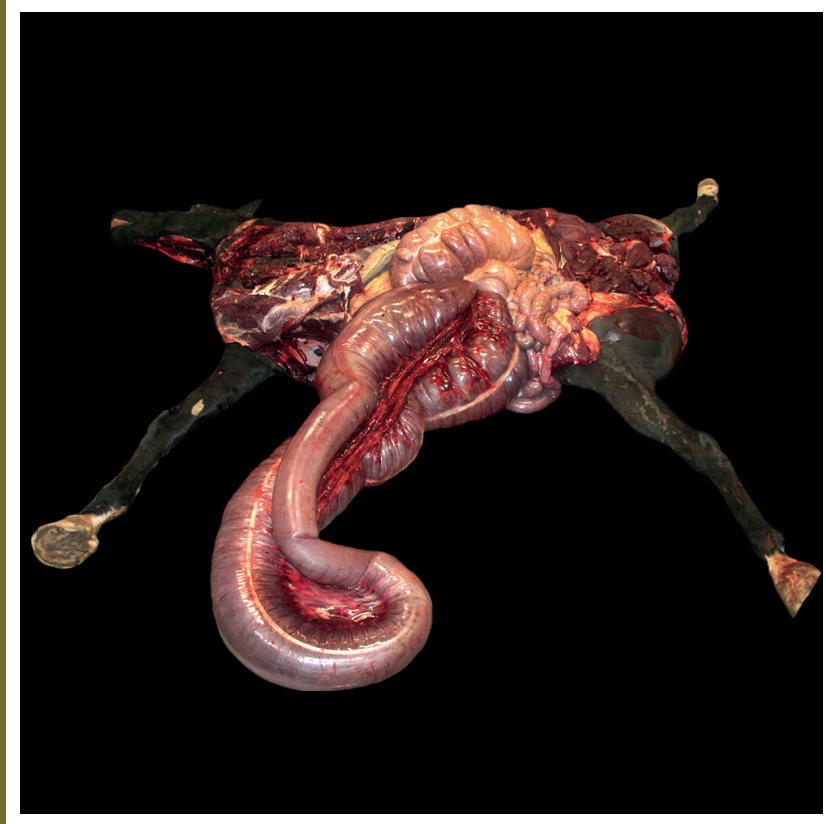
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Cover illustration: Volvulus in a horse (Bianchi M.V. et al., p.245)

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Original Article
Livestock Diseases
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Cattle rabies: the effect of clinical evolution, viral genetic lineage, and viral load on the severity of histological lesions[1]

Claudia S. Wisser[2]*, André Thaler Neto[3], Helena B.C.R. Batista[4], Enio Mori[4], Maria E.R. Chierato[4], Marcélia E.S. Fernandes[4] and Sandra D. Traverso[2]

ABSTRACT.- Wisser C.S., Thaler Neto A., Bastista H.B.C.R., Mori E., Chierato M.E.R, Fernandes M.E.S. & Traverso S.D. 2020. **Cattle rabies: the effect of clinical evolution, viral genetic lineage, and viral load on the severity of histological lesions.** *Pesquisa Veterinária Brasileira 40(4):227-233*. Centro de Ciências Agroveterinárias, Universidade do Estado de Santa Catarina, Av. Luiz de Camões 2090, Bairro Conta Dinheiro, Lages, SC 88520-000, Brazil. E-mail: claudiawisser@hotmail.com

Our objective was the characterization and staging of histological lesions in different anatomical sites of the central nervous system (CNS) of rabid cattle. The severity of the lesions was compared with the clinical stages of the disease, the variants of viral isolates, and with the load of virus. Thirty-one spontaneously affected rabid cattle the state of Santa Catarina underwent clinical follow-up and were eventually necropsied. CNS tissues were sampled and submitted to direct fluorescent antibody technique (DFAT), immunohistochemistry (IHC), routine histopathology with hematoxylin and eosin stain (HE), reverse transcriptase polymerase chain reaction (RT-PCR), and polymerase chain reaction in quantitative reverse transcriptase in real time (qRT-PCR). Affected cattle were allotted in four groups according to their clinical stage when euthanized: G1, euthanized while standing; G2, euthanized when in sternal recumbence; G3, euthanized when in lateral recumbence; and G4, affected cattle with natural death. In order to evaluate the degree of severity of the lesions and the presence of Negri bodies (NBs), the brain was sectioned at 9 sites. Additionally, spinal cord and trigeminal ganglion sections were examined. The intensity of the lesions was graded as either absent, mild, moderate, or marked, and the presence or absence of the NBs was noted. Histological lesions were characterized by lymphocytic and monocytic meningoencephalitis with NBs in 28 cases. In all analyzed groups, intensities of histological lesions ranging from mild to severe were observed. Brain regions with the highest inflammatory lesion intensity were the medulla at the level of obex, followed by the colliculus and thalamus. NBs were observed in a higher percentage in the cerebellum, followed by medulla at the obex level, striatum complex, and frontal telencephalon. The duration of the clinical course of the disease did not influence the intensity of the inflammatory lesion, but it did influence the presence of NBs, with a higher percentage of these inclusions in cattle that died naturally than in euthanized cattle. All isolated rhabdovirus included in this study were genetically compatible with samples from hematophagous bats *Desmodus rotundus*. The evaluation by qRT-PCR did not demonstrate a correlation between lesion intensity and the amount of virus.

INDEX TERMS: Cattle rabies, clinical course, virus, genetic lineage, histology, lesion severity, RT-PCR.

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RESUMO.- [**Raiva bovina: relação do curso clínico, da linhagem genética do vírus e carga viral com a intensidade de lesões histológicas.**] Nosso objetivo foi a caracterização e estadiamento de lesões histológicas em diferentes locais anatômicos do sistema nervoso central (SNC) de bovinos raivosos. A gravidade das lesões foi comparada com os estágios clínicos da doença, as variantes dos isolados virais e com a quantidade de vírus. Trinta e um bovinos do estado de Santa Catarina, afetados naturalmente por raiva, foram acompanhados clinicalmente e, ao final, necropsiados. Os tecidos do SNC foram amostrados e submetidos a imunofluorescência direta, imunohistoquímica, histopatologia de rotina, reação em cadeia da polimerase via transcriptase reversa (RT-PCR) e reação em cadeia da polimerase em transcriptase reversa quantitativa em tempo real (qRT-PCR). Os bovinos afetados foram distribuídos em quatro grupos, de acordo com sua fase clínica: G1, eutanasiados quando ainda se mantinham em pé; G2, eutanasiados quando em decúbito esternal; G3, eutanasiados quando em decúbito lateral; e G4, bovinos afetados com morte natural. Para avaliar o grau de gravidade das lesões e a presença de corpúsculos de Negri (CNs), o cérebro foi seccionado em 9 locais. Além disso, seções da medula espinhal e do gânglio trigêmeo foram examinadas. A intensidade das lesões foi graduada como ausente, leve, moderada ou acentuada, e a presença ou ausência dos CNs foi anotada. Lesões histológicas foram caracterizadas por meningoencefalite linfocítica e monocítica com CNs em 28 casos. Em todos os grupos analisados foram observadas intensidades de lesões histológicas variando de leve a grave. As regiões cerebrais com maior intensidade de lesão inflamatória foram o bulbo no nível do obex, seguido do colículo e tálamo. CNs foram mais prevalentes no cerebelo, seguido pelo bulbo ao nível do óbex, corpo estriado e telencéfalo frontal. A duração do curso clínico da raiva não influenciou a intensidade da lesão inflamatória, mas influenciou a presença de CNs, com maior porcentagem dessas inclusões em bovinos que morreram naturalmente do que em bovinos sacrificados. Todos os isolados rabdovírus obtidos neste estudo eram geneticamente compatíveis com amostras provenientes de morcegos hematófagos *Desmodus rotundus*.

TERMOS DE INDEXAÇÃO: Raiva bovina, evolução clínica, linhagem genética, vírus, histologia, intensidade das lesões, RT-PCR, bovinos.

INTRODUCTION

Rabies is an infectious, notifiable disease that affects the central nervous system (CNS) of several species (Barros et al. 2006, Beigh et al. 2015, Faizee et al. 2012). It causes acute encephalomyelitis in all warm-blooded animals, including humans and many wild species, which can serve as reservoirs for the virus (Rupprecht & Gibbons 2004).

The disease is caused by RNA viruses, belonging to the genus Lyssavirus of the family Rhabdoviridae (Buchen-Osmond 2003). Based on phylogenetic analysis, the genus Lyssavirus is currently divided into seven genotypes (Heaton et al. 1999). The classical *rabies lyssavirus* (RABV) and its strains are known worldwide as the cause of rabies in humans and animals (Paweska et al. 2006), and so far Genotype 1, is the only one isolated in the Americas (Schaefer et al. 2005)

The RABV circulates within two interrelated epidemiological cycles, the urban and the wild cycle (Escobar et al. 2013). In Brazil, rabies prevalence is maintained by the main reservoirs, the dog in the urban cycle and bats in the wild cycle (Dietzschold et al. 1988, Schaefer et al. 2005). Evaluation of isolates from different regions of Brazil has identified that most cattle samples are related to the rabies virus from hematophagous bats (Schaefer et al. 2005). The isolated variant of hematophagous bats (*Desmodus rotundus*) is important throughout Latin America not only due to economic livestock losses but also to the impact on public health by transmission to humans (Rupprecht et al. 2018). In 2007, six cases of human rabies were recorded in Brazil; all of which were caused by variant 3 of hematophagous bats (*D. rotundus*) (Brasil 2018).

A recent case of autochthonous human rabies has been reported from Santa Catarina and the last reported cases of human and canine rabies in this southern Brazilian State were diagnosed respectively in 1981 and 2016 (DIVE 2019). Cases of rabies in herbivores are frequently reported from Santa Catarina (CIDASC 2019) but, there are insufficient data informing about different viral variants circulating the State.

Nervous lesions are characterized by lymphocytic and monocytic infiltration in the perivascular spaces an meninges, gliosis, and neuronal degeneration and necrosis (Beck et al. 2017, Rupprecht et al. 2018). Characteristic eosinophilic inclusion bodies, the Negri bodies (NBs) may be present in the neuronal cytoplasm (Beck et al. 2017). It is estimated that in up to 30% of cases of rabies in cattle, NBs are not visible in routine histological staining (Barros et al. 2006).

The intensity of the lesions is quite variable (Summers et al. 1995). It is believed that several factors can influence the lesion intensity, such as the type and concentration of the virus (Fernandes & Riet-Correa 2007), the incubation period, the duration of the clinical course of the disease, individual reaction of the infected animal (Germano et al. 1977). Although data linking clinical stages with histological changes in cattle are scarce, more severe lesions are often related to longer clinical courses (Langohr et al. 2003, Lima et al. 2005). Thus, it is believed that the severity of the lesions increases with disease progression (Langohr et al. 2003).

This study provides information on the characterization and staging of histological lesions in the different anatomical sites of the CNS in rabid, comparing with the clinical stages of the disease, the variants of circulating rabies virus in Santa Catarina.

MATERIALS AND METHODS

Tissue samples from central nervous system were obtained during necropsies of cattle with naturally acquired rabies after a follow-up of clinical cases in the State of Santa Catarina, southern Brazil. Affected cattle were euthanized only after the first positive diagnosis for rabies in a bovine involved in the outbreak. Affected cattle were allotted in four groups according to their clinical stage when euthanized: G1, euthanized while standing; G2, euthanized when in sternal recumbence; G3, euthanized when in lateral recumbence; and G4, affected cattle with natural death. The procedures for the current study were submitted to and approved by the Ethics Committee in Experimentation of the Agroveterinary Sciences Center (CETEA), of the State University of Santa Catarina, under protocol number 1.06.15.

Samples of the cerebellum, hippocampus, and thalamus were submitted to DFAT and IHC for confirmation of the diagnosis. A

pool containing samples of cerebellum, brainstem and cortex was submitted to molecular characterization. The remained CNS tissue was preserved in 10% formaldehyde for histopathological evaluation.

DFAT was performed according technique previously described (Dean et al. 1996), for the use of antinucleocapsid polyclonal antiragic conjugate, produced at Instituto Pasteur of São Paulo/SP. The IHC was applied in the samples according to a protocol adapted from Pedroso et al. (2008) using Chemicon #5199 polyclonal antirabies antibody.

For histological evaluation, the following portions were sectioned: the trigeminal ganglion, medulla at the level of the obex, cerebellum, rostral colliculus, frontal telencephalon, corpus striatum, parietal telencephalon, thalamus, temporal telencephalon, hippocampus, and cervical spinal cord. Hematoxylin and eosin (HE) stained sections were evaluated for the intensity of inflammatory lesions and classified as: (0) without alterations, (1) mild perivascular mononuclear infiltrate with up to two layers, and mild gliosis, (2) moderate perivascular mononuclear infiltrate with 3-4 cell layers and moderate gliosis, (3) marked perivascular mononuclear inflammation with 3-4 cell layers and marked gliosis or more than 4 layers of perivascular inflammatory cells. All regions were also evaluated for the presence of NBs.

Table 1. Severity of the histological lesions, by brain area, in the different groups of clinical evolution

Bov	Group	Day of death	Severity of inflammation in each region										
			ME	TR	OB	CR	CO	TF	CE	TP	TA	TT	HI
1	G1	4	2	3	3	1	3	0	3	1	3	1	1
2	G1	3	3	1	3	1	3	1	0	0	2	0	0
3	G1	3	NA	3	2	1	3	1	2	1	2	0	1
4	G1	4	1	NA	2	1	2	1	2	2	1	1	1
5	G1	5	2	1	3	3	2	2	3	2	2	1	2
6	G1	2	0	0	2	1	2	1	1	1	3	1	1
7	G2	6	0	NA	1	0	1	1	0	0	1	0	0
8	G2	5	1	1	0	0	0	0	0	0	1	0	0
9	G2	6	NA	NA	1	1	2	1	1	1	2	0	0
10	G2	2	2	2	2	2	1	0	1	1	1	1	0
11	G2	4	1	NA	2	1	2	NA	NA	1	1	0	1
12	G2	5	1	1	2	1	2	0	1	0	2	1	1
13	G3	8	1	1	2	2	2	1	1	1	2	NA	1
14	G3	8	1	NA	3	2	2	2	2	1	2	2	2
15	G3	8	NA	NA	1	1	1	0	0	0	1	0	0
16	G3	3	NA	NA	2	1	2	NA	1	1	1	0	1
17	G3	5	1	NA	2	1	2	1	2	1	2	1	1
18	G3	7	NA	2	2	1	2	NA	1	0	1	0	0
19	G3	7	NA	2	3	1	1	0	1	0	1	0	1
20	G3	4	NA	1	2	1	1	0	1	0	1	0	0
21	G3	5	0	NA	2	1	3	2	2	1	2	1	1
22	G3	2	3	2	3	3	2	1	1	1	3	0	0
23	G3	5	2	NA	3	2	2	2	2	2	2	2	2
24	G3	3	2	NA	3	3	3	2	2	1	2	1	2
25	G3	7	3	1	3	2	3	2	2	1	3	1	2
26	G3	4	1	1	3	3	2	2	2	1	3	0	2
27	G3	4	2	1	2	1	2	0	2	1	1	0	1
28	G4	5	1	3	3	1	3	1	1	1	2	0	1
29	G4	6	2	NA	3	1	2	1	2	1	3	1	0
30	G4	3	NA	NA	3	2	3	2	3	2	3	1	2
31	G4	3	2	NA	3	2	3	3	3	2	3	2	3

G1 = Cattle euthanized while standing, G2 = euthanized when in sternal recumbence, G3 = euthanized when in lateral recumbence, G4 = affected cattle with natural death, NA = unrecorded region, 0 = absence of lesions, 1 = mild inflammation, 2 = moderate inflammation, 3 = marked inflammation, ME = spinal cord, TR = ganglion trigeminal, OB = medulla at the obex level, CR = cerebellum, CO = rostral colliculus, TF = frontal telencephalon, CE = corpus striatum, TP = parietal telencephalon, TA = thalamus, TT = temporal telencephalon, HI = hippocampus.

For molecular characterization, RT-PCR followed by genetic sequencing was performed, using a pool containing samples of cerebellum, brainstem, and cortex. The total RNA from each of the samples was extracted using TRIzol Reagent (Invitrogen™). For the reverse transcription, 5µl of the RNA extracted from each sample was utilized, 21G (sense) (5'-ATGTAACACCTCTACAATG-3') and 304 (antisense) primers (5'-TTDACGAAGATCTTGCTCAT-3') directed to the RABV nucleoprotein gene and the kit Superscript™ II Reverse Transcriptase (Invitrogen™). PCR primers 21G, 304 and the recombinant Taq DNA polymerase kit (Invitrogen™) were used. Both RNA extraction and RT-PCR were performed according to the manufacturer's instructions. For each amplified sample, the genetic sequencing with each primer previously described was separately performed using 4µl of BigDye 3.1 (Applied Biosystems), 3.2pmol of each primer, 30-50ng of target DNA and water DNAse/RNAse free qsp for a final reaction of 10µl. The product of this reaction was further purified with Sephadex (GE Healthcare™) in a 96-well plate following the manufacturer's instructions. Subsequently, the plate was taken to the ABI-3130 automatic gene analyzer (Applied Biosystems™) to obtain the electropherograms of the sequences of DNA. The nucleotides of the electropherograms were checked manually with the program Chromas v.2.23 (©1998-2002 Technelysiumm Pty LTD) and the final sequence obtained with the Cap-Contig application of the BIOEDIT v.5.0.9 (Hall 1999), which was then compared with others retrieved by BLASTn[5] to obtain the identities of the genetic lines of the samples. The sequences were aligned by the CLUSTAL/W method, for which the BIOEDIT program was used, v.5.0.9. Phylogenetic trees were obtained using the Neighbor-Joining algorithm and Kimura-2-parameter evolutionary model, with 1000 bootstrap repetitions with the MEGA program (Kumar et al. 2001).

The qPCR was performed using the SyBR Green I system and the antisense primers 304 and sense 504 (5'-TATACTCGAATCATGATGAATGGAGGTCGACT-3'). For the real-time RT-PCR, 2µL per sample was used, plus 9µL of free nuclease water, 12.5µL of 2X Power SYBR® Green PCR Master Mix (Applied Biosystems) and 0.75µL of each primer and antisense (504 and 304) at 10µM. Each sample was analyzed in duplicate using MicroAmpOptical 96-well Reaction Plate in the Applied Biosystems 7500 Real-Time PCR System apparatus. The results were analyzed using the 7500 Software v.2.0.5. The specificity of the reaction was verified by the analysis of the dissociation or melting curve. Samples with quantification cycle (Cq) <35 were considered positive by RT-qPCR. When samples showed Cq>35 or Cq<40, they were considered as inconclusive results, requiring 1% agarose gel electrophoresis. When the samples presented Cq>40, they were considered negative.

The data obtained in the histological evaluation were subjected to statistical analysis, with a completely randomized experimental design. The data of the quantitative variables (intensity of the inflammatory lesion and NBs presence) were subjected to the analysis of variance using the mixed procedure of the statistical package SAS. The intensity data were analyzed as repeated measures in the space for each animal (random variable) The statistical model includes the effects of the group with the region of the nervous system and the interaction between these variables. The Kolmogorov-Smirnov test evaluated the normality of the residues, and the means were compared by the Tukey test at the 5% level of statistical significance.

[5] Available at <http://www.ncbi.nlm.nih.gov/BLAST>

RESULTS

All samples were confirmed as rabies positive through IHC and DFAT. Histological lesions were characterized by lymphocytic and macrophagic meningoencephalitis associated with gliosis, with intensities ranging from mild to severe and NBs present in most cases. Table 1 and 2 describe the data regarding the group and day of clinical evolution of each cattle, as well as the intensity of inflammatory lesions and the presence of inclusions in each anatomic CNS site

The encephalic regions with the highest intensity of inflammatory lesions were the obex, followed by the colliculus, thalamus, and trigeminal ganglion, with no statistical difference between them (p>0.005). The average intensity of actions in the 11 regions evaluated can be observed in Table 3. NBs were observed in 28 of the 31 cattle (90.3%). The cerebellum was the region with the highest percentage of inclusions, followed by the obex, corpus striatum and frontal telencephalon, with no statistical difference between them (Table 3).

Table 4 shows the relationship between the clinical evolution of the disease, the intensity of the histological lesion and the presence of NBs. It was observed that the clinical evolution of rabies had no effect on the intensity of the inflammatory lesion, as the difference was not statistically significant (P>0.05) between cattle euthanized in standing (G1) and those naturally dead (G4). There was also no statistically significant difference between cattle euthanized in standing (G1) and those euthanized in lateral recumbency (G3). On the other hand, there was a correlation with the presence of NBs, since animals with spontaneous death had a higher percentage of inclusions, with a statistically significant difference in relation to those that were euthanized for examination.

The comparative analysis between the sequences generated and the sequences retrieved from GenBank showed that all the isolated RABV samples included in the present study show a genetic lineage characteristic of hematophagous bats *Desmodus rotundus*. Of the 31 samples collected, nine amplicated. The highest viral load was not found not to be related to a greater mean intensity of the lesion, nor to differences in clinical evolution. The ratio of the cycles threshold (CT) in RT-PCR with clinical evolution and lesion intensity can be observed in Table 5. For example, Cattle BOV2 and BOV26, with higher viral load, had lesion intensity similar to BOV3 and BOV13, with a lower virus replication rate.

DISCUSSION

The intensity of the histological lesions of rabies can be quite variable (Summers et al. 1995) and it is believed that the stage and course of the disease, together with the viral strain and load, can influence both the onset of inflammatory lesions and of the development of NBs (Fernandes & Riet-Correa 2007).

The most prominent inflammatory lesions in this study were observed in the medulla, colliculus and thalamus which is similar to the distribution described by others (Langohr et al. 2003) and are possibly related to the characteristics of the route of viral infection, since the virus spreads from the site of replication by rapid retrograde axonal transport (Wang et al. 2013) reaching first the trunk (spinal cord) and then the brain. More pronounced inflammation in the brainstem could prevent viral spread to the cerebral hemispheres (Shuangshoti et al. 2013).

Table 2. Presence of Negri bodies (NBs), by brain area, in the different groups of clinical evolution, in the different animals

Bovine	Group	Day of death	Negri inclusion bodies in each region										
			ME	TR	OB	CR	CO	TF	CE	TP	TA	TT	HI
1	G1	4	-	+	+	+	-	-	-	-	-	-	-
2	G1	3	-	-	-	-	-	-	-	-	-	-	-
3	G1	3	+	+	+	+	+	+	+	+	-	+	+
4	G1	4	-	NA	+	+	-	-	-	-	-	-	-
5	G1	5	-	-	+	+	+	+	+	+	+	+	+
6	G1	2	-	-	-	-	-	-	-	-	-	-	-
7	G2	6	+	NA	+	+	+	+	+	+	+	+	+
8	G2	5	+	-	+	+	+	-	+	-	+	-	-
9	G2	6	NA	-	-	+	-	-	-	-	-	-	-
10	G2	2	-	-	-	-	-	-	-	-	-	-	-
11	G2	4	-	NA	+	+	+	NA	NA	-	+	-	-
12	G2	5	-	-	-	-	-	-	-	-	+	-	-
13	G3	8	+	+	+	+	+	+	+	+	+	-	+
14	G3	8	+	NA	+	+	+	+	-	+	-	+	+
15	G3	8	+	NA	+	+	-	+	+	+	-	+	+
16	G3	3	-	NA	-	+	-	-	-	-	-	-	-
17	G3	5	+	NA	+	-	-	+	+	-	+	+	+
18	G3	7	NA	-	+	+	NA	+	+	-	-	-	+
19	G3	7	+	+	+	+	+	+	+	+	-	-	-
20	G3	4	-	+	-	-	-	-	-	-	-	-	-
21	G3	5	-	NA	+	-	+	+	+	+	-	+	+
22	G3	2	-	+	-	+	-	+	-	+	-	-	-
23	G3	5	-	NA	-	+	-	+	+	-	-	-	-
24	G3	3	-	NA	-	+	-	+	+	+	-	-	+
25	G3	7	+	-	+	+	+	+	+	+	+	+	+
26	G3	4	-	-	+	+	-	+	+	+	+	+	+
27	G3	4	-	-	-	-	-	-	+	-	-	-	-
28	G4	5	+	+	+	+	+	+	+	+	-	+	+
29	G4	6	NA	-	-	+	-	-	-	-	-	-	-
30	G4	3	NA	NA	+	+	+	+	+	+	+	+	+
31	G4	3	+	NA	+	+	+	+	+	+	+	+	+

G1 = Cattle euthanized while standing, G2 = euthanized when in sternal recumbence, G3 = euthanized when in lateral recumbence, G4 = affected cattle with natural death, NA = unrecorded region; (+) presence of Negri inclusions, (-) no inclusion of Negri; ME = spinal cord, TR = ganglion trigeminal, OB = medulla at the obex level, CR = cerebellum, CO = rostral colliculus, TF = frontal telencephalon, CE = corpus striatum, TP = parietal telencephalon, TA = thalamus, TT = temporal telencephalon, HI = hippocampus.

Table 3. Estimation of lesion intensity and percentage of Negri inclusion bodies in the different encephalic regions

Region	Intensity of lesion	Percentage of inclusions of Negri
Obex	2.3083[a]	61.29[ab]
Colliculus	2.1458[a]	43.33[b]
Thalamus	2.0125[a]	35.48[b]
Trigeminal	1.9089[ab]	36.84[b]
Striated complex	1.5502[bc]	60.00[ab]
Spinal cord	1.4569[bc]	40.74[b]
Cerebellum	1.3333[cd]	74.19[a]
Frontal telencephalon	1.0787[de]	60.00[ab]
Parietal telencephalon	0.9917[e]	48.39[b]
Hippocampus	0.9750[e]	48.39[b]
Temporal telencephalon	0.6421[f]	38.71[b]

[a, b, c, d, e, f] Averages followed by the same letter do not differ from each other (P>0.05) by the Tukey test.

Table 4. Effect of the clinical evolution of rabies in the presence of the Negri inclusion bodies and the severity of inflammatory lesion

Group	Percentage of inclusions of Negri	Intensity of lesion
G1	36.92[c]	1.81[ab]
G2	36.07[c]	1.06[c]
G3	55.13[b]	1.66[b]
G4	75[a]	2.3[a]

G1 = Cattle euthanized while standing, G2 = euthanized when in sternal recumbence, G3 = euthanized when in lateral recumbence, G4 = affected cattle with natural death; [a, b, c] Averages followed by the same letter do not differ from each other (P>0.05) by the Tukey test.

However, when it comes to the clinical course of the disease, previous studies report cattle with a 3-4 days of evolution without histological lesions in the CNS (Marcolongo-Pereira et al. 2011) and suggest that more severe lesions are observed in cases with more prolonged evolution (Lima et al. 2005). In the current study, even cattle that were euthanized in the early stages of the disease, with 2-3 days of evolution and were still able to stand, presented marked histological lesions in some encephalic anatomical sites. In addition, it was observed that even cattle that died after a clinical course of 7-8 days or longer had similar or even milder lesions than those that were euthanized in the early stages. These data suggest that clinical evolution cannot be considered as the only determinant factor for the severity of inflammatory histological lesions.

Regarding NBs, this study showed that their presence was influenced by the duration of the clinical course. Out of the 31 cases analyzed, only three samples did not present NBs; those were from cattle euthanized in the early stages of the disease. According to Summers et al. (1995), the premature euthanasia of affected animals can result in cases with absence of NBs. According to Langohr et al. (2003) inclusions are most frequently detected in animals which survived for more than 4 days after the onset of clinical signs. This was also observed when we analyzed each case individually, since cattle without NBs were euthanized up to 3 days after the onset of clinical signs.

However, controversial data may be inferred if one considers that two cattle that died spontaneously (without euthanasia) in the present study had a very acute clinical course (up to 3 days) and yet presented inclusion bodies in all the analyzed areas of the encephalon, indicating that a short clinical course does not necessarily equate with lack of development of NBs. Moreover, a bovine that died after a clinical course of 6 days in the current study had NBs only in the cerebellum. NBs developed more often in the cerebellum of cattle from the current study corroborating the findings from others (Langohr et al. 2003, Silva et al. 2010). However, in one bovine NBs were found only in the trigeminal ganglion. This finding indicates that a more careful analysis of other areas of the nervous system should be made when there is no NBs found in the cerebellum, cortex, or hippocampus. A higher frequency of NBs were demonstrated in portions of the spinal cord of horses when compared to other regions (Bassuino et al. 2016) concluding that the analysis of this portion of the CNS will increase the chances of establishing a rabies diagnosis.

Table 5. Relationship among clinical evolution, inflammatory lesion and viral load of cattle in different groups

Bovine	Groups	Day of death	Mean of inflammatory lesion	CT-qRT-PCR
2	G1	3	1.27	28.05
3	G1	3	1.6	13.074
9	G2	6	1	19.431
13	G3	8	1.4	18.29
14	G3	8	1.9	19.092
23	G3	5	2.1	22.7
26	G3	4	1.8	32, 396
29	G4	6	1.6	22.41
31	G4	3	2.6	21.348

G1 = Cattle in standing, G2 = sternal recumbency, G3 = lateral recumbency, G4 = natural death.

According to Maxie & Youssef (2006), NBs tend to be scarcer win sites where the inflammation is more severe. In this study, no relationship was established between NBs the intensity of the inflammatory infiltrate, as was previously observed (Langohr et al. 2003, Beck et al. 2017), since the region with the more severe inflammatory lesions, the medulla, was also the second most likely region to find NBs. The strain or type of virus is another factor that has been shown to influence the histological changes of rabies (Fernandes & Riet-Correa 2007). The phylogenetic analysis of the samples obtained from cattle in the State of Santa Catarina were all identified as genetic lineage of hematophagous bats *Desmodus rotundus* (1B). Thus, the same strain presented lesions of varying intensity suggesting that lineage alone is not a determinant factor for the severity of the inflammatory histological lesions. Likewise, the evaluation of qRT-PCR did not show a relationship of lesion intensity with a greater viral load. This corroborates the findings of Appolinário et al. (2015) who observed that there was no relation of replication with different clinical presentations. According to Zhao et al. (2009) the difference in pathogenicity would be related to longer duration of cytokine and chemokine expression than to the effects of viral replication.

CONCLUSIONS

The duration of clinical course of rabies in cattle does not significantly affect the intensity of the inflammatory lesions in the different areas of the brain, but it does influence the development of the NBs, and there is a greater tendency to find NBs in rabid cattle that die spontaneously cattle when compared to those the were euthanized.

This study's viral evaluation indicated that for cattle infected with the genetic strain Desmodus rotundus (1B), variation in viral loads did not directly affect the intensity of the histological lesions.

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Livestock rabies in Pará state, Brazil: a descriptive study (2004 to 2013[1])

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ABSTRACT- Andrade E.A., Monteiro F.D.O., Solorio M.R., Raia V.A., Xavier D.A., Colino E., Guimarães R.J.P.S. & Abel I. 2020. **Livestock rabies in Pará state, Brazil: a descriptive study (2004 to 2013)**. *Pesquisa Veterinária Brasileira 40(4):234-241.* Laboratório de Epidemiologia e Geoprocessamento, Instituto de Medicina Veterinária, Universidade Federal do Pará, Campus Castanhal, BR-316 Km 61, Saudade II, Castanhal, PA 68740-970, Brazil. E-mail: isisabel@ufpa.br

Rabies is an important zoonosis to public health associated with lethal encephalitis and economic losses. Analysis of its spatial distribution is a meaningful tool in understanding its dispersion, which may contribute to the control and prophylaxis of the disease. This study analyzed the spatial-temporal distribution of rabies outbreaks in livestock in Pará state, Brazil, from 2004 to 2013. We used records of neurological syndromes obtained from the state's livestock authority (Adepará). The analysis recorded 711 neurological syndromes reports in livestock, of which 32.8% were positive for rabies. In 8% of the neurological syndromes (n=57) was not possible to perform the analysis because of bad-packaging conditions of the samples sent. Outbreaks involved at least 1,179 animals and cattle were the most affected animal species (76.8%). The numbers of reported neurological syndromes and of rabies outbreak shad strong positive correlation and exhibited decreasing linear trend. Spatially, most outbreaks occurred in two mesoregions in Pará (Northeast and Southeast). One of the justifications for this spatial distribution may be related with the distribution of the animals in the state, since these mesoregions are the largest cattle producers in Pará and have most of their territory deforested for pasture implementation.

INDEX TERMS: Rabies, surveillance, livestock, Eastern Amazon, Pará, Brazil, cattle.

RESUMO.- [**Raiva em herbívoros no estado do Pará, Brasil: estudo descritivo (2004 a 2013).**] A raiva é uma zoonose importante para a saúde pública associada à encefalite letal e às perdas econômicas. A análise de sua distribuição espacial é uma ferramenta importante no entendimento de sua dispersão, o que pode contribuir para o controle e a profilaxia da doença. Este estudo analisou a distribuição espaço-temporal do surto de raiva em rebanhos no estado do Pará, Brasil, entre 2004 e 2013. Foram utilizados registros de síndromes neurológicas obtidas junto à agência de defesa agropecuária do estado (Adepará). A análise revelou 711 notificações de síndromes neurológicas em herbívoros, das quais 32,8% foram positivas para raiva. Em 8% das síndromes neurológicas (n=57) não foi possível realizar as análises devido às más condições das amostras enviadas. Surtos envolveram pelo menos 1.179 animais e os bovinos foram a espécie animal mais afetada

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(76,8%). Os números de síndromes neurológicas relatadas e de surtos de raiva apresentam forte correlação positiva e exibem tendência linear decrescente. Espacialmente, a maioria dos surtos ocorreu em duas mesorregiões no Pará (Nordeste e Sudeste). Uma das justificativas para essa distribuição espacial pode estar relacionada à distribuição dos animais no estado, uma vez que essas mesorregiões são os maiores produtores de gado do Pará e possuem grande parte do seu território desflorestado para implantação de pastagens.

TERMOS DE INDEXAÇÃO: Raiva, vigilância, herbívoros, Amazônia Oriental, Pará, Brasil, bovinos.

INTRODUCTION

Rabies virus (RabV) belongs to the genus *Lyssavirus*, family Rhabdoviridae, and affects the central nervous system (CNS), causing fatal encephalitis. In majority countries, the rabies virus reservoirs are domestic and wild carnivores, although some Chiroptera species and other mammal species may also host the pathogen (Mollentze et al. 2014). The hematophagous bat species, *Desmodus rotundus*, is known to transmit rabies to livestock in Latin America, causing economic losses in the continent (Schneider et al. 2009). The abundance of *D. rotundus* in Brazil has been associated with deforestation and increase of bovine density. Deforestation reduces the availability of natural preys, increasing the chances of contact of RabV with humans. Although, there are few reports about RabV transmission to humans by handling of farm animals, these assume greater importance in the surveillance of viral circulation, being considered sentinels of disease outbreaks, alerting the circulation of the virus in a particular region (Wada et al. 2011).

Covering an area of approximately 1,248,000km^2 in the Amazon region, the Pará state, Brazil, has 144 municipalities distributed in 22 micro-regions and six mesoregions (IBGE 2015). The state has 21 million of farmed animals bred in the state and a diversified landscape that includes environmental protection areas, indigenous peoples' reserves, pastures, and agricultural zones that many times are all adjacent to forest fragments. From 1999 to 2004, rabies cases in livestock in the state occurred mainly in the mesoregion Southeastern Pará (Fernandes et al. 2013). According to Braga et al. (2014), mesoregions Northeastern and Southeastern Pará were considered areas of intermediate to high risk for rabies, where the landscape is favorable to the survival of the *D. rotundus*, since almost all municipalities are considered as receptive to the bat. Other environmental conditions, as proximity to highways and rivers, were cited as important in this scenario (Andrade et al. 2016). However, a study with more details, taking in consideration regional peculiarities is necessary to comprehend this distribution and to be an important tool to establish control strategies.

In Pará state, few studies about this topic were realized. A descriptive, temporal, and spatial investigation more detailed would prove its importance in the effort towards understanding rabies occurrence, making clear the reality of this disease in the region. Therefore, the present study aims to describe the spatial and temporal patterns of rabies distribution from 2004 to 2013 in livestock in Pará state, Brazil.

MATERIALS AND METHODS

This study used data obtained by the "Agência de Defesa Agropecuária do Estado do Pará" (Adepará) in two forms: Early Disease Investigation Report (FORM-IN) and Complementary Disease Investigation Report (FORM-COM). Adepará uses this report to record neurological syndromes in farmed animals and this study used reports registered from January 1, 2004, to December 31, 2013. The following variables were used: year, month, and municipality where outbreaks occurred, purpose of farming (dairy, beef or mixed), agent reporting of suspected case, affected species, productive system (technified or not) and farming activity. The analyses considered only the accuracy of the data provided, that is, unclearly stated information and boxes left blank in forms were not considered.

After every notification of a suspected neurological disease, Adepará agents collected fragments of the CNS of animals, including portions of the frontal cortex, cerebellum, and brainstem. Furthermore, according to recommendations of the "Ministério da Agricultura, Pecuária e Abastecimento" (MAPA), fragments of the spinal cord of equids were also collected (Brasil 2009). All material was kept under refrigeration for posterior analysis in the "Laboratório Nacional Agropecuário" (Lanagro/PA) from MAPA, in Belém, the capital city of Pará state. Diagnosis was conducted using direct immunofluorescence (DIF) and intracerebral inoculation of mice (IIM). According to Lanagro's diagnosis, cases were classified in three categories: inappropriate sample, positive for rabies or negative for rabies. Inappropriate samples were considered those in putrefaction stage, when received by Lanagro. According to Portaria nº 168/2005 from Animal Health Department (SDA), a notified neurological condition case is considered as rabies outbreak when at least one sample was positive during the tests realized.

A databank was created with Adepará's data in Microsoft Excel software to perform the linear regression analysis and temporal trends of the reports and of the rabies outbreaks. After that, Pearson's correlation coefficient (ρ) was calculated considering $p \leq 0.05$ as significant. Subsequently, these data were exported to the software SPSS v.20.0, to realize a descriptive statistical analysis of the variables selected. The Chi-square test was applied for that, if necessary, the odds ratio would be calculated. A 95% confidence interval was considered significant.

Mapping and spatial analysis were conducted in ArcGis 10.3, using a Geographical Databank and the Chart Encyclopedia published by the "Instituto Brasileiro de Geografia e Estatística" (IBGE). The layers representing municipality, hydrography, animal density, indigenous areas, sustainable use areas and integral conservation areas were superimposed on the distribution map of rabies outbreaks.

RESULTS

During the study period 711 notifications of neurological syndromes were recorded in 101 municipalities of Pará state. Of total, 233 reports (32.8%) were positive to rabies and for 8% of the notifications, the final diagnosis was inconclusive. Only 633 reports were completely filled and 684 reports contained information about the origin of notification (defense agency). Positive cases were reported by farmers, private veterinarians and Adepará (28.4%, 29.8% and 56.7%, respectively) (Table 1).

In total, 730 samples were collected, once in some properties fragments were collected in more than one animal, as recommended by Portaria nº 168/2015. So, rabies outbreaks affected at least 1,179 animals. Moreover, 65% of

the rabies outbreaks occurred in animals that integrate the main activity of the farm. Regarding productive system, 78,6% of properties showed non-technified and 57% recorded beef cattle, followed by mixed production animals (33.3%), and dairy cattle (7.8%). The results obtained did not reveal any correlation between the variables associated with farming activity and the results of rabies tests ($p>0.05$).

The majority positive rabies cases were registered in cattle (182, 76.8%), followed by equids (44, 18.6%), bubalines (5, 2.1%), and pigs (4, 1.7%). Goats and sheep accounted one positive case each (0.4%).

During the study period, the number of reported neurological syndromes in livestock showed a declining trend, with fewer 5.01 cases notified per year. In the same way, the positive cases for rabies decreased in 2.6 cases per year, showing a strong positive correlation between these two variables ($\rho=0.81$) (Fig.1). Furthermore, rabies cases occurred mainly in March and July, 16.0% and 13.5%, respectively, showing no seasonal trend.

The spatial analysis showed that rabies outbreaks incidence during the studied period were distributed in 59 of the 101 municipalities wherein a neurological syndrome was reported, and 52.4% of rabies outbreaks were concentrated in nine municipalities, mainly in Northeast and Southeast mesoregions. However, the municipalities with the most animal density did not show the highest incidence for rabies (Fig.2).

Table1. Source of notification from fully filled reports

All notifications (711)	n	%	Positive (%)
Completely filled	633	89	-
Origin of the notification	684	96.2	-
Farmers	457	70.5	28.4
Private veterinarians	124	19.3	29.8
Agents of Adepará	67	10.2	56.7

The outbreaks were distributed along the main roads and/or evident deforestation areas, as observed for Transamazonian Highway and in Northeast mesoregion of state (Fig.3). There were no cases in Marajó mesoregion. Furthermore, other silent regions were recognized as indigenous territory or as conservation areas, although cases were registered surrounding these localities (Fig.4).

DISCUSSION

The present study applied spatial and temporal trend analysis to comprehend livestock rabies distribution during 10 years of surveillance observation in Pará state in Amazon region.

In general, the notification of outbreaks registered was done principally by the farm owners, corroborating with the study developed in Paraíba state, Brazil (Andrade et al. 2014). According to results, Pará's surveillance strategy for livestock was characterized as predominantly passive. The higher number of reports done by farmers may be related to the fact that two human's rabies outbreaks were recorded in 2004 and 2005, registering 39 died people by hematophagous bats aggression. Of these deaths, 22 occurred in 2004, in Portel municipality (Marajó mesoregion) and Viseu municipality (Northeast mesoregion). The others 17 deaths were recorded in 2005, in Augusto Corrêa municipality (Northeast mesoregion) (Brasil 2004, 2015). Possibly, the news of rabies cases in the state broadcast by the media promoted awareness and, in turn, influenced the numbers of reports by farmers, registering the highest numbers from 2005 to 2008, with 59.7% of the cases notified during the whole study period. This result demonstrated the important role of awareness campaigns to maintain the adequate level of watchfulness across farmers.

All CNS samples of rabies suspected animals in the period considered in the present study (n=730) were analyzed. In some farms, CNS samples were collected from more than

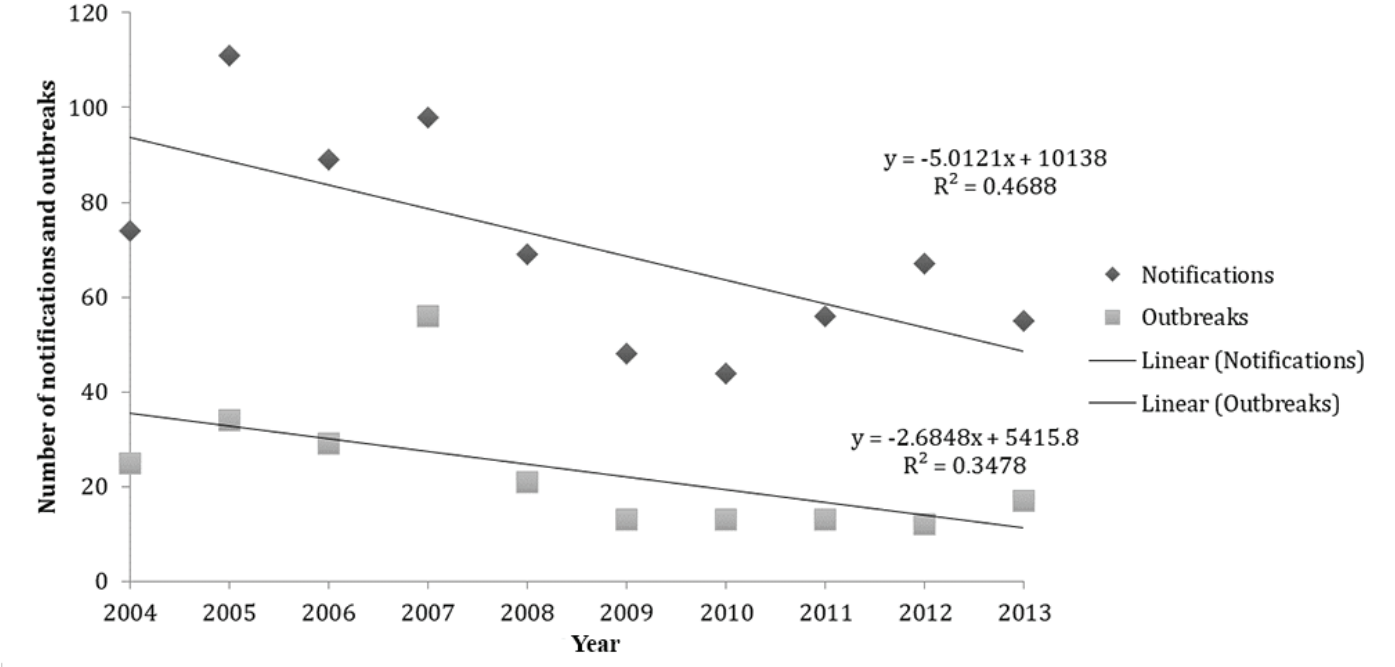


Fig.1. Distribution of notified cases of neurological syndromes in livestock in the state of Pará, Brazil, and rabies outbreaks from 2004 to 2013.

one animal. Of this number, 8% (n=57) were considered inappropriate for analysis. Possibly, some logistic problems as transportation of refrigerated samples, farms localization and transportation duration could be related with this problem. Importantly, in addition to problems in highway infrastructure, the extensive territorial size and the diverse rivers that flow across the state are obstacles for maintaining the appropriate condition of a sample. In some cases, the samples collected were more than 800km of distance from Belém, where the analyses were realized. More than 50% of these inappropriately preserved samples were transported by barges (which is a rather slow transportation), moreover,

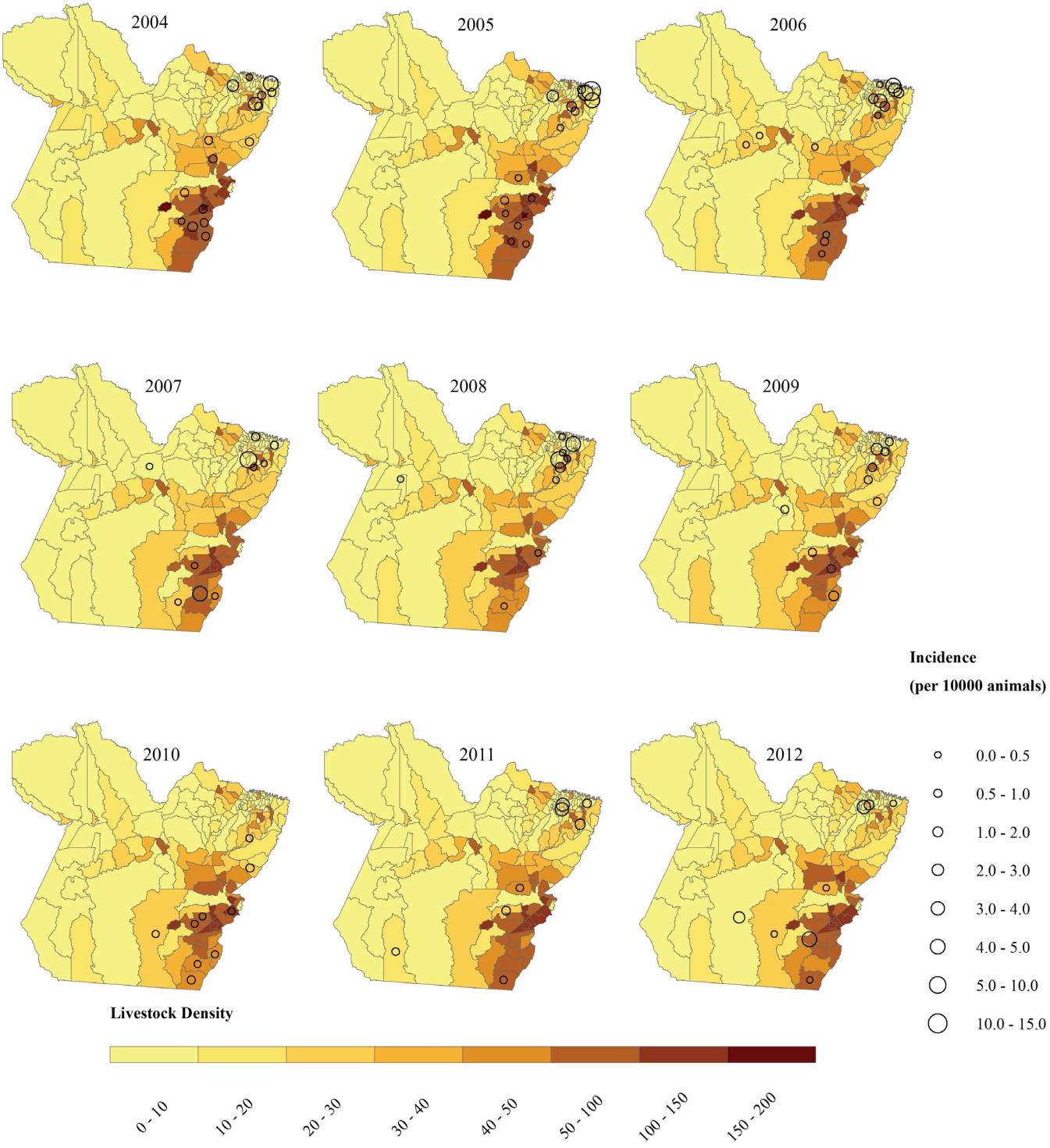


Fig.2. Annual maps of the incidence of rabies cases (cases per 10,000) and average density of livestock animals according to municipalities in the state of Pará from 2004 to 2012.

the climatic conditions of the region can also contribute to samples deterioration. In this way, histopathological and immunohistochemical methods should be considered as complementary techniques to detect rabies in large regions like Pará state. These methods present good correlation with DIF findings, and may improve the possibility of preserving biological samples for RabV analysis. These methods may be conserved in formalin, by passing the logistic difficulties discussed (Abreu et al. 2012).

Once rabies outbreaks were confirmed, our attention should be directed to the role of these animals in farm activity. In 65% of the rabies outbreaks, the animals of the main activity in the given farm were more affected. It leads to a reflection about economic impacts of losing these animals to the disease, in specifically for small farms, since 78.6% of the outbreaks identified during the studied period were rural properties without technician production systems in place, adopting extensive cattle farming techniques.

Although we did not detect any association between rabies results and the variables describing production systems (farming activity, technification of farming, purpose of farming) ($p>0.05$), the way cattle farming has been conducted in Pará state in the last decades showed suitable conditions for reproduction of hematophagous bat populations in these farms. This may be explained because of livestock farming takes place in areas that vary in terms of receptivity, and are surrounded by permanent forest fragments (Braga et al. 2014).

The pattern of distribution of rabies cases observed, considering the species affected by the disease, was described in other regions of the country (Wada et al. 2011, Lopes et al. 2015). The main nourishment source for *Desmodus rotundus*, especially, is the pig's blood (Bobrowiec et al. 2015). However, pig farming is not a significant economic activity in Pará state, explaining the low percent number of samples positive for rabies from these farmed animals.

Although cattle breeding is the main activity in Pará state, presenting areas with high bovine density, Braga et al. (2014) showed that there is not association between bovine density and hematophagous bat distribution. In the same way, authors believe that there are not associations between bovine density and rabies cases that occurred in these animals. Andrade et al. (2016) showed analysis that oppose those data, observing that high-risk areas for bovine rabies were related to large herds cattle in Pará state. Nevertheless, in an observation of ten years, this study showed no evidences that rabies cases occur where there is a high livestock density. It seems that bats prefer cattle blood even when wild animals are present in the same environment, possibly because cattle become easy prey due to confinement restrictions (Voigt & Kelm 2006).

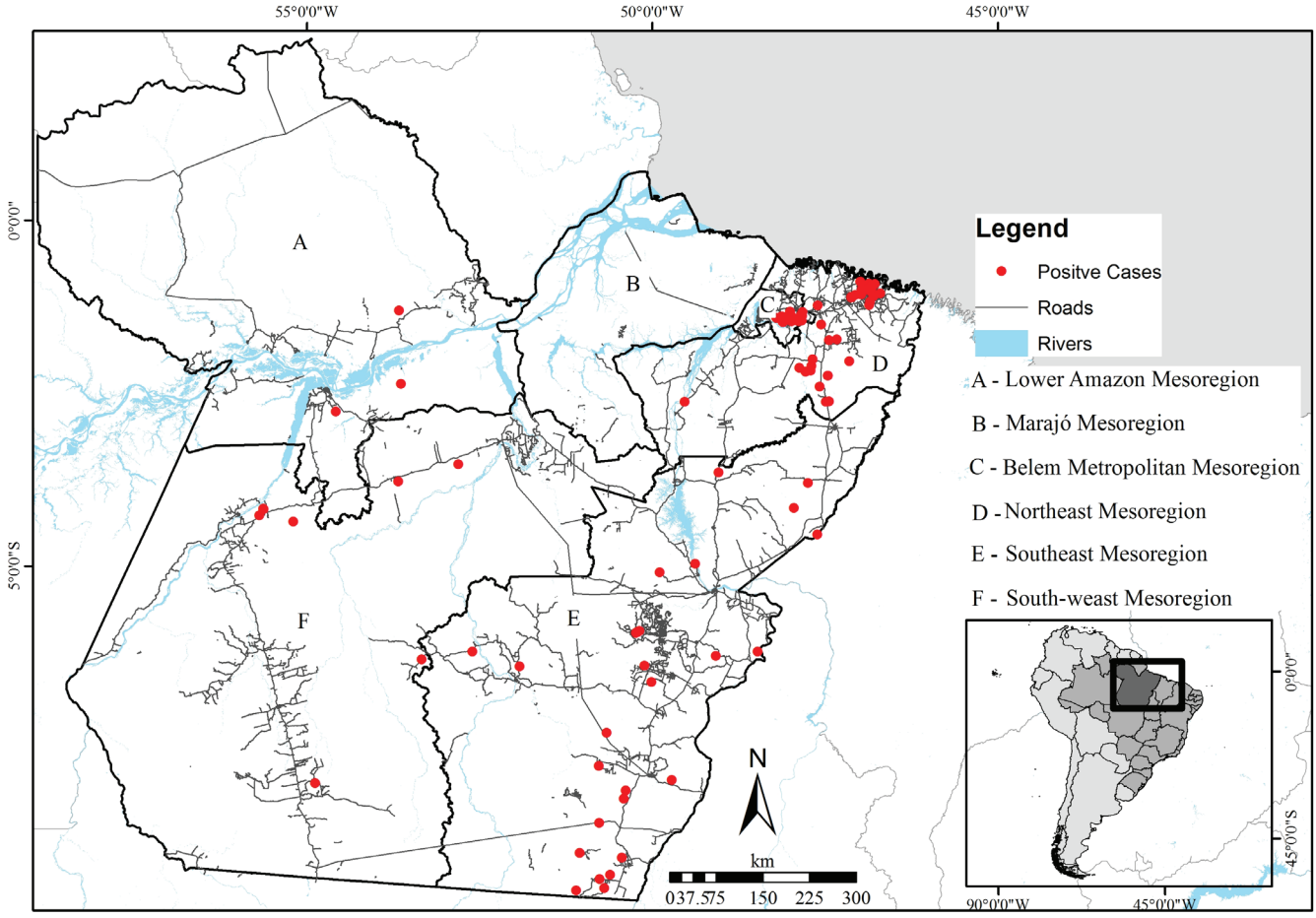


Fig.3. Livestock rabies cases, rivers and roads distribution in the state of Pará from 2004 to 2013.

Equids were the second species affected by rabies during the investigation period, showing 18.6% of cases (44/237), corroborant with other studies carried out in the region (Casseb et al. 2006, Póvoas et al. 2012). However, proximity 50% of the notified cases of neurological syndromes in equids were positive for rabies (47%). Compared with equids, the most preventive measures are directed to cattle due to the greater economic importance of bovine herds. The data obtained in this study point to the need for more attention to equids in rabies surveillance programs.

In Brazil, 50% of the bubaline herds are reared in Pará state. Despite that, just 2.9% of notified rabies cases affected this species. It is believed that the physiological and behavioral traits of buffaloes, besides the use of extensive farming methods in farms with no fences, especially in the Marajó mesoregion, difficult that the bats feed on these herds and the geographic features of the Marajó island also difficult the Adepará to inspect these animals.

Most rabies outbreaks were reported in mesoregions Northeastern and Southeastern Pará, with some cases registered in Southwestern Pará, classifying these areas as posing intermediate to high risk for rabies (Braga et al. 2014). Andrade et al. (2016) showed that rabies cases in bovines, especially in Pará may be associated with the expansion of cattle farming in the state, reflecting in the increment of deforestation areas. These areas have been deforested in the last decades with highly anthropic actions and they are included in the Eastern Amazon Arc of deforestation (Domingues & Bermann 2012).

Marajó mesoregion was characterized as a silent area. This region does not present roads to provide access to farms where rabies cases may occur. Other geographical characteristics is the presence of large rivers, acting as barriers to surveillance and playing an important role in rabies transmission (Mungrue & Mahabir 2011). Possibly, the absence of positive cases may be related with the difficulties in transport, reflecting in register of notifications. The possibility of rabies occur in these areas in a silent way may not be discharged, once animals are bred extensively without limits between properties, offering favorable conditions to bat attacks.

Conservation areas or indigenous reserves are, possibly, other silent areas observed in this study. These areas are located along the borders with the neighboring Amazonas, Amapá and Mato Grosso states and were classified as low risk areas for human and bovine rabies by Andrade et al. (2016). The surveillance actions are limited in these areas, especially in indigenous areas, that are under the jurisdiction of Brazilian governmental protection agency for Indian interests and their culture, the "Fundação Nacional do Índio" (FUNAI). This agency is the responsible to authorize the entry of surveillance

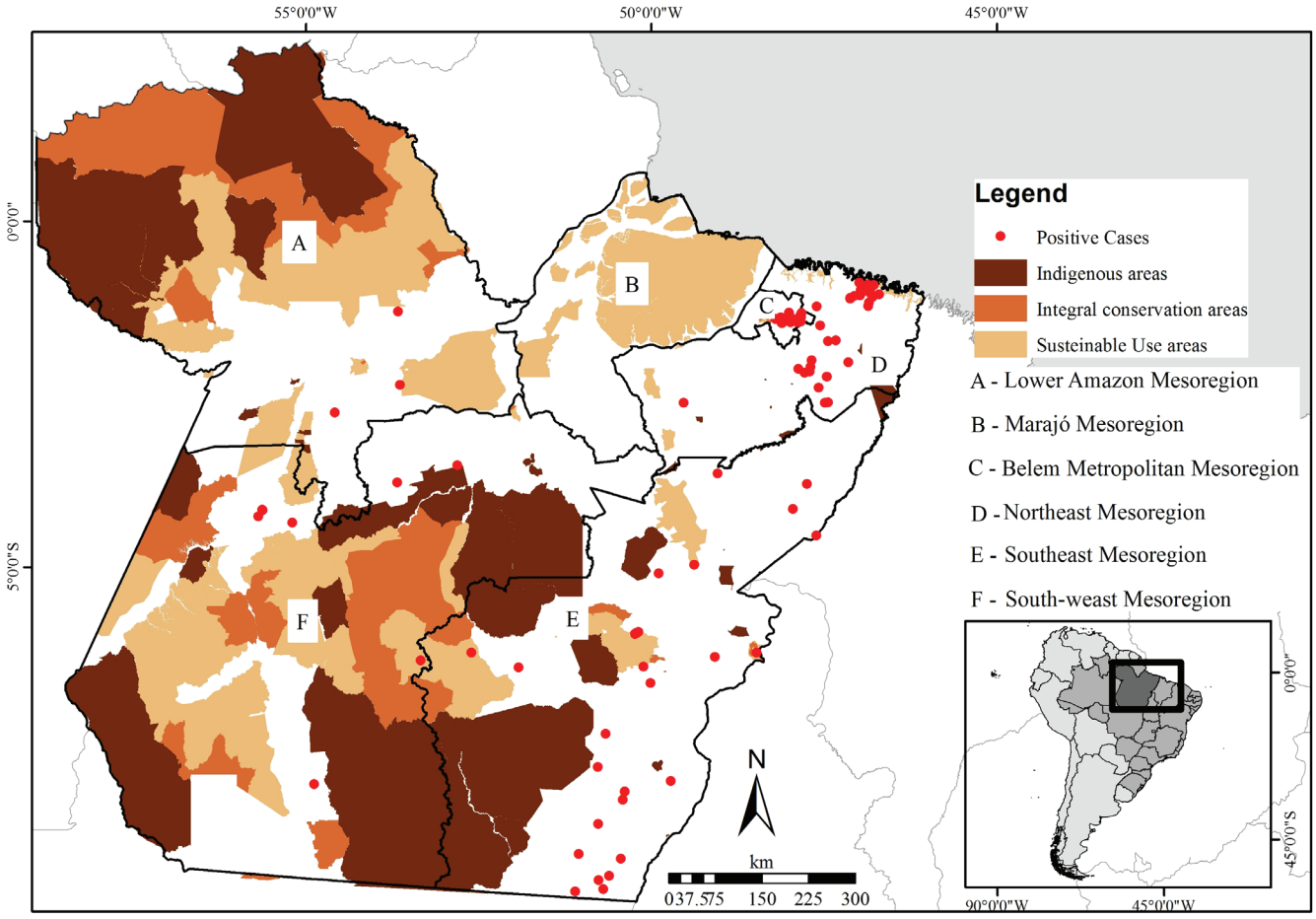


Fig.4. Livestock rabies cases and conservations areas in the state of Pará from 2004 to 2013.

services. Rabies cases were observed in deforested areas next to indigenous and conservation reserves, probably, because lose shelter and primarily food source. In this scenario, the conservation and indigenous areas show favorable conditions for installation of bats (Dias et al. 2011). This may explain areas with sporadic rabies cases in this study, although without description in forest areas (Mungrue & Mahabir 2011).

The positive correlation between notified cases and outbreaks indicates the importance of report in the identification of cases. Furthermore, the data demonstrate that cases follow a decreasing trend. However, in fact, notifications are decreasing in the studied period. Added for this, probably the sanctioning of the 'Technical Guide for the Control of Rabies in Herbivores' in 2005 may also have influenced in these results, affording to adopt standardized measures to control the disease in Brazil (Lopes et al. 2015). The present investigation revealed that the most cases were notified by breeders and veterinarians. Therefore, sanitary education initiatives should be directed for these people, searching to improve surveillance measures.

The data analyzed showed that most rabies cases were reported in March and June. Pará state is located in Eastern Amazon and its location experiment a complex climate influenced by the large rivers and the heterogeneous vegetation. The pluviometric indices varies from 1000 to 4500 mm along the region. The state registers two season: the rainy season, known as amazon winter (from December to June) and the less rainy season, called amazon summer (from July to November). In this study, seasonality and rabies incidence in cattle did not show correlation. However, some researchers suggest that depending on the climate conditions bats change roosting sites and reorganize their colonies, explaining rabies outbreaks during the cold, rainy seasons (Gomes et al. 2011).

The results obtained may explain that even under adverse conditions, the bats exhibit a considerable adaptation skill for both natural and artificial shelters, especially in the Amazon region, where there is a wide variety of natural habitats, providing shelter during all year round. This casts doubts as to the actual relationship between seasonality and bat migration habits (Gomes & Uieda 2004). In fact, more studies are necessary to shed more light on these aspects, especially the population dynamic of *D. rotundus* in Pará and its relationship with rabies outbreaks in livestock at given times during the year, considering regional peculiarities.

CONCLUSIONS

The present study confirmed that rabies is endemic and does not present seasonality in Pará state, showing how Northeastern and Southeastern mesoregions stand out with the highest rabies cases in the region. However, the municipalities with higher animal density did not show the highest rabies incidence. Furthermore, the spatial dynamics of the disease accompanies areas where there are more roads.

The roads absence associated with the presence of large rivers may difficult surveillance in the more distant properties.

An important fact is the need for improved surveillance measures against rabies in equines.

Although, the parameters analyzed and positive rabies cases did not show any association, it was possible to observe that other diagnosis tools are required to consider regional peculiarities in rabies dynamic. Similarly, educational initiatives to explain the role of livestock owners in the reporting of neurological diseases in livestock should be implemented as part of a wider surveillance strategy in Pará state, in the Brazilian Amazon.

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Epidemiological and pathological aspects of noninfectious diseases of the gastrointestinal tract in 114 horses in Southern Brazil[1]

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ABSTRACT.- Bianchi M.V., Ribeiro P.R., Stolf A.S., Bertolini M., Laisse C.J.M., Sonne L., Driemeier D. & Pavarini S.P. 2020. **Epidemiological and pathological aspects of noninfectious diseases of the gastrointestinal tract in 114 horses in Southern Brazil.** *Pesquisa Veterinária Brasileira 40(4):242-253.* 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

Equine colic is one of the most common cause of death in horses, but few studies have investigated specifically the conditions at the necropsy. This study aimed to describe the epidemiological and pathological features of noninfectious diseases of the gastrointestinal tract in horses. A retrospective study was conducted in search of cases of these diseases affecting horses from 2005 to 2017. During this period, 114 horses died of noninfectious diseases of the gastrointestinal tract, and the main causes were: primary gastric dilation (27/114), volvulus (27/114), enterolithiasis (20/114), rectal (colonic) perforation (15/114), gastric or cecocolonic impaction (10/114), incarcerations (6/114), intussusception (4/114), and others (5/114). Mixed breeds horses (56/114) and males (69/114) were mostly affected. The horses had a median and mean age of 10 and 10.9-years old, respectively. Primary gastric dilation was characterized by distension of the stomach by moderate to large amounts of content, which in 21 cases caused tearing of the stomach wall at the greater curvature (peritonitis), and the main predisposing factor was alimentary overload (17/27). Intestinal volvulus occurred within the small intestine (14 cases) and within the large intestines (13 cases). Grossly, there was intestinal ischemia with reddened to deep-black serosa and diffusely red mucosae. Enterolithiasis caused partial or complete obstruction of the right dorsal colon (9/20), transverse colon (4/20), small colon and right dorsal colon (3/20), rectum and right dorsal colon (2/20), and small colon (2/20). Viscera perforation and peritonitis occurred in 11 cases. Rectal (colonic) perforation involved the rectum (10/15), rectum/small colon (4/15), and the small colon (1/15). It was characterized by a focally extensive transmural tearing, associated with reddened borders, and retroperitoneal to diffuse peritonitis. Palpation related iatrogenic injuries (11/15) were the main cause. Impactions affected the large colon (7/10), the cecum (2/10), and the stomach (1/10). Incarcerations consisted of inguinoscrotal hernias (2/6), small intestine entrapment by a mesenteric failure (2/6), diaphragmatic hernia, and umbilical eventration. Grossly, the organs were constricted by a hernial ring, with intestinal ischemia and reddened to dark-red serosa. Predisposing factors included previous surgeries (2/6) and patent inguinal ring (1/6). Intussusception involved the small intestine (3/4) and ileocecum (1/4). Foals with lack of colostrum intake and concomitant pneumonia was a characteristic presentation (3/4). Grossly, the intussusceptum slipped into intussuscipiens, with diffuse deep black-red discoloration. Other causes included large

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colon displacement (2/5), extrinsic and intrinsic obstruction of the small intestine (2/5), and an intestinal adenocarcinoma. Noninfectious gastrointestinal diseases are major causes of death in horses. Epidemiological and gross features of the conditions should be accounted to obtain a final diagnosis of the cause of the colic.

INDEX TERMS: Epidemiology, pathology, noninfectious diseases, gastrointestinal tract, horses, Brazil, colic, gastric dilation, volvulus, enterolithiasis, rectal perforation, impaction, incarceration, intussusception.

RESUMO.- [**Aspectos epidemiológicos e patológicos de doenças não infecciosas do trato gastrointestinal de 114 equinos no sul do Brasil.**] A cólica em equinos é considerada como a principal causa de morte de cavalos, porém poucos estudos têm investigado especificamente as condições envolvidas através da necropsia. O objetivo desse estudo foi descrever os aspectos epidemiológicos e patológicos de doenças não infecciosas do trato gastrointestinal de equinos. Foi conduzido um estudo retrospectivo em busca de casos dessas doenças envolvendo cavalos de 2005 a 2017. Durante esse período, 114 equinos morreram devido a doenças não infecciosas do trato gastrointestinal, e as principais causas foram: dilatação gástrica (27/114), vólvulos (27/114), enterolitíase (20/114), ruptura retal (colônica) (15/114), compactação gástrica ou cecocolônica (10/114), encarceramentos (6/114), intussuscepções (4/114), e outros (5/114). Animais sem raça definida (56/114) e machos (69/114) foram mais afetados. Os equinos apresentavam uma média e mediana de idade de 10 e 10,9 anos, respectivamente. A dilatação gástrica primária era caracterizada por distensão do estômago por moderada a grande quantidade de conteúdo, que em 21 casos provocava ruptura da parede gástrica na curvatura maior (peritonite), e o principal fator predisponente foi sobrecarga alimentar (17/27). Vólvulo intestinal ocorreu no intestino delgado e no intestino grosso (14 e 13 casos, respectivamente). Macroscopicamente, havia isquemia intestinal com serosa avermelhada a enegrecida e mucosa difusamente avermelhada. A enterolitíase causou obstrução parcial ou completa do cólon dorsal direito (9/20), cólon transverso (4/20), cólon menor e cólon dorsal direito (3/20), cólon menor (2/20), e reto e cólon maior direito (2/20). Perfuração de vísceras e peritonite foram observadas em 11 casos. A ruptura retal (colônica) envolveu o reto (10/15), reto/cólon menor (4/15) e cólon menor (1/15). Essa era caracterizada por ruptura transmural focalmente extensa, com bordos avermelhados e peritonite retroperitoneal a difusa. Traumas relacionados à palpação (11/15) foram a principal causa. Compactações afetaram o cólon maior (7/10), ceco (2/10) e estômago (1/10). Encarceramentos consistiram em hérnias inguinoescrotais (2/6), encarceramento de alças intestinais por falha no mesentério (2/6), hérnia diafragmática e eventração umbilical. Macroscopicamente, os órgãos estavam constritos por um anel hernial, com isquemia intestinal e serosa avermelhada a vermelho-escura. Fatores predisponentes incluíram cirurgias prévias (2/6) e anel inguinal patente (1/6). Intussuscepções envolveram o intestino delgado (3/4) e íleoceco (1/4). A apresentação característica foi em potros com falta de colostro e concomitante pneumonia. Macroscopicamente, o intussuscepto deslizava em direção ao intussuscepiente, exibindo coloração vermelho-enegrecida difusa. Outras causas incluíram deslocamento de cólon maior (2/5), obstrução extrínseca e intrínseca do intestino delgado (2/5), e um caso de adenocarcinoma intestinal. Doenças não infecciosas são importantes causas de morte em equinos. Os aspectos epidemiológicos e macroscópicos das condições devem ser considerados para o que o diagnóstico final da causa da cólica seja obtido.

TERMOS DE INDEXAÇÃO: Epidemiologia, patologia, doenças não infecciosas, trato gastrointestinal, equinos, Brasil, cólica, dilatação gástrica, vólvulo, enterolitíase, ruptura retal, compactação, encarceramento, intussuscepção.

INTRODUCTION

Horses are monogastric grazers, with a gastrointestinal tract that may be divided into three segments: foregut (stomach and esophagus), midgut (small intestine), and hindgut (large colon and cecum). The foregut and midgut receive continuous amounts of food that finally reach the hindgut, which is, essentially, a fermentation chamber. However, modern management practices have replaced the horse's natural grazing habit with a diet high in grains and fats, which may not be properly digested (Bland 2016). Colic is an acute gastrointestinal disease characterized by a clinical syndrome of abdominal pain in horses, which can be infectious or noninfectious (Tinker et al. 1997). Almost 10 to 11% of horses will present clinical signs of colic at some point in their lives, and mortality rates are usually high (Bland 2016). Surgical intervention is sometimes necessary to treat colic in horses when a noninfectious cause is defined, but this is expensive, extremely invasive, and has a relatively high postoperative mortality rate (Immonen et al. 2017, Mair & Smith 2005a, Proudman et al. 2002).

Colic has been considered the most common cause of death in horses, accounting for at least 28% of deaths (Tinker et al. 1997). Numerous studies have described the alimentary system as the most commonly involved in the cause of death of horses, followed by the locomotor, nervous, cardiovascular, and respiratory systems (Baker & Ellis 1981, Pierezan et al. 2009, USDA 2017). Most studies have listed the noninfectious diseases of the alimentary tract in horses (Baker & Ellis 1981, Pierezan et al. 2009, Marcolongo-Pereira et al. 2014), but few have specifically investigated its conditions, focusing on the epidemiological and pathological features. Nonetheless, nonspecific colic cases (unknown causes) are often clinically referred to as spasmodic, flatulent, or mild colic, especially because of lack of proper surgery or necropsy (Tinker et al. 1997).

The knowledge of common causes and outcomes of equine colic may aid veterinarians to consider appropriate diagnostic methods, therapeutics, and determine the prognosis for the individual horse (Voigt et al. 2009). Furthermore, equine colic might have a substantial economic impact, as sport or reproductive horses are becoming more valuable, and insurance expenses have increased. In 2001, by instance, the United States horse insurance industry paid over US$ 115

million in claims due to horse's deaths (Bland 2016). With current trends of insuring horses in Brazil, this knowledge is becoming increasingly important, as insurance companies may need detailed necropsy reports into the cause of death in order to reimburse the horse owner (Immonen et al. 2017). Therefore, this study aimed to describe the epidemiological and pathological features of noninfectious diseases affecting the gastrointestinal tract in 114 horses necropsied in Southern Brazil.

MATERIALS AND METHODS

A retrospective study of noninfectious diseases affecting the gastrointestinal tract of horses was conducted at the necropsy database of the veterinary pathology laboratory from January 2005 to December 2017. All horses were from properties located in the state of Rio Grande do Sul, especially in the metropolitan region of Porto Alegre. The necropsy protocols were reviewed, and information regarding the cause of death, predisposing factors (one or multiple per horse), location of lesions, breed, age (age range, mean and median age), and sex were registered. Gross lesions were reevaluated through photographs and combined with the previous descriptions available on the necropsy reports. The cases were grouped into categories: primary gastric dilation, volvulus, enterolithiasis, rectal (colonic) perforation, gastric or cecocolonic impaction, incarceration, and intussusception. Displacements, extrinsic obstruction, intrinsic obstruction, and neoplasm did not fit into any of the above categories, and, thus, were listed as other causes. Each category was later evaluated in terms of secondary gastrointestinal abnormalities, such as ruptures, peritonitis, gastric dilation, and impactions. Additionally, enteroliths were classified according to location, aspect, distribution (single or multiple), and composition of the central *nidus* (foreign body that originated the enterolith). Furthermore, the causes of rectal (colonic) perforation were characterized as iatrogenic (reproductive tract or colic related palpation) or natural mating. As gross lesions were characteristic of many of these categories, histological features (stained with hematoxylin and eosin, HE) were reevaluated only for the neoplasm.

RESULTS

From January 2005 to December 2017, 654 horses were necropsied in the veterinary pathology laboratory. The alimentary system was related to the cause of death in 201 horses, of which 114 (56.7%) were associated with noninfectious diseases of the gastrointestinal tract. These included mainly gastric dilation (27 cases), volvulus of the small or large intestines (27 cases), enterolithiasis (20 cases), rectal (colonic) perforation (15 cases), gastric or cecocolonic impaction (10 cases), incarcerations or hernias (six cases), intussusception (four cases), and other causes (five cases). Mixed breed horses were mostly affected (56/114), followed by Criollo breed (32/114), Ponies (6/114), Quarter Horse (5/114), Brazilian Sport Horse or "Brasileiro de Hipismo" (5/114), Thoroughbred (5/114), American Trotter (2/114), and Appaloosa, Dutch Warmblood, and Brazilian Saddle Horse or "Mangalarga Marchador" with one case each. Males were more affected (69/114) than females (45/114). The horses had a median age of 10-years old and a mean age of 10.9-years old, with an age range of 3-days old to 35-years old. The frequency of diagnosis, as well as information regarding the sex, age range, mean and median age of the affected animals, according to each category, is presented in Table 1.

Gastric dilation

Grossly, the stomach was distended by moderate to large amounts of liquid and, often, fetid content, which in 21 cases caused a tearing of the stomach wall at the greater curvature. This led to stomach rupture, which was characterized by small to large amounts of feed content released into the abdominal cavity causing peritonitis (Fig.1A-B). In addition, the edges of the rupture were reddened, while frequently the serosa was lacerated, with adjacent hemorrhages, which dissected the muscular layer (Fig.1C). The main predisposing factor related to this condition was alimentary overload (17/27) by corn (10/17), grains (3/17), or both (4/17). Other lesions frequently observed in these horses were gastric ulcers (8/27) and poor dentition (5/27).

Intestinal volvulus

Intestinal volvulus was diagnosed in 27 horses, and the small intestine (mainly jejunum and ileum) and large intestine were involved in 14 and 13 cases, respectively. Of these 13 cases, the entire large colon and cecum were rotated in five cases, the large colon in three cases, the left dorsal and ventral colon in three cases, and only the cecum in two cases. Grossly, independently of the affected tissue, there was intestinal ischemia with reddened to deep black-red serosa of the compromised portion (Fig.1D-E), as well as moderate to severe luminal distension and diffusely red mucosae (Fig.1F). The small intestinal contents were reddish and liquid (14/14),

Table 1. Frequency of diagnosis of noninfectious diseases of the gastrointestinal tract in horses, as well as information regarding the sex, age range, mean and median age of the affected animals

Categories	Frequency	Sex (M:F)	Age range	Mean/median age (y)
Gastric dilation	23.7%	20:7	3 m - 26 y	10.6/11
Volvulus	23.7%	13:14	1.5 m - 35 y	9/8
Enterolithiasis	17.5%	13:7	5 y - 18 y	10.3/10
Rectal (colonic) perforation	13.2%	6:9	2 m - 20 y	10.5/12
Impactions	8.8%	6:4	2 y - 25 y	11.1/10
Incarcerations or hernias	5.3%	5:1	5 y - 15 y	11/12
Intussusception	3.5%	4:0	3 d - 8 y	2/0.04

M = male, F = female, d = days old, y = years old, m = months old.

while the large intestinal contents were frequently dry and impacted (5/13). Other secondary gastrointestinal changes included secondary gastric impaction in three cases, and ruptures with peritonitis in two cases. In 11 cases, single or multiple predisposing factors per horse were identified: poor dentition in four cases, previous hospitalization/surgery in three, alimentary overload in three, feed changes in two, and recurrent colic in one case.

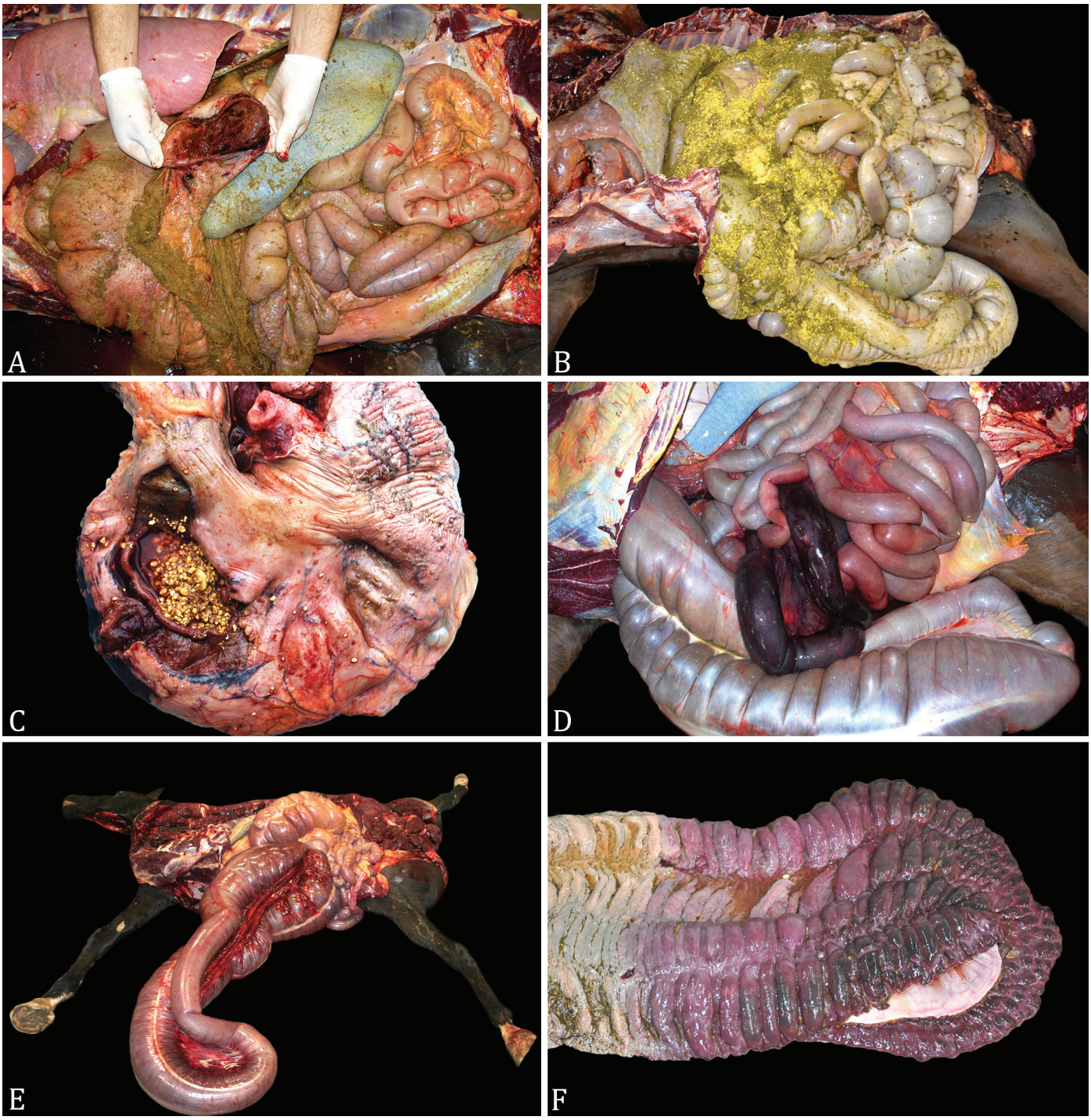


Fig.1. Gastric dilation and intestinal volvulus in horses in Southern Brazil. (**A**-**B**) Gastric dilation. Rupture of the stomach wall at the greater curvature with reddened edges and release of small to large amounts of feed content into the abdominal cavity, causing peritonitis. (**C**) The serosa of the stomach at the greater curvature was lacerated, with adjacent hemorrhages, and the muscular layer was dissected. (**D**) Intestinal volvulus. A segment of the small intestine twisted around its mesenteric axis, which caused intestinal ischemia, with deep black-red serosa of this segment. (**E**) The large colon had a 360° twist (volvulus) which caused complete intestinal ischemia with diffusely dark-red serosa. (**F**) The left large colon (both ventral and dorsal) mucosa was diffusely reddish due to left large colon volvulus.

Enterolithiasis

Enterolithiasis was the cause of death in 20 horses, leading to partial or complete intestinal obstruction in all cases. Enterolithiasis was followed by visceral perforation and peritonitis (11/20), as well as cecocolic impaction (6/20), and secondary gastric dilation (5/20). The right dorsal colon was the most affected segment (nine cases) (Fig.2A), followed by the transverse colon in four cases (Fig.2B), small

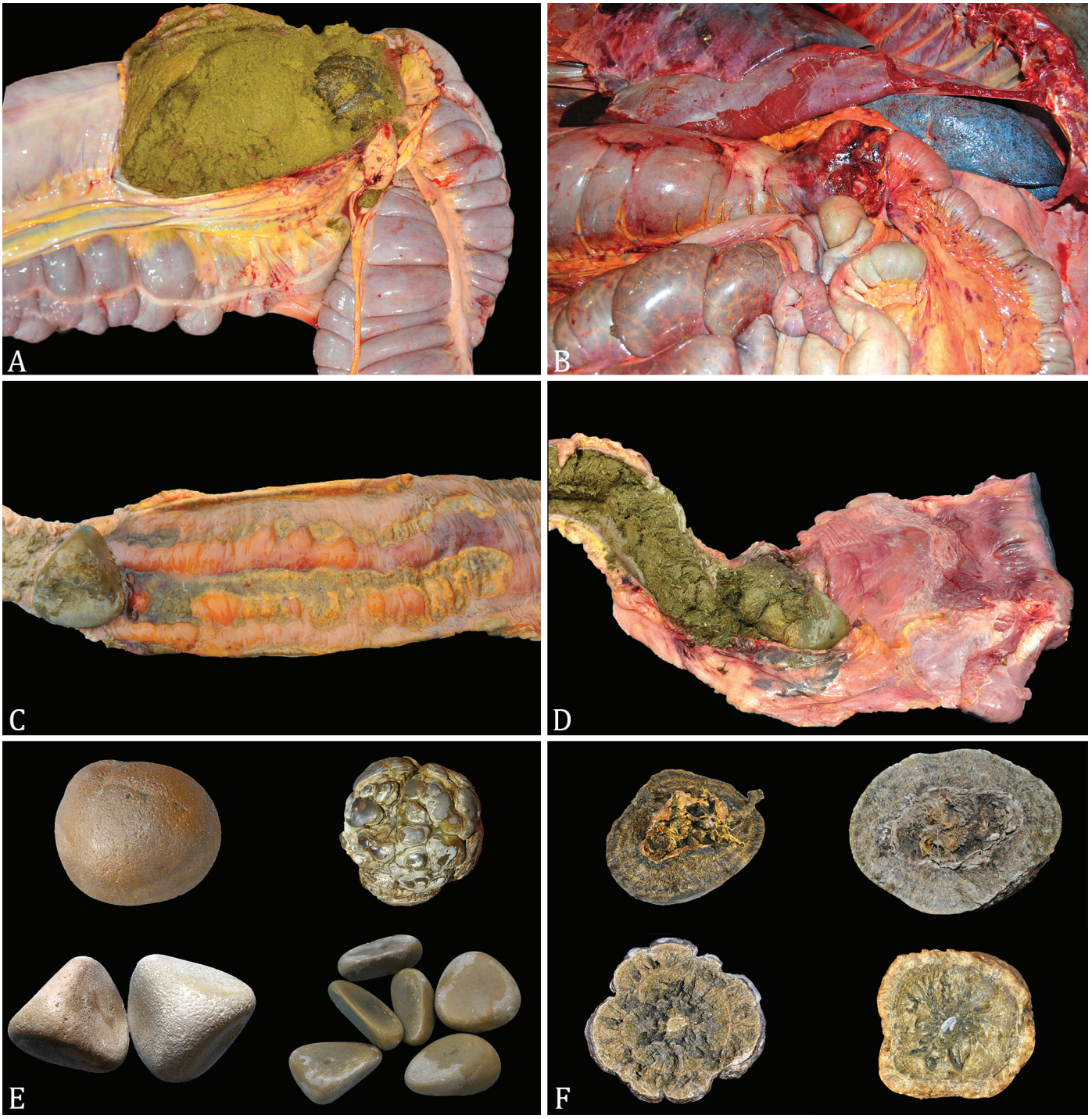


Fig. 2. Enterolithiasis in horses in Southern Brazil. (**A**) A rounded enterolith partially obstructed the lumen of the right dorsal large colon, and led to secondary anterior large colon impaction. (**B**) The transverse colon was another common location for enteroliths, as observed in this case, where it completely obstructed the lumen of the organ. (**C**) This pyramidal to flattened enterolith obstructed the final segment of the small colon, as well as it caused a severe focally extensive ulcer at the mucosa while it was moved by the guts. (**D**) This flattened enterolith was located at the rectum, where it caused a complete obstruction, in addition to secondary anterior impaction. (**E**) Clockwise, beginning at the top left corner, concretions were rounded, irregular, and flattened to pyramidal. (**F**) Clockwise, beginning at the top left corner, the enteroliths had concentric layers centered on rope (two cases), wire, and stone.

colon and right dorsal colon simultaneously in three cases (perforation of the small colon was the cause of death), small colon in two cases (Fig.2C), and rectum and right dorsal colon simultaneously in two cases (rectal perforation was the cause of death; Fig 2D). Enteroliths varied in size, being smaller than 10 cm in diameter (11/20) or larger than 10.1cm in diameter (9/20). These concretions were mostly single (11/20), but also multiple (9/20) per horse, with varying shapes (Fig.2E): rounded (13), irregular (7), pyramidal to flattened (4), and elongated (1). On the cut surface, some enteroliths exhibited concentric layers centered on a *nidus*: rope in seven cases, stone in two cases, and wire in one case (Fig.2F).

Rectal (colonic) perforation

Rectal (colonic) perforation was diagnosed in 15 cases, with involvement of the rectum in ten cases, transition between rectum and small colon in four cases, and small colon in one case. Grossly, these organs had focally extensive transmural tearing, associated with reddened borders, and retroperitoneal to diffuse peritonitis (Fig.3A-B). Iatrogenic injuries were the most common (11/15) causes, with colic related palpation in six cases (males and females) and reproductive tract palpation in five cases (only females), followed by one case of perforation that occurred after natural mating in a mare. In three cases, the clinical history was insufficient to establish a possible cause for the perforation.

Impactions

Impactions were diagnosed in ten cases, of which seven affected the large colon, two affected the cecum, and one affected the stomach. Grossly, cecocolonic impactions were caused by large amounts of impacted and dry content (6/9; Fig.3C), variable amounts of sand (2/9; Fig.3D), or plastic material within the lumen (1/9), while gastric impaction was caused solely by large amounts of impacted and dry ingesta, with a concomitant severe distal esophageal muscle hypertrophy. Locations affected within the large colon varied greatly from flexures (Fig.3E) to the entire organ. Other gastrointestinal lesions associated with cecocolonic impactions included intestinal rupture and peritonitis (3/9), as well as secondary gastric impaction (3/9). Predisposing factors included previous colic surgery (four cases), poor dentition (three cases; Fig.3F), water deprivation (three cases), dehydration (two cases), and anti-inflammatory treatment in one case. The horse affected by gastric impaction was found in a ditch without any food or water supply.

Incarcerations

Incarcerations or hernias were detected in six cases, of which two consisted of inguinoscrotal hernias (Fig.4A), other two of small intestine entrapment by a mesenteric failure, and two cases of diaphragmatic hernia and umbilical eventration (Fig.4B). Grossly, the small intestine was involved in five cases, and the large colon was involved in one case (diaphragmatic hernia). In all cases, the organs were constricted by a hernial ring, which led to intestinal ischemia with reddened to dark-red serosa. The intestinal contents were liquid and red. In the case of diaphragmatic hernia, the large colon was ruptured within the thoracic cavity. Predisposing factors included previous surgeries (two cases of small intestine entrapment within a mesenteric failure), and patent inguinal ring in one case.

Intussusception

Intussusception was diagnosed in four cases, of which three involved the small intestine (two in the jejunojejunum and one in the jejunoileum), while the other involved the ileocecum (Fig.4C-D). Three cases occurred in foals and were associated with lack of colostrum intake and concomitant pneumonia, while the other case occurred in an eight-year-old horse with poor dentition. In all cases, grossly, a segment of the small intestine slipped (intussusceptum) into another portion of the small/large intestine (intussuscipiens), causing a focal area of luminal obstruction and ischemia, with the intussusceptum presenting a diffuse deep black-red discoloration.

Other causes

Other causes of noninfectious diseases of the gastrointestinal tract included two cases of left dorsal displacement of the colon, one case of extrinsic obstruction of the small intestine, one case of intrinsic obstruction at the jejunum, and one case of primary intestinal adenocarcinoma. The extrinsic obstruction of the small intestine was caused by foreign material (surgical dressing that led to fibrous adhesions between intestinal loops), and the intrinsic obstruction was caused by a linear foreign body (rope) with complete obstruction of the intestinal lumen. The neoplasm was composed by multifocal firm whitish masses (6x4x3cm), which partially occluded the intestinal lumen, and was also associated with multifocal areas of intestinal stenosis. On the cut surface, it had a friable aspect (Fig.4E). In addition, the mesenteric lymph node was enlarged and firm. Histologically, there was an unencapsulated neoplastic proliferation of epithelial cuboidal cells arranged in tubules and papillae, occasionally filled by a faint basophilic content, extending from the mucosa into the internal muscular layer (suggestive of intestinal papillary adenocarcinoma). Additionally, the neoplastic cells were intermixed with a severe desmoplastic reaction (Fig.4F), inflammatory infiltrate of lymphocytes, and multifocal areas of necrosis. These cells were also observed within lymphatic vessels and within the mesenteric lymph node.

DISCUSSION

The diagnosis of the cases of the present study was obtained mainly by an association of the epidemiological and gross findings. Histological findings were restricted to the neoplasm case in order to obtain a differential diagnosis to other tumoral conditions, such as pythiosis (Uzal et al. 2016). Noninfectious gastrointestinal diseases were related to the cause of death mostly in adult horses with a median age of 10-years old. This is similar to the median age of horses submitted to colic surgeries (8.5-years old) in a retrospective study conducted in Finland (Immonen et al. 2017) and in South Africa (Voigt et al. 2009), and it is equal to another investigation that evaluated the survival and complication rates of 300 horses submitted to surgical treatment of colic (Mair & Smith 2005a). Regarding the affected breeds, there was an overrepresentation of mixed breeds and Criollo breed, that accounted for 76.8% of the cases, which may be a simple

reflection of the horse population in Rio Grande do Sul state, as well as of the population examined at the hospital.

Volvulus of the small and large intestines and primary gastric dilation were the most common diagnoses in this work. This is distinct from that observed in a previous study, in which gastric dilation was the fourth leading cause of death related to the alimentary system in the same state (Pierezan et al. 2009), but similar to another investigation conducted in horses previously submitted to celiotomy, of which 11% of the deaths were linked to gastric rupture (Todhunter et al. 1986). Gastric dilation is often considered to be secondary to obstruction of the stomach, small bowel, or of colic with

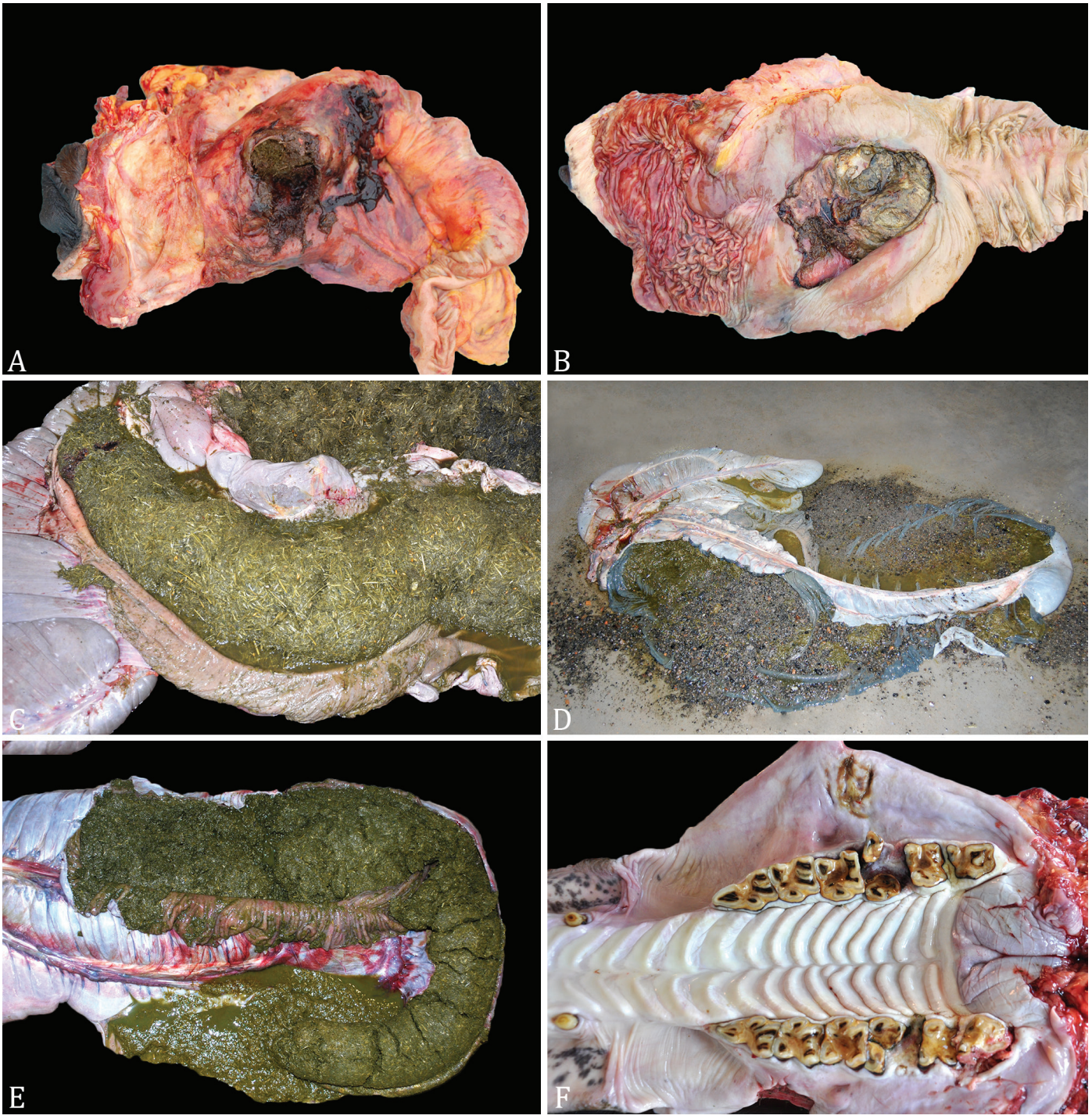


Fig. 3. Rectal (colonic) perforation and cecocolonic impaction in horses in Southern Brazil. (**A**) The rectum displays a focally extensive area of rupture characterized by transmural tearing, associated with reddened borders. (**B**) The mucosa of this ruptured area was severely ulcerated and covered by large amounts of fibrin and feces. (**C**) Impaction of the right ventral large colon by large amounts of dry contents. (**D**) Impaction was also caused by variable amounts of sand material within the lumen of the large colon. (**E**) Locations affected within the large colon were variable, as in this case, wherein the pelvic flexure was involved. (**F**) Poor dentition was a common predisposing factor to the development of impactions in horses, with shallow or absent maxillary teeth.

adynamic (paralytic) ileus, which frequently follows abdominal surgery, especially when the intestines are roughly handled or traumatized (Uzal et al. 2016). Nonetheless, all cases of the present study were characterized as primary because of the absence of other gastrointestinal disturbances and to the identification of predisposing factors. Alimentary overload was the most common predisposing factor, wherein excess of corn, grain or both in the horse's diet were commonly detected. In addition, other associated findings included gastric ulcers, which in horses are often incidental findings

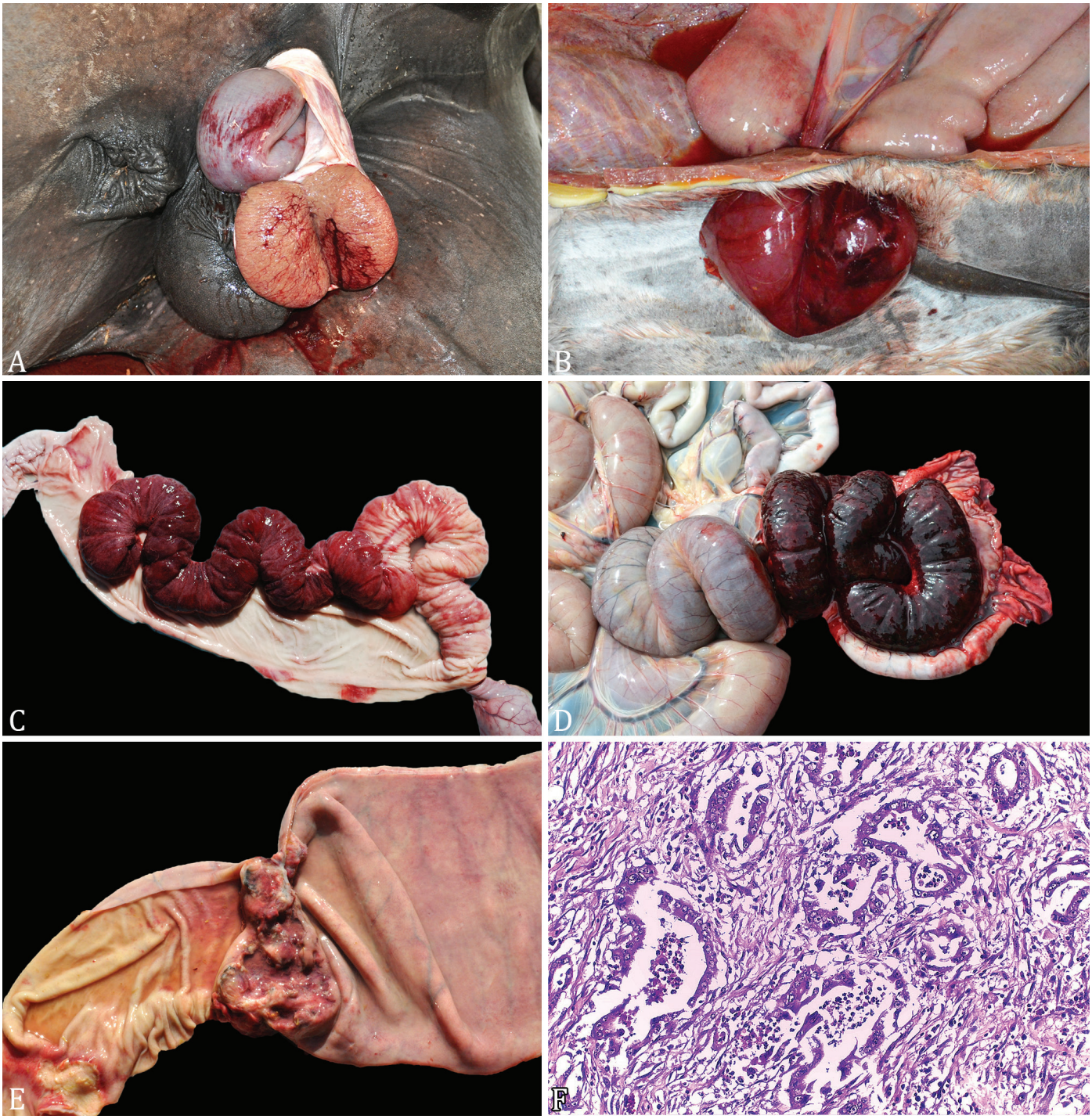


Fig. 4. Incarcerations or hernias, intussusceptions, and neoplasms in horses in Southern Brazil. (**A**) A segment of the small intestine has passed through the inguinal canal and reached the scrotum, where these intestinal loops were constricted and presented a reddened serosa. (**B**) Umbilical eventration of a small bowel segment caused intestinal ischemia with dark-red serosa. (**C-D**) Small intestine intussusception characterized by passage of an intussusceptum into an intussuscipiens, with the intussusceptum presenting a diffuse deep black-red discoloration. (**E**) A focal whitish and friable mass (6x4x3cm) partially occluded the intestinal lumen. (**F**) Histologically, this mass was composed by a neoplastic proliferation of epithelial cuboidal cells arranged in tubules and papillae extending into the muscular layer, often intermixed with desmoplastic reaction. HE, obj.20x.

at the necropsy, but may also be rarely related to perforation (Winfield & Dechant 2015, Uzal et al. 2016). Nevertheless, gastric rupture and subsequent peritonitis were the most common pathological pictures observed in association with primary gastric dilation, unlike previous studies (Pierezan et al. 2009). Diffuse peritonitis in horses is often acute and fatal, as observed, owing to the small omentum of this species, which is probably related to the poor capacity of healing contaminated areas (Uzal et al. 2016). Grossly, all cases were differentiated from postmortem lesions based on the reddened edges at the ruptured sites at the greater curvature, which is the most common gastric rupture location in horses (Winfield & Dechant 2015). Moreover, the lesions seemed to begin at the serosa, which was frequently stretched, as has been previously described in gastric ruptures, wherein, generally, the gastric seromuscular tears are larger than the mucosal tears, indicating that the seromuscular layer is weakened first (Todhunter et al. 1986).

Mesenteric volvulus of the small intestines, also referred to as mesenteric torsion, occurs frequently in species with a long small intestinal tract, such as in horses (Pierezan et al. 2009, Buergelt & Del Piero 2014). Moreover, in horses, strangulating obstructions of the small bowel are associated with reduced short-term survival rates after surgery (Mair & Smith 2005a). Similarly, in the present investigation, small intestine volvulus was the most common pathological presentation of volvulus and affected mainly adults older than six-years old, in accordance to a previous study in which small intestine volvulus occurred in animals with a median age of five-years old (Stephen et al. 2004). Furthermore, in horses, the large colon and cecum are predisposed to volvulus owing to the lack of mesenteric anchorage and potential mobility of the large intestines within the abdominal cavity (Uzal et al. 2016), as frequently observed in the present work, in which the large intestine was almost equally affected by volvulus as the small intestine. Colon volvulus has been considered to be one of the most painful and fatal cause of colic in the horse (Suthers et al. 2013), in addition to a relatively common complication of previous colic surgeries leading to relaparotomy (Mair & Smith 2005c), but in the present investigation this predisposing factor was not identified. It is estimated that between 10 to 20% of horses submitted to exploratory celiotomy present large colon volvulus (Suthers et al. 2013), similarly to the current study in which large intestine volvulus represented 11.4% of the total of causes of death (13/114). Grossly, small and large intestinal volvulus shared similar gross aspects as well as similar secondary gastrointestinal changes, in accordance to that previously described (Uzal et al. 2016).

Enterolithiasis has been previously identified in 15.1% of horses presenting for colic and in 27.5% of patients undergoing celiotomy for colic treatment (Hassel et al. 1999), while Corrêa et al. (2006) found that approximately 15.4% of the horses submitted to surgical abdominal procedures had enterolithiasis, which is very similar to the frequency of causes of death observed in the current work (17.5%). This reinforces that this may be an important condition in horses with clinical signs of colic in Southern Brazil. Enteroliths are mineral concretions (calculi) usually formed in the ampulla coli of the right dorsal colon (Hassel 2002), and are composed by distinct amounts of magnesium, ammonium, and phosphate, which are often arranged in concentric lamellae around a central *nidus* (Uzal et al. 2016), as in the present study. These concretions are most commonly solitary (Hassel et al. 1999), as in most of the cases in this work (55%), but may also be multiple within the guts. Enterolithiasis is a common cause of obstructive colic in horses with records dating up to 140 years (Hassel 2002). It occurs when the concretion passes through the intestinal tract until it causes intestinal obstruction with subsequent luminal distention, mucosal necrosis, and, possibly, perforation of the guts (Uzal et al. 2016). This was often observed in the current study as all enteroliths were partially or completely obstructing the intestinal lumen, whereas in 55% (11/20) of the cases there was also intestinal perforation and peritonitis, which is distinct from a previous investigation that reported only 15% of intestinal rupture in association with enterolithiasis (Hassel et al. 1999). This difference probably occurred due to delay on the decision by the clinicians to perform a celiotomy in the present cases.

Common locations for the enteroliths to become entrapped are the transition into the transverse colon (Buergelt & Del Piero 2014), right dorsal colon, and descending colon (small colon) (Hassel et al. 1999, Hassel 2002), similarly to the present study in which the right dorsal large colon and the transverse colon were the most common locations. Moreover, an uncommon location (rectum) was also affected, while the pelvic flexure was not affected, suggesting that this is not a common location, unlike previously described (Buergelt & Del Piero 2014). Similar to other studies (Hassel et al. 1999, Pierezan et al. 2009), adult horses with a mean and median age of 10.3 and 10-years old , respectively, were most commonly affected. This was expected as the pathogenesis of enterolith formation is longstanding owing to the fact that it depends on many factors: salts, a nidus for precipitation of triple phosphates, and some degree of intestinal hypomotility (Buergelt & Del Piero 2014). *In vivo* diagnosis of enterolithiasis relies mainly on abdominal radiography, rectum palpation, and/or exploratory laparotomy. Still, abdominal radiographs may fail if the enterolith is located in the small colon, as well as palpation per rectum may provide limited information mainly because of the size of the examined horse, with enteroliths located at the terminal right dorsal colon being rarely detected (Hassel 2002). This reinforces that concretions located at the right dorsal colon, transverse colon, and small colon, such as in most of the cases of this work, may be related to poor prognosis if not properly detected early. Furthermore, smaller enteroliths, such as the ones involving the small colon and rectum in this study, are potentially more pathogenic than similar concretions present in the large dorsal colon, as they may obstruct the intestinal lumen (Hassel et al. 2008), while larger enteroliths are always contained within the right dorsal colon (Hassel et al. 1999). Several different shapes of enteroliths may occur in horses, but most commonly these are spherical or tetrahedral, in which the latter is frequently multiple (Hassel 2002). In the current investigation, most were rounded to spherical and often irregularly shaped. In six cases, poor dentition was observed and interpreted as a possible consequence of imbalanced diets, similarly to a previous study in Criollo horses which reported 56.25% of dentition problems occurring in association with enterolithiasis (Santos et al. 2017).

Rectal (colonic) perforation in horses are job-related risks for equine veterinarians, as accidental iatrogenic events may

occur, such as obstetric interventions and/or rectal palpation (Buergelt & Del Piero 2014, McMaster et al. 2015, Uzal et al. 2016). This was observed in the present study, wherein iatrogenic injuries were reported in 73.3% of the cases (11/15). Most perforation-related tears occur 25 to 30 cm away from the anus, involve the rectum mainly at the dorsal aspect (Uzal et al. 2016), and are, thus, difficult to be surgically accessed (McMaster et al. 2015). However, in four cases of the current work, the tears/perforations were located at the limit between the rectum and small colon, and at the small colon in another horse. Therefore, a better nomenclature for these would be rectal-colonic perforation in cases wherein the boundaries between these two organs are not clear. Rectal tears may be classified into grades 1 to 4 based on the extent of disruption, with full-thickness rupture observed only in grade 4 (Watkins et al. 1989), as all the cases of this study would be classified. Moreover, only 6% of the horses with grade 4 lesions survive (McMaster et al. 2015), which is mainly because of secondary peritonitis. This was a constant finding associated with these lesions in this investigation, occurring either as a located or disseminated lesion. As expected based on previous studies (Claes et al. 2008), females were mostly affected by injuries related to reproductive tract palpation. Horses older than nine-years old, similarly to the ones of the current study, are more predisposed to these perforations (Claes et al. 2008), as the reproductive tract becomes more pendulous at these ages and the frequency of palpation in subfertile older mares is increased (LeBlanc et al. 1998). Moreover, colic related palpation affected both males and females, but especially males, which probably occurred because stallions or geldings are less used to palpation per rectum than mares, and other factors, such as restless behavior, small rectum, inadequate restraint, application of inadequate amounts of lubricants and absence of spasmolytic drugs, may have played a role in these cases (Stauffer 1981). Interestingly, a case of rectal perforation following natural mating in a mare was detected, which appears to be an unusual presentation, that was not observed in a previous investigation of 99 cases of rectal perforation in the USA (Claes et al. 2008), but was previously reported in a mare following accidental penetration of a stallion's penis into the rectum (Arnold et al. 1978).

Large intestine and stomach impaction was the fifth most common category of gastrointestinal noninfectious disease detected as the cause of death in horses of the present work, which is distinct from previous clinical studies that identified impactions as the most common causes of colic (Abutarbush et al. 2006, Voigt et al. 2009). Indeed, this difference may be based on the fact that secondary impactions are often clinically detected without proper identification of the primary cause. Predisposing factors to this condition in horses are water deprivation, poor dentition, and dietary changes (Uzal et al. 2016), as observed in the current study. Dental problems were evidenced in three cases, but, still, a previous work was not able to establish an association between dental pathology, fecal particle size, and impaction colic in horses (Gunnarsdottir et al. 2014). Furthermore, another important predisposing factor identified was previous colic surgery in four cases. Postoperative colic during the first year after surgery may reach an incidence of 20% in horses (Immonen et al. 2017), and these surgeries may act by modifying the colonic or cecal peristalsis, causing abnormal motility (Uzal et al. 2016). In horses, this pathogenesis is usually related to adynamic or paralytic ileus when intestines are not properly handled at surgery (Uzal et al. 2016), but may also apply to the present cecocolonic impaction cases. Unlike previous investigations, impactions affected not only locations where the lumen was narrowed, such as the pelvic flexure and the transverse/small colon (Uzal et al. 2016), but also the entire large colon and cecum. In these cases, probably the association of predisposing factors, which were frequently multiple per horse, worsened the condition.

Sand impaction was detected in two cases and is commonly related to horses grazing in poorly covered sandy soils (Uzal et al. 2016). The ingested sand tends to settle down within the cecum and the ventral large colon, rendering these organs susceptible to rupture (Buergelt & Del Piero 2014), as occurred in one of the cases of the present study. Nonetheless, the mortality rate for horses with sand impaction is reported to be low, as observed in this work in which a low frequency was detected (two cases), and early surgical intervention may provide a good prognosis (Granot et al. 2008). Furthermore, the stomach was involved in one case of primary impaction, which is a rare cause of colic in horses (Bird et al. 2012), and in this situation it was caused by inadequate water supply, since the horse was found in a ditch without any food or water available for three days. Primary gastric impactions are usually characterized by large quantities of ingesta (15-35kg) associated with gross thickening of the muscle layers of the stomach wall, in addition to variable thickening of the pylorus and hypertrophy of the distal esophageal muscles (Bird et al. 2012), which was observed grossly in this case.

Incarcerations or hernias occurred mainly as internal and external hernias, such as inguinoscrotal and diaphragmatic, but also as internal failures of the mesentery and a case of eventration. Grossly, these lesions were typical of intestinal ischemia and infarction (Uzal et al. 2016), exhibiting reddened to dark-red serosa. The pathogenesis of this condition may be related to fixation of the hernial/entrapped contents within a location, which may be followed by distention of the viscera with accumulated gas or ingesta, and later the lesion becomes nonreducible (Uzal et al. 2016). Inguinal hernias may evolve to scrotal hernia when the herniated pass down the inguinal canal. These lesions are usually observed in foals because the inguinal ring tends to close as the animal grows up (Uzal et al. 2016). Unlike that, two cases of inguinoscrotal hernias were detected in adult horses in the present investigation, and a patent inguinal ring-opening was detected in one of these. Moreover, an umbilical eventration was observed in an adult horse, but it could have resulted from a rare enterocutaneous fistula related to an umbilical hernia (Uzal et al. 2016). Another interesting finding were two cases of small intestine entrapment by a mesenteric failure, which were most likely related to previous surgeries that caused tissue gaps within the mesentery, allowing the small intestinal loops to slip into this failure and become entrapped (Buergelt & Del Piero 2014).

Intussusceptions were not frequently detected in this study, corresponding to only 3.5% of the diagnosis, as was observed in another study, which pointed out a prevalence of 8.7% of intussusceptions in horses undergoing laparotomy owing to abdominal pain (Edwards 1986). This condition is usually related to preceding intestinal hypermotility or endoparasitism (Buergelt & Del Piero 2014), as well as it may be associated

with previous intestinal surgery, enteritis, and tumors (Uzal et al. 2016). In two of the present cases, decreased intake of colostrum and concomitant pneumonia were identified as predisposing factors, and probably resulted in hypermotility of the guts. Still, in horses ileocecal or cecocolic intussusceptions are most commonly detected (Bell & Textor 2010, Buergelt & Del Piero 2014), while in this investigation jejunojejunal and jejunoileal intussusceptions were more frequent. This difference probably occurred because of the lower age of the animals affected, with a median age of 0.04-years old (which corresponds to almost 15-days old), when compared to another study that investigated cecal intussusceptions in horses with a median age of 14.5-months old (Bell & Textor 2010). Grossly, intussusception may present as a simple obstruction if mural blood flow is preserved, or as a necrotic lesion when there is occlusion of the blood flow (Nelson & Brounts 2012). This second pattern was the most commonly observed in the current work, with ischemic lesions affecting the intussusceptum, which probably occurs in most severe cases wherein this condition is related to the cause of death of horses.

Intestinal neoplasms are rare in horses (Munday et al. 2017), and are usually mesenchymal, mainly represented by fibrosarcomas, leiomyosarcomas, and gastrointestinal stromal tumors (GIST) (Buergelt & Del Piero 2014), additionally to lymphoma and adenocarcinoma (Taylor et al. 2006). However, in this investigation a primary intestinal adenocarcinoma was detected, and, similarly to that previously described (Uzal et al. 2016), it represented less than 1% of the cases. These neoplasms usually involve the small intestines of adult horses and most of them invade the intestine wall, rarely causing complete obstruction of the lumen (Taylor et al. 2006), similarly to the one observed in the present work. Histologically, neoplastic cells tend to be well differentiated and intermixed with marked fibrous metaplasia (desmoplastic reaction) (Munday et al. 2017), as in this case. Another finding of this investigation was one case of extrinsic obstruction of the small intestine caused by a foreign material that led to focal adhesion in a similar manner that previously reported in long-term complications of previous colic surgeries in horses (Mair & Smith 2005b). Adhesions become a clinical problem only when they mature into fibrous adhesions, which may compress or anatomically distort the intestine, causing an obstruction (Mair & Smith 2005b), which occurred in this case.

CONCLUSIONS

Noninfectious diseases of the gastrointestinal tract are major causes of colic and death of horses in general, and are the main diseases affecting the alimentary system in this species. Gastric dilation and volvulus were the most common conditions. Enterolithiasis was the third most common diagnosis and visceral perforation with peritonitis frequently occured. Rectal (colonic) perforation was most commonly related to iatrogenic injuries associated with colic related and reproductive tract palpation, affecting more males and females, respectively.

Impactions affected mostly the large colon and cecum, with previous colic surgery, poor dentition, water deprivation, and dehydration identified as important predisposing factors.

Incarcerations were mostly related to hernias, mesenteric failures, and eventration.

Intussusception was observed mainly in the small intestine of foals and it was often related to other concomitant diseases.

Other causes were less common and included a multifocal primary intestinal papillary adenocarcinoma causing a partial occlusion of the intestinal lumen, as well as multifocal areas of stenosis.

Epidemiological and gross lesions are essential to obtain a final diagnosis of these conditions.

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

Animal welfare/ethical statement.- We authors of the article entitled "Epidemiological and pathological aspects of noninfectious diseases of the gastrointestinal tract in 114 horses in Southern Brazil" declared, for all due purposes, that the project that gave rise to the present data of this manuscript was submitted to the Research Commission of the "Universidade Federal do Rio Grande do Sul" (UFRGS) for evaluation, and was approved under the number 33501 on December 7th, 2017.

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Original Article
Livestock Diseases
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Infectious diseases dynamics in growing/finishing pigs in Southern Brazil (2005-2016)[1]

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ABSTRACT.- 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).** *Pesquisa Veterinária Brasileira 40(4):254-260*. Setor de Patologia Veterinária, Departamento de Patologia Clínica Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil. E-mail: saulo.pavarini@ufrgs.br

This study aimed to determine the frequency and distribution of infectious diseases diagnosed through necropsy examination and histopathological analysis in growing/finishing pigs along 12 years (2005-2016) in Southern Brazil. We evaluated 1906 anatomopathological exams of pigs at growing/finishing phases, of which the infectious diseases corresponded to 75.6% of the cases (1,441/1,906). Porcine circovirus type 2 (PCV2) infections were the most frequent, accounting for 51.3% of the cases (739/1,441) with a higher frequency from 2005 to 2007, characterizing an epidemic distribution, with a gradual decline after 2008. Infectious diseases affecting the respiratory system were the second major cause with 30.1% of the cases. Among these, necrotizing bronchiolitis caused by swine Influenza (15.1%, 218/1,441) and bacterial pneumonia (15%, 216/1,441) were the main conditions. Influenza was mostly diagnosed from 2010 to 2013, accounting for 43.1% (167/387) of the cases. After this period, both respiratory infectious diseases were endemic. Digestive system infectious diseases accounted for 10.5% of the diagnoses (151/1,441), with the following main conditions: *Salmonella* spp. enterocolitis (43.7%, 66/151), *Lawsonia* spp. proliferative enteropathy (41.7%, 63/151), and *Brachyspira* spp. colitis (14.6%, 22/151). The latter had a higher incidence from 2012 to 2014 with all cases detected in this period. Polyserositis and bacterial meningitis represented, respectively, 5.8% (84/1,441) and 2.3% (33/1,441) of the cases diagnosed, with a constant endemic character.

INDEX TERMS: Infectious diseases, growing pigs, finishing pigs, Brazil, swine diseases, porcine circovirus, swine influenza, bacterial pneumonia, enterocolitis, polyserositis.

RESUMO.- [**Dinâmica de ocorrência das doenças infecciosas em suínos nas fases de crescimento e terminação no sul do Brasil (2005-2016).**] O objetivo deste estudo consistiu em determinar a frequência e a distribuição das doenças infecciosas diagnosticadas através de exame de necropsia e análise histopatológica em suínos nas fases de crescimento/terminação ao longo de 12 anos (2005-2016) no sul do Brasil. Foram avaliados 1906 laudos anatomopatológicos de suínos nas fases de crescimento/terminação, dos quais as doenças infecciosas corresponderam a 75,6% (1441/1906) do total. As infecções por circovírus suíno tipo 2 (PCV2) foram as mais frequentes, contabilizando 51,3% (739/1441) dos casos, com uma alta frequência de 2005 a 2007 caracterizando uma distribuição epidêmica neste período, e um declínio gradual após o ano de 2008. A segunda principal causa incluiu as doenças infecciosas que afetam o sistema respiratório (30,1% dos casos). Dentre essas, destacaram-se a influenza suína (15,1%; 218/1441) e pneumonias bacterianas (15%; 216/1441). O diagnóstico de influenza apresentou uma frequência elevada de 2010 a 2013, totalizando 43,1% (167/387) dos casos. Após este período, ambas doenças infecciosas respiratórias exibiram caráter endêmico. As

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doenças infecciosas do sistema digestório totalizaram 10,5% (151/1441) dos diagnósticos, com as seguintes principais condições: enterocolite por *Salmonella* spp. (43,7%; 66/151), enteropatia proliferativa por *Lawsonia* spp. (41,7%; 63/151) e colite por *Brachyspira* spp. (14,6%; 22/151). A colite por *Brachyspira* spp. apresentou uma alta incidência de 2012 a 2014 com todos os casos detectados no período. As polisserosites e meningites bacterianas representaram 5,8% (84/1441) e 2,3% (33/1441) dos casos diagnosticados, respectivamente, com um caráter endêmico constante.

TERMOS DE INDEXAÇÃO: Doenças infecciosas, suínos, fase de crescimento, fase terminal, Brasil, doenças dos suínos, circovirose suína, influenza suína, pneumonias bacterianas, enterocolites, polisserosites.

INTRODUCTION

Swine industry represents an important economic activity in Brazil, which has a worldwide leading position in pig production and export (Brasil 2018). Pig health is one of the main challenges in swine industry and it represents, along with biosecurity measures, one of the main focus to improve productivity (Amaral et al. 2006, Sobestiansky & Barcellos 2012). In this context, infectious diseases (ID) have a direct impact in mortality indexes, as well as it may cause losses in alimentary conversion in finishing pigs (Maes et al. 2003, Neumann 2012). Porcine circovirus type 2 associated disease (PCV-SD) is one of the main conditions affecting swine health (Segalés 2012), with mortality indexes ranging from 4 to 20% (Segalés et al. 2005, Segalés 2012). Besides this condition, porcine respiratory disease complex (PRDC) causes substantial sanitary impacts in swine industry (Schaefer et al. 2013), with a myriad of viral and bacterial pathogens involved. Primary infectious agents, which are able to induce severe respiratory/lung lesions, are included among these causes, as well as secondary/opportunistic infectious agents, which are mainly related to noninfectious cofactors at the swine farms (Opriessnig et al. 2011). Among these, swine influenza A, *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, *Glaesserella* (*Haemophilus*) *parasuis*, and *Pasteurella multocida* are the main agents involved (Opriessnig et al. 2011, Schaefer et al. 2013). Nevertheless, digestive system diseases are also highlighted at this phase, with *Lawsonia* spp. proliferative enteropathy, swine salmonellosis, and swine dysentery as the main conditions involved. These are often related to profuse diarrhea, with similar clinical-pathological findings, and a frequency of occurrence of 19.56%, 6.52%, and 4.36%, respectively (Suh & Song 2005, Kich & Cardoso 2012, Viott et al. 2013, Burrough 2016). This study aimed to determine the frequency and distribution of infectious diseases diagnosed through necropsy examination and histopathological analysis in growing/finishing pigs along 12 years (2005-2016) in Southern Brazil.

MATERIALS AND METHODS

A retrospective study of ID affecting growing/finishing pigs (age range of 63 to 160 days) was conducted in the anatomopathological database of the veterinary pathology laboratory from January 2005 to December 2016. The necropsy and anatomopathological protocols were reviewed, and information contained within these reports was registered. These were later classified regarding the diagnosis, annual distribution, and seasonal occurrence. All pigs were originated from farms located in Southern Brazil (Rio Grande do Sul, Santa Catarina, and Paraná states), and were raised in growing-finishing units with variable herds of 200 to 5,000 pigs. Cases with a conclusive diagnosis (obtained through histopathological, immunohistochemical, or Warthin-Starry histochemical exams) were grouped into eight categories of ID: porcine circovirus type 2 infections, swine influenza A infection, bacterial pneumonias, polyserositis, *Salmonella* spp. enterocolitis, *Lawsonia* spp. proliferative enteropathy, *Brachyspira* spp. colitis, and bacterial meningitis. Cases wherein epidemiological, clinical, or pathological data were scarce to obtain a final diagnosis were excluded from this study. Moreover, materials obtained from scientific experiments, abattoirs analysis, and locations outside the scope of this study were excluded of the analysis. Distribution, frequency, and seasonal occurrence of the ID were evaluated and determined through a descriptive statistical analysis. This analysis was represented as n (%) for the frequency of diseases diagnosed along the years. A contingency analysis was used to evaluate an association between categorical variables (disease and affected system) along the years. A p-value <0.05 was considered statistically significant. All analysis were performed with the Software Jmp 14 (SAS, Cary/NC).

RESULTS

A total of 1,906 anatomopathological exams of growing-finishing pigs in Southern Brazil were evaluated in this study. Among these, ID corresponded to 75.6% of the diagnoses (1,441/1,906). All cases were from samples originated in the Southern Brazil, mainly represented by Rio Grande do Sul state with 44.6% of the cases (643/1,441), followed by Santa Catarina state with 42.3% (610/1,441), and Paraná state with 13.1% (188/1,441) of the cases.

Porcine circovirus type 2 (PCV2) infections were the most frequent and accounted for 51.3% of the ID (739/1,441), of which 88.6% were associated with PCV-SD, followed by porcine dermatitis and nephropathy syndrome (PDNS) in 11.4% of the cases (84/739). PCV2 infections had a higher frequency from 2005 to 2007, with 54.7% of the diagnoses during this period (404/739), and, thus, an epidemic distribution was observed. Later on, there was a gradual decline after 2008, until it reached an endemic status. A seasonal occurrence was not observed ($p>0.1$) either for spring/summer (358/739) or autumn/winter (381/739). The annual distribution for PCV2 infections is shown in Figure 1.

ID that affected the respiratory system accounted for 30.1% of the cases. These were mostly represented by necrotizing bronchiolitis caused by swine influenza A infection, which accounted for 15.1% of the ID in this study and represented almost 50% of the pneumonia samples. This condition, which was initially diagnosed by the end of 2009, had an epidemic distribution with an increased frequency of diagnosis from 2010 to 2013, accounting for 43.1% of the cases (167/387). After this period, the disease occurred with an endemic character along with other bacterial pneumonia. Similarly, these accounted for 15% of the ID (216/1,441) diagnosed, in which 79.6% of the cases (172/216) were compatible with suppurative bronchopneumonia, while 20.4% (44/216) were compatible with fibrinonecrotizing pleuropneumonia. Both conditions (necrotizing bronchiolitis by swine influenza A and bacterial pneumonia) did not vary along the distinct seasons.

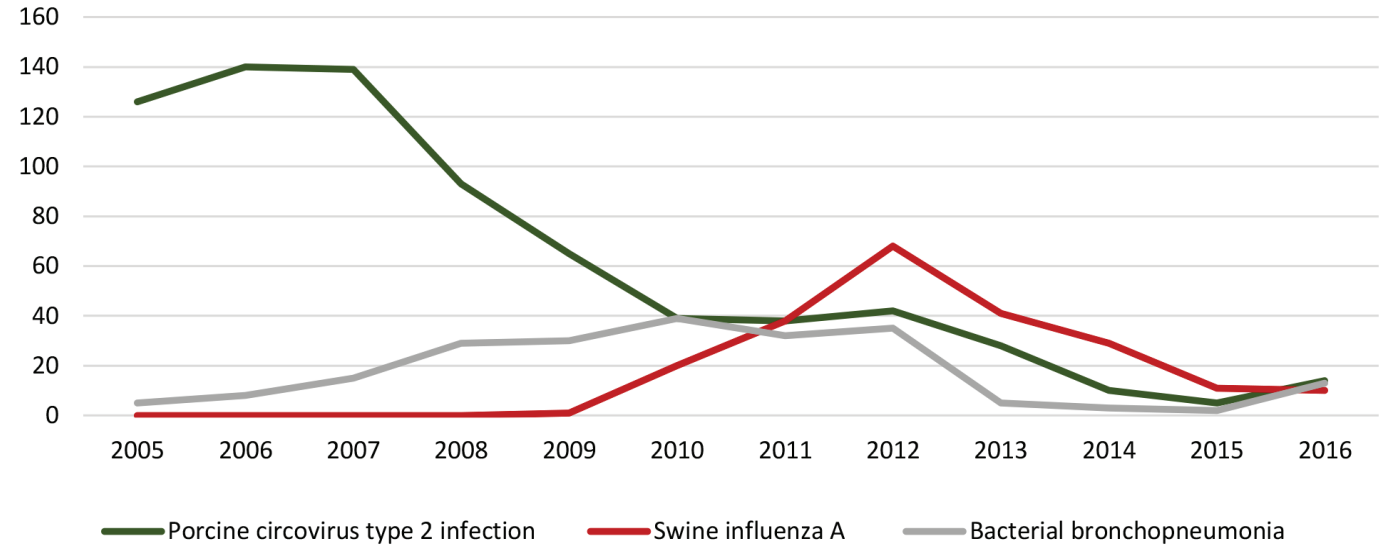


Fig.1. Annual distribution of porcine circovirus type 2 infection, swine influenza A infection, and bacterial bronchopneumonia along 12 years (2005-2016) in Southern Brazil.

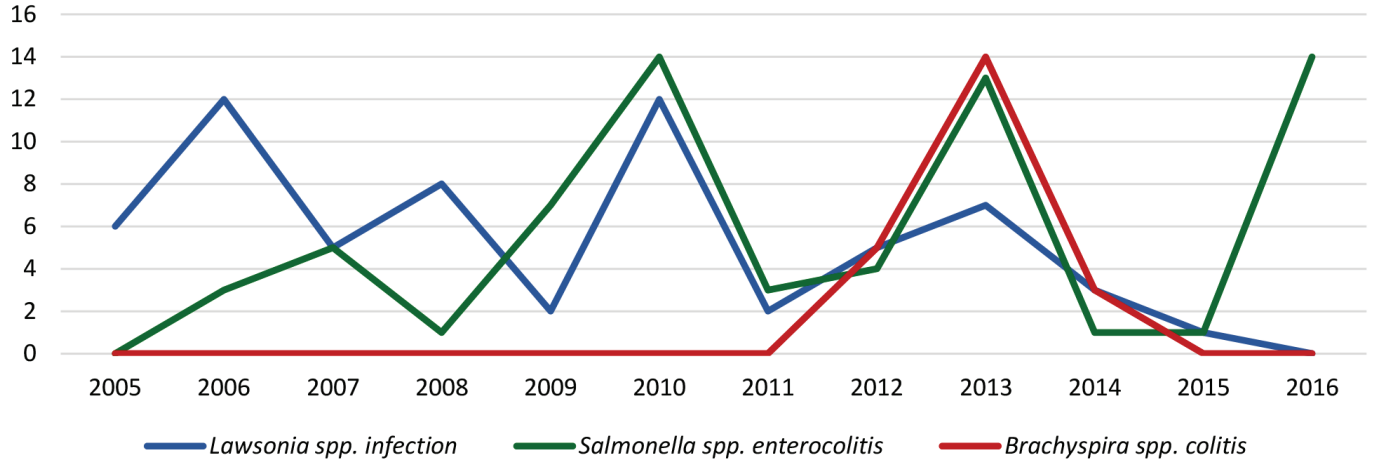


Fig.2. Annual distribution of *Lawsonia* spp. proliferative enteropathy, *Salmonella* spp. enterocolitis, and *Brachyspira* spp. colitis along 12 years (2005-2016) in Southern Brazil.

Moreover, an etiology for bacterial pneumonias could not be determined as these samples were previously fixed in 10% formalin. The annual distribution for swine influenza A and bacterial pneumonia is shown in Figure 1.

ID affecting the digestive system accounted for 10.5% of the cases (151/1,441). The most common conditions were: *Salmonella* spp. enterocolitis (43.7%, 66/151), *Lawsonia* spp. proliferative enteropathy (41.7%, 63/151), and *Brachyspira* spp. colitis (14.6%, 22/151). *Salmonella* spp. enterocolitis and *Lawsonia* spp. proliferative enteropathy had an endemic character distribution in this study, with a higher frequency in 2010, 2013, and 2016. On the contrary, *Brachyspira* spp. colitis had a higher frequency from 2012 to 2014, with all cases diagnosed within this period. Additionally, *Brachyspira* spp. colitis had a tendency for seasonal occurrence (p=0.08), with 68% of the cases (15/22) diagnosed during spring/summer. The annual distribution of ID affecting the digestive system is shown in Figure 2.

Polyserositis accounted for 5.8% of the cases (84/1,441), and it occurred with an endemic character along all the analyzed period. Seasonal occurrence was not observed. Bacterial suppurative meningitis represented 2.3% of the cases diagnosed (33/1,441), which had an endemic distribution along the analyzed years. The pathological findings consisted of suppurative meningitis involving the leptomeninges (29/33), and in 4/33 of the cases, besides the leptomeningeal lesions, there were also pleuritis and suppurative pericarditis.

DISCUSSION

Swine ID affecting growing-finishing pigs accounted for 75.6% of the cases diagnosed in general, which is slightly above the data reported previously by Brum et al. (2013) in Brazil (68.1%) and below the data reported by Pelliza et al. (2007) and Tateyama et al. (2000), with 77.2% and 90% in Argentina and Philippines, respectively. The highest number of ID in the

present study may have been influenced by the type of samples analyzed, since this investigation was conducted with fixed samples submitted to a pathology laboratory. Noninfectious diseases affecting growing-finishing pigs, such as gastric ulcers and mesenteric torsions/volvulus, are not often submitted for analysis, because these conditions are easily diagnosed at the gross examination (Morés 2009).

PCV-SD was initially described in 1991 in western Canada, wherein it was associated with postweaning multisystemic wasting syndrome (Allan & Ellis 2000). Since then, it has been widely described (Opriessnig et al. 2007, Gillespie et al. 2009, Ciacci-Zanella et al. 2015, Konradt et al. 2018), and it was the most frequently diagnosed condition in the current work, accounting for 51.3% of the cases. These were mostly diagnosed between 2005 and 2007, similarly to previous studies (Opriessnig et al. 2007, Gillespie et al. 2009). After commercial vaccines containing subunits of PCV2 expressed in baculovirus were employed and noninfectious cofactors were reduced, there was a significant decay of the disease in Brazil (Ciacci-Zanella et al. 2015), which was also observed in this study after 2008, when the condition passed to occur with an enzootic character. Therefore, since commercial available vaccines (effective against PCV2 infections) were introduced, there was a marked reduction in PCV associated disease in brazilian swine herds (Ciacci-Zanella 2017).

Respiratory infections in pigs may result in significant economic losses to the production systems, aside from causing negative effects on animal welfare (VanAlstine 2012). These conditions are usually multifactorial and associated with the interaction between multiple infectious agents, as well as noninfectious cofactors, such as environmental, handling, age, genetics, and nutrition (Rech et al. 2018). Swine influenza, which was the main respiratory condition diagnosed in the present study, stood out in Brazil after the human pandemic influenza took place in 2009 concomitantly with a similar clinical disease in pigs, which was caused by similar viral subtypes to the worldwide distributed (Hofshagen et al. 2009, Howden et al. 2009, Pasma & Joseph 2009, Moreno et al. 2010, Pereda et al. 2010, Schaefer et al. 2011, Watanabe et al. 2012, Njabo et al. 2012, Holyoake et al. 2011). Similarly, in the present study there was a higher frequency of diagnosis between 2010 and 2013, with indexes that reached 43%. This fact may be explained by viral introduction in naïve swine herds to influenza; therefore, the disease had an epidemic character, with higher morbidity indexes (Van Reeth et al. 2012). Biosecurity measures to prevent influenza occurrence in the herds, as well as reducing noninfectious cofactors (environmental factors, handling, stress, and higher infection pressure) are important measures to avoid viral dissemination, in addition to reduce infections by other important respiratory pathogens at this phase (FAO 2010, Barcellos et al. 2017). These were also observed in the present study, as, along with the reduction of swine influenza from 2012 to 2013, a concomitant decrease of bacterial pneumonias was also observed in growing-finishing pigs. Nowadays, swine influenza A virus is frequently found in postweaning pigs in association with *Glaesserella* (*Haemophilus*) *parasuis*, and in finishing pigs in association with *Mycoplasma hyopneumoniae* and *Pasteurella multocida* (Barcellos et al. 2017). In Brazil, bacterial pneumonias are important conditions diagnosed in growing and finishing pigs owing to the intensive production systems employed, which are characterized by high animal farming densities in addition to mixture of animal lots with distinct origins (Opriessnig et al. 2011, Mores et al. 2015). The prevalence of this condition varies from 19 to 79% of the diagnoses in pigs, and, among the bacterial agents, *P. multocida*, *M. hyopneumoniae*, and *Actinobacilus pleuropneumoniae* are highlighted (Enoe et al. 2002, Fraile et al. 2010, Meyns et al. 2011, Morés et al. 2015). In this study, suppurative bronchopneumonia accounted for 79.6% of the cases of bacterial pneumonias, which is similar to a previous result (77.3%) obtained by Mores et al. (2015) in Brazil. These authors have also highlighted from an immunohistochemical and bacterial isolation analysis perspective that 80.3% and 56.7% of these cases were caused, respectively, by *M. hyopneumoniae* and *P. multocida*, suggesting that these agents were often coinfecting the pigs. Fibrinonecrotizing pleuropneumonia accounted for 20.4% of the cases, and these conditions are frequently caused by *A. pleuropneumoniae* infection, as well as are associated with the production of four bacterial exotoxins (ApxI, ApxII, ApxIII, and ApxIV) (Kamp et al. 1997, Opriessnig et al. 2011, Gottschalk 2012a).

Lawsonia intracellularis proliferative enteropathy, *Salmonella* spp. enterocolitis, and *Brachyspira* spp. colitis are important worldwide distributed conditions in growing-finishing pigs with similar clinical and pathological features (Jacobson et al. 2003, Suh & Song 2005, Kich et al. 2011, Viott et al. 2013, Burrough 2016). These conditions represented 10.5% of the diagnoses in the present study, which are unequal to previous results of 31.7% and 15.4% in South Korea and Argentina, respectively (Suh & Song 2005, Pelliza et al. 2007). *Lawsonia* spp. enteropathy and *Salmonella* spp. enterocolitis were endemic diseases in the current work with a frequency of 43.7% and 41.7%, respectively, when enteric diseases were analyzed. Kich et al. (2011) and Jacobson et al. (2003) have reported results of 39% and 67% for *Salmonella* spp. and *L. intracellularis* isolation from samples of pigs with diarrhea and poor development, which are, respectively, below and above to the ones obtained in this study. Although *Brachyspira* spp. colitis presented an overall low frequency of diagnosis, a higher incidence was observed from 2010 to 2014. Two possible explanations may be applied to this increase in number of cases of *Brachyspira* spp. colitis: it may be related to a bacterial resistance to some of the antimicrobials commonly applied to control clinical disease; or it may have occurred due to the introduction of clinically health females, which could have carried subclinical infections to many sow farms of different integration companies (Daniel et al. 2013, Daniel et al. 2017). Strains of *Brachyspira* spp. resistant to commonly used antimicrobials have been detected in several studies (Duinhof et al. 2008, Pringle et al. 2012), and, recently, in Brazil in a investigation that evaluated the sensitivity of *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* to different classes of antimicrobials (Daniel et al. 2017). *Brachyspira* species may survive in organic material, such as feces diluted in water, for as long as 48 days in environmental temperatures ranging from 0 to 10°C (Hampson & Burrough 2019); however, this study has detected a higher incidence of clinical disease during spring/summer, which may have occurred because of a higher infection pressure, as well as due to problems of bay drainage and asymptomatic pigs acting as carriers and infection sources (Hampson & Burrough 2019).

Polyserositis results with significant economic impact in swine industry owing to increased mortality indexes and carcass condemnation at slaughterhouses (Kang et al. 2012, Uzal et al. 2016). In this study, these lesions corresponded to 5.8% of the diagnoses, and presented an enzootic distribution along the analyzed years, corroborating with previous data (Nedbalcova et al. 2006, Uzal et al. 2016). Moreover, a previous study by Kang et al. (2012) identified *G.* (*H.*) *parasuis* (85%), *Streptococcus suis* (10.7%), and *Mycoplasma hyorhinis* (3.6%) as the main causes of polyserositis in growing-finishing pigs. The low frequency of this condition may be explained by the infection dynamics of *G.* (*H.*) *parasuis* in swine, since this infection occurs mainly at the postweaning phase in pigs from 4 to 8 weeks of age, wherein there is a decay of colostrum passive immunity (Oliveira & Pijoan 2004, Nedbalcova et al. 2006).

Suppurative meningitis in pigs is frequently associated with acute infections by *Streptococcus suis* serotype 1 and 2 (Cantile & Youssef 2016). The condition is usually related to neurological clinical signs in 5-10-weeks old piglets (Gottschalk 2012b, Cantile & Youssef 2016), which may justify the low frequency of diagnosis of this condition in the present work (2.3%), as all animals were older than 63 days. Etiological diagnosis was not obtained in this study because samples were previously fixed in 10% formalin; even though, the association of the epidemiological and pathological features (suppurative meningitis) led us to believe that these cases are, indeed, related to *S. suis*. *G.* (*H.*) *parasuis* is another common agent associated with suppurative meningitis in pigs (Oliveira & Pijoan 2004); however, fibrinosuppurative polyserositis is a classical lesion in this condition, and, only, in severe cases the meninges are involved (Nedbalcova et al. 2006).

CONCLUSIONS

Infectious diseases accounted for 75.6% of the diagnoses in growing-finishing pigs in Southern Brazil.

Although PCV2 is considered an important agent detected, a significant reduction of the frequency of cases has been detected as commercially available vaccines were employed since 2008 in Southern Brazil.

Among respiratory diseases, swine influenza A infection was highlighted, reaching a higher frequency at 2012, and, nowadays, it represents the main respiratory disease in pigs along with bacterial bronchopneumonia.

Lawsonia spp. proliferative enteropathy and *Salmonella* spp. enterocolitis had an endemic distribution among all years of the present study, while *Brachyspira* spp. colitis had a higher frequency from 2010 to 2014.

Polyserositis and bacterial meningitis had a low frequency of diagnosis with an endemic distribution along the 12 years of this study.

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Serological survey of bluetongue virus in sheep from Minas Gerais[1]

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ABSTRACT.- Biihrer D.A., Albuquerque A.S., Romaldini A.H.C.N., Pituco E.M., Matos A.C.D., Lobato Z.I.P., Varaschin M.S. & Raymundo D.L. 2020. **Serological survey of bluetongue virus in sheep from Minas Gerais.** *Pesquisa Veterinária Brasileira 40(4):261-265*. Departamento de Medicina Veterinária, Universidade Federal de Lavras, Campus Histórico, Cx. Postal 3037, Av. Doutor Sylvio Menicucci 1001, Lavras, MG 37200-000, Brazil. E-mail: djeison.raymundo@dmv.ufla.br

Bluetongue is an infectious, non-contagious disease that affects domestic and wild ruminants, caused by a virus from the *Orbivirus* genus, Reoviridae family, transmitted by arthropod vectors of the *Culicoides* genus. This paper aims to be the first serological survey of bluetongue in sheep from the Meso-regions of Campo das Vertentes and South and Southeast of Minas Gerais. Samples were collected from sheep from different properties. The serum samples were submitted to Agar Gel Immunodiffusion (AGID) and competitive Enzyme-Linked Immunosorbent Assay (cELISA). 303 serum samples were submitted to AGID and cELISA. In these samples, 164 (54.13%) were positive in the AGID technique, and 171 (56.44%) positive in the cELISA technique, with an almost perfect agreement between the techniques (kappa index = 0.887). In all visited properties, positive animals have been found in the herd. Animals acquired from properties of the studied mesoregions were more likely to be positive in IDGA and cELISA tests than animals acquired from properties in other regions of Brazil (p<0.001). These results suggest that bluetongue virus (BTV) is widespread in the mesoregions of Campo das Vertentes and South and Southeast of Minas Gerais.

INDEX TERMS: Serological survey, bluetongue virus, sheep, Minas Gerais, Brazil, *Orbivirus*, diagnosis, serology, sheep farming, ovine.

RESUMO.- [**Inquérito sorológico do vírus da língua azul em ovinos de Minas Gerais.**] A língua azul (LA) é uma doença infecciosa, não contagiosa, que acomete ruminantes domésticos e silvestres, causada por um vírus do gênero *Orbivirus* da família Reoviridae, transmitida por vetores artrópodes do gênero *Culicoides*. O presente estudo representa o primeiro trabalho a realizar um inquérito sorológico da língua azul em rebanhos ovinos nas Mesorregiões de Campo das Vertentes e Sul e Sudoeste de Minas Gerais. Foram coletadas amostras de soro de ovinos de diferentes propriedades. As amostras de soro foram submetidas aos testes de imunodifusão em gel de ágar (IDGA) e ensaio de imunoadsorção enzimática por competição (cELISA). Ao todo 303 amostras de soro foram submetidas ao IDGA e cELISA. Dessas amostras, 164 (54,13%) foram positivas na técnica de IDGA e 171 (56,44%) positivas na técnica de cELISA, havendo concordância quase perfeita entre as técnicas (índice kappa = 0,887). Em todas as propriedades visitadas, foram encontrados animais positivos no rebanho. Animais adquiridos de propriedades das Mesorregiões estudadas, tiveram mais chances de serem positivos nos testes de IDGA e cELISA do que animais adquiridos de propriedades de outras Regiões do Brasil (p<0,001). Esses resultados sugerem que o vírus da língua

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azul encontra-se disseminado em ovinos nas Mesorregiões de Campo das Vertentes e Sul e Sudoeste de Minas Gerais.

TERMOS DE INDEXAÇÃO: Inquérito sorológico, vírus da língua azul, ovinos, Minas Gerais, Brasil, *Orbivirus*, diagnóstico, sorologia, ovinocultura.

INTRODUCTION

The bluetongue (BT) disease is caused by a virus of the genus Orbivirus of the family Reoviridae, transmitted by arthropods of the genus Culicoides, especially in the hot and humid times of the year, which favor the proliferation of these vectors (Maclachlan 2011). Currently, 26 serotypes of the bluetongue virus (BTV) are recognized worldwide (OIE 2018), grouped according to their serological reactivity. BTV is capable of infecting several domestic and wild ruminants, including sheep, goats, cattle, buffalo, and deer. The clinical form of the disease is most commonly seen in sheep and deer (Sánchez-Cordón et al. 2013, Ruiz-Fons et al. 2014).

Several serological surveys carried out in cattle, sheep, and goats in different states of Brazil have shown that the Southeast (Lobato et al. 2001, Konrad et al. 2003), Midwest (Tomich et al. 2009, Dorneles et al. 2012) and North regions (Abreu 1982) have a high number of seropositive animals. The South (Costa et al. 2006) and Northeast (Melo et al. 2000, Silva 2002, Souza et al. 2010) regions have meager seroprevalence rates. The low rates of seropositivity observed in the Northeast and South Regions may be associated with the unfavorable climate of the semi-arid region and more severe winters in these two regions, which would make it difficult to multiply or maintain the vector (Melo et al. 2000, Costa et al. 2006).

From the diagnosis of BTV, serotype 4, in clinical disease in a sheep herd in the Mesoregion of Campo das Vertentes in Minas Gerais (Lima et al. 2016), it was noticed that the virus is present in sheep in the state. However, serological data on the virus presence in the Mesoregions of Campo das Vertentes and South and Southwest of Minas Gerais were not known until then. This study aimed to be the first serological survey of the BTV in these mesoregions.

MATERIALS AND METHODS

This work is duly registered with the Ethics Committee on the Use of Animals of the "Universidade Federal de Lavras" (CEUA-UFLA) under number 023/2013.

Sample collection locations. Serum collection was performed on sheep farms in the Mesoregions of Campo das Vertentes and South and Southwest of Minas Gerais during the period from August 2015 to October 2016. A preliminary survey with the "Instituto Mineiro de Agropecuária" (IMA) highlighted 22 properties that develop sheep farming in the regions close to the municipality of Lavras, with a herd that varies from 10 to 1000 sheep. The properties with the highest number of adult animals were selected for the collection of serum samples. From the contact with the breeders, the collection was carried out in eight properties in the municipalities of Santo Antônio do Amparo, São João Del Rey, Nazareno, Ritápolis, Cambuquira, Soledade de Minas and two properties in Nepomuceno. At each property, blood samples were collected from at least 30% of the herd or the entire herd when it was less than 50 animals, in addition to information about the breeding system, access to veterinary assistance, and the animals' place of origin.

Search for antibodies against BTV in serum samples. Sample collection was performed after the physical containment of the animals, with blood drawn from the jugular vein in blood collection tubes without anticoagulants. The samples were transported refrigerated to the "Universidade Federal de Lavras" where they were centrifuged. The serum was stored, in duplicate, in 1.5ml microtubes, and frozen at -20°C until the exams were performed.

The serum samples of each animal were sent to the Animal Virology Research Laboratory of the "Departmento de Medicina Veterinária Preventiva" at the "Universidade Federal de Minas Gerais" (UFMG) and to the Bovine Virus Laboratory of the "Instituto Biológico de São Paulo". Then, it was performed, respectively, the tests of IDGA and cELISA, for the detection of antibodies against BTV.

cELISA. To perform cELISA, the commercial Bluetongue Virus Antibody Test Kit VMRD® Kit was used, following the manufacturer's instructions. For each sample, 25μL of the serum to be tested was used. The samples, positive (present in the kit), and negative controls were incubated at room temperature for 15 minutes. Then, 25μL of the antibody-peroxidase conjugate was added to each well. The plate was incubated for another 15 minutes at room temperature. After three washing steps, 50μL of the substrate solution was added for a 10-minute incubation period. Then 50μL of stop solution was added, and the plates were read in an ELISA reader model Thermo Labsystem Multiskan Ascent with 620nm filter to obtain the optical density (OD) values. The samples were considered positive when their OD values corresponded to less than 50% of the average OD values of the negative controls, using two standard deviations (two negative and two positive controls).

IDGA. To perform the IDGA technique, 0.85% sodium chloride (NaCl) solution and agarose at a final concentration of 0.9% in deionized water, deposited on circular acrylic plates, were used. Seven rosettes with seven holes each were drilled on each plate. The antigen was placed in the central well and the positive control serum in three other alternating wells in the amount of 30 μL in each well. In the remaining wells, 30 μL of the serum samples to be tested were placed. The plates remained in a humid chamber with 0.3% magnesium sulfate for 48 hours at room temperature. Then, the plates were observed with the aid of indirect light in a dark field. The antigen and positive control serum came from the UFMG Veterinary School.

Statistical analysis. Statistical analyzes were performed using the Epi-info ™ 7 program. The agreement between the cELISA and IDGA techniques was determined using the kappa coefficient (Thrusfield 2004). The Chi-square test was used to associate the results of the serology of sheep with the origin of animals (Thrusfield 2004).

RESULTS AND DISCUSSION

Of the 303 sheep serum samples analyzed, 171 (56.4%) were positive in cELISA (Table 1) and 164 (54.1%) in IDGA (Table 2). The results obtained from serological tests show that all properties were positive - the virus is not only present but also has already circulated in all properties visited in this study; this shows how the virus is spread in the regions close to the municipality of Lavras.

This work was the first serological survey carried out in the Mesoregions of Campo das Vertentes and South and Southwest of Minas Gerais. Other studies carried out in the state of Minas Gerais (Lobato et al. 2001, Laender 2002), also show that the Blue Tongue virus can be found in other Mesoregions of the state. The positivity values found in this study (56.44% and 54.13%) are similar to those observed in

other studies carried out in Minas Gerais, in the Mesoregions of Norte de Minas, Jequitinhonha and Vale do Mucuri, 58.6% by Laender (2002) and 65% by Lobato and collaborators (2001).

The wide distribution of this virus in the state is a characteristic of the Southeast region of Brazil. In this region, the climate and the sparse rains throughout the year, guarantee adequate humidity and temperature for the proliferation of the vector mosquito (Laender 2002). The large cattle herd in the region may be responsible for the maintenance of the virus since this species works as a reservoir of the virus. Studies in the north and northeast regions of Minas Gerais show a high prevalence of antibodies against BTV in cattle (Konrad et al. 2003). However, there are no serological surveys for BTV in this species in the regions studied in this work.The value of the kappa index between the results of the techniques of IDGA and cELISA (Table 3) demonstrated an almost perfect agreement between the techniques (Thrusfield 2004). This value was higher than other values found in the literature, which showed moderate (Venditti 2009) and substantial (Nogueira 2008) agreement. Although the OIE has prescribed the IDGA technique since 1982, it recommends the use of cELISA to confirm positive results to exclude possible cross-reactions with other Orbiviruses, in addition to being a more sensitive technique (OIE 2018). However, the agreement value shows that the AGID technique can still be used as an important method of seroepidemiological surveillance.

The eighth property had the lowest number of positive animals (22.31%). The large number of animals collected was

Table 1. Distribution of the number and percentage of reactive sheep in the cELISA test to detect antibodies against BTV, according to the properties of Campos das Vertentes Mesoregions and South and Southwest of Minas Gerais

Properties	No. of analyzed samples	Total adult sheep by property	cELISA results		% of positives
			Positive	Negative	
P1	29	91	18	11	62.07%
P2	12	12	6	6	50.00%
P3	16	16	16	0	100.00%
P4	24	79	17	7	70.83%
P5	44	134	43	1	97.73%
P6	16	16	12	4	75.00%
P7	32	32	30	2	93.75%
P8	130	400	29	101	22.31%
Total	303	780	171	132	56.44%

Table 2. Distribution of the number and percentage of reactive sheep in the IDGA exam for detection of antibodies against BTV, according to the properties of Campos das Vertentes Mesoregions and South and Southwest of Minas Gerais

Properties	No. of analyzed samples	Total adult sheep by property	IDGA results		% of positives
			Positive	Negative	
P1	29	91	16	13	55.17%
P2	12	12	6	6	50.00%
P3	16	16	16	0	100.00%
P4	24	79	15	9	62.50%
P5	44	134	42	2	95.45%
P6	16	16	12	4	75.00%
P7	32	32	28	4	87.50%
P8	130	400	29	101	22.31%
Total	303	780	164	139	54.13%

Table 3. Comparison of the results obtained between the cELISA and IDGA techniques against BTV of 303 sheep serum collected in the Campos das Vertentes Mesoregions and South and Southwest of Minas Gerais

	IDGA positive	IDGA negative	Total	Kappa	IC 95%	P
cELISA positive	159	12	171			
cELISA negative	5	127	132	0.887	0.774; 0.999	<0.001
Total	164	139	303			

Table 4. Positive and negative sheep using the cELISA and IDGA techniques, considering the region of origin of 303 sheep collected in the Campo das Vertentes Mesoregions and South and Southwest of Minas Gerais

	Origin of the sheep	Serological result		Total	Odds ratio	CI (95%)	p value*
		Positive	Negative				
cELISA	Own region	114	27	141	7.8	4.58; 13.2	<0.001
	Other regions	57	105	162			
IDGA	Own region	107	34	141	5.8	3.51; 9.59	<0.001
	Other regions	57	105	162			

* Chi-square test.

due to the large herd of this property, which had sheep raised intensively for milk production. This creation had a more advanced degree of technification, hygiene, and veterinary assistance for the sheep, in addition to fans for air circulation in the animals' confinement area. Better hygiene at the animals' location was associated with a protective factor against BT (Alves et al. 2009). It may be related to the smaller number of positive animals on this property.

Regarding the origin of the animals purchased on each property, 141 animals (46.5%) came from other producers in the region itself (Mesoregions of Campo das Vertentes and South and Southwest of Minas Gerais). In comparison, 162 animals (53.5%) were acquired from the Regions of Paraná, Santa Catarina, and Goiás. Table 4 compares these data with the animals' positivity in the IDGA and cELISA tests.

As noted, in both tests, there was a significance for animals from the region itself having more chances of being positive than animals acquired from other regions. This fact may be related to the lack of control in the transit of these animals between the properties. Much of the obtaining of new animals takes place among the region's producers, without any type of veterinary supervision or serological surveillance. Besides, there are a large number of positive animals in the Mesoregions of Campo das Vertentes and South and Southwest of Minas Gerais. The property with the lowest number of positive animals (P8) frequently acquires its animals from Santa Catarina, Southern Region of Brazil, which, according to previous serological surveys, has low animal positivity (Melo et al. 2000, Costa et al. 2006).

CONCLUSIONS

Bluetongue virus (BTV) circulates in the sheep herd in the Mesoregions of Campo das Vertentes and South and Southwest of Minas Gerais state.

All of the properties studied in the region show positive animals for antibodies against BTV, and most of these properties demonstrate high values of positive animals in the techniques of IDGA and cELISA, with an almost perfect agreement between the techniques.

Besides, animals acquired among producers in the studied Mesoregion are more likely to be positive, which highlights the importance of good practices in animal transit to help control the spread of BTV.

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Effect of different doses of dexamethasone on labor in Santa Inês ewes and its influence on the onset of labor and the expulsion of fetal membranes[1]

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ABSTRACT.- Santos E.S., Bittencourt R.F., Ribeiro Filho A.L., Dantas F.T.D, Alves A., Carvalho J.V.G.S. & Ayres M.C.C. 2020. **Effect of different doses of dexamethasone on labor in Santa Inês ewes and its influence on the onset of labor and the expulsion of fetal membranes**. *Pesquisa Veterinária Brasileira 40(4):266-270.* Setor de Reprodução, Universidade Federal da Bahia, Av. Adhemar de Barros, Ondina, Salvador, BA 40170-110, Brazil. E-mail: rfb@ufba.br

The induction of labor aims to concentrate births to follow up better the parturient and the first care to the neonates. However, even if the labor induction technique with dexamethasone administration has been successfully described since the late 1970s, few studies report the technique of birth development and neonatal vitality in Santa Inês sheep. This study aimed to evaluate the efficiency of dexamethasone use in two doses (8 and 16mg) in labor induction of Santa Inês ewes at 145 days of gestation and to evaluate its effects on the birth characteristics. In this study, 58 ewes were used, raised in an extensive system in the experimental farms of UFBA, with confirmation pregnancy after fixed-time artificial insemination or controlled breeding. These female ewes were separated into three groups according to the dose of dexamethasone administered (G1 = 0mg, G2 = 8mg, and G3 = 16mg). From these births, 79 lambs were born. This study analyzed the period from induction of labor to birth, fetal presentation at birth, the weight of the placenta, and the period for placenta expulsion. The data were analyzed by the Statistical Analysis System (SAS v.9.1.3®, 2002), and the significance level considered for all analyzes was 5%. Births of induced groups occurred on average at 48.4±22.17 hours after induction, while the females with non-induced labor gave birth 131.96±41.9 hours on average after the placebo application ($P<0.05$), confirming the efficiency of both doses for induction of labor. The period from induction to birth did not differ ($P>0.05$) between the doses used. There were no differences in delivery about the fetal static relation, time to placental attachment, and weight. With this study, it can be concluded that the induction at 145 days of gestation with eight or 16mg of dexamethasone is a useful technique and does not alter the labor in Santa Inês sheep.

INDEX TERMS: Dexamethasone, labor, Santa Inês ewe, fetal membranes, sheep, parturient, corticoid, reproduction biotechnology, ewe.

RESUMO.- [**Efeito de diferentes doses de dexametasona para a indução do parto em ovelhas Santa Inês e sua influência sobre as características do parto.**] A indução do parto visa concentrar os nascimentos para melhor acompanhamento das parturientes e primeiros cuidados aos neonatos. Contudo, mesmo que a técnica de indução de parto, com administração de dexametasona, tenha sido descrita com sucesso desde o final da década de 70, existem estudos escassos que relatam a influência desta técnica sobre o parto em ovinos da raça Santa Inês. Dessa forma, o objetivo do estudo foi avaliar a eficácia da dexametasona em duas doses (8 e 16mg), para a indução do parto de ovelhas Santa Inês com 145 dias de gestação e avaliar os seus efeitos nas características de desencadeamento e finalização do parto. Para este estudo foram utilizadas 58 ovelhas, criadas em sistema extensivo nas

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fazendas experimentais da UFBA, com prenhez confirmada após inseminação artificial em tempo fixo ou monta controlada. Essas fêmeas foram separadas em três grupos, de acordo com a dose de dexametasona administrada (G1 = 0mg, G2 = 8mg e G3 = 16mg). Destes partos nasceram 79 cordeiros. Foram avaliados o período em horas da indução do parto aos nascimentos, a apresentação fetal ao nascimento, assim como o peso da placenta e o período para o delivramento. Os dados foram analisados pelo pacote estatístico Statistical Analysis System (SAS v.9.0®, 2002) sendo considerado para todas as análises o nível de significância de 5%. Os nascimentos dos grupos induzidos ocorreram em média com 48,4±22,1 horas após a indução, enquanto que as fêmeas com parto não induzido pariram em média 131,96±41,9 horas após aplicação do placebo (P<0,05), confirmando a eficácia de ambas as doses para indução do parto. O período da indução até o parto não diferiu (P>0,05) entre as doses utilizadas. Não ocorreram diferenças no parto em relação à estática fetal, tempo para o delivramento e peso da placenta nos diferentes grupos. Com este estudo, conclui-se que a indução de parto em ovelhas aos 145 dias de gestação com oito e 16 mg de dexametasona é uma técnica eficaz e que não altera o trabalho de parto nas ovelhas da raça Santa Inês.

TERMOS DE INDEXAÇÃO: Dexametasona, indução de parto, parto, ovelhas Santa Inês, ovinos, parturiente, corticoide, biotecnologia da reprodução.

INTRODUCTION

Among the benefits achieved with labor induction, there is the optimization of labor available on the farm, reduced time in observing the parturient ewes, and assisting females in labor and neonates (Özalp et al. 2017). The technique aims to reduce economic losses by offering assistance to dystocia that occurs in approximately 25% of sheep deliveries (Dwyer & Bünger 2012). These actions minimize the mortality of mothers and newborns (Santana et al. 2010).

Among the labor-inducing drugs is the glucocorticoid dexamethasone (Kastelic et al. 1996, Ingoldby & Jackson 2001). A 16mg dose (Ingoldby & Jackson 2001) was used from the 137th day of gestation of the sheep, with births occurring approximately 34 to 50 hours after the administration of this corticosteroid (Sir & Bartlewski 2010).

Exogenous glucocorticoids can induce birth in sheep by mimicking the action of cortisol produced by the fetal adrenal cortex, acting as an initial trigger for a hormonal cascade that triggers birth (Zoller et al. 2015). Corticosteroids still promote fetal maturation, acting mainly on pulmonary maturation (Liggins et al. 1969, Fowden et al. 1995, Ballard et al. 1997, Zaremba, et al. 1997), in addition to activating the maturation of brown adipose tissue, making lambs born with a week less gestation capable of promoting thermoregulation (Bispham et al. 2002).

The labor induction technique with dexamethasone administration has been successfully described since the late 1970s. However, few studies report the influence of this technique on Santa Inês sheep, as well as the efficacy of doses lower levels of dexamethasone for inducing labor and its influence on the onset and completion of labor. In this context, the objective of this study is to evaluate the efficacy of dexamethasone in two doses (8 and 16mg) for the birth induction of Santa Inês ewes at 145 days of gestation and to analyze its effects on delivery (triggering and termination).

MATERIALS AND METHODS

Experiment location. The experiment was carried out on experimental farms belonging to the Federal University of Bahia, located in the municipalities of Entre Rios and São Gonçalo dos Campos. The farm located in the municipality of Entre Rios is at latitude 11°56'31" South and longitude 38°05'04" West, at an altitude of 162m. It has an average annual rainfall of 1,251mm and a hot, semi-humid climate. The farm located in the municipality of São Gonçalo dos Campos is located at latitude 12°25'58'' South and longitude 38°58'01" West, at an altitude of 234m and has an average annual rainfall of 1,079mm and a hot, semi-humid climate.

Animals and management. The study was carried out according to ethical precepts recommended by the National Council for Animal Experimentation Control (CONCEA), after approval by the Ethics Committee on the Use of Animals (CEUA) of the "Escola de Medicina Veterinária e Zootecnia" of the "Universidade Federal da Bahia" (UFBA), under protocol no. 23/2018.

For this study, 58 sheep were used, 38 sheep from the São Gonçalo farm, and 20 from the Entre Rios farm. The animals were initially submitted to clinical, gynecological, and ultrasound examinations. The transrectal ultrasound was performed with the aid of a multifrequency linear transducer at a 7.5MHz frequency (Mindray Z5, Shenzhen, China). Clinically healthy females were considered able to participate in the experiment, which at the time of the evaluation did not present any abnormalities in the reproductive tract and absence of pregnancy.

The females were subjected to extensive breeding, maintained on pasture (mixed pasture) with water and mineral salt ad libitum. Before the experiment started, all animals were vaccinated against clostridiosis and dewormed.

Estrus synchronization protocol and return to heat. After the ultrasound evaluation, the animals were submitted to a protocol of estrus synchronization and ovulation in October 2016, in both experimental farms. Initially, on a random day of the estrous cycle (Day 0), all animals received an intravaginal device with 0.33g of progesterone (CIDR®, Pfizer, Guarulhos, Brazil) with which they remained for eight consecutive days. The intramuscular (IM) administration of 300IU of equine chorionic gonadotropin (eCG, Novormon®, MSD Animal Health, São Paulo, Brazil), and 0.125mg of sodium cloprostenol (PGF2α, Ciosin®, MSD Animal Animal, São Paulo, Brazil) was performed on the eve of the removal of the devices. On the ninth day, 200IU of human chorionic gonadotrophin (hCG, Chorulon®, MSD Animal Health, São Paulo, Brazil) was applied via IM, 32 hours after the removal of the CIDR. Laparoscopic intrauterine inseminations were performed, with frozen semen from a breeding center, on the tenth day (approximately 56 hours after removal of the intravaginal device). The parameters were above those recommended by the Brazilian College of Animal Reproduction (CBRA) (Henry & Neves 2013).

From the 14th to the 18th day after the fixed-time artificial insemination (TAI) protocol, the return to estrus of the registered sheep was observed, and a Santa Inês breeder covered the females that presented estrus. The date of the payment was duly registered. Before the beginning of the reproductive program, the male underwent a clinical and andrological evaluation, when it was confirmed that the ejaculation was within the standards recommended by CBRA.

After 30 days of TAI or coverage, the sheep were evaluated by ultrasound examination to confirm pregnancy with a multifrequency linear transducer (7.5 MHz, Mindray Z5, Shenzhen, China).

Labor induction. At 145 days after coverage or insemination, all females with confirmed pregnancy at 30 days were reassessed for body condition, mammary gland, absence of reproductive diseases, and systemic diseases. Also, to transrectal ultrasound examination, with a multifrequency linear transducer (7.5 MHz, Mindray Z5, Shenzhen, China), for obstetric and fetal viability assessment. The sheep included in the labor induction group had a body condition between 2.5 and 3.5 (Jefferies 1961), were healthy and with pregnancy compatible with the gestational period.

Right after the evaluations, during the period of induction of birth, the sheep were kept in closed facilities, equipped with drinking fountains and troughs, with water, hay, and mineral salt ad libitum, to facilitate the division of groups, observation, and assistance to parturients and neonates.

The females were divided into three groups under a completely randomized design (CRD). At 145 days of pregnancy, the G1 group of sheep (control) received 0.9% NaCl solution intramuscularly (n=19). G2 (n=20) was administered 8mg of dexamethasone (Azium®, MSD) and G3 (n=19) received 16mg of dexamethasone (Azium®, MSD). From 24 hours after the administration of dexamethasone, the animals were monitored continuously until the 11th day after induction. There was no human interference between the expulsion of the fetus and maternal recognition, except when there was dystocic delivery.

Labor evaluation. Labor was assessed in hours, from induction with 8 and 16mg dexamethasone, at 145 days of gestation, until birth (BIT). In the fetal expulsion phase, the static presented by the fetus was observed. The time interval, in minutes, between births and complete expulsion of the placenta (IEP), was also evaluated. Then the placenta had its weight measured on a semi-analytical scale with a capacity of up to 4200g, fractioned every 0.01g.

Statistical analysis. For the analysis of the variables, the statistical program Statistical Analysis System (SAS v. 9.1.3®, 2002) was used. Data consistency and descriptive analysis (means and standard deviation) of the characteristics of interest to the study were performed using the MEANS Procedure (PROC MEANS). The Kruskal-Wallis test was used to observe differences between groups (G1 = control, G2 = 8mg, and G3 = 16mg dexamethasone) for the variables that did not show normal distribution by the Shapiro-Wilk test (labor induction interval in minutes and placental elimination). The values were adjusted and compared by the Bonferoni test, in case of significant differences in Kruskal. For variables with a normal distribution (placenta weight), analysis of variance (ANOVA) was performed using the "General Linear Model" Procedure (PROC GLM). For the comparison between the groups studied, it was performed the Tukey-Kramer test. For all analyzes, a significance level of 5% was considered.

RESULTS AND DISCUSSION

Fifty-eight sheep participated in this study. Of these, 39 underwent dexamethasone-induced labor protocols, and all responded to the induction protocol. The births in the induction groups occurred on average at 147 days of gestation (48.4±22.1 hours after the application of the drug). In total, 79 lambs were born. There was no effect of the site (Experimental Farms of Entre Rios and São Gonçalo) on the studied parameters (P>0.05). Doses of 8 and 16mg of dexamethasone were effective in inducing labor in Santa Inês ewes at 145 days of gestation. Induction labor groups G2 (8mg) and G3 (16mg) were similar to each other (P>0.05) and different from the non-induced group G1 (0mg) (P<0.05) about the time interval, in hours, between the day of the induction and the births (BIT) (Table 1).

The G1 animals gave birth on average at 150 days of gestation (132.0±41.0 hours after induction of labor, at 145 days of pregnancy), which corresponds to the physiological behavior for the species. Silva et al. (1995) and Machado & Simplício (1998) found that, for the Santa Inês breed, delivered at 151.66 and 149.80 days of gestation, respectively.

The first delivery of G1 was recorded 63 hours after administration of the placebo (at 145 days of gestation), that is, a sheep, even though it did not belong to the induction groups, had the delivery at 147 days of gestation. The last delivery in this group was recorded at 203 hours after the placebo, at 154 days of gestation. Thus, the animals belonging to G1 had a variation of 7 days from the first to the last birth, which would require the mobilization of a working group to monitor all parturients and newborns for approximately three times the period of the groups that had induced childbirth, burdening this type of service.

The animals in G2 and G3 calved on average at 147 days of gestation (48.4±22.1h), demonstrating that the dexamethasone used in both doses was efficient in inducing and concentrating labor. It was also observed that, under the conditions of this study, the administration of dexamethasone decreased the delivery date considered physiological by an average of 3 days.

The sheep of G2 that were treated with 8mg of dexamethasone had an interval between the first and the last birth of 75.8 hours. Three ewes calved within 17-19 hours after induction, that is, at 146 days of gestation, 45% (9/20) of births that occurred in the first 48 hours after induction and 70% (14/20) of ewes gave birth within 72 hours, and the last delivery in this group took place 93.3 hours after induction. The G3 treated with 16mg of dexamethasone showed a 39.1 hours interval from the first to the last birth. Three ewes calved in the first 24 hours (146 days of gestation), the other 16 ewes (84.2%) calved within 25-53 hours after induction. Sixty-three percent (63%, 13/19) of births took place between 40 and 53 hours, demonstrating that induction of labor with 16mg dexamethasone concentrates births 24 to 53 hours after

Table 1. Mean values ± standard deviation of the time between labor induction and induction of births, expulsion and placental weight in Santa Inês ewes undergoing labor induction with dexamethasone at 145 days of gestation

Treatment	LDD (day)	BIT (hours)	Placental detachment (min)	Placental weight (g)
G1 (0mg) N=19 sheep	155	131.96 ±41.9[a]	233.8±59.0	375.5±97.4
G2 (8 mg) N=20 sheep	149	54w.35±26.239[b]	230.7±50.9	389.5±142
G3 (16 mg) N=19 sheep	148	40.97±12.47[b]	239.3±43.8	310.0±97.4

G1 = Control group, G2 = labor induction with 8mg dexamethasone group, G3 = labor induction with 16mg dexamethasone group, BIT = birth induction time interval in hours, LDD = group's last day of delivery in days; a,b Values followed by distinct lowercase letters in the same column differ from each other by the Kruskal-Wallis test, adjusted by the Bonferoni test (P<0.05).

induction, with a higher frequency of births in the last 13 hours of this interval.

The dose used in G3, 16mg dexamethasone per animal, was chosen according to studies carried out previously by Kastelic et al. (1996) and Ingoldby & Jackson (2001), establishing themselves as the standard dose for induction of labor in sheep (Sir & Bartlewski 2010). In this sense, the present study also evaluated in G2 whether the administration of half the conventional dose (8mg) would have the potential to induce labor with the same efficiency. Tsiligianni et al. (2008) were able to induce the birth process with just two mg and one and a half mg of dexamethasone at 146 days of gestation, which suggests that induction of labor in ewes with advanced gestation, close to the date of physiological birth, may be performed with low doses. However, the same authors observed dystocia in some births, which made it necessary to use obstetric maneuvers. There was also the occurrence of stillbirths and placental retention in a 10% average among induced groups, which justified the use of dexamethasone. In the present study, it was used a higher dose than that reported by Tsiligianni et al. (2008) in an attempt to avoid occurrences of the problems above.

The mean time intervals between administration of dexamethasone and births in hours were close to those reported by Kastelic et al. (1996), Balios et al. (2008), Santana et al. (2010). They found 51, 59.7, and 49.38 hours, respectively.

Kastelic et al. (1996), when inducing the birth of 53 Suffolk ewes at 140 days of gestation with 16mg of dexamethasone associated or not with cloprostenol (CLOP), observed mean intervals (hours) between administration and births: 147.4 hours for ewes with uninduced deliveries, 51 hours for dexamethasone-induced deliveries, and 62.6 hours for dexamethasone and CLOP induced animals. These results demonstrated that the use of CLOP (PG synthetic) does not bring benefits to the induction of labor program in sheep. The corpus luteum (CL) of sheep at 90-100 days of gestation is functional and sensitive to prostaglandins. However, luteolysis is not enough to induce abortion (Weems et al. 1992), since, from the second trimester; the placenta becomes competent to secrete progesterone at levels sufficient to maintain pregnancy, even without the presence of CL (Purohit et al. 2012). Corticosteroids such as dexamethasone, flumetazone, betamethasone, or cortisol acetate perform labor induction effectively (Ingoldby & Jackson 2001, Purohit et al. 2012). Alternatively, by progesterone antagonists such as Aglepristone (Özalp et al. 2017), the latter has a high cost, which limits its use for this purpose.

Santana et al. (2010) also evaluated the use of dexamethasone to induce labor in Santa Inês ewes, at 144 days of gestation, using doses of 16 and 20mg, in addition to a control group. These authors reported intervals until birth (in hours) of 49.0 (±6.8), 78.0 (±7.2) and 122.0 (±37.2), respectively, without reports of placenta retain or labor dystocia, reinforcing the positive results obtained in previous studies with the dose of 16mg, not differing from the upper dose.

Labor induction with dexamethasone simulates the natural pathway: the glucocorticoid mimics the cortisol produced by the fetal adrenal cortex, acting as the trigger for the hormonal cascade that initiates labor (Silver 1992). Fetal cortisol induces the transformation of placental progesterone (P4) into estrogen (E2), resulting in a reduction in P4 and an increase in E2 levels. Estrogen induces the release of prostaglandins (PGs), mainly PGF 2α, PGE_2, and PGI_2, from the uterus. These promote luteolysis, increased placental vascular perfusion, myometrial contractions, and relaxation of the cervix and vaginal canal, triggering childbirth (Silver 1992, Jenkin & Young 2004).

Around 140 days of gestation, there is an increase in fetal cortisol, and important processes modulated by the cortisol released by the fetus occur, which promotes the neonate's maturity, preparing it for post-uterine life (Ingoldby & Jackson 2001). At 146 days of gestation, there is the peak of fetal cortisol, suggesting that even a small dose of dexamethasone would be enough to reinforce endogenous cortisol and trigger delivery in periods close to this (Tsiligianni et al. 2008), which explains the performance equivalent between G2 and G3.

As for fetal statics, 94.1% of the animals were born with the physiological fetal statics for the sheep species, and of these, a lamb (1.3%) was born with a posterior longitudinal presentation, in a twin birth female product belonging to the induction group with 8mg. The others were born with an anterior longitudinal presentation, dorsal position. These findings are under the normality of the species (Dwyer 2008); they demonstrate that dexamethasone-induced labors did not influence fetal statics.

Four sheep presented dystocia (6.9%). In these cases, the fetal statics of the dystocic births were the anterior longitudinal, dorsal position, and flexed attitude of the carpal joint. All dystocias occurred in uncomplicated deliveries of male products. Therefore 10.25% of simple deliveries and 10.5% of males were born in dystocic delivery, with two births in G1 and two in G2. In these deliveries, obstetric interventions were necessary to correct the attitude, and later, forced traction was performed. All products were born alive. Dwyer & Bünger (2012) observed a higher percentage of dystocia in simple delivery when the product is male. According to Dwyer (2003), male products are twice as prone to dystocia as females, attributing this finding to weight, since neonates from uncomplicated birth and males are the heaviest. The dystocia values of the present study are below the numbers described by Dwyer & Bünger (2012), who found in 3,252 animals born in uninduced births 25.1% of dystocia due to an abnormal fetal presentation.

The placental detachment occurred on average 4 hours after birth (234.6±51.3min) and did not differ between groups ($P>0.05$). Detachment time was similar to that found by Tsiligianni et al. (2008). Placental weight was the same in both groups ($P>0.05$). It is essential to mention that no female in this study had retained placenta, being considered as "retained placenta" the non-delivery of the organ eight hours after delivery (Jainudeen & Hafez 2004). Previous studies describe that in sheep, induction of labor with dexamethasone rarely promotes retention of the placenta (Rubianes et al. 1991, Kastelic et al. 1996, Ingoldby & Jackson 2001, Santana et al. 2010).

CONCLUSION

The use of dexamethasone in labor induction of Santa Inês ewes at 145 days of gestation, with 8 and 16mg of dexamethasone, was efficient for the concentration and labor induction, without promoting adverse effects concerning expulsion and fetal static, delivery, and placental weight.

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Uncommon anatomical sites and anatomopathological presentations of lymphoma in dogs[1]

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ABSTRACT.- Mazaro R.D., Luz F.S., Herbichi A., Lorenzetti D.M., Cogliati B. & Fighera R.A. 2020. **Uncommon anatomical sites and anatomopathological presentations of lymphoma in dogs.** *Pesquisa Veterinária Brasileira 40(4):271-283*. Departamento de Patologia, Universidade Federal de Santa Maria, Av. Roraima 1000, Santa Maria, RS 97105-900, Brazil. E-mail: anemiaveterinaria@yahoo.com.br

Lymphomas are the tumors most frequently associated with the death or euthanasia of dogs in most parts of the world. In dogs, they almost always occur as disseminated (multicentric lymphoma), gastrointestinal (alimentary lymphoma), or nodal mediastinal (mediastinal lymphoma) diseases. However, other uncommon presentations can occasionally occur. This study aimed to establish the prevalence of these unusual types of lymphoma and demonstrate to veterinary pathologists how they present pathologically. From a total of 100 cases of lymphoma in dogs diagnosed between 1965 and 2017, 16 cases (16/100) were considered by us as non-traditional presentations of the disease: follicular lymphoma, (5/100), peripheral T-cell lymphoma, NOS (2/100), angiocentric lymphoma (2/100), intravascular large T-cell lymphoma (2/100), lymphomatoid granulomatosis (1/100), anaplastic large-cell lymphoma (1/100), hepatosplenic T-cell lymphoma (1/100), and chronic small B-cell lymphocytic lymphoma, intermediate type (1/100). We hope that the results presented here can help veterinary pathologists to recognize such cases of "atypical lymphoma" in their diagnostic routines.

INDEX TERMS: Anatomical sites, anatomopathological, lymphoma, dogs, non-traditional lymphomas, atypical lymphomas.

RESUMO.-[Sítios anatômicos e apresentações anatomopatológicas incomuns do linfoma em cães.] Linfomas são os tumores mais associados a morte ou eutanásia de cães na maior parte do mundo. Nessa espécie animal ocorrem quase sempre como uma doença disseminada (linfoma multicêntrico), gastrintestinal (linfoma alimentar) ou nodal mediastinal (linfoma mediastínico), entretanto, ocasionalmente, outras apresentações bem menos comuns podem ser encontradas. O objetivo deste artigo é estabelecer a prevalência desses pouco usuais tipos de linfoma e demonstrar aos patologistas veterinários como eles se apresentam anatomopatologicamente. De um total de 100 casos de linfoma em cães diagnosticados entre os anos de 1965 e 2017, 16 casos (16/100) foram considerados como apresentações não tradicionais da doença: linfoma folicular (5/100), linfoma de células T periférico inespecífico (2/100), linfoma angiocêntrico (2/100), linfoma intravascular de grandes células T (2/100), granulomatose linfomatoide (2/100), linfoma de grandes células anaplásicas (1/100), linfoma hepatoesplênico de células T (1/100) e linfoma linfocítico crônico de pequenas células B – tipo intermediário (1/100). Esperamos que os resultados aqui demonstrados auxiliem patologistas

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veterinários a reconhecerem tais casos de "linfomas atípicos" em suas rotinas diagnósticas.

TERMOS DE INDEXAÇÃO: Sítios anatômicos, anatomopatológicas, linfoma, cães, linfomas raros, linfomas não tradicionais, linfomas atípicos.

INTRODUCTION

Lymphoma, also known as lymphosarcoma, is a malignant neoplasm originating from B lymphocytes, T lymphocytes, or null cells (non-B and non-T, natural killer cells - NK) that, except for the bone marrow, most commonly arise from organs/hematopoietic tissues, including lymph nodes or spleen, and mucosa-associated lymphoid tissue (MALT) (Valli 2007, Vezzali et al. 2009, Ponce et al. 2010, Swerdlow et al. 2017). In dogs, the annual prevalence of lymphoma in the United States is 13 to 24 cases per 100,000 animals (Dorn et al. 1987). In Europe, lymphoma accounts for 20.1% and 8.4% of cancers in males and females, respectively (Merlo et al. 2008). In Brazil, lymphoma represents 8.8% of all cancers affecting dogs, observed at necropsy, and accounts for 1% of the causes of death or reasons for euthanasia in this animal species (Fighera 2008), being more critical in males (13% of tumors responsible for spontaneous death or euthanasia) than in females (6% of tumors responsible for spontaneous death or euthanasia) (Flores 2016).

The most common presentation of lymphoma in dogs is characterized by widespread involvement of superficial and/or deep lymph nodes (generalized lymphadenopathy), concomitant or not with involvement of the spleen, liver and, less frequently, tonsils and bone marrow (multicentric lymphoma (Vail 2004, Couto 2010, Helfand & Kisseberth 2010, Fry & McGavin 2012, Vail et al. 2013, Fighera & Graça 2016). Macroscopically, in this form of presentation, lymph nodes are diffusely enlarged (lymphadenomegaly), whitish or variably reddish, soft and succulent, not allowing corticomedullary delimitation to the cut surface (diffuse pattern) (Vail 2004, Couto 2010, Helfand & Kisseberth 2010, Fry & McGavin 2012, Vail et al. 2013, Fighera & Graça 2016). The spleen and liver show diffuse swelling (diffuse hepatosplenomegaly) (Helfand & Kisseberth 2010, Fry & McGavin 2012, Fighera & Graça 2016). When the tonsils are affected, they lose their pleated conformation and stand out as bilaterally symmetrical, non-ulcerated masses (diffuse tonsilomegaly) (Fighera & Graça 2016). By highlighting the final stage of lymphoma, neoplastic lymphocytes released from the affected organs can colonize the bone marrow, leading to medullary involvement (Fighera et al. 2002).

The second most common presentation of lymphoma in dogs occurs when there is diffuse enlargement of the mesenteric lymph nodes (localized lymphadenomegaly), with a gross lesion pattern similar to the one previously described, and/or when segments of the gastrointestinal tract (GIT) present nodules of transmural, mucosal to serous infiltration, or diffuse segmental thickening of the mucosa or submucosa (alimentary lymphoma) (Vail 2004, Couto 2010, Helfand & Kisseberth 2010, Fry & McGavin 2012, Vail et al. 2013, Fighera & Graça 2016). Secondarily, due to neoplasm progression, the liver and regional lymph nodes may be affected (Fry & McGavin 2012).

The third but less commonly observed presentation of lymphoma in dogs involves the mediastinal lymph nodes and/or the thymus (Vail 2004, Couto 2010, Helfand & Kisseberth 2010, Fry & McGavin 2012, Vail et al. 2013, Fighera & Graça 2016). The lymph nodes appear diffusely enlarged with a gross pattern similar to that previously described, or as only a large homogeneously yellowish white, soft tumor mass occupying the mediastinum and, sometimes, a large part of the thoracic cavity (mediastinal lymphoma) (Helfand & Kisseberth 2010, Fry & McGavin 2012, Vail et al. 2013, Fighera & Graça 2016).

Regarding the histopathology of canine lymphoma, the diffuse architectural pattern is predominant over the nodular pattern, representing 87.7 to 98.8% of the diagnoses in the literature, indicating that follicular lymphoma, which so common in humans, is rare in dogs (Sueiro et al. 2004, Arespacochaga et al. 2007, Vezzali et al. 2009, Ponce et al. 2010). Based on the different lymphoma classification systems, a large microscopic variety of types and subtypes has been described (Valli et al. 2017). However, most lymphomas already diagnosed in dogs are represented by five recognized types and/or subtypes according to the histological criteria established by the World Health Organization (WHO) for the classification of hematopoietic tumors in animals (Valli 2002) as: 1) diffuse large B-cell lymphomas (47 [Ponce et al. 2010] to 54.4% [Vezzali et al. 2009]), which include centroblastic, immunoblastic, and B-cell rich T-cell histiocyte lymphomas; 2) lymphoblastic lymphomas (2.8 [Ponce et al. 2010] to 10.7% [Vezzali et al. 2009]), which may arise from B or T cells; 3) small B-cell lymphocytic lymphomas (1..8 [Ponce et al. 2010] to 7.8% [Vezzali et al. 2009]), which include chronic lymphocytic lymphoma and lymphoplasmocytic lymphoma ("immunocytoma"); 4) marginal zone lymphomas (10.8% [Ponce et al. 2010]); 5) peripheral T-cell lymphomas, not otherwise specified (NOS) (6.8 [Vezzali et al. 2009] to 13.6% [Ponce et al. 2010]).

Based on the information previously described, veterinary pathologists working in the field of dog disease diagnosis will often encounter mainly cases of multicentric lymphoma and, occasionally, cases of alimentary and mediastinal lymphoma. More rarely, however, these same pathologists may encounter unusual cases of lymphoma in their routines, in which: 1) only a single lymphoid organ other than the lymph node is affected (solitary lymphoma), 2) one or more non-lymphoid organs are affected (extranodal lymphoma), 3) an uncommon combination of lymphoid and non-lymphoid organs (mixed lymphoma), or 4) a gross presentation of lymphoma different from that traditionally described in the literature, i.e., lymphomas that do not occur as "diffuse organomegaly". Generally, these cases reflect distinct microscopy, which bears no resemblance to the traditional histopathological patterns previously considered common to the species (diffuse large B-cell lymphomas, lymphoblastic lymphomas, small B-cell lymphomas, marginal zone lymphomas, and peripheral T-cell lymphomas, NOS). Thus, lymphomas that occur in unusual anatomical sites and/or present anatomopathologically (macroscopically and/or at histopathology) are infrequently described in the medical and veterinary literature, according to each author, by the adjectives "unusual" or "rare". Therefore, this study aims to demonstrate to veterinary pathologists interested in canine oncopathology that, although such lymphomas are not very prevalent, they do occur, and their recognition is fundamental for excellent diagnostics in their necropsy or surgical pathology routines.

MATERIALS AND METHODS

Reports of the dog necropsies performed at the "Laboratório de Patologia Veterinária" of the "Universidade Federal de Santa Maria" (LPV-UFSM) between 1965 and 2017 were used to collect data on cases of lymphoma. For this study, we considered only cases in which the presentation of lymphoma was not classically observed i.e., 1) unusually affecting anatomical sites; 2) gross patterns little or not described in the literature, and 3) histological types or subtypes reported only occasionally in dogs. From the necropsy reports selected for the study, we collected macroscopic and histopathological information about each case. When possible, the cases were reassessed macroscopically using images from the LPV-UFSM. Subsequently, all cases were histologically reevaluated by making slides from the cut-out hematoxylin-eosin (HE)-stained, paraffin-embedded tissues. The definitive diagnosis of the tumor was based on the World Health Organization (WHO) histological criteria for the classification of hematopoietic tumors in humans (Swerdlow et al. 2017) and for the classification of hematopoietic tumors in domestic animals published by Armed Forces Institute of Pathology (AFIP) (Valli 2002), including its most recent update (Valli et al. 2017).

In order to determine the origin of the neoplastic lymphocytes and for classification purposes, at least one affected tissue from each of these cases was selected and analyzed by immunohistochemistry (IHC). The technique employed was B-lymphocyte immunostaining using CD79 (Clone JCB117, Dako Cytomatic, mouse-produced) or CD20 (RB9013-P, ThermoScientific, rabbit-produced) antibodies and T-lymphocyte immunostaining CD3 (Clone F7.2.38, Dako Cytomatic, mouse-produced) antibody. IHC was performed according to the following protocol: After tissue deparaffinization and rehydration, antigen recovery using Tris-EDTA solution (pH 9.0) was performed in microwave oven at high power for 10 min. Endogenous peroxidase blockade was performed using 3% hydrogen peroxide for 20 min. Nonspecific reactions were blocked using Protein Block at room temperature for 10 min. The primary antibody was anti-CD79 monoclonal (prediluted) or anti-CD20 polyclonal (1:800 dilution) (ScyTek - ATG 125), and anti-CD3 monoclonal antibody (prediluted). Both antibodies were incubated in oven at 37 °C for 60 min or in refrigerator at 7 °C for 16 h (overnight). Secondary antibodies and Horseradish peroxidase (HRP) polymer were used consecutively incubated at 25 °C for 30 min and developed by adding 3-3'diaminobenzidine (DAB) tetrachloride chromogen for 5 min. Counterstaining was performed using Harris Hematoxylin. Dog lymph nodes and tonsils were used as positive and negative controls, respectively. Negative control was obtained by omitting the primary antibody using only the antibody diluent.

RESULTS

All cases included in this study occurred in the municipality of Santa Maria, located in the midwestern mesoregion of the state of Rio Grande do Sul (29°32'52''; 53°42'10''), popularly known as central region. From 1965 to 2017, 9020 dogs were necropsied at the VPL-UFSM. Of these, 100 died or were euthanized, and lymphoma was diagnosed in approximately 1% at necropsy. Of the 100 necropsied dogs, 16 cases (16/100) were considered by us as "nontraditional" lymphoma presentations, featuring eight lymphoma types and/or histological subtypes, namely, follicular lymphoma (5/100), peripheral T-cell lymphoma, NOS (2/100), angiocentric lymphoma (2/100), intravascular large T-cell lymphoma (2/100), lymphomatoid granulomatosis (2/100), anaplastic large-cell lymphoma (1/100), hepatosplenic T-cell lymphoma (1/100), and B-cell chronic small lymphocytic lymphoma, intermediate type (1/100). The anatomopathological findings of each case will be described below.

Follicular lymphoma

Of all dogs diagnosed with lymphoma (n=100), five (5/100) corresponded to follicular lymphoma. All necropsied dogs presented diffuse and markedly enlarged superficial and deep lymph nodes (generalized lymphadenomegaly), whitish to slightly reddish, and soft to the touch. When cut, they were equally soft and allowed a minimal amount of translucent liquid to flow. The cut surface was damp, shiny, white or mottled white and red, but characteristically dotted with tens to hundreds of white nodules of varying size, multifocal or coalescing (nodular pattern) that did not allow corticomedullar delimitation (Fig.1). In all cases, the spleen was diffuse and markedly enlarged (diffuse splenomegaly), which was evidenced by the rounding of its edges and displacement of its anatomical position in the abdominal cavity. Three gross patterns were identified on the natural surface. In one of them, observed in three of the five cases, the surface was red, smooth, and filled with myriads of multifocal or coalescing white dots of varying sizes (nodular pattern). In another pattern, observed in one of the five cases, the surface was homogeneously red and smooth, but without the white dots described in the previous three cases. In the last pattern, also observed in one of the five cases, the surface was markedly irregular, dotted with hemispherically elevated white dots. Despite these three distinct patterns, at the cutting surface, the spleen was always characterized by a white or pinkish, bright or glassy, random, multifocal, or coalescing dotted line, macroscopically similar to that described for diffuse white pulp hyperplasia (Fig.1).

At histopathology, all cases presented, both in the lymph nodes and the spleen, a dense cell population, with an organization pattern characterized by nodules of variable dimensions, moderately or markedly defined, not encapsulated, absence of mantle zone, and without noticeable stroma (Fig.2). In the lymph nodes, these nodules invaded the capsule and obliterated the subcapsular and peritrabecular sinuses. Carefully arranged neoplastic nodules, divided or not by delicate connective tissue trabeculae, extended from the cortical to the medullary region, almost entirely replacing the parenchyma and the lymphoid follicles, including their polarization, and the hematopoietic components of the cord could not be visualized. In the spleen, the parenchyma was similarly replaced by closely related and identical nodules. These nodules were composed of neoplastic lymphocytes distributed in the form of a mantle and separated by splenic structures, sometimes by discrete remaining segments of the red pulp. Predominantly, neoplastic lymphocytes had a moderate amount of homogeneous and eosinophilic cytoplasm. Rounded oval nuclei consisting of loose or vesicular chromatin, including one to four prominent, mainly peripheral, large eosinophilic nuclei, often in contact with a nuclear membrane, were observed. At IHC, neoplastic cells were immunostained for B lymphocytes (anti-CD20), and small groups of normal lymphocytes distributed randomly throughout the remaining tissue showed positivity for the T lymphocyte marker (anti-CD3).

Peripheral T-cell lymphoma, NOS

Two cases of peripheral T-cell lymphoma were found in this study (2/100). In one case (1/100), there was concomitant involvement of the lungs, stomach, liver, and lymph nodes (hepatic, gastric, and pancreatic). At necropsy, pulmonary involvement stood out compared with those of the other affected organs. Neither lung collapsed upon opening of the thoracic cavity. They were heavy, moist, shiny, reddish, and soft. The middle lobe of the right lung was homogeneously reddish, opaque, and firm to the touch (pulmonary hepatization). Additionally, there were multifocal nodules distributed throughout the natural surface of the pulmonary lobes, except for the consolidated lobe, as well as several non-elevated and few elevated white, soft umbilicated annular parenchyma areas ranging from 0.1 to 1.5cm in diameter (Fig.3). At cut, the lungs were soft with homogeneously red surface close or not to multifocal or coalescing whitish hemispheric nodules. The consolidated lobe (middle lobe) was firm at cut, with consistency similar to flesh ("pulmonary carnification").

In the other case (1/100), the heart was the only organ affected. Macroscopically, there were non-elevated yellowish white areas with multifocal distribution or coalescing in the epicardium, both on the left (atrial) and right (atrial) chambers, but sparing the vessels. Transverse sectioning at the ventricle level showed that the externally observed areas presented an equally yellowish white cut surface and extended through the myocardium, including the papillary muscle of both ventricles and, in some places, reached the endocardium (transmural involvement) of right atrium and ventricle (Fig.4). The proliferated tissue caused moderate diffuse thickening of the left atrium.

Histologically, in both cases, peripheral T-cell lymphoma, NOS was characterized by dense proliferation of neoplastic lymphocytes organized in a mantle infiltrated, dissected and/or diffusely replaced in the affected tissues. In the lungs, cell proliferation partially or totally obliterated the alveolar spaces (Fig.5), the bronchiole-alveolar junctions, and the bronchioles, presenting discrete angiocentricity in



Fig.1. Follicular lymphoma, spleen and lymph node (cut surface), dog. Presence of a multifocal and coalescing white and bright dotted.

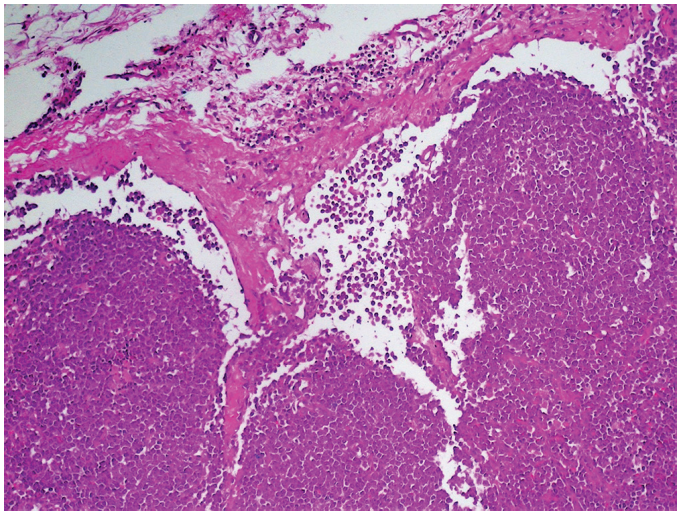


Fig.2. Follicular lymphoma, lymph node, dog. Parenchyma replaced by large coalescing lymphoid nodules. HE, obj.4x.



Fig.3. Peripheral T-cell lymphoma, NOS, lung, dog. Absence of collapse ("armed lungs") and intense reddening give the organ a hepatized appearance. Note the various white, non-elevated annular parenchyma areas, some similar to a target.

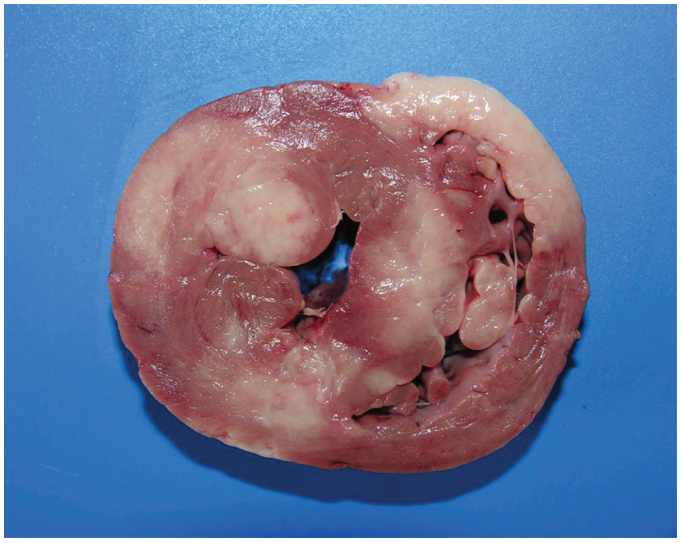


Fig.4. Peripheral T-cell lymphoma, NOS, heart (ventricular cut surface), dog. Yellowish white tissue replaces much of the myocardium in both ventricles and ventricular septum.

this case. In the heart, cells containing nuclei in the form of a homogeneous, condensed, hyperchromatic mass (apoptosis) next to cardiac fibers (Fig.6), sometimes fragmented, markedly eosinophilic, and surrounded by macrophages containing cell debris (necrosis) were observed in large quantities. The neoplastic lymphocytes presented intermediate volume and were composed of a small amount of homogeneous and eosinophilic cytoplasm. The nuclei were predominantly round, formed by loosely arranged chromatin, containing multiple basophilic, inconspicuous nucleoli (2-4) mostly located peripherally. Moderate nuclear pleomorphism and several mitosis figures, including some atypical, were observed. Both cases showed intense T lymphocyte (anti-CD3) immunostaining and absence of B lymphocyte immunostaining (anti-CD79).

Angiocentric lymphoma

Of all the dogs diagnosed with lymphoma (n=100), two (2/100) were classified as having angiocentric lymphoma. In one of these cases, the affected organs included the liver, spleen, and submandibular, precapular, axillary, inguinal, mesenteric and popliteal lymph nodes (generalized lymphadenopathy). Macroscopically, the liver was diffuse and markedly enlarged (diffuse hepatomegaly), with rounded lobe borders, and protruding beyond the costal arch. The natural surface was filled with numerous yellowish dots. The organs was firm at cut, with red cut surface containing multifocal yellow dots. The spleen was also diffuse and markedly enlarged (diffuse splenomegaly), and soft to the touch and cut. The cutting surface was homogeneously red. All lymph nodes were diffuse and markedly enlarged in volume (generalized lymphadenomegaly), slightly red, succulent, shiny, and soft. When cut, it was equally soft, with damp cut surface, red and filled with white dots, and with loss of corticomedullar delimitation. In the other case, macroscopically, the liver was the only organ affected, but histologically the bone marrow was also affected. At necropsy, the liver was diffuse and markedly enlarged (diffuse hepatomegaly), brownish, with

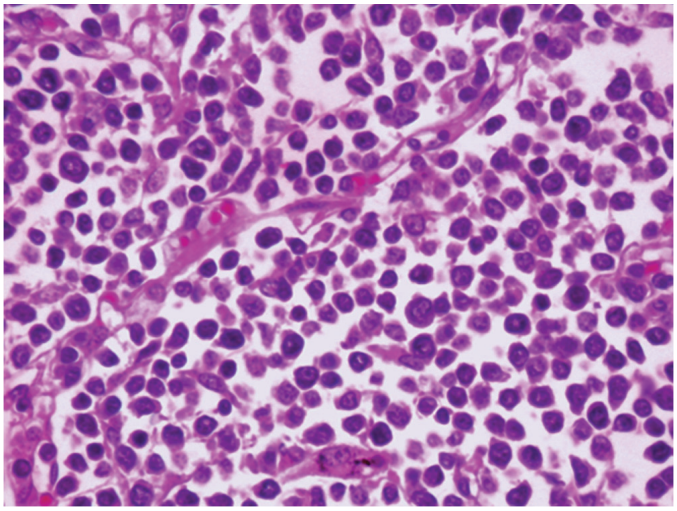


Fig.5. Peripheral T-cell lymphoma, NOS, lung, dog. Alveolar spaces completely obliterated by a mixed population of intermediate volume lymphocytes. HE, obj.20x.

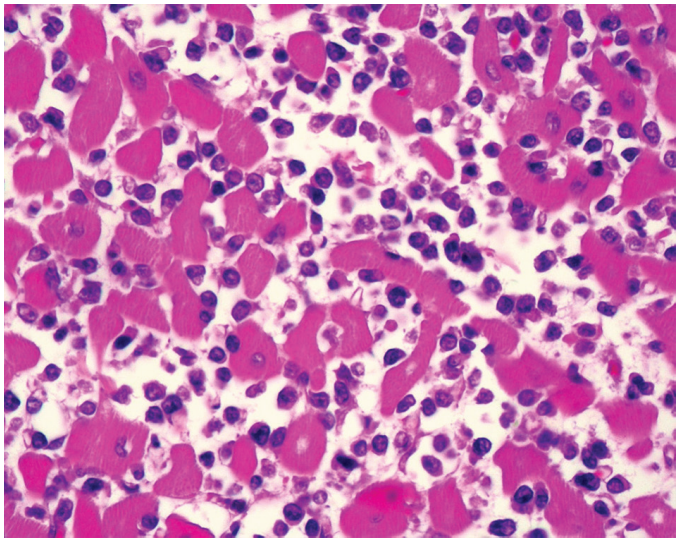


Fig.6. Peripheral T-cell lymphoma, NOS, heart, dog. Marked lymphocyte infiltrate that dissects the cardiomyocytes HE, obj.20x.

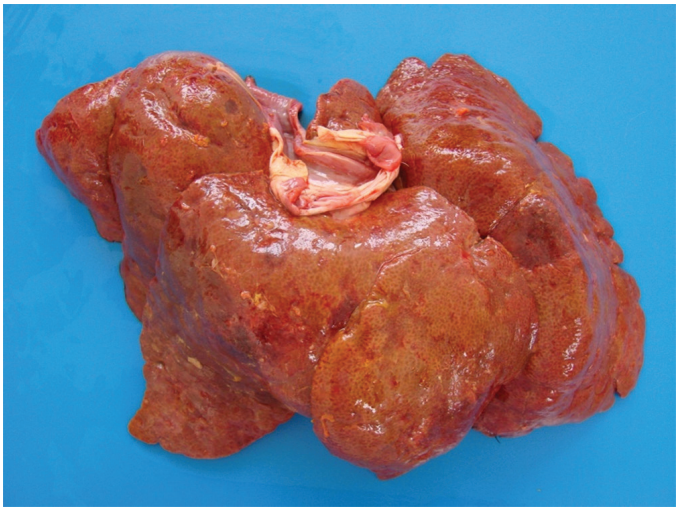


Fig.7. Angiocentric lymphoma, liver, dog. Markedly irregular natural surface, accentuated lobular pattern, areas of parenchymal collapse, and deposition of fibrin films.

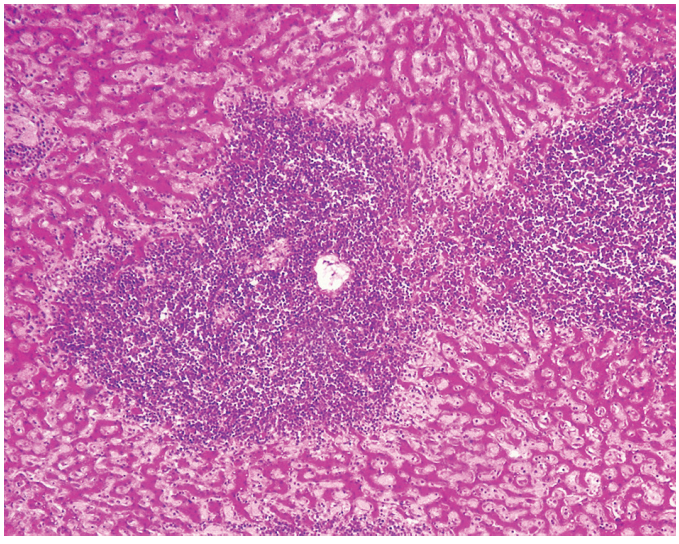


Fig.8. Angiocentric lymphoma, liver, dog. Perivascular lymphoid proliferation that completely destroys the portal area. HE, obj.4x.

lobe edges, and intensely irregular natural surface, including marked evidence of the lobular pattern (Fig.7), observed on both the natural and cut surfaces. Multifocal dark red areas were observed in some lobes (areas of necrosis) (Fig.7). A moderate amount of filamentous material in the form of a friable yellow film was adhered to the diaphragmatic surface (fibrin) (Fig.7). Areas of parenchymal collapse could be noticed randomly in all lobes. (Fig.7).

Histologically, in both cases, there was dense proliferation of neoplastic lymphocytes forming large cell clusters, predominantly around vessels (angiocentric pattern), causing luminal compression, but with absence or minimal invasion of the vascular wall. In the liver, these cells mainly surrounded the portal spaces, thoroughly dissected, and replaced the connective tissue between the structures (Fig.8). Sometimes neoplastic cells extended from a portal space to the center of the lobe, surrounding the centrilobular vein. In the subcapsular region, along its entire length, clustered neoplastic cells were also observed, forming a cell band. Occasionally, hepatic lobes were observed as areas composed of a grossly flocculent and markedly eosinophilic material near cellular debris (areas of necrosis). In the spleen and lymph nodes, the angiocentric pattern was maintained; however, due to marked cell proliferation, much of the parenchyma had been replaced. Additionally, in the lymph nodes, the subcapsular and peritrabecular sinuses were entirely obliterated by the neoplastic cells. In the bone marrow, capillaries surrounded by cell clusters were more easily identified when they were interspersed with areas of necrosis (Fig.9). The neoplastic lymphocytes had intermediate volumes and a moderate amount of homogeneous and eosinophilic cytoplasm. They contained round or oval, rarely reniform nuclei, located in the central region of the cell, and consisting of loose chromatin. Within these nuclei, single or multiple nucleoli were observed, mostly small, eosinophilic, and variably evident. Such cells were positive for the T lymphocyte marker (anti-CD3) and negative for the B lymphocyte marker (anti-CD79).

Intravascular large T-cell lymphoma

In this study, diagnosis of intravascular large T-cell lymphoma was observed in two cases (2/100). Of these, one showed exclusive renal involvement and the other presented hepatic nodal (periportal lymph nodes) and cutaneous (scrotum skin) involvement. At necropsy, in the first case, when opening the abdominal cavity, the kidneys protruded from the other organs because they were diffuse and markedly enlarged (diffuse nephromegaly); they were homogeneously yellow and firm to the touch. When cut, they were equally firm, with yellow surface and marked corticomedullary dissociation in which the medulla was approximately five times thicker than the cortex.

In the other case, macroscopically, the liver was diffuse and markedly enlarged (diffuse hepatomegaly), which was evidenced by displacement in its anatomical position in the abdominal cavity, projecting beyond the costal arch by rounding the edges of the hepatic lobes and imprinting the ribs on the diaphragmatic surface. On the natural surface, it was discreetly mottled in white and reddish black (Fig.10), smooth, and soft. The cutting surface was homogeneously red. The periportal lymph nodes were also enlarged (lymphadenomegaly), red, shiny, succulent and soft to the touch. When cut, they were soft, with moist, friable, homogeneously red cut surface, not allowing corticomedullar delimitation. The scrotum was markedly enlarged and a significant amount of a faintly reddish liquid was discharged at cut (hemorrhagic edema - hydrocele).

In both cases, histopathology showed dense round cell proliferation that partially obliterated the lumen of small or medium-sized vessels, but spared the hepatic and renal parenchyma. In the kidneys, proliferation of neoplastic lymphocytes in the lumen of interstitial vessels, exclusively from the medullary region, caused vascular distension and, consequently, collapsed by compression of most of the renal tubules, with only a few collections of collapsed tubules remaining as a reference for organ identification. In the lymph nodes, these cells also obliterated the subcapsular and peritrabecular sinuses. However, there was loss of corticomedullar delimitation macroscopically, but this delimitation was preserved histologically. The neoplastic cells were round, presented distinct cell boundaries and a moderate amount of homogeneous and eosinophilic cytoplasm. The nuclei were round, oval or cleaved, centrally located, and consisting of loosely arranged chromatin. The nucleoli were predominantly single, large, eosinophilic, and conspicuous. Only anisocytosis and anisokaryosis were observed. In the liver, nodal/cutaneous pleomorphism and atypia were accented, and many cells presented beveled, horseshoe shape, or multilobulated nuclei (Fig.11). Multinucleated cells were frequent. At IHC, neoplastic lymphocytes were markedly positive for the T lymphocyte marker (anti-CD3) and negative for the B lymphocyte marker (anti-CD79).

Lymphomatoid granulomatosis

Lymphomatoid granulomatosis corresponded to two (2%) of the 100 cases of canine lymphoma in this study. In necropsied dogs, the lungs, lymph nodes, spleen and liver were jointly affected. The lungs did not collapse upon opening of the thoracic cavity. They were heavy, moist, shiny, pink or reddish black (Fig.12), and soft to the touch. When cut, they were soft, and a small amount of faintly reddish fluid was discharged next to the foam (hemorrhagic edema). The cut surface was homogeneously pink or red. Regarding the lymph nodes affected, they were variably submandibular, retropharyngeal, prescapular, axillary, inguinal, mesenteric and popliteal (generalized lymphadenopathy). All lymph nodes were diffuse and markedly enlarged, red or yellowish white, succulent, shiny, and soft. At cut, they were equally soft, moist, with a homogeneously yellow or reddish black surface, not allowing corticomedullar delimitation. In both cases, the spleen was diffuse and markedly enlarged (diffuse splenomegaly), occupying almost the entire central portion of the abdominal cavity. It was soft at cut, homogeneously red and opaque, with protrusion of the parenchyma over the capsule. The liver was also diffuse, markedly enlarged (diffuse hepatomegaly) and firm, protruding beyond the costal arch. Both the natural and cut surfaces were homogeneously brownish. In one of the two cases (1%), two other anatomical sites were involved, including the tonsils and esophagus. The tonsils were diffuse and markedly enlarged (diffuse tonsillomegaly), yellowish white, moist and shiny, with loss of pleated conformation, partially obstructing the pharynx. In the esophagus, there were some whitish hemispherical

nodules, 0.3 cm in diameter, distributed multifocally through the esophageal mucosa, mainly at the level of the thoracic esophagus. At cut, such nodules were homogeneously white.

In the lungs of both cases (2/100), microscopically, there was dense proliferation of round cells arranged on the periphery of vessels (angiocentric pattern) (Fig.13) and bronchioles (peribronchiolar pattern), but also free, to a lesser extent, within the alveolar spaces, bronchi, and bronchioles. These proliferated cells characterized a mixed population consisting of small mature lymphocytes (scarce cytoplasm with round nuclei formed by dense chromatin), intermediate-sized neoplastic lymphocytes, and histiocytes or macrophages containing a brownish, granular pigment (hemosiderin). Cells with two or more nuclei (binucleated or multinucleated, respectively) were occasionally observed. The neoplastic lymphocytes had scarce, homogeneous, and eosinophilic cytoplasm. Round or oval nuclei formed by loose chromatin were centrally located. Single, small, basophilic, and variably evident nucleoli were observed. In some places, proliferated cells invaded the vessel wall (angioinvasive pattern) causing it to condense into a homogeneous and markedly eosinophilic material, characterizing fibrinoid necrosis. In both cases, the other affected organs were infiltrated by the same cell population observed in the lungs; however, in the lymph nodes, tonsils, spleen and esophagus, the cells proliferated intensely, coalesced, and replaced the parenchyma, giving the impression of proliferation in a mantle. Differently from the liver, where the angiocentric pattern was maintained, leading to multifocal nodules, especially in the portal spaces. At IHC, neoplastic lymphocytes were markedly positive for the B lymphocyte marker (anti-CD79) and negative for the T lymphocyte marker (anti-CD3). Small mature lymphocytes were immunostained for T lymphocytes (anti-CD3) but not for B lymphocytes (anti-CD79).

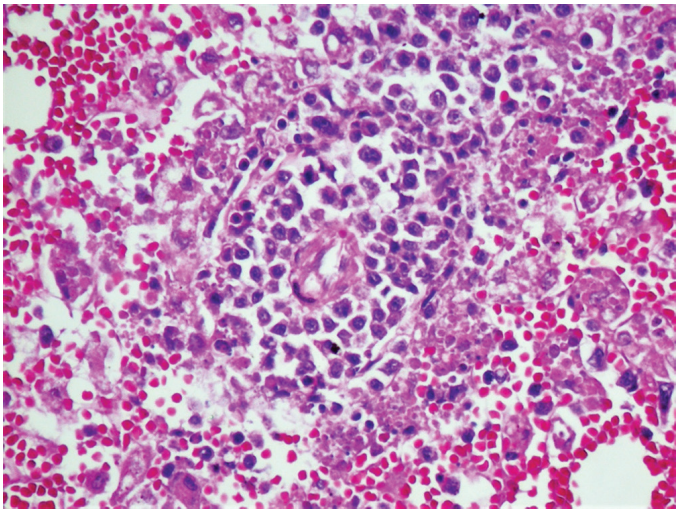


Fig.9. Angiocentric lymphoma, bone marrow, dog. Proliferation of neoplastic lymphocytes forming a cluster around an arterial capillary. There is extensive necrosis and bleeding around these areas. Note the accumulation of macrophages filled with tingible bodies HE, obj.20x.

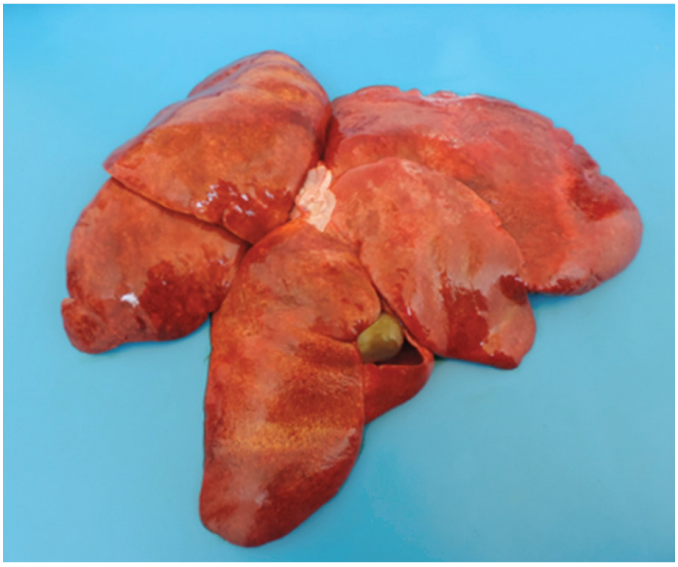


Fig.10. Intravascular large T-cell lymphoma, liver, dog. Hepatomegaly with discoloration and mottle. Note the multiple areas of costal impression.

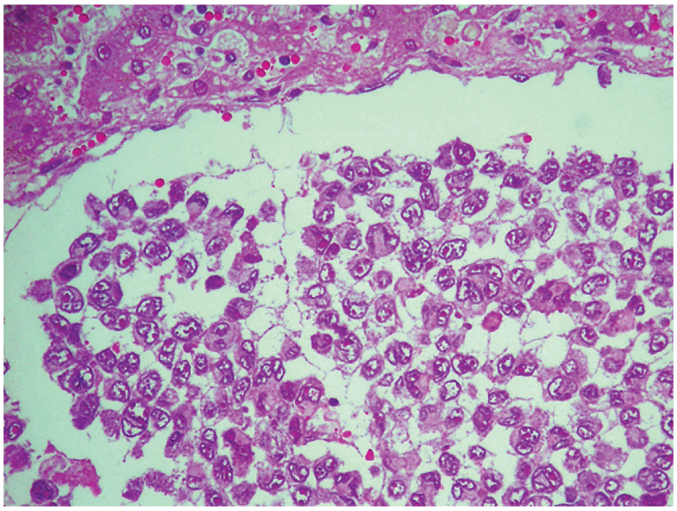


Fig.11. Intravascular large T-cell lymphoma, liver, dog. Intraluminal proliferation of markedly anaplastic neoplastic lymphocytes. HE, obj.20x.

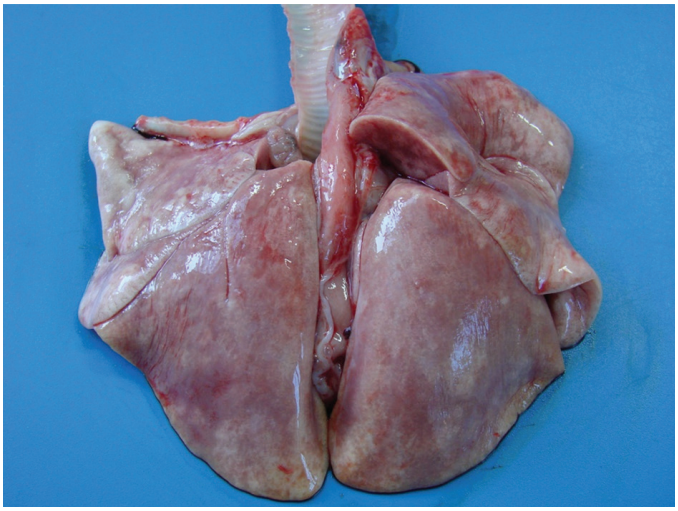


Fig.12. Lymphomatoid granulomatosis, lung, dog. Lack of collapse ("armed lungs") and intense grayness give the organ a hepatized appearance.

Anaplastic large-cell lymphoma

Of all dogs with lymphoma (n=100), only one (1/100) was diagnosed with anaplastic large-cell lymphoma. In this case, the liver, skin and left axillary lymph node were affected concurrently. At necropsy, distributed multifocally on the natural surface of all hepatic lobes, some non-elevated parenchymal annular areas, whitish and shiny, ranging from 0.3 to 3cm in diameter (Fig.14) were observed. They were soft to the touch and cut and presented homogeneously white cutting surface. In the skin of the ventral abdominal region, to the left of the median plane and craniolateral to the prepuce opening, there was an irregular mass measuring 6 x 4 x 2.5cm. In the skin of the right inguinal region, a similar mass measuring 5 x 2 x 1.5cm was observed. Both masses were soft to the touch and cut and presented homogeneously white cutting surface. The left axillary lymph node was diffuse and markedly enlarged, succulent shiny, and soft. At cut, it was equally soft with multilobulated surface, white with yellowish multifocal areas (areas of necrosis) and red dots (hemorrhage), not allowing corticomedullar delimitation.

The whitish areas observed on the natural surface of the liver, the cutaneous masses, and the left axillary lymph node showed dense proliferation of mantle-organized neoplastic lymphocytes histologically. In the liver, these lymphocytes formed nodules delineated by hepatocytes. In the skin, they replaced much of the superficial and deep dermis, including the cutaneous appendages, but sparing the epidermis. In the lymph node, they obliterated the subcapsular and peritrabecular sinuses, completely replacing the parenchyma, causing "nodal effacement. Neoplastic lymphocytes characterized the small cell variant, represented predominantly by small neoplastic lymphocytes called "fried egg" cells" (Fig.15). Such lymphocytes were composed of scarce, homogeneous and eosinophilic cytoplasm. They had centrally located round nuclei formed by loose chromatin with multiple, small, basophilic and inconspicuous nucleoli. Interspersed with these small neoplastic lymphocytes, but more intensely surrounding vessels, there were large lymphocytes with horseshoe-shaped or reniform eccentric nuclei, sometimes giving the cell an embryo appearance ("hallmark cells") (Fig.16). These cells were

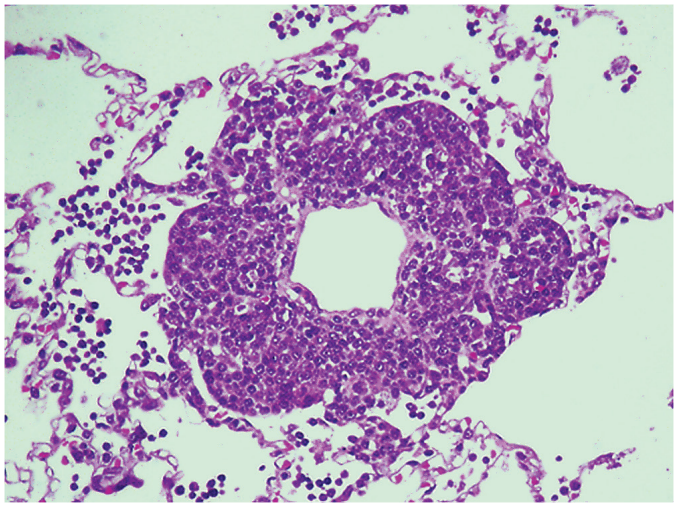


Fig.13. Lymphomatoid granulomatosis, lung, dog. Perivascular lymphoid infiltrate (angiocentric pattern). HE, obj.10x.

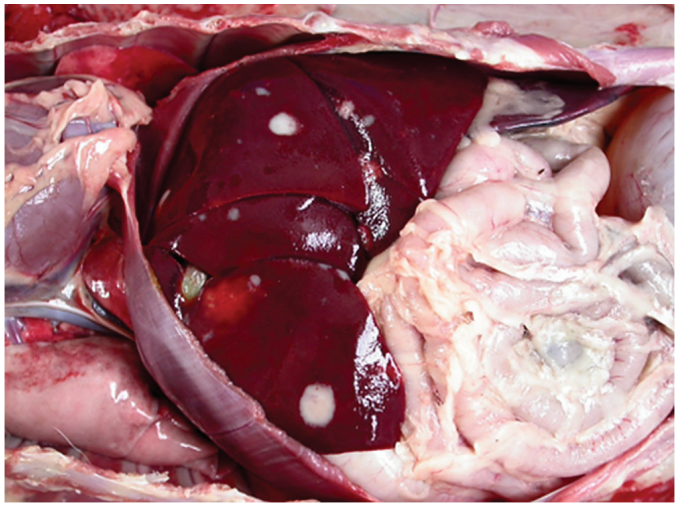


Fig.14. Anaplastic large cell lymphoma, liver, dog. White and non-elevated patches randomly distributed throughout the parenchyma.

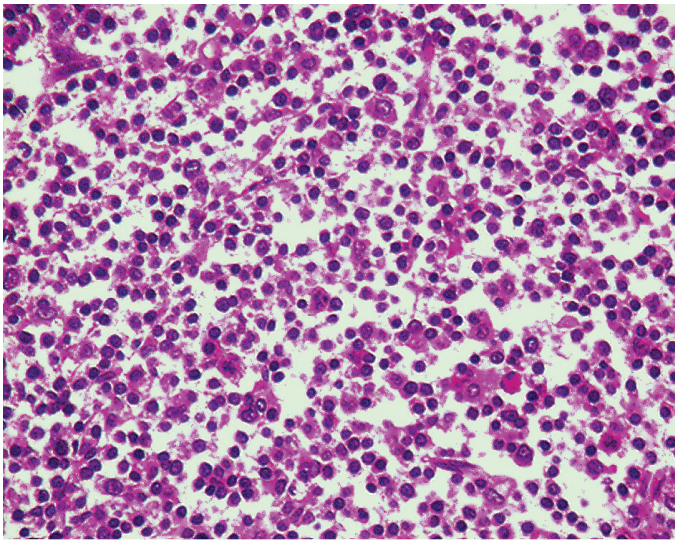


Fig.15. Anaplastic large cell lymphoma, liver, dog. Intensely condensed nucleus lymphocytes ("fried egg" cells"). HE, obj.20x.

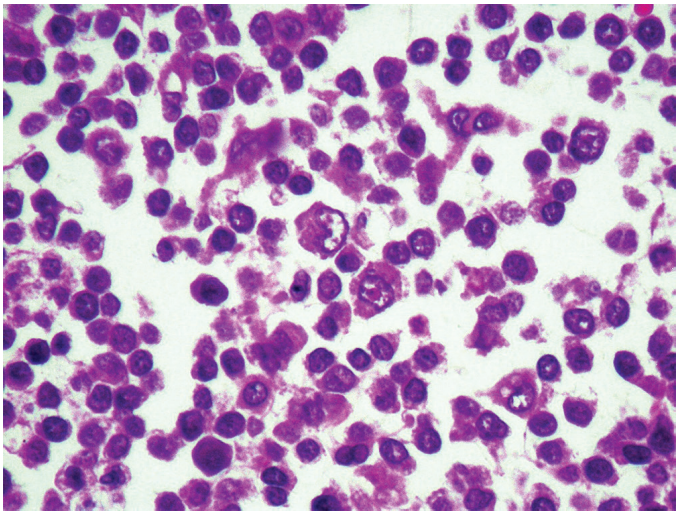


Fig.16. Anaplastic large cell lymphoma, liver, dog. Note a "hallmark cell" in the center of the field amidst variably anaplastic lymphocytes. HE, obj.40x.

characterized by abundant, homogeneous and eosinophilic cytoplasm, containing nuclei, consisting of loose chromatin. The nucleoli were multiple, medium-sized, basophilic, and more easily observed compared with those seen in small neoplastic lymphocytes. Cells whose horseshoe-shaped nuclei had joined at the ends, presenting the appearance of a donut ("donut cells"), were occasionally visualized. Some large neoplastic cells were binucleated, whereas others were multilobulated or even multinucleated. In some binucleated cells, the nuclei were identical and closely related, arranged as a "mirror image," and large conspicuous nucleoli of "owl-eye shape," much like the Reed-Sternberg cells, were observed. Depending on the cut incidence, some of these hallmark cells showed irregular and eosinophilic intranuclear structures, characterizing false inclusions (pseudoinclusions). Cells whose nuclei were moved to the periphery due to the presence of a large cytoplasmic vacuoles ("signet ring cells") were also randomly observed. At IHC, neoplastic lymphocytes were immunostained for T lymphocytes (anti-CD3) but not for B lymphocytes (anti-CD79).

Hepatosplenic T-cell lymphoma

Hepatosplenic T-cell lymphoma corresponded to only one case (1/100) in this study. In this case, the ocular, oral and genital mucosae, as well as the skin and subcutaneous tissue, were markedly yellow (jaundice) and, in addition to concomitant involvement of the spleen and liver, there was involvement of duodenal and pancreatic lymph nodes (deep localized lymphadenopathy). (Fig.17). Macroscopically, the spleen was diffuse and markedly enlarged (diffuse splenomegaly), occupying much of the abdominal cavity. Multifocal distribution over the diaphragmatic surface was observed in several variable and blackened parenchyma areas (areas of necrosis) (Fig.17). At cut, the spleen was soft with homogeneously red surface, including those blackened areas. The liver was also diffuse and markedly enlarged (diffuse hepatomegaly) with the edges of the lobes markedly bulging, presenting brownish and intensely irregular natural surface (Fig.17). At cut, it was soft with an equally brownish surface. The lymph nodes (duodenal and pancreatic) were diffuse and markedly enlarged (lymphadenomegaly), reddish, showing some elevated blackish parenchyma areas (hemorrhage), succulent, shiny (Fig.17), and soft to the touch and cut. On the cutting surface, they were equally succulent and bright, mottled in white and red, not allowing corticomedullar delimitation.

At histopathology, both organs showed dense proliferation of neoplastic lymphocytes that diffusely infiltrated the parenchyma. In the spleen, the sinusoids and splenic cords (red pulp), as well as the lymphoid nodules, the periarteriolar lymphoid sheaths (white pulp) and the capillary macrophage sheaths were completely replaced by a mantle of neoplastic cells. Multifocal or coalescing areas composed of a grossly floccular and eosinophilic material along with cellular debris and erythrocytes (necrosis areas) were randomly observed. In the liver, the sinusoids were diffusely distended by a large number of neoplastic lymphocytes, forming large areas composed exclusively of neoplastic cells in several places (Fig.18). A large number of these cells also surrounded the portal spaces and dissected the connective tissue between their structures (Fig.18). The nodal parenchyma was diffusely replaced by neoplastic proliferation, similar to what was observed in the spleen, characterizing "nodal effacement. Microscopic evaluation of the bone marrow and lungs revealed the presence of neoplastic cells in both organs. In the bone marrow, a small population of neoplastic lymphocytes was observed interspersed with the normal hematopoietic population. In the lungs, a moderate number of these cells were observed inside the alveolar septal capillaries, suggesting occurrence of leukemization of lymphoma. The population of neoplastic lymphocytes was classically monomorphic, composed of lymphocytes of intermediate volume, with scarce, homogeneous and eosinophilic cytoplasm. The nuclei of these cells were round, centrally located, and composed of dense chromatin. The nucleoli were unique, small, basophilic and inconspicuous. At IHC, such cells were immunostained for T lymphocytes (anti-CD3) but not for B lymphocytes (anti-CD79).

Chronic small B-cell lymphocytic lymphoma, intermediate type

Chronic small B-cell lymphocytic lymphoma, intermediate type corresponded to one case in this study (1/100). At necropsy, the upper and lower eyelids covering the free surface of both eye bulbs were diffusely thickened to the fullest extent, but with greater intensity over the left eye, not allowing their visualization. The posterior (conjunctival) face of the left lower eyelid was exposed due to marked increase in volume. The skin covering the eyelids, especially at the level of the medial angle of the left eye, had hair thinning (hypotrichosis), including the absence of cilia. A moderate amount of mucopurulent discharge partially covered both eyelids and condensed the hairs of the lower left periocular region. Cross sectioning of the skull at the level of the orbits showed that the peri- and intra-orbital adipose body, fasciae and extrinsic muscles of the ocular bulb were diffusely replaced by a homogeneously white tissue, succulent (Fig.19) and soft to the touch, in both eyes. At cut, this tissue was equally soft and presented homogeneously white cut surface (Fig.19). In that same patient, three other sites were affected, including the nasal cavity, tongue and tonsils. Regarding the nasal cavity, cross section of the skull at orbit level showed that the nasal conchae, especially those on the left side of the cavity, were partially replaced by a tissue similar to that observed in the eyelids (Fig.19). As for the tongue, on the dorsal surface, there was a moderate increase in unilateral root volume near the left tonsil and, on the cutting surface, a large amount of the aforementioned tissue largely replaced the lingual musculature.

Microscopically, this white tissue (eyelids, tongue, nasal cavity, and tonsils) was characterized by dense proliferation of neoplastic lymphocytes organized in a mantle that infiltrated and diffusely replaced the affected tissues on both sides. Although macroscopically the ocular architecture was maintained, microscopically, only the sclera and few portions of the choroid coat remained, and the latter presented by some pigmented cells. In the tongue, cell proliferation dissected much of the musculature, but spared the epithelium. In the nasal conchae, the respiratory epithelium was largely replaced, but the bony base of the nasal turbines was maintained. In the tonsils, neoplastic cells formed an extensive mantle that compressed and atrophied but did not invade the lining epithelium. The neoplastic lymphocytes were small and composed of scarce,

homogeneous and eosinophilic cytoplasm (Fig.20). The nuclei were predominantly round, eccentric, and formed by dense chromatin. Nucleoli were not observed. At IHC, neoplastic lymphocytes were positive for the B lymphocyte marker (anti-CD79) and negative for the T lymphocyte marker (anti-CD3).

DISCUSSION

Most of the scientific literature considers follicular lymphoma in dogs as an uncommon or rare condition, as it accounts for only 6.6 (4/60) to 7.5% (5/66) of non-Hodgkin's lymphomas in the USA (Valli et al. 2006, Flood-Knapik et al. 2012) and between 0.5 (3/608) and 1.2% of these diseases in Europe (Arespacochaga et al. 2007, Ponce et al. 2010), with a total of only 13 cases described. These data are similar to the prevalence found in this study, which corresponded to only 5% of the lymphoma presentations observed in dogs. Regarding the affected organs, a significant difference was observed between our results and the data published in the literature consulted. According to some authors (Valli et al. 2006, Arespacochaga et al. 2007, Ponce et al. 2010, Flood-Knapik et al. 2012), lymph nodes were the only organs affected in all cases diagnosed as follicular lymphoma, unlike our findings, considering that all cases also presented splenic involvement, observed macroscopically as diffuse splenomegaly, including three different gross presentation patterns. In humans, follicular lymphomas also affect the spleen, bone marrow, Waldeyer's lymphatic ring (formed by the pharyngeal, palatal and lingual tonsils), and peripheral blood, manifesting as lymphocytosis in approximately 10% of patients (Harris & Ferry 1992,

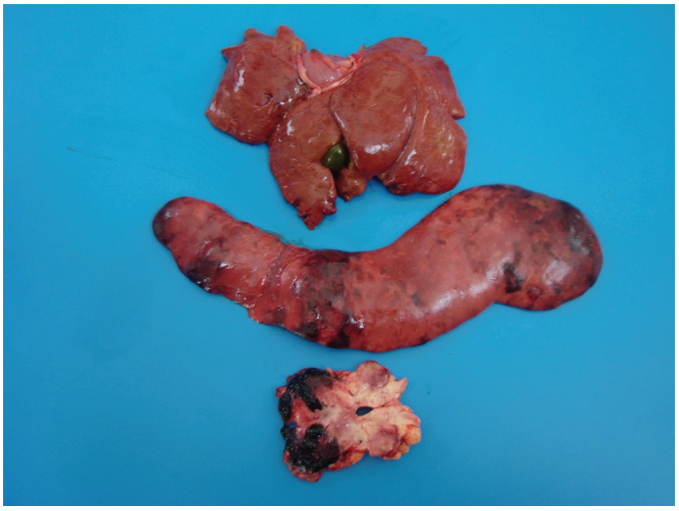


Fig.17. Hepatosplenic T-cell lymphoma, liver, spleen and lymph node, dog. The three affected organs showing diffuse organomegaly Note the areas of hemorrhagic necrosis in the spleen and lymph node.

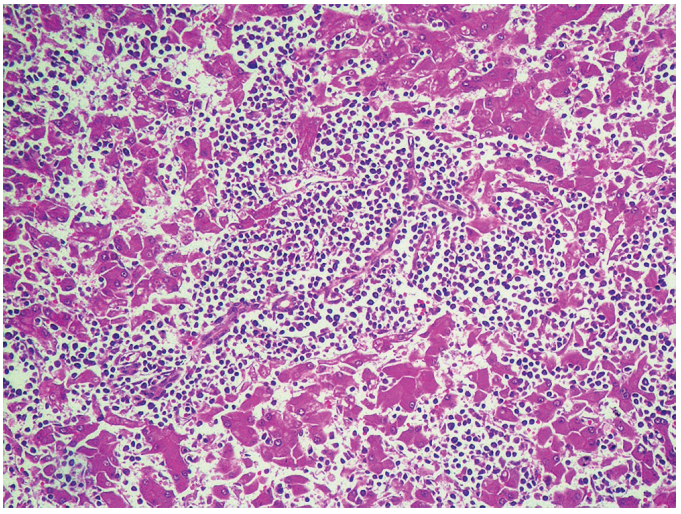


Fig.18. Hepatosplenic T-cell lymphoma, liver, dog. Lymphoid infiltrate that distends the sinusoids, obliterates the portal areas and dissociates the hepatocyte cords. HE, obj.10x.



Fig.19. Chronic small B-cell lymphocytic lymphoma, intermediate type, head (cut surface), dog. Whitish mass that replaces intra-orbital adipose tissue, fasciae, and extrinsic muscles of the ocular bulb causing bilateral ocular infiltration and compression. Additionally, there is partial replacement of the nasal conchae, especially on the left side of the nasal cavity, by the same tissue seen in the peri-ocular region.

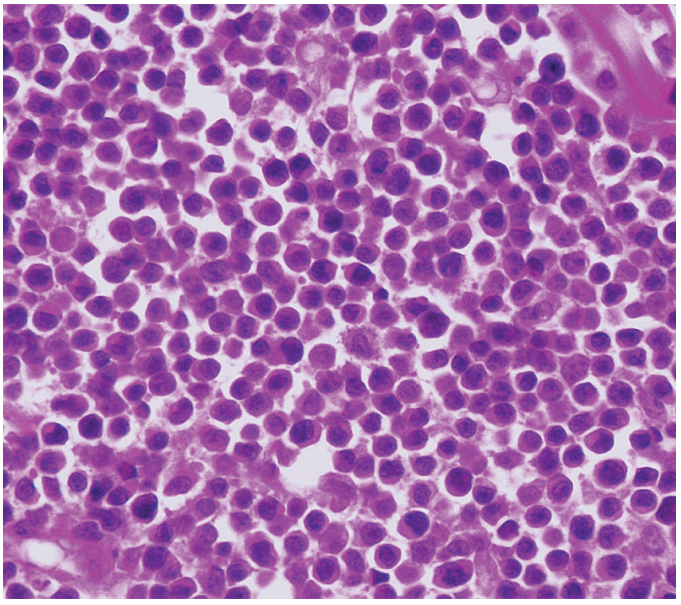


Fig.20. Chronic small B-cell lymphocytic lymphoma, intermediate type, skin (eyelid), dog. Lymphoid proliferation consists of small cells with round and slightly eccentric nuclei formed by aggregate chromatin without nucleoli. HE, obj.40x.

Harris et al., 2008). Gastrointestinal tract (GIT) and soft tissue involvement may occur as part of a multicenter follicular lymphoma, and other non-hematopoietic organs are rarely primarily affected, including the skin, eyeball attachments, mammary glands and testes (Harris et al 2008). In addition to the evident involvement of the spleen and lymph nodes in follicular lymphoma, the other changes were not observed in the dogs included in this study.

Peripheral T-cell lymphoma, NOS is a group of T-cell lymphomas that have not yet been fully classified and characterized (Valli 2002, 2007, Swerdlow et al. 2017). In human medicine, lymphoma subtypes belonging to this large group have been characterized (according to anatomopathological patterns, biological behavior, and molecular characteristics) reducing the number of lymphomas of this category called nonspecific (Swerdlow et al. 2017); however, this has been slowly performed through immunohistochemical profiles in veterinary medicine, given the limited availability of economic resources for such characterization in animals (Valli 2007). In humans, most patients have a generalized disease characterized by nodal, hepatic, splenic, and cutaneous involvement, but any organ can be affected (Swerdlow et al. 2017). In dogs, involvement is described primarily as cutaneous and/or subcutaneous with or without nodal involvement, with a wide histological variety (Valli 2007, Vezzali et al. 2009). In this study, peripheral T-cell lymphoma, NOS corresponded to only 2% of the lymphoma presentations diagnosed in dogs in our routine, affecting anatomical sites similar to what is observed in the human literature, but not yet described in the veterinary literature, characterizing a mixed lymphoma (with concomitant involvement of the lungs, stomach, liver, and lymph nodes) and a solitary lymphoma (exclusive cardiac involvement).

The classification of hematopoietic tumors in animals, established by WHO in 2002 and endorsed by the AFIP (Valli 2002), characterizes angiocentric lymphoma as a neoplasm originating from T lymphocytes, corroborating what was observed using IHC in our two cases. However, only lymphomatoid granulomatosis and lymphomas that develop after skin injections have been established by WHO as angiocentric (Valli 2002). In addition, the most current classifications suggested for hematopoietic tumors in dogs include only lymphomatous granulomatosis as an angiocentric lymphoma (Valli 2007, Valli et al. 2016, 2017), similarly to cases reported in dogs (Berry et al. 1990, Fitzgerald et al. 1991, Shimazaki et al. 2010, Hatoya et al. 2011). Given that the lungs and skin were not affected in either of the two cases of angiocentric lymphoma described here, which would enable the diagnoses of lymphomatoid granulomatosis or post-vaccine lymphoma, respectively, and that the neoplastic lymphocyte population not mixed as classically observed in lymphomatoid granulomatosis, but rather monomorphic, anatomical sites of angiocentric T-cell lymphoma not previously reported in the veterinary literature are described in this study. In humans, nasal extranodal NK/T-cell lymphoma corresponds microscopically (Swerdlow et al. 2017) to the angiocentric lymphoma described in the two cases of this study.

Currently, intravascular lymphoma of dogs is classified as one of the neoplasms originating from mature T cells or NK-cells (Valli 2007, Valli et al. 2017), considering the majority of cases already reported in this species (McDonough et al. 2002, Kent et al. 2001, Machado et al. 2011, Ridge & Swinney 2004), including the cases described herein. In addition to the classification, when cases originating from B cells are occasionally diagnosed, intravascular lymphoma is considered a subtype of diffuse large B-cell lymphoma (Valli et al. 2017). Based on a considerable number of descriptions (Kent et al. 2001, Lane et al. 2012, McDonough et al. 2002, Machado et al. 2011), intravascular lymphoma is, for dogs, a proven condition that presents a high central nervous system (CNS) predilection, affecting it exclusively or concomitantly with several other organs. Nevertheless, in the cases observed in this study, CNS was spared, with concomitant involvement of the liver, periportal lymph nodes, and scrotum in one case, and only of the kidneys in the other.

Lymphomatoid granulomatosis is a mixed lymphoproliferative disorder of primarily neoplastic and non-neoplastic cells, primarily pulmonary, characterized histologically by a pattern of angiocentric and angioinvasive cell proliferation (Berry et al. 1990, Fitzgerald et al. 1991, Park et al. 2007, Song et al. 2015, Swerdlow et al 2017). In both humans and dogs, it is considered a rare presentation of lymphoma, since the lungs do not represent one of the anatomical sites classically affected by this neoplasm (Fry & McGavin 2012, Fighera & Graça 2016, Swerdlow et al. 2017, Valli et al. 2017). Corroborating the rarity of its occurrence, lymphomatoid granulomatosis corresponded to 2% of the lymphoma presentations in dogs in the present study. The macroscopic pattern observed in the lungs and the location of the metastases described herein are similar to what has been reported in the veterinary literature (Berry et al. 1990, Fitzgerald et al. 1991, Park et al. 2007, Hatoya. et al., 2011). However, although lymph nodes, liver, and spleen comprise the major sites of metastasis, any organ or tissue can be affected (Shimazaki et al. 2010, Valli et al. 2017), as it was observed in one case described herein, in which the esophagus and tonsils in addition to the lymph nodes, liver and spleen were sites of metastasis.

In our routine, anaplastic large-cell lymphoma represented only 1% of diagnosed lymphoma presentations in dogs. This is a rare manifestation of lymphoma, characterized in both humans and dogs by a primarily cutaneous and systemic forms (Valli et al. 2017). In the systemic form, lymph nodes are usually affected concurrently with other sites, including bones, lungs and liver, but skin seems to be the most frequently involved extranodal site (Valli 2007, Swerdlow et al. 2017, Valli et al. 2017). Perhaps the case of anaplastic large-cell lymphoma presented in this study represents the systemic form of this neoplasm, similar to that described in the veterinary and human literature, concomitantly affecting the axillary lymph node, skin and liver; however, based on the findings, it cannot be defined whether the neoplasm could have started on the skin and later involved the axillary lymph node and liver.

Hepatosplenic lymphoma in both humans and dogs is a rare condition characterized by concomitant involvement of the liver and spleen, in the form of diffuse hepatosplenomegaly, and often of the bone marrow, but with no peripheral nodal involvement (superficial lymphadenopathy) (Weidmann 2000, Keller et al. 2012, Swerdlow et al. 2017). In this study, this presentation of lymphoma corresponded to 1% of the forms diagnosed in dogs in our routine, similar to what is observed in the veterinary literature, which describes only eight cases in dogs (Fry et al. 2003, Cienava et al. 2004, Keller

et al. 2012). Regarding the affected organs, the case described herein was identical to that observed in the literature, because in seven of the eight reported cases (Cienava et al. 2004, Keller et al. 2012), hepatosplenic lymphoma was invariably characterized by diffuse hepatosplenomegaly with microscopic involvement of the bone marrow and lungs. Based on the human literature (Weidmann 2000, Swerdlow et al. 2017), pulmonary involvement possibly occurred as a result of the advanced stage of the disease and its aggressive behavior. More rarely, jaundice due to diffuse infiltration of the liver by neoplastic lymphocytes and deep localized lymphadenopathy, respectively, has been described in only one case (Keller et al. 2012), corroborating the necropsy findings in our case, in which accentuated jaundice and deep localized lymphadenomegaly of the pancreatic lymph nodes were observed.

Chronic small B-cell lymphocytic lymphoma is one of the five most common types and/or histological subtypes reported in dogs, ranging from 1.8 (Ponce et al. 2010) to 7.8% (Vezzali et al. 2009) of the cases. However, its so-called an "intermediate type" variant, which is characterized by the presence of lymphocytes with plasmocytic differentiation, is much less common than the classical form (Valli 2002). In our routine, this type of lymphoma, which is considered by many authors, both in humans and dogs, as an indolent form of the disease (Valli 2007, Swerdlow et al. 2017, Valli et al. 2017), accounted for only 1% of the presentations of lymphoma diagnosed in dogs. Its slowly progressive course is due to a much more cumulative than progressive lymphocyte proliferation, providing longer survival times for patients with this condition (Valli 2002, 2007, Swerdlow et al. 2017, Valli et al. 2017). Usually, the affected sites include the lymph nodes, spleen and liver. Rarely, other sites may be affected, such as the kidneys, adrenals, CNS, eyes, and eye bulb attachments (Valli 2007, Swerdlow et al. 2017, Valli et al. 2017). Given this, the case of chronic small B-cell lymphocytic lymphoma, intermediate type presented in this study represents one of the unusual or rare anatomical sites of this type of lymphoma. In addition, this study presents three other sites not previously described in the veterinary literature as possible sites of this type neoplasm (tonsils, tongue, and nasal cavity [nasal concha]).

CONCLUSIONS

Although lymphoma is an easily recognized neoplasm among veterinary pathologists, uncommon (16% of the time) non-traditional cases of this important form of cancer can be found. These cases correspond not only to solitary, extranodal and mixed lymphomas, but also to distinct microscopic presentations.

We hope that the results presented here can assist veterinary pathologists with the classification and recognition of cases of "atypical lymphoma" in their anatomopathological routines.

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

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Original Article
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Progesterone and estrogen receptor expression by canine cutaneous soft tissue sarcomas[1]

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ABSTRACT.- Loures F.H., Conceição L.G., Amorim R.L., Nobrega J., Neto R.T., Costa E.P. & Fonseca e Silva F. 2020 **Progesterone and estrogen receptor expression by canine cutaneous soft tissue sarcomas**. *Pesquisa Veterinária Brasileira 40(4):284-288*. Departamento de Veterinária, Universidade Federal de Viçosa, Avenida PH Rolfs s/n, Campus Universitário, Viçosa, MG 36570-000, Brazil. E-mail: fabriciadermatopato.vet@gmail.com

Canine soft tissue sarcomas (STS) comprise a heterogeneous group of malignancies that share similar histopathological features, a low to moderate recurrence rate and low metastatic potential. In human medicine, the expression of estrogen receptors (ER) and progesterone receptors (PR) in sarcomas has been studied to search for prognostic factors and new treatment targets. Similar studies have yet to be conducted in veterinary medicine. The objective of this study was therefore to investigate by immunohistochemistry (IHC) the ER and PR expression in a series of 80 cutaneous and subcutaneous sarcomas in dogs with histopathological features of peripheral nerve sheath tumor (PNST) and perivascular wall tumor (PWT). All cases were positive for PR and negative for ER. Tumors of high malignancy grade (grade III) exhibited higher PR expression than low-grade tumors (grade I). Tumors with mitotic activity greater than 9 mitotic figures/10 high power fields also exhibited higher PR expression. In addition, there was a positive correlation between cell proliferation (Ki67) and PR expression. Therefore, it is possible that progesterone plays a greater role than estrogen in the pathogenesis of these tumors. Future studies should explore the potential for selective progesterone receptor modulators as therapeutic agents in canine STS, as well as evaluating PR expression as a predictor of prognosis.

INDEX TERMS: Progesterone, estrogen, receptor expression, canine, cutaneous tissue, soft tissue sarcomas, dogs.

RESUMO.- [**Expressão de receptores para progesterona e estrógeno em sarcomas de tecidos moles cutâneos em cães.**] Sarcomas de tecidos moles (STM) caninos compreendem um grupo heterogêneo de neoplasias malignas, que apresentam alterações histopatológicas similares, baixa a moderada taxa de recorrência e baixo potencial metastático. Em medicina humana, a expressão de receptor para estrógeno (RE) e receptor para progesterona (RP) nos sarcomas tem sido estudada, visando a busca por fatores prognósticos e novos alvos para tratamentos. Na medicina veterinária, ainda não foram realizados estudos similares. O objetivo deste trabalho foi investigar por imuno-histoquímica a expressão de RE e RP em uma série de 80 sarcomas cutâneos e subcutâneos de cães, com características histopatológicas de tumor de bainha de nervo periférico e tumor de parede perivascular. Todos os casos foram positivos para RP e negativos para RE. Tumores de alto grau de malignidade (grau III) exibiram maior expressão deste receptor que os tumores de baixo grau (grau I). Tumores com atividade mitótica maior que 9 figuras mitóticas/10 campos de grande aumento também exibiram

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maior expressão do RP. Em adição, houve correlação positiva entre o índice de proliferação celular (Ki67) e a expressão de RP. Assim, é possível que a progesterona desempenhe maior papel que o estrógeno na patogênese desses tumores. Futuros trabalhos poderão explorar o potencial dos moduladores seletivos de RP como agente terapêutico em STM caninos, bem como avaliar a expressão de RP como preditiva de prognóstico.

TERMOS DE INDEXAÇÃO: Receptores, progesterona, estrógeno, sarcomas, tecidos moles cutâneos, cães, caninos.

INTRODUCTION

Soft tissue sarcomas (STS) comprise a heterogeneous group of malignant neoplasms, derived from extraskeletal mesenchymal tissues, that share similar histopathological findings and biological behaviour (Ettinger 2003, Dennis et al. 2011). These neoplasms frequently occur in cutaneous and subcutaneous tissue and usually exhibit low metastatic potential and a variable recurrence rate after surgical excision (Dennis et al. 2011, Avallone et al. 2014).

For prognostic purposes and in search of new treatment targets, recent studies in human medicine have evaluated the expression of estrogen receptors (ER) and progesterone receptors (PR) in STS (Ioffe et al. 2009, Valkov et al. 2011, Akashi et al. 2013, Li et al. 2013, Sorbye et al. 2013, Thanopoulou et al. 2014). In dogs, the prognosis related to ER and PR expression was evaluated mainly in mammary neoplasms (Millanta et al. 2005, Millanta et al. 2006). Understanding the influence of steroid hormones on the development of neoplasms may be important for the development of new therapeutic approaches. In addition, investigation of the expression of these receptors in spontaneous animal STS may provide additional information for cancer research in humans (Vail & Macewen 2000).

The objective of this study was to evaluate by immunohistochemistry (IHC) the expression of ER and PR in canine STS, with morphological compatibility with peripheral nerve sheath tumor (PNST) and perivascular wall tumor (PWT).

MATERIALS AND METHODS

This study was approved by the Ethics Committee for Animal Use (CEUA/UFV, proccess no. 95/2014).

Inclusion criteria and case selection. Microscopic slides of canine STS from the Pathology Service were reviewed. Cases with morphological and immunohistochemical findings compatible with PNST and/or PWT were included in the study. The cases showed positive immunostaining for neural markers and muscular markers, according to the panel of antibodies presented below. Canine STS with morphological findings suggestive of other sarcomas (e.g. fibrosarcoma, liposarcoma, myxosarcoma, leiomyosarcoma and rhabdomyosarcoma) were not included in the study.

Clinical records information. The clinical information regarding sex, age, breed, location and size of the lesions was obtained from the clinical and pathological records.

Histopathology and immunohistochemistry (IHC). Hematoxilin and eosin (HE) stained slides were reviewed under light microscopy for tumor grading. Histological grade was based on the sum of scores attributed to the mitotic rate, percentage of necrosis and differentiation of the neoplasm as previously described (Dennis et al. 2011).

For IHC analysis, the paraffin blocks were cut into 3μm thick sections and placed on electrically charged plates (Starfrost®). The antibody dilutions and clones were as follows: Progesterone Receptor Ab-2 Mouse Monoclonal Antibody (Thermo Scientific, Runcorn, UK; hPRa2; 1:50); Monoclonal Mouse Anti-Human Estrogen Receptor (Dako, Glostrup, Denmark; ID5; 1:20); Monoclonal Mouse Anti-Human Ki-67 (Dako, Glostrup, Denmark; MIB-1; 1:50); Antigen CD56 (Biocare Medical, Concord/CA, USA; BC56C04; 1:200); Monoclonal Mouse Anti-Human Smooth Muscle Actin (Dako, Glostrup, Denmark; A4; 1:1000); Monoclonal Mouse Anti-Human Desmin (Dako, Glostrup, Denmark; D33; 1:50); Polyclonal Rabbit Anti-Human Von Willebrand Factor (Dako, Glostrup, Denmark; Polyclonal; 1:1000); Monoclonal Mouse Anti-Human Neuron-Specific Enolase (NSE) (Dako, Glostrup, Denmark; BBS/NC/VI-H14; 1:3000); Glial Fibrillary Acidic Protein Rabbit Polyclonal Antibody (Diagnostic BioSystems, Pleasanton/CA; Polyclonal; 1:300).

After incubation at 54°C for 24h, tissue sections were deparaffinized in xylene, hydrated in ethanol and washed with deionized water. For antigen retrieval, citrate was used at pH 6.0 in a pressure cooker. The endogenous peroxidase blockage was performed with 3% hydrogen peroxide for 10min. After washing, the primary antibody and slides were kept in a moist chamber at 4°C for 18h. After washing with Tris at pH 7.4, a peroxidase-bound secondary antibody polymer system was used according to the manufacturer's recommendations (EnVision, DakoCytomation®). Visualization was accomplished with 3,3'-diaminobenzidine tetrachloride (DakoCytomation®) and counterstaining with Harris hematoxylin. Internal positive controls (muscle tissue and endothelial cells) were used to smooth muscle actin, desmin, and Von Willebrand factor. Canine cystic endometrial hyperplasia tissue was used as the positive control for ER and PR**.** For other antibodies, the positive control consisted of tumors known to be positive for that particular antibody. For all the used antibodies, negative controls were performed by omitting the primary antibodies.

The IHC interpretation was performed under light microscopy. Areas with intense inflammation or necrosis were avoided, since inflammatory cells can be positive for Ki67. For Ki67, the percentage of positive nuclei was obtained by manual count of at least 1000 cells in 10 high-power fields (400X) (Image Pro Plus® program). For ER and PR expression, scores were defined in relation to the percentage of positive cells (Score 0 = negative, Score 1 = positivity in 1-33% of cells, Score 2 = positivity in 34-66%, Score 3 = positivity in 67-100%). Other antibodies were used to exclude hemangiosarcoma (factor Von Willebrand), and to confirm the morphological diagnosis of TBNP and TWP (smooth muscle actin, desmin, GFAP, S100 and CD56).

Statistical analysis. Clinical information (sex and age of animals, size and location of lesions) was analysed using descriptive statistics.

Relative risk (Odds Ratio), with a confidence interval of 95%, was used to evaluate breed predisposition (MedCalc statistical program - easy to use statistical software[7]). To evaluate gender predisposition, the proportion of occurrence was calculated with a confidence interval of 95%; statistically equal proportions were identified by overlapping intervals (package prop; test of the statistical software R) (R Development Core Team 2008).

ER and PR qualitative variables were compared between tumor grades and mitotic scores, using the non-parametric Wilcoxon test. The cellular proliferation index (KI67) was correlated with extent of PR immunostaining using Spearman's correlation (Statistical and Genetic Analysis System) (SAEG 1999).

[7] Available at <https://www.medcalc.org/calc/odds_ Ratio.Php>

RESULTS

Clinical records information

Eighty cases of canine STS with histopathological characteristics of PNST and/or PWT were selected. Thirty-four (42.5%) cases occurred in males and 46 (57.5%) in females. The male/female ratio was 1:1.3. The age (known in 72 cases) ranged from 2 to 16 years, with an average of 10 years (±2.93) and mode of 12 years. The location of the tumors was mentioned in 74 cases, with more than half (40 cases, 54.0%) occurring in the limbs (thoracic 24.3% and pelvic 29.7%). Other anatomical regions were involved less frequently: trunk (20 cases, 27%); face (6 cases, 8.1%); digits (6 cases, 8.1%); perineum (1 case, 1.35%) and tail (1 case, 1.35%). The size of the lesions (information obtained in 35 cases) ranged from 1.5 to 25cm in diameter, with an average of 5.5cm (±4.9) and mode of 2.0cm.

Mongrel dog, Labrador Retriever, Maltese, Poodle, Boxer, Golden Retriever and Siberian Husky were the most frequently affected breeds. However, Siberian Husky (OR: 7.68, P = 0.0003) and the Maltese (OR: 2.60, P = 0.048) were the most predisposed for the development of the disease; they showed a seven-fold and two-fold higher risk of disease development than the other breeds, respectively.

Histopathology and Immunohistochemistry (IHC)

The morphological diagnosis of PNST and PWT was confirmed, by positive immunostaining for neural and smooth muscle markers (GFAP, NSE, CD56, smooth muscle actin and desmin). All cases were negative for Von Willebrand factor.

Twenty-six cases (32.5%) were histologically classified as grade I, 43 (53.75%) as grade II and 11 cases (13.75%) as grade III.

All 80 cases exhibited positive nuclear immunostaining for PR (Fig.1 and 2). Most of the tumors (53 cases, 66,25%) showed positivity in at least 66% of the tumor cells (score 3).

All 80 cases were negative for ER.

There was a significant difference between the histological grades of the tumors in relation to PR expression. Grade III tumors exhibited greater expression of this receptor than grade I tumors (P=0.015). The same difference occurred when grade I and II tumors were grouped and compared to grade III (P=0.028).

The PR expression varied significantly according to the mitotic index scores. Tumors with score 1 for mitotic activity (0-9 mitosis/10hpf) exhibited lower expression of this receptor than tumors with score 2 (10-19 mitosis/10hpf, P=0.011) and tumors with score 3 (P=0.018, >19 mitosis/10hpf).

Finally, there was a positive correlation between the cellular proliferation index (ki67) and PR expression (P=0.05).

DISCUSSION

Receptors for steroid sex hormones regulate the transcription of genes involved in cellular proliferation and apoptosis, and act in cellular differentiation and as growth factors (Buffet et al. 2005, Fishbein et al. 2007, Sorbye et al. 2013).

The highest level of expression of PR in high-grade sarcomas (grade III) and those with high mitotic activity shows that this hormone may also be associated with tumor growth. Consistent with this, the Ki67 index correlated positively with the expression of PR as well. The positivity for PR across large extents of tissue in all cases points to the possible participation of progesterone in the development of these sarcomas.

Progesterone stimulates axon myelination during development and remyelination after injury (Schumacher et al. 2008). Schwann cells express PR and synthesize progesterone in response to neuronal signals (Patel et al. 2008). In vitro studies demonstrated that the Schwann cells derived from neurofibromas showed high proliferation in the presence of this steroid hormone (Overdiek et al. 2008). Here, all cases were positive for neural markers, and Schwann cells may be the main cellular component of these sarcomas. This explains the presence of PR in a high proportion of the cellular population, as well as its possible participation in cellular proliferation.

Similar to the IHC pattern found in the present study, neurofibromas in humans showed diffuse immunostaining for PR, with rare positivity for ER (McLaughlin & Jacks 2003, Geller et al. 2008). Negativity for ER was also observed in

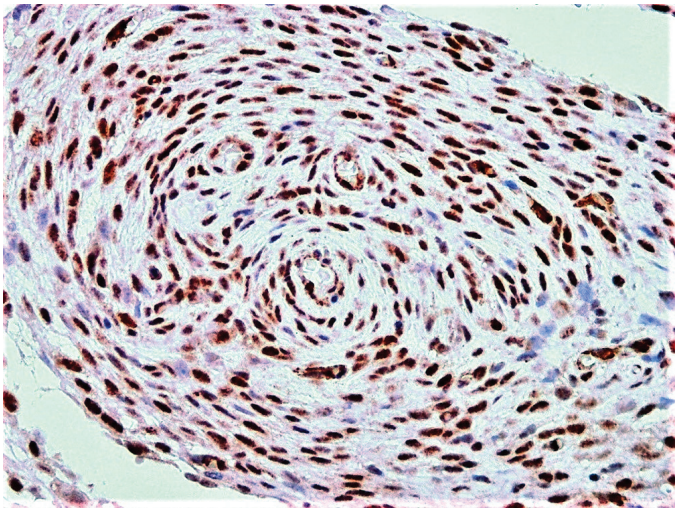


Fig.1. Canine Soft Tissue Sarcoma. Positive nuclear immunostaining for PR in cellular whorls. IHC counterstaining with Harris hematoxylin, obj.40x.

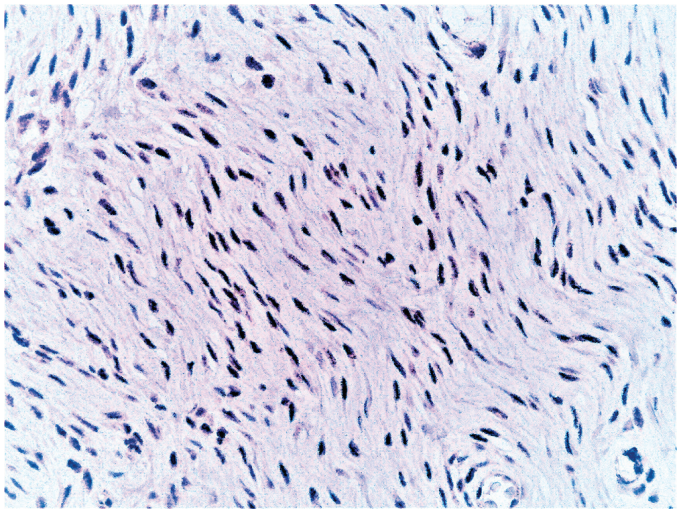


Fig.2. Canine Soft Tissue Sarcoma. Positive nuclear immunostaining for PR in undulating cellular bundles. IHC counterstaining with Harris hematoxylin, obj.40x.

human vestibular schwannomas (Cafer et al., 2008, Jaiswal et al., 2009). On the other hand, STS of different histogenesis located at the extremities, positivity for ER was observed in 30% of 89 cases (Valkov et al., 2011). It is possible that there are differences in ER expression between species, individuals, histological type and even between the stages of neoplasm development. In the current study, the ER apparently did not participate in the growth and proliferation of these tumors.

PR may act as a possible predictive molecular marker of prognosis and therapeutic targets in canine spindle cell sarcomas, considering that in humans PR and ER expression in STS has been associated with variable survival times (Ioffe et al. 2009, Valkov et al. 2011, Li et al. 2013, Sorbye et al. 2013). Human patients with IHC profiles similar to that of this study (ER negative and PR positive) presented reduced survival (Valkov et al. 2011, Sorbye et al. 2013). When each sex was evaluated separately, PR expression in men was associated with poor prognosis and reduced survival (Valkov et al. 2011). Furthermore, the influence of steroid sex hormones on the growth of neurofibroma and vestibular schwannoma is well documented, with greater prevalence in women; there was an increase in the size, number of lesions or malignant transformation during periods of hormonal changes, particularly during puberty and pregnancy (Geller et al. 2008, Patel et al. 2008, Jaiswal et al. 2009, Brown et al. 2011). In dogs, future researches are needed to assess the relationship of STS RP expression, gender, reproductive status and prognostic factors (eg, recurrence, metastasis, disease-free interval and survival time).

Also, the response to hormonal treatment (eg. selective ER or PR modulators, aromatase inhibitors) has also been described for several types of human STS (Ioffe et al. 2009, Byer et al. 2011, Meijer et al. 2011, Akashi et al. 2013, Li et al. 2013, Thanopoulou et al. 2014), which may prove to be useful in dogs with the evolution of the pathogenesis understanding.

Finally, in the present study, neoplasm size, location, and gender distribution did not differ from those described previously in the literature (Goldschmidt & Shofer 1992, Ettinger et al. 2006, McSporran 2009, Bray 2016). A predisposition of the Siberian Husky for hemangiopericytomas was also noted, but the Maltese has not previously been reported as a predisposed breed (Goldschmidt & Shofer 1992).

CONCLUSION

The presence of progesterone receptors (PR) in all cases reveals a promising area for future investigations, new therapeutic modalities and new molecular biomarkers of prognosis. In particular, selective PR modulators should be investigated as a possible approach in the treatment of canine soft tissue sarcomas (STS).

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Original Article
Small Animal Diseases
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Effect of pre-anesthetic fasting on gastric emptying and plasma glucose in healthy dogs of different age groups[1]

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ABSTRACT.- Lecheta D.R., Silva D.K.M., Santos G.A., Cunha M.S., Gaspar T.T., Lopes B.A., Deboleto S.G.C. & Braz P.H. 2020. **Effect of preanesthetic fasting on gastric emptying and plasma glucose in healthy dogs of different age groups.** *Pesquisa Veterinária Brasileira 40(4):289-292*. Centro Universitário da Grande Dourados, Rua Balbina de Matos 2121, Jardim Universitário, Dourados, MS 79824-900, Brazil. E-mail: paulo.braz@iffarroupilha.edu.br

Gastric emptying and plasma glucose were evaluated in young and adult dogs, fed with dry and wet food, submitted to different periods of pre-anesthetic fasting (6, 8, and 12 hours). Forty healthy dogs were selected, which were segmented into four groups according to the age group and type of diet. It was evaluated the gastric emptying by ultrasound and serum glycemia. Only 17.5% presented complete gastric emptying, and no significant differences were found between the 6 and 8-hour fasting evaluations, or between the age groups and the diets, considering significance level $p<0.05$. Mean plasma glucose values from the groups indicated normal glycemia at all times of evaluation. A significant difference was found between the means of glycemia in young and adult dogs, with the 8-hour fasting with wet diet ($p=0.03$) and with 12 hours with dry diet ($p=0.04$). Healthy young and adult dogs, in physiological equilibrium, maintain average values of plasma glucose despite prolonged periods of pre-anesthetic fasting, which may be necessary, since 8-hour fasting for solid food is not enough to provide complete gastric emptying.

INDEX TERMS: Preanesthetic fasting, gastric emptying, plasma glucose, healthy dogs, age, anesthesia, hypoglycemia, dogs.

RESUMO.- [**Efeito do jejum pré-anestésico sobre o esvaziamento gástrico e a glicemia plasmática em cães hígidos de diferentes faixas etárias.**] Avaliou-se o esvaziamento gástrico e a glicemia plasmática em cães jovens e adultos, alimentados com ração seca e úmida, submetidos a diferentes períodos de jejum pré-anestésico (6, 8 e 12 horas). Foram selecionados 40 cães hígidos, os quais foram segmentados em 4 grupos de acordo com a faixa etária e o tipo de dieta administrada. Foi avaliado o esvaziamento gástrico por ultrassonografia e a glicemia sérica. Apenas 17,5% apresentaram completo esvaziamento gástrico, não sendo encontradas diferenças significativas entre as avaliações com 6 e 8 horas de jejum, ou entre as faixas etárias e dietas, considerando nível de significância $p<0,05$. Os valores médios da glicose plasmática dos grupos indicaram normoglicemia em todos os momentos de avaliação. Foi encontrada diferença significativa entre as médias da glicemia dos cães jovens e adultos, no período de 8 horas de jejum com dieta úmida ($p=0,03$) e com 12 horas nos animais com dieta seca ($p=0,04$). Conclui-se que cães hígidos jovens e adultos, em equilíbrio fisiológico, mantêm valores normais de glicemia plasmática apesar de períodos prolongados de jejum pré-anestésico, os quais podem ser necessários, tendo em vista que 8 horas de jejum alimentar de sólidos não é suficiente para proporcionar completo esvaziamento gástrico.

TERMOS DE INDEXAÇÃO: Jejum pré-anestésico, esvaziamento gástrico, glicemia plasmática, cães hígidos, anestesia, hipoglicemia, caninos.

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INTRODUCTION

There are few studies on the effect of pre-anesthetic fasting after the administration of different types of diet and its glycemic and physiological relationship. Preanesthetic fasting is a common practice in both medicine and veterinary medicine. The fasting aimed to reduce the volume and acidity of stomach contents, decreasing the risk of reflux and aspiration of gastric contents into the respiratory tract during surgery. Also, reducing the risk of postoperative complications, such as gastroesophageal reflux, esophagitis, and aspiration pneumonia (Brady et al. 2009, Castro et al. 2016).

The short fasting period in surgical patients can lead to metabolic disorders that directly interfere with the animal's response to inhalation anesthesia and surgical intervention (Castro et al. 2016). Prolonged fasting increases the risk of hypoglycemia (Nogueira et al. 2003).

Hypoglycemic animals may experience depression, prolonged recovery from anesthesia, weakness, muscle tremors, changes in blood pressure, changes in behavior, and seizures (Cortopassi et al. 2002, Futema 2002). Blood glucose concentrations depend on the interaction of multiple factors, including the time since the last meal, hormonal influences, and the use of glucose by peripheral tissues, such as skeletal muscle (Allison 2015).

The fasting time varies according to the type of diet, the volume of food, and variations between patients of different species and digestive conditions (Castro et al. 2016). Another relevant factor for the definition of adequate pre-anesthetic food fasting time is the age of the animal. Food should not be restricted to young animals for more than 4 to 6 hours, which can cause hypoglycemia to the patient (Fossum 2014).

The objective was to evaluate the glycemic variations of healthy dogs, of different age groups, with the supply of dry and wet diets and their influence on gastric emptying time.

MATERIALS AND METHODS

This research was approved by the Ethics Committee for Animal Use of the "Centro Universitário da Grande Dourados" (CEUA-Unigran), under protocol 005/16. Forty dogs were selected, regardless of breed, of both sexes, with the following inclusion criteria: be on the waiting list for the Castration Project; be aged between six months and eight years old; weigh between two and 15kg; be healthy, according to the results of hematological, biochemical (renal, hepatic and pancreatic) and ultrasound examinations (abdominal and pelvic evaluation); and, having authorization from the dog's tutor in an informed consent form.

Animals under treatment for any previously diagnosed disease or condition, using any medication, or animals that did not feed spontaneously on the day of the experiment were excluded from the study.

For experimental design, four groups of 10 animals were formed, according to the age group and the type of diet administered, as shown below. The group was defined according to the order of arrival for surgical care:

Group I - Young dogs, from 6 months to 2 years old, with a wet diet;

Group II - Young dogs, from 6 months to 2 years old, with a dry diet;

Group III - Adult dogs, from 2 years and 1 month to 8 years old, with a wet diet;

Group IV - Adult dogs, from 2 years and 1 month to 8 years old, with a dry diet.

All animals were hospitalized the day before the experiment. At this time, the patients underwent physical examination, with measurement of rectal temperature, heart rate, and respiratory rate, as well as the assessment of mucosal color and capillary refill time. Each dog was kept in individual stalls, with free access to water, having received the last meal of the day at 6 p.m.

The following morning, at 7 a.m., all dogs were fed wet or dry food, according to the group to which they belonged. The dry food offered was Golden® (PremierPet, Brazil), for dogs of small to medium size, composed of 10% moisture, 23% crude protein, 12% fat, 3.0% fiber, 0.2% sodium, and 0.5% potassium. The wet food offered was the same food previously described, but added with water in an amount equivalent to 70% moisture, and later homogenized in a blender to achieve the desired consistency. The amount of feed offered was equivalent to half of the daily recommendation, according to the manufacturer (Golden - PremieR Pet®), since the dog's food is offered in one to three times a day.

After the ingestion of the feed, all animals remained fasting. Then, dogs were submitted to three blood samples by venipuncture in the jugular vein for glycemic analysis, six, eight, and 12 hours after the beginning of the fast. For the glycemic test, the blood was placed in a glass tube containing sodium fluoride, being centrifuged for 5 minutes at 2,000rpm. The determination of the concentration of serum glucose was made using an enzymatic-colorimetric method, at an absorbance of 505nm, with a reagent of the Gold Analisa® brand and reading on a semi-automatic spectrophotometer.

After 6 hours of solid fasting and 2 hours of water fasting, the ultrasound study was performed through abdominal scanning with Mindray® ultrasound device, model M5-VET, with microconvex transducer and frequencies between 5.0, 6.5 and 8.0MHz, according to the size of the animal.

After the general examination of the organs, the dog's gastric emptying was evaluated for the presence or absence of any luminal content in the stomach, and gastric motility. The transducer was placed in a sagittal plane in the epigastric region. Then, the antrum and gastric body were scanned by moving the transducer from right to left, to obtain an overall qualitative impression of the cavity and gastric content.

The den has a wall characterized by multiple layers, and its visibility has been assessed in a binary way (visible or not). A single sonographer performed qualitative assessments of the gastric antrum. The antrum was considered empty if it appeared with the anterior and posterior walls juxtaposed. It was considered as containing liquid if it had an endocavity with a hypoechoic content inside and with distended walls. It was considered to have solid content if it appeared distended with an echogenic content similar to the liver parenchyma. If the dog had food content in the stomach, the ultrasound was repeated after 2 hours, then totaling 8 hours of fasting.

Statistical analysis was performed with the aid of the BioEstat software version 5.3, using Fisher's Exact Test to assess gastric emptying, comparing the groups of young and adult dogs, as well as those with dry food and wet food. As for blood glucose, we calculated the means and standard deviations of each group concerning the different periods of pre-anesthetic fasting, and then the Friedman test compared the values over time. When variables with a statistically significant difference, the Chi-square test verified the pairs of groups in which a difference was found. The Mann Whitney non-parametric test assessed the differences in plasma glucose between pairs of groups. For all, we considered a significance level of $p<0.05$.

RESULTS

As for gastric emptying, 25% of the animals (n=5) with a wet diet (Groups I and III) had no food content with 6 hours of fasting. Dogs submitted to the dry diet (Groups II and IV) were represented by only 10% (n=2), with no significant difference (p=0.40) between groups.

When assessing the influence of age on this variable, among the 20 animals under 2 years old (Groups I and II), only 20% (n=4) had complete gastric emptying in 6 hours, while in the groups of adult animals (Groups III and IV) were 15% (n=3), not being statistically significant (p=0.70).

Animals that had food content during abdominal ultrasound after 6 hours of fasting were reevaluated after 2 hours. In all cases, 8 hours of waiting were not enough for the dogs to have complete gastric emptying, with no difference between gastric emptying occurring after 6 or 8 hours of fasting.

The mean values of glycemia indicated normoglycemia at all times of assessment (Table 1). Among the 40 animals evaluated, only six dogs presented hypoglycemia, two animals from Group I (the first with 8 hours and the second with 12 hours of fasting), one animal from Group III (with 12 hours of fasting) and three dogs from Group IV (with 6, 8 and 12 hours of fasting, respectively). When comparing the mean blood glucose values of each group over time, considering the different fasting periods (6, 8, and 12 hours), we found a statistically significant difference only in Group III, between the mean values of 8 and 12 hours.

When comparing the different groups to assess the interference of age or diet in the glycemia of the animals (Table 2), a significant difference was found in dogs with 8 hours of fasting. In Groups I and III, age interfered in their blood glucose in dogs fed with a wet diet. Between Groups III and IV, the diet interfered with the glycemia of adult animals. Also, in individuals with 12 hours of fasting, we found a significant difference between Groups II and IV, indicating age interference in the plasma glucose of dogs fed a dry diet.

When observing the results of the four experimental groups with the same fasting time, we noticed that after 8 hours of fasting, the dogs tend to present significant glycemic changes. The most considerable changes were found when comparing Groups I and III, taking into account the changes due to age, and between Groups III and IV due to the type of diet offered.

DISCUSSION

Recommendations regarding fasting before anesthetic and surgical procedures vary according to some authors, who report a minimum of 6 hours (Bednarski 1996), 6 to 12 hours (Fossum 2014), 8 to 12 hours (Futema 2002), 12 to 16 hours (Massone 2011) and up to 18 hours of fasting (Guimarães et al. 2007). However, regarding water restriction, there is a recommendation to suspend water for 2 to 3 hours before the intervention (Massone 2011).

Fasting induces low levels of plasma glucose, mobilizes the liver's glycogen stores, and reduces the circulation of fatty acids, which can alter the rate of biotransformation of drugs (Thurmon et al. 1996). Prolonged fasting can result in severe hypoglycemia and metabolic acidosis, the acid-base imbalance, more common in carnivores (Luna 2002), in addition to stress and discomfort (Guimarães et al. 2007).

According to Savvas et al. (2009), the standard compound paste ration, in the portion of half of the daily recommendation, administered 3 hours before anesthesia does not significantly increase the gastric content of dogs and keeps the pH of this content high. Dogs fasted for 10 hours have more acidic gastric contents than dogs fasted for 3 hours. After 10 hours of food consumption, stomach contents are not found to exert its known buffer effect on gastric acidity (Herdt 1999).

The high acidity of gastric contents can contribute to the increase in the incidence of gastroesophageal reflux in dogs during anesthesia, after prolonged pre-anesthetic fasting, as it decreases the pressure of the lower esophageal sphincter (Savvas et al. 2009). However, when comparing gastric emptying with eating canned meats and dry cereals, gastric emptying occurs in about 10 hours and water after 52 minutes of ingestion, with observations of individual variations (Ambrósio 2002), which can reach this value 18 hours of solid food fasting (Guimarães et al. 2007).

The presence of pain, stress, and the animals' emotional condition can delay gastric emptying (Guimarães et al. 2007). It is possible that animals in a hospital environment, under confinement, and undergoing procedures that are not part of their routine have the physiological function of the gastrointestinal tract altered, in addition to the absence of the tutor, reflecting on the speed of gastric emptying.

Regarding glycemia, the maintenance of stable blood glucose concentrations involves finely regulated mechanisms

Table 1. Mean plasma glucose values in dogs submitted to different periods (DP) of pre-anesthetic fasting

Groups	Plasma glycemia (mg/dl) - DP			
	Fasting 6h	Fasting 8h	Fasting 12h	p-value
Group I	69.5 ± 7.11	72.7 ± 10.37	73.6 ± 12.95	0.49
Group II	75.2 ± 19.57	80.1 ± 9.10	75.5 ± 7.43	0.20
Group III	74.4[a,b] ± 9.79	78.8[a] ± 5.92	71.4[b] ± 7.97	0.036
Group IV	69.5 ± 9.14	73.6 ± 11.17	68.9 ± 10.80	0.20

[a,b] Different superscript letters have statistical differences; equal letters have no statistical difference.

Table 2. Comparison of plasma glucose between groups

Fasting period	Groups analyzed	p-value
6 hours	G1 x G3	0.16
	G2 x G4	0.41
	G1 x G2	0.36
	G3 x G4	0.17
8 hours	G1 x G3	0.03
	G2 x G4	0.05
	G1 x G2	0.07
	G3 x G4	0.01
12 hours	G1 x G3	0.23
	G2 x G4	0.04
	G1 x G2	0.38
	G3 x G4	0.12

G1 = Group 1, young dogs with wet diet; G2 = Group 2, young dogs on a dry diet; G3 = Group 3, adult dogs with a wet diet; G4 = Group 4, adult dogs on a dry diet.

in which the liver, extrahepatic tissues, and various hormones, such as insulin, glucagon, epinephrine, glucocorticoids, and thyroid hormone, play a fundamental regulatory role (Beitz 2012). Glucose is provided by the intestinal absorption of this monosaccharide present in the diet or by the liver production from its precursors, for example, carbohydrates and amino acids. After intestinal absorption, liver production is the main source of glucose input for maintaining healthy blood glucose (Nogueira et al. 2003).

Animals use different proportions of the body's stock of carbohydrates, fat, and protein to maintain glycemic levels during fasting, maintaining vital functions for as long as possible (Remillard, 2000). The hormones epinephrine and glucagon promote the release of glucose from glycogen. In fasting situations, insulin levels decrease, proteolysis increases, and the release of amino acids increases. Fatty acids are also mobilized during fasting (Nogueira et al. 2003).

Hypoglycemic changes are essential for anesthetic evaluation since animals in these conditions may have changes in blood pressure and prolonged anesthetic recovery (Cortopassi et al., 2002; Futema, 2002). There were no evident clinical signs of hypoglycemia in anesthetized animals, which can only be diagnosed by measuring blood concentration. That is why it is crucial to detect hypoglycemia during the preoperative period (Nogueira et al., 2003).

There are undoubtedly individual variations in physiological adaptation to fasting. When Nogueira et al. (2003) evaluated 31 dogs at different times of pre-surgical fasting, 6 to 8 hours of fasting, 12 to 14 hours, or above 16 hours, there were no differences in the glycemic level in the pre- and post-anesthetic, while Guimarães et al. (2007) found glycemic changes in animals with 12 hours of pre-anesthetic fasting.

Regarding the glycemia variation due to the age of the animals, it is vital to have different clinical monitoring of the animal's glycemic condition. No previous studies were found that emphasize the differentiation of pre-anesthetic fasting according to the age of the animal. However, it is recommended to avoid restricting food to young animals for more than 4 to 6 hours, due to the risk of hypoglycemia (Fossum 2014).

The handling of the patient produces a typical stress response with hormonal and metabolic changes in animals, including elevated glycemic values (Luna 2002). However, it appears that in this study, the manipulation of animals for serial venipuncture and ultrasound, in addition to the confinement they were subjected to during the experiment period, did not interfere with neuroendocrine responses to stress, as described by Guimarães et al. (2007). In this way, the selection of healthy individuals contributed to the physiological balance of dogs, resulting in efficient compensatory mechanisms concerning glucose metabolism.

CONCLUSIONS

A period of 8 hours of pre-anesthetic fasting is not enough to provide complete gastric emptying in dogs fed both dry and wet food.

Healthy young animals and adults, in physiological balance, maintain average plasma glucose values despite prolonged periods of pre-anesthetic fasting.

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

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Hemoplasma infection in wild captive carnivores[1]

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ABSTRACT.- Carneiro F.T, Scalon M.C., Amorim G., Silva W.A.C, Honorato S.M., Pereira F.M.A.M, Silva L.H.R., Aquino L.C. & Paludo G.R. 2020. **Hemoplasma infection in wild captive carnivores.** *Pesquisa Veterinária Brasileira 40(4):293-299.* Laboratório de Patologia Clínica Veterinária, Faculdade de Agronomia e Medicina Veterinária, Universidade de Brasília, Av. L4 Norte, Brasília, DF 70910-900, Brazil. E-mail: giane@unb.br

Hemoplasmas are bacteria able to adhere themselves loosely to the plasma membrane of erythrocytes and may parasitize several species of mammals. There are three known species of hemoplasmas that parasitize domestic and wild cats: *Mycoplasma haemofelis*, '*Candidatus* Mycoplasma haemominutum' and '*Candidatus* Mycoplasma turicensis'. Dogs are infected by at least two species of hemoplasmas: '*Candidatus* Mycoplasma haematoparvum' and *Mycoplasma haemocanis*. The hemoplasmoses are very important in veterinary clinics, either because of its worldwide distribution and severity of clinical signs, depending on parasite species and host immune competence, or due to its zoonotic potential and capability of infecting endangered species. This study set out to investigate which hemoplasmas species parasitize different captive wild carnivores in order to clarify the epidemiology of hemoplasmoses in wild animals. Furthermore, the research intended to characterize the hematological changes caused by different species of hemotropic mycoplasmas infection in order to establish their clinical importance to wild species and the capacity of these species to become a reservoir of studied agents. Samples of 33 wild felids and 18 wild canids were investigated using polymerase chain reaction (PCR) to detect hemoplasmas DNA and it was observed that the occurrence of infection in these species is 45.5% and 83.3%, respectively. Factors such as age, gender or anaemia are not more frequent in animals positive for the infection. Therefore, it is concluded that infection caused by hemoplasmas in wild carnivores has high prevalence, and either agent pathogenicity is low, or chronic stage is more frequent, resulting in a low rate of diagnosis.

INDEX TERMS: Hemoplasma, infection, wild captive, carnivores, hemotropic mycoplasmas, hemoparasites, anaemia, diagnose, polymerase chain reaction, wildlife animals.

RESUMO.- [Infecção por hemoplasmas em carnívoros selvagens cativos.] Hemoplasmas são bactérias capazes de aderir frouxamente à membrana plasmática de eritrócitos e que podem parasitar diversas espécies de mamíferos. São conhecidas três espécies de hemoplasmas que parasitam felídeos domésticos e selvagens: *Mycoplasma haemofelis*, '*Candidatus* Mycoplasma haemominutum' and '*Candidatus* Mycoplasma turicensis'. Cães são infectados por ao menos duas espécies de hemoplasmas: *Candidatus* Mycoplasma haematoparvum' and *Mycoplasma haemocanis*. As hemoplasmoses são de grande importância na clínica veterinária, tanto pela sua distribuição ubíqua e severidade dos sinais clínicos, a depender da espécie do parasita e imunocompetência do hospedeiro, quanto pelo seu potencial zoonótico e capacidade de infectar espécies ameaçadas. Este estudo visa investigar quais espécies de hemoplasmas parasitam diferentes carnívoros selvagens de cativeiro, a fim de esclarecer a epidemiologia das hemoplasmoses em animais selvagens. Além disso, o trabalho objetivou caracterizar as alterações hematológicas causadas pela infecção por diferentes espécies de micoplasmas hemotrópicos visando estabelecer sua importância clínica para espécies selvagens e a capacidade destas espécies de

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se tornar reservatórios dos agentes estudados. Amostras de 33 felídeos selvagens e de 18 canídeos selvagens foram investigadas por meio da reação em cadeia da polimerase (RCP) para detectar o DNA dos agentes e foi observado que a ocorrência da infecção por hemoplasmas nestas espécies é de 45,5% e 83,3%, respectivamente. Fatores como idade, sexo ou anemia não são mais frequentes em animais positivos para a infecção. Dessa forma, conclui-se que a infecção causada por hemoplasmas em carnívoros selvagens possui alta prevalência, no entanto ou a patogenicidade dos agentes é baixa ou o estágio crônico da infecção é mais frequente, resultando em uma baixa frequência diagnóstica.

TERMOS DE INDEXAÇÃO: Infecção, hemoplasma, carnívoros, selvagens cativos, micoplasmas hemotrópicos, hemoparasitas, anemia, diagnóstico, reação da polimerase em cadeia, animais selvagens.

INTRODUCTION

Hemoplasmas, or hemotropic mycoplasmas are important causative agents of infectious anaemia. which have been found in all continents, except Antarctica, leading to the belief that its distribution is linked to urbanization. However, the epidemiology and transmission of these agents remains somewhat obscure (Tasker et al. 2003, Biondo et al. 2009).

There are three known species of hemoplasmas that parasitize domestic and wild cats: *Mycoplasma haemofelis*, '*Candidatus* Mycoplasma haemominutum' and '*Candidatus* Mycoplasma turicensis'. It is known that *Mycoplasma haemofelis* induces anemia in immunocompetent hosts, whereas '*Candidatus* Mycoplasma haemominutum' and '*Candidatus* Mycoplasma turicensis' appear to be less pathogenic (Almosny 1998, André et al. 2010, Allison & Little 2013).

Dogs may be infected by at least two species of mycoplasma: *Mycoplasma haemocanis* and '*Candidatus* Mycoplasma haematoparvum' (O'Dwyer et al. 1997, Biondo et al. 2009). In addition, a canine hemoplasma more similar to '*Candidatus* Mycoplasma haemominutum' than to '*Candidatus* Mycoplasma haematoparvum' was detected by polymerase chain reaction (PCR) in a dog in Londrina, Brazil, and another hemoplasma with 98-100% homology to '*Candidatus* Mycoplasma turicensis found in cats also amplified in 7 out of 10 splenectomized dogs in Porto Alegre, Brazil (André et al. 2012). It is unclear if these are new canine hemoplasmas or if dogs are susceptible to infection by feline hemoplasmas.

Parasitism by hemoplasmas has been reported in different wild species, such as: *Leopardus geoffroyi*, *Leopardus wiedii*, *Leopardus pardalis*, *Puma concolor, Panthera* onca, *Lynx lynx*, *Felis silvestris silvestris*, *Puma yagouaroundi*, *Canis lupus* and *Speothos venaticus*, including vulnerable species like *Panthera leo,* and *Leopardus tigrinus*, or endangered like *Panthera tigris*, and L*ynx pardinus* (Birkenheuer et al. 2003, Bayliss et al. 2009, André et al. 2010, Almeida et al. 2011, Santos et al. 2013).

Furthermore, *Anaplasma platys*, *Bartonella henselae* and '*Candidatus* Mycoplasma haematoparvum' coinfection of a veterinarian who moved amongst Grenada, Ireland and South Africa has been described in literature. The woman reported contact with several domestic and wild animals, as well as fleas, ticks, lice, biting flies, mosquitoes, spiders and mites. She developed symptoms such as fainting, headaches, photophobia, muscle twitching, tonic-clonic seizures, ataxia and memory loss (Maggi et al. 2013). Also, a 34-year-old HIV-positive patient has tested positive for hemoplasmas in Brazil (Dos Santos et al. 2008). These cases alert to the zoonotic potential of hemoplasmosis and severity of its clinical signs in humans, especially combined with other infectious agents or in immunocompromised patients.

In dogs and cats, infection of red blood cells by hemoparasites can result in intravascular or extravascular anaemia. Other clinical signs may include depression, splenomegaly, fever, anorexia, dehydration, wasting, lethargy, icterus, and sudden death. Anaemia is regenerative, unless there is a primary disease to inhibit erythropoiesis. For cats, hemoplasmosis commonly is secondary to feline leukemia virus (FeLV) or feline immunodeficiency virus (FIV) (Willi et al. 2007, Thrall 2012).

Hemoparasite identification traditionally occurs by viewing blood smear under light microscope. Nevertheless, this diagnostic method is rather limited since it greatly depends on the amount of parasite circulating, as well as technician experience to distinguish the parasite from other structures such as Howell-Jolly boddies, basophilic stippling or even artifacts caused by poor staining. Therefore, the development of a PCR technique highly sensitive and specific assures an effective diagnosis, even in situations where there is a small amount of the microorganism (Thrall 2012).

Even though hemoplasmas can infect different species of wild carnivores and cause clinical signs extreme enough to compromise the conservation of endangered species, there is little information on the occurrence of hemoplasma infection in these species. Moreover, considering the zoonotic potential of the disease, it is necessary to assess whether there is a likelihood of wild animals to act as reservoirs and transmit it to humans and domestic animals.

The objectives of this study were to determine the occurrence and identify the species of hemoplasmas that affect captive wild canids and felids by conventional PCR. Additionally, our aim was to verify haematological changes caused by hemoplasmas and study the epidemiology of hemoplasma infection in carnivores, correlating the occurrence of the disease in different wild species.

MATERIALS AND METHODS

Blood samples of 33 wild felids and 18 wild canids from both Brasília Zoo and a conservationist breeding facility were used. The zoo provided samples from three jaguars (*Panthera onca*), one ocelot (*Leopardus pardalis*), two pampas cats (*Leopardus pajerus*), three pumas (*Puma concolor*), one margay (*Leopardus wiedii*), one oncilla (*Leopardus tigrinus*), four tigers (*Panthera* tigris), three jaguarundis (*Puma yagouaroundi*), two lions (*Panthera leo*), six maned wolves (*Chrysocyon brachyurus*), five hoary foxes (*Lycalopex vetulus*), five crab-eating foxes (*Cerdocyon thous*) and two bush dogs (*Speothos venaticus*). The breeding facility provided samples of eight jaguars, one ocelot, a pampas cat and three pumas.

Samples were collected under authorization of the Ethics Committee on Animal Use (CEUA-UnB, Doc. no. 130988/2015) and the "Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis" (IBAMA, SISBIO no. 46097).

Samples from two natural infected domestic dogs and four domestic cats from the Veterinary Hospital of "Universidade de Brasília" (UnB) were used as positive controls for the PCR techniques. These animals had been previously diagnosed with hemoplasmas through real-time PCR (qPCR) and their samples had been sequenced

and stored in a biobank at the Laboratory of Veterinary Molecular Microbiology and Clinical Pathology at the UnB.

In order to reduce stress caused by physical and chemical restraint, all wildlife samples were collected during routine veterinary procedures of the host institution.

Samples of venous blood were taken by puncturing either the cephalic vein or jugular ventral caudal. These samples were collected in an atraumatic manner and quickly packed into tubes containing ethylenediaminetetraacetic acid (EDTA).

Samples from the breeding facility were sent to the Veterinary Clinical Pathology Laboratory at the University of Brasilia, where an automated haematologic counter for veterinary use (Horiba ABX Micros ESV 60) was used to determine red blood cell (RBC) and white blood cell (WBC) count. Samples from the zoo were sent to the zoo's Veterinary Hospital Laboratory Nucleus, where it was held manual RBC and WBC count with a Neubauer chamber.

The difference in methodology for WBC and RBC counts for samples from different origins took place in order to ensure that processing was carried out as quickly as possible. However, the same technician processed and analyzed all samples from both institutions in order to reduce variation to a minimum. Further processing was identical for samples from both origins. The packed cell volume (PCV) was determined by microhematocrit technique and whole blood smears were prepared and stained with a Romanowsky type stain (Diff-Quick) in order to perform leukocyte differential count, platelets count, morphological observation of blood cells and hemoparasites search.

After performing complete blood counts, samples were frozen and sent to the Laboratory of Veterinary Molecular Microbiology and Clinical Pathology at the University of Brasilia, where the DNA was extracted using commercial kits (Illustra blood genomicPrep Mini Spin Kit®, GE Healthcare of Brazil Ltda, São Paulo/SP), following the manufacturer's recommendations. The DNA samples were kept at -20°C until the PCR.

In order to confirm DNA extraction and attest the quality and integrity of extracted material, and absence of inhibitors, a PCR was carried out to detect the gene encoding glyceraldehyde 3-phosphate dehydrogenase using the primers GAPDH- F and GAPDH-R (Birkenheuer et al. 2003).

Specific primers for the 16S rRNA gene were used to identify hemoplasmas. For the standardization of PCR techniques, domestic dogs and cats with a positive laboratory diagnosis using qPCR and sequencing were used. The conditions of PCR techniques were determined experimentally. Samples from domestic animals known to be positive were used as positive controls, while miliQ water was used as a negative control in all experiments.

Six different standard PCR techniques were carried out for the detection of positive samples of hemoplasmas: for hemoplasmas genus using primers HBT-F and HBT-R , for *Mycoplasma haemofelis* with HF-F1 and HF-R3 primers and for '*Candidatus* Mycoplasma haemominutum' 1183-F and 1290R primers (Criado-Fornelio et al. 2003). Primers CMtFw2 and CMtRev2 was used for '*Candidatus* Mycoplasma turicensis' detection (Aquino et al. 2014). For *Mycoplasma haemocanis,* HCAN-F and HCAN-R primers was used and, for '*Candidatus* Mycoplasma haematoparvum', HPARV-F and HPARV -R (Torkan et al. 2014). Rickettsiae was also identified through PCR by using one technique to identify Anaplasmataceae family, capable of identifying *Ehrlichia canis*, *E. chaffeensis, E. muris, Anaplasma equi, A. phagocytophila, A. platys, A. marginale, A. centrale, Wolbachia pipientis, E. sennetsu, Neorickettsia risticii* and *N. helminthoeca* with EHR16sd and EHR16sr primers, and another for *Anaplasma platys* using PLATYS as foward primer (Inokuma et al. 2000). Also, other PCR specific for identification of *E. canis,* using ECAN5 and He3 primers was performed (Murphy et al. 1998). The final volume of each PCR mix was 25µl, containing 1X PCR buffer, primers, MgCl2, dNTPs, Taq DNA polymerase (Invitrogen® Brasil Ltda, São Paulo, Brazil) and DNA sample.

The results of the PCRs were analysed in agarose electrophoresis gel stained with ethidium bromide and observed under fluorescence transilluminator.

Data was entered into Microsoft Office Excel® and statistical analysis was performed with SPSS software (SPSS Inc., Chicago/IL, USA). Categorical variables (gender, origin, species, age, presence of anaemia and PCR result) were evaluated using chi-square test, following a 95% confidence interval. Kolmogorov-Smirnov and Shapiro-Wilk tests were used to test the normality of continuous variables (PCV, number of erythrocytes and leukocytes). Mann-Whitney U test was used to analyze the data did not follow normal distribution. Results were considered significant when $P<0.05$.

RESULTS

Initially we compared complete blood cell count (CBC) results of animals from different origins, in order to investigate if the difference in processing methodology resulted in some kind of bias. No differences were found, suggesting that this disagreement was not a determining factor in the results.

Considering the 33 wild felids tested, fifteen cats (45.5%) were positive for hemoplasmas, being two jaguars, two ocelots, three pampas cats, four pumas, one margay, two jaguarundis and a lion (Table 1). There was no statistical difference between the positive and negative animals when compared to origin, institution, gender, age (greater or less than 2 years old) or anaemia (Table 2-4). There was also no difference between

Table 1. Results of the PCR for hemoplasma by felid species

	Number (%) of positives	Number (%) of negatives	Total
Leopardus wiedii	1(3)	1(3)	2 (6)
Panthera onca	2 (6.1)	9 (27.3)	11 (33.4)
Leopardus pardalis	2 (6.1)	0 (0)	2 (6.1)
Leopardus pajerus	3 (9.1)	0 (0)	3 (9.1)
Puma concolor	4 (12.1)	2 (6.1)	6 (18.2)
Panthera tigris	0 (0)	4 (12.1)	4 (12.1)
Puma yagouaroundi	2 (6.1)	1 (3)	3 (9.1)
Panthera leo	1 (3)	1 (3)	2 (6)
TOTAL	15 (45.5)	18 (54.5)	33 (100)

Table 2. Number (%) of positive and negative animals for hemoplasma infection compared to gender by Pearson's Chi-Square

Hemoplasma PCR	Gender			
	Felids		Canids	
	Male	Female	Male	Female
Negative	7 (21.2)[a]	11 (33.3)[a]	1 (5.6)[a]	2 (11.1)[a]
Positive	10 (30.3)[a]	5 (15.2)[a]	9 (50)[a]	6 (33.3)[a]
Total	17 (51.5)	16 (48.5)	10 (55.6)	8 (44.4)

Felids: *p=0.112,* Canids: *p=0.396;* [a] Equal letters in different columns indicate proportions are not significantly different.

Table 3. Number (%) of positive and negative animals for hemoplasma infection compared to age by Pearson's Chi-Square

Hemoplasma PCR	Age			
	Felids		Canids	
	<2 years old	>2 years old	<2 years old	>2 years old
Negative	2 (6.1)[a]	16 (48.5)[a]	1 (5.6)[a]	2 (11.1)[a]
Positive	1 (3)[a]	14 (42.4)[a]	5 (27.8)[a]	10 (55.5)[a]
Total	3 (9.1)	30 (90.9)	6 (33.4)	12 (66.6)

Felids: *p=0.658*; Canids: *p=1.000;* [a] Equal letters in different columns indicate proportions are not significantly different.

Table 4. Number (%) of positive and negative animals for hemoplasma infection compared to presence or not of anaemia by Pearson's Chi-Square

Hemoplasma PCR	Anemia			
	Felids		Canids	
	PCV≥27	PCV<27	PCV≥38	PCV<38
Negativo	12 (50)[a]	1 (4.2)[a]	2 (12.5)[a]	1 (6.3)[a]
Positivo	10 (41.7)[a]	1 (4.2)[a]	4 (25)[a]	9 (56.2)[a]
Total	22 (91.7)	2 (8.4)	6 (37.5)	10 (62.5)

Felids: *p=0.902,* Canids: *p=0.247;* PCV = packed cell volume; [a] Equal letters in different columns indicate proportions are not significantly different.

PCV, RBC, WBC and platelet count of positive and negative animals. No hemoplasmas were found in blood smears from both positive and negative animals.

Two jaguars were positive for '*Candidatus* Mycoplasma haemominutum' and '*Candidatus* Mycoplasma haematoparvum', a pampas cat for *Mycoplasma haemocanis* and an ocelot and a pampas cat for '*Ca*. M. haematoparvum'. The other ten felids positive for hemoplasmas had no species detected by PCR techniques used in this study (Table 5).

Among the positive felines, a jaguar and an ocelot were positive for *Anaplasma platys,* a puma was positive for *Ehrlichia canis* and one jaguar, one ocelot, two pampas cats, one puma and one oncilla were also tested positive for some other Anaplasmataceae family agent. Since the animals were naturally infected, it is difficult to say which agent caused the infection first. However, it is quite likely that one of the agents has compromised the hosts immune system and favoured the infection of other.

Fifteen canids (83.3%) were positive for hemoplasmas out of the 18 tested, being six maned wolves, four hoary foxes, four crab-eating foxes and one bush dog (Table 6). There was no difference between positive and negative animals when compared regarding gender, age or the presence of anaemia (Tables 2-4). Likewise, there was no difference in comparison between PCV, RBC, WBC and platelet count from positive and negative animals.

Two crab-eating foxes were tested positive for '*Candidatus* Mycoplasma haemominutum', one hoary fox, and two crab-eating foxes were positive for *Mycoplasma haemocanis* and one bush-dog had the presence of '*Candidatus* Mycoplasma haematoparvum' in its sample. Even so, nine positives did not have the species of hemoplasma detected by PCR techniques used here (Table 5).

Table 5. Number (%) of positive and negative samples for the different PCR techniques

	Felids		Canids	
	PCR positive	PCR negative	PCR positive	PCR negative
Hemoplasma genus	15 (45.5)	18 (54.5)	15 (83.3)	3 (16.7)
Mycoplasma haemofelis	0 (0)	33 (100)	0 (0)	18 (100)
Candidatus Mycoplasma haemominutum	2 (6.1)	31 (93.9)	2 (11.1)	16 (88.9)
Ca. Mycoplasma turicensis	0 (0)	33 (100)	0 (0)	18 (100)
Mycoplasma haemocanis	1 (3)	32 (97)	3 (16.7)	15 (83.3)
Ca. Mycoplasma haematoparvum	4 (12.1)	29 (87.9)	1 (5.6)	17 (94.4)

Table 6. Results of the PCR for hemoplasma by canid species

	Number (%) of positives	Number (%) of negatives	Total
Chrysocyon brachyrurus	6 (33.3)	0 (0)	6 (33)
Lycalopex vetulus	4 (22.2)	1 (5.6)	5 (27.8)
Cerdocyon thous	4 (22.2)	1 (5.6)	5 (27.8)
Speothos venaticus	1 (5.6)	1 (5.6)	6 (11.2)
Total	15 (83.3)	3 (16.7)	33 (100)

No co-infection between hemoplasmas species was encountered in any of the studied samples from canids. Nevertheless, among positive hemoplasma samples one maned wolf and a hoary fox were positive for *A. platys* and *E. canis*; a crab-eating fox, a hoary fox and three maned wolves were positive for *A. platys*; a hoary fox and a maned wolf tested positive for *E. canis*; and a crab-eating fox, two hoary foxes and a maned wolf were positive for some unspecified Anaplasmataceae family agent. In the blood smear belonging the maned wolf positive for *E. canis* morula was found in platelets, but hemoplasmas were not found in smears of any of the studied canids.

DISCUSSION

Other studies have been conducted investigating hemoplasma infection in wild felines in recent years and the agent occurrence differs considerably. In a study with 54 felines in captivity Illinois and Florida, in the United States, there were only two (0.04%) positive tigers (André et al. 2010). This value is much below the 45.5% found in this study, although it also samples from captive animals.

Brasília Zoo is located in a major metropolitan center and, therefore, faces a relevant problem with domestic and synanthropic animals such as stray cats and capybaras (*Hydrochoerus hydrochaeris*). Cats and capybaras have free access in and out the institution and may be responsible for

vector traffic, therefore, maintaining high infection rates in wild specimens. Study regarding these animals and their vectors is essential in order to gather information about their role in this cycle.

In another study, conducted with 165 felines from zoos in São Paulo, Mato Grosso and the Distrito Federal, the occurrence of hemoplasma infection was 13.9% in felids, totalling 23 infected animals. Of all the animals, ten belonged to Brasilia Zoo and three (30%) of which tested positive for '*Candidatus* Mycoplasma haemominutum' (Birkenheuer et al. 2003). Though the total occurrence is much lower, when considering only the animals located in the Distrito Federal, despite only ten animals having been sampled, there is greater proximity to the occurrence found in this study. This suggests that both hemoplasma infections in wild felines can be more frequent in the Federal District than in Mato Grosso and São Paulo, and that it has grown over the years, going from 30% to 45.5%. This problem may be once again related to stray animals, increasingly frequent in the vicinity of Brasilia Zoo.

A third study evaluated blood samples and serosanguinous fluids of 257 free-living or captive wild felids of 15 different species, originating from Africa, South America and Europe, achieved 96 positive animals (37%) for hemoplasma infection. The felids were divided into five groups with different frequencies of infection: Serengeti lions (98%), Eurasian lynxes (44%), European wild cats (39%), Iberian lynxes (37%) and Brazilians wild felids (10%) (Willi et al. 2007). Although the total occurrence is very close to that found in this paper, there is considerable disparity when comparing only Brazilian wild felids. The authors imply that the difference in the frequency of infection among the five groups may be due to differences in habitat, either from one captivity to another, or from captivity to free-living. It should be considered, therefore, that the samples of Brazilian wild felids were taken from the Zoological Park of São Paulo, located in Brazilian Atlantic Forest, while samples of the current paper were collected from animals that live in Brazilian Cerrado. The differences between these two ecosystems can influence factors such as temperature variation, facilitating or hindering that animals suffer heat stress, as well as altering susceptibility to ectoparasites that potentially convey the agent in question. This may account for the variation of 35.5% in the frequency of infection between papers.

The infection of domestic cats is potentially pathogenic as evaluated previously (Dumler et al. 2001, Ebani & Bertelloni 2014), but this has not often been reported in wild felids (Birkenheuer et al. 2003, Almeida et al. 2011). One can reach the hypothesis that animals are in the chronic stage of infection, less pathogenic strains of the agent caused the infection, or the hosts had better immune competence and thus did not have anemia (Foley et al. 1999, Filoni et al. 2006, Fortes et al. 2011). One of the pumas positive to '*Ca*. M. haemominutum' and '*Candidatus* Mycoplasma haematoparvum' had its sample tested twice in one year apart and were positive in both tests. This finding confirms the theory of this animal being in the chronic phase of infection and increases the likelihood that the same is happening with the other test subjects.

Researches with domestic cats indicate that males are more prone to hemoplasma infection than females, and *Mycoplasma haemofelis* is more common in young animals, whereas '*Ca.* M. haemominutum' is most reported in older animals (Fournier et al. 2007, Fortes et al. 2011). However, just like in this paper, Willi et al. (2007) found no correlation between gender and hemoplasma infection in other research investigating wild felids. Co-infection by different species of hemoplasmas is often reported in domestic cats.

Co-infection by different species of hemoplasmas is often reported in domestic cats (Filoni et al. 2006, Fortes et al. 2011, Adaszek et al. 2013). The percentage of animals concurrently infected with more than one specie of hemoplasma was 21.4% in a research that only investigated the presence of *M. haemofelis*, '*Ca.* M. haemominutum' and '*Ca.* M. turicensis' in wild felids (Almeida et al. 2011). Interestingly only two pumas had simultaneous infections with more than one species of hemoplasma, in both cases '*Ca.* M. haemominutum' and '*Ca.* M. haematoparvum'. It is worth noting that there is a very close proximity between these two species and the two PCR techniques may be identifying the same agent (Fortes et al. 2011).

Hemoplasma infection in domestic dogs has been described in the literature, particularly associated with splenectomized or immunosuppressed animals. A study of 889 dogs in Switzerland found an occurrence of infection by *Mycoplasma haemocanis* or '*Ca.* M. haematoparvum' of 1.2%, being the former 0.9% and latter 0.3% (Hegarty et al. 2015). Another study surveyed the same agents in 154 dogs from Ribeirão Preto and found a canine hemoplasma occurrence of 2.5%, 1.9% of infections by *M. haemocanis* and 0.6% by '*Ca. M.* haematoparvum' (Inokuma et al. 2000). The occurrence found in the present study significantly outweighs that which was found in the other papers, either because of the difference of host species evaluated or because of the variation in the availability of vectors in the environment.

Another study investigated the occurrence of hemoplasmas in 100 wild canids, three European wolves (*Canis lupus*) and 97 Brazilian wild canids kept in captivity in zoos from São Paulo, Mato Grosso and the Distrito Federal. The frequency of hemoplasma infection was estimated at 4%, a mark well below the 83.3% found in this study. Nevertheless, two wolves (2%) tested positive for '*Ca.* M. haemominutum', and two bush dogs (2%) were infected with '*Ca.* M. haematoparvum'. In the present paper two crab-eating foxes (11.1%) were also positive for '*Ca.* M. haemominutum' and a bush dog was positive for '*Ca.* M. haematoparvum' (Birkenheuer et al. 2003).

It should be noted that all assessed canids came from the same institution and that the high rate of infection may be the result of not only regional ecological conditions, but also local health conditions. Again, the difficulty to perform efficient vector control of wandering and synanthropic animals may have been decisive in this case and will need more targeted studies.

Interestingly, all the maned wolves were positive for hemoplasmas and rickettsiae. The animals live in enclosures side by side and communicate through railings, which is certainly a factor that facilitates the transmission of pathogens. Yet, the species of hemoplasma infecting them was not detected, but three individuals were positive for *A. platys*, one for *E. canis,* and one for *A. platys* and *E. canis*, showing a triple infection. This animal, a female cub, presented anaemia and died in about two months. Another maned wolf, born in the same litter, but only positive for hemoplasma and *A. platys*, presented thrombocytopenia e anaemia. Four months later

the animal was diagnosed with an osteometabolic disease and was euthanized. Among the studied animals there was also a hoary fox with a triple infection. Unlike the maned wolf, it showed only thrombocytopenia without any signs on physical examination. This study converges with another investigating hemoplasma infection in dogs, both domestic and wild, by showing that hemoplasmas have low pathogenicity in these species, showing no correlation between anaemia, or any other hematologic finding, and infection (Inokuma et al. 2000, Birkenheuer et al. 2003, Hegarty et al. 2015). It is noteworthy that even animals co-infected with *Rickettsia* showed no statistically significant haematological abnormalities compared to uninfected animals.

Studies with domestic dogs also point out that there is no correlation between hemoplasma infection and gender or age group (Hegarty et al. 2015), but no other study with wild canids tried this comparison.

The most frequent hemoplasma species found in felids was '*Ca.* M. haematoparvum', while the most frequent in canids was *M. haemocanis*, with an incidence of 12.1% and 16.7%, respectively. On the other hand, the less frequent species were *M. haemofelis* and '*Ca.* M. turicensis', which were found in none of the specimens analysed.

CONCLUSIONS

Hemoplasma infections are frequent in captive wild carnivores, however, significant haematological findings have not been found, leading us to believe that the hemoplasmosis does not usually appear as a disease in these animals, possibly because they are chronically infected or parasitized by less pathogenic species or strains of the agent. Furthermore, the high incidence of hemoplasmas in wild animals from major population centers implies that they can act as reservoirs and perpetuate the infection of domestic animals.

This seems to be the first study to report *Mycoplasma haemocanis* and '*Candidatus* Mycoplasma haematoparvum infecting wild felids, and *M. haemocanis* infecting wild canids. Nevertheless, we must emphasize the similarity between the genomes *M. haemocanis* and *M. haemofelis*, especially in the 16S rRNA gene, and that sequencing of the positive samples must be carried out in order to distinguish between species.

The use of other molecular techniques, such as gene sequencing, is essential to elucidate if infected individuals without species identification regard to small changes in the genome of the parasite or if there are new species infecting wild carnivores.

Future researches on the infection of synanthropic animals that have contact with both wild captive animals and domestic animals, as well as on their vectors, are also crucial to understanding the disease better.

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Markers of inflammation and insulin resistance in dogs before and after weight loss versus lean healthy dogs[1]

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ABSTRACT.- Jeremias J.T., Takeara P., Vendramini T.H.A., Rodrigues R.B.A., Perini M.P., Pedrinelli V., Teixeira F.A., Brunetto M.A. & Pontieri C.F.F. 2020. **Markers of inflammation and insulin resistance in dogs before and after weight loss versus lean healthy dogs.** *Pesquisa Veterinária Brasileira 40(4):300-305.* Departamento de Nutrição e Produção Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Duque de Caxias Norte 225, Pirassununga, SP 19635-900, Brazil. E-mail: mabrunetto@usp.br

Chronic low-grade inflammation in obesity is characterized by an increased production of pro-inflammatory cytokines that contribute to insulin resistance. For this study body composition, markers of inflammation and of insulin resistance in dogs before and after weight loss were compared to those of lean dogs. Eleven client-owned obese adult dogs underwent a weight loss program with commercial dry food for weight loss and reached an ideal body condition score (BCS) six months after the beginning of the weight loss program. A Control Group of nine dogs with ideal BCS were selected for the comparison. Shapiro-Wilk test was used to test for normality, Mann Whitney were used for non-normally distributes data, and Student t-test was used for normally distributed parameters. In the Obese Group body fat decreased from 41.6% (30.7-58.6) to 29.1% (18.6-46.3) ($P<0.01$) and dogs maintained lean body mass throughout the weight loss program ($P>0.05$). Obese dogs presented higher concentration of fructosamine, triglycerides, insulin, IGF-1 and leptin than the Control Group before weight reduction ($P<0.05$). Serum concentrations of triglycerides, IL-2, IL-6, TNF-α, insulin, leptin and IGF-1 decreased after weight loss ($P<0.01$), and these concentrations were similar to the Control Group ($P>0.05$), except for leptin ($P<0.001$). No alteration on peptide YY was found. Leptin ($r=0.60$, $P=0.01$), fructosamine ($r=0.44$, $P=0.02$) and triglycerides ($r=0.40$, $P=0.04$) concentrations correlated with the reduction of body fat. Weight loss reduced the concentrations of inflammatory and insulin resistance markers and most parameters became similar to dogs that have always been lean, reinforcing the importance of weight loss in small animal practice.

INDEX TERMS: Inflammation, insulin resistance, dogs, weight loss, healthy dogs, adipocytokines, canine, cytokines, leptin, obesity.

RESUMO.- [Marcadores de inflamação e resistência à insulina em cães antes e depois da perda de peso versus cães saudáveis e magros.] A inflamação crônica de baixo grau na obesidade é caracterizada pela produção aumentada de citocinas pró-inflamatórias que contribuem para a resistência à insulina. Para este estudo a composição corporal e os marcadores de inflamação e de resistência à insulina em cães antes e após a perda de peso foram comparados aos de cães magros. Onze cães adultos obesos pertencentes a tutores foram incluídos em um programa de perda de peso com ração comercial hipocalórica e alcançaram escore de condição corporal (ECC) ideal seis meses após o início do

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regime. Um Grupo Controle de nove cães com ECC ideal foi selecionado para a comparação. O teste de Shapiro-Wilk foi usado para testar a normalidade, Mann Whitney foi usado para análise dos dados que não atenderam distribuição normal e o teste t de Student foi usado para parâmetros que atenderam a normalidade. No Grupo Obesidade, a gordura corporal diminuiu de 41,6% (30,7-58,6) para 29,1% (18,6-46,3) (P<0,01) e os cães mantiveram massa magra ao longo do programa de perda de peso (P>0,05). Cães obesos apresentaram maior concentração de frutosamina, triglicérides, insulina, IGF-1 e leptina do que o Grupo Controle antes da redução de peso (P<0,05). As concentrações séricas de triglicerídeos, IL-2, IL-6, TNF-α, insulina, leptina e IGF-1 diminuíram após a perda de peso (P<0,01) e, essas concentrações foram semelhantes ao Grupo Controle (P>0,05), com exceção da leptina (P<0,001). Nenhuma alteração no peptídeo YY foi encontrada. As concentrações de leptina (r=0,60; P=0,01), frutosamina (r=0,44; P=0,02) e triglicerídeos (r=0,40; P=0,04) correlacionaram-se com a redução da gordura corporal. A perda de peso reduziu as concentrações de marcadores inflamatórios e de resistência à insulina e a maioria dos parâmetros tornaram-se semelhante aos dos cães que sempre foram magros, reforçando a importância da perda de peso na prática clínica de pequenos animais.

TERMOS DE INDEXAÇÃO: Inflamação, insulina, cães, perda de peso, cães saudáveis, adipocitocinas, caninos, citocinas, leptina, obesidade.

INTRODUCTION

The adipose tissue in addition to serving as a reservoir of energy, can protect organs from direct impact and is responsible for the synthesis and secretion of various hormones and cytokines called adipokines (Zoran 2010). Chronic low-grade inflammation in obese dogs is characterized by increased secretion of pro-inflammatory cytokines that reduce glucose tolerance, increase insulin resistance, and may cause hyperlipidemia and several other significant health problems, such as cardiorespiratory and orthopedic alterations (Gayet et al. 2004, German 2006, German et al. 2009, Brunetto 2010, Brunetto et al. 2011).

Most studies with humans and rodents indicate that obese individuals present higher levels of pro-inflammatory cytokines (IL-6 and TNF-α), IGF-1 and leptin than non-obese individuals (Hotamisligil et al. 1995, Shoelson et al. 2007). However, studies in obese dogs have shown that the values of IL-6 and TNF-α are often below the lower limit of detection (Tvarijonaviciute et al. 2012, Bastien et al. 2015), although some studies suggest that canine obesity is also correlated with low-grade inflammation (Eirmann et al. 2009, German et al. 2009, Frank et al. 2015).

In humans, IL-6, TNF-α and IGF-1 are blood markers of insulin resistance, and their overproduction is related to this syndrome (Leahy & Vandekerkhove 1990, Hotamisligil et al. 1995). Elevated IGF-1 concentration has an impact on insulin secretion, which could lead to insulin resistance over time (Leahy & Vandekerkhove 1990). In obese dogs, impairment in both TNF-α and IGF-1 plasma concentration is associated with insulin resistance (Gayet et al. 2004).

The purpose of this study was to assess inflammatory adipokines profile and insulin resistance in obese dogs and to determine the effect of subsequent weight loss, as well as to compare these findings with lean adult dogs.

MATERIALS AND METHODS

Ethics statement. All experimental procedures agreed with the ethical principles in animal experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and was approved and agree with Ethical Principles in Animal Research adopted by Ethic Committee on Animal Use of the Grandfood Indústria e Comércio LTDA, Brazil.

Animals and experimental design. A total of twenty dogs were used in this study and were divided in two groups. The Experimental Group consisted of eleven client-owned obese adult dogs of different breeds (4 mixed-breed, 4 Labrador Retrievers, 2 Dachshunds and 1 Cocker Spaniel), three males and eight females (5 neutered females, 3 intact females and 3 intact males), with body condition score (BCS) 7, 8 or 9 on a scale of 1 to 9 (Laflamme 1997) which were submitted to a weight loss program. All dogs of the Experimental Group were housed in their owner's home in the city of Dourado, Brazil.

The Control Group consisted of nine healthy kennel dogs from an animal nutrition center (Dourado, Brazil). They were of different breeds (2 Labrador Retrievers, 3 Cocker Spaniels, 2 American Pit bulls, 1 Beagle and 1 Collie) with ideal BCS of 5 on a scale of 1 to 9 (Laflamme 1997), five males and four females (2 neutered females, 2 intact females and 5 intact males).

Median age of the Obese Group was 4 years (range 2-12 years), and median age of the Control Group was 7 years (range 2-12 years). Median body weight of the Obese Group before weight loss was 36kg (range 9.1-64.5kg) and median body weight for the Control Group was 26kg (range 9.8-46.9 kg). The animals of both Control and Experimental Groups were referred to the Nutritional Development Center (PremieR pet, Dourado, Brazil) for evaluation during the study's period. All dogs were evaluated by the same veterinarian for determination of BCS; assessed by visual inspection and palpation of strategic anatomical areas: ribs, subcutaneous fat and waist silhouette (Laflamme 1997). Animals from both Control and Obese Groups were considered healthy by complete blood cell count, biochemical parameters, and clinical examination. Dogs with previously diagnosed ongoing diseases other than obesity were not considered for this study.

Weight loss program and experimental design. The Control Group animals were evaluated and had blood samples collected in the beginning of the study period. They were fed a maintenance diet (Premier Formula Raças Grandes Adultos, Premier Pet), with metabolizable energy (ME) of 3.9kcal/g and macronutrient profile of 66.3g/1000kcal of crude protein, 35.7g/1000kcal of fat, 100.7g/1000kcal of starch and 9.0g/1000kcal of total dietary fiber based on ME provided by manufacturer. The mean energy requirement of the Control Group was 117kcal per $kg^{0.75}$, based on intake that maintained body weight.

The Experimental Group underwent a 6-month weight loss program to reach an ideal BCS of 5 on a scale of 1 to 9 (Laflamme 1997). They were fed twice daily with a commercial dry food for weight loss (Premier Nutrição Clínica Obesidade Cães, Premier Pet). The ME of the dry diet was 2.7kcal/g as informed by manufacturer, and the macronutrient profile was 119g/1000kcal of crude protein, 27g/1000kcal of fat, 54g/1000kcal of starch and 50g/1000kcal of total dietary fiber based on ME provided by manufacturer. The energy intake was restricted, corresponding to 60% of the maintenance energy requirements calculated for an individual target weight of minus 15% of the first assessment weight (Brunetto et al. 2011). Throughout the experimental period, the obese dogs were kept in their homes, being cared for by their owners. Records of body weight and BCS were updated every fifteen days by the same person, and

adjustments to food amount were made to keep weight loss rate around 1 to 2% of body weight per week (Brunetto et al. 2011).

Average weekly body weight loss rate was calculated and sessions of exercise of at least 20 minutes per day were recommended. Body composition was determined in the beginning of the trial for both lean and obese dogs and after the weight loss program for the obese dogs, the method used (deuterium oxide dilution) and the values of fat mass and lean mass were calculated. according to Brunetto et al. (2011). Serum concentrations of fructosamine, triglycerides, interleukin-2 (IL-2), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), insulin, leptin, insulin-like growth factor-1 (IGF-1) and peptide YY (PYY) were analyzed before and after weight loss for the Obese Group and in the beginning of the trial for the Control Group.

Blood collection and storage. All dogs were fasted overnight, with free access to water, and blood samples were collected by jugular venipuncture the following morning, once before the beginning and once after weight loss period. Immediately after the collection, samples were centrifuged at 3000 x g for 10 minutes, and the serum was divided into 4 aliquots and then placed into -20°C for immediate analysis of fructosamine and triglycerides and into -70°C storage freezer until other assays were performed.

Serum analysis of fructosamine and triglycerides. Fasting serum fructosamine and triglycerides were measured with commercial kit by colorimetric methods (Labtest Diagnóstica S.A., Lagoa Santa, Brazil). The assays were performed according to manufacturer's protocol at the research laboratory.

Circulating concentrations of cytokines, insulin, leptin, PYY and IGF-1. Serum IL-2, IL-6 and TNF-α were measured with Luminex-based canine cytokine assay according to manufacturer's instructions (Milliplex® MAP canine cytokine Magnetic Bead Panel, Millipore, Billerica, Massachusetts, USA). The intra and inter assay coefficients of variation were 7.5% and 17.0% for IL-2 (minimum limit of detection = 3.5pg/mL), 7.3% and 15.0% for IL-6 (minimum limit of detection = 3.7pg/mL) and 6.1% and 15.0% for TNF-α (minimum limit of detection = 6.1pg/mL). Serum insulin, leptin and PYY were measured using a Luminex-based assay (Milliplex® Canine Gut Hormone Magnetic Panel, Millipore, Billerica, Massachusetts, USA). The intra and inter assay coefficients of variation were 6.7% and 7.0% for insulin (minimum limit of detection = 52.8pg/mL), 4.7% and 8.0% for leptin (minimum limit of detection = 80.6pg/mL) and 3.9% and 7.0% for PYY (minimum limit of detection = 11.2pg/mL). Serum IGF-1 was measured using a commercial enzyme immunoassay (Enzyme-linked Immunosorbent Assay kit, Uscn Life Science Inc., Wuhan, China). The intra and inter assay coefficients of variation were 9.9% and 12.0% (minimum limit of detection = 0.9pg/mL). The assays were performed at the IgAc (Institute Genese of Scientific Analyses, São Paulo, Brazil) according to manufacturer's protocol. All samples analyses were made in duplicate.

Statistical analysis. All parameters were tested with the Shapiro-Wilk normality test. For non-normally distributed data the Wilcoxon matched paired test was used to compare obese dogs before and after weight loss and Mann Whitney rank test to compare obese dogs with the Control Group. A paired t test was performed for normally distributed parameters to compare obese dogs before and after weight loss and an unpaired t test was performed to compare obese dogs with the Control Group. Spearman (non-normally distributed data) and Pearson (normally distributed data) rank correlation with a two-tailed p-value were used to evaluate association between body fat (%) and other parameters. Differences were considered significant if $P<0.05$. Data are presented as medians (with range). Statistical analysis was performed with Graph-Pad Prism 5.02 for Windows (GraphPad Software, San Diego, USA).

RESULTS

Weight loss program. The obese dogs presented BCS 9 (4 dogs), BCS 8 (5 dogs) and BCS 7 (2 dogs) (Laflamme 1997), with a median BCS of 8. All the obese dogs achieved an ideal BCS of 5 within 6 months. The weight loss program resulted in a median weekly weight loss rate of 0.86% (0.49-1.19%) and dogs achieved a 21.8% (14.8-28.6%) weight reduction in 180 (126-281) days. Body fat mass decreased from 41.6% (30.7-58.6%) to 29.1% (18.6-46.3%) ($P<0.01$). All obese dogs maintained lean body mass after achieving ideal body condition score ($P>0.05$). Body composition of obese and of Control Group are shown in Table 1.

Metabolic parameters and markers of inflammation and insulin resistance. The concentrations of all the serum parameters evaluated can be observed in Table 2. Obese dogs presented higher concentration of fructosamine and triglycerides before weight loss when compared to the Control Group ($P<0.05$). There was a decrease in serum concentrations of triglycerides when comparing obese dogs before and after weight loss ($P<0.001$), but no differences in serum fructosamine were observed ($P=0.185$). However, after the weight loss program there was no difference in fructosamine and triglycerides when the Obese Group was compared to the Control Group (Figure 1).

Insulin, leptin and IGF-1 serum concentrations after weight loss of obese dogs decreased ($P<0.05$), and were significantly higher in obese dogs when compared to the Control Group ($P<0.05$). After weight loss, these concentrations were similar to the ones of the Control Group ($P>0.05$), except for leptin, which was still higher in the obese dogs after weight loss than the dogs in the Control Group ($P<0.01$). Serum concentrations of cytokines IL-2 and IL-6 decreased after weight loss ($P<0.01$), but were no different from the Control Group ($P>0.05$). No differences on fasting serum concentration of PYY were found between groups of dogs studied ($P>0.05$)

Table 1. Body composition of obese dogs before and after weight loss program, and Control Group

	Obese dogs Group (n=11)		Control Group (n=9) Median (range)
	Before weight loss Median (range)	After weight loss Median (range)	
Body fat (%)	41.25[a] (30.71-58.62)	28.56[b] (18.64-46.35)	27.34[b] (10.25-34.97)
Body fat (kg)	10.53[a] (3.45-37.81)	6.44[b] (2.47-19.75)	4.44[b] (2.69-13.60)
Lean body mass (%)	58.75[a] (41.38-69.29)	71.44[b] (53.65-81.36)	72.66[b] (65.03-89.75)
Lean body mass (kg)	23.77[a] (5.05-36.83)	22.16[a] (4.68-35.33)	18.65[a] (7.20-40.12)

[a,b] Means followed by the same letter in the rows do not differ by the Student t-test ($P<0.05$), being t-paired test for before weight loss vs after weight loss and t test for independent samples for before weight loss vs Control Group and after weight loss vs Control Group.

(Fig.1). TNF-α concentration was below the detection level on most samples (21 of 31 serum samples evaluated), and therefore it was not possible to compare the groups.

Of the parameters studied, the ones that most correlated with the reduction of fat mass were leptin (r=0.61, P=0.001), fructosamine (r=0.44, P=0.028) and triglycerides (r=0.40, P=0.048).

DISCUSSION

The weight loss program of obese animals resulted in decrease of fat mass and maintenance of lean mass, which indicates that calorie intake and dietary protein levels of the diet were effective in promoting healthy weight loss. The mean weight loss rate was 0.86% of body weight per week, an adequate rate for client-owned dogs (Carciofi et al. 2005, German 2006).

Table 2. Blood concentrations of metabolic parameters and markers of inflammation and insulin resistance in obese dogs before and after weight loss program, and in the Control Group of lean dogs

	Obese dogs Group (n=11)		Control Group (n=9) Median (range)
	Before weight loss Median (range)	After weight loss Median (range)	
Triglycerides (mg/dl)	124 (71-376)	62 (29-120)	71.5 (51-101)
Fructosamine (µmol/l)	230 (200-353)	223 (141-271)	189 (158-218)
Insulin (pg/ml)	376 (11-1117)	260 (96-419)	219 (145-393)
Leptin (ng/ml)	13.3 (1.9-34.1)	5.5 (0.82-13.9)	3.8 (1.3-8.6)
IGF-1 (ng/ml)	717 (318-1180)	437 (206-730)	332 (177-908)
IL-2 (pg/ml)	23.8 (0.54-656)	5.6 (2.5-106)	*
IL-6 (pg/ml)	31.9 (4.5-594)	11.3 (1.3-164)	*
TNF-α (pg/ml)	3.11 (0.5-46.9)	1.06 (0.11-59.8)	*

* Most sample values were below detection.

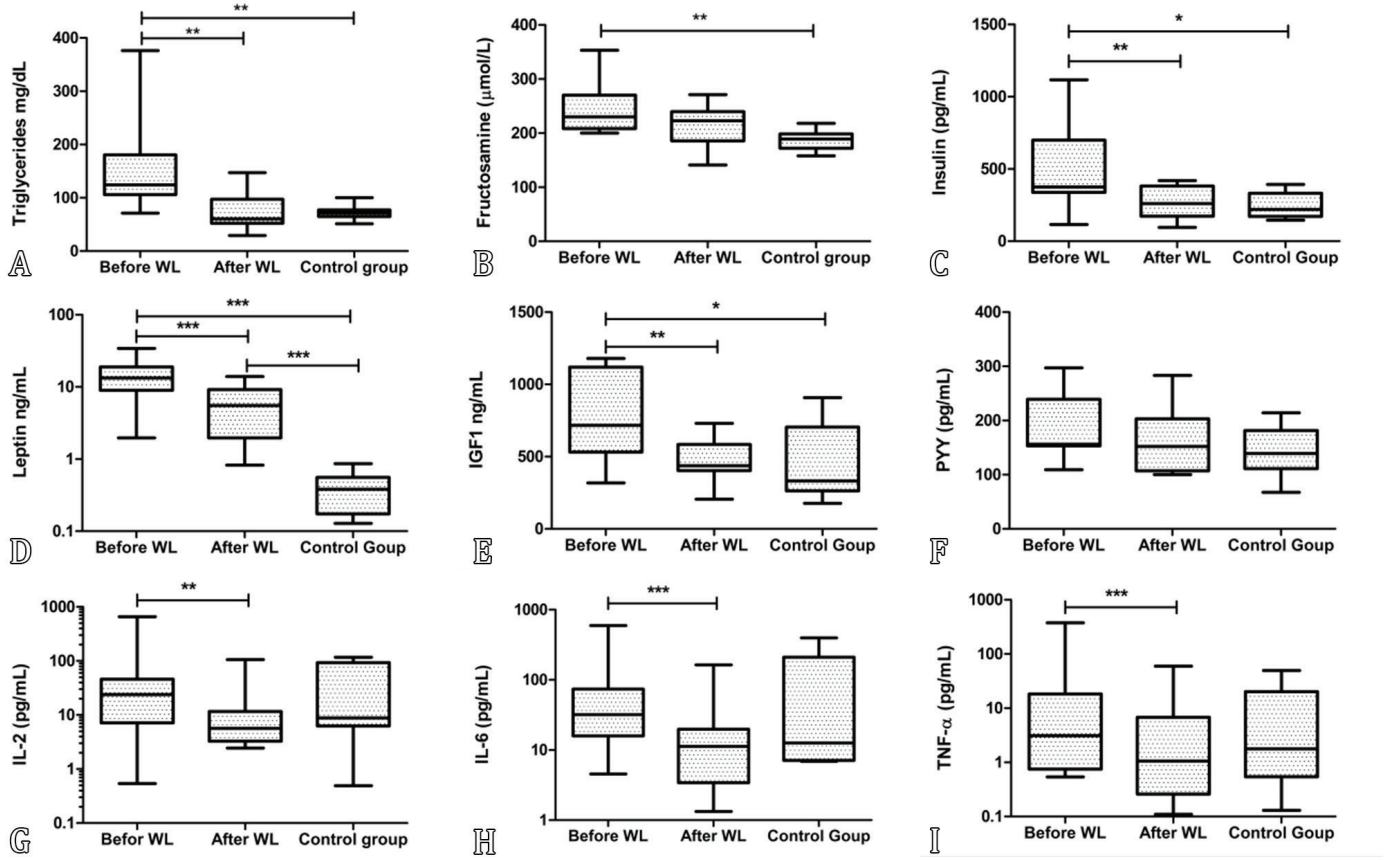


Fig.1. Concentrations of triglycerides, fructosamine, insulin, leptin, IGF-1, PYY, IL-2, IL-6 and TNF-α in dogs before weight loss (Before WL, n=11), after weight loss (After WL, n=11) and Control Group (n=9). Values are expressed as median (range) and the scale is logarithmic for leptin, IL-2, IL-6 and TNF-α (*); significantly differences are expressed as p<0.05 (**); significantly differences are expressed as p<0.001 (***).

In addition to the beneficial changes in body composition, reduction of body fat mass promoted significant decrease in the serum concentrations of triglycerides, IL-2, IL-6, insulin and IGF-1, as previously demonstrated in dogs, rodents and in humans (Hotamisligil et al. 1995, Blanchard et al. 2004, Jeusette et al. 2005, Shoelson et al. 2007, Eirmann et al. 2009, German et al. 2009, Tvarijonaviciute et al. 2012, Bastien et al. 2015, Frank et al. 2015). Unfortunatly, most samples analyzed for TNF-α concentrations were below detection levels, making it hard to properly take a conclusion about the changes in this parameter before and after weight loss.

Higher serum concentration of triglycerides in obese dogs can be associated with higher body fat mass, as observed in a previously study comparing obese and lean dogs (Eirmann et al. 2009). The results of the current study suggests that insulin resistance is present in obese dogs, as evidenced by elevated serum insulin concentration in this group before weight loss. The concentration of serum insulin decreased significantly in obese dogs after weight loss, suggesting improved insulin sensitivity after dogs lost weight, which supports prior results observed in dogs (German et al. 2009, Bastien et al. 2015).

Previous research found positive correlation between leptin concentration and body fat content and observed that plasma leptin is a good biochemical index of adiposity/obesity in dogs (Ishioka et al. 2002). Other studies observed higher leptin concentration in obese dogs, suggesting that obese dogs, like obese humans, are leptin resistant (Gayet et al. 2004, Jeusette et al. 2005). In the present study, leptin serum concentration was high in obese dogs and had a significant correlation with body fat content. Furthermore, leptin concentrations remained 44% higher even after achieving ideal BCS when compared to the Control Group, suggesting that immediately after weight loss dogs' metabolism may still present leptin resistance. In contrast, Jeusette et al. (2005) observed that dogs that just lost weight had similar concentration of leptin than lean dogs. It is important to understand that in the current study the composition of the diets used for both groups was different, and along with different housing spaces, this may have influenced markers' concentration levels and so results must be considered with caution.

Receptors that bind insulin and IGF-1 show immunological, structural, and functional analogies, which could explain why IGF-1 has an insulin-like activity, and participates in insulin resistance (Gayet et al. 2004). This present study's results showed that the treatment of obesity may have improved markers of insulin resistance. Blanchard et al. (2004) observed a decrease in IGF-1 concentration after 6 weeks of restriction of energy intake, suggesting that this quick reduction was due to caloric restriction and not to a decrease in body fat mass. The data obtained in the current study corroborates this statement since the concentration of IGF-1 decreased after weight loss, however, it did not correlate with body fat mass ($r=0.07$, $P=0.7128$). A more recent study showed a significant reduction in IGF-1 concentration after 3 months of energy restriction to induce rapid weight loss (2-3% of BW/week) (Tvarijonaviciute et al. 2012).

Reference ranges for circulating cytokines in healthy dogs have not been established yet and many of the studies in obese dogs have shown that the values of common inflammatory biomarkers, such as IL-2, IL-6, and TNF-α, are often below the lower limit of detection (Eirmann et al. 2009, German et al. 2009, Wakshlag et al. 2011, Tvarijonaviciute et al. 2012, Bastien et al. 2015, Frank et al. 2015). In this study, IL-2 concentrations were below the kit sensibility in 6 of the 31 serum samples evaluated, 4 of them in obese dogs after weight loss, and IL-6 concentrations in 3 of 31, all of them in obese dogs after weight loss. On the other hand, most of TNF-α concentrations were below the level of detection (21 of 31 serum samples evaluated), suggesting that normal circulating levels of TNF-α are lower since the studied dogs were all healthy, despite obesity.

Even though part of the samples were close to the minimum limit of detection, a significant decrease in concentration of all cytokines studied after weight loss was found, suggesting some influence of adipose tissue on this cytokines secretion. The median values found in Obese Group were higher than the Control Group in all inflammatory cytokines evaluated. However, significant differences were not detected, possibly due to the great variation within Control Group concentrations.

Growth factors have not been related to rodent and human obesity. The growth-factor IL-2 is more related to possible immune status and less likely to be influenced by obesity status. However, a recent study of obese dogs performed by Bastien et al. (2015) evaluated IL-2 and found decrease in this parameter after weight loss, similar to this study.

Results are in accordance with a recent study that showed fasting serum IL-6 concentrations are elevated in Labrador Retrievers that were overweight or obese when compared to lean dogs from the same breed (Frank et al. 2015). It has been hypothesized that in absence of acute inflammation, fifteen to thirty percent of circulating IL-6 level comes from adipose tissue, including the macrophages and stromal cells contained in the tissue (Mohamed-Ali et al. 1997). However, it is important to highlight that in other studies the concentration of IL-6 was below the minimum limit of detection (German 2006, Eirmann et al. 2009, Tvarijonaviciute et al. 2012).

Studies have reported increase in TNF-α concentration in obese humans and rodents (Hotamisligil et al. 1995, Shoelson et al. 2007), and TNF-α has been found to also be higher in dogs (Eirmann et al. 2009, German et al. 2009, Bastien et al. 2015, Frank et al. 2015). However, only one study observed that concentrations of TNF-α were higher in obese dogs and decreased significantly after weight loss (Gayet et al. 2004).

No studies that assessed serum PYY concentrations in obese dogs were found in the literature. The lack of response observed in this study may be due to the fact that samples were collected during fasting. In humans, PYY levels are low in the fasting state, rapidly increasing in response to food intake, reaching a peak at 1-2h after a meal and then remaining high for several hours (Adrian et al. 1985).

CONCLUSIONS

The treatment of obesity allows the return of triglycerides, insulin and IGF-1 concentrations to normal levels and the reduction of the concentrations of IL-2 and IL-6.

The lack of convergent results in the veterinary literature available suggests that more research is needed to characterize the pro-inflammatory effects of canine obesity and the role of cytokines in insulin resistance and related co-morbidities.

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

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Effect of refrigeration at -1°C on spermatozoa quality of domestic cats[1]

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ABSTRACT.- Souza A.K., Trautwein L.G.C., Paranzini C.S., Schnitzer J.F., Perencin F.M., Cardoso G.S. & Martins M.I.M. 2020. **Effect of refrigeration at -1°C on spermatozoa quality of domestic cats.** *Pesquisa Veterinária Brasileira 40(4):306-314*. Universidade Estadual de Londrina, Rodovia Celso Garcia Cid PR-445 Km 380, Campus Universitário, Cx. Postal 10.011, Londrina, PR 86057-970, Brazil. E-mail: imartins@uel.br

The objective of this study was to evaluate the sperm quality obtained of domestic cats by electroejaculation and recovery of the tail of the epididymis after cooling at -1°C and 4°C for 24 and 48 hours. Twenty-nine adult cats (2 to 6kg) were used. Sperm collection was performed by electroejaculation (EEJ), and after 48 hours, the cats were orchiectomized, and sperm sample was obtained from the vas deferens and epididymis tail (EPD). The samples were diluted in ACP-117® extender, and the sperm characteristics were evaluated at three different moments: when still fresh, 24 and 48 hours after cooling. In order to compare the two refrigeration temperatures, the first stage was to analyze if there was a difference between the harvesting techniques. After this, two experiments were conducted: in the first, sperm sample from 14 cats were used and the cooling was performed at -1°C; and in the second, sample from 15 cats were used and the sperm were refrigerated at 4°C. Sperm kinetics were evaluated by computerized analysis (CASA) and concentration by Neubauer chamber, spermatic morphology was evaluated by modified Karras staining, and membrane integrity was evaluated by eosin nigrosine. The results obtained were analyzed in R software, version 3.2.5 using the Mann-Whitney test for variables with abnormal distributions, considering significance at the level of 5%. In ejaculate samples, higher values of total morphological defects were observed after 24 and 48 hours of refrigeration at 4°C ($P<0.022$) compared to refrigeration at -1°C, using Friedman test. To quantify the decrease in sperm quality, parameter reductions were calculated among time points (F-24h/F-48h/24h-48h). In EPD samples, a greater reduction in sperm quality was detected after 24 hours of refrigeration at 4°C, both in motility and sperm kinetics and in the movement and velocity indices, compared to refrigeration at -1°C. Based on the results, it can be concluded that cooling of feline spermatozoa at -1°C for up to 48 hours was efficient in maintaining spermatic quality collected by EEJ and EPD, and it could be an alternative to spermatozoa cryopreservation in domestic felines.

INDEX TERMS: Refrigeration, spermatozoa, domestic cats, cryopreservation, coconut water extender, electroejaculation, epididymis, cats.

RESUMO.- [**Efeito da refrigeração a -1°C na qualidade de espermatozoides de gatos domésticos.**] O objetivo deste trabalho foi avaliar a qualidade espermática de gatos domésticos obtidos por eletroejaculação e recuperação da cauda do epidídimo após a refrigeração a -1°C e a 4°C por 24 e 48 horas. Vinte e nove gatos adultos (2 a 6kg) foram utilizados. A colheita de espermatozoides foi realizada por eletroejaculação (EEJ) e, após 48 horas, os gatos foram orquiectomizados, e as amostras espermáticas foram obtidas a partir do ducto deferente e da cauda do epidídimo (EPD). As amostras foram diluídas em ACP-117® e as características espermáticas foram avaliadas em três momentos distintos:

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fresco, 24 e 48 horas após a refrigeração. Para ser possível comparar as duas temperaturas de refrigeração, a primeira etapa foi analisar se havia diferença entre as técnicas de colheita. Após isto, dois experimentos foram conduzidos: no primeiro, espermatozoides de 14 gatos foram utilizados e a refrigeração foi realizada a -1°C; e no segundo, amostras de 15 gatos foram utilizados e os espermatozoides foram refrigerados a 4°C. A cinética espermática foi avaliada por análise computadorizada (CASA), a concentração por câmara de Neubauer, a morfologia espermática foi avaliada pela coloração de Karras modificada, e a integridade da membrana foi avaliada por eosina nigrosina. Os resultados obtidos foram analisados no software R, versão 3.2.5, utilizando o teste de Mann-Whitney para variáveis com distribuições anormais, considerando significância ao nível de 5%. No ejaculado, maiores valores de defeitos morfológicos totais foram observados após 24 e 48 horas de refrigeração a 4°C (P<0,022) em comparação com refrigeração a -1°C, usando o teste de Friedman. Para quantificar a diminuição na qualidade espermática, as reduções dos parâmetros foram calculadas entre os pontos de tempo (F-24h/F-48h/24h-48h). Na EPD, uma maior redução na qualidade espermática foi detectada após 24 horas de refrigeração a 4°C, tanto na motilidade e na cinética espermática quanto nos índices de movimento e velocidade, em comparação com a refrigeração a -1°C. Com base nos resultados, pode concluir-se que a refrigeração dos espermatozoides felino a -1°C, até 48 horas, foi eficaz na manutenção da qualidade espermático colhidos por EEJ e EPD, e pode ser uma alternativa para a criopreservação de espermatozoides em felinos domésticos.

TERMOS DE INDEXAÇÃO: Refrigeração, espermatozoides, gatos domésticos, criopreservação, diluente de água de coco, eletroejaculação, epidídimo.

INTRODUCTION

The survival and maintenance of species are facilitated by assisted reproduction, however, there are restrictions on genetic material preservation. One important tool is the cryopreservation of gametes (Farstad 2000), which enables the conservation of genetic material of domestic species, including those threatened with extinction. Semen refrigeration is used to allow the genetic material to be transported over long distances because with decreased temperature, the metabolic activity of sperm decreases, thus avoiding the formation of free radicals (Buranaamnuay 2017).

The domestic cat is the experimental model for wild feline species, so understanding of factors that interfere with sperm viability is necessary for the use of reproductive biotechniques in felids, such as the study of sperm cryopreservation conditions (Bronson 1985, Meyer 2013, Martins et al. 2014, Martins & Justino 2015).

The viability of cryopreserved spermatozoa is affected by several factors, such as the composition and concentration of extender, temperature and velocity of cooling processes and, mainly, fresh sperm quality (Buranaamnuay 2017). Although there have been promising results with refrigerated feline semen (Luvoni et al. 2003), there is still no definition of a cooling temperature with high success rates. There is evidence that a large proportion of spermatozoa that undergo the cryopreservation process acquire acrosomal damage, with compromise fertilization capacity (Tebet et al. 2006).

Only one study of artificial insemination in cats (Tsutsui 2006) reported the use of semen at a lower refrigeration temperature of 0°C, in which a conception rate of 57% was obtained. Garcia-Olivares et al. (2016) a boar spermatozoa refrigeration study comparing temperatures less than zero (-3°C, -5°C, -7°C), and they found that negative temperatures allowed the sperm plasma membrane to adapt better to changes in fluidity without loss of selective permeability, thus improving cell quality.

Based on the assumption that physiological spermatozoa characteristics are species specific, it is necessary to study refrigeration methodologies for cat semen, the cells of which are more resistant to low temperatures compared to other species and which can maintain their quality throughout three weeks (Harris et al. 2001, Chatdarong et al. 2009).

Alternative extenders for sperm cooling, such as coconut water, have achieved promising results both because it is a sterile, natural solution and because it presents longevity in powdered form (Barros & Toniolli 2011, Lima et al. 2016). It has been used in a standardized manner (ACP-117® - Biotechnology, Fortaleza, Brazil), being free of variability of biological origin, and because it does not contain egg in its composition, it reduces sanitary barriers.

The objective of this work was to evaluate the sperm quality of domestic felines, comparing cooling temperatures of -1°C and 4°C and the storage times of 24 and 48 hours for spermatozoa obtained from ejaculate and recovery from the epididymis tail.

MATERIALS AND METHODS

Ethical and local aspects. This study was approved by the Ethical Commission on the Use of Institutional Animals (CEUA), number 7099.2015.53.

Animals. Spermatozoa samples of twenty-nine domestic cats adult, mongrel, semi-domiciled domestic cats in good nutritional status, weighing from 2 to 6 kg and clinically healthy, were used from November 2015 to February 2016.

Experimental design. In order to compare the two cooling temperatures, the first step was to analyze if there was a difference between the sperm harvesting techniques of the 29 animals. After this, two experiments were conducted: in the first, sperm samples of 14 cats were used, and sperm cooling was performed at -1°C; and in the second, samples of 15 cats were used, and the spermatozoa were refrigerated at 4°C. Both were refrigerated in a domestic refrigerator which the thermostat was set to proper temperatures and checked daily.

In both experiments the harvesting and processing of sperm was performed in the same way. Initially, electroejaculation was performed and than, 48 hours later, elective orchiectomy was done and spermatozoa were recovered from the epididymis tail. The samples were diluted in ACP-117® extender (Biotechnology, Fortaleza, Brazil) and spermatozoa characteristics were evaluated at three different moments: when still fresh; after 24 and 48 hours of cooling.

Materials collected. By electroejaculation (EEJ): an anesthetic protocol with ketamine (12mg/kg - Dopalen®, Ceva, Brazil) associated with medetomidine (30µg/kg - Domitor®, Pfizer, France) was used. After anesthesia, urethral catheterization was performed with a 24G catheter coupled to a 1.5mL polypropylene microtube. The electrical impulse was produced by a transrectal probe, with two

longitudinal electrodes (0.9cm in diameter and 11cm in total length, inserted 2cm into the rectum) positioned above the prostate. The electroejaculation protocol was based on that described by Howard et al. (1990).

By epididymal sperm recovery (EPD): after elective orchiectomy, the vascularization of the duct deferens was isolated and with the aid of a hemostatic clamp, the epididymal tail was compressed to obtain the epididymal fluid in a Petri dish containing 100µL of 0.9% NaCl solution for each epididymis, with a final volume of 200µL per animal.

Sperm processing. The spermatic evaluation was performed at three different moments (when still fresh; after 24 and 48 hours of cooling. After fresh analysis, the samples were centrifuged at 300g for 10 minutes (MiniSpin Plus® Microcentrifuge - Eppendorf, São Paulo/SP, Brazil). The supernatant was discarded and the pellet resuspended with ACP-117 extender (pH 7.43, 297mOsm/kg H_2O, PL = 3.43g), prepared according to the manufacturer's recommendations, to the final concentration of 40 x 10^6 sperm/mL. The samples were aliquoted into 1.5 mL plastic tubes, which were packed in a plastic container containing 200 mL of water at room temperature so that the contents of tubes were submerged and cooled to -1°C (Experiment 1) or 4°C (Experiment 2). It is important to note that at the temperature at -1°C, only a thin layer of ice crystals is formed on the surface of the liquid solution, not enough to freeze the spermatozoid.

Computed Sperm Analysis: the parameters of spermatic kinetics were analyzed by the CASA system (HTR-IVOS, version 14.0), according to feline setup (Table 1). Total motility (% - MT) were obtained; progressive motility (% - MP); average path velocity (µm/s - VAP); straight-line velocity (µm/s - VSL); curvilinear velocity (µm/s - VCL); amplitude of lateral head displacement (µm - ALH); straightness (% - STR); and linearity (% - LIN) (Niżański et al. 2016). Subsequently, the sperm movement index (SMI = (VSL x 0.59) + (VAP x 0.37) + (LIN x 0.95) + (STR x 0.89) + (WOB x 0.83)) and sperm velocity index (SVI = (VCL x 0.87) + (VSL x 0.76) + (VAP x 0.90) + (ALH x 0.92)) were calculated according to Agarwal et al. (2003), modified by Núñez-Martinez et al. (2006), and the oscillation of spermatic movement, wobble (WOB) was calculated by the index VAP/VCL x 100.

Table 1. Setup used in HTR-IVOS 14.0 for the evaluation of feline spermatozoa samples

Parameters	Values
Chamber type	Cell-Vu®
Temperature, set (°C)	37
Frames acquired	30
Frame rate (hz)	60
Number of frames	5
Minimum contrast	40
Minimum cell size (pixels)	4
Straightness (STR), Threshold (%)	80
Vap cutoff (µm/s)	30
Prog. min VAP (µm/s)	70
VSL cutoff (µm/s)	20
Cell intensity	50
Magnification	1.89

STR = straightness, VAP = average path velocity, VSL = straight-line velocity

Concentration by the Neubauer Chamber: a 1:200 dilution of the semen sample was used in distilled water and was read in a Neubauer chamber under a phase contrast optical microscope at 200x magnification. The result was expressed as sperm count x 10^6/mL.

Spermatic Morphology: for sperm morphology evaluations, smears of sperm samples were performed on glass slides and stained with modified Karras (Papa 1988). A total of 100 sperm cells were evaluated using a 1000x magnification optical microscope. The morphological alterations were classified into major and minor defects and divided into defects of the head, intermediate piece, tail and acrosome. The results were expressed as a percentage.

Membrane Integrity: for the evaluation of membrane integrity, smears were performed with 3µL of sample plus 3µL of eosin nigrosine dye. A total of 100 cells per slide were counted under light microscopy (1000x). Sperm cells were classified as having an intact membrane when translucent or damaged when they had heads with pink coloration. The results were expressed as percentages of spermatozoa.

Statistical analysis. The results obtained were analyzed in R software, version 3.2.5. The non-parametric Mann-Whitney test was used to compare the refrigeration temperatures of -1°C and 4°C and the collection techniques used (EEJ and EPD), and Friedman's test was used to compare cooling times (when still fresh, at 24h and at 48h). The level of significance considered for all of the tests was 5%.

RESULTS

For the two experiments the ejaculate volume obtained was on average 50µL (ranging from 10 to 90µL) and the volume for the spermatic samples collected by EPD was predetermined at 200µL.

First stage: comparison with sperm harvesting techniques. The ejaculate (EEJ) and epididymal tail (EPD) sperm samples of both temperatures were compared. There was no difference in motility with the use of ACP-117®, as presented in Table 2. While fresh, there was a significant difference in the sperm between EEJ and EPD regarding the mean movement of cells in ALH, STR, LIN and SMI, as well as in the acrosome integrity evaluation. After 24 hours of refrigeration, significant differences were detected in BCF and STR, while after 48 hours, in addition to significant differences in BCF and STR, differences in slow and static sperm motility, SVI and total morphological defects were identified.

Second stage: comparison between the cooling temperatures of the two experiments. Regarding the kinetic parameters evaluated, after refrigeration at -1 and 4°C the spermatozoa collected by EEJ showed no difference. In the sample EPD harvest, spermatozoa showed a more progressive and linear movement characteristic at 4°C (Table 3), compared to cooling at -1°C (Table 4). However, when the morphology was evaluated, a significant difference was observed in the percentages of normal cells ($P<0.026$), major defects ($P<0.022$) and total defects ($P<0.026$) between the two refrigeration temperatures, and the results at -1°C above 4°C.

To identify the decrease in sperm quality, the reduction calculation of parameters among the moments (F-24h/F-48h/24 h-48h) at each (-1°C and 4°C) cooling temperatures (Tables 5 and 6) was performed. In samples from ejaculate, the percentage of minor defects showed a more pronounced reduction in the group refrigerated at 4°C. In epididymis spermatozoa, the parameters of MT, MP, velocity, ALH, SVI and SMI were difference between fresh and 24 hours of cooling,

and the sperm quality reduction was superior in the group refrigerated at 4°C in all of the parameters.

DISCUSSION

First stage: comparison with sperm harvesting techniques. Concerning the spermatozoa collection method, in the ejaculate, it was possible to obtain better results regarding rectilinearity (STR) at three different moments (when still fresh; at 24h and at 48h). Epididymal spermatozoa were significantly fewer than those in ejaculate, likely because they were recovered in physiological solution and initially presented lower sperm quality. It was necessary for a period to equilibrate the solution, and although it did not alter the pH or osmotic pressure, because no membrane changes were observed, the solution decreased the motility in a non-deleterious manner, and after addition of the diluent, it stabilized.

The higher percentage of damaged cells in the membrane integrity assessment of the ejaculate in the fresh samples could be associated with the time that the spermatozoa were in contact with seminal plasma since it is already known that there are proteins of higher molecular weight that can promote spermatic membrane lesions (Martins et al. 2004), in contrast to what occurred with epididymal spermatozoa, which were recovered in a shorter period of time and in physiological solution (Prochowska et al. 2015).

Second stage: comparison between the cooling temperatures of the two experiments. The results of the evaluations of kinetic parameters after cooling of samples feline spermatozoa for up to 48 hours at -1°C or 4°C suggest that the sperm viability is maintained at both cooling temperatures (-1 and 4°C). Authors recommend cooling to 4°C (Pukazhenthi et al. 1999, Zambelli et al. 2002, Tebet et al. 2006, Nunes et al. 2008, Nunes 2010, Buranaamnuay 2017). However the present article has shown that cooling at -1°C is an option especially when there are reproducers with the need to preserve the spermatic morphology after cooling. A greater percentage of total sperm defects was detected when the sperm samples were cooled at 4°C ($P<0.026$), showing that the temperature at -1°C caused less cryolysis, which could be explained by the rapid adaptability of the cells to lower temperatures (Rijsselaere et al. 2007, Buranaamnuay 2015).

The results of the reduction in the percentage of sperm parameters showed a decrease after cooling at both temperatures. It was observed that there was a significant reduction when comparing fresh/24 hours of refrigeration and fresh/48 hours of refrigeration, but there was no difference between 24 and 48 hours of refrigeration, suggesting cell stabilization at low temperature after the first few hours of refrigeration. Similar results were obtained by VillaVerde et al. (2006),

Table 2. Comparison of samples spermatozoa kinetics cooled using coconut water extender (ACP-117®) obtained by electroejaculation (EEJ) and retrieved from the epididymis tail (EPD) at three moments (fresh, 24 and 48 hours)

Sperm characteristics	Refrigeration time					
	Fresh		24 hours		48 hours	
	EE	EPD	EE	EPD	EE	EPD
MT (%)	67.6 ± 17.7	69 ± 15.3	33.9 ± 22	34.1 ± 21.7	30.3 ± 22.0	22.9 ± 20.4
MP (%)	43.5 ± 17.5	32.2 ± 13.5	21.1 ± 16.4	15.1 ± 12.6	11.9 ± 11.7	9.3 ± 11.9
VAP (µm/s)	139.2 ± 29.6	121.7 ± 31.8	98.7 ± 18.6	97.5 ± 25.8	75.8 ± 18.6	81.9 ± 36.4
VSL (µm/s)	120 ± 30.1	99.6 ± 28.9	85 ± 15.9	77.6 ± 21.8	62.2 ± 13.9	63.2 ± 30.4
VCL (µm/s)	184.5 ± 61.7	190.5 ± 34.6	162 ± 26	162.2 ± 28.9	138.4 ± 29.9	142.8 ± 50.2
ALH (µm)	6.1 ± 0.6*	6.6 ± 0.6*	5.2 ± 1.1	5.2 ± 1.4	5.2 ± 0.7	4.2 ± 2.6
BCF (Hz)	34.3 ± 4.9	32.9 ± 4.3	41.5 ± 4.1*	36.6 ± 5.4*	37.8 ± 3.6*	31.7 ± 10*
STR (%)	83.7 ± 5.3*	79.9 ± 3.9*	85.1 ± 3.7*	79.1 ± 7.9*	81.8 ± 4.8*	71.9 ± 21.4*
LIN (%)	59.2 ± 10.1*	51.6 ± 8.1*	53.3 ± 8.8	48.9 ± 10.6	46.6 ± 5.2	43 ± 17.2
AREA	6.8 ± 0.7	6.9 ± 1.4	5.6 ± 0.6	5.6 ± 0.7	5.9 ± 1	5 ± 1.5
WOB	69.3 ± 9.2	63.2 ± 7.9	61 ± 7.45	59.9 ± 10.7	54.6 ± 3.6	57.1 ± 13.9
SVI	395.6 ± 65.1	357 ± 78.5	299.2 ± 48.4	292.6 ± 59.4	240.6 ± 52.4*	292.6 ± 59.4*
SMI	310.5 ± 48.1*	276.3 ± 48.1*	263.7 ± 28.3	276.3 ± 43.4	227.1 ± 19.3	232.3 ± 47.8
Morphology						
Normal cells	51.5 ± 26.1	54.2 ± 15.9	43.8 ± 23.9	42.9 ± 18.7	39 ± 20.5	39.5 ± 20.8
Major defects	31.1 ± 23.1	19.7 ± 11.7	36 ± 20.1	28.7 ± 14.4	37 ± 20.1	32.2 ± 20
Minor defects	17.4 ± 11	27.2 ± 14.6	20.2 ± 8.9	28.3 ± 14.3	47 ± 28.7	28.1 ± 11,9
Total defects	48.7 ± 26.1	44.9 ± 17.7	56.2 ± 23.9	57.1 ± 18.7	38 ± 19.8*	60.4 ± 20.8*
Integrity membrane						
Injured	10.7 ± 6.8*	5.9 ± 3.4*	13.9 ± 7.7	8.9 ± 5.1	13.9 ± 8.5	11.1 ± 6.1

* Significant difference between the corresponding columns ($P<0.05$); MT = total motility, MP = progressive motility, VAP = average path velocity, VSL = straight-line velocity, VCL = curvilinear velocity, ALH = amplitude of lateral head displacement, STR = straightness, LIN =linearity, SMI = sperm movement index, SVI = sperm velocity index, WOB = wobble.

who observed decreases in total and progressive motility 24 hours after refrigeration.

The SVI and SMI indices showed greater reductions in refrigerated spermatozoa at 4°C. Considering that the best results with cryopreservation are related to higher rates of movement and velocity, since they represent the biological status of spermatozoa (Núñez-Martínez et al. 2006, Niżański et al. 2016) it can be inferred that the results obtained in the cooled epididymal spermatozoa at 4°C could compromise the results of feline breeding biotechniques. Spermatozoa cooled to -1°C presented acceptable results according to standards CBRA (2013) in both sample sperm kinetics and normal cell percentage, demonstrating superior quality in analyzed parameters.

It is believed that the results obtained from cooling at -1°C were due to the physiological characteristics of the feline spermatozoa, the plasma membrane of which is better adapted and provides membrane fluidity at lower temperatures (Garcia-

Table 3. Results of the median (minimum-maximum) analyses of samples domestic feline spermatozoa collected by electroejaculation (EEJ) and from the epididymal tail (EPD), fresh and after refrigeration at 4°C for 24 hours and 48 hours

Sperm characteristics	Refrigeration 4°C					
	Electroejaculation			Epididymis		
	Fresh	24 h	48h	Fresh	24 h	48 h
MT (%)	61.5 (34 - 72)	17 (8 - 40)	17 (6 - 20)	71 (46 - 92)	23 (2 - 68)	10 (0 - 61)
MP (%)	34.5 (16 - 44)	8.5 (5 - 21)	4.5 (2 - 8)	38 (22 - 59)*	8 (1 - 41)	2 (0 -39)
VAP (µm/s)	121.3 (99.8 - 148.7)	98.3 (82.7 - 126)	70.7 (56.9 - 100.4)	142.4 (109.5 - 170.3)*	101.1 (61.4 - 151.4)	76.4 (0 - 132.4)
VSL (µm/s)	104.8 (82.2 - 123)	89 (68.8 - 101.5)	59.2 (50.1 - 73.1)	120.1 (89 - 144.5)*	76 (46.4 - 127.6)	60.6 (0 - 112.5)
VCL (µm/s)	186.3 (16.5 - 203.9)	151 (137.9 - 205.3)	128.3 (100.9 - 184.9)	217.1 (171.1 - 253.8)*	162.1 (106.4 - 193.6)	148.7 (0 - 191.6)
ALH (µm)	6 (5.6 - 6.4)	5.2 (2.7 - 5.9)	4.8 (4.2 - 5.4)	6.7 (5.6 - 7.7)	4.8 (3.4 - 6.5)	4 (0 - 7,2)*
BCF (Hz)	37.4 (26.7 - 40.2)	42.1 (37.7 - 47.4)	38.9 (37.1 - 41.1)	32.5 (21.8 - 39.7)	38 (28.9 - 50.3)	30.8 (0 - 41.2)
STR (%)	82 (79 - 86)	84.5 (80 - 91)	82.5 (73 - 88)	80 (76 - 87)	83 (68 - 92)	77 (0 - 95)
LIN (%)	56 (50 - 58)	57.5 (43 - 68)	48.5 (41 - 53)	55 (45 - 66)*	56 (36 - 71)*	44 (0 - 75)
Area	6.9 (6.5 - 8.2)	17 (8 - 40)	17 (6 - 20)	6.3 (5.2 - 7.9)	5.1 (4.4 - 6.8)	5 (0 - 6,5)*
WOB	61.5 (34 - 72)	65.8 (51.3 - 70.7)	55.1 (54.2 - 56.3)	67.3 (58.1 - 79.3)*	62.8 (46.1 - 85.1)	68.1 (39.2 - 85.5)*
SVI	357.9 (302.2 - 407.8)	280.8 (272.1 - 374.5)	224.7 (181.2 - 311.4)	402.7 (325.4 - 482.7)*	296.4 (198.9-392.2)	288 (97.2 - 346.4)
SMI	288.8 (254.7 - 314.4)	280.3 (227.6 - 295)	226.3 (226 - 229.2)	312.9 (258.8 - 350.9)*	246.4 (185.4-343.3)	236.7 (170.6 - 332.7)
Morphology						
Normal cells	30 (14 - 55)*	26.5 (13 - 37)*	28 (7 - 36)*	55 (36 - 73)	46 (14 - 66)	46 (12 - 71)
Major defects	55.5 (22 - 72)*	52.5 (36 - 65)*	46.5 (37 - 77)	25 (9 - 48)	29 (13- 60)	24 (7 - 60)
Minor defects	19 (4 - 24)	22 (14 - 33)	22 (16 - 34)	18 (9 - 29)*	25 (17 - 44)	28 (12 - 53)
Total defects	70 (45 - 86)*	73.5 (63 - 87)*	72 (64 - 93)*	45 (27 - 64)	54 (34 - 86)	54 (29 - 88)
Integrity membrane						
Injured	8 (3 - 16)	8 (5 - 16)	5 (3 - 18)	4 (1 - 13)	8 (1 - 21)	12 (1 - 30)

* Significant difference between the corresponding columns of Table 3 and 4 (P<0.05); MT = total motility, MP = progressive motility, VAP = average path velocity, VSL = straight-line velocity, VCL = curvilinear velocity, ALH = amplitude of lateral head displacement, STR = straightness, LIN = linearity, SMI = sperm movement index, SVI = sperm velocity index, WOB = wobble.

Olivares et al. 2016), because during cryopreservation, the plasma membrane of the spermatozoon undergoes a series of changes related to temperature changes, progressively changing from the crystalline liquid phase to the gel phase, thus increasing the cell protection (Hazel 1995).

The superior results obtained with cooling at -1°C suggested that feline spermatozoa can be resistant to lower temperatures. This resistance of sperm cells to thermal stress could be attributed to the lower damage of domestic cats' spermatozoa, as well as that identified in dogs, likely caused by small proportions of polyunsaturated and saturated phospholipid fatty acids (White 1993, Rodenas et al. 2014). Lower cooling temperatures reduce the cells' metabolism, reaching near basal, allowing for greater survival of the more cold-resistant cells. In addition, considering that the

Table 4. Results of median (minimum-maximum) analyses of samples domestic cats spermatozoa collected by electroejaculation (EEJ) and from the epididymal tail (EPD), fresh and after refrigeration evaluations at -1°C for 24 hours and 48 hours

Sperm characteristics	Refrigeration -1°C					
	Electroejaculation			Epididymis		
	Fresh	24 h	48h	Fresh	24 h	48 h
MT (%)	77.5 (50 - 91)	46 (10 - 75)	43.5 (4 - 64)	66 (32 - 92)	44 (7 - 77)	24 (6 - 68)
MP (%)	49.5 (27 - 75)	26 (2 - 56)	14.5 (1 - 36)	29.5 (6 - 60)*	17.5 (0 - 37)	5 (34 - 0)
VAP (µm/s)	160.6 (109.8 - 185.2)	106.6 (60.1 - 115.1)	67.1 (60.1 - 109.6)	96.7 (68.6 - 152)*	95.1 (60.2 - 126.4)	83.7 (53.8 - 163.6)
VSL (µm/s)	140.5 (81.1 - 171.8)	91.9 (52.7 - 99.3)	54.5 (49.4 - 91.6)	78.3 (52.4 - 124.2)*	71.9 (44.3 - 106.4)	59 (40.3 - 136.4)
VCL (µm/s)	208.5 (178 - 234.7)	167.5 (115.2 - 189.3)	133.4 (107.9 - 175.3)	160.7 (125.1 - 222.2)*	176.2 (111.1 - 204.6)	166.7 (108.2 - 217.5)
ALH (µm)	5.9 (5.5 - 7.6)	5.3 (4.5 - 6.8)	5.6 (4.2 - 6.1)	6.6 (5.6 - 7.5)	6.1 (0 - 7.3)	5.7 (0 - 7.3)*
BCF (Hz)	33.8 (26.9 - 40.6)	40.2 (36.4 - 46.7)	37.2 (31.4 - 42.9)	33.9 (31 - 41.3)	37.5 (23.9 - 41.2)	35.4 (27.6 - 41.1)
STR (%)	86 (72 - 91)	84.5 (81 - 90)	82.5 (75 - 87)	77.5 (74 - 88)	77.5 (63 - 90)	78.5 (63 - 89)
LIN (%)	66 (39 - 72)	51 (43 - 61)	45 (37 - 53)	45 (38 - 60)*	44 (33 - 56)*	42 (28 - 61)
Area	6.5 (6 - 7.6)	46 (10 - 75)	43.5 (4 - 64)	7 (5.5 - 10.7)	6 (4.4 - 6.7)	5.4 (4.7 - 6.3)*
WOB	76.1 (52.7 - 80.7)	59.7 (52.1 - 66.4)	53.1 (48.2 - 62.5)	55.5 (50.9 - 69.7)*	54.8 (44.1 - 65.8)	50.5 (41.7 - 75.2)*
SVI	436.2 (342.3 - 506.8)	315.4 (198.5 - 337.6)	222.7 (194 - 325.5)	296 (216.9 - 426.1)*	299.1 (198.3 - 368.3)	277.3 (178.6 - 445)
SMI	344.3 (233.3 - 384.7)	267.1 (217.7 - 289.1)	222.5 (198.1 - 270.7)	246.7 (204.4 - 313.6)*	232 (182.2 - 290.9)	211.7 (171.3 - 333.4)
Morphology						
Normal cells	63.5 (38 - 92)*	58 (29 - 84)*	59 (20 - 65)*	58.5 (16 - 84)	38.5 (9 - 81)	28.5 (5 - 78)
Major defects	17.5 (4 - 32)*	16 (9 - 45)*	19 (12 - 41)	12 (0 - 36)	25.5 (8 - 63)	37.5 (10 - 82)
Minor defects	18 (4 - 37)	17 (7 - 31)	20 (17 - 39)	40.5 (5 - 60)*	22 (10 - 71)	29 (10 -56)
Total defects	36.5 (8 - 62)*	42 (16 - 71)*	41 (35 - 80)*	46.5 (6 - 84)	61.5 (19 - 91)	71.5 (22 - 95)
Integrity membrane						
Injured	10 (5 - 25)	15 (11 - 29)	17 (14 - 30)	6.5 (3 - 13)	7.5 (3 - 24)	9.5 (4- 19)

* Significant difference between the corresponding columns of Table 3 and 4 (P<0.05); MT = total motility, MP = progressive motility, VAP = average path velocity, VSL = straight-line velocity, VCL = curvilinear velocity, ALH = amplitude of lateral head displacement, STR = straightness, LIN =linearity, SMI = sperm movement index, SVI = sperm velocity index, WOB = wobble

extender used caused membrane stabilization during the cooling process, it reduced the damage related to cold shock.

The present study demonstrated that the use of ACP-117® maintained the viability of the refrigerated spermatozoa obtained by electroejaculation, similar to those obtained from the epididymis tail, suggesting that it could be another low-cost and easy to obtain alternative for cooling feline semen since coconut water-based extender has been described only for the preservation of spermatozoa recovered from the epididymis tail or collected with an artificial vagina (Silva 2008, Lima et al. 2014, Lima et al 2016).

Cooling of feline semen at a temperature of -1°C resulted in lower cryolysis, mainly in total morphological defects, allowing for a smaller reduction in sperm quality after 48 hours of cooling, different from results available in the literature that determined 4°C as the ideal temperature for spermatic cooling (Goodrowe & Hay 1993, Pukazhenthi et al 1999, Harris et al. 2001).

Table 5. Percentage reduction in median values (min-maximum) among fresh (F), 24 hours and 48 hours of refrigeration at -1°C of samples domestic feline spermatozoa collected by electroejaculation (EEJ) and from the epidydimal tail (EPD)

Sperm characteristics refrigeration at -1°C	Electroejaculation			Epididymis		
	F - 24h	F - 48h	24h - 48h	F - 24h	F - 48h	24h - 48h
MT (%)	29.5 (7 - 56)	24.5 (14 - 62)	4.5 (-23 - 25)	26.5 (-1 - 59)*	33.5 (15 - 72)*	9.5 (-7 - 33)
MP (%)	23.5 (-3 - 50)	36 (-1 - 59)	35 (-1 - 30)	7 (-16 - 38)*	11 (-10 - 41)*	5 (-9 - 19)
VAP (µm/s)	61.6 (0.4 - 92.5)	75.9 (15.9 - 119.5)	20.2 (-7.6 - 46.6)	-1.1 (-30.9 - 44.3)*	4.8 (-32.3 - 58)*	11.3 (-51 - 31.2)
VSL (µm/s)	55.7 (3.6 - 81.1)	68.1 (3.6 - 115.9)	20.5 (-7.3 - 46.9)	3.8 (-29.7 - 38.2)*	7.2 (-34.2 - 49.4)*	9.8 (-46.6 - 29.7)
VCL (µm/s)	41 (2.2 - 88.1)	68.1 (33 - 109.9)	24 (10.5 - 53.9)	-5.9 (-60.9 - 69.9)*	-6.2 (-55.7 - 86.5)*	11.8 (-18.9 - 32.4)
ALH (µm)	1 (-0.6 - 1.4)	0.6 (-0.10 - 1.5)	-0.3 (-1.5 - 1.4)	0.8 (-1.3 - 7.5)*	0.6 (-0.7 - 7.5)*	0 (-1.1 - 4.8)
BCF (Hz)	-6.2 (-15.1 - -1)	-1.5 (-16 - 3.4)	2.9 (-4.3 - 15.3)	-2.3 (-7 - 9.4)	-0.8 (-7.7 - 8.2)	0.5 (-10 - 6.9)
STR (%)	1 (-9 - 4)	4 (-10 - 13)	3 (-1 - 9)	0.5 (-6 - 14)	1 (-8 - 13)	-0.5 (-4 - 11)
LIN (%)	13 (-4 - 20)	18 (-6 - 27)	4 (-5.0 - 18)	5.5 (-7 - 14)	7 (-11 - 13)	1.5 (-18 - 9)
AREA	0.9 (-0.3 - 1.2)	0.4 (-1.3 - 2.1)	-0.6 (-1.7 - 1.3)	1.2 (-0.6 - 4.6)	1.5 (0.1 - 5.5)	0.4 (-0.4 - 1.4)
WOB	15.1 (-0.6 - 22.9)	18 (-0.8 - 28.2)	4.6 (-5.9 - 17.2)	3.6 (-4.3 - 14.7)	6.4 (-8.5 - 16.3)	3.4 (-20.1 - 14.1)
SVI	127 (5.7 - 222.8)	180.98 (47.1 - 291.5)	54.2 (21.4 - 121.6)	-0.9 (-87.1 - 122.3)*	-4.1 (-81.5 - 166)	22.5 (-90.7 - 75.5)
SMI	89.1 (3.6 - 119.8)	101.8 (-7.3 - 165.3)	28.2 (-10.9 - 84.3)	17.1 (-44.2 - 63.9)*	23.1 (-56.8 - 70.4)*	16 (-83.7 - 48.1)
Morphology						
Normal cells	11.5 (-5 - 55)	20.5 (-3 - 55)	5.5 (-1 - 20)	3.5 (-22 - 72)	10 (-20 - 62)	10.5 (-15 - 25)
Major defects	- 5 (-20 - 27)	-7 (-16 - 27)	-0.5 (-10 - 4)	-10.5 (-55 - 3)	-20 (-65 - 11)*	-5.5 (-26 - 15)
Minor defects	3.5 (-13 - 18)	-6.5 (-16 - 18)	-5 (-13 - 2)*	11 (-63 - 93)*	14.5 (-48 - 32)*	0.5 (-23 - 31)
Total defects	-11.5 (-55 - 5)	-20.5 (-55 - 3)	-5.5 (-20 - 1)	-3.5 (-72 - 22)	-10 (-62 - 20)	-10.5 (-25 - 15)
Integrity membrane						
Injured	-4 (-14 - 15)	-6.5 (-12 - 15)	-2 (-6 - 7)	-3 (-20 - 8)	-3 (-13 - 8)*	0 (-7 - 7)*

* Significant difference between the corresponding columns of Table 5 and 6 (P<0.05); substituir por MT = total motility, MP = progressive motility, VAP = average path velocity, VSL = straight-line velocity, VCL = curvilinear velocity, ALH = amplitude of lateral head displacement, STR = straightness, LIN = linearity, SMI = sperm movement index, SVI = sperm velocity index, WOB = wobble.

Table 6. Percentage reduction in median values (minimum-maximum) among fresh, 24 hours and 48 hours of refrigeration at 4°C of samples domestic feline spermatozoa collected by electroejaculation and from epididymis tail (EPD)

Sperm characteristics refrigeration at 4°C	Electroejaculation			Epididymis		
	F - 24h	F - 48h	24h - 48h	F - 24h	F - 48h	24h - 48h
MT (%)	31.5 (26 - 58)	44 (28 - 53)	3.5 (-5 - 20)	51 (-20 - 68)*	60 (-15 - 76)*	9 (-1 - 35)
MP (%)	23.5 (11 - 28)	30.0 (14 - 36)	20 (3 - 13)	33 (-12 - 52)*	38 (-17 - 51)*	7 (-5 - 20)
VAP (µm/s)	17 (2.3 - 49.6)	38.8 (28.9 - 91.8)	26.4 (11.4 - 42.2)	56.6 (-32.2 - 108.7)*	76.9 (-22.9 - 168.9)*	15.2 (-12.4 - 73)
VSL (µm/s)	19.7 (-3.6 - 30.8)	38.6 (23.1 - 72.9)	27.5 (9.4 - 42.1)	42 (-34.3 - 91.1)*	60.1 (-22.9 - 144.3)*	14.8 (-7.3 - 64.2)
VCL (µm/s)	4.7 (-121.4 - 59.8)	31 (-112 - 99.8)	26.6 (9.4 - 40)	61.9 (-17.1 - 118.8)*	74.2 (17.7 - 212.8)*	21.9 (-55.6 - 106.7)
ALH (µm)	1 (0.1 - 2.9)	1.4 (0.2 - 1.9)	0.5 (-2.7 - 1.5)	1.6 (0.4 - 3.8)*	2.7 (-0.1 - 7.1)*	0.9 (-3 - 6.5)
BCF (Hz)	-6.6 (-11.0 - -3.3)	-0.35 (-14.4 - 0.9)	4.3 (-3.4 - 8.1)	-6.1 (-23.6 - 5.9)	0.7 (-16.1 - 33)	4.8 (-6.3 - 41.3)
STR (%)	-1.5 (-12 - 4)	1 (-9 - 10)	3.5 (0 - 7)	-2 (-10 - 14)	6 (-16 - 85)	5 (-7 - 92)
LIN (%)	-3.5 (-14 - 15)	7.5 (1 - 13)	12.5 (-5 - 15)	2 (-25 - 24)	8 (-21 - 66)	5 (-15 - 57)
AREA	1.4 (1.1 - 3.2)	1.65 (1.2 - 3.2)	0.05 (-0.1 - 0.6)	0.8 (-0.1 - 2.8)	0.9 (-0.3 - 5.8)	0.2 (-1.5 - 5.1)
WOB	2.9 (-10.7 - 14.6)	9.7 (5.3 - 17.6)	10.5 (-4.2 - 16)	5.5 (-26.8 - 30.1)	14 (-21.3 - 79.3)	6.1 (-12.1 - 61.3)
SVI	43.7 (8.6 - 121.5)	92.6 (71.7 - 226.5)	58.8 (47.4 - 105)	154.4 (-57.2 - 251.9)*	185.6 (-20.9 - 452.3)	36.2 (-55.1 - 200.3)
SMI	16.4 (-28.8 - 59.4)	61.1 (28.1 - 88.4)	52.4 (1.4 - 68.9)	36.6 (-84.4 - 138.1)*	79.6 (-64 - 350.9)*	19.2 (-27.2 - 246.4)
Morphology						
Normal cells	1.5 (-1 - 24)	3.5 (-5 - 28)	2.5 (-7 - 6)	12 (-48.0 - 45)	8 (-49 - 47)	1 (-31 - 31)
Major defects	-1.5 (-14 - 16)	-5 (-27 - 35)	-3.5 (-13 - 19)	-7 (-29 - 21)	1 (-38 - 31)*	0 (-19 - 27)
Minor defects	-4.5 (-18 - 2)	-3.5 (-28 -5)	-49.5 (-71 - -40)*	-9 (-37 - 5)*	-9 (-39 - 11)*	3 (-30 - 16)
Total defects	-1.5 (-24 - 1)	43 (21 - 70)	-2.5 (-6 - 7)	-12 (-45 - 48)	-8 (-47 - 49)	-1 (-31 - 31)
Integrity membrane						
Injured	0.5 (-13 - 10)	4.5 (-15 - 10)	1 (-2 - 6)	-3 (-16 - 5)	-4 (-25 - 1)*	-2 (-19 - 7)*

* Significant difference between the corresponding columns of Table 5 and 6 (P<0.05); MT = total motility, MP = progressive motility, VAP = average path velocity, VSL = straight-line velocity, VCL = curvilinear velocity, ALH = amplitude of lateral head displacement, STR = straightness, LIN =linearity, SMI = sperm movement index, SVI = sperm velocity index, WOB = wobble.

CONCLUSION

The refrigeration of cat spermatozoa samples at -1°C for up to 48 hours was efficient to maintain the quality of sperm cells obtained from ejaculate and the epididymis tail and could be an alternative temperature to the cooling of domestic feline spermatozoa.

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Vascularization of the *Alouatta belzebul* brain base[1]

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ABSTRACT.- Sabec-Pereira D.K., Lima F.C., Melo F.R., Melo F.C.S.A., Pereira K.F. & Vulcani V.A.S. 2020. **Vascularization of the *Alouatta belzebul* brain base.** *Pesquisa Veterinária Brasileira 40(4):315-323*. Escola de *Veterinária* e Zootecnia, Universidade Federal de Goiás, Avenida Esperança s/n, Campus Samambaia, Goiânia, GO 74690-900, Brazil. E-mail: daya_ks@hotmail.com

We studied the arterial circle in the brain of five specimens of the *Alouatta belzebul* primate. The material had the arterial system perfused (water at 40°C), injected with stained latex (Neoprene 650), fixed in aqueous formaldehyde solution (10%) and dissected for vessel verification. The arterial circle of this primate is composed of two vascular systems: the vertebra-basilar and the carotid ones, which anastomose to close the arterial circuit. In the caudal portion of the arterial circle, there are the vertebral arteries and their branches: the rostral spinal artery and the caudal inferior cerebellar artery. The anastomosis of the vertebral arteries gives rise to the basilar artery. It presented an anatomical variation at the beginning of its path, forming a double basilar artery, called arterial island. In its course, it emitted branches giving rise to the rostral inferior cerebellar artery, the pontine arteries, the rostral cerebellar arteries, the satellite rostral cerebellar arteries and its terminal branch, the caudal cerebral artery, which presented itself in two segments: the pre-communicating one and post-communicating, joining the internal carotid artery and originating the caudal communicating artery. This group of arteries and anastomoses enclose the caudal portion of the arterial circle. From the right and left internal carotid arteries begins the rostral portion of the arterial circle, which consists of the right and left rostral cerebral arteries and the right and left middle cerebral arteries. The rostral cerebral arteries anastomose into a single trunk, giving rise to the interhemispheric artery, and in *A. belzebul* and *Sapajus libidinosus*, the rostral communicating artery is absent. The interhemispheric artery goes to the midbrain region and the corpus callosum knee divides into pericalous artery and callosarginal artery, which will supply the pre and post-central regions of the cerebral hemispheres of this species, as well as other non-human and human primates. It is noted that in the first part of the left rostral cerebral artery, there is a direct inosculation between the recurrent branch of the rostral cerebral artery and left middle cerebral artery to supply the entorhinal region. This fact also occurs in *Pongo* spp. The middle cerebral artery travels along the lateral sulcus where it emits several superficial branches to irrigate the superior and inferior lateral cortical regions of the frontal, parietal and temporal lobes. It is not part of the arterial circle but is the terminal branch of the internal carotid artery. *A. belzebul* can be considered to depend on two sources of supply to the brain: the vertebra-basilar and carotid systems, contributing to the intervention of veterinarians during clinical and surgical procedures in other primates, as well as the preservation of wild animals.

INDEX TERMS: Vascularization, *Alouatta belzebul*, brain base, arterial circle, neotropical primate, carotid system.

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RESUMO- [**Vascularização da base do encéfalo de *Alouatta belzebul*.**] Estudamos o círculo arterial no encéfalo de cinco espécimes do primata *Alouatta belzebul*. O material teve o sistema arterial perfundido (água a 40°C), injetado com látex corado (Neoprene 650), fixado em solução aquosa de formaldeído (10%) e dissecado para verificação dos vasos. O círculo arterial deste primata é composto por dois sistemas vasculares: vértebro-basilar e o sistema carotídeo, que se anastomosam para fechar o circuito arterial. Na porção caudal do círculo arterial encontra-se as artérias vertebrais e seus ramos: artéria espinal rostral e a cerebelar inferior caudal. A anastomose das artérias vertebrais dá origem a artéria basilar. Esta apresentou uma variação anatômica no início do seu trajeto, formando uma dupla artéria basilar, denominada ilha arterial. Em seu trajeto emitiu ramos dando origem a artéria cerebelar inferior rostral, as artérias pontinas, as artérias cerebelares rostrais, as artérias cerebelares rostrais satélites e o seu ramo terminal, a artéria cerebral caudal, que apresentou-se em dois segmentos: o pré-comunicante e pós-comunicante, unindo-se a artéria carótida interna e originando a artéria comunicante caudal. Este grupo de artérias e anastomoses encerram a porção caudal do círculo arterial. Das artérias carótidas internas direita e esquerda, inicia-se a porção rostral do círculo arterial, ao qual é constituído pelas artérias cerebrais rostrais direita e esquerda e as artérias cerebrais médias direita e esquerda. As artérias cerebrais rostrais se anastomosam em um tronco único dando origem a artéria inter-hemisférica e em *A. belzebul* e *Sapajus libidinosus*, a artéria comunicante rostral se encontra ausente. A artéria inter-hemisférica segue para região média do encéfalo e no joelho do corpo caloso se divide em artéria pericalosa e artéria calosomarginal, que vão suprir as regiões pré e pós-central dos hemisférios cerebrais desta espécie, assim como outros primatas não humanos e humano. Nota-se que na primeira parte da artéria cerebral rostral esquerda, ocorre uma inosculação direta entre o ramo recorrente da artéria cerebral rostral e artéria cerebral média esquerda para suprir a região entorrinal, esse fato também ocorre em *Pongo* spp. A artéria cerebral média segue seu trajeto pelo sulco lateral onde emite vários ramos superficiais para irrigar as regiões corticais supero e ínfero lateral do lobo frontal, parietal e temporal, esta não faz parte do círculo arterial mas é o ramo terminal da artéria carótida interna. Pode-se considerar que *A. belzebul* depende de duas fontes de suprimento para o encéfalo: os sistemas vértebro-basilar e carotídeo, contribuindo na intervenção de médicos veterinários durante os procedimentos clínicos e cirúrgicos em outros primatas, assim como na preservação de animais silvestres.

TERMOS DE INDEXAÇÃO*:* Vascularização, base do encéfalo, *Alouatta belzebul*, encéfalo, círculo arterial, primata neotropical, sistema carotídeo.

INTRODUCTION

Alouatta belzebul (Linnaeus, 1766) is popularly known as bugio or guariba and, for being part of a group of neotropical primates, it presents exclusive characteristics, such as limbs and body adapted to suspending behavior and a truly prehensile long tail (Gregorin 2006). Despite the extensive amount of information available about primates, there are still gaps in knowledge about endemic or rare animals, especially anatomical studies. (Nunes & Catão-Dias 2006). The anatomical approach of nonhuman primates is given to Old World species, such as *Papio* spp., popularly known as baboon and *Macaca mulatta*, known as monkey rhesus (Ribeiro 2006). Studies in animals of the New World began in the last century (Tilney 1928) which becomes restricted to the scientific community and the clinical and surgical routine of veterinary medicine.

Anatomical studies in different species provide information that add up and become an important source of consultation for society and the scientific community. This way, it is understood that the knowledge of the various aspects of neuroanatomy of nonhuman primates, scarce in the literature, are important not only for their intrinsic value, but also for providing a better understanding of the evolutionary process of these animals, which represents a relevant factor for its preservation and protection (Aversi-Ferreira et al. 2005).

The arterial circuit of the base of the brain has been described by other authors aiming at detailing the brain vascularization in comparative character between human, non-human primates and other vertebrates (Ferreira & Prada 2009, Prada 2014). The arterial circle is a very important vascular pathway of the brain. Anatomocomparative studies of vessels responsible for cerebral blood flow and their anatomical variations that occur in different species of fauna may facilitate the understanding of the mechanisms responsible for the proper functioning or dysfunction of this organ (Kapoor et al. 2003, Kuchinka et al. 2008). Studies that allow for the determination of the organization, distribution, and potential differences in blood vessels at the base of the brain may also contribute to phylogenesis and ontogenesis of the brain artery (Bugge 1974). The aim of this paper is to describe the vascularization of the brain base of the Neotropical primate *A. belzebul*, focusing on the anatomical aspects of the arterial circle of this species.

MATERIALS AND METHODS

For this study, we used five *Alouatta belzebul* brains, collected during the rescue of terrestrial fauna during vegetation suppression activities for the implementation of Belo Monte hydroelectric exploitation, Brasília/DF, conducted by IBAMA process number 02001.001848/2006-75 and authorization number 473/2014 - 2nd rectification. After rescue, the animals were frozen and sent to the Human and Comparative Anatomy Laboratory of the "Universidade Federal de Goiás" (UFG), being kept frozen until the beginning of the experiment. The procedure involved macroscopy and dissection techniques, where the Neoprene 650 latex was injected, stained with red pigment, using as access to the thoracic aorta. Then, the pieces were fixed in 10% aqueous formaldehyde solution by intramuscular, subcutaneous and intracavitary injections. The animals were kept in this solution for at least 72h and, after this period, the brains were removed from the skull with the aid of an oscillating saw (Dremel® 3000). After removal of the brains, they returned to the 10% formaldehyde solution for a further 72h, so that the descriptive analyzes were then performed and photographed. All nomenclature adopted was based on Veterinary Anatomical Nomina (International Committee on Veterinary Gross Anatomical Nomenclature, ICVGAN 2017). The experimental procedure was approved by the Animal Use Ethics Committee of the UFG (protocol number 083/17).

RESULTS

The analysis of the results of this study made it possible to observe the origin, the course and the collateral branches, as well as the terminations of the arteries that participate, in or part of it, in the formation of the arterial circle of the base of the brain of *Alouatta belzebul*. We have identified a division of these arteries into three vascular pedicles: the basilar artery, the right internal carotid artery and the left internal carotid artery, which have morphological features reminiscent of a hexagonal figure, arranged ventrally in relation to the brain base and rostrally to the optic chiasm (Fig.1). In the ventral region of the brain is a carotid system and in the caudal region the vertebra-basilar system.

The vertebra-basilar system forms the caudal part of the arterial circle of the brain of *A. belzebul*. The subclavian arteries originate the right and left vertebral arteries, which in the rostral region emits the rostral spinal artery, which supplies the anterior and lateral spinal cord and funiculi, and in the lateral region emits the caudal inferior cerebellar artery, the largest branch of the vertebral artery to supply the inferior and caudal region of the cerebellum (Fig.2A). Then they anastomose, originating the basilar artery.

In the basilar artery, in 100% of the specimens, we observed anatomical variation in its path, forming a double basilar artery in the transition region between the bridge and the brainstem bulb, called the arterial island. This double vessel is approximately 2mm and has a single segment over the bridge extension (Fig.2B).

The first large branch of the basilar artery is the right and left rostral inferior cerebellar artery, which supplies the rostral inferior portion of the cerebellum. The pontine arteries in the mid-lateral region of the basilar artery supply the bridge and the right and left rostral cerebellar arteries and satellite rostral cerebellar arteries, terminal branches of the basilar artery, supply the cranial part of the cerebellum (Fig.3).

The caudal cerebral artery is divided into two segments: the pre-communicating artery, this part of the artery between the basilar artery bifurcation and the caudal communicating artery, and the post-communicating artery between the caudal communicating artery and the temporal branches, which supply the medial and lateral regions of the midbrain and the occipital pole and inferior margin of the temporal lobe. The caudal communicating artery, formed by the union of the internal carotid artery and the caudal cerebral artery, runs laterally to supply the pituitary and the nipple bodies.

The other vascular system we identified in *A. belzebul* is the carotid system, consisting of the right and left internal carotid arteries, which emits two main branches: the rostral cerebral artery that ends the arterial circle to the base of the brain and the middle cerebral artery (Fig.4).

The rostral cerebral artery runs through the anterior encephalic region at the base of the cerebral peduncle, infundibular and chiasmatic region. Moreover, near the longitudinal cleft of the brain is an anastomosis between the rostral cerebral artery with its contralateral homonymous, forming a single trunk giving rise to the interhemispheric artery (Fig.4). This artery was observed in 100% of the specimens analyzed and with symmetrical vascular gauges, sending branches to supply

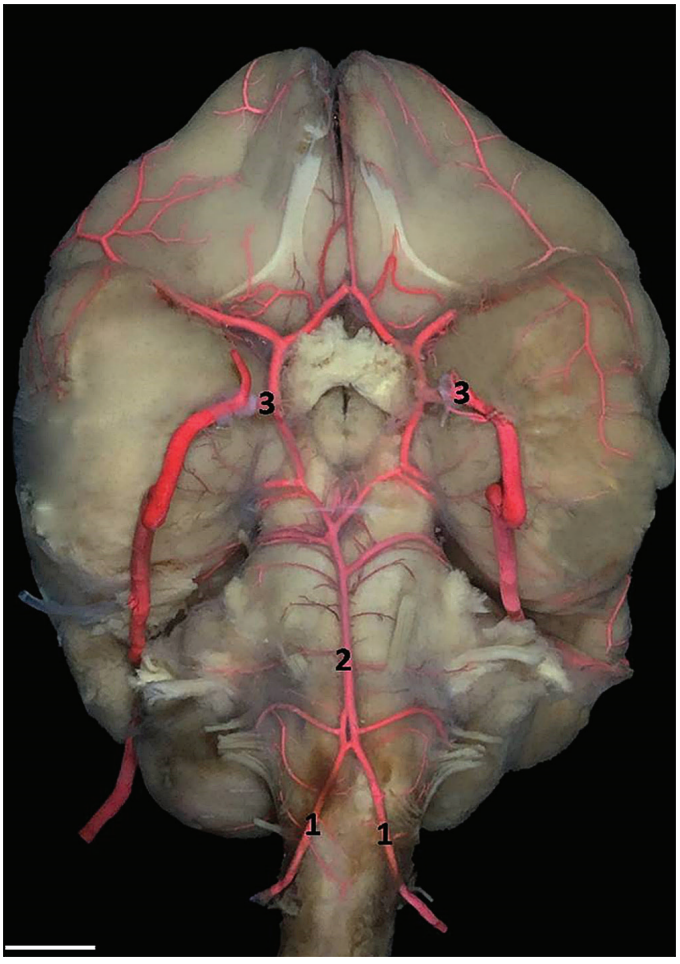


Fig.1. Arterial circle formation by right and left vertebral arteries (1), basilar artery (2) and left and right internal carotid arteries (3). Bar = 1cm.

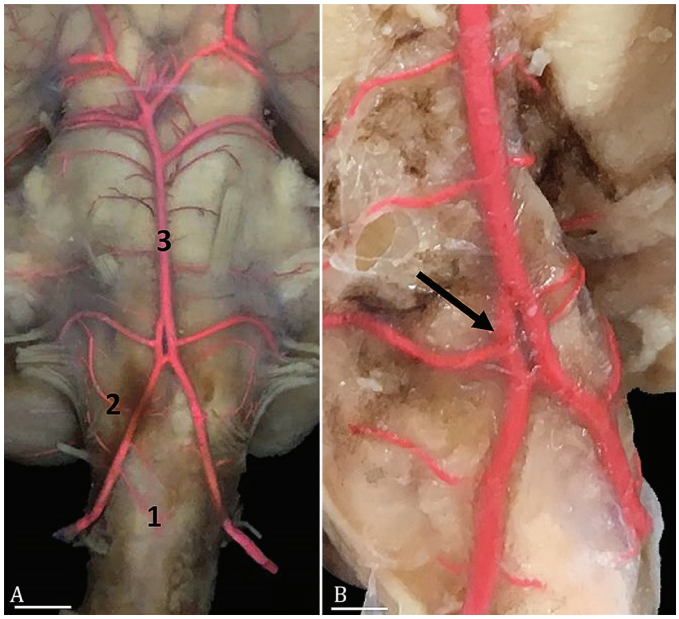


Fig. 2. (**A**) Brainstem of *Alouatta belzebul* highlighting the rostral spinal artery (1), caudal inferior cerebellar artery (2) and basilar artery (3). Bar = 0.8cm. (**B**) Double basilar artery (arrow) of *A. belzebul* forming an arterial island at the beginning of vertebral artery anastomosis. Bar = 0.4cm.

cortical areas of the anterior perforated substance, orbital region, straight gyrus, medial surface of the sub-rostral sulcus, frontal pole, anterior frontal, middle and posterior regions, corpus callosum and grooves, pre-central region, upper part of pre- and post-central gyrus and pre-cuneal region (Fig.5).

The union of the rostral cerebral arteries gives rise to the interhemispheric artery and it forks in the knee region of the corpus callosum into two terminal branches called the pericalous artery and the calosmarginal artery (Fig.5). These two terminal branches continue the path by branching into the brain gyrus from the main trunk at acute angles, directed to the medial surface of *A. belzebul*'s cerebral hemisphere. The branches of the calosomarginal artery go to the regions of the cortex in the upper and pre-central frontal branches, and the branches of the pericalous artery go to the post-central and precununal regions (Fig.5). In the first part of the left rostral cerebral artery, there is a direct inosculation between the recurrent branch of the rostral cerebral artery and the left middle cerebral artery to supply the entorhinal region (Fig.6).

The even-sized middle cerebral artery branches from the internal carotid artery and laterally traverses the lateral sulcus between the inferior frontal gyrus and the superior temporal gyrus without forming part of the contour formation of the arterial circle. Along the way, it emits branches that vascularize most of the inferolateral face of each cerebral hemisphere,

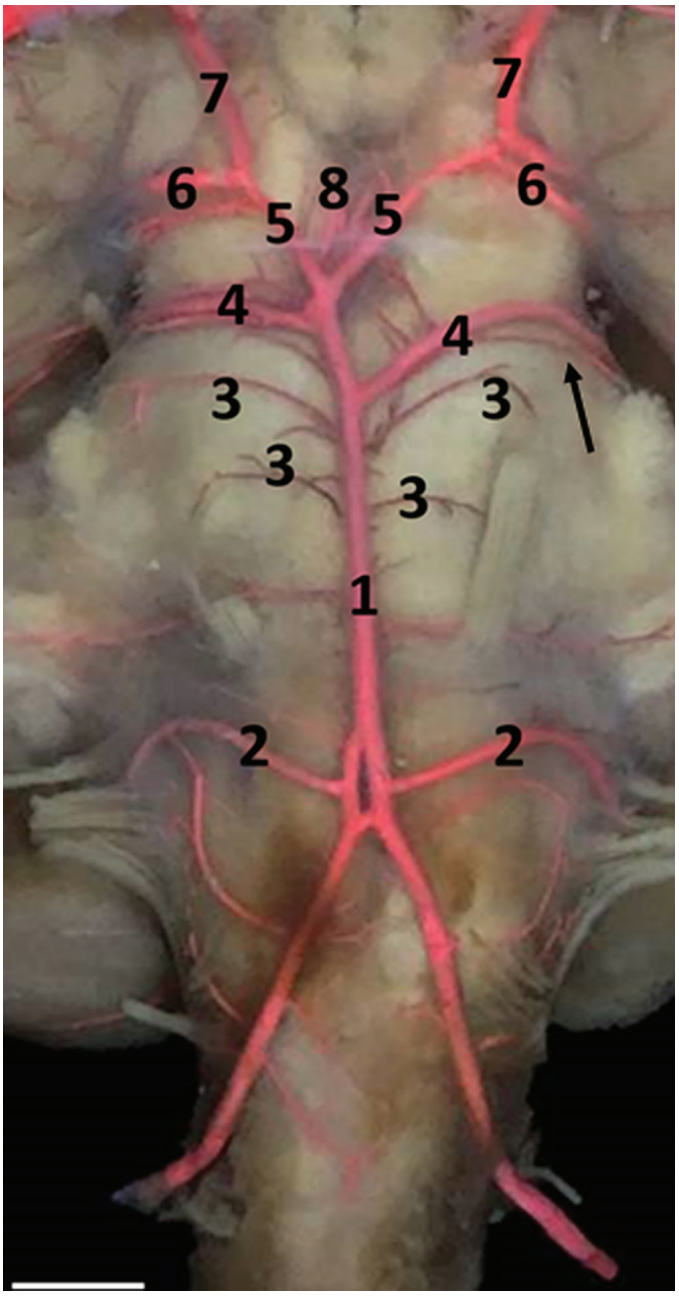


Fig.3. Basilar artery (1) and its branches in the vertebra-basilar system of *Alouatta belzebul*. Right and left rostral inferior cerebellar artery (2), right and left pontine arteries (3), right and left rostral cerebellar artery (4), satellite rostral cerebellar artery (arrow), pre-communicating caudal cerebral artery (5), cerebral artery post-communicating caudal flow (6), caudal communicating artery (7) and branches for the chiasmatic region (8). Bar = 0.8cm.

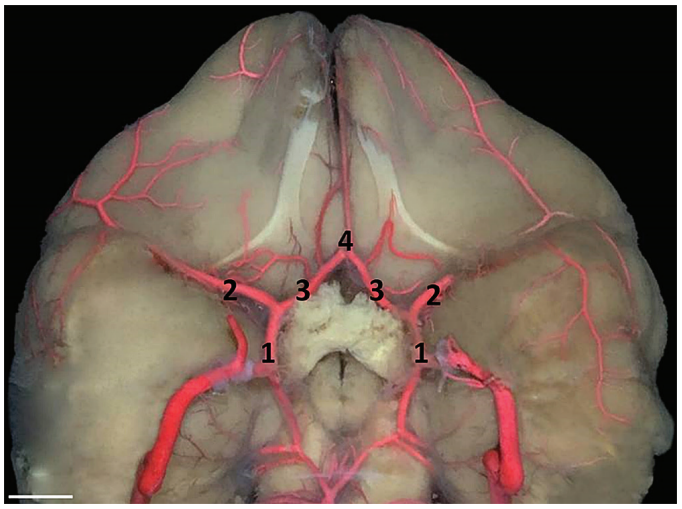


Fig.4. Alouatta *belzebul* carotid system formed by the right and left internal carotid arteries (1), right and left middle cerebral artery (2), right and left rostral cerebral artery (3) and interhemispheric artery (4). Bar = 0.6cm.

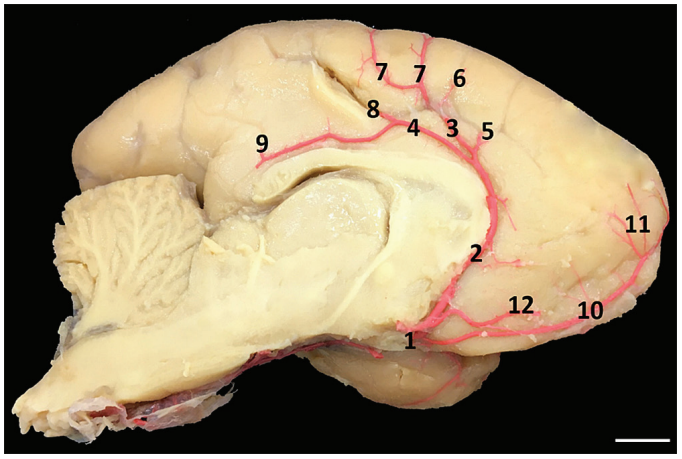


Fig.5. Left rostral cerebral artery (1), interhemispheric artery (2), callosalginal artery (3), pericalous artery (4), rostral frontal artery (5), middle frontal artery (6), caudal frontal artery (7), post-central artery (8), pre-cuneal artery (9), frontal polar artery (10), frontal polar artery branches (11) and anteromedial frontal branch of the interhemispheric artery (12). Bar = 1.2cm.

namely: the lateral basilar frontal artery, pre-central sulcus artery, central sulcus artery, postcentral sulcus artery, parietal artery rostral, temporo-occipital branch, caudal temporal branch, middle temporal branch and rostral temporal branch (Fig.7). The main vessels that form the arterial circle of Old and New World primates are shown in Table 1.

DISCUSSION

In this study, it was observed that in *Alouatta belzebul*, the vertebra - basilar system occurs through an irrigation anastomosis between the right and left vertebral arteries, the basilar artery and the right and left caudal cerebral arteries, corroborating the findings in *Pongo* spp. (Shellshear 1927), *Pan* spp. and family Hylobatidae (Watts 1934b), *Macaca mulatta* (Kassel & Langefitt 1965), *Homo* spp. (Gardner et al. 1978) and *Sapajus libidinosus* (Ferreira & Prada 2001, 2009). The caudal arterial supply comes from the vertebral arteries, to which they anastomose and form the caudal portion of the arterial circle. The intracranial vertebral artery ascends medially rostral to the spinal cord and the inferior border of the bridge, and the two vertebral arteries join to form the basilar artery.

The vertebral artery in *A. belzebul* originates at the level of the inferior border of the olivary eminence and joins with its counterpart to form the basilar artery. Branching from the vertebral arteries is the rostral spinal artery, which

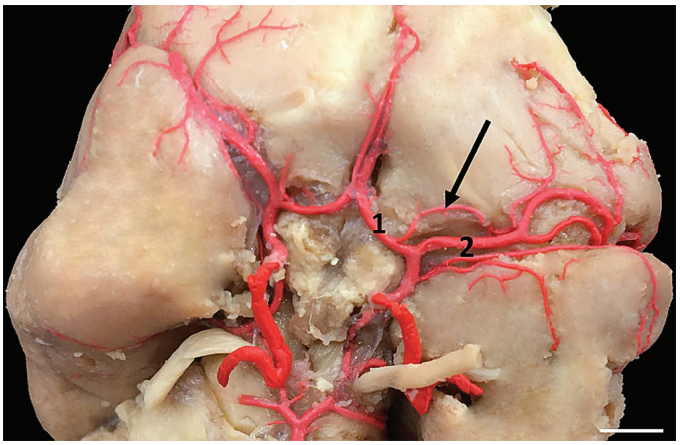


Fig.6. Inoculation (arrow) of the rostral cerebral artery (1) with the middle cerebral artery (2) in the anterior part. Bar = 0.6cm.

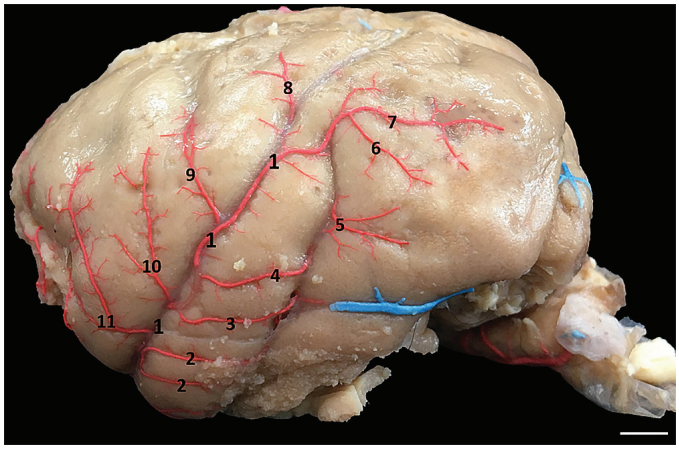


Fig.7. Middle cerebral artery (1) and its branches: rostral temporal branch (2), middle temporal branch (3), caudal temporal branch (4), temporo-occipital branch (5), inferior parietal artery (6), rostral parietal artery (7), post-central sulcus artery (8), central sulcus artery (9), pre-central sulcus artery (10) and lateral basilar frontal artery (11). Bar = 1.2cm.

Table 1. Comparative data on arterial circle formation in primates

Taxonomic group	Taxon	Popular name	Vertebral artery	Basilar artery	Caudal cerebral artery	Caudal communicating artery	Internal carotid artery	Rostral cerebral artery	Rostral communicating artery	Interhemispheric artery
New World	*Alouatta belzebul*	Bugio	X	X (arterial island)	X	X	X	X	Absent	X
New World	*Sapajus libidinosus*	Capuchin monkeys	X	X (arterial island)	X	X	X	X	Absent	X
Old World	*Pongo* spp.	Orangutan	X	X	X	X	X	X	X	Absent
Old World	*Pan* spp.	Chimpanzee	X	X	X	X	X	X	Absent	Absent
Old World	Hylobatidae	Gibbon	X	X	X	X	X	X	Absent	
Old World	*Homo sapiens*	Human	X	X	X	X	X	X	X	Absent
Old World	*Macaca mulata*	Rhesus	X	X	X	X	X	X	X	Absent
Old World	*Papio ursinus*	Baboon	X	X	X	X	X	X	Absent	X
Old World	*Cercopithecus pygerithrus*	Vervet	X	X	X	X	X	X	Absent	X
Old World	*Galago senegalensis*	Bushbaby	X	X	X	X	X	X	Absent	X

Source: Lake et al. 1990 (modified).

presents a ventral downward pathway to the spinal cord and anastomoses with its counterpart to form a median trunk that supplies the lateral and dorsal regions of the spinal cord. This morphological arrangement is similar in *Pongo* spp. (Shellshear 1927) and *Homo* spp. (Amato & Stolf 2015). In these species, it was also observed the presence of perforating, segmental and longitudinal rostral spinal arteries that form an irrigation channel connecting the perforating and segmental with the same function. These branches were not identified in *A. belzebul.*

The caudal and rostral inferior cerebellar arteries were observed in 100% of the analyzed specimens. The caudal inferior cerebellar artery is a branch of the vertebral artery and irrigates the posteroinferior surface of the cerebellum. Its caliber is smaller than the rostral inferior artery, unlike that observed in *S. libidinosus* (Silva & Ferreira 2002b), which presented a larger caliber in the caudal inferior cerebellar artery. The rostral inferior cerebellar artery is a branch of the basilar artery, its path runs posteriorly around the olives, between the roots of the hypoglossal nerve, and caudally to the roots of the vagus and glossopharyngeal nerves, vascularizing the inferior surface of the cerebellum, the inferior worm, the tonsil and the inferolateral surface of the cerebellar hemisphere. In *Papio ursinus*, *Cercopithecus pygerithrus* and *Galago senegalensis*, the caudal and rostral inferior cerebellar arteries originate from a single trunk called the common inferior cerebellar artery (Lake et al. 1990). This common vessel is absent in *A. belzebul*, as well as in *S. libidinosus* (Silva et al. 2003, Silva & Ferreira 2003) and *Homo* spp. (Meneses 2015). Elze (1910) described that in *Ateles paniscus* one or two caudal inferior cerebellar arteries and one or two rostral inferior cerebellar arteries (or internal auditory artery) are present, which was not observed in *A. belzebul*, where only a single right and left branch was identified from each artery.

By joining the right and left vertebral arteries there is the formation of the basilar artery. In *A. belzebul*, the path begins at the lower edge of the bridge passing through the longitudinal groove, differing from the findings of Shellshear (1927), who reports that in *Pongo* spp. the origin is at the lower edge level of the olive eminence. In all species found in the literature and in *A. belzebul,* this artery terminates at the upper edge of the bridge, emitting four branches: the pontine arteries, which supply the brainstem bridge; the inferior and superior cerebellar even arteries, which supply the bridge and cerebellum; and the caudal cerebral arteries, which are the terminal branches of the basilar artery, supplying most of the temporal and occipital lobes and joining with the caudal communicating arteries to form the vertebra-basilar system of the arterial circle. This pattern has also been described in other nonhuman primates such as *Pongo* spp. (Shellshear 1927), *Pan* spp. and Hylobatidae (Watts 1934b), *M. mulatta* (Kassel & Langefitt 1965), *S. libidinosus* (Ferreira & Prada 2001, Prada 2014) and human (Gardner et al. al. 1978).

In the initial course of the basilar artery, in *A. belzebul* was observed the formation of a double basilar artery, which is called arterial island. This data was also described in *S. libidinosus* (Ferreira & Prada 2001), which describes the arterial island as a variation in the fusion of this blood vessel.

The rostral cerebellar artery of *A. belzebul* is a collateral branch dependent on the large basilar artery. Reaches the upper part of the cerebellum, its branches go to the upper worm and to the remaining hemispherical portions of the upper and lateral region. Its origin, path and distribution corroborate the findings for *P. ursinus*, *C. pygerithrus* and *G. senegalensis* (Lake et al. 1990), *S. libidinosus* (Silva & Ferreira 2002b) and *Homo* spp. (Gardner et al. 1978).

The rostral cerebellar arteries of *A. belzebul* are often accompanied at their origins by a smaller caliber satellite vessel, the rostral satellite cerebellar artery, also observed in *S. libidinosus* (Ferreira 1997, Silva & Ferreira 2002a, Silva & Ferreira 2003), and absent in other primates. This satellite vessel is mainly arboreal in the bridge region and anastomoses with the lateral branch of the rostral cerebellar artery in the lateral vicinity of the superior semilunar lobe. It is noteworthy that, according to the De Vriese's classification (De Vriese 1905), primates present a well-elaborated vascularization model, where the cerebellum vessels are dependent on the vertebra-basilar system, with few variations. This has been confirmed for *A. belzebul*, which we understand to be indicative of a relative morphological and functional stability, which allows us to consider that this richness of interconnections may mean an important aspect of the adaptation of the cerebellum to the tree locomotion of this species, whose rapid dislocations on the ground and at the height of the forests where they are found require extremely flexible and adapted cerebellar blood flow.

The caudal cerebral artery in *A. belzebul* was observed in 100% of the specimens. This artery is divided into two segments: the pre-communicating artery, located between the basilar artery bifurcation and the caudal communicating artery, and the postcommunicating artery, between the caudal communicating artery and the temporal branches, which supply cortical collateral branches, the medial and lateral regions of the midbrain, parietal, occipital pole and inferior border of the temporal lobe.

In all analyzed brain, the origin of the caudal cerebral artery was forked from the basilar artery as a terminal branch, corroborating with other authors who described this artery in *P. ursinus*, *C. pygerithrus* and *G. senegalensis* (Lake et al. 1990), *S. libidinosus* (Siqueira & Ferreira 2002), *Pan* spp. (Sperino 1897, Shellshear 1930); *M. mulatta* (Weinstein & Hedges 1962, Kassel & Langefitt 1965); *Pongo* spp. (Shellshear 1927); in Hylobatidae (Watts 1934b); and *Homo* spp. (Testut & Latarjet 1954, Gillilan 1969, Machado & Haertel 2014).

The caudal communicating artery in *A. belzebul* presents itself as a branch of the union of the internal carotid artery with the caudal cerebral artery, and its course runs laterally to the cerebral hemisphere to supply the pituitary and nipple bodies. In *S. libidinosus* (Ferreira & Prada 2009), the caudal communicating artery has the same configuration, which delimits the caudal part of the arterial circle with the vertebra-basilar system.

The other system described for *A. belzebul*'s arterial circle was the carotid artery. This occurs through an irrigation anastomosis between the right and left internal carotid arteries that emit two main branches: the right and left rostral cerebral arteries, which terminate the arterial circle in the rostral portion, and the middle cerebral artery to the medial region of the brain.

The internal carotid artery of *A. belzebul*, in the intracranial segment, follows its path laterally to the optic nerve and medially to the oculomotor nerve. This vessel gives rise to its two terminal branches: the rostral cerebral artery and

the middle cerebral artery, according to findings in *Pan* spp. (Shellshear 1930), Cereophitecidae and Cebidae (Watts 1934b); *M. mulatta* (Dyrud 1944, Lineback 1961, Weinstein & Hedges 1962), *Gorilla* spp. (Raven & Hill 1950), *Homo* spp. (Testut & Latarjet 1954), *S. libidinosus* (Ferreira & Pires 2004, Ferreira & Prada 2009), *Pongo* spp. (Watts 1934b) and *P. ursinus*, *C. pygerithrus* and *G. senegalensis* (Lake et al. 1990).

The rostral cerebral artery in *A. belzebul* is a terminal branch of the right and left internal carotid arteries. Its origin is medially related to the optic chiasm, passing dorsally to the optic tract and caudally to the olfactory bulbs. After emitting the olfactory arteries, the rostral cerebral artery goes to the longitudinal fissure of the brain where it anastomoses with its contralateral homonymous forming a single trunk to which the interhemispheric artery originates. This segment follows the longitudinal fissure of the brain passing dorsally to the knee of the corpus callosum, where it emits collateral branches to the two cerebral hemispheres. In the knee region of the corpus callosum, it ends by bifurcating into the calosmarginal artery and pericalous artery, both with directions to the right and left antimers. This arterial distribution favors the cerebral cortical regions: prefrontal, frontopolar, superior frontal, pre-central and postcentral, similar to the findings in *S. libidinosus* (Ferreira & Pires 2004, Ferreira et al. 2005).

In *Homo* spp. (Meneses 2015), *Pongo* spp. and *Pan* spp. (Watts 1934a), the rostral cerebral artery bifurcates from the internal carotid artery and runs medially and anteriorly to the longitudinal fissure of the brain, where it makes an anastomosis with its contralateral counterpart through the rostral communicating artery, and then follow separated to the cortical areas. This pattern differs in *M. mulatta* and *P. cynocephalus*, where the arteries join and then separate into a spiral loop to supply the cerebral hemispheres (Kassel & Langefitt 1965, Lake et al. 1990). This communicating branch is absent in *A. belzebul*, as well as in *S. libidinosus* (Ferreira et al. 2005, Ferreira & Prada 2009).

Another important consideration in *A. belzebul* is that a direct inosculation with the middle cerebral artery was observed in the first portion of the left rostral cerebral artery. This inoculation has also been found in *Pongo* spp. (Shellshear 1927) on the same brain antigen, which gives rise to the striated arteries for the endorhinal fissure. The usual anatomical condition of this inoculation is to be present on the right side, where the striated branches of the two arteries are separated from each other.

The pericalous artery and callosarginal artery represent the terminal branches of the interhemispheric artery, with a bifurcation at the level of the caudal half of *A. belzebul*'s corpus callosum. In *S. libidinosus* (Pires & Ferreira 2004), other arrangements for bifurcation of the interhemispheric artery were observed. However, in both species the arteries supply the polar front region, the pre-central region and the post-central region, which resembles the arterial supply model described for *Pan* spp., *Pongo* spp., *M. mulatta*, Hylobatidae and *Ateles paniscus* (Watts 1934b).

The calosmarginal artery in *A. belzebul* is a branch of the interhemispheric artery that bifurcated at the level of the caudal half of the corpus callosum, following the cingulate groove, where it gave rise to three frontal arteries: superior frontal artery, middle frontal artery, and posterior frontal artery. They are important for supplying pre- and post-central cerebral cortical areas, linked to motor activity. In *Homo* spp. (Martin 2013, Meneses 2015) and *Pongo* spp. (Lemos 1984), the calosomarginal artery presents anatomical and angiographic variations, which may become a direct branch of the pericalous artery. Due to this variability in arterial arrangements, Perlmutter & Rhoton (1978) report that in microsurgery in humans, it is possible to define the calosomarginal artery only as the artery that runs through the cingulate groove and gives rise to two or more cortical branches.

The pericalous artery in *A. belzebul* is also a branch of the interhemispheric artery that bifurcates parallel to the callosarginal artery in the caudal part of the knee of the corpus callosum and follows the groove of the corpus callosum. This vessel branches into the post-central and pre-cuneal arteries to supply the post-central and pre-cuneal areas, responsible for overall sensitivity and primary visual function, respectively. In these regions, the pericalous arteries overlap in the border areas of the vascularization territory, which may reinforce the importance of the pericalous arteries and their branches in the blood supply of these cerebral cortical areas in *A. belzebul*. This same description was found in *S. libidinosus* (Pires & Ferreira 2004).

The middle cerebral artery in *A. belzebul* is the largest caliber branch of the internal carotid artery bifurcation, laterally following the lateral sulcus between the inferior frontal gyrus and the superior temporal gyrus without forming part of the contour formation of the arterial circle. Along the way, it emits deep branches that encompass the perforating branches and, consequently, deep brain areas such as the inner capsule, base nuclei and superficial branches. These deep and superficial branches vascularize most of the superinferior and inferior lateral face of each cerebral hemisphere through the arteries: lateral basilar frontal, pre-central sulcus artery, central sulcus artery, postcentral sulcus artery, and rostral parietal artery in the previous part, and in the inferior region of the temporal lobe the arteries: temporo-occipital branch, caudal temporal branch, middle temporal branch and rostral temporal branch.

In *S. libidinosus*, the middle cerebral artery did not participate in the formation of the arterial circuit of the base of the brain. It can be interpreted as a terminal branch of constant and symmetrical disposition and, after its origin, is insinuated in the lateral sulcus, going through it to subdivide into the corresponding hemispheres (Ferreira & Prada 2009). The authors describe that after detaching from the carotid trunk the middle cerebral artery emits a branch collateral to the chorioid artery.

In *P. ursinus*, *C. pygerithrus* and *G. senegalensis* (Lake et al. 1990), the middle cerebral artery follows the same path as in *A. belzebul* and emits the first branches, called medial striated arteries. These ascend through the lentiform nucleus to irrigate the caudate nucleus head and the lateral striated arteries that ascend into the outer capsule, supplying the caudate nucleus capsule and body, and the branches of these arteries supply the thalamus. The middle cerebral artery in these three species also supplies the insular lobe through its branches and travels to the surface of the cerebral hemispheres where it provides convex branches for irrigation of cortical areas. In *Homo* spp. (Meneses 2015), the middle cerebral artery also presents itself as a large vessel of the internal carotid artery bifurcation and follows the same path observed in *A. belzebul* to irrigate the superficial and deep regions of the cerebral hemispheres.

CONCLUSIONS

The brain vascularization of *Alouatta belzebul* depends on two irrigation systems: the vertebra-basilaris and the carotid, where an artery anastomosis occurs and form the closed circuit that supplies the brain. The vertebral arteries of *A. belzebul* give rise to the basilar artery, which forms an arterial island at its origin by demonstrating a double basilar artery and then follows its single path through the basilar sulcus to emit its terminal branch, the caudal cerebral artery.

The caudal cerebral artery is subdivided into arterial segments: pre-communicating and post-communicating and will supply the medial and lateral areas of the brain. The right and left internal carotid arteries emit their terminal branches, being the rostral cerebral artery and the middle cerebral artery, which make an anastomosis and close the rostral part of the arterial circle. The right and left rostral cerebral arteries unite to form the interhemispheric artery that follows its path through the cerebral hemispheres, giving rise to the pericalous artery and the calosomarginal artery to supply the pre and post central areas of the brain cortex. The middle cerebral artery and rostral cerebral artery anastomose by direct inoculation indicating uniform flow. In addition, in its path emits several superficial branches to supply the frontal and temporal areas of the cerebral cortex.

Alouatta belzebul presents a blood supply model considered efficient for the exercise of the function and complex, due to the various aspects discussed in this paper. The blood supply dependent on the arterial circle presents a constancy of blood flow, which suggests experimental models for the treatment of vascular disorders. The similarity of Old and New World primate species can provide experimental data in clinical trials for aneurysm treatment, for example. The constancy of the vessels and absence of abnormalities in the *A. belzebul*'s cerebral arterial circuit also suggest a complex model and may contribute to the intervention of veterinarians during clinical and surgical procedures in other primates.

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As opiniões e conceitos emitidos nos artigos submetidos são de responsabilidade dos autores. O Conselho Editorial da PVB, com a assistência da Assessoria Científica, pode sugerir ou solicitar modificações. Os artigos submetidos são avaliados pelos pares (*peer review*) e, aceitos para publicação com dois pareceres favoráveis, ou rejeitados por dois pareceres desfavoráveis.

Os direitos autorais dos artigos aceitos para publicação permanecem com os autores.

1. **Os artigos devem ser organizados em TÍTULO, ABSTRACT, RESUMO, INTRODUÇÃO, MATERIAL E MÉTODOS, RESULTADOS, DISCUSSÃO, CONCLUSÕES (de preferência os últimos três separadamente), Agradecimentos, Declaração de conflito de interesse e REFERÊNCIAS:**

a) O **TÍTULO** deve ser conciso e indicar o conteúdo do artigo; pormenores de identificação científica devem ser colocados em MATERIAL E MÉTODOS.

b) O(s) Autor(es) com numerosos primeiros nomes e sobrenomes, deve(m) padronizar o seu "nome para publicações científicas", como por exemplo: Cláudio Severo Lombardo de Barros, escreve Cláudio S.L. Barros ou Barros C.S.L.; Franklin Riet-Correa Amaral escreve Franklin Riet-Correa ou Riet-Correa F. **Os artigos devem ter no máximo 8 (oito) autores**. O autor para correspondência deve ser um autor que garanta o contato com o Conselho Editorial da PVB. Asteriscos de chamadas para o rodapé não devem ser sobrescritos.

c) O **Cabeçalho do ABSTRACT** deve conter além dos nomes dos autores abreviados invertido, o ano, o TÍTULO, o endereço postal do laboratório (inclusive o CEP) ou instituição principal onde foi desenvolvida a pesquisa. Endereços postais brasileiros

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3. **Apresentação das Figuras:**

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

b) Enviar cada figura separadamente.

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

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

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

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

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

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

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

Exemplos de Referências

➢ Artigos publicados em periódicos:

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

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

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

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

- Livros:

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

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

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

- Capítulos de livros:

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

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

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

- Dissertações e Teses:

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

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

- Resumos publicados em eventos:

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

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

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

Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

GUIDE FOR AUTHORS

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

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

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

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

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

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

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

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

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

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

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

The author rights of the accepted papers are preserved.

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

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

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

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

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

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

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

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

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

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

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

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

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

m) Conflict of interest or none should be mentioned.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3. **Figure presentation:**

a) Save images at 300 dpi, TIF files.

b) Send each figure separately.

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

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

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

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

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

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

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

Exemples for References:

➢ Articles published in scientific journals:

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.

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