cell carcinoma, p.1044-1046. In: Calnek B.W., Barnes H.J., Beard C.W., McDougald L.R. & Saif Y.M. (Eds), Diseases of Poultry. 10th ed. Iowa State University Press, Iowa.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16(2):111-120. <https://dx.doi.org/10.1007/BF01731581> <PMid:7463489>

Kunert-Filho H.C., Cibulski S.P., Finkler F., Grassotti T.T., Jaenisch F.R.F., Brito K.C.T., Carvalho D., Lovato M. & Brito B.G. 2016. First phylogenetic analysis of Avipoxvirus (APV) in Brazil. Pesq. Vet. Bras. 36(5):357-362. <https://dx.doi.org/10.1590/S0100-736X2016000500001>

Langheinrich K. 1991. Pathology of squamous cell carcinomas in broilers, p.58-62. In: Langheinrich K. (Eds), Proceedings of the Symposium on Avian Tumor Virus, Seattle, Washington.

Lawson B., Lachish S., Colvile K.M., Durrant C., Peck K.M., Toms M.P., Sheldon B.C. & Cunnhingham A.A. 2012. Emergence of a novel avian pox disease in British tit species. PLoS One 7(11):e40176. <https://dx.doi.org/10.1371/journal.pone.0040176> <PMid:23185231>

Lee L.H. & Lee K.H. 1997. Application of the polymerase chain reaction for the diagnosis of fowl poxvirus infection. J. Virol. Methods 63(1/2):113-119. <https://dx.doi.org/10.1016/s0166-0934(96)02119-2> <PMid:9015281>

Luna L.G. 1968. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd ed. American Registry of Pathology, New York. 258p.

Manarolla G., Pisoni G., Sironi G. & Rampin T. 2010. Molecular biological characterization of avian poxvirus strains isolated from different avian species. Vet. Microbiol. 140(1/2):1-8. <https://dx.doi.org/10.1016/j.vetmic.2009.07.004>

McFerran J. & McNulty M. 1993. Poxviridae, p.1-15. In: McFerran J. & McNulty M. (Eds), Virus Infections of Birds. Elsevier Scientific Publishing, Oxford.

Mete A., Borst G.H. & Dorrestein G.M. 2001. Atypical poxvirus lesions in two Galapagos doves (Nesopelia g. galapagoensis). Avian Pathol. 30(2):159-162. <https://dx.doi.org/10.1080/03079450120044560> <PMid:19184890>

Morton J.K. & Dieterich R.A. 1979. Avian pox infection in an American green-winged teal (Anas crecca carolinensis) in Alaska. J. Wildl. Dis. 15(3):451-453. <https://dx.doi.org/10.7589/0090-3558-15.3.451>

Nayeri Fasaei B., Madadgar O., Ghalyanchi Langeroodi A. & Ghafari M. 2014. Molecular detection and phylogenetic analysis of Avipoxvirus strains isolated from different bird species. Iran. J. Vet. Res. 15(1):40-44. <https://dx.doi.org/10.22099/IJVR.2014.1980>

Pereira W.L.A., Gabriel A.L.M., Monger S.G.B., Moraes L.A., Queiroz D.K.S. & Souza A.J.S. 2014. Lesões cutâneas tipo tumorais associadas à infeção por Avipoxvirus em uma marreca-cabocla (Dendrocygna autumnalis). Ciênc. Anim. Bras. 15(2):234-238. <https://dx.doi.org/10.1590/1809-6891v15i217202>

Pesaro S., Biancani B., Fabbrizi G. & Rossi G. 2009. Squamous cell carcinoma with presence of poxvirus-like inclusions in the foot of a pink-backed pelican (Pelecanus rufescens). Avian Pathol. 38(3):229-231. <https://dx.doi.org/10.1080/03079450902912176> <PMid:19468940>

Ritchie B.W. & Carter K. 1995. Poxviridae, p.285-311. In: Ritchie B.W. (Ed.), Avian Viruses: function and control. Winger's Publishing, Lake Worth, Florida.

Saitou N. & Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4(4):406-425. <https://dx.doi.org/10.1093/oxfordjournals.molbev.a040454> <PMid:3447015>

Schafer K. 1998. The cell cycle: a review. Vet. Pathol. 35(6):461-478. <https://dx.doi.org/10.1177/030098589803500601> <PMid:9823588>

Şevik M. 2012. Oncogenic viruses and mechanisms of oncogenesis. Turk. J. Vet. Anim. Sci. 36(4):323-329. <https://dx.doi.org/10.3906/vet-1104-2>

Tamura K., Stecher G., Peterson D., Filipski A. & Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30(12):2725-2729. <https://dx.doi.org/10.1093/molbev/mst197> <PMid:24132122>

Thompson J.D., Higgins D.G. & Gibson T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22(22):4673-4680. <https://dx.doi.org/10.1093/nar/22.22.4673> <PMid:7984417>

Tripathy D. & Reed W. 2008. Pox, p.291-307. In: Saif Y.M., Fadly A.M., Glisson J.R., McDougald L.R., Nolan L.K. & Swayne D.E. (Eds), Diseases of Poultry. 12nd ed. Iowa State Press, Iowa.

Tsai S.S., Chang T.C., Yang S.F., Chi Y.C., Cher R.S., Chien M.S. & Itakura C. 1997. Unusual lesions associated with avian poxvirus infection in rosy-faced lovebirds (Agapornis roseicollis). Avian Pathol. 26(1):75-82. <https://dx.doi.org/10.1080/03079459708419195> <PMid:18483891>

Van Riper C. III. & Forrester D. 2007. Avian pox, p.131-176. In: Van Riper C. III & Forrester D. (Eds), Infectious Diseases of Wild Birds. Blackwell Publishing, Iowa.

Werner P.R., Chiquito M. & Pachaly J.R. 1998. Estudo retrospectivo das neoplasias diagnosticadas em animais selvagens ou exóticos pelo Serviço de Patologia do Hospital Veterinário da Universidade Federal do Paraná entre 1974 e 1996. Arch. Vet. Sci. 3(1):39-44. <https://dx.doi.org/10.5380/avs.v3i1.3737>

PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(10):824-829, October 2020
DOI: 10.1590/1678-5150-PVB-6597

Original Article
Animal Morphophysiology
BY-NC

Description of brachial plexus of sloth (*Bradypus variegatus*)[1]

Silvia F. Alcântara[2]*, Marleyne J.A.L. Amorim[2], Priscilla V. Albuquerque[2], Emanuela P. Mesquita[2], Maria Eduarda L.C. Miranda[2], Gilcifran P. Andrade[2], Nathalia E.O. Nascimento[3] and Júlio C.S. Nascimento[4]

ABSTRACT.- Alcântara S.F., Amorim M.J.A.L, Albuquerque P.V., Mesquita E.P., Miranda M.E.L.C., Andrade G.P., Nascimento N.E.O. & Nascimento J.C.S. 2020. **Description of brachial plexus of sloth (*Bradypus variegatus*)**. *Pesquisa Veterinária Brasileira 40(10):824-829*. Departamento de Morfologia e Fisiologia Animal, Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros s/n, Recife, PE 52171-900, Brazil. E-mail: alcantarabio35@gmail.com

The species *Bradypus variegatus* is known as the common sloth, an endemic mammal from neotropical regions, which has been suffering from devastating anthropogenic activities. Our study aimed to describe the brachial plexus of *B. variegates,* regarding the origin and distribution of nerves, through the sampling of 10 adult females. Analyses were carried out at the Anatomy Section, "Departamento de Morfologia e Fisiologia Animal", "Universidade Federal Rural de Pernambuco", under license no. 034/2015 of the Ethics Committee on the Use of Animals. The results determined that the brachial plexus of the common sloth starts from the fifth cervical spine segment until the second thoracic segment. This area contains the long and suprascapular thoracic nerves, which originate immediately from the medullary segment 5 and 6, respectively, and from the pectoral, subscapular, axillary, radial, musculocutaneous, medial, forearm and ulnar medial cutaneous nerves, arising from a trunk comprised of cervical spine nerves (C) 7, C8, C9, and thoracic (T) 1 and T2. Regarding other wild and domestic animals, different suggestions were observed about the origin of the plexus in *B. variegatus*, however, the constituent nerves and their innervation areas did not demonstrate any discrepancies.

INDEX TERMS: Brachial plexus, sloth, *Bradypus variegatus*, Bradypodidae, thoracic members, spinal nerves, neuroanatomy, nervous system, morphology.

RESUMO.- [**Descrição do plexo braquial do bicho-preguiça (*Bradypus variegatus*).**] A espécie *Bradypus variegatus* é conhecida como preguiça-comum. Trata-se de um mamífero endêmico de regiões neotropicais que vem sofrendo com a ação antrópica devastadora. Esses Bradipodídeos possuem três dedos nos membros torácicos e pélvicos, são arborícolas consagrados e descem ao solo apenas para excretar e trocar de árvore. O estudo teve como objetivo descrever o plexo braquial de *B. variegatus* em relação à origem e distribuição dos nervos. Para tal, utilizou-se 10 fêmeas adultas. As análises foram realizadas no Pavilhão de Anatomia do Departamento de Morfologia e Fisiologia Animal da Universidade Federal Rural de Pernambuco, sob a licença nº 034/2015 do Comitê de Ética no Uso de Animais. Os cadáveres foram obtidos após morte natural, fixados em formaldeído a 20%, conservados em solução salina a 30% em tanques e dissecados para a visualização dos nervos destinados a inervar os membros e músculos torácicos. Uma vez feito, constatou-se que o plexo braquial da preguiça-comum se origina do quinto segmento espinal cervical, se estendendo até o segundo segmento torácico. Sendo formado pelos nervos torácico longo e supraescapular, de origem imediatamente do segmento medular 5 e 6, respectivamente, e pelos nervos peitorais, subescapulares, axilar, radial, musculocutâneo, mediano, cutâneo medial do antebraço e ulnar, decorrentes de um tronco formado a partir de nervos espinais cervicais (C) 7, C8, C9, e torácicos (T) 1 e T2. Em comparação a outros animais silvestres e domésticos

[1] Received on June 2, 2020.
Accepted for publication on July 12, 2020.
[2] Departamento de Morfologia e Fisiologia Animal, Universidade Federal Rural de Pernambuco (UFRPE), Rua Dom Manoel de Medeiros s/n, Recife, PE 52171-900, Brazil. *Corresponding author: alcantarabio35@gmail.com

[3] Departamento de Biologia, Universidade Federal Rural de Pernambuco (UFRPE), Rua Dom Manoel de Medeiros s/n, Recife, PE 52171-900, Brazil.

[4] Departamento de Zootecnia, Universidade Federal Rural de Pernambuco (UFRPE), Rua Dom Manoel de Medeiros s/n, Recife, PE 52171-900, Brazil.

foram observadas diferentes disposições em relação à origem do plexo de *B. variegatus*, todavia, os nervos constituintes e suas áreas de inervação não apresentaram discrepâncias.

TERMOS DE INDEXAÇÃO: Plexo braquial, bicho-preguiça, *Bradypus variegatus*, Bradipodídeos, membro torácico, nervos espinais, neuroanatomia, sistema nervoso, morfologia.

INTRODUCTION

Sloths are placental mammals that have a herbivorous diet and low metabolism. They belong to the order Pilosa, superorder Xenarthra, which is considered as the most distinct group among the Eutheria, encompassing animals with distinct behaviours, habitats and morphologies (Fernandez & Miranda 2007, Adami et al. 2013). Sloths are distributed across two genera due to the quantity of data available on their thoracic members, the genus *Choloepus*, two-toed and the genus *Bradypus*, three-toed. These animals are easily found in the neotropical regions. Bradypodidae are represented by four species: *Bradypus variegatus, Bradypus tridactylus, Bradypus torquatus* and *Bradypus pygmaeus*. The latter species is endemic to the Escudo de Veraguas Island, located in Panama, whereas the first species presents an extensive distribution and for this reason is known as the common sloth (Azarias 2005, Wilson & Reeder 2005, Kaviar et al. 2012).

Characterised by their peculiar habitats, sloths live in the forest canopy, suspended by their claws and their bodies are covered by hairs which are covered by green algae which aids in their camouflage. They descend to the ground only to change tree or to defecate, as on the ground they become more susceptible to predators such as snakes and jaguars, due to their slow movements in this environment, which has contributed to the decrease in the number of individuals (Pauli et al. 2014). The introduction of exotic species and fragmentation of environments as a result of anthropic activities, culminate in their increased risk of extinction (Cullen Júnior et al. 2001). The genus *Bradypus*, has especially suffered from these impacts and according to the International Union for Conservation of Nature (IUCN 2019), some species are threatened such as *B. torquartus* which is classified as vulnerable and *B. pygmaeus* which is classified as critically endangered.

In terms of anatomical characteristics, sloths present differences in the number of cervical vertebrae, with *Choloepus hoffmanni* having five to six vertebra, *Choloepus didactylus* having six to seven, and *B. variegatus* having between six and nine vertebrae. This characteristic allows for these animals to rotate their heads up to 270 degrees, facilitating the search for food and better identification of potential predators. This skeletal variation has been associated with the number of spinal segments. However, between seven and eight cervical vertebrae have been found in domestic mammals (Hautier et al. 2010, Cruz et al. 2013).

It is already known that each pair of cervical spinal nerves enter through the intervertebral foramina above each corresponding vertebra, however the eighth pair of nerves pass below the seventh cervical vertebra (Getty et al. 1986, Adami et al. 2013). The ventral and dorsal roots are inserted into the intervertebral foramen and join together to form the brachial and lumbosacral plexuses (Dyce et al. 2010). Considering that this is a fragile region of the peripheral nervous system, with regard to trauma exposure, a lesion in the brachial plexus can cause serious damage, such as the paralysis of thoracic members, decreasing an individual's quality of life and impairing, principally, the locomotion of tree species (Nowak 1999, Pereira 2015). As this is an area of anatomical relationships with the mobile structures of the neck and shoulders, tractions can occur, resulting in lesions or even penetrating injuries (Flores 2006).

During the evolutionary process there was an ascension in the cranial direction of the spinal nerve trunks which contributed to the formation of the brachial plexus, where the fourth spinal nerve was reached in monkeys (Parada et al. 1989). Just as with these primates, it is expected that there will be a pathway coming off the brachial plexus trunk in the first cervical vertebrae in sloths, in a cranial direction, since monkeys and sloths are both tree-living animals, inhabiting similar habitats. In this context, we aim to describe the anatomy of this plexus in *B. variegatus* indicating the origin and distribution of constituent nerves, with the aim of providing clinical medical data for this species.

MATERIALS AND METHODS

In order to carry out this research, the project was approved under the license no. 034/2015 from the Ethics committee on the Use of Animals at the "Universidade Federal Rural de Pernambuco" (UFRPE) and SISBIO no. 46665-3, for which 10 adult female individuals of the species *Bradypus variegatus* Schinz, 1825 (Mammalia: Pilosa) were used. The animals were donated post natural death by the "Centro de Triagem de Animais Silvestres", CETAS-Tangará. The animals were then sent to the collection of the Area of Anatomy of the "Departamento de Morfologia e Fisiologia Animal" (DFMA) of the UFRPE. The analyses performed were properly described and the photos documented with the aid of a Canon T6 camera and a schematic drawing was produced to facilitate the understanding of the results. The names of the structures were based on the determinations of the Nomina Anatomica Veterinaria in 2017.

The corpses were fixed by the cannulation of the common left carotid artery, using 20% formaldehyde and conserved in 30% saline solution in tanks. The dissection was performed through a medial sagittal ventral incision, from the larynx to the xiphoid process of the sternum bone and through transverse insertions with the formation of lateral windows. A medial incision was then made from the root of the thoracic limb to the wrist, with the opening of skin and musculature for the identification of the nerves that constitute the brachial plexus.

RESULTS

The brachial plexus of the common sloth did not demonstrate anatomical variation between study specimens. The specimens presented eight cervical vertebrae and seven pairs of spinal nerves forming the plexus, with the emergence of the first pair between the fourth and fifth cervical vertebrae and the last between the second and third thoracic vertebrae. The plexus in both antimeres was derived from the fifth to the ninth cervical medullary segment and from the first and second thoracic segments, corresponding to the cervical spinal nerves (C) 5, C6, C7, C8 and C9 and the thoracis spinal nerves (T) 1 and T2 (Fig.1).

As for the brachial plexus arrangement in *Bradypus variegatus*, it was found that the long thoracic nerve emerged from the fifth cervical spinal segment, responsible for innervating the lateral portion of the chest. The suprascapular nerve originated

from the sixth segment and supplied the deep musculature of the lateral region of the scapula (Fig.1 and 2).

A pattern comprised of two trunks was observed in the study animals, a cranial trunk originating from the junction of the convergence of the C7 and C8 nerves with the C9 nerve. The nerve originating from C7 and C8 emitted subscapular pathways, responsible for innervating the medial scapular muscles. The other trunk was comprised of T1 and T2 with the contribution of T3. The trunks, cranial and caudal, were joined together in a common trunk, from which two fascicles appeared, the ventral and dorsal, and from these came the innervation of the chest muscles and limbs (Fig.1 and 2).

From the common trunk came two pectoral nerves which innervated the pectoral muscles. The axillary nerve originated from the dorsal fascicle and ran through the medial scapular muscles, the thoracodorsal nerve (which innervated the dorsal portion of the chest and the radial nerve), extending in the direction of the deep lateral region of the arm. From the ventral fascicle came the medial cutaneous nerves of the forearm, the ulnar nerve, the median and musculo cutaneous nerves, the last two originated from a single nerve that suffered a bifurcation. The medial cutaneous nerves of the forearm, ulnar nerve and median nerve were distributed up to the forearm (Fig.2).

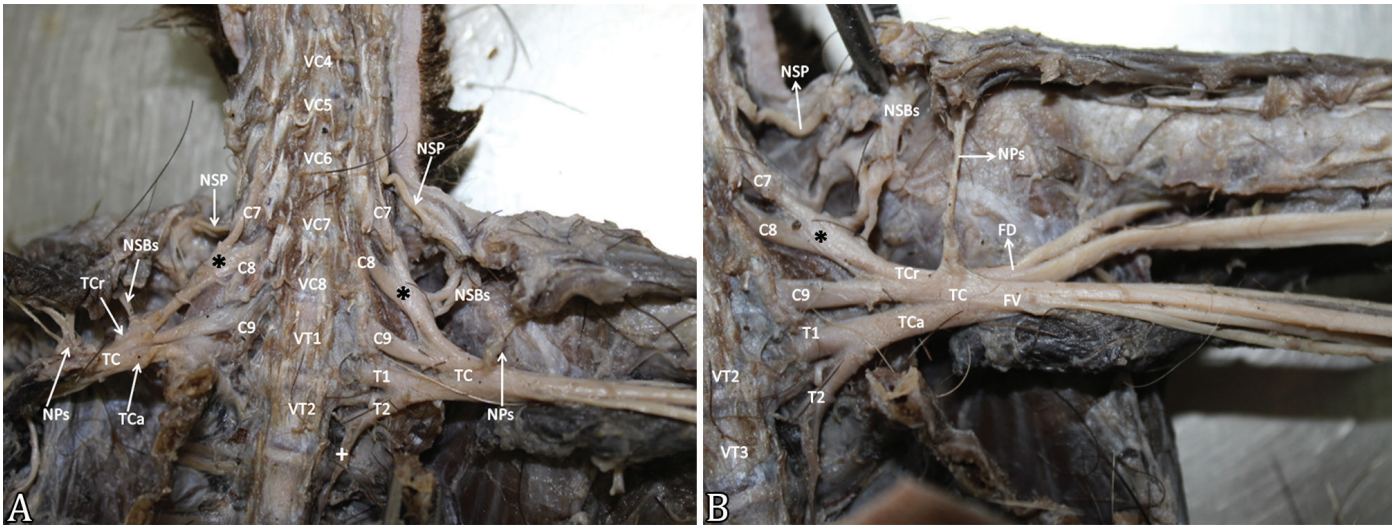


Fig.1. Photomacroscopy of the origin of the brachial plexus of the adult sloth (*Bradypus variegatus*). (**A**) Identification of the positioning of the cranial (TCr) and caudal (TCa) trunks forming the common trunk (TC). (**B**) Origin of the ventral (FV) and dorsal (FD) fascicles. The cervical vertebrae (VC), thoracic vertebrae (VT), cervical spinal nerves (C), thoracic spinal nerves (T), the nerve formed by the joining of the seventh and eighth cervical spinal nerves (*), the suprascapular nerve (NSP), the subscapular nerves (NSBs), the pectoral nerves (NPs), the contribution of the third pair of thoracic spinal nerves to the second pair (+).

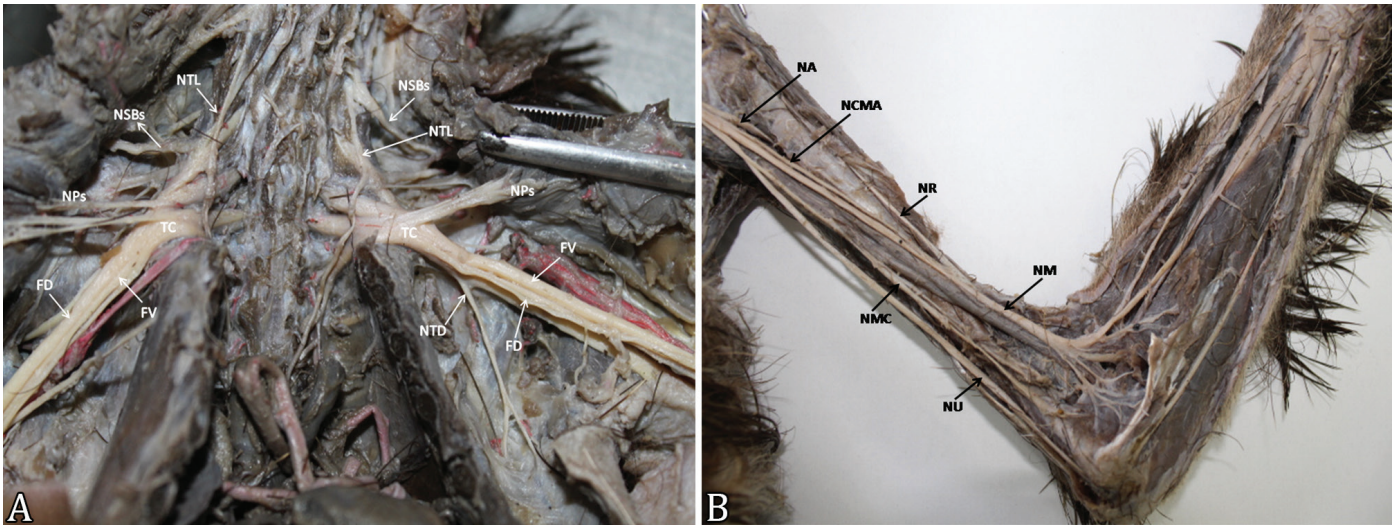


Fig.2. Photomacroscopy of the brachial plexus of the adult sloth (*Bradypus variegatus*). (**A**) Identification of the pathways near the common trunk (TC) and the dorsal fascicle (FD). (**B**) Identification of the distal pathways of the ventral (FV) and dorsal fascicles (FD). The subscapular nerves (NSBs), the long thoracic nerve (NTL), pectoral nerves (NPs), the thoracodorsal nerve (NTD), the axillary nerve (ND), the radial nerve (NR), the medial cutaneous nerve of the forearm (NCMA), the median nerve (NM), the musculocutaneous nerve (NMC) and the ulnar nerve (NU).

The pattern observed for the distribution of the brachial plexus of the studied Bradypodidae can be seen schematically in Figure 3.

DISCUSSION

The brachial plexus of the common sloth originated from C5 to T2, with the contribution of T3. This information is different to that found in previous studies on other eutherians. In domestic animals, for example, the brachial plexus is more commonly formed by the C6 to T2 nerves (Ghoshal 1986). In studies by (Souza Junior et al. 2014) and (Vieira et al. 2013), they found that in *Cerdocyon thous* and *Mazama gouazoubira* the plexuses were comprised of the C6 to T1 nerves, whereas, for horses and cattle the plexuses were comprised of the C6 to T2 nerves. For wild mammals such as *Hydrochoerus hydrochaeris*, the plexus is formed by C4 to T1 nerves (Fioretto et al. 2003). Whereas, for *Hystrix cristata*, *Agouti paca* and *Cebus apella*, the brachial plexus was observed to be comprised of C5 to T2 nerves (Aydin 2003, Ribeiro et al. 2005, Scavone et al. 2008). C4 to T2 combinations were described for *Tayassu tajacu* (Moura et al. 2007), whereas Kawashima et al. (2007), Cruz & Adimi (2010) and Santos et al. (2016) noted that for *Leopardus geoffroyi, Pongo pygmaeus, Logothrix lagotricha, Callithrix jacchus* and *Callithrix penicillata* the nerves extend from C5 to T1. According to (Moura et al. 2007), the variation in the origin of the brachial plexus may be related to embryological factors due to the position of limb bud development in relation to the neuro axis. According to (Hautier et al. 2010) and (Cruz et al. 2013), these alterations occur in relation to the variation in the number of cervical vertebrae. The formation of each nerve varies notably between species, due to the differences in their ability to use of thoracic limbs for defence, locomotion or searching for food (Dyce et al. 2010) (Table 1).

In regard to the Bradypodidae, previous literature notes that for the species *Bradypus torquartus* and *Bradypus tridactylus*, the brachial plexuses occurred from C7 to T2 nerves (Bielik 1837, Cruz et al. 2013), which is in concordance with the formation of nerve trunkes found in this study for *B. variegatus*. The sloths in this study presented plexuses formed by nerves that are distributed from a common trunk, formed by cranial and caudal pathways, which has also been described for *B. torquartus, H. cristata,* marsupials and lemurs (Miller 1934, Aydin 2003, Cruz et al. 2013, Ricci et al. 2013). However, this characteristic differs from that observed for other Xenarthrans, such as *Tamandua tetradactyla*, whose plexus is comprised of three trunks, as well as primates such as *P. pygmaeus, L. lagotricha, C. jacchus and C. penicillata* (Kawashima et al. 2007, Cruz & Adimi 2010, Santos et al. 2016), and further demonstrates a difference from the description of *Chinchilla lanigera* which has four trunks and for pacas that do not present trunks (Gamba et al. 2007, Scavone et al. 2008, Ricci et al. 2013).

In terms of the pectoral nerves, in *B. variegatus* they originated from a common trunk, supplying the pectoral muscles, however, in *B. torquartus* and in *H. cristata*, these nerves emerged from the caudal trunk (Aydin 2003, Cruz et al. 2013). In dogs and cats, the pectoral nerves are divided into cranial and caudal nerves (Stüpp-De-Souza et al. 2010), which was not observed in *B. variegatus*. In this species, the suprascapular nerve came from the sixth cervical medullary segment and was inserted into the shoulder musculature, differing to *B. torquartus*, since the nerve presented a variation in terms of origin, emerging from the seventh cervical medullary segment or the cranial trunk (Cruz et al. 2013), which is similar to the primates *L. lagothricha*, *C. jacchus* and *C. Penicillata* (Cruz & Adimi 2010, Santos et al. 2016).

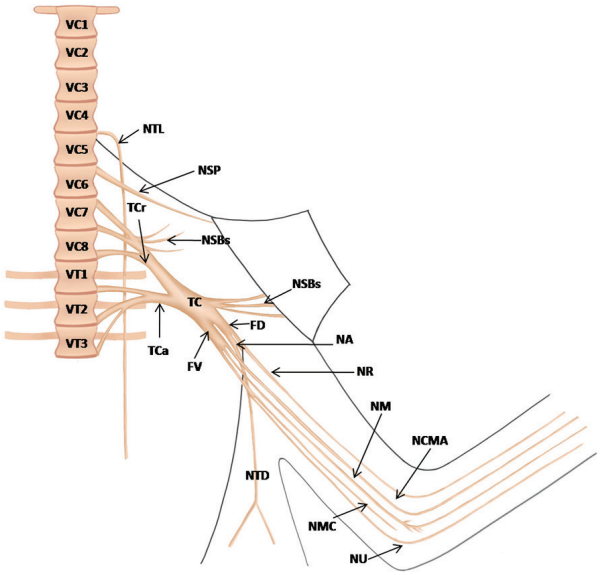


Fig.3. Schematic drawing of the brachial plexus of the adult sloth (*Bradypus variegatus*). Cervical vertebrae (VC), thoracic vertebrae (VT), cervical spinal nerves (C), thoracic spinal nerves, the nerve formed by the joining of the seventh and eighth cervical spinal nerves (*), the long thoracic nerve (NTL), the suprascapular nerve (NSP), subscapular nerve (NSBs), cranial (TCr), caudal (TCa) and common (TC) trunks, contribution of the third pair of thoracic spinal nerves to the second pair (+), pectoral nerves (NPs), ventral (FV) and dorsal (FD) fascicles, thoracodorsal nerve (NTD), axillary nerve (ND), radial nerve (NR), medial cutaneous nerve of the forearm (NCMA), median nerve (NM), musculocutaneous nerve (NMC) and the ulnar nerve (NU).

Table1. The origin of nerves that comprise the brachial plexus of mammals

Specie	Origin	Reference
Agouti paca	C5 to T2	Scavone et al. (2008)
Bradypus trydactylus	C7 to T2	Bielik (1937)
Bradypus torquartus	C7 to T2	Cruz et al. (2013)
Bovines	C6 to T2	Ricci et al. (2013)
Cerdocyon thous	C6 to T1	Souza Junior et al. (2014)
Cebus apella	C5 to T2	Ribeiro et al. (2005)
Equines	C6 to T2	Ricci et al. (2013)
Hystrix cristana	C5 to T2	Aydin (2003)
Hydrochoerus hydrochaeris	C4 to T1	Fioretto et at. (2003)
Leopardus geoffroxi	C5 to T1	Souza Junior et al. (2018)
Logothix logotricha	C5 to T1	Cruz & Adimi (2010)
Mazama gouazoubira	C6 to T1	Vieira et al. (2013)
Pongo pygmaeus	C5 to T1	Kawashima et al. (2007)
Tyassu tajuca	C4 to T2	Moura et al. (2007)

The results indicate that four nerves originated from the ventral fascicle in *B. variegatus*: the medial cutaneous nerve of the forearm, the ulnar nerve, the median nerve and the musculocutaneous nerve. A common origin was observed for these last two nerves, however, they formed distinct nerves, which differs from other mammals where a separation does not occur, resulting in the formation of the median musculocutaneous nerve as with *B. torquartus*, the *Capricornis crispus* and *H. hydrochaeris* (Atoji et al. 1987, Fioretto et al. 2003, Cruz et al. 2013). In *T. tetradactyla*, *H. cristata* and *Papio ursinus* the median musculocutaneous nerve forks in the distal third of the arm and thereby forms the median and musculocutaneous nerves. However, in the anteater, the origin of the median musculocutaneous nerve occurs before the formation of the trunks, with T1 also contributing to this (Booth et al. 1997, Aydin 2003, Cruz et al. 2012). It was found that the medial cutaneous nerve of the forearm and the ulnar nerve, continued to the forearm, as was observed by (Cruz et al. 2013) for *B. torquartus*.

In the dorsal fascicle, the axillary nerve emerged from the caudal trunk, similar to *B. torquartus* and *M. Gouazoubira* (Cruz et al. 2013, Vieira et al. 2013). However, this differs to *H. cristata*, *P ursinus, T. Tetradactyla* and *L. lagotricha* where the origin of the axillary nerve came from the cranial and middle trunks (Cruz & Adimi 2010, Cruz et al. 2012). In regard to the radial nerve of the study specimens, it is comparable to those analysed for *B. torquartus, H. Crisyaya, C. jacchus* and *C. penicillata* with the nerve originating from the caudal trunk (Aydin 2003, Cruz et al. 2013, Santos et al. 2016). However, this characteristic is different from that described for *C. lanigera*, where the nerve was observed to come from the middle and caudal trunks (Gamba et al. 2007). In their studies on brachial plexuses in *T. tetradactyla* (Cruz et al. 2012), confirmed that the thoracodorsal nerve received contributions from all three trunks, cranial, middle and caudal, which differs from that found for the common sloth, where this nerve was found to originate exclusively from the caudal trunk.

CONCLUSIONS

The brachial plexus of *Bradypus variegatus* arose from the fifth cervical spinal segment, extending until the second thoracic segment, constituting the cranial and caudal trunks. Different anatomical dispositions were observed for the common sloth when compared to domestic and wild mammals in terms of constituent medullary segments of the brachial plexus. However, this is likely associated with the number of cervical vertebrae which varies between different species, since the nerves that comprise the plexus and the structures innervated by them, do not present discrepancies.

Changes in the origin of the plexus are due to variations in the insertion position of limb buds in relation to the neuro axis, since cervical vertebrae vary in terms of their quantity and shape, both for wild and domestic animals, which contributes to variation in the origin of nerves that comprise the brachial plexus.

Acknowledgements.- We thank the "Departamento de Morfologia e Fisiologia Animal" at the "Universidade Federal Rural de Pernambuco", Recife/PE, for the provision of the corpses and the space for the dissection of the specimens.

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

REFERENCES

Adami M., Cruz G.A.M. & Oliveira V.L. 2013. Características anatômicas do plexo braquial de bicho-preguiça-de-coleira (*Bradypus torquatus* Illiger, 1811). Biotemas 26(3):195-201. <https://dx.doi.org/10.5007/2175-7925.2013v26n3p195>

Atoji Y., Suzuki Y. & Sugimura M. 1987. The brachial plexus of the Japanese serow (*Capricornis crispus*). Anatomischer Anzeiger 163(1):25-32. <PMid:3565786>

Aydin A. 2003. Brachial plexus of the porcupine (*Hystrix cristata*). Vet. Med. 48(10):301-304. <https://dx.doi.org/10.17221/5783-VETMED>

Azarias R.E.G.R. 2005. Morfologia dental da preguiça-de-coleira *(Bradypus torquatus* Illiger, 1858). Doctoral Dissertation in Sciences, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo.

Bielik P. 1937. Constituição do Plexus Brachialis no *Bradypus tridactylus*. Arq. Anat. Antropol., Lisboa, 18:1-4.

Booth K.K., Baloyi F.M. & Lukhele O.M. 1997. The brachial plexus in the Chacma baboon (*Papio ursinus*). J. Med. Primatol. 26(4):196-203. <https://dx.doi.org/10.1111/j.1600-0684.1997.tb00052.x> <PMid:9416570>

Cruz G.A.M. & Adami M. 2010. Anatomia do plexo braquial de macaco-barrigudo (*Lagothrix lagothricha*). Pesq. Vet. Bras. 30(10):881-886. <https://dx.doi.org/10.1590/S0100-736X2010001000012>

Cruz G.A.M., Adami M. & Oliveira V.L. 2013. Características anatômicas do plexo braquial de bicho-preguiça-de-coleira (*Bradypus torquatus* Illiger, 1811). Biotemas 26(3):195-201. <https://dx.doi.org/10.5007/2175-7925.2013v26n3p195>

Cruz G.A.M., Adami M., Almeida A.E.F.S., Silva E.A.A.C., Faria M.M.M.D., Pinto M.G.F. & Silva R.D.G. 2012. Características anatômicas do plexo braquial de tamanduá-mirim (*Tamandua tetradactyla* Linnaeus, 1758). Revta Bras. Saúde Prod. Anim. 13(3):712-719. <https://dx.doi.org/10.1590/S1519-99402012000300011>

Cullen Júnior L., Bodmer E.M. & Valderes-Padua C. 2001. Ecological consequences of hunting in Atlantic forest patches, São Paulo, Brazil. Oryx 35(2):137-144. <https://dx.doi.org/10.1046/j.1365-3008.2001.00163.x>

Dyce K.M., Sack W.O. & Wensing C.J.G. 2010. Tratado de Anatomia Veterinária. Guanabara Koogan, Brasil. 856p.

Fernandez F. & Miranda F. 2007. Preguiças: conhecer para preservar. Nosso Clínico 10:44-48.

Fioretto E.T., Castro M.F.S., Guid W.L., Mainardi R., Souza R.R. & Ribeiro A.A.C.M. 2003. Grossan atomic organization of thecapybara's (*Hydrochaeris hydrochaeris*) brachial plexus. Anat. Histol. Embryol. 32(3):169-174. <https://dx.doi.org/10.1046/j.1439-0264.2003.00453.x> <PMid:12823103>

Flores P.L. 2006. Estudo epidemiológico das lesões praumáticas de plexo braquial em adultos. Arq. Neuro-Psiquiatria 64(1):88-94. <https://dx.doi.org/10.1590/S0004-282X2006000100018>

Gamba C.O., Castro T.F., Rickes E.N. & Pereira M.A. 2007. Sistematização dos territórios nervosos do plexo braquial em chinchila (*Chinchillalanigera*). Braz. J. Vet. Res. Anim. Sci. 44(4):283-289. <https://dx.doi.org/10.11606/issn.1678-4456.bjvras.2007.26629>

Getty R.S.S. & Grossman J.D. 1986. Anatomia dos Animais Domésticos. Guanabara Koogan, Brasil. 2052p.

Ghoshal N.G. 1986. Nervos espinhais, p.1597-1600. In: Sisson S. & Grossman J.D. (Eds), Anatomia dos Animais Domésticos. Guanabara Koogan, Brasil.

Hautier L., Weisbecker V., Sanchez-Villagra M.R., Goswami A. & Asher R.J. 2010. Skeletal development in sloths and the evolution of mammalian vertebral patterning. Proc. Natl Acad. Sci. 107(44):18903-18908. <https://dx.doi.org/10.1073/pnas.1010335107>

IUCN 2019. Red List of Threatened Species, 2019. International Union for Conservation Nature. Available at <http://www.iucnredlist.org> Accessed on Aug. 10, 2019.

Kaviar S., Shockey J. & Sundberg P. 2012. Observations on the endemic pygmy three-toed sloth, *Bradypus pygmaeus* of Isla Escudo de Veraguas, Panamá. Plos One 7(11):e49854. <https://dx.doi.org/10.1371/journal.pone.0049854> <PMid:23185461>

Kawashima T., Yoshitomi S. & Sasaki H. 2007. Nerve fibre tracing of branches to the coracobrachialis muscle in a bornean orangutan (*Pongo pygmaeus pygmaeus*). Anat. Histol. Embryol. 36(1):19-23. <https://dx.doi.org/10.1111/j.1439-0264.2006.00714.x>

Miller R.A. 1934. Comparative studies upon the morphology and distribution of the brachial plexus. Am. J. Anat. 54(1):143-175. <https://dx.doi.org/10.1002/aja.1000540106>

Moura C.E.B., Albuquerque J.F.G., Magalhães M.S., Silva N.B., Oliveira M.T. & Papa P.C. 2007. Análise comparativa da origem do plexo braquial de catetos (*Tayassu tajacu*). Pesq. Vet. Bras. 27(9):357-362. <https://dx.doi.org/10.1590/S0100-736X2007000900001>

Nowak R.M. 1999. Walker's Mammals of the World. Johns Hopkins University Press, Baltimore. 1936p.

Parada H., Pineda U.H., Lagunas E.M. & Vidal H.A. 1989. Variaciones anatômicas de las ramas raquídeas que constituyen los troncos de origen del plexo braquial. Anal. Anat. Normal 7:32-36.

Pauli J.N., Mendoza J.E., Steffan S.A., Carey C.C., Weimer P.J. & Peery M.Z. 2014. A syndrome of mutualism reinforces the lifestyle of a sloth. Proc. R. Soc. B 281(1778):20133006. <https://dx.doi.org/10.1098/rspb.2013.3006> <PMid:24452028>

Pereira K.F. 2015. Antrozoologia e hematologia de preguiças comuns (*Bradypus variegatus*) de áreas urbanas. Master's Thesis in Animal Biology, Universidade Federal de Viçosa, Minas Gerais. 58p.

Ribeiro A.R., Prada I.L.S., Silva Z., Barros R.A.C. & Silva D.C.O. 2005. Origem do plexo braquial do macaco *(Cebus apella)*. 2005. Braz. J. Vet. Res. Anim. Sci. 42(2):143-149. <https://dx.doi.org/10.11606/issn.1678-4456.bjvras.2005.26445>

Ricci G.D., Guazzelli Filho J., Silva J.R.CP., Matheus S.M.M. & Filadelpho A.J. 2013. Plexo braquial de mamíferos e aves- Revisão de literatura. Revta Cient. Eletrôn. Med. Vet. 11(20):1-15.

Santos P.R.S., Silva M.H.R., Rodrigues A.R. & Assis Neto A.C. 2016. Anatomic description of brachial plexus of *Callithrix jacchus* and *Callithrix penicillata.* Pesq. Vet. Bras. 36(9):901-904. <https://dx.doi.org/10.1590/s0100-736x2016000900017>

Scavone A.R.F., Machado M.R.F., Guimarães G.C., Oliveira F.S. & Gerbasi S.H.B. 2008. Análise da origem e distribuição dos nervos periféricos do plexo braquial da paca (*Agouti paca*, Linnaeus, 1766). Ciênc. Anim. Bras. 9(4):1046-1055.

Souza Junior P., Carvalho N.C., Mattos K. & Santos A.L.Q. 2014. Origens e ramificações do plexo braquial no cachorro-do-mato *Cerdocyon thous* (Linnaeus, 1766). Pesq. Vet. Bras. 34(10):1011-1023. <http://dx.doi.org/10.1590/S0100-736X2014001000015>

Souza Junior P., Wronski J.G., Carvalho N.C. & Abidu-Figueiredo M. 2018. Brachial plexus in the *Leopardus geoffroyi*. Ciênc. Anim. Bras. 19:e-50805. <http://dx.doi.org/10.1590/1809-6891v19e-51240>

Stüpp-De-Souza D.A., Castro T.F., Franceschi R.C., Silva Filho R.P. & Pereira M.A.M. 2010. Formação do plexo braquial e sistematização dos territórios nervosos em membro torácico de lobos-marinhos *(Arctocephalus australis).* Braz. J. Vet. Res. Anim. Sci. 47(2):168-174.

Vieira L.G., Ribeiro P.R.Q., Lima M.O., Souza R.R., Valdes S.A.C. & Santos A.L.Q. 2013. Origens e ramificações do plexo braquial do veado-catingueiro *Mazama gouazoubira* (Artiodactyla: Cervidae). Biotemas 26(1):137-146. <https://dx.doi.org/10.5007/2175-7925.2013v26n1p137>

Wilson D.E. & Reeder D.M. 2005. Mammals Species of the World: a taxonomic and geographic reference. John Hopkins University Press, Baltimore. 2142p.

Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

INSTRUÇÕES AOS AUTORES

A submissão de artigos à revista "Pesquisa Veterinária Brasileira" (PVB) deve ser feita em Word, através do Sistema ScholarOne, *link* <https://mc04.manuscriptcentral.com/pvb-scielo>

A tramitação somente pode ter início se o seu artigo estiver rigorosamente dentro das normas de apresentação da revista, de acordo com as Instruções aos Autores, o modelo no site da revista e os últimos fascículos publicados (www.pvb.com.br). Na verificação de falhas de apresentação, o artigo será devolvido aos autores para as devidas correções.

Os autores podem submeter seus artigos em **Inglês** ou em **Português**, mas sempre com um Resumo em português. No caso de artigos aceitos escritos em **Português**, estes serão traduzidos para o **Inglês** pela Editora Cubo; pois todos os artigos publicados na PVB serão em inglês. Para os artigos já submetidos em **Inglês**, os autores devem apresentar via ScholarOne um Certificado de Tradução de uma empresa habilitada ou de um Tradutor Nativo. **Essa regra vale para artigos submetidos a partir de 1 de janeiro de 2018.**

Os pagamentos da taxa de publicação (*Paper Charge*) serão cobrados na ocasião do envio da comunicação de aceite por e-mail:

(1)Artigos submetidos em inglês, R$ 1.500,00 (*US$ 480.00*) por artigo;

(2)Artigos submetidos em português, R$ 1.500,00 (*US$ 800.00*) mais R$ 0,32 por palavra (o valor total cobrado por será enviado por e-mail pela tesouraria do CBPA).

A partir de 2019, **todos os autores deverão criar um registro no ORCID** (Open Researcher and Contributor ID - https://orcid.org/register) e vinculá-lo ao seu perfil no ScholarONE. O vínculo pode ser feito editando o perfil do usuário no ScholarONE na opção **Associate your existing ORCID iD**. Os identificadores ORCID contribuem para a identificação única dos autores e, portanto, para os processos interoperacionais e bibliométricos que envolvem autores. Usando um ORCID, os pesquisadores são fácil e corretamente conectados com seus resultados de pesquisa, publicações e afiliações.

O **texto** deve ser formatado, em todos os pormenores, de acordo com as normas de apresentação da revista (www.pvb.com.br).

Se o artigo for submetido fora das normas de apresentação da PVB, a tramitação somente ocorrerá após as devidas correções feitas pelo autor.

A PVB publica Artigos Originais, Artigos de Revisão Crítica e Tópicos de Interesse Geral; não publica artigos com a denominação de *Short Communications*.

Relatos de Caso serão aceitos somente em artigos classificados como pertencentes à área de Animais Selvagens (*Wildlife Medicine*).

Os Artigos Originais devem conter resultados de pesquisa ainda não publicados ou submetidos para outros periódicos.

Artigos de Revisão de Literatura, submetidos a convite, devem constituir-se de análise crítica, de assuntos na área de experiência dos autores, isto é, quando os autores já tiverem publicado anteriormente artigos sobre o assunto.

Os raros Tópicos de Interesse Geral devem constituir-se de assuntos de grande importância atual baseado na vasta experiência dos autores.

As opiniões e conceitos emitidos nos artigos submetidos são de responsabilidade dos autores. O Conselho Editorial da PVB, com a assistência da Assessoria Científica, pode sugerir ou solicitar modificações. Os artigos submetidos são avaliados pelos pares (*peer review*) e, aceitos para publicação com dois pareceres favoráveis, ou rejeitados por dois pareceres desfavoráveis.

Os direitos autorais dos artigos aceitos para publicação permanecem com os autores.

1. **Os artigos devem ser organizados em TÍTULO, ABSTRACT, RESUMO, INTRODUÇÃO, MATERIAL E MÉTODOS, RESULTADOS, DISCUSSÃO, CONCLUSÕES (de preferência os últimos três separadamente), Agradecimentos, Declaração de conflito de interesse e REFERÊNCIAS:**

a) O **TÍTULO** deve ser conciso e indicar o conteúdo do artigo; pormenores de identificação científica devem ser colocados em MATERIAL E MÉTODOS.

b) O(s) Autor(es) com numerosos primeiros nomes e sobrenomes, deve(m) padronizar o seu "nome para publicações científicas", como por exemplo: Cláudio Severo Lombardo de Barros, escreve Cláudio S.L. Barros ou Barros C.S.L.; Franklin Riet-Correa Amaral escreve Franklin Riet-Correa ou Riet-Correa F. **Os artigos devem ter no máximo 8 (oito) autores**. O autor para correspondência deve ser um autor que garanta o contato com o Conselho Editorial da PVB. Asteriscos de chamadas para o rodapé não devem ser sobrescritos.

c) O **Cabeçalho do ABSTRACT** deve conter além dos nomes dos autores abreviados invertido, o ano, o TÍTULO, o endereço postal do laboratório (inclusive o CEP) ou instituição principal onde foi desenvolvida a pesquisa. Endereços postais brasileiros

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3. **Apresentação das Figuras:**

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

b) Enviar cada figura separadamente.

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

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

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

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

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

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

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

Exemplos de Referências

➢ Artigos publicados em periódicos:

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

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

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

Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

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

- Livros:

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

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

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

- Capítulos de livros:

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

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

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

- Dissertações e Teses:

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

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

- Resumos publicados em eventos:

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

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

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

Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

GUIDE FOR AUTHORS

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

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

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

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

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

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

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

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

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

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

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

The author rights of the accepted papers are preserved.

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

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

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

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

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

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

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

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

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

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

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

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

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

m) Conflict of interest or none should be mentioned.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3. **Figure presentation:**

a) Save images at 300 dpi, TIF files.

b) Send each figure separately.

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

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

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

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

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

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

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

Exemples for References:

➢ Articles published in scientific journals:

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

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

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

➢ Books:

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

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

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

➢ Chapters of books:

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

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

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

➢ Dissertations and Theses:

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

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

➢ Abstracts published in Events:

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

Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

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

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

(Note: Consult entire papers instead of only Abstracts)

Pesquisa Veterinária Brasileira

Brazilian Journal of Veterinary Research

CONTENTS Volume 40, Number 10, October 2020

Livestock Diseases

Research and extension action for parasitic control in pig breeding families located in Tanguá, Rio de Janeiro [Pesquisa e ação de extensão para controle das parasitoses em propriedades familiares de criação de suínos localizadas em Tanguá, Rio de Janeiro].Class C.S.C., Silveira R.L., Palmer J.P.S., Fialho P.A., Lobão L.F., Dib L.V., Uchôa C.M.A. & Barbosa A.S. 739-749

Embryonic loss, abortion, and malformations in ewes caused by the ingestion of hay leaves of Cenostigma pyramidale [Perda embrionária, aborto e malformações em ovinos por ingestão de Cenostigma pyramidale na forma fenada]. Reis S.D.S., Macêdo J.T.S.A., Pereira A.L., Jesus R.S., Pimentel L.A., Pivato I., Peixoto T.C. & Pedroso P.M.O. 750-757

Causes of death in growing-finishing pigs in two technified farms in southern Brazil [Causas de morte em suínos de crescimento e terminação em duas granjas tecnificadas no Sul do Brasil]. Piva M.M., Schwertz C.I., Bianchi R.M., Kemper R.T., Henker L.C., Nagae R.Y., Cê T.R.M., Barcellos D.E.S.N., Driemeier D. & Pavarini S.P. 758-775

Clostridium perfringens α and β recombinant toxoids in equine immunization [Toxóides recombinantes α e β de Clostridium perfringens na imunização de equinos].Freitas N.F.Q.R., Barbosa J.D., Otaka D.Y., Ferreira M.R.A., Rodrigues R.R., Moreira Jr C., Conceição F.R. & Salvarani F.M. 776-780

Investigation of Listeria monocytogenes, Salmonella enterica and Yersinia enterocolitica in pig carcasses in Southern Brazil [Pesquisa de Listeria monocytogenes, Salmonella enterica and Yersinia enterocolitica em carcaças de suínos no sul do Brasil]. Kich J.D., Souza A.I.A., Montes J., Meneguzzi M., Costa E.F., Coldebella A., Corbellini L.G. & Cardoso M. 781-790

Small Animal Diseases

Mast cells in the pathophysiology of Duchenne muscular dystrophy in Golden Retriever dogs [O papel dos mastócitos na patogênese da distrofia muscular de Duchenne em cães Golden Retriever]. Martins I.M., Malvestio L.M.M., Engracia-Filho J.R., Claudiano G.S., Moraes F.R. & Moraes J.R.E. 791-797

Use of bone marrow for detection of toxic chemicals for the elucidation of poisoning in forensic veterinary medicine [Uso de medula óssea para detecção de produtos químicos tóxicos para a elucidação de intoxicação em patologia veterinária forense]. Marcelino S.A.C., Serakides R., Castro-Silva V.N., Ramos M.L., Ocarino, N.M. &. Melo M.M. 798-803

Antimicrobial resistance evaluation of bacteria isolated from infections in small animals in the Umuarama region, Paraná [Avaliação da resistência antimicrobiana de bactérias isoladas de infecções em pequenos animais na região de Umuarama, Paraná]. Souza M.M., Bordin J.T., Pavan A.C.L., Rodrigues R.G.A., Sfaciotte R.A.P., Vignoto V.K.C., Ferrante M. & Wosiacki S.R. 804-813

Wildlife Medicine

Rickets in white-eared opossums (Didelphis albiventris) [Raquitismo em gambás-de-orelha-branca (Didelphis albiventris]. Ferreira Júnior J.A., Landi M.F., Passos A.O., Nascimento K.A., Macêdo J.T.S.A., & Pedroso P.M.O. 814-817

Avipoxvirus detected in tumor-like lesions in a white-faced whistling duck (Dendrocygna viduata) [Avipoxvirus detectado em lesões tipo tumorais em uma marreca-irerê (Dendrocygna viduata)]. Braga J.F.V., Couto R.M., Rodrigues M.C. & Ecco R. 818-823

Animal Morphophysiology

Description of brachial plexus of sloth (Bradypus variegatus) [Descrição do plexo braquial do bicho-preguiça (Bradypus variegatus)]. Alcântara S.F., Amorim M.J.A.L, Albuquerque P.V., Mesquita E.P., Miranda M.E.L.C., Andrade G.P., Nascimento N.E.O. & Nascimento J.C.S. 824-829

Grillo Tecnologia

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