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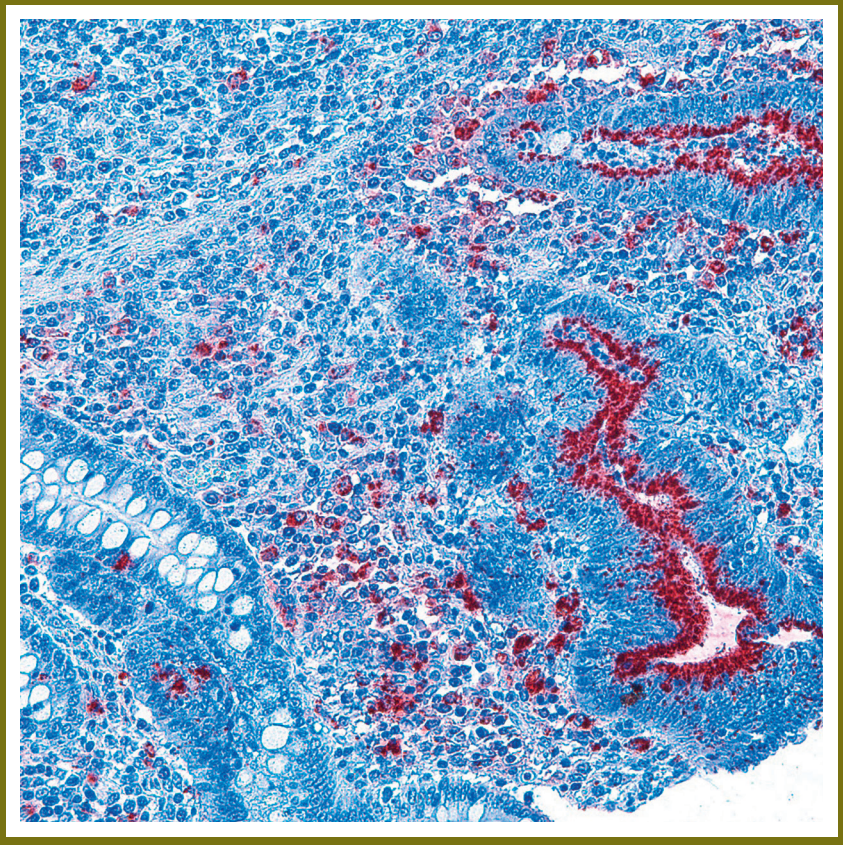

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Cover illustration: Ileum of pig with anti-*Lawsonia intracellularis* immunostaining, obj.20x. (Otoni et al., p.172)

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Original Article
Livestock Diseases

Neonatal mortality associated with sodium monofluoracetate in kids fed with colostrum from goats ingesting *Amorimia septentrionalis*[1]

José R.G. Lopes[2]*, José A.S. Araújo[3], Danielle A.N. Pessoa[2], Stephen Lee[4], Daniel Cook[4], Franklin Riet-Correa[2,5] and Rosane M.T. Medeiros[2]

ABSTRACT.- Lopes J.R.G., Araújo J.A.S., Pessoa D.A.N., Lee S., Cook. D., Riet-Correa F. & Medeiros R.M.T. 2019. **Neonatal mortality associated with sodium monofluoride in kids fed with colostrum from goats ingesting *Amorimia septentrionalis*.** *Pesquisa Veterinária Brasileira 39(3):163-167*. Hospital Veterinário, Centro de Saúde e Tecnologia Rural, Universidade Federal de Campina Grande, Avenida Universitária s/n, Bairro Santa Cecília, Patos, PB 58780-110, Brazil. E-mail: macyo_mv@hotmail.com

Sudden deaths after colostrum ingestion in kids and lambs born to mothers grazing in areas with *Amorimia septentrionalis* have been reported in the Brazilian northeastern semi-arid region, in Paraíba state. This study aimed to determine whether the sodium monofluoracetate (MF) contained in *A. septentrionalis* is eliminated in milk, causing the death of kids. After confirming gestation on the 25th day after mating, 26 goats were randomly distributed into three groups. In Group 1, eight goats received fresh leaves of *A. septentrionalis* in daily doses of 1g/kg body weight, administered at three different periods during gestation: from days 91 to 100, 116 to 125, and from day 140 until delivery day. In Group 2, consisting of 10 females, eight goats received 1g/kg body weight of *A. septentrionalis* dried and milled leaves, fed daily from the 140th day of gestation until delivery. The other two goats of this group did not ingest the plant during gestation and after delivery the colostrum supplied to their kids was replaced by colostrum of goats from that same group that had ingested the plant. Eight goats from Group 3 (control) did not ingest *A. septentrionalis*. Seven goats from Group 1 showed signs of poisoning from 2nd to 8th days of plant administration, in all periods, and recovered within 7 to 12 days. Another goat presented severe clinical signs and was submitted to euthanasia *in extremis*. Two goats aborted. Four kids, from two goats, received colostrum and, after 15 minutes, presented depression, breathing wheezing, lateral recumbence, bleating, and death. Two goats gave birth at night; the two kids were found dead and, at necropsy, it was verified that they were born alive. The last goat in this group gave birth to two kids which showed no signs of poisoning after colostrum ingestion. In Group 2, the eight goats that ingested dry leaves of the plant presented tachycardia and engorgement of the jugular veins; six aborted, and the kids of the other two goats died immediately after delivery without ingesting colostrum. The three kids of the two goats that did not ingest the plant during gestation did not show signs of poisoning after ingesting colostrum from the goats that had ingested the plant. In Group 3, all females kidded normally and the kids showed no signs of poisoning. Ten leaf

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samples of *A. septentrionalis* contained 0.00074% ±0.00018 MF. These results demonstrate that the MF of *A. septentrionalis* is eliminated in colostrum and may cause the death of kids. As in previous reports, the plant also caused abortion.

INDEX TERMS: Neonatal mortality, sodium monofluoracetate, colostrum, goats, kids, *Amorimia septentrionalis*, toxins in milk, abortion, plant poisoning, toxicoses.

RESUMO.- [**Mortalidade neonatal associada ao monofluoroacetato de sódio em cabritos alimentados com colostro de cabras ingerindo *Amorimia septentrionalis*.**] Mortes súbitas, após a ingestão do colostro, em cabritos e cordeiros nascidos de mães que pastejam em áreas com *Amorimia septentrionalis* são relatadas no semiárido da Paraíba. O objetivo deste trabalho foi determinar se o monofluoracetato de sódio (MF) contido em *Amorimia septentrionalis* é eliminado pelo leite, causando a morte dos cabritos. Após a confirmação da gestação no 25º dia após a cobertura, 26 cabras foram aleatoriamente distribuídas em três grupos. No Grupo 1, oito cabras receberam folhas frescas de *A. septentrionalis* em doses diárias de 1g/kg de peso vivo, administradas em três períodos diferentes durante a gestação: entre os dias 91 a 100, 116 a 125 e do 140º dia até o parto. No Grupo 2, composto por 10 fêmeas, oito cabras receberam 1g/kg de peso vivo de folhas secas e trituradas de *A. septentrionalis*, fornecida diariamente do 140º dia de gestação até o parto. As outras duas cabras desse grupo não ingeriram a planta durante a gestação e, ao parirem, o colostro fornecido aos seus cabritos foi substituído pelo colostro de cabras, desse mesmo grupo, que ingeriram a planta. Oito cabras do Grupo 3 (controle) não ingeriram *A. septentrionalis*. Sete cabras do Grupo 1 apresentaram sinais de intoxicação entre o 2º e 8º dia de administração da planta, em todos os períodos, e se recuperavam em 7 a 12 dias. Outra apresentou sinais clínicos graves e foi eutanasiada *in extremis*. Duas cabras abortaram. Quatro cabritos, oriundos de duas cabras, receberam colostro e, após 15 minutos, apresentaram depressão, respiração ofegante, decúbito lateral, berros e morte. Dois cabritos, nascidos de duas cabras que pariram durante a noite, foram encontrados mortos e os achados de necropsia permitem afirmar que nasceram vivos. A outra cabra desse grupo pariu dois cabritos que, mesmo mamando o colostro, não apresentaram sinais de intoxicação. No Grupo 2, as oito cabras que ingeriram a planta seca apresentaram taquicardia e ingurgitamento das veias jugulares; seis abortaram e os cabritos das outras duas morreram imediatamente após o parto, sem ingerir colostro. Os três filhotes das duas cabras que não ingeriram a planta durante a gestação não apresentaram sinais de intoxicação após ter ingerido colostro das cabras que tinham ingerido a planta. No Grupo 3, todas as fêmeas pariram normalmente e os filhotes não apresentaram sinais de intoxicação. Dez amostras de folhas de *A. septentrionalis* continham 0,00074% ±0,00018 de MF. Estes resultados demonstram que o MF de *A. septentrionalis*, além de causar abortos, é eliminado pelo colostro podendo causar a morte dos cabritos.

TERMOS DE INDEXAÇÃO: Mortalidade neonatal, monofluoroacetato de sódio, cabritos, colostro, *Amorimia septentrionalis,* toxinas no leite, aborto, caprinos, intoxicação por planta, toxicoses.

INTRODUCTION

Sodium monofluoracetate (MF) has been identified as a toxic agent in several plants, causing sudden death associated with exercise (Tokarnia et al. 2012). In Brazil, 12 toxic plant species belonging to families Rubiaceae, Bignoniaceae, and Malpighiaceae are known to cause this syndrome (Carvalho et al. 2009). Dueto their acute toxicity, good palatability, and wide geographical distribution, these plants are among the most important toxic plants for ruminants in the Country (Tokarnia et al. 2012).

In the Brazilian northeastern semi-arid region, mainly in the states of Ceará, Paraíba, and Pernambuco (Albuquerque et al. 2014), poisoning with *Amorimia septentrionalis* is the most known, widespread and important cause of sudden death in ruminants (Duarte et al. 2013). There are reports of poisoning outbreaks by this plant in sheep and goats in the state of Paraíba, with poisoning occurring mainly at the beginning of the rainy season, when these plants sprout before other forage plants, or after that period, when some forage plants dry (Vasconcelos et al. 2008).

The following clinical signs have been observed in experimentally poisoned goats: dyspnea, tachycardia, and sternal decubitus evolving to lateral decubitus with peddling movements followed by death. Some less affected animals recover. No significant lesion has been described in the necropsy of poisoned animals (Paraguassu 1983), although, histologically, hydropic-vacuolar degeneration of the renal tubular epithelial cells have been observed in some poisoned ruminants (Tokarnia et al. 2012); and in smaller doses ingested for prolonged periods, plants containing MF may cause cardiac fibrosis (Soares et al. 2011).

In addition to causing sudden death syndrome, ingestion of *A. septentrionalis* results in embryonic mortality and abortions in goats (Silva et al. 2017). Goat and sheep raisers in the semi-arid region of Paraíba state have reported that kids and lambs, born to animals that grazed on areas with plants of the *Amorimia* genus during gestation, have died suddenly after colostrum ingestion, suggesting that MF can be excreted in milk, causing the death of kids. This route of elimination of the active principle constitutes a risk to public health, because of the possibility of human consumption of milk from animals that ingest *A. septentrionalis*.

Aiming to experimentally investigate the elimination of MF in milk, the objective of this study was to verify the occurrence of poisoning of kids fed with colostrum from goats that ingested *Amorimia septentrionalis*.

MATERIALS AND METHODS

The experiment was conducted at the Hospital Veterinário of Universidade Federal de Campina Grande (UFCG). Twenty-six goats and two male goats, crossbred, at breeding age, were used in this study. Initially, the females were submitted to ultrasound examination for negative diagnosis of pregnancy and, subsequently, hosted in stalls

with the bucks for mating. Each male was marked daily with red paint on the chest. When red spots were observed in the lumbar region of the females, indicating possible mating, ultrasound examination was performed 25 days after the observation to confirm gestation by visualization of fetal heartbeat. After a positive diagnosis of gestation, pregnant goats were randomly assigned to three groups (Groups 1, 2, and 3).

Animals were fed with *Amorimia septentrionalis* collected weekly in the municipality of Teixeira (07°12'24"S; 37°15'11"W), Paraíba state. One specimen of the plant was dried and deposited in the Herbarium of the Universidade Federal de Campina Grande, campus of Patos, Paraíba state, Brazil (checking copy no. 6702). Ten samples of leaves of *A. septentrionalis* were sent to the Poisonous Plant Laboratory, Agricultural Research Service, United States Department of Agriculture, Logan, USA, for detection and quantification of MF in its composition using the Gas Chromatograph-Mass Spectrometry (GC-MS) method described by Santos-Barbosa et al. (2017).

Group 1, composed of eight goats, received fresh leaves of *A. septentrionalis* in daily doses of 1g/kg body weight administered at three different periods during gestation: between days 91 to 100, 116 to 125, and from day 140 till delivery. The 15-day interval between each administration period was observed in order to detoxify MF, reducing the risk of death of females, and to induce their increased resistance to poisoning, as described by Duarte et al. (2014). The plant leaves were stored under refrigeration for up to one week, and supplied daily to the goats by placing small volumes directly in the animals' mouths.

In Group 2, composed of 10 goats, eight which also received 1g/kg body weight of *A. septentrionalis*, but the leaves had been previously left to dry in the shade for 10 days, and then milled. Dry matter percentage was 34.5%. The dry plant was supplied to the goats from the 140th day of gestation till delivery. The other two female goats of this group did not ingest the plant during gestation and after delivery the colostrum supplied to their kids was replaced by that of goats of the same group that had ingested the dry plant in the end of gestation. The dried and milled plant leaves were stored in plastic buckets at room temperature and supplied to the animals mixed with the concentrated feed to facilitate ingestion.

Group 3, composed of eight goats, served as control, and animals in this group did not ingest *A. septentrionalis*.

During the experiment, each animal received daily food equivalent to 3% body weight, with 1% of concentrated feed and 2% of bulky feed (Tifton hay, *Cynodon dactylon*), and water *ad libitum*.

All pregnant females were monitored for viability of gestation through ultrasound examinations performed every 15 days. Prior to supply of *A. septentrionalis*, all animals were weighed and had their heart rate and respiratory and ruminal movements measured. Goats were monitored daily for observation of delivery or abortion. After delivery, all goats, except for the two goats in Group 2 that did not ingest the plant, had their colostrum collected and supplied to the respective kids, which were observed for a period of six hours after ingestion.

Colostrum that was not offered to the kids (in the case of abortion) or any surplus of it was frozen and subsequently administered to the kids from goats that did not ingest the plant during pregnancy. In the event of death or abortion, the animals were necropsied, samples of the organs were collected from the thoracic and abdominal cavities and the central nervous system, fixed in 10% formalin, embedded in paraffin, sectioned at 4-6µm, and stained with hematoxylin-eosin for histopathological examination.

RESULTS

All goats in Group 1 showed signs of poisoning, namely, depression, tachycardia, engorgement of the jugular veins, and reluctance to walk, remaining most of the time in sternal decubitus. These clinical signs began between the 2nd and 8th days of each of the three periods of plant administration. Recovery of the animals occurred within seven to 12 days after the end of the plant administration period, except in one of the goats that showed aggravated poisoning and, in addition to the clinical signs already described, showed peddling movements in lateral decubitus, nystagmus, and opisthotonus; euthanasia *in extremis* was performed on the 2nd day of the third plant administration period. At necropsy, congestion of the encephalon vessels and distended bladder were observed. No significant histopathological lesion was found. Two goats had bloody vaginal discharge after eight and 28 days of plant ingestion respectively, and abortion of one fetus each. Four kids, from two goats, received colostrum and, after approximately 15 minutes, presented with depression, wheezing, lateral decubitus, and bleating, and evolved to death. Pulmonary edema and hydropericardium were found at necropsy. Two kids born during the night were found dead and, at necropsy, there were signs that they had been born alive: presence of colostrum in the abomasum and pulmonary aeration. No significant lesions were observed in the histopathological studies. One of the goats in Group 1 delivered two kids that, even after being fed with colostrum, showed no signs of poisoning (Table 1).

In Group 2, the eight goats that ingested dry plant leaves as of the 140th day of gestation presented with tachycardia and engorgement of the jugular veins, delivering 14 kids between two and five days after the beginning of plant consumption. Five deliveries were monitored, resulting in nine stillborn kids. Three goats delivered during the night, and the five resulting kids were found dead in the morning; necropsy showed that three of kids had air in the lungs, indicating that they were born alive. The other two animals had no pulmonary aeration. No colostrum was found in the abomasum of any of the five kids found dead in the morning, and no significant changes were observed during necropsy or histopathology. The three kids of the two goats that did not ingest the plant during gestation received colostrum, immediately after delivery, from the other three goats that had ingested the plant leaves in the end of gestation. The offspring were observed for six hours and showed no signs of poisoning (Table 1).

In Group 3 (control), all female goats kidded normal. The kids ingested colostrum and showed no sign of poisoning (Table 1).

The average amount of MF contained in the *A. septentrionalis* leaf samples was 0.00074% ±0.00018.

DISCUSSION

Hyperacute death of kids, with clinical signs characteristic of sodium monofluoracetate (MF) poisoning, after consumption of colostrum from goats that ingested *Amorimia septentrionalis* confirm the hypothesis of elimination of MF in milk as a cause of poisoning. In contrast, in Group 2, administration of plant leaves in larger doses caused neonatal death, and it was not possible to demonstrate elimination of MF in milk.

Table 1. Effect of ingestion of colostrum from goats that consumed *Amorimia septentrionalis* or not during gestation on their kids

Group	Goat	Plant ingestion (days)	Abortion	Stillborn	Death soon after birth	Death after feeding with colostrum	Alive even after feeding with colostrum
Group 1 (1g/kg of fresh leaves)	1	22[a]					
	2	8	1				
	3	28	1				
	4	30				2	
	5	29				2	
	6	28				1[b]	
	7	28				1[b]	
	8	32					2
Group 2 (1g/kg of dry leaves)	1	4		2			
	2	2		2			
	3	5		2			
	4	4		1			
	5	5		2			
	6	4		2			
	7	5			2[c]		
	8	5			1[c]		
	9	0					2
	10	0					1
Group 3 (control)	1	0					2
	2	0					1
	3	0					2
	4	0					2
	5	0					2
	6	0					2
	7	0					1
	8	0					1

[a] Kid showed clinical signs of poisoning and was submitted to euthanasia *in extremis*, [b] kids that were born during the night and were found dead the next morning (colostrum observed in the abomasum and airways during necropsy), [c] kids found dead and, at necropsy, air was observed in the lungs, indicating that they were born alive.

In a similar study, Vasconcelos et al. (2008), administered *A. septentrionalis* at a dose of 2g of fresh plant leaves per kg of body weight to two goats and five sheep during the last 15 days of gestation. One kid from one of the goats died five minutes after being fed with colostrum and the other goat delivered a kid that died soon after birth without ingesting colostrum. As in the present study, the authors also observed abortion, which occurred with sheep that consumed the plant for 10 days. The clinical signs observed in the kids of Group 1 that died immediately after ingesting colostrum are similar to those described by Vasconcelos et al. (2008) in natural poisoning with *A. septentrionalis* in goats occurred in the municipality of Cabaceiras, Paraíba state, which included reluctance to walk, falls, lateral decubitus, peddling movements, tachypnea, bleating, and death. Absence of significant histopathological findings in intoxicated kids was also observed by Paraguassu (1983) in a series of experiments conducted with goats. The clinical signs and absence of histopathological findings observed in the kids that died immediately after ingesting colostrum are consistent with poisoning by *A. septentrionalis*, with milk from the goats as the only route of poisoning, considering that it was the only food source of the kids.

It has already been demonstrated that other plants in the semi-arid region also have phytotoxins eliminated in milk, causing poisoning of offspring that ingest it. Using mice as an experimental model, Lopes et al. (2014) demonstrated that the tremorgenic toxin present in *Ipomoea asarifolia*, or its metabolite, is eliminated in the milk of females that consume ration containing this plant, causing tremors and even the death of the offspring. Posteriorly, Carvalho de Lucena et al. (2014) also confirmed the occurrence of poisoning in lambs that ingested milk from ewes that fed exclusively on *I. asarifolia*.

Crotalaria spectabilis also affects the offspring in mice, either when the plant seeds are supplied together with the ration to lactating female mice or when monocrotaline (the plant toxic principle) is added to the feed, causing toxic effects such as weakness, ascites, and anasarca, both in the female mice and pups (Medeiros 1994).

Some active plant principles have also been found in dairy by-products. Alvarenga (2015) fed mice with ration containing cheese produced from the milk of cows that received *Pteridium aquilinum*, which contains ptaquiloside, a known carcinogen. At necropsy, the cows did not show any changes associated with poisoning, but the mice showed development of pre-neoplastic lesions in the stomach, intestines, and bladder.

Regarding the occurrence of abortion due to ingestion of *A. septentrionalis*, Silva et al. (2017) supplied 5g/kg body weight of fresh *A. septentrionalis* leaves, at the beginning of the fetal phase (36 days of gestation) and at the middle third (93 days of gestation) of the same phase, to four pregnant

goats in each of these periods. Of the animals that ingested the plant at the beginning of the fetal phase, 75% aborted; whereas of the animals that received the plant in the middle third of the fetal phase, 50% aborted. The researchers suggested that the toxic effects on fetus decrease as gestation progresses. However, in the present experiment, the plant was ingested for a shorter time, in smaller amounts, and in the end of gestation, causing abortion in 75% of the goats in Group 2, which suggests that the effect of plant on reproduction is associated with the concentration of MF present in it, and not with gestational age. Sodium monofluoracetate was not quantified in plant samples used in the experiment conducted by Silva et al. (2017). In the present study, the amount of MF in the *A. septentrionalis* samples was 0.00074% ±0.00018 and, although at a lower concentration than that observed by Lee et al. (2012) and Albuquerque et al. (2014), which was 0.002% of MF in the plant leaves, was able to cause poisoning, most likely by the repeated ingestion of small doses.

CONCLUSION

Amorimia septentrionalis causes abortion and its toxic active principle, sodium monofluoracetate (MF), is eliminated in milk and can result in neonatal deaths.

Ethics Committee.- This study was conducted in agreement with ethical principles on animal experimentation and was approved by the Ethics Committee on Animal Use of the UFCG under protocol no. 69-2013.

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

REFERENCES

Albuquerque S.S.C., Rocha B.P., Almeida V.M., Oliveira J.S., Riet-Correa F., Lee S.T., Evêncio Neto J. & Mendonça F.S. 2014. Fibrose cardíaca associada à intoxicação por *Amorimia septentrionalis* em bovinos. Pesq. Vet. Bras. 34(5):433-437. <http://dx.doi.org/10.1590/S0100-736X2014000500008>

Alvarenga T.M.P. 2015. Avaliação clinicopatológica de ratos alimentados com queijo de leite de vacas que consumiam *Pteridium aquilinum*. Masters Thesis, Universidade Federal de Lavras, Lavras, MG. 88p.

Carvalho G.D., Nunes L.C., Bragança H.B.N. & Porfírio L.C. 2009. Principais plantas tóxicas causadoras de morte súbitas em bovinos no estado do Espírito Santo, Brasil. Arch. Zootec. 58:87-98.

Carvalho de Lucena K.F., Rodrigues J.M., Campos É.M., Dantas A.F., Pfister J.A., Cook D., Medeiros R.M. & Riet-Correa F. 2014. Poisoning by *Ipomoea asarifilia* in lambs by the ingestion of milk from ewes that ingest the plant. Toxicon 92:129-132. <http://dx.doi.org/10.1016/j.toxicon.2014.10.019> <PMid:25448387>

Duarte A.L., Medeiros R.M.T. & Riet-Correa F. 2013. Intoxicação por *Amorimia* spp. em ruminantes. Ciência Rural 43(7):1294-1301. <http://dx.doi.org/10.1590/S0103-84782013005000081>

Duarte A.L., Medeiros R.M., Carvalho F.K., Lee S.T., Cook D., Pfister J.A., Costa V.M. & Riet-Correa F. 2014. Induction and transfer of resistance to poisoning by *Amorimia (Mascagnia) septentrionalis* in goats. J. Appl. Toxicol. 34(2):220-223. <http://dx.doi.org/10.1002/jat.2860> <PMid:23400835>

Lee S.T., Cook D., Riet-Correa F., Pfister J.A., Anderson W.R., Lima F.G. & Gardner D.R. 2012. Detection of monofluoroacetate in *Palicourea* and *Amorimia* species. Toxicon 60(5):791-796. <http://dx.doi.org/10.1016/j.toxicon.2012.05.029> <PMid:22699106>

Lopes J.R.G., Riet-Correa F., Cook D., Pfister J.A. & Medeiros R.M.T. 2014. Elimination of the tremorgenic toxin of *Ipomoea asarifolia* by milk. Pesq. Vet. Bras. 345(11):1085-1088. <http://dx.doi.org/10.1590/S0100-736X2014001100009>

Medeiros R.M.T.M. 1994. Veiculação do princípio tóxico de *Crotalaria spectabilis* (Roth) em leite de cabra e rata: uma avaliação toxicológica. Masters Thesis, Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, São Paulo. 143p.

Paraguassu A.A. 1983. Intoxicação experimental por *Mascagnia rigida* Grisebach (Malpighiaceae) em caprinos no Nordeste do Brasil. Masters Thesis, Universidade Federal Rural do Rio de Janeiro, Itaguaí. 65p.

Santos-Barbosa J.M., Lee S.T., Cook D., Gardner D.R., Viana L.H. & Ré N. 2017. A gas chromatography-mass spectrometry method for the detection and quantitation of monofluoroacetate in plants toxic to livestock. J. Agricult. Food Chem. 65(7):1428-1433. <http://dx.doi.org/10.1021/acs.jafc.7b00294> <PMid:28132508>

Silva L.C.A., Pessoa D.A.N., Lopes R.G., Santos J.R.S., Olinda R.G. & Riet-Correa F. 2017. Embryonic death and abortions in goats caused by the ingestion of *Amorimia septentrionalis.* Pesq. Vet. Bras. 37(12):1401-1404. <http://dx.doi.org/10.1590/s0100-736x2017001200007>

Soares M.P., Pavarini S.P., Adrien Mde.L., Quevedo Pde.S., Schild A.L., Peixoto P.V., da Cruz C.E. & Driemeier D. 2011. *Amorimia exotropica* poisoning as a presumptive cause of myocardial fibrosis in cattle. J. Vet. Diagn. Invest. 23(6):1226-1229. <http://dx.doi.org/10.1177/1040638711425586> <PMid:22362807>

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.27-71.

Vasconcelos J.S., Riet-Correa F., Dantas A.F.M., Medeiros R.M.T., Galiza G.J.N., Oliveira D.M. & Pessoa A.F.A. 2008. Intoxicação por *Mascagnia rigida* (Malpighiaceae) em ovinos e caprinos. Pesq. Vet. Bras. 28(10):521-526. <http://dx.doi.org/10.1590/S0100-736X2008001000013>

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Original Article
Livestock Diseases

Tylosin injectable for the treatment of porcine proliferative enteropathy in experimentally inoculated pigs[1]

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ABSTRACT.- Otoni L.V.A., Gabardo M.P., Macêdo N.R., Wagatsuma M.M., Pereira M.M. & Guedes R.M.C. 2019. **Tylosin injectable for the treatment of porcine proliferative enteropathy in experimentally inoculated piglets.** *Pesquisa Veterinária Brasileira 39(3)168-174.* Departamento de Clínica e Cirurgia Veterinárias, Escola de Veterinária, Universidade Federal de Minas Gerais, Avenida Antônio Carlos 6627, Belo Horizonte, MG 31270-901, Brazil. E-mail: guedes@vet.ufmg.br

Porcine proliferative enteropathy (PPE) is one of the most common enteric diseases in growing and finishing pigs. PPE is characterized by reduced growth performance, accompanied or not by diarrhea. PPE is highly prevalent in several countries of the Americas, Europe and Asia, causing high economic losses in swine herds. The most common form of PPE control in pigs is antibiotic therapy. The objective of this study was to evaluate a new product based on tylosin injectable (Eurofarma Laboratórios S.A.) to control PPE in experimentally inoculated animals. Sixty 5-week-old pigs with mean weight of 9.5kg were divided into two experimental groups of 30 animals: medication and control. All pigs were challenged with *Lawsonia intracellularis*, the etiologic agent of PPE, on day zero. Fecal score, body condition score, and behavior were daily evaluated. Pigs were weighted on days -2, 13 and 21 of the experiment. Pigs in the Medication Group received tylosin injectable 13 days after inoculation, in three doses with a 12-hour interval between them. Pigs in the Control Group received injectable saline solution following the same protocol. In the Control Group, 23pigs presented with diarrhea before day 13. After day 13, the number of diarrheic animals in this group was reduced to 17. In the Medication Group, 26 pigs presented with diarrhea in the initial period, and in the period after medication, only 11 animals had diarrhea. The score of gross intestinal PPE lesions in the Medication Group was lower than that in the Control Group (p=0.031). The Medication Group also showed lower score for *Lawsonia intracellularis* antigen-labeling by immunohistochemistry compared with that of the Control Group (p=0.032), showing lower level of infection. These results demonstrate that tylosin injectable (Eurofarma Laboratórios S.A.), administrated in three doses (1mL/20kg) every 12 hours, was effective for the control of PPE in experimentally inoculated pigs.

INDEX TERMS: Tylosin injectable, treatment, porcine proliferative enteropathy, pigs, macrolides, ileitis, antimicrobial, *Lawsonia intracellularis*, metaphylactic, diarrhea, pigs, practice, clinics.

RESUMO.- [Tilosina injetável no tratamento da enteropatia proliferativa suína em leitões experimentalmente inoculados.] Enteropatia proliferativa suína (EPS), causada pela bactéria *Lawsonia intracellularis,* é uma das doenças entéricas mais comuns em suínos de recria e terminação. A EPS caracteriza-se por redução no desempenho dos animais, acompanhada ou não por diarreia. É uma doença altamente prevalente em diversos países da América, Europa e Ásia, provocando elevados prejuízos econômicos nos rebanhos suínos. A forma de controle da EPS mais adotada em rebanhos suínos é a antibioticoterapia. O objetivo deste estudo foi avaliar um novo produto à base de tilosina (Eurofarma Laboratórios S.A.) na forma injetável para controlar a EPS em animais

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experimentalmente inoculados. Foram utilizados 60 leitões, de cinco semanas de idade, com peso médio de 9,5kg, divididos em dois grupos experimentais (n=30), medicados e não medicados. Todos os leitões foram desafiados com *Lawsonia intracellularis* no dia zero. Avaliações clínicas de escore fecal, escore corporal e comportamento foram realizadas diariamente além da pesagem individual dos animais realizada nos dias -2, 13 e 21 do experimento. Os leitões do grupo medicado receberam tilosina injetável 13 dias após a inoculação em três doses com intervalo de 12 horas cada. Já os leitões do grupo não medicado receberam solução salina injetável com o mesmo protocolo. O grupo não medicado apresentou 23 animais com diarreia antes do dia 13 e 17 após este período. No grupo medicado, 26 animais apresentaram diarreia previamente à medicação e apenas 11 após a medicação a partir do dia 13. Os leitões medicados apresentaram extensão de lesão macroscópica, caracterizada por espessamento de mucosa intestinal, menor em comparação com o grupo não medicado (p=0,031). A imunomarcação para *Lawsonia intracellularis* foi menor no grupo medicado (p<0,032), mostrando redução no grau de infecção por *L. intracellularis* nos animais medicados. Estes resultados demonstram que a tilosina injetável (Eurofarma Laboratórios S.A.) (1mL/20kg) em três doses, a cada 12 horas, foi eficaz no tratamento da enteropatia proliferativa suína em animais experimentalmente inoculados.

TERMOS DE INDEXAÇÃO: Tilosina injetável, tratamento, enteropatia proliferativa, leitões, macrolídeos, antimicrobianos, *Lawsonia intracellularis*, metafilático, diarreia, suínos, clínica.

INTRODUCTION

Porcine proliferative enteropathy (PPE) is an infectious disease caused by the obligate intracellular bacterium *Lawsonia intracellularis*. PPE is characterized by thickening of the intestinal mucosa, and it affects mainly growing -finishing pigs. Its main clinical manifestations include three forms: acute or hemorrhagic, chronic, and subclinical (Guedes 2012). In hemorrhagic PPE, animals present with bloody diarrhea, apathy, and death (McOrist & Gebhart 2012). The chronic form affects growing pigs and is characterized by failure to gain weight and transient diarrhea, whereas in the subclinical form animals also show reduction in weight gain, but with no evident diarrhea (Guedes 2012).

PPE is of great importance in swine production, and it causes significant economic losses resulting from diarrhea, increased mortality, decreased growth performance of animals, as well as from expenses with medicine, reaching an annual cost of US$ 20 million in the USA (McOrist 2005). PPE can be controlled through administration of antimicrobial drugs, mainly macrolides, tetracyclines, lincosamides, and pleuromutilins (França & Guedes 2008). Among them, tylosin, chlortetracycline, and tiamulin are the most frequently used (Burch 2000). Tylosin is a macrolide antibiotic that inhibits bacterial protein synthesis (Kim et al. 2008), acting as a bacteriostatic agent, and may also act as a bactericide when used in high concentrations (Barcellos et al. 2012).

McOrist et al. (1997) demonstrated that in-feed tylosin phosphate is effective in the prevention and treatment of PPE. In another study addressing experimental inoculation of *L. intracellularis* conducted with 114 swine, the authors showed the efficacy of tylosin injected twice daily for three consecutive days in improving clinical signs, reducing elimination of bacteria in feces, enhancing growth performance, and reducing macro- and microscopic lesions (Marsteller et al. 2001).

Indiscriminate use of in-feed antimicrobial drugs at low doses is associated with bacterial resistance (Maron et al. 2013). Some studies conducted in the USA have shown that, in order to preserve the efficacy of antibiotics for human and animal treatment, it is necessary to limit the use of antimicrobial drugs (Levy & Marshall 2004, Silbergeld et al. 2008). As a result, many countries have restricted the use of antibiotics as growth promoters (Maron et al. 2013). The European Union banned the use of antimicrobial growth promoters in pig feed in 2006 (Gaggia et al. 2010); a new regulation on the use of antibiotics in animal feed was enacted in the USA in 2017 (Beek 2017). Consequently, the use of metaphylactic or water-soluble and/or injectable therapeutic medication has become increasingly common to the detriment of additive or preventive use (Callens et al. 2012). In this context, this study aimed to assess the effect of tylosin injectable in the treatment of PPE in pigs experimentally inoculated with *L. intracellularis*.

MATERIALS AND METHODS

Animals and facilities. This study was approved by the Research Ethics Committee of the Universidade Federal de Minas Gerais (UFMG) under protocol no. 250/2015. Sixty male pigs aged five weeks, weighing 9.5kg on average, were used. The animals were purchased from a swine farm free of toxigenic *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, *Brachyspira hyodysenteriae*, *Brachyspira pilosicoli*, *Salmonella enterica* sorovar Choleraesuis, *Pasteurella multocida*, and suid herpesvirus type I.

The pigs were identified with ear tags and housed in an experimental barn of the College of Veterinary Medicine of the UFMG in 10 nursery pens (1.4x14m, 0.33m^2/animal density) with slated plastic floors, artificial heating system, a nipple drinker, and a two-hole deposit feeder equipped with a stainless steel pan in its lower part to collect feed waste. The animals received feed and water *ad libitum* throughout the experiment.

Study design. Two days before inoculation (day-2), all animals were weighed and had their feces collected to be used as samples for Polymerase Chain Reaction (PCR) testing for *Lawsonia intracellularis* (Jones et al. 1993) in order to confirm their negativity for this bacterium prior to inoculation.

The 60 pigs were divided into two experimental groups of 30 animals each, distributed in five pens with six pigs each, with all animals allocated in each pen of the same treatment. The groups were balanced by weight as follows: light (7.99 and 7.89kg of mean weight, in pens of the Control and Medication Groups), moderately light (8.83 and 8.91kg), medium (9.58 and 9.54kg), moderately heavy (10.16 and 10.22kg), and heavy (10.88 and 10.91kg). Pigs in the Control Group were inoculated but not medicated, whereas those in the Medication Group were inoculated and medicated.

Inoculum production. Fragments of the small intestine of naturally infected pigs with typical lesions of proliferative enteropathy were submitted to bacteriological evaluation to discard the presence of other pathogens. Presence of moderate-to-severe infection was confirmed in histological sections by hematoxylin and eosin (HE) staining and by immunohistochemistry (IHC) using specific antibodies against *L. intracellularis* (Guedes & Gebhart 2003a). The selected intestinal samples were frozen at -80°C until inoculation.

On inoculation day, the scraped mucosa from the intestines was defrosted and blended with sucrose-potassium-glutamate (SPG) solution (1:1 w/v), as described in Guedes et al. (2009) and Guedes et al. (2009). The final product was also examined bacteriologically to ensure absence of enterotoxigenic *Salmonella* sp. and *Escherichia coli* species through detection of pathogens by multiplex PCR panels (Macedo et al. 2007).

Inoculation. On day 0, all pigs were individually inoculated intragastrically with 43mL of a homogenate of intestinal mucosa inoculum of swine known to be infected by *L. intracellularis*, as described in Guedes (2002). Each animal received $1.6x10^7$ *L. intracellularis* organisms. This quantification was performed through serial dilution and immunoperoxidase staining using leporine polyclonal antibodies, as described in Guedes & Gebhart (2003a).

Clinical assessment and growth performance. Individual clinical evaluations of all pigs were performed daily, from day-2 to the end of the experiment. The following parameters were observed: behavior, body score, and grade of diarrhea (grade 0 = without diarrhea, grade 1 =pasty feces, grade 2 = liquid feces, grade 3 = bloody diarrhea). Also, feed waste was collected and actual dietary intake per pen was evaluated daily. These data were divided into two periods: pre- and post-treatment. All animals were weighed individually on days -2, 13, and 21.

Therapy. On day 13 after inoculation, when at least 25% of the pigs showed diarrhea caused by *L. intracellularis*, the Medication Group was treated with tylosin (Tilosina 20%, Eurofarma Laboratórios S.A.), 1mL/20kg p.v., injected intramuscularly in the region of the neck, in three doses every 12 hours. All animals were previously weighed on day 13 to calculate the individual dose of the drug. Pigs in the Control Group received volume of sterile saline solution (0.9% NaCl) proportional to their body weight following the same protocol of the medication.

Euthanasia and *post-mortem* evaluation. All animals were weighed and euthanized by electrocution followed by bleeding on day 21 after inoculation, when a higher index of gross PPE lesions is expected (Guedes et al. 2017). In the *post-mortem* assessment, the macroscopic lesions compatible with PPE were graded and measured individually according to the following score: grade 0 = normal mucosa; grade 1 = hyperemia and thickened mucosa; grade 2 = thickened and necrotic mucosa; grade 3 = thickened mucosa with blood clots in the intestinal lumen (Guedes 2002). For histopathology and immunohistochemistry, samples of the ileum, cecum, proximal colon, and mesenteric lymph node were fixed in 10% formalin (Guedes & Gebhart 2003b).

Immunohistochemistry (IHC). The formalin-fixed intestine samples were routinely processed for histology, embedded in paraffin, and sectioned 3µ thick. The sections of ileum were stained immunohistochemically by the labeled Streptavidin method (Dako - Vila Real Carpinteria, EUA, K675) with leporine polyclonal antibodies to *L. intracellularis* (Guedes & Gebhart 2003a) and Harris hematoxylin. Immunostaining was quantified as follows: grade 0 = no positive antigen for *L. intracellularis* labeled, grade 1 = positive antigen for *L. intracellularis* labeled in up to 25% of intestinal crypts, grade 2 = positive antigen labeled in up to 50% of the crypts, grade 3 = positive antigen labeled in up to 75% of the crypts; grade 4 = positive antigen labeled in 100% of the mucosa (Guedes et al. 2009).

Statistical analysis. In the present study, statistical analysis of the data was processed using the SPSS Statistics 25 software with confidence interval of 95% ($p<0.05$). The Chi-squared test was applied to compare the frequency of animals with diarrhea between the Control and Medication Groups in the post-treatment period and the frequency of animals with intestinal lesions according to macroscopic features, histology, and grade of infection based on IHC. The Student's *t*-test was used to compare the mean weight of the groups on days -2, 13, and 21 of the experiment, as well as the daily weight gain between the groups on days -2 to 13 and 14 to 21. Poisson regression was used to compare the number of days with diarrhea between the groups in the post-treatment period. The Mann-Whitney test was applied to compare the mean daily dietary intake between the groups in the pre- (days 0-13) and post-treatment (days 14-20) periods. Binomial regression was employed to compare data on the length of gross intestinal lesion between the groups.

RESULTS

Clinical findings

All fecal samples collected before the pigs were inoculated (day 2) tested negative for the presence of *Lawsonia intracellularis* by the Polymerase Chain Reaction (PCR) technique. Results of the bacteriological examinations of the inoculum were negative for enterotoxigenic *Salmonella* sp. and *Escherichia coli*. However, a total of 27 pigs (45%), 13 from the Control Group and 14 from the Medication Group, presented with liquid and yellowish diarrhea in the first four days after inoculation. As the period after inoculation was too short for occurrence of diarrhea as a result of infection by *L. intracellularis*, infection by enterotoxigenic *E. coli* was suspected. Fecal samples were collected for bacteriological examination and beta-hemolytic *E. coli* was isolated and tested positive for the *Sta* and *Stb* genes. Based on these results, zinc oxide (3.000ppm) was added to the feed of all animals for three days.

As of day 6, the number of pigs showing diarrhea associated with *E. coli* began to decline, and on day-9 the animals began to present pasty diarrhea compatible with that caused by *L. intracellularis*. On day12 of the experiment, 19 of the 60 pigs showed diarrhea: 10 (33.3%) in the Control Group and nine (30%) in the Medication Group, reaching the expected minimum of 25% of animals with diarrhea to begin treatment (Fig.1).

After treatment with tylosin injectable, which occurred on days 13 and 14, clinical evaluations continued to be performed in the same manner, and a gradual reduction of diarrhea was observed in both groups, more numerically accentuated in the Medication Group. In the Control Group, 23 and 17 pigs showed diarrhea before and after day 13, respectively; whereas,

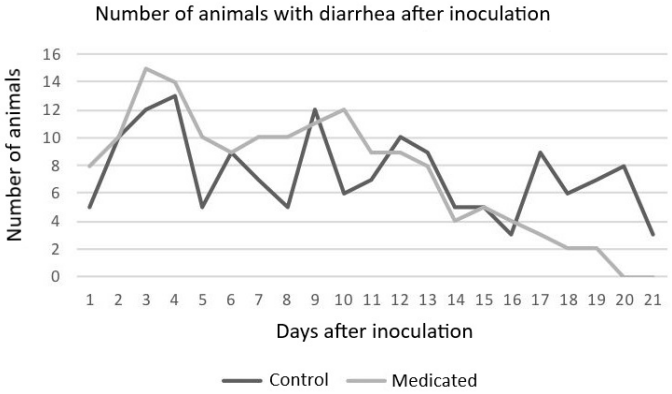

Fig.1. Number of pigs with diarrhea in each experimental group (Control and Medication) on the days after inoculation (days 1 to 21).

in the Medication Group, 26 and 11 animals showed diarrhea in the pre- and post-treatment periods, respectively.

Poisson regression analysis showed statistical difference (p=0.001) between the groups relative to the sum of the number of animals with and without diarrhea in the post-treatment period (Table 1). Pigs in the Medication Group presented, on average, one day (0.85 days) less without diarrhea than those in the Control Group (Table 1).

On day 18 of the experiment, one of the pigs in the Medication Group was found dead. The animal had not been presented with any clinical signs before death. Necropsy identified that the death was caused by septicemia due to mitral valve endocarditis and the animal did not have gross lesions of proliferative enteropathy. The weight gain data of this animal were considered until day 18, and the final calculation was adjusted until the end of the experiment. At the end of the experiment, five pigs in the Control Group were thin, showing lack of uniformity of the group.

Growth performance

Although the pigs in the Medication Group were, on average, 730g heavier than those in Control Group 21 days after inoculation, no significant difference was found between the groups regarding the variables mean weight and mean daily weight gain (Table 2). Mean daily dietary intake was 39g higher in the Medication Group compared with that in the Control Group, but with no significant difference (Table 2).

Table 1. Total number of days with and without diarrhea between the animals in the Control and Medication Groups in the post-treatment period (days- 14 to 21 after inoculation)

Groups	Days with diarrhea	Days without diarrhea	Total
Control	49	191	240
Medication	20	220	240
p	0.001		

Gross lesions

At necropsy, typical PPE grade 1 lesions were observed in the ileum of 16 animals, with the Control Group showing a larger number (10 pigs) compared with that (6 pigs) of the Medication Group (p>0.05). The lesions comprised discrete thickening of the intestinal mucosa in the ileum with mild hyperemia, and their length for each animal ranged from 6 to 65cm. The total lesion length observed in pigs in the Control Group (366cm of intestinal lesion) was statistically larger compared with that in pigs in the Medication Group (97cm of intestinal lesion) (p=0.031). The mean lesion lengths per affected pig were 16.16 and 36.6cm in the Medication and Control Groups, respectively (p=0.093). The mean lesion lengths by the total number of animals were 3.2 and 12.2cm in the Medication and Control Groups, respectively (p=0.151) (Table 3).

IHC

Statistically significant difference (p<0.032) in immunostaining was observed between the groups, present in 16 pigs in the Control Group (53.3%) and in eight pigs in the Medication Group (26.6%). All labels were observed in the ileum, and were classified as grade 1 (positive antigen labeled in up to 25% of the intestinal crypts) (Figs.2 and 3).

DISCUSSION

Based on clinical signs, gross lesions, and immunostaining for *Lawsonia intracellularis* observed in the pigs in the Control Group, it can be stated that the experimental inoculation

Table 2. Comparison between mean weight of animals in the Control and Medication Groups on days -2, 13, and 21. Comparison between mean daily weight gain (MDWG) in the Control and Medication Groups in the periods between days -2 and 13 and days 13 and 21. Comparison between mean daily dietary intake (MDDI) per animal, calculated by the mean of the pen, in the Control and Medication Groups in the periods: total (days 0 to 20), pre-treatment (days 0 to 13), and post-treatment (days 14 to 20)

Date		Weight			MDDI		MDWG		
		-2	13	21	-2 a 13	14 a 21	0 a 13	14 a 20	0 a 20
Groups	Medication	9.49	13.53	16.51	0.27	0.37	0.48	0.76	0.60
	Control	9.49	13.32	15.78	0.25	0.30	0.48	0.8	0.13
Standard deviation	Medication	1.06	2.58	3.31	0.12	0.13	0.05	0.09	0.06
	Control	1.10	2.33	3.24	0.14	0.17	0.10	0.15	0.13
	p	0.99	0.75	0.39	0.68	0.93	1.0	0.34	0.84

Table 3. Grade of gross lesions, number of animals with gross lesion, mean lesion length (in cm), and immunostaining in the Control and Medication Groups

Groups	Grade 0*	Grade 1	Grade 2	Grade 3	Number of animals with gross lesion	Total lesion length (cm)	Mean lesion length/affected pig (cm)	Mean lesion length/total of pigs (cm)	Immunostaining
Control	20	10	0	0	10	366	36.3	12.2	16 (53.5%)
Medication	24	6	0	0	6	97	16.16	3.2	8 (26.6%)
p						0.031**	0.093	0.151	<0.032**

* Grade 0 = normal, Grade 1 = thickened mucosa, Grade 2 = thickened and necrotic mucosa, Grade 3 = thickened mucosa with blood clots in the intestinal lumen; **statistically significant difference.

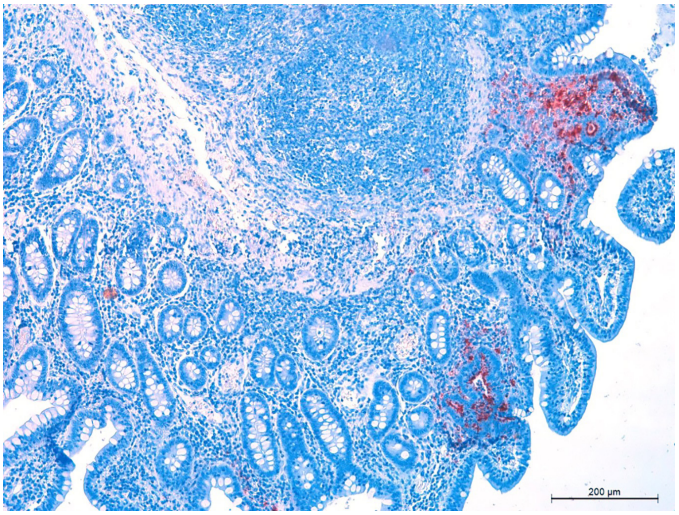

Fig.2. Histology section of ileum of Pig 37, Control Group (PAb 1999, Guedes & Gebhart 2003a). Grade 1, positive antigen labeled in up to 25% of the crypts. IHC anti-*Lawsonia intracellularis*, obj.10x.

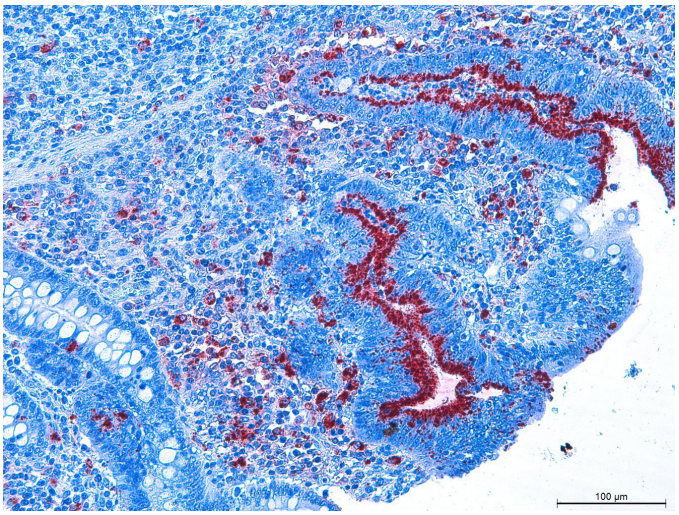

Fig.3. Histology section of ileum of Pig 8, Control Group (PAb 1999, Guedes & Gebhart 2003a). IHC anti-*Lawsonia intracellularis*, obj.20x.

model was effective to reproduce the disease. The fecal sample collection from all animals in the study prior to inoculation and the negative result for *L. intracellularis* in all of them, characterize the absence of infection in these animals at the beginning of the study and show that a non-inoculated restricted Control Group is not necessary.

Considering that it takes at least 7 to 8 days for the onset of a clinical condition caused by infection with *L. intracellularis* (Guedes et al. 2017), despite the presence of diarrhea caused by enterotoxigenic *Escherichia coli* on the first days after inoculation, the higher incidence of diarrhea from day 9 to day 12 after inoculation is in agreement with findings of other studies that used a similar experimental infection model (Paradis et al. 2004).

For zinc oxide, used in the first days after inoculation to contain the diarrhea caused by enterotoxigenic *E. coli*, there are no studies that clinically assessed animals infected with *L. intracellularis.* However, it is known that zinc oxide, at the doses applied in the present study, can be used during the administration of the live attenuated vaccine of *L. intracellularis* (Enterisol Ileitis, Boehringer Ingelheim VetMedica) without compromising its effectiveness, according to the manufacturer's information. Thus, we strongly believe that the use of zinc oxide did not affect infection by *L. intracellularis,* but it was effective in controlling the initial diarrhea induced by *E. coli.*

Although the growth performance results were numerically different between the experimental groups of this study, no significant statistical difference was observed, which can be justified by the high coefficient of variation of the analyzed variables (body weight, mean daily weight gain, and mean daily dietary intake) (Veenhuizen et al. 1998, Paradis et al. 2005).

Gross lesions were more frequently observed in pigs in the Control Group, which showed greater total length than that of pigs in the Medication Group ($p<0.05$). The lesions found on the day of euthanasia (day 21 post-inoculation) are consistent with those described in another study, in which most gross lesions were found between days 15 and 24 (Guedes et al. 2017).

Pigs in the Medication Group had less ileum immunostaining ($p<0.032$) compared with those in the Control Group, showing that the presence of the *L. intracellularis* antigen was more frequent in non-medicated animals. Similar findings were reported by Marsteller et al. (2001) using tylosin injectable, in a different presentation and formulation, in experimentally inoculated pigs. It is worth noting that the number of applications was half that used by Marsteller et al. (2001), which demonstrates clear practical advantage based on the time spent with handling animal medication. With respect to the active principle used for the treatment of PPE in the present study, tylosin is a macrolide, bacteriostatic agent that can act as a bactericide when in high concentrations (Kim et al. 2008). Macrolides bind to the subunit (50s) of the bacterial ribosome by inhibiting bacterial protein synthesis (Barcellos et al. 2012). Particularly important for the case of intracellular microorganisms, as *L. intracellularis*, in this class of antimicrobial drugs, it is the liposolubility that enables crossing of cell barriers and reaching the target agent more easily (Spinosa et al. 2002).

Regarding previous studies addressing tylosin and *L. intracellularis*, despite showing values of minimum inhibitory concentration (MIC) *in vitro*, that is, little effective action against PPE in an *in vitro* study (McOrist et al. 1995), in-feed tylosin was effective to treat the disease in pure culture experimental inoculation when administered for 14 days (100ppm) (McOrist et al. 1997). As previously mentioned, tylosin also showed satisfactory results in the treatment of PPE in another study (Marsteller et al. 2001), in which it was injected twice daily for three consecutive days, twice as much as in the present study. The difference between *in vivo* and *in vitro* results may be justified by the fact that *L. intracellularis* is an obligate intracellular bacterium (McOrist et al. 2000).

In addition to management measures, medication is the most used form for the treatment and control of PPE (França & Guedes 2008). Metaphylactic use corresponds to the application of medication at therapeutic doses in the whole batch of animals, indicated when diseases begin to manifest in a small percentage of animals (Barcellos et al. 2012). Metaphylactic

antibiotic therapy and treatment are the most indicated, because the use of antimicrobial drugs in inadequate doses and times, as growth promoters, may increase the chance of outbreaks of enteric diseases (Bane et al. 2001), in addition to favoring the risk of bacterial resistance (Silbergeld et al. 2008, Dosen et al. 2014). Administration of in-feed medication has been more associated with increased risk of bacterial resistance when compared with individual treatment (Dunlop et al. 1998, Haese & Silva 2004).

Indiscriminate use of antimicrobial drugs in low dose diet is associated with bacterial resistance (Maron et al. 2013). Some studies conducted in the USA have shown that in order to preserve the efficacy of antibiotics for human and animal treatments, it is necessary to limit the use of antimicrobial drugs (Levy & Marshall 2004, Silbergeld et al. 2008). As a result, many countries have restricted the use of antibiotics as growth promoters (Maron et al. 2013). The European Union banned the use of antimicrobial growth promoters in pig feed in 2006 (Gaggia et al. 2010) and a new regulation proposed by the FDA (Food and Drugs Administration) on the use of human antibiotics in domestic animal feed was enacted in the USA in 2017 (FDA 2017, Beek 2017). Therefore, the individual use of injectable antimicrobial drugs, as in this study, can assist with reversing the frequency of high bacterial resistance, as well as preventing the emergence of new resistant bacteria (Levy & Marshall 2004).

Most in-feed antibiotics provide low plasma levels of the drug compared with those of injectable drugs, especially macrolides and pleuromutilins, which also reduces bioavailability. In order to achieve treatment efficacy, the drug should be at the site of infection for sufficient time and concentration, otherwise it might favor development of bacterial resistance (Burch 2012).

Presentation of medication in injectable form, as used in the present study, is advantageous, because it enables its complete absorption, ensuring that the animal receives the entire necessary dose (Karriker et al. 2012). Animals infected with *L. intracellularis* present with atrophy and fusion of the villi, with reduction of digestive enzymes, and inhibition of membrane transporters, mechanisms that lead to malabsorption diarrhea (Argenzio 1980, Vannucci & Guedes 2009), suggesting that it may result in low antibiotic uptake when this is administered orally. Intramuscular medication has another advantage compared with in-feed medication, because ill animals show lower feed intake (Apley et al. 2012). It is true that intramuscular application is more laborious in larger animals, but long-acting formulations that do not need to be applied more than once have been increasingly growing (Burch 2012).

CONCLUSION

Tylosin injectable (Eurofarma Laboratórios S.A), in the conditions of the present study, was effective in treating porcine proliferative enteropathy (PPE) in experimentally inoculated pigs, because it significantly reduced lesion length and grade of infection by *Lawsonia intracellularis.*

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Conflict of interest statement.- Marina Mendonça Pereira has a commercial interest in tylosin injectable; however, it did not interfere with the experiment or analysis and interpretation of results.

REFERENCES

Apley M.D., Bush E.J., Morrison R.B., Singer R.S. & Snelson H. 2012. Use estimates of In-feed antimicrobials in swine production in the United States. Foodborne Pathog. Dis. 9(3):1-8. <http://dx.doi.org/10.1089/fpd.2011.0983> <PMid:22324295>

Argenzio R.A. 1980. Glucose-stimulated fluid absorption in the pig small intestine during the early stage of swine dysentery. Am. J. Vet. Res. 41(12):2000-2006. <PMid:7212433>

Bane D.P., Neumann E., Gebhart C.J., Gardner I.A. & Norby B. 2001. Porcine proliferative enteropathy: a case-control study in swine herds in the United States. J. Swine Health Prod. 9(4):155-158.

Barcellos D., Sobestiansky J., Linhares D. & Sobestiansky T.B. 2012. Uso de antimicrobianos, p.839-845. In: Sobestiansky J. & Barcellos D. (Eds), Doenças dos suínos. 2ª ed. Cânone Editorial, Goiânia.

Beek V.T. 2017. US: Vaccines and biosecurity to replace antibiotics. Pig Progress. Available at <http://www.pigprogress.net/Health/Articles/2017/1/US-vaccines and biosecurity to replace antibiotics 81584E/> Accessed on Sept. 23, 2017.

Burch D.G.S. 2000. Controlling ileitis in the "colitis" complex. Pig J. 45:131-149.

Burch D.G.S. 2012. Examination of the pharmacokinetic/pharmacodynamic (PK/PD) relationships of orally administered antimicrobials and their correlation with the therapy of various bacterial and mycoplasmal infections in pigs. Royal Colege of Veterinary Surgeons, London, p.1-130.

Callens B., Persoons D., Maes D., Laanen M., Postma M., Boyen F., Haesebrouck F., Butaye P., Catry B. & Dewulf J. 2012. Prophylactic and metaphylactic antimicrobial use in Belgian fattening pig herds. Prev. Vet. Med. 106(1):53-62.

Dosen R., Prodanov-radulovic J., Pusic I., Ratajac R., Stojanov I. & Grubac S. 2014. The uncontrolled use of antibiotics in pig production, a threat to public health. XVI International Symposium "Feed Technology", p.20-24. (Resumo)

Dunlop R.H., McEwen S.A., Meek A.H., Clarke R.C., Black W.D. & Friendship R.M. 1998. Associations among antimicrobial drug treatments and antimicrobial resistance of fecal *Escherichia coli* of swine on 34 farrow-to-finish farms in Ontario, Canada. Prev. Vet. Med. 34(4):283-305. <http://dx.doi.org/10.1016/S0167-5877(97)00095-0> <PMid:9618742>

FDA 2017. Guidance for Industry 203: New animal drugs and new animal drug combiation products administered in or on medicated feed or drinking water of food-producing animals, recommendations for drugs sponsors for voluntarily aligning product use conditions with GFI #209. Center for Veterinary Medicine, Food and Drug Administration, U.S. Department of Health and Human Services, USA. 18p.

França S.A. & Guedes R.M.C. 2008. Antimicrobianos para o controle da enteropatia proliferativa suína. Ciência Rural 38(1):288-296. <http://dx.doi.org/10.1590/S0103-84782008000100050>

Gaggìa F., Mattarelli P. & Biavati B. 2010. Probiotics and prebiotics in animal feeding for safe food production. Int. J. Food Microbiol. 141(Suppl.1):1-14. <http://dx.doi.org/10.1016/j.ijfoodmicro.2010.02.031> <PMid:20382438>

Guedes R.M.C. 2002. Porcine proliferative enteropathy: diagnosis, immune response and pathogenesis. Doctoral Dissertation, University of Minnesota, St Paul. 261p.

Guedes R.M.C. & Gebhart C.J. 2003a. Preparation and characterization of polyclonal and monoclonal antibodies against *Lawsonia intracellularis.* J. Vet. Diagn. Invest. 15(5):438-446. <http://dx.doi.org/10.1177/104063870301500506> <PMid:14535543>

Guedes R.M.C. & Gebhart C.J. 2003b. Comparison of intestinal mucosa homogenate and pure culture of the homologous *Lawsonia intracellularis*

isolate in reproducing proliferative enteropathy in swine. Vet. Microbiol. 93(2):159-166. <http://dx.doi.org/10.1016/S0378-1135(03)00013-0> <PMid:12637004>

Guedes R.M.C., França S.A., Machado G.S., Blumer M.A. & Costa Cruz Junior E.C. 2009. Use of tylvalosin-medicated feed to control porcine proliferative enteropathy. Vet. Rec. 165(12):342-345. <http://dx.doi.org/10.1136/vr.165.12.342> <PMid:19767637>

Guedes R.M.C., Machuca M.A., Quiroga M.A., Pereira C.E.R., Resende T.P. & Gebhart C.J. 2017. *Lawsonia intracellularis* in pigs: progression of lesions and involvement of apoptosis. Vet. Pathol. 54(4):620-628. <http://dx.doi.org/10.1177/0300985817698206> <PMid:28622490>

Haese D. & Silva B.A.N. 2004. Antibióticos como promotores de crescimento em monogástricos. Revta Eletrôn. Nutritime 1(1):7-19.

Jones G.F., Ward G.E., Murtaugh M.P., Lin G. & Gebhart C.J. 1993. Enhanced detection of intracellular organism of swine proliferative enteritis, ileal symbiont intracellularis, in feces by polymerase chain reaction. J. Clin. Microbiol. 31(10):2611-2615. <PMid:8253956>

Karriker L.A., Coetzee J., Friendship R.M. & Prescott J.F. 2012. Drug pharmacology, therapy and prophylaxis, p.438-443. In: Zimmerman J.J., Karriker L.A., Ramirez A., Schwartz K.J. & Stevenson G.W. (Eds), Diseases of Swine. 10th ed. John Wiley and Sons, Iowa.

Kim M., Gebru E., Chang Z., Choi J., Hwang M., Kang E., Lim J., Yun H. & Park S. 2008. Comparative Pharmacokinetics of Tylosin of Florfenicol after a single intramuscular administration at two different doses of tylosin-florfenicol combination pigs. J. Vet. Med. Sci. 70(1):99-102. <http://dx.doi.org/10.1292/jvms.70.99> <PMid:18250580>

Levy S.B. & Marshall B. 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nature 10(Suppl.12):122-129. <PMid:15577930>

Macêdo N.R., Menezes C.P., Lage A.P., Ristow L.E., Reis A. & Guedes R.M.C. 2007. Detecção de cepas patogênicas pela PCR multiplex e avaliação da sensibilidade a antimicrobianos de *Escherichia coli* isoladas de leitões diarreicos. Arq. Bras. Med. Vet. Zootec. 59(5):1117-1123. <http://dx.doi.org/10.1590/S0102-09352007000500005>

Maron D.F., Smith T.J.S. & Nachman K.E. 2013. Restrictions on antimicrobial use in food animal production: an international regulatory and economic survey. Globalization Health 9(48):1-11. <PMid:24131666>

Marsteller T., Winkelman N., Gebhart C., Armbruster G., Weldon W., Muller R., Weatherford J. & Symanowski J. 2001. Efficacy of intramuscular tylosin for the treatment and control of porcine proliferative enteropathy caused by *Lawsonia intracellularis*. Vet. Therap. 2(1):51-60.

McOrist S. 2005. Prevalence and impact of proliferative enteropathy (ileitis) in East Asia. Proceedings II Asian Pig Veterinary Society Congress, Pasig City, Philippines, p.24-37. (Resumo)

McOrist S. & Gebhart C.J. 2012. IV: Bacterial diseases, proliferative enteropathy, p.2976-3004. In: Zimmerman J.J., Karriker L.A., Ramirez A., Schwartz K.J. & Stevenson G.W. (Eds), Diseases of Swine. 10th ed. John Wiley and Sons, Iowa.

McOrist S., Mackie R.A. & Lawson G.H.K. 1995. Antimicrobial susceptibility of ileal symbiont intracellularis isolated from pigs with proliferative enteropathy. J. Clin. Microb. 33(5):1314-1317. <PMid:7615747>

McOrist S., Morgan J., Veenhuizen M.F., Lawrence K. & Kroger H.W. 1997. Oral administration of tilosin phosphate for treatment and prevention of porcine proliferative enteropathy. Am. J. Vet. Res. 58(2):136-139. <PMid:9028475>

McOrist S., Muller Wager A., Kratzer D. & Sjösten C.G. 2000. Therapeutic efficacy of water-soluble lincomycin-spectinomycin powder against porcine proliferative enteropathy in a European field study. Vet. Rec. 146(3):61-65. <http://dx.doi.org/10.1136/vr.146.3.61> <PMid:10674691>

Paradis M.A., Pauling G.E., Brennan J., Winkelmand N.L., Bagg R.N., Dick C.P. & Wilson J. 2004. Evaluation of tylosin tartrate in drinking water for treatment of porcine proliferative enteropathy (ileitis). J. Swine Health Prod. 12(4):177-181.

Paradis M.A., Mcya R.I., Wilson J.B., Vessie G.H., Winkelman N.L., Gebhart C.J. & Dick C.P. 2005. Subclinical ileitis produced by sequential dilutions of *Lawsonia intracellularis* in a mucosal homogenate challenge model. Am. Assoc. Swine Vet. 2005:189-191.

Silbergeld E.K., Graham J. & Price L.B. 2008. Industrial Food Animal Production, antimicrobial resistance and human health. Annual Rev. Publ. Health 29(1):151-169. <http://dx.doi.org/10.1146/annurev.publhealth.29.020907.090904> <PMid:18348709>

Spinosa H.S., Gorniak S.L. & Bernardi M.M. 2002. Farmacologia Aplicada à Medicina Veterinária. 3ª ed. Guanabara Koogan, Rio de Janeiro. 646p.

Vannucci F.A. & Guedes R.M.C. 2009. Fisiopatologia das diarreias em suínos. Ciência Rural 39(7):2233-2242. <http://dx.doi.org/10.1590/S0103-84782009005000163>

Veenhuizen M.F., Mowrey D.H., Moore G.M. & Watkins L.E. 1998. Evaluating a natural outbreak of porcine proliferative enteropathy and treatment with tylosin in the grow-finish phase. J. Swine Health Prod. 6(2):67-72.

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Original Article
Livestock Diseases

Molecular detection of albinism gene in Brazilian buffalo herds (*Bubalus bubalis*)[1]

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ABSTRACT.- Bernardino P.N., Martins A.F.A., Barbosa J.D., Schild A.L., Damé M.C.F., Borges A.S. & Oliveira-Filho J.P. 2019. **Molecular detection of albinism gene in Brazilian buffalo herds (*Bubalus bubalis*).** *Pesquisa Veterinária Brasileira 39(3):175-178*. Departamento de Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Rua Prof. Doutor Walter Mauricio Correa s/n, Cx. Postal 560, Botucatu, SP 18618-681, Brazil. E-mail: jose.oliveira-filho@unesp.br

Albinism is a genetic disease characterized by deficient melanin production making affected animals more susceptible to skin problems, negatively influencing production systems of the same. In buffalo, a nonsense mutation (c.1431G>A) in the tyrosinase gene was already described, which is responsible for the oculocutaneous albinism buffalo phenotype. However, prevalence studies have never been performed for this anomaly in Brazil. Therefore, the objective of this study was to investigate this mutation in buffalo herd in Brazil. Of the 315 buffalo tested with no albinism phenotype evident, 11 (3.5%) were heterozygous for the mutation and none were mutated homozygous, showing the existence of the albinism gene in buffalo production herds and proving the importance of prevalence studies for hereditary diseases in order to prevent the dissemination of these same genes and their negative productivity consequences.

INDEX TERMS: Molecular detection, albinism gene, buffalo, herds, *Bubalus bubalis*, mutation, tyrosinase, prevalence, genetics.

RESUMO.- [**Detecção molecular do gene do albinismo em rebanhos de búfalos (*Bubalus bubalis*) do Brasil.**] O Albinismo é uma doença genética caracterizada pela deficiência na produção de melanina, o que torna os animais afetados mais susceptíveis a problemas cutâneos e influencia negativamente a criação destes animais. A mutação *nonsense* (c.1431G>A) no gene da tyrosinase já foi descrita como responsável pelo albinismo oculocutâneo em búfalos, entretanto estudos prévios sobre a prevalência dessa mutação ainda não foram realizados no Brasil. Portanto, o objetivo deste estudo foi avaliar a presença desta mutação em uma população de búfalos brasileiros. Foram genotipados 315 búfalos clinicamente normais, ou seja, sem o fenótipo albino evidente. Desses, 11 (3,5%) eram heterozigotos para a mutação (N/TYR) e os demais eram homozigotos selvagens (N/N). Este resultado demonstra que o alelo mutado para o albinismo em búfalo está presente no rebanho brasileiro e aponta a importância de estudos de prevalência de enfermidades hereditárias com o objetivo de prevenir a disseminação desses alelos mutados, minimizando os prejuízos.

TERMOS DE INDEXAÇÃO: Detecção molecular, gene do albinismo, rebanhos, búfalos, *Bubalus bubalis*, mutação, tirosinase, prevalência, genética.

INTRODUCTION

Genetic Type 1 oculocutaneous albinism (OCA) is an autosomal recessive genetic anomaly in which the production of melanin is reduced or inexistent due to a point mutation that decodes the tyrosinase gene (*TYR*), enzyme that takes part on the melanin synthesis process (Oetting 2000). It was already described in

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multiple species: albino Wistar lab rats present a nucleotide shift in the *TYR* gene (Blaszczyk et al. 2005), in albino cats it is observed a deletion of a cytosine in the *TYR* gene producing a premature stop codon (Imes et al. 2006), bovines showed a insertion of a cytosine in the same gene also originating a premature stop codon (Schmutz et al. 2004), and yet, albino ferrets presented a deletion in exon 4 of the *TYR* gene (Blaszczyk et al. 2007). In buffalo, a nonsense mutation in the nucleotide 1431 (G to A) was pointed to produce an inactive TYR protein resulting in animals with photophobia and skin, hair, hooves, horns, iris and mucous membranes depigmentation, characteristic of OCA (Damé et al. 2012).

According to the Brazilian Association of Buffalo Farmers, the Brazilian buffalo herd was around 3.5 million animals in 2007. This population started in the late XIX century with only 200 animals, adding a few pure buffalo from India in 1962 and 8 from Italy in 1989 (Bernarde 2007). The large population growth based on few starters indicates high probabilities of consanguinity, which allows the propagation of mutated genes for hereditary diseases, as the *TYR* mutation for albinism.

Individuals without adequate pigmentation (Fig.1) are more susceptible to skin and eyes problems, such as sun burnt, retinal lesions and melanomas (Damé et al. 2015, Fuller & Hay 2015). Those could affect negatively the buffalo production system, making the albinism an undesirable characteristic. No prevalence data concerning this disease in Brazil exists so far, only case reports (Damé et al. 2012) proving the presence of this mutation in the herds and making the selection for breeding based on genetic aspects more important in this specie. Therefore, the objective of this study was to investigate this mutation in buffalo herd in Brazil.

MATERIALS AND METHODS

Ethics statement. All procedures were approved by the Board of Ethics and Animal Experimentation of the institution (Protocol no. 54/2016 - CEUA).

Experimental samples. For the present molecular study, hair, blood or semen samples were collected from 315 Murrah buffalo from Southern (states of Rio Grande do Sul and Paraná), Northern

Fig.1 (**A**) Oculocutaneous albinism (OCA) Murrah buffalo, showing white skin and the stratum corneum of the horns; mucosa and periocular region (eyelashes, conjunctiva and iris) with absence of pigmentation. (**B**) Normal Murrah buffalo with black skin. (**C**) Depigmented skin and hairs of the dorsal region of an OCA buffalo. (**D**) An OCA albino bull and normal cow buffalo.

(state of Pará) and South-Eastern (state of São Paulo) Brazilian properties, with no sex, age or breed predilection. Blood and semen samples were refrigerated until the extraction.

DNA purification and genotyping analysis. Genomic DNA was purified from hair root samples using an in-house method and from blood and semen samples using the GenElute™ Genomic Blood DNA Kit (Sigma-Aldrich®) according to the manufacturer's instructions. The DNA obtained was used to genotype the mutation c.1431G>A in the *TYR* gene. The PCR was performed using ASBTYR-F3 and ASBTYR-R3 primers previously described for OCA in buffalo (Dame et al. 2012). The PCR amplifications were performed in a total volume of 25µl, which contained 0.3µM each forward and reverse primer, 2.5µl of template cDNA, 12.5µl of GoTaq® Green PCR Master Mix (Promega), and nuclease-free water q.s.p. In addition, a no-template control reaction was performed in duplicate to check for the possible presence of contamination in the preparations, besides that, a positive control (DNA from OCA buffalo) was used in order to ensure that the reaction worked well. The obtained PCR products were analyzed via 1.5% agarose gel electrophoresis (Invitrogen™, Carlsbad, CA), stained with the Sybr® Safe DNA Gel Stain (Invitrogen™) and then purified using the QIAquick® PCR Purification Kit (Qiagen®).

Sequencing analysis. To sequence the DNA, 10µL of each PCR product, 5µL of the purified forward primer and the BigDye® Terminator Cycle Sequencing Kit were used (Life Technologies™ CA, USA). The sequences were determined using the ABI 3500 Genetic Analyzer (Life Technologies™ CA, USA). The obtained sequences and the electropherograms (Fig.2) were analyzed using Geneious® 10.0.9 (Biomatters Ltd, Auckland, New Zealand). The sequences were compared with the normal *Bubalus bubalis TYR* gene sequence using BLAST (Basic Local Alignment Search Tool, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The genotypic frequency was estimated and the data were analyzed descriptively.

RESULTS AND DISCUSSION

None of the buffalo included in the study presented the phenotype for albinism, fitting the result of no mutated homozygous individual (0%). Despite not having albino animals, 11 out of the 315 buffalo (3.5%) were heterozygous for the *TYR* gene mutation as seen in the electropherogram (Fig.2), the other 304 (96.5%) are non-mutated homozygous individuals (Table 1). The heterozygous animals were present in two of the properties that took part on this research: 6 out of 111 animals (5.4%) from the state of Rio Grande do Sul and 5 out of 46 (10.8%) from the state of Para. The other properties presented 100% of the sampled animals as non-mutated homozygous.

Knowing which animals have the mutation for the *TYR* gene, the farmers of each property can take this information in consideration during the selection of breeders, avoiding these anomalies in new animals. In addition, if buffalo are purchased from other properties, the genetic test can help to show if the incomes carry the mutation or not in order to select them as breeders as well.

Besides albinism, other buffalo diseases have been confirmed to be hereditary or have strong evidences of being carried through the genes like arthrogryposis, hereditary myotonia, and hydranencephaly (Damé et al. 2013). Precautions should be taken to avoid the propagation of those diseases and their adverse effect on buffalo production system (Damé et al. 2015). For this reason, prevalence studies on genetic disorders aiming in the identification of heterozygous animals give information for future references on which animals should be used or not for reproduction.

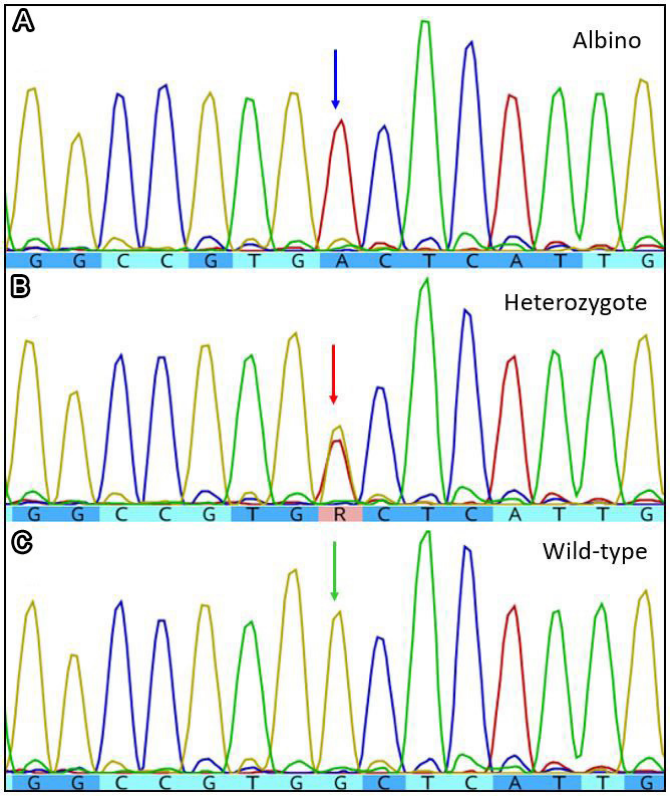

Fig.2 (**A**) Partial electropherogram obtained from albino (positive control), (**B**) heterozygote and (**C**) wild-type buffalo. It is possible to observe a double peak (R, G/A) in the heterozygous (red arrow), and a single peak in albino (A nucleotide, blue arrow) and in wild-type buffalo (G nucleotide, green arrow). Image obtained in the Geneious® 10.0.9 software (Biomatters Ltd, Auckland, New Zealand).

Table 1. Prevalence of heterozygous (N/TYR) and wild-type (N/N) Murrah buffalo for the mutation c.1431G>A in the *TYR* gene responsible for albino buffaloes

	Heterozygotes (N/TYR)	Wild types (N/N)	Total sampling
Frequency	3.5%	96.5%	100%
Number of animals	11	304	315

CONCLUSIONS

The study shows the existence of a gene mutation without phenotype manifestation for one of many genetic diseases among buffalo of more than one herd in different properties of the same country.

This proves the importance of molecular prevalence studies both for the production system that should be careful not to propagate the mutation and for the scientific community that can understand more about the disease's epidemiology.

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

REFERENCES

Bernarde O. 2007. Bubalinocultura no Brasil: situação e importância econômica. Revta Bras. Reprod. Anim. 31:293-298.

Blaszczyk W.M., Arning L., Hoffmann K.P. & Epplen J.T. 2005. A tyrosinase missense mutation causes albinism in the Wistar rat. Pigment Cell Res. 18(2):144-145. <http://dx.doi.org/10.1111/j.1600-0749.2005.00227.x> <PMid:15760344>

Blaszczyk W.M., Distler C., Dekomien G., Arning L., Hoffmann K.P. & Epplen J.T. 2007. Identification of tyrosinase (TYR) exon 4 deletion in albino ferrets (*Mustela putorius furo*). Anim. Genet. 38(4):421-423. <http://dx.doi.org/10.1111/j.1365-2052.2007.01619.x> <PMid:17655555>

Damé M.C.F., Riet-Correa F. & Schild A.L. 2013. Doenças hereditárias e defeitos congênitos diagnosticados em búfalos (*Bubalus bubalis*) no Brasil. Pesq. Vet. Bras. 33(7):831-839. <http://dx.doi.org/10.1590/S0100-736X2013000700001>

Damé M.C.F., Marcolongo-Pereira C., Fiss L., Adrién M.L. & Schild A.L. 2015. Malignant melanoma in albino water buffalo (*Bubalus bubalis*). Semina, Ciênc. Agrárias 36:3239-3244.

Damé M.C.F., Xavier G.M., Oliveira-Filho J.P., Borges A.S., Oliveira H.N., Riet-Correa F. & Schild A.M. 2012. A nonsense mutation in the tyrosinase gene causes albinism in water buffalo. BMC Genet. 13(1):62-68. <http://dx.doi.org/10.1186/1471-2156-13-62> <PMid:22817390>

Fuller C. & Hay R. 2015. Albinism: a cause for concern. Community Dermatol. J. 11:1-12.

Imes D.L., Geary L.A., Grahn R.A. & Lyons L.A. 2006. Albinism in the domestic cat (*Felis catus*) is associated with tyrosinase (TYR) mutation. Anim. Genet. 37(2):175-178. <http://dx.doi.org/10.1111/j.1365-2052.2005.01409.x> <PMid:16573534>

Oetting W.S. 2000. The tyrosinase gene and oculocutaneous albinism type I (OCA1): a model for understanding the molecular biology of melanin formation. Pigment Cell Res. 13(5):320-325. <http://dx.doi.org/10.1034/j.1600-0749.2000.130503.x> <PMid:11041207>

Schmutz S.M., Berryere T.G., Ciobanu D.C., Mileham A.J., Schmidtz B.H. & Fredholm M. 2004. A form of albinism in cattle is caused by a tyrosinase frameshift mutation. Mammalian Genome 15(1):62-67. <http://dx.doi.org/10.1007/s00335-002-2249-5> <PMid:14727143>

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Original Article
Livestock Diseases

Bovine rabies: economic loss and its mitigation through antirabies vaccination[1]

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ABSTRACT.- Mello A.K.M., Brumatti R.C., Neves D.A., Alcântara L.O.B., Araújo F.S., Gaspar A.O & Lemos R.A.A. 2019. **Bovine rabies: economic loss and its mitigation through antirabies vaccination**. *Pesquisa Veterinária Brasileira 39(3):179-185*. Laboratório de Patologia Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul, Avenida Senador Filinto Muller 2443, Jd. Monte Alegre, Campo Grande, MS 79074-460, Brazil. E-mail: lap.famez@ufms.br

Rabies is among the most common neurological disease in cattle in Brazil, causing significant economic losses. Data on the economic impact of rabies in livestock are available in several countries. However, in Brazil, these data focus mainly on the public health point o view, emphasizing the costs related to the prevention of rabies in humans, in dogs, or wildlife. Specific studies carried out in different regions of Brazil indicate critical economic losses caused by rabies in cattle in this country. However, the studies on the losses caused by the disease in cattle lack a detailed analysis of the affected rural properties based on data from official disease control agencies. The objective of this work was to evaluate the economic impact of bovine rabies, and its mitigation through antirabies vaccination in rural properties in Mato Grosso do Sul, Midwestern Brazil.

INDEX TERMS: Bovine rabies, economic loss, antirabies vaccination, cattle, clinics.

RESUMO.- [**Raiva em bovinos: perdas econômicas e sua mitigação através da vacinação antirrábica.**] A raiva é uma das doenças neurológicas mais comuns em bovinos no Brasil, causando perdas econômicas significativas. Dados sobre o impacto econômico da raiva em bovinos de vários países estão disponíveis. No entanto, no Brasil, esses dados enfocam principalmente o ponto de vista de saúde pública, enfatizando os custos relacionados à prevenção da raiva em humanos, em cães ou animais silvestres. Estudos pontuais realizados em diferentes regiões do Brasil indicam perdas econômicas importantes causadas pela raiva em bovinos no país. No entanto, os estudos sobre as perdas causadas pela doença em bovinos carecem de uma análise detalhada das propriedades rurais afetadas com base em dados das agências oficiais de controle de doenças. O objetivo deste trabalho foi avaliar o impacto econômico da raiva bovina e sua mitigação através da vacinação antirrábica em propriedades rurais de Mato Grosso do Sul, no Centro-Oeste do Brasil.

TERMOS DE INDEXAÇÃO: Raiva em bovinos, perdas econômicas, vacinação antirrábica, bovinos, clínica.

INTRODUCTION

Rabies is a neurological disease caused by a *Lyssavirus* of the Rhabdoviridae familyand can affect all mammals (Swanepoel 2004). In cattle, it is an acute disease, invariably fatal, transmitted through the saliva of the vampire bat *Desmodus rotundus* (Barros et al. 2006). It is one of the most prevalent neurological diseases in ruminants (Barros et al. 2006), causing significant economic losses, especially for developing countries (King & Turner 1993, Rupprecht et al. 2002, Lima et al. 2005).

Although the economic significance of rabies in cattle in Brazil can be inferred by studies on the prevalence of the disease in different regions of the country (Langohr et al. 2003,

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Marcolongo-Pereira et al. 2011, Ribas et al. 2013), there are no detailed studies on the economic losses caused by the disease. An estimation of these losses is hampered by the difficulty in obtaining reliable data on the occurrence of the disease (Kotait et al. 1998, Braga et al. 2014, Taylor & Knopf 2015). The Brazilian National Herbivore Rabies Control Program (PNCRH), implemented a solid system of information and surveillance in areas or properties at risk, closely following the outbreaks of the disease. It aims to effectively control rabies in domestic herbivores through strategic vaccination of the susceptible species and control of the vampire bat. The data generated by the PNCRH are an essential source for the study of the economic impact caused by rabies in cattle in Brazil.

In 1988 The World Health Organization (WHO) recognized the lack of data on the economic significance of rabies and suggested the development of a model that would be the basis for determining the costs required for controlling the disease, but this model did not appear in the literature (Meltzer & Rupprecht 1998).

Studies on the economic impact of rabies are conducted in several countries, but the focus of these studies is mainly on public health, emphasizing the costs related to the prevention of human (Shwiff et al. 2007, Dhankhar et al. 2008, Anyiam et al. 2017), canine or wild animals (Knobel et al. 2005, Sterner et al. 2009, Hampson et al. 2015) rabies. Wild stock is a potential transmitter of the disease to humans (Swanepoel 2004). Particularly concerning bovine rabies, a detailed study was conducted in Mexico (Anderson et al. 2012) analyzing and comparing the efficiency of two methods of rabies control: vampire bat control and vaccination of cattle at risk.

However, studies analyzing losses occurring on rural properties, based on data from an official disease control program, were not found in the literature. Analysis of the economic impact of diseases is relevant to highlight the importance of agricultural defense policies. It also contributes to identifying priority health policies and also to support decision making by rural producers.

Using data provided by PNCRH, this study was aimed to evaluate the economic impact of bovine rabies on rural properties of Mato Grosso do Sul (MS) and the mitigation of this impact by antirabies vaccination.

MATERIALS AND METHODS

A retrospective study was carried out consulting the data contained in the initial formulary (FORM IN) on bovine rabies of the State Agency of Animal and Plant Health Protection of MS (IAGRO). The study covered all state area from 2010 to 2016. The following data were analyzed: size of the property, its location, the notifications of rabies occurrence, the number of bovine in the herd, the number of cattle deaths and the herd rabies vaccination status.

For the calculations, the criterion for considering a rabies case was any sick or dead cattle from a herd with an ongoing outbreak with a definite diagnosis of rabies in one or more components of the herd. Tests accepted for detecting a positive case were direct fluorescent antibody test (dFA) and intracerebral inoculation of suckling mice (IISM), following the Brazilian Herbivore Rabies Control Manual (BHRCM) (Brasil 2009). An event was considered an outbreak of bovine rabies there was the death of more than one bovine per herd.

The market value of the kg of beef was used for the calculation of the stockholders' equity considering the number of cattle in the property studied. The calculations were made using the average of the prices reported by CEPEA/ESALQ and BM&F Bovespa (CEPEA 2017), for July 2017, which is the official source for the price of the kilo of fat steers in Brazil, which was converted to dollars using the Brazilian Central Bank quotations for the same period.

Morbidity (morb.) was calculated by the following formula (Thrusfield 2004):

$$\text{Morb.} = \left(\frac{\text{Total of sick cattle}}{\text{Total cattle population at risk}} \right) \times 100$$

The patrimony estimated (PEs) for each property was based on the number of cattle in the herd. The value of each cattle category was estimated by the value paid per kg of beef multiplied by the estimated weight of individual cattle in each category multiplied for the yield of carcass:

$$\text{PEs} = (\text{N.cattle}) \times (\text{E.weight} \times \text{E.Y.C})) \times \text{US\$ / kg}$$

Where *N.cattle* = number of cattle, *E.w*eight = Estimated weight, and *E.Y.C* = Estimated yield of carcass.

The estimated total equity was calculated by the sum of the estimated equity in each cattle category. The economic losses per category (EconLCat) were estimated by the sum of the dead animals in the category multiplied by the estimated weight and estimated yield, multiplied by the amount paid per kg of beef according to the following formula:

$$\text{EconLCat} = \left(\sum \text{head cat} \times (\text{E.weight} \times \text{EYC}) \right) \times \text{US\$ / kg}$$

Whre *head cat*=heads per category,*E.w*eight = Estimated weight, and *EYC* = Estimated yield of carcass.

The total economic loss was calculated using the sum of the economic losses per category. For the calculation of the dose of rabies vaccine, prices practiced in the resales market of the Capital of MS were surveyed. The cost of vaccination by category of cattle was calculated using the total number of cattle per category multiplied by the number of times they were vaccinated and then multiplied by the amount paid per vaccine dose. Three strategic vaccinations were considered, one at three months, one booster after 30 days and annual revaccinations, according to the BHRCM. The total cost of vaccination was calculated by adding up the cost of vaccination in the different cattle categories.

$$\text{Cvac / cat.} = (\sum \text{head catx numbers of vaccine doses}) \times \text{US\$ / dose}$$

Where *Cvac* = Cost of vaccination, *head cat* = heads per category.

The following formula estimated the relationship between the cost of herd vaccination and the economic loss caused by rabies-induced death in cattle:

$$\text{C.Vac. / P.Econ} = \left((\text{TVacC}) / (\text{TEconL}) \right) \times 100$$

Where *Cvac* = Cost of vaccination, *TvacC* cost, *TEconL*= total economic loss.

The resultant values were analyzed to obtain the descriptive statistics with the determination of the histograms of occurrences.

RESULTS

From January 2010 to December 2016, there were 52 outbreaks of bovine rabies in 23 out the entire 79 municipalities forming the state of Mato Grosso do Sul (Fig.1), with a total of 305 deaths of rabid cattle. The size cattle herd of Mato

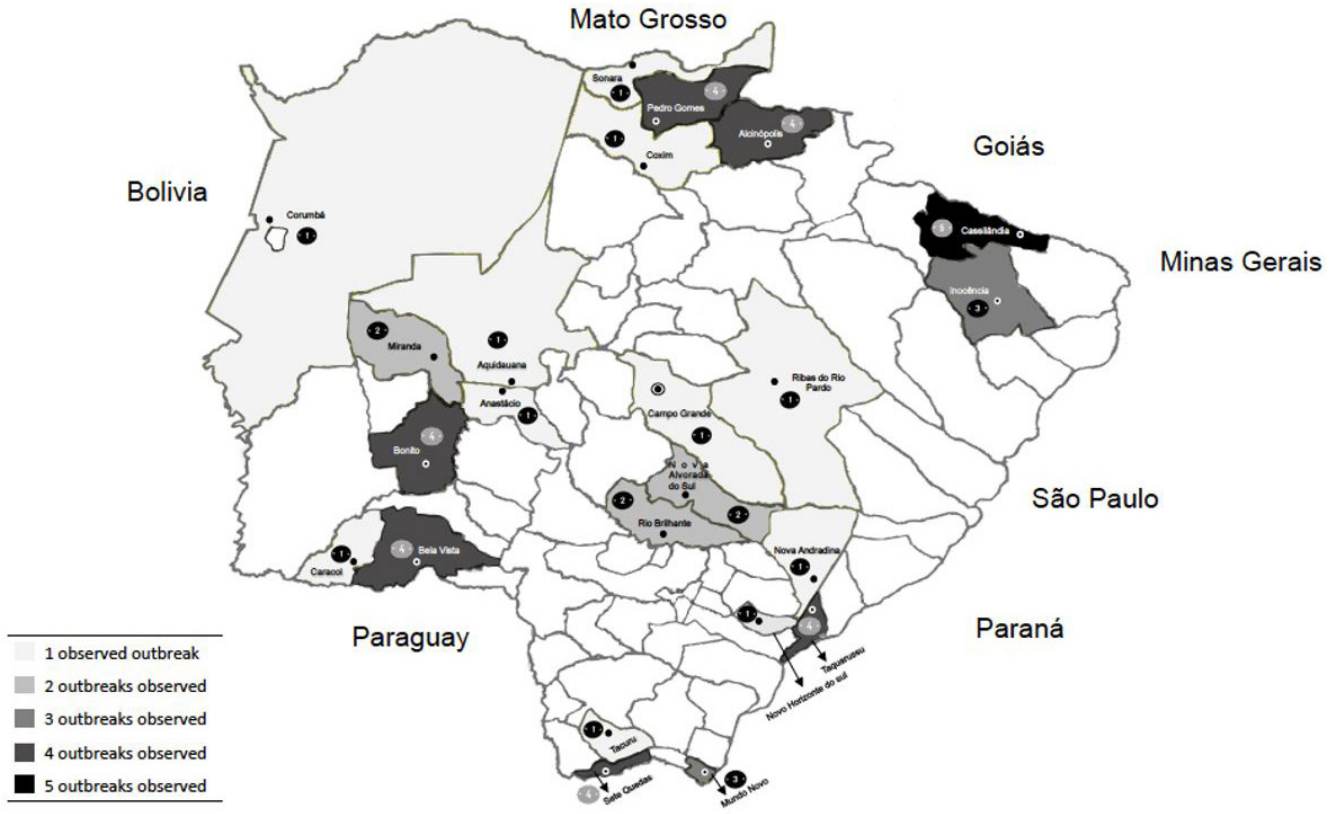

Fig.1. Map of Mato Grosso do Sul highlighting the municipalities where one or more rabies outbreak occurred from 2010 to 2016. The number of outbreaks for each municipality is noted.

Grosso do Sul is of 21.8 million bovines (IBGE 2016), of which 9.50 million (43.57% of the herd of the state of MS) are in areas where rabies occurs and therefore are exposed to the risk of bovine rabies. Epidemiological data on outbreaks are shown in Table 1.

Cases of bovine rabies were observed in large and small farms, with herds consisting of 10 to 6,210 cattle. The estimated value of the assets ranged from US$ 4,307.00 to US$ 3,005,948.00. The average value of the equity was estimated at US$ 402,528.00. The total value of the assets of properties that had outbreaks of rabies was valued at approximately US$ 20,931,466.00.

In 28 properties, the number of deaths per herd varied from 1-2 cattle, in ten the number was 3-5 cattle and in fourteen properties the number of dead cattle died was above six. The morbidity varied widely from 0.04-20%; the lethality was 100%.

In 39 properties, the estimated patrimony was up to US$ 500,000.00; other thirteen properties presented values above that (Fig.2), indicating that most of the properties fit as small and medium rural properties. In 47 properties, the estimated economic loss was less than US$ 5,000.00; the others sustained losses heavier than this (Fig.3).

Currently, MS has 12 municipalities where vaccination is mandatory, namely: Aquidauana, Anastácio, Corumbá, Miranda, Bonito, Coxim, Corguinho, Bodoquena, Rio Verde de Mato Grosso, Rio Negro, Jardim, and Ladário (Fig.1). The criterion adopted for the inclusion of a municipality in an area of obligatory vaccination is in the number of outbreaks that occurred historically in this municipality (Brasil 2009).

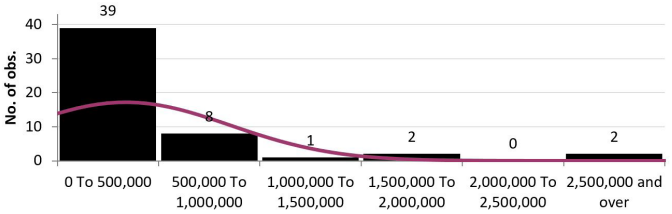

Fig.2. Histogram of the distribution of the values in US dollars of estimated stockholders' equity of the rural properties where outbreaks of bovine rabies occurred.

The average dose price of anti-rabies vaccine practiced in MS in July 2017 was $ 0.12. The estimated amounts spent on vaccination ranged from US$ 2.00 to US$ 1,437.00 per property, depending on the number of cattle. In 45 properties this cost was less than US$ 200.00. The average cost of vaccination was $ 148.00. Total vaccination expenditures of all herds at all properties where outbreaks of rabies were observed were US$ 7,716.12 (Fig.4).

The ratio of the estimated cost of rabies vaccination of the entire herd to the economic loss per property was, on average, 9.74%. In 37 properties, this relation was less than 10% (Fig.5). The relation between the total cost of vaccination and the total economic loss, adding up to all the properties studied, was 5.8%.

Table 1. Epidemiological data on bovine rabies outbreaks in Mato Grosso do Sul, from 2010 to 2016

Outbreak	Month	Year	Municipality	Number of cattle in the herd	Number of deaths	Morbidity (%)
1	January	2010	Alcinópolis	5,970	76	1.27
2	February	2010	Pedro Gomes	1,609	6	0.37
3	March	2010	Alcinópolis	584	2	0.34
4	March	2010	Alcinópolis	39	3	7.69
5	August	2010	Alcinópolis	338	2	0.59
6	December	2010	Taquarussu	37	1	2.7
7	December	2010	Taquarussu	150	2	1.33
8	December	2010	Taquarussu	85	1	1.18
9	March	2011	Aquidauana	996	6	0.6
10	March	2011	Rio Brilhante	1,282	5	0.39
11	February	2011	Nova Alvorada do Sul	499	2	0.4
12	March	2011	Cassilândia	234	6	2.56
13	March	2011	Cassilândia	258	1	0.39
14	October	2011	Cassilândia	182	1	0.55
15	October	2011	Cassilândia	488	1	0.2
16	October	2011	Cassilândia	57	1	1.75
17	December	2011	Nova Alvorada do Sul	441	3	0.68
18	March	2012	Inocência	419	2	0.47
19	May	2012	Anastácio	903	12	1.33
20	June	2012	Miranda	421	3	0.71
21	October	2012	Miranda	1430	1	0.07
22	January	2013	Caracol	572	5	0.87
23	May	2013	Inocência	636	4	0.63
24	May	2013	Mundo Novo	21	1	4.76
25	June	2013	Mundo Novo	22	1	4.55
26	June	2013	Mundo Novo	20	1	10
27	September	2013	Sonora	5,297	33	0.62
28	February	2014	Bela Vista	2,482	1	0.04
29	March	2014	Taquarussu	10	2	20
30	April	2014	Bonito	1,388	6	0.43
31	April	2014	Coxim	19	2	10.53
32	April	2014	Bonito	1,024	10	0.97
33	May	2014	Bonito	318	4	1.26
34	May	2014	Bonito	18	2	11.11
35	June	2014	Sete Quedas	1,196	3	0.25
36	June	2014	Bela Vista	533	9	1.69
37	July	2014	Sete Quedas	1,106	2	0.18
38	June	2014	Inocência	139	3	2.16
39	July	2014	Pedro Gomes	595	2	0.34
40	September	2014	Sete Quedas	1,321	1	0.08
41	October	2014	Tacuru	430	7	1.63
42	October	2014	Sete Quedas	731	2	0.27
43	November	2014	Bela Vista	231	2	0.87
44	May	2015	Rio Brilhante	68	2	2.94
45	April	2015	Pedro Gomes	661	9	1.36
46	June	2015	Bela Vista	1,200	33	2.75
47	July	2015	Ribas do Rio Pardo	577	2	0.35
48	July	2015	Campo Grande	150	1	0.67
49	March	2015	Corumbá	27	3	11.11
50	March	2015	Pedro Gomes	150	1	0.67
51	April	2016	Nova Andradina	4,268	6	0.14
52	September	2016	Novo Horizonte do Sul	6,210	8	0.13
TOTAL				47,842	305	

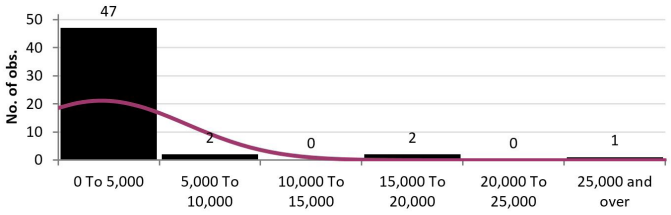

Fig3. Histogram of the distribution of the values in US dollars of estimated economic losses of the rural properties where outbreaks of bovine rabies occurred.

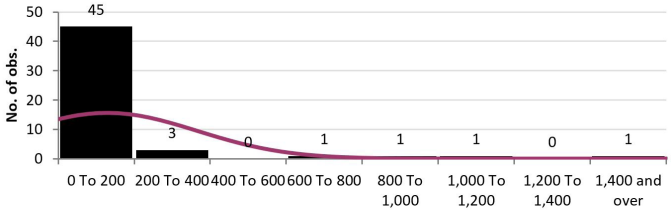

Fig.4. Histogram of the distribution of the values in US dollars of the estimated cost with vaccination in rural properties where outbreaks of bovine rabies occurred.

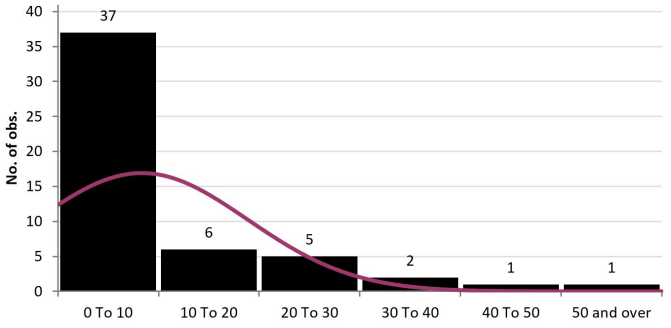

Fig.5. Histogram of distribution of the values of the ratios (%) between estimated cost with vaccination and estimated economic losses for rural properties where outbreaks of bovine rabies occurred.

DISCUSSION

The results of the present study demonstrate that rabies is a frequent disease in bovines in MS occurring in every year of the studied period. The area where the outbreaks were diagnosed is where of the cattle population of MS is concentrated (IBGE 2016). It is noteworthy that of the 23 municipalities with the occurrence of bovine rabies, 12 are mandatory anti-rabies vaccination zone (IBGE 2016). The persistence in the occurrence of outbreaks in this zone indicates a failure in the immunization process of the cattle.

In five of the seven years studied, the number of outbreaks was 6-9, with one peak and significant declines in the number of outbreaks in one and two years respectively. The highest number of outbreaks diagnosed in certain years can be attributed to a more effective surveillance in those years (Brasil 2009, Oliveira et al. 2013). The decline is considered a standard pattern in rabies epidemiology (Mori & Lemos 1998, Teixeira et al. 2008) and it is attributed to cyclic dynamics of vampire bat population.

When analyzed in their entirety, the economic losses of the reported outbreaks are below US$ 5,000.00. In a few outbreaks, the losses were between US$ 15,000.00 and US$ 25,000.00. These data demonstrate the importance of analyzing stratified losses by focusing on the occurrences of each property. Data evaluated without considering the morbidity ratios of each outbreak do not reflect the potential risk for each property and may convey the wrong message that the disease poses no risk of significant economic losses for individual farmers.

This misinterpretation is apparent when analyzing the total herd of farms with rabies outbreaks: properties with the lowest number of cattle had the highest morbidity ratios.

Lethality is invariably 100%; in order to calculate the losses, all sick cattle at the time of data collection should be considered as a loss.

The data presented here markedly differ from those of other rabies Brazilian studies (Sanches et al. 2000, Lemos 2005, Lima et al. 2005). Some authors mention that 30,000 to 40,000 cattle die each year of rabies in Brazil (Silva et al. 2000, Heinemann et al. 2002). It should be noted, however, that these studies are not based on reliable surveys, and the authors mention lack of accurate official data on deaths caused by bovine rabies.

The difficulty in obtaining accurate data on the losses caused by bovine rabies in Brazil is mentioned by several authors (Lemos 2005, Oliveira et al. 2013, Andrade et al. 2014). Studies based on surveys involving historical series reveal great variation according to the region of the country where the study was done. In a thirty-five-year retrospective study conducted in the Central region of Rio Grande do Sul, 151 cases of bovine rabies were diagnosed, in a total of 6,021 examined materials (49.5%) in the routine diagnostic service. A retrospective 16-year study conducted in the state of Paraná (Dognani et al. 2016) describes the occurrence of 2,331 (30.6%) cases in a total of 7,627 bovine samples examined. Another 16-year retrospective study was conducted in Rio Grande do Sul (Teixeira et al. 2008), in which 670 cases of rabies were diagnosed within a total of 1,729 samples (38.7%). In Minas Gerais (Silva et al. 2001), in a period of 7 years, 1,540 cases of rabies were found out of a total of 3,073 samples examined (50.1%),

Despite the relevance of these studies to demonstrate the importance of rabies as a cause of mortality in cattle, the description of the morbidity ratios of the outbreaks is fundamental for estimating the economic losses caused by the disease. This is evident in the present study; of 28 outbreaks diagnosed on farms with up to 500 cattle, 18 had morbidity ratios higher than 1%, and of these, in six, it was higher than 5%. When properties with more than 500 cattle were analyzed, in only five the morbidity ratio exceeded 1%, and in no instance, this ratio was higher than 3%.

The methodology used to estimate the economic losses due to bovine rabies-related deaths used in this study is similar to those used in other studies to estimate the economic losses caused by a specific disease in cattle (Heckler et al. 2018)

Another methodology used to estimate economic losses caused by a particular disease is the database from veterinary diagnostic laboratories - VDLs (Lima et al. 2005). In this approach, the percentage of d cases of a particular disease in cattle diagnosed in a given VDL is calculated over the total number of diagnosis performed in cattle in that VDL and this

percentage is extrapolated as the percentage of deaths caused by this disease in the region of the VDL. In a survey of bovine diseases carried out in Mato Grosso do Sul between 2008 and 2012 (Ribas et al. 2013), 15.92% were cases of rabies. Considering that the annual mortality of cattle in Brazil is estimated at 5% and that the herd size of MS at the time of the study was 23 million cattle, then 183,080 of these would die from rabies. However, the data of the present study do not support this, since deaths attributed to rabies in almost seven years totaled 305 cattle. Such a discrepancy should not be expected in the face of the existence of an official bovine rabies control program in Brazil, which determines the compulsory notification of cases of the disease (Brasil 2009). However, this notification depends on the farmer's initiative; therefore, the accuracy of the methodologies used depends on the improvement of data collection by the official veterinary service.

Data on the losses caused by bovine rabies in other countries are also very imprecise. In Central and South America, the estimated loss for the disease is 100,000 to 500,000 cattle per year, but there is insufficient data to accurately determine these figures and fatality cases are estimated to be greater than the figures presented (Swanepoel 2004). There is a shortage of data on bovine rabies; in several countries where more accurate epidemiological information on canine rabies and rabies in wild animals, exist (Swanepoel 2004). An earlier study shows that in the Americas, the annual cattle loss varies from two to 32,200 animals, with an economic impact of US$ 5,000 to US$ 22 million (Acha & Malaga-Alba 1968). The economic impact of the disease is also evaluated from public health, mainly the cost with pre and post-exposure treatment in humans.

The total cost of vaccination would be less than US$ 200.00 in 45 of the 52 properties surveyed in the current report. When the cost of vaccination is compared to the losses caused by rabies-associated deaths, regardless of the size of the herd, the cost of the vaccination averaged 9.74% of the estimated economic loss, demonstrating that vaccination is an efficient and economically feasible procedure for rabies control. Although rabies vaccination is widely recommended in Brazil (Brasil 2009), there are not detailed reports on the efficiency of this procedure in the mitigation of the economic losses. In a study carried out in Mexico (Anderson et al. 2012), which compared the economic impact of bovine rabies-cost with two methods of its prevention, it was concluded that vaccination of cattle is more economically beneficial to the farmer than the control of vampire bats.

The data obtained in the present study demonstrate that the correct evaluation of the damages caused by rabies requires an adequate methodology based on the collection of individualized data on each occurrence of the disease. Considering that 43.57% of the MS cattle herd is in an area of risk for rabies, the morbidity ratios cannot be extrapolated to the total State herd.

The morbidity ratios can be higher than those described in the present study since our data considered deaths reported at the time of the outbreak, which is most likely lower than if the final outbreak data. The absence of standardization in data collection procedures is also a limiting factor for the elaboration of an efficient model for estimating the economic losses caused by bovine rabies.

CONCLUSIONS

Rabies is a cause of economic losses for the cattle industry in Mato Grosso do Sul. Vaccination is an economically feasible sanitary measure to minimize losses regardless of the size of the herd.

It is necessary to improve the efficiency in data collection by the rabies surveillance system in order to better evaluated the economic losses in the outbreaks.

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

REFERENCES

Acha P.N. & Malaga-Alba A. 1968. Economic losses due to *Desmodus rotundus*, p.207-214. In: Greenhall A.M. & Schmidt U. (Eds), Natural History of Vampire Bats. CRC Press, Boca Raton.

Anderson A.M., Shwiff S.A., Gebhardt K., Ramırez A.J., Shwiff S., Kohler D. & Lecuona L. 2012. Economic evaluation of vampire bat (*Desmodus rotundus*) rabies prevention in Mexico. Staff Publications 1091, USDA National Wildlife Research Center. Available at <http://digitalcommons.unl.edu/icwdm_usdanwrc/1091> Accessed on Mar. 1, 2018.

Andrade J., Azevedo S., Peconick A.P., Pereira S., Barçante J., Vilar A.L. & Silva M. 2014. Estudo retrospectivo da raiva no Estado da Paraíba, Brasil, 2004 a 2011. Braz. J. Vet. Res. Anim. Sci. 51(3):212-219. <http://dx.doi.org/10.11606/issn.1678-4456.v51i3p212-219>

Anyiam F., Lechenne M., Mindekem R., Oussigéré A., Naissengar S., Alfaroukh I.O., Mbilo C., Moto D.D., Coleman P.G., Probst-Hensch N. & Zinsstag J. 2017. Cost-estimate and proposal for a development impact bond for canine rabies elimination by mass vaccination in Chad. Acta Tropica 175:112-120. <http://dx.doi.org/10.1016/j.actatropica.2016.11.005> <PMid:27889225>

Barros C.S.L., Driemeier D., Dutra I.S. & Lemos R.A.A. 2006. Rabies, p.21-28. In: Ibid. (Eds), Doenças do Sistema Nervoso de Bovinos no Brasil. Valée, Montes Claros.

Braga G.B., Grisi-Filho J.H.H., Leite B.M., Sena E.F. & Dias R.A. 2014. Predictive qualitative risk model of bovine rabies occurrence in Brazil. Prev.Vet. Med. 113(4):536-546. <http://dx.doi.org/10.1016/j.prevetmed.2013.12.011> <PMid:24433635>

Brasil. 2009. Controle da Raiva dos Herbívoros: manual técnico. 2ª ed. Secretaria de Defesa Agropecuária, Ministério da Agricultura, Pecuária e Abastecimento, Brasília. 124p.

CEPEA 2017. Centro de Estudos Avançados em Economia Aplicada. Available at <http://www.cepea.esalq.usp.br/br/indicador/boi-gordo.aspx> Accessed on Mar. 1, 2018.

Dhankhar P., Vaidya S.A., Fishbien D.B. & Meltzer M.I. 2008. Cost effectiveness of rabies post exposure prophylaxis in the United States. Vaccine 26(33):4251-4255. <http://dx.doi.org/10.1016/j.vaccine.2008.05.048> <PMid:18599167>

Dognani R., Pierre E.J., Silva M.C.P., Patrício M.A.C., Costa S.C., Prado J.R. & Lisbôa J.A.N. 2016. Epidemiologia descritiva da raiva dos herbívoros notificados no estado do Paraná entre 1977 e 2012. Pesq. Vet. Bras. 36(12):1145-1154. <http://dx.doi.org/10.1590/s0100-736x2016001200001>

Hampson K., Coudeville L., Lembo T., Sambo M., Kieffer A., Attlan M., Barrat J., Blanton J.D., Briggs D.J., Cleaveland S., Costa P., Freuling C.M., Hiby E., Knopf L., Leanes F., Meslin F.X., Metlin A., Miranda M.E., Müller T., Nel L.H., Recuenco S., Rupprecht C.E., Schumacher C., Taylor L., Vigilato M.A., Zinsstag J. & Dushoff J. 2015. Estimating the global burden of endemic canine rabies. PLoS Negl. Trop. Dis. 9(4):e0003709. <http://dx.doi.org/10.1371/journal.pntd.0003709> <PMid:25881058>

Heckler R.F., Lemos R.A.A., Gomes D.C., Dutra I.S., Silva R.O.S., Lobato F.C.F., Ramos C.A.N. & Brumatti R.C. 2018. Blackleg in cattle in the state of Mato

Grosso do Sul, Brazil: 59 cases. Pesq. Vet. Bras. 38(1):6-14. <http://dx.doi.org/10.1590/1678-5150-pvb-4964>

Heinemann M.B., Fernandes-Matioli F.M.C., Cortez A., Soares R.M., Sakamoto S.M., Bernardi F., Ito F.H., Madeira A.M.B.N. & Richtzenhain L.J. 2002. Genealogical analyses of rabies virus strains from Brazil based on N gene alleles. Epidemiol. Infect. 128(3):503-511. <http://dx.doi.org/10.1017/S095026880200688X> <PMid:12113496>

IBGE 2016. Tabela 3939, efetivo de rebanhos. Sistema IBGE de Recuperação Automática (SIDRA), Intituto Brasileiro de Geografia e Estatística. Available at <https://sidra.ibge.gov.br/tabela/3939#resultado> Accessed on Mar. 1, 2018.

King A.A. & Turner G.S. 1993. Rabies: a review. J. Comp. Pathol. 108(1):1-39. <http://dx.doi.org/10.1016/S0021-9975(08)80224-1> <PMid:8473555>

Knobel D.L., Cleaveland S., Coleman P.G., Fèvre E.M., Meltzer M.I., Miranda M.E., Shaw A., Zinsstag J. & Meslin F.X. 2005. Re-evaluating the burden of rabies in Africa and Asia. Bull. World Health Org. 83(5):360-368. <PMid:15976877>

Kotait I., Gonçalves C.A., Peres N.F., Souza M.C.A.M. & Tarqueta M.C. 1998. Controle da Raiva dos Herbívoros: manual 1. Instituto Pasteur, São Paulo. 15p.

Langohr I.M., Irigoyen L.F., Lemos R.A.A. & Barros C.S.L. 2003. Aspectos epidemiológicos, clínicos e distribuição das lesões histológicas no encéfalo de bovinos com raiva. Ciência Rural 33(1):125-131. <http://dx.doi.org/10.1590/S0103-84782003000100020>

Lemos R. 2005. Enfermidades do sistema nervoso de bovinos de corte das Regiões Centro-Oeste e Sudeste do Brasil. Doctoral Dissertation, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Jaboticabal, SP. 149p.

Lima E.F., Riet-Correa F., Castro R.S., Gomes A.B. & Lima F.S. 2005. Sinais clínicos, distribuição das lesões no sistema nervosos e epidemiologia da raiva em herbívoros na região Nordeste do Brasil. Pesq. Vet. Bras. 25(4):250-264. <http://dx.doi.org/10.1590/S0100-736X2005000400011>

Marcolongo-Pereira C., Sallis E.S.V., Grecco F.B., Raffi M.B., Soares M.P. & Schild A.L. 2011. Raiva em bovinos na Região Sul do Rio Grandedo Sul: epidemiologia e diagnóstico imuno-histoquímico. Pesq. Vet. Bras. 31(4):331-335. <http://dx.doi.org/10.1590/S0100-736X2011000400010>

Meltzer M.I. & Rupprecht C.E. 1998. A review of the economics of the prevention and control of rabies. Part 2: rabies in dogs, livestock and wildlife. Pharmacoeconomics 14(5):481-498. <http://dx.doi.org/10.2165/00019053-199814050-00003> <PMid:10344914>

Mori A.E. & Lemos R.A.A. 1998. Raiva, p.47-58. In: Ibid (Eds), Principais Enfermidades de Bovinos de Corte do Mato Grosso do Sul: reconhecimento e diagnóstico. Universidade Federal de Mato Grosso do Sul, Campo Grande, MS.

Oliveira M.S., Frota F.P., Carvalho M.P., Bersano P.R.O., Dias F.E.F., Cavalcante T.V., Santos H.D. & Minharro S. 2013. Frequência da raiva em herbívoros e humanos no estado do Tocantins de 1999 a 2010: relatório técnico. Acta Vet. Bras. 7(3):180-183.

Ribas N.L.K.S., Carvalho R.I., Santos A.C., Valençoela R.A., Gouveia A.F., Castro M.B., Mori A.E. & Lemos R.A.A. 2013. Doenças do sistema nervoso de bovinos no Mato Grosso do Sul: 1082 casos. Pesq.Vet. Bras. 33(10):1183-1194. <http://dx.doi.org/10.1590/S0100-736X2013001000003>

Rupprecht C.E., Hanlon C.A. & Hemachudha T. 2002. Rabies re-examined. Lancet Infect. Dis. 2(6):327-343. <http://dx.doi.org/10.1016/S1473-3099(02)00287-6> <PMid:12144896>

Sanches A.W.D., Langohr I.M., Stigger A.L. & Barros C.L.S. 2000. Doenças do sistema nervoso central em bovinos no Sul do Brasil. Pesq. Vet. Bras. 20(3):113-118. <http://dx.doi.org/10.1590/S0100-736X2000000300005>

Shwiff S.A., Sterner R.T., Jay M.T., Parikh S., Bellomy A., Meltzer M.I., Rupprecht C.E. & Slate D. 2007. Direct and indirect costs of rabies exposure: a retrospective study in Southern California (1998-2002). J. Wildl. Dis. 43(2):251-257. <http://dx.doi.org/10.7589/0090-3558-43.2.251> <PMid:17495309>

Silva A.C.R., Caporale G.M.M., Gonçalves C.A., Targueta M.C., Comin F., Zanetti C.R. & Kotait I. 2000. Antibody response in cattle after vaccination with inactivated and attenuatedrabies vaccines. Revta Inst. Med. Trop. S. Paulo 42(2):95-98. <http://dx.doi.org/10.1590/S0036-46652000000200006>

Silva J.A., Moreira E.C., Haddad J.P.A., Modena C.M. & Tubaldini M.A.S. 2001. Distribuição temporal e espacial da raiva bovina em Minas Gerais, 1976 a 1997. Arq. Bras. Med. Vet. Zoot. 53(3):263-272. <http://dx.doi.org/10.1590/S0102-09352001000300001>

Sterner R.T., Meltzer M.I., Shwiff S.A. & Slate D. 2009. Tactics and economics of wildlife oral rabies vaccination, Canada and the United States. Emerg. Infect. Dis. 15(8):1176-1184. <http://dx.doi.org/10.3201/eid1508.081061> <PMid:19757549>

Swanepoel R. 2004. Rabies, p.1123-1182. In: Coetzer J. A. W., Tustin R.C. (Eds), Infectious Diseases of Livestock. Vol.2. 2nd ed. Oxford University Press, Cape Town.

Taylor L.H. & Knopf L. 2015. Surveillance of human rabies by national authorities, a global survey. Zoonoses Public Health 62(7):543-552. <http://dx.doi.org/10.1111/zph.12183> <PMid:25683444>

Teixeira T.F., Holz C.L., Caixeta S.P.M.B., Dezen D., Cibulski S.P., Silva J.R., Rosa J.C.A., Schmidt E., Ferreira J.C., Batista H.B.C.R., Caldas E., Franco A.C. & Roehe P.M. 2008. Diagnóstico de raiva no Rio Grande do Sul, Brasil, de 1985 a 2007. Pesq. Vet. Bras. 28(10):515-520. <http://dx.doi.org/10.1590/S0100-736X2008001000012>

Thrusfield M.V. 2004. Descrevendo a ocorrência de doenças, p.47-73. In: Ibid. (Ed), Epidemiologia Veterinária. Roca, São Paulo.

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Original Article
Small Animal Diseases

Protein-to-creatinine urinary in the early diagnosis of renal injury in canine pyometra[1]

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ABSTRACT.- Sant'Anna M.C., Martins G.F., Flaiban K.K.M.C., Trautwein L.G.C. & Martins M.I.M. 2019. **Protein-to-creatinine urinary in the early diagnosis of renal injury in canine pyometra.** *Pesquisa Veterinária Brasileira 39(3):186-191*. Departamento de Clínicas Veterinárias, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, Londrina, PR 86061-900, Brazil. E-mail: imartins@uel.br

Kidney disease that affects bitches with pyometra may lead patients to develop chronic renal failure even after pyometra treatment. Therefore, several studies have sought to clarify the gaps in the understanding of the pathogenesis of renal injury in pyometra. Identification of early detection markers for renal damage, which can predict and identify the prognosis of the disease, is very important. Proteinuria analysis can diagnose kidney damage, since proteins such as albumin are not filtered through the glomerulus and those that undergo glomerular filtration are almost completely reabsorbed by tubular cells. The objective of this study was to evaluate whether the urinary protein-to-creatinine ratio (UPC) can detect renal injury in bitches with pyometra before development of azotemia. For this, 44 bitches with pyometra were divided into two groups: bitches with azotemic piometra (A, n=15, creatinine >1.7) and bitches with non-azotemic pyometra (NA, n=29). The two groups were compared to the control group (CG, n=12), which had no signs of systemic disease. All animals underwent blood and urine tests. Leukocytosis was more evident in bitches in the A group than in the other groups. This shows that the inflammatory response may be associated with the pathogenesis of renal injury. The median UPC in bitches with pyometra was significantly higher than in the CG, with a median above the reference values. In conclusion, the UPC can be used in bitches with pyometra to detect renal damage before the development of azotemia. It has been suggested that the UPC of bitches with pyometra should be followed through during the postoperative period so that permanent renal lesions secondary to pyometra can be diagnosed and treated properly before the development of azotemia.

INDEX TERMS**:** Protein, creatinine, urinary, renal injury, canine pyometra, UPC, cystic endometrial hyperplasia, proteinuria, bitches, dogs, surgery.

RESUMO.- [**Relação proteína-creatinina-urinária no diagnóstico precoce de lesão renal em cadelas com piometra.**] A doença renal que afeta cadelas com piometra pode levar a insuficiência renal crônica mesmo após o tratamento. Portanto, vários estudos procuraram esclarecer as lacunas na compreensão da patogênese da lesão renal na piometra. A identificação de marcadores de lesão renal precoce, que podem prever e identificar o prognóstico da doença é muito importante. A análise da proteinúria pode diagnosticar lesão renal, uma vez que proteínas como a albumina não são filtradas através do glomérulo e aquelas que sofrem filtração glomerular são quase completamente reabsorvidas pelas células tubulares. O objetivo deste estudo foi avaliar se a relação proteína-creatinina urinária (UPC) pode detectar lesão renal em cadelas com piometra antes do desenvolvimento de azotemia. Para isso, 44 cadelas com piometra foram divididas em dois grupos: cadelas com piometra azotêmica (A, n=15, creatinina >1,7) e cadelas com piometra não azotêmica (NA, n=29). Os dois grupos foram comparados

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ao grupo controle (CG, n=12), que não apresentaram sinais de doença sistêmica. Todos os animais foram submetidos a exames de sangue e urina. A leucocitose foi mais evidente nas cadelas do grupo A do que nos outros grupos. Isso mostra que a resposta inflamatória pode estar associada à patogênese da lesão renal. A mediana da UPC em cadelas com piometra foi significativamente maior que no CG, com uma mediana acima dos valores de referência. Em conclusão, a UPC pode ser usada em cadelas com piometra para detectar lesões renais antes do desenvolvimento de azotemia. Sugeriu-se que a UPC de cadelas com piometra deve ser acompanhada durante o pós-operatório, de modo que as lesões renais permanentes secundárias à piometra possam ser diagnosticadas e tratadas adequadamente antes do desenvolvimento de azotemia.

TERMOS DE INDEXAÇÃO: Proteína, creatinina, sistema urinário, lesão renal, cadelas, piometra, UPC, hiperplasia endometrial cística, proteinúria, caninos, cirurgia.

INTRODUCTION

Canine pyometra mainly affects adult and elderly bitches during diestrus after uterine contamination by bacteria of the vaginal microbiota during estrus. In this period of the estrous cycle, the bacteria find a favorable uterine environment that is affected by cystic endometrial hyperplasia (Hagman 2004, Pretzer 2008, Verstegen et al. 2008). *Escherichia coli* is the Gram-negative bacteria most frequently isolated, since it has virulence factors that facilitate its adhesion in the endometrium under the influence of progesterone (Hagman & Kuhn 2002, Bassessar et al. 2013, Hagman 2017).

Renal damage is common in bitches with pyometra, but it is not fully understood yet. The theory that glomerulonephritis occurs secondary to the deposition of immunocomplexes that are formed because of the inflammatory response, which are triggered by the excess of *E. coli* antigens, has been accepted for a long time (Johnston et al. 2001, Fieni 2006). However, with the development of new renal injury markers that have the capacity to identify the affected renal compartment, this pathogenesis has been contested. The hypothesis that has been most accepted in the last decade is that involving glomerular and tubular renal injury, but there is no evidence of glomerulonephritis (Heiene et al. 2007, Maddens et al. 2010, 2011).

Renal insufficiency that developed during pyometra is one of the main prognostic factors of the affection (Kuplulu et al. 2009, Sant' Anna et al. 2014). Therefore, the search for markers of early renal damage that may predict the evolution of the disease is necessary, since serum creatinine, which has low sensitivity, is still the most commonly used marker in the clinical routine to detect this change in bitches with pyometra (De Loor et al. 2013, Pressler 2013).

In this context, the urine is easy to collect, and contains small amounts of protein; persistent proteinuria is related to a worse prognosis in patients with chronic kidney disease (Grauer 2005, 2011). The study of proteinuria during the development of pyometra and the comparison of patients with different levels of renal damage can provide important information about the evolution of the disease, since a portion of patients remain proteinuria even after clinical recovery, being at elevated risk in the development of chronic renal failure.

The aim of this study was to evaluate whether urinary protein-to-creatinine (UPC) can detect renal injury in bitches with pyometra before the development of azotemia.

MATERIALS AND METHODS

Ethics statement. All the procedures in this study were submitted and approved by the animal ethics and experimentation committee.

Animals. The study included 44 bitches with pyometra and 12 bitches with no systemic symptomatology (control group). No breed was over-represented. The bitches with pyometra received clinical support and surgical treatment according to the clinical routine adopted for the treatment.

The diagnosis of the infection was based on patient history and clinical examination, and hematological exams and abdominal ultrasonography performed at the admission of the patients and confirmed during the surgical procedure.

The inclusion criterion for the pyometra group was diagnosis based on the tests used. Exclusion was based on suspected or confirmed concomitant disease.

The inclusion criteria for the control group were intact bitches, adult or elderly, who sought hospital care for surgeries, such as elective ovariohysterectomy (OH), unilateral mastectomy due to single mammary neoplasia (less than 3cm), and periodontal treatment. Exclusion criteria for this group were when any systemic clinical abnormality was identified during anamnesis, general physical examination, blood count and plasma biochemistry (creatinine, alamino aminotransferase and glucose) performed at admission.

Bitches with pyometra were divided into two groups based on plasma creatinine levels and were considered azotemic when creatinine was greater than 1.7mg/dL. The pyometra non-azotemic group (NA) consisted of 29 bitches and the pyometra azotemic group (A) contained 15 animals. A third group was formed by 12 bitches in the control group (CG).

The mean age of the NA dogs was 8.0±4.0 years, the A dogs was 10.0±3.0 years and the CG was 9.5±3.0 years. The mean weight of the NA dogs was 16.0kg, the A dogs was 14.5kg and the CG was 11.0kg. Both age and weight were not significantly different between groups.

All patients underwent blood collection by puncture of the external jugular vein at admission. The sample was placed in a tube containing EDTA and sent to the laboratory for testing (complete blood count and creatinine, alanine aminotransferase and glucose dosage). The blood count result was used in the tabulation of the data and the biochemical results for the inclusion and exclusion criteria of the animals. Subsequently, all animals underwent a new blood collection after anesthetic induction with propofol. This sample was used to obtain the serum, after centrifugation for 5 minutes at 1,500g. The resulting supernatant was stored at -20°C for evaluation of serum biochemistry.

Urine collection was performed by trans-surgical cystocentesis for bitches with pyometra or bitches of the CG group that passed through OH. Ultrasound-guided cystocentesis was used in the other bitches of the CG. After urinalysis, the supernatant was stored at -20°C, which was used for UPC.

The UPC was performed only on urine samples without sediment. As an exclusion criterion, samples with more than five leukocytes and/or erythrocytes per field in the urinary sediment analysis were not included in the UPC assessment. The urinary density analysis was not performed in this study, since bitches with pyometra were given fluid therapy before the surgical procedure.

Hematology, biochemistry, urinalysis and UPC. The globular volume, hemoglobin, total red cell count and total leukocyte count were performed in an automated hematological analyzer. The differential leukocyte count was performed on a blood smear with Romanowisky-type staining (Fast Panoptic, Laborclin®, Pinhais,

Brazil) together with platelet estimation, which was averaged over five observed fields.

The determination of alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea, creatinine and urinary creatinine were performed by the kinetic method. Proteins were determined by the biuret method for total protein, the colorimetric method using pyrogallol red for urinary protein and the colorimetric method for albumin, all with spectrophotometer reading (BS-120 Mindray®, China). The UPC was calculated by dividing the urinary protein by the urinary creatinine (Thrall 2007).

The urinalysis was performed to evaluate the urinary sediment, which was obtained after centrifugation for 5 minutes at 1,500g and visualization of the sediment under a Neubauer camera microscopy. RBCs, leukocytes and bacteria were expressed in number per field.

Statistical analysis. The variables were analyzed using the statistical program Bioestat 5.0. Variables were tested for distribution by the Shapiro Wilk test. ANOVA followed by Tukey's test compared the variables with normal distribution (red blood cells, hematocrit and hemoglobin). Variables that did not present a normal distribution (albumin, total proteins, ALT, FA, urea, creatinine, urinary protein, urinary creatinine and UPC) were compared by the Kruskal Wallis test followed by the Dunn test. The level of significance was $p<0,05$.

RESULTS

Hematological parameters were different between the groups of bitches with pyometra and the CG (Table 1). Among the hematological variables of the red series, the number of red blood cells, the hemoglobin level and the globular volume were smaller in the NA and A groups compared to the control group, but there was no difference between the NA and A groups. The median, total leukocytes, segmented neutrophils and band neutrophils were significantly higher in bitches with pyometra than in the CG. In addition, the median total and segmented leukocytes of group A were higher than in the NA group. The number of lymphocytes and platelets was not different between the groups, although the median number of platelets in group A was lower than the normal reference value for the canine species (Thrall 2007).

Among the biochemical parameters, serum albumin was significantly lower in dogs with pyometra than in the CG group, but there was no difference between the NA and A groups (Table 2).

The urinary creatinine concentration was significantly higher in the control group than in the NA group, but there was no difference between the NA and A groups. On the other hand, the concentration of the urinary protein of group A was higher than in the NA group and in the CG group (Table 3).

The median of the UPC in the control group was 0.23, ranging from 0.14 to 0.49. Therefore, all patients presented with a UPC within the reference values (Table 3). The NA group had a median UPC of 0.95, ranging from 0.02 to 5.53. In this group, 8/21 (38.1%) presented a UPC <0.5, 4/21 (19%), ranging from 0.5 to 1, while 9/21 (42.8%) presented UPC >1.

DISCUSSION

Studies comparing the erythrogram parameters between bitches with pyometra and healthy bitches also have found similar findings (Hagman et al. 2006, Emanuelli et al. 2012). Bitches with pyometra developed mild to moderate anemia, since the mean globular volume of the NA and A groups were smaller than the bitches of the CG and lower than the reference values (Table 1). Anemia in canine pyometra is due to endotoxemia and sepsis secondary to uterine infection, which promotes decreased red blood cell survival and decreased bone marrow response to erythropoietin and is usually classified as moderate, aregenerative, normocytic and normochromic anemia (Pretzer 2008, Verstegen et al. 2008).

Leukocytosis in pyometra is characterized by an important regenerative deviation marked by the presence of rods in the circulation in response to infection, although in severe and/or chronic cases, leucopenia with degenerative deviation can be observed, indicating bone marrow depletion (Emanuelli et al. 2012, Hagman 2017). Acute renal disease may be due to decreased tissue perfusion associated with septic shock, and ischemic processes usually lead to tubular renal damage, as this part of the nephron is more metabolically active (Grauer 2005). Therefore, the association of more

Table 1. Erythrogram variables, expressed as the mean and standard deviation and leukocytes, expressed as median, minimum and maximum, and compared between the non-azotemic pyometra, azotemic pyometra and control groups

Hematologic parameters	Non-azotemic (n = 29)	Azotemic (n = 15)	Control (n = 12)
Blood cells ($x10^6$)	5.05 (±1.90)^A	5.45 (±1.50)^A	7.27 (±1.09)^B
Hemoglobin (g/dl)	10.7± 4.2^A	11.6 ± 3.1^A	16.1 ± 2.1^B
Globular volume (%)	33.8 ± 8.2^A	33.4 ± 9.0^A	47.1 ± 5.9^B
Total leukocytes (m/mm^3)	26,400^A (9,100-75,600)	43,500^B (18,700-101,300)	8,650^C (5,500-16,660)
Segmented (m/mm^3)	18,427^A (3,549-70.560)	35,002^B (10,285-75,975)	6,142^C (3,294-13,446)
Bands (m/mm^3)	760^A (0-11,022)	1.347^A (0-15,195)	0^B (0-282)
Lymphocytes (m/mm^3)	2,526 (1,088-7,384)	2,694 (959-9,117)	1,494 (880-2,852)
Platelets (m/mm^3)	225,000 (28,000-990,000)	166,000 (39,000-897,000)	250,000 (102,000-382,000)

$^{A, B, C}$ Different letters between columns show significant difference.

Table 2. Biochemical variables expressed in median, minimal and maximal and compared between the non-azotemic, azotemic and control groups

Biochemical parameters	Non-azotemic (n = 29)	Azotemic (n = 15)	Control (n = 12)
Albumin (g/dl)	1.6[A] (1.2-3.7)	1.7[A] (1.0-2,7)	2.2[B] (1.3-2.7)
Total proteins (g/dl)	6.3 (4.4-10.5)	6.3 (3.6-9.7)	5.4 (3.6-6.8)
FA (U/L)	81 (36-1,173)	142 (30-942)	33.5 (22-500)
ALT (U/L)	11[A] (4-95)	7[A] (2-316)	32.5[B] (16-168)
Creatinine (mg/dl)	0.9[A] (0.5-1.5)	3.3[B] (1.5-11.1)	0.7[A] (0.3-1.6)
Urea (mg/dl)	27[A] (19-59)	115[B] (53-445)	40[C] (25-96)

[A, B, C] Different letters between columns show significant difference.

Table 3. Urinary parameters, expressed as median, minimum and maximum, and compared between the non-azotemic, azotemic, and control groups

Urinary parameters	Non-azotemic (n = 21)	Azotemic (n = 6)	Control (n = 11)
Urinary creatinine (mg/dl)	42.5[A] (7.5-175)	85[AB] (27.5-217.5)	145[B] (40-345)
Urinary protein (mg/dl)	34.4[A] (1.2-167.2)	114.4[B] (79,4-252,3)	38.3[A] (10.4-97.6)
UPC	0.95[A] (0.02-5.53)	1.67[A] (0.52-3.02)	0.23[B] (0.14-0.49)

[A, B, C] Different letters between columns show significant difference.

severe leukocytosis in azotemic patients found in this study may indicate that renal hypo-perfusion secondary to sepsis is an important mechanism of worsening renal injury in bitches with pyometra.

Dogs with sepsis may develop thrombocytopenia due to the formation of platelet aggregates secondary to the action of lipopolysaccharides and interaction with neutrophils (Li & Chan 2016), justifying the findings about lymphocytes and platelets.

Hypoalbuminemia in bitches is an inflammatory condition of infectious origin, mainly by Gram-negative bacteria, which may occur secondary to decreased liver production and/or increased vascular permeability, both due to the release of endotoxins (Greiner et al. 2008).

The difference found in creatinine and urea values between groups NA and A was expected, since the groups were formed based on creatinine values (Table 2). This is the differential of this study, with a significant number of bitches with non-azotemic and azotemic pyometra. Therefore, this allows the evaluation of UPC as a marker for early renal damage in bitches with pyometra.

Kidney damage caused during canine pyometra has long been credited to the formation of immune complexes in the circulation and subsequent deposition in the basement membrane of the glomerulus, causing glomerulonephritis (Johnston et al. 2001, Fieni 2006). However, this concept has been well-studied. Through the histopathological analysis of renal biopsies and the use of biomarkers of renal injury, studies have found that the renal compartment that is most affected in bitches with pyometra are tubular cells, and that the glomerular changes are similar to those found in healthy bitches of the same age group (Heiene et al. 2001, Zaragoza et al. 2004, Heiene et al. 2007, Maddens et al. 2010).

Serum creatinine and urinalysis are the methods used in clinical practice to detect and assess the extent of renal damage. However, serum creatinine increase and renal inability to concentrate urine only occurs after severe renal impairment. In addition to being less sensitive methods, they do not differentiate tubular and glomerular renal damage (Pressler 2013). On the other hand, proteinuria may indicate renal damage prior to the development of azotemia in dogs with chronic kidney disease and serves as an indicator to determine the severity of renal disease, which can be determined by UPC (Grauer 2005). In this study, the urinary creatinine concentration was significantly higher in the control group than in the NA group, but there was no difference between the NA and A groups. On the other hand, the concentration of the urinary protein of group A was higher than in the NA group and in the CG group (Table 3). These results have shown that proteinuria in bitches with pyometra and renal insufficiency is more evident, and reflects the severity of renal damage in group A. Renal proteinuria may be triggered by increased glomerular filtration of plasma proteins associated with intraglomerular hypertension, the

presence of immunocomplexes, vascular inflammation in the glomerular capillaries, or structural defects in the basement membrane of the glomerulus. In addition, proteinuria may be tubular in origin, resulting in decreased tubular reabsorption of the plasma filtrate (De Loor et al. 2013).

Most of the time, dogs are considered to have proteinuria when the UPC >0.5. A UPC of 0.5 to 1 is usually associated with proteinuria of tubular origin and UPC >1 is associated with glomerular proteinuria (Grauer 2011, De Loor et al. 2013). The UPCs results show that bitches with pyometra without renal insufficiency have high UPC, most of them suggestive of glomerular lesion (42.8%) and 19% suggestive of tubular lesions. However, 38.1% were not considered proteinuric when we evaluated the UPC. This demonstrates the need for the use of more sensitive renal injury markers than the UPC for such patients, preventing false negative results.

In group A, the median UPC was 1.67 and ranged from 0.52 to 3.02. In this group A, none of the bitches presented UPC <0.5, 2/6 (33.3%) presented UPC between 0.5 and 1, and 4/6 (66.6%) presented UPC >1. These results demonstrate that when azotemic, bitches with pyometra show more evident proteinuria of glomerular origin.

Most bitches with pyometra of in both groups (NA and A) presented a UPC above the reference values for the species and significantly higher than the CG. Therefore, the UPC can be used to detect early renal damage in bitches with pyometra. Since UPC is a fast method and has lower cost, it should be used in the clinical routine for both early diagnosis of kidney injury in bitches with pyometra and in follow-up after clinical recovery from the infectious disease. As such, the bitches may become proteinuric and are more likely to develop chronic kidney failure if not treated correctly. Maddens et al. (2011) studied some markers of early renal damage in bitches with pyometra, and among them UPC. They also found a significant difference between the pyometra and control groups. The pyometra group was not separated in azotemic and non-azotemic dogs and it was not possible to evaluate if the UPC could detect renal damage before the development of azotemia.

CONCLUSIONS

The UPC can be used in bitches with pyometra to detect renal damage before the development of azotemia.

In addition, we suggest that bitches that had pyometra are followed up by urinalysis and UPC during the postoperative period, aiming at the diagnosis of permanent renal lesions.

As such, these patients will be treated properly prior to the development of azotemia, increasing the survival and quality of life of patients.

REFERENCES

Bassessar V., Verma Y. & Swamy M. 2013. Antibiogram of bacterial species isolated from canine pyometra. Vet. World 6(8):546-549. <http://dx.doi.org/10.5455/vetworld.2013.546-549>

De Loor J., Daminet S., Smets P., Maddens B. & Meyer E. 2013. Urinary biomarkers for acute kidney injury in dogs. J. Vet. Intern. Med. 27(5):998-1010. <http://dx.doi.org/10.1111/jvim.12155> <PMid:23952327>

Emanuelli M.P., Martins D.B., Wolkmer P., Antoniazzi A.Q., Emanuelli T., de Vargas A.C. & dos Anjos Lopes S.T. 2012. Complete blood count, total plasma protein, neutrophil oxidative metabolism, and lipid peroxidation in female dogs with pyometra associated with *Escherichia coli.* Comp. Clin. Pathol. 21(3):309-313. <http://dx.doi.org/10.1007/s00580-010-1097-z.

Fieni F. 2006. Patologia de los ovários y el útero, p.75-89. In: Wanke M.M. & Gobello C. Reproduccion en Caninos y Felinos Domesticos. Ed. Inter-Médica, Buenos Aires.

Grauer G.F. 2005. Early detection of renal damage and disease in dogs and cats. Vet. Clin. N. Am., Small. Anim. Pract. 35(3):581-596. <PMid:15833560>

Grauer G.F. 2011. Proteinuria: measurement and interpretation. Topics Companion Anim. Med. 26(3):121-127. <http://dx.doi.org/10.1053/j.tcam.2011.04.002> <PMid:21782142>

Greiner M., Wolf G. & Hartmann K. 2008. A retrospective study of the clinical presentation of 140 dogs and 39 cats with bacteraemia. J. Small Anim. Pract. 49(8):378-383. <http://dx.doi.org/10.1111/j.1748-5827.2008.00546.x> <PMid:18422500>

Hagman R. 2004. New aspects of canine pyometra: studies on epidemiology and pathogenesis. Doctoral Dissertation, Swedish University of Agricultural Sciences, Uppsala. 55p.

Hagman R. 2017. Canine pyometra: what is new? Reprod. Domest. Anim. 52(Suppl.2):288-292. <PMid:27807901>

Hagman R. & Kuhn I. 2002. *Escherichia coli* strains isolated from the uterus and urinary bladder of bitches suffering from pyometra: Comparison by restriction enzyme digestion and pulsed-field gel electrophoresis. Vet. Microbiol. 84(1/2):143-153. <http://dx.doi.org/10.1016/S0378-1135(01)00449-7> <PMid:11731167>

Hagman R., Kindahl H., Fransson B.A., Bergstrom A., Holst B.S. & Lagerstedt A.S. 2006. Differentiation between pyometra and cystic endometrial hyperplasia/mucometra in bitches by prostaglandin F2α metabolite analysis. Theriogenology 66(2):198-206. <http://dx.doi.org/10.1016/j.theriogenology.2005.11.002> <PMid:16375957>

Heiene R., Kristiansen V., Teige J. & Jansen J.H. 2007. Renal histomorphology in dogs with pyometra and control dogs, and long term clinical outcome with respect to signs of kidney disease. Acta Vet. Scand. 49(1):13. <http://dx.doi.org/10.1186/1751-0147-49-13> <PMid:17480218>

Heiene R., Moe L. & Molmen G. 2001. Calculation of urinary enzyme excretion, with renal structure and function in dogs with pyometra. Res. Vet. Sci. 70(2):129-137. <http://dx.doi.org/10.1053/rvsc.2000.0451> <PMid:11356092>

Johnston S.D., Kustritz M.V.R. & Olson P.N.S. 2001. Canine and Feline Theriogenology. W.B. Saunders, Philadelphia. 592p.

Kuplulu S., Vural M.R., Demirel A., Polat M. & Akçay A. 2009. The comparative evaluation of serum biochemical, haematological, bacteriological and clinical findings of dead and recovered bitches with pyometra in the postoperative process. Acta Vet. 59(2/3):193-204.

Li R.H.L. & Chan D.L. 2016. Evaluation of platelet function using multiple electrode platelet aggregometry in dogs with septic peritonitis. J. Vet. Emerg. Crit. Care, San Antonio, 26(5):630-638. <PMid:27428542>

Maddens B., Daminet S., Smets P. & Meyer E. 2010. *Escherichia coli* pyometra induces transient glomerular and tubular dysfunction in dogs. J. Vet. Intern. Med. 24(6):1263-1270. <http://dx.doi.org/10.1111/j.1939-1676.2010.0603.x> <PMid:21039862>

Maddens B., Heiene R., Smets P., Svensson M., Aresu L., van der Lugt J., Daminet S. & Meyer E. 2011. Evaluation of kidney injury in dogs with pyometra based on proteinuria, renal histomorphology, and urinary biomarkers. J. Vet. Intern. Med. 25(5):1075-1083. <http://dx.doi.org/10.1111/j.1939-1676.2011.0772.x> <PMid:21848947>.

Pressler B.M. 2013. Clinical approach to advanced renal function testing in dogs and cats. Vet. Clin. N. Am., Small Anim. Pract. 43(6):1193-1208. <http://dx.doi.org/10.1016/j.cvsm.2013.07.011> <PMid:24144085>

Pretzer S.D. 2008. Clinical presentation of canine pyometra and mucometra: a review. Theriogenology 70(3):359-363. <http://dx.doi.org/10.1016/j.theriogenology.2008.04.028> <PMid:18513791>

Sant'Anna M.C., Giordano L.G.P., Flaiban K.K.M.C., Muller E.E. & Martins M.I.M. 2014. Prognostic markers of canine pyometra. Arq. Bras. Med. Vet. Zootec. 66(6):1711-1717. <http://dx.doi.org/10.1590/1678-6859>

Thrall M.A. 2007. Hematologia e Bioquímica Clínica Veterinária. Roca, São Paulo. 592p.

Verstegen J., Dhaliwal G. & Verstegen-Onclin K. 2008. Mucometra, cystic endometrial hyperplasia, and pyometra in the bitch: advances in treatment and assessment of future reproductive success. Theriogenology 70(3):364-374. <http://dx.doi.org/10.1016/j.theriogenology.2008.04.036>. <PMid:18501959>.

Zaragoza C., Barrera R., Centeno F., Tapia J.A. & Mane M.C. 2004. Canine pyometra: a study of the urinary proteins by SDS-PAGE and Western blot. Theriogenology 61(7/8):1259-1272. <http://dx.doi.org/10.1016/j.theriogenology.2003.07.019> <PMid:15036960>

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Original Article
Small Animal Diseases

Causes of death and euthanasia in domestic cats in the Santa Catarina plateau (1995-2015)[1]

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ABSTRACT.- Withoeft J.A., Cristo T.G., Biezus G., Costa L.S., Dal Pont T.P., Freitas, A.C., Traverso S.D. & Casagrande R.A. 2019. **Causes of death and euthanasia in domestic cats in the Santa Catarina plateau (1995-2015)**. *Pesquisa Veterinária Brasileira 39(3):192-200*. Laboratório de Patologia Animal, Centro de Ciências Agroveterinárias, Universidade do Estado de Santa Catarina, Av. Luís de Camões 2090, Bairro Conta Dinheiro, Lages, SC 88520-000, Brazil. E-mail: renata.casagrande@udesc.br

Knowledge about the causes of death in felines constitutes important information to owners, veterinarians, and researchers, aiming at reducing the number of deaths in this species. In order to determine the main causes of death or euthanasia in cats in the Santa Catarina plateau, data from 1995 to 2015 available in necropsy files of the Laboratory of Animal Pathology (LAPA) of the State University of Santa Catarina (UDESC) were collected and evaluated. In that period, 1,728 cats were necropsied, mainly males (46.12%) and adults (50.11%). The mean ages at death for kittens, adults, and elderly were 5.07 months, 3.9 years, and 13.9 years, respectively. Of the 1,728 necropsy reports assessed, the cause of death was identified in 1,184 (68.52%) cases. The main cause of death was associated with infectious diseases (15.8%), with prevalence of feline infectious peritonitis (29.76%), followed by neoplasms (11.98%) with lymphoma (44.93%) and leukemia (16.91%) as the most common, and traumas (11.81%) mainly caused by motor vehicle accidents. These results show the need for owner awareness, as well as establishment of prophylaxis and vaccination programs, aimed at reducing the number of deaths and thus increasing life expectancy in the feline population.

INDEX TERMS: Euthanasia, domestic cats, Santa Catarina, mortality, infectious diseases, neoplasm, trauma, cats, necropsy.

RESUMO.- [**Causas de morte e eutanásia em felinos domésticos no Planalto de Santa Catarina (1995-2015).**] O conhecimento a respeito da *causa mortis* em felinos é importante para que se construa um informativo para proprietários, médicos veterinários e pesquisadores, objetivando a redução no número de mortes na espécie. Com o intuito de determinar as principais causas de morte ou eutanásia em felinos domésticos no planalto catarinense foram avaliados os arquivos de registro das necropsias do período de 1995 a 2015 do Laboratório de Patologia Animal da Universidade do Estado de Santa Catarina. No período, foram necropsiados 1.728 felinos, principalmente machos (46,12%), adultos (50,11%). A idade média para filhotes foi de 5,07 meses, enquanto para adultos foi 3,9 anos e para idosos 13,9 anos. Das 1.728 necropsias de felinos, a enfermidade que levou o animal a morte foi determinada em 1.184 (68,52%). As doenças infecciosas foram a principal causa de morte (15,8%), dentre as quais a peritonite infecciosa felina (29,76%) foi a mais frequente; seguida das neoplasias (11,98%), sendo o linfoma (44,93%) e a leucemia (16,91%), as mais comuns; e dos traumatismos (11,81%), principalmente atropelamentos por veículos automotivos. Estes resultados refletem a necessidade da conscientização dos proprietários, bem como da instituição de programas de profilaxia e vacinação, visando a redução de mortes e o aumento na expectativa de vida para a população felina.

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TERMOS DE INDEXAÇÃO: Eutanásia, felinos domésticos, Santa Catarina, mortalidade, doenças infecciosas, neoplasia, traumatismos, gatos, necropsia.

INTRODUCTION

Information on the causes of death of a given species constitutes an important tool to provide epidemiological studies with data, and thus enable the planning of methods for prevention and treatment of high-prevalence diseases (Santo 2007). The preparation of a set of statistical data on the causes of death of individuals is usually based on the primary cause of death, and do not include other associated factors that contribute to the death of the animal, as in chronic diseases. For this reason, few studies addressing the comprehensive causes of mortality of a given species have been conducted, which can be justified by the difficulty in obtaining reliable data for epidemiological analysis (Fighera et al. 2008).

Pets, particularly dogs and cats, are raised according to the routine of humans, which often leads to incorporation of bad habits into their way of life, and can deprive them of their natural behavior. These inflicted influences can contribute to reduction of their life expectancy (Berzins 2000). Estimates of survival for a given animal population is informative for current and prospective owners, veterinarians, and researchers, comparing differences and similarities in mortality rates across breed or gender preferences and suggesting theories about the causes and possible evolutions of a disease (Bonnett et al. 2005).

Knowledge about mortality in felines is applicable to the understanding and correlation between associated factors and specific characteristics, such as eating and living habits, breed, gender, age, and origin. Still from the epidemiological standpoint, it suggests patterns of occurrence of certain diseases, facilitating the establishment of differential diagnoses based on clinical manifestations or complementary tests, such as laboratory and histopathological analyses. From these findings, it provides subsidies to the establishment of prophylactic measures, promoting a better quality of life and reducing the percentage of deaths due to a specific illness, such as infectious diseases (Fighera et al. 2008).

In general, studies addressing the causes of death in felines in Brazil are scarce (Trapp et al. 2010, Togni et al. 2018), as well as epidemiological data on the prevalence of conditions associated with feline death in the Santa Catarina plateau region. Therefore, this study aimed to determine the most prevalent diseases in felines that died in the aforementioned region from data obtained through a retrospective analysis of the causes of death or euthanasia in animals necropsied in the Laboratory of Animal Pathology (LAPA), at the State University of Santa Catarina (UDESC), located in the municipality of Lages, Santa Catarina state, Brazil.

MATERIALS AND METHODS

Data on cats necropsied at the LAPA/UDESC from January 1995 to December 2015 were obtained through a retrospective analysis of necropsy files. Individuals of all age groups, breeds, and genders were included in the study. Due to lack of information on the records, data such as size, weight, coat, castration, and origin of the animals were not considered. The data were organized into spreadsheets that included diagnosis and classification of the diseases. Regarding age, animals were classified into three categories according to Trapp et al. (2010): kittens (≤1 year of age), adults (≥1 year <11 years), and elderly (>11 years of age).

The cause of death documented and assessed for the study corresponded to the macroscopic, as in trauma, or histopathological diagnoses. Aiming to facilitate the descriptive analysis of the data and interpretation of the results, all documented causes of death were classified as follows: degenerative diseases (including natural death); malformations; endocrine or metabolic diseases; infectious diseases, subdivided into viral, bacterial and fungal; intoxications and toxi-infections (of accidental, drug and criminal origin, in addition to disturbances caused by accumulation of endogenous toxins in the organism); neoplasms; nutritional disorders; parasitic diseases; traumas; other (diseases not included in the previously determined categories). Cases were considered inconclusive when the necropsy findings did not enable a concise diagnosis of the cause of death. Their justifications included absence of lesions or only observation of incidental lesions that did not result in death, as well as corpses in advanced state of postmortem changes, which hindered interpretation of the macro- and microscopic aspects of disease.

RESULTS

In the study period 1,728 felines were necropsied at the LAPA/UDESC. Of these, 797 (46.12%) were males, 680 (39.35%) were females, and 251 (14.52%) did not have their gender informed in the reports. Regarding breed, the numbers and percentages of necropsies were as follows: 1,107 (64,06%) crossbred (CB), 267 (15.45%) Siamese, 130 (7.52%) Persian, five (0.29%) Angora, five (0.29%) Himalayan, and four (0.23%) Brazilian shorthair. The remainder of the cases (210; 12.15%), did not have their breed informed in the necropsy reports. Table 1 shows the age group distribution according to gender.

Of the total of 1,728 records assessed, 1,184 (68.52%) cases showed defined diagnoses of the cause of death (Table 2),

Table 1. Frequency distribution of gender and age group of cats necropsied at the LAPA/UDESC from 1995 to 2015

Gender	Age group	Frequency		Age (years)			
		(N)	(%)	Mean	SD[a]	Min.[b]	Max.[c]
Males	Kittens*		19.77	0.46	0.25	0.04	0.92
	Adults	454	73.58	3.6	2.52	1	10
	Elderlies	41	6.65	14.25	2.87	11	24
Females	Kittens*	120	23.12	0.42	0.24	0.02	0.92
	Adults	325	62.62	4.37	2.92	1	10
	Elderlies	74	14.26	13.5	2.33	11	26

[a] Standard deviation, [b] minimum age, [c] maximum age; * All kittens aged <1 year.

Table 2. Classification of diseases diagnosed in cats necropsies at the LAPA/UDESC from 1995 to 2015

Cause of death or euthanasia	Total	Cause of death or euthanasia	Total
Bacterial diseases	152	Degenerative diseases	160
Sepsis	36	Chronic kidney disease	94
Septic peritonitis	30	Chronic liver disease	40
Purulent bronchopneumonia	29	Cardiomyopathy	21
Mycoplasmosis	20	Osteoporosis	2
Bacterial enteritis	9	Chronic enteropathy	2
Pyometra	8	Hydronephrosis	1
Purulent myocarditis	6	Intoxications and toxi-infections	51
Purulent pleuritis	6	Sodium fluoracetate intoxication	21
Pyelonephritis	2	Intoxication by rodenticides	18
Purulent meningoencephalitis	4	Intoxication by cresols	3
Purulent nephritis	1	Intoxication by anticoagulants	2
Bacterial necrotizing esophagitis	1	Strychnine intoxication	2
Viral diseases	111	Aflatoxin intoxication	1
Feline infectious peritonitis	89	Benzyl benzoate intoxication	1
Panleukopenia	12	Cypermethrin intoxication	1
Feline respiratory disease complex	10	Sodium hypochlorite intoxication	1
Fungal diseases	10	Intoxication by NSAIDs	1
Fungal bronchopneumonia	10	Endocrine or metabolic diseases	27
Neoplasms	207	Hepatic lipidosis	13
Lymphoma	93	Diabetes mellitus	10
Leukemia	35	Fibrous osteodystrophy	3
Mammary carcinoma	25	Endogenous lipoid pneumonia	1
Squamous cell carcinoma	22	Parasitic diseases	26
Bronchoalveolar carcinoma	5	Verminotic bronchopneumonia	15
Hepatocellular carcinoma	4	Intestinal parasitosis	9
Cholangiocarcinoma	4	Toxoplasmosis	2
Lung adenocarcinoma	3	Nutritional disorders	25
Fibrosarcoma	3	Protein-calorie malnutrition	24
Osteosarcoma	2	Malabsorption syndrome	1
Nasal adenocarcinoma	1	Malformations	9
Apocrine sweat gland carcinoma	1	Hydrocephalus	2
Chondrosarcoma	1	Renal hypoplasia	2
Pancreatic adenocarcinoma	1	Renal dysplasia	1
Transitional cell carcinoma[a]	1	Rectal stricture	1
Undifferentiated carcinoma	1	Pulmonary hypoplasia	1
Uterine leiomyoma	1	Patent ductus arteriosus	1
Uterine leiomyosarcoma	1	Patent foramen ovale	1
Melanoma	1	Other	202
Infrequent sarcoma	1	Shock syndrome	91
Basal cell carcinoma	1	FLUTD[c]	57
Traumas	204	Acute renal failure	24
Car accident	138	Gastroduodenal ulcer	17
Dog attack	30	Aspiration bronchopneumonia	4
Fall	17	Intestinal torsion	4
Fight with other felines	9	Fecaloma of unknown cause	4
Firearm	4	Masticatory muscle myositis	1
Indeterminate cause	6	TOTAL	1184

[a] Originated in the urinary bladder, [b] nonsteroidal anti-inflammatory drugs, [c] feline lower urinary tract disease.

whereas 544 (31.48%) cases showed inconclusive diagnoses, of which 454 (83.45%) lacked data to confirm diagnosis, 39 (7.17%) showed no micro or macroscopic changes, 30 (5.51%) showed no diagnostic data, and 21 (3.86%) was in advanced state of postmortem changes. Table 3 shows the classification of diseases according to age group.

Infectious diseases were the main causes of death in the cats, with prevalence of adult males (67/273; 24.54%) over adult females (44/273; 16.11%). Although bacterial diseases were the most frequent, feline infectious peritonitis (FIP) (Fig.1A), caused by Coronavirus, accounted for 32.6% (89/273) of the deaths, with prevalence of adult cats (38/89; 42.70%) aged 2.3 years on average, with 39 (43.82%) males and 36 (40.45%) females. Sepsis was the second cause of death (36/273; 13.18%) observed in the necropsy reports assessed in this disease category, most of them caused by fetal dystocia (12/36; 3.33%), with prevalence of adults (20/36; 55.55%). Bacterial septic peritonitis was the third most frequently observed diagnosis (Fig.1B), comprising 10.99% (30/273) of the deaths. In this case, there was higher occurrence in females than in males, with 46.66% (14/30) and 43.33% (13/30) of the deaths, respectively, with predominance of adults (mean age of 3.2 years) with a death rate of 43.33% (13/30). As primary causes of septic peritonitis, perforated gastroduodenal ulcers were identified in 53.33% (16/30) of the cases; the other causes included, in all, nine cases (30%), with postoperative wound dehiscence, rupture of the urinary bladder, puerperal metritis, pyometra, onfalitis, and liver abscess syndrome among the most significant. It was not possible to identify the main cause of peritonitis in five cats (16.66%). Purulent bronchopneumonia was the fourth most commonly diagnosed cause of death (29/273; 10.62%), with higher incidence in adult males (7/29; 24.14%). Mycoplasmosis caused by *Mycoplasma haemofelis* was detected in 7.33% (20/273) of the cases, with prevalence of adult male cats (11/20; 55.0%). Feline panleukopenia was responsible for 4.39% (12/273) of the deaths, affecting mostly female (7/12; 58.33%) and adult (6/15; 40.0%) cats. Table 2 shows the other deaths resulting from infectious diseases.

Neoplasms were the second most frequent cause of death among the necropsy reports assessed, with prevalence of females (93/207; 44.93%) compared with males (91/207; 43.96%), and age group incidence as follows: adults (121/207; 58.45%), elderly (41/207; 19.81%), and kittens (7/207; 3.38%). Lymphoma was the most frequently diagnosed neoplasm, responsible for 44.93% (93/207) of the deaths, with predominance of males (49/93; 52.69%) aged 4.4 years on average. The most affected organs, in isolation or associated with the infiltrate of neoplastic lymphocytes in more organs, were liver (49/93), kidneys (23/93), mediastinum (19/23), lymph nodes (17/93), spleen (17/93; Fig.1C), bone marrow (10/93), intestines (9/93), heart (8/93), lungs (6/93), and thymus (5/93). Leukemia (Fig.1D) followed lymphoma, accounting for 16.91% (35/207) of the deaths, with males (19/35, 54.28%) aged 4.4 years on average as the most affected. Mammary carcinoma was diagnosed in 12.07% (25/207) of the necropsied cats, with prevalence of elderly females (23/25, 92%) with mean age of 11.7 years. The following sites were the most affected by single or multiple metastases: lungs (24/25; 96%), lymph nodes (6/25; 24%), liver (5/25; 20%), kidneys (5/25; 20%), heart (4/25; 16%), and spleen (4/25; 16%). Squamous cell carcinoma was a significant cause of euthanasia observed in the necropsy files evaluated, accounting for 10.63% (22/207) of the cases, with males (10/22; 45.45%) with mean age of 7.3 years as the most affected. The neoplasms were mainly located in the nasal plane (12/22, 54.5%), followed by the lip commissure (6/22, 27.27%), eyelid region (3/22; 13.63%), and ear pinna (3/22; 13.63%). Squamous cell carcinoma metastases were found in seven cats, located in the liver (4), lungs (1), lymph nodes (1), and kidneys (1). The other neoplasms were responsible for a number of deaths ≤5 each, totaling 15.46% (32/207) of all death caused by neoplasia (Table 2).

Deaths caused by trauma were more frequent in males (96/204; 47.06%) than in females (84/204; 41.18%) cats, and was most often caused by car accidents (138/204; 67.65%), dog attacks (30/204; 14.70%), falls (17/204; 8.33%), fights with other felines (9/204; 4.41%), and firearm (4/204; 1.96%) (Fig.1E). Regarding the type of trauma, traumatic brain injury was

Table 3. Categories of diseases of cats necropsied at the LAPA/UDESC from 1995 to 2015 with their respective age group distribution

Disease classification	Frequency		Age (years)[a]			
	(N)	(%)	Mean	SD[b]	Min.[c]	Max.[d]
Infectious[‡]	273	15.8	2.89	3.13	0.03	15
Neoplasms	207	11.98	6.7	4.6	0.08	20
Traumas	204	11.81	2.5	3.04	0.04	14
Degenerative	160	9.26	6.5	5.5	0.16	24
Toxic*	51	2.95	1.9	1.3	0.5	7
Metabolic[†]	27	1.56	4.72	4.1	0.16	15
Parasitic	26	1.5	2.6	2.9	0.04	12
Nutritional	25	1.45	1.33	2.3	0.04	10
Malformations	9	0.52	2.1	4.18	0.04	13
Other	202	11.68	3.27	3.14	0.05	19
Inconclusive	544	31.48	3.98	4.15	0.008	26

[a] Distribution defined only for animals whose age was reported, [b] standard deviation, [c] minimum age, [d] maximum age; [‡] Bacterial, fungal and viral, *Toxic, including intoxications and toxi-infections, [†] Metabolic, comprising endocrinologic and metabolic disorders.

the most prevalent (45/204, 22.06%), followed by abdominal rupture with evisceration and thoracic trauma, with 11.27% (23/204) of the cases each. Pelvic fractures accounted for 10.29% (21/204) of the trauma cases, followed by fractures of pelvic limbs (19/204; 9.13%); spleen rupture (12/204; 5.88%), and fractures of thoracic limbs (11/204; 5.39%). The remainder of trauma cases was responsible for a number ≤10 cases each, totaling 24.51% (50/204) of the deaths (Table 2).

Degenerative diseases affected a larger number of adults (87/160; 54.37%) compared with those of elderly (27/160;

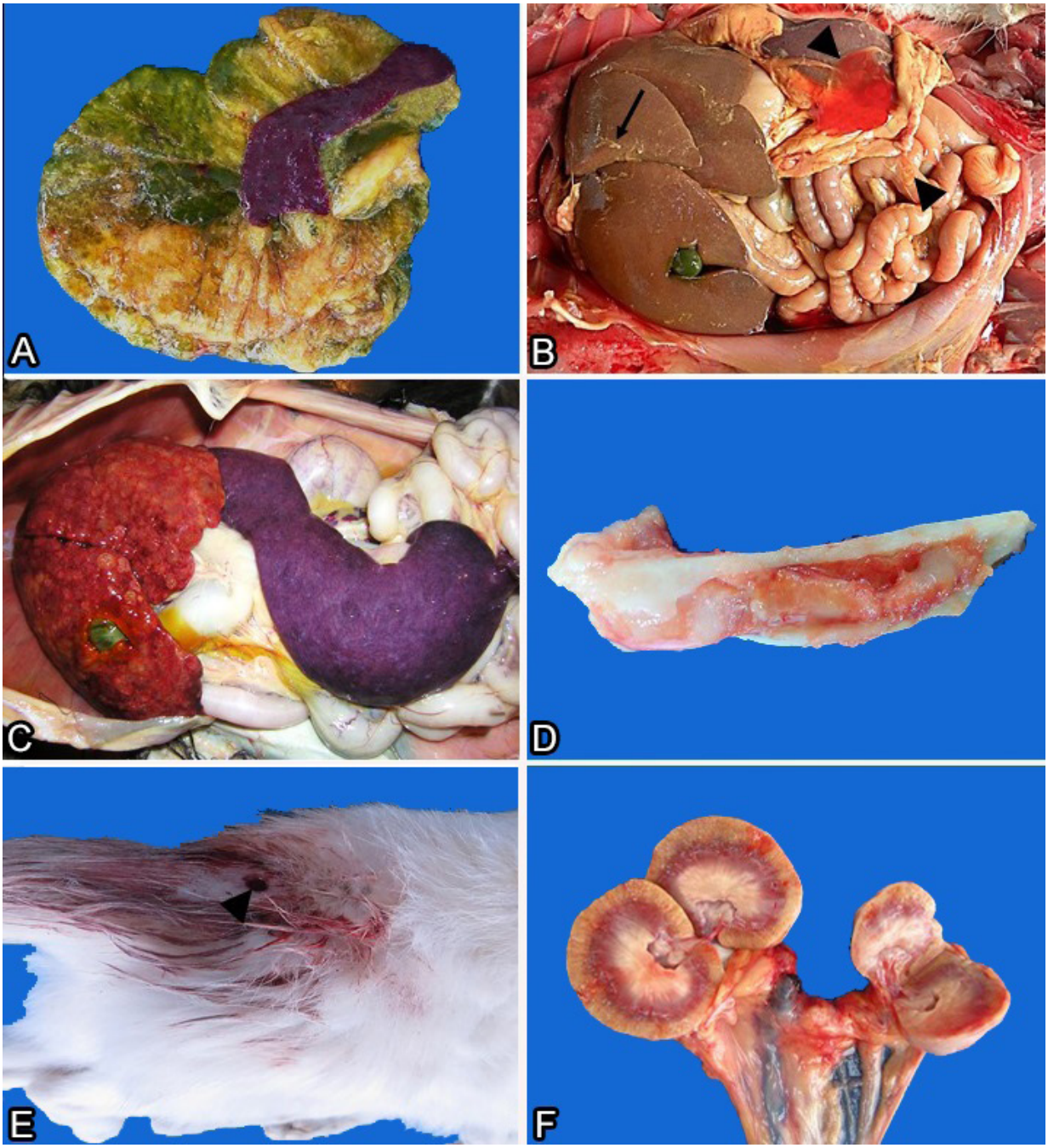

Fig.1. Causes of death and euthanasia in domestic cats in the Santa Catarina plateau. (**A**) Feline infectious peritonitis (FIP); spleen with an intensely irregular surface, next to diffusely swollen omentum, with multifocal fibrin bundles. (**B**) Bacterial peritonitis; abdominal cavity filled with bloody fluid and fibrin bundles adhered to the surface of the hepatic (thin arrow) and splenic capsules, omentum and intestinal serosa (arrow heads). (**C**) Lymphoma; diffuse, pronounced splenomegaly and multiple hepatic nodulations resulting from infiltration of neoplastic lymphocytes into the parenchyma. (**D**) Chronic lymphocytic leukemia; bone marrow with diffuse, pale, opaque parenchymal mass. (**E**) Injury caused by perforating instruments; perforation caused by firearm bullets (arrow head) in the sacrococcygeal region. (**F**) Chronic kidney disease; irregular kidney surface with whitish lines and intense retraction of the cortex in the left renal parenchyma.

16.87%) and kittens (13/160; 8.12%). Chronic renal disease was most prevalent (94/160; 58.75%) (Fig.1F) among cats aged 6.8 years on average, followed by chronic liver disease (40/160; 25%) in the group with mean age of 6.9 years, and cardiomyopathies (21/160; 13.12%), with prevalence of idiopathic hypertrophic cardiomyopathy (11/21; 52.38%), in the group with mean age of 4.3 years. There were two cases of dilated cardiomyopathy (9.5%), and the remaining eight cases regarded congestive heart failure with no determined primary cause (38.09%). The other degenerative diseases were responsible for ≤2 deaths each, totaling 3.75% (6/160) of the cases (Table 2).

Among the deaths caused by intoxications or toxi-infections, sodium fluoracetate intoxication was the most prevalent with 41.17% (21/51) of the cases, followed by intoxication by rodenticides (18/51, 35.29%), and strychnine and anticoagulants (4/51; 7.84%). The other informed cause intoxications accounted for 15.68% (8/51) of the deaths, including intoxications by ectoparasiticides (benzyl benzoate) and medications (nonsteroidal anti-inflammatory drugs - NSAIDs). The largest number of deaths in this category occurred in males (28/51; 54.9%); it was reported in 29.41% (15/51) of the females, with 50.98% (26/51) of the cases in adult cats.

Among the endocrine or metabolic diseases, idiopathic hepatic lipidosis was responsible for 48.15% (13/27) of the deaths, with predominance in females (7/13; 53.85%) and adults (7/13; 53.85%) aged five years on average. Diabetes mellitus was diagnosed in 37.03% (10/27) of the necropsied cats. Fibrous osteodystrophy, which was attributed to food origin in all cases, occurred in 11.11% (3/27) of the deaths. Verminotic bronchopneumonia caused by *Aelurostrongylus abstrusus* was the most prevalent parasitic disease, with 57.69% (15/26) of the cases. Nutritional disorders included mainly protein-calorie malnutrition, and accounted for 96% (24/25) of the deaths, with higher incidence observed in kittens (12/24; 50.0%) and adults (6/24; 25.0%). Congenital malformations predominantly affected kittens (6/9; 66.66%), and the most frequently observed diagnoses were hydrocephalus and renal hypoplasia, responsible for two deaths each (44.44%). Rectal stricture, pulmonary hypoplasia, patent ductus arteriosus, and patent foramen ovale were also found in the necropsy files, accounting for one case each, contributing to 55.55% (5/9) of the cases of death in this disease category.

The category "Other" comprised animals with no history but with lesions compatible with shock syndrome, including pulmonary edema and congestion, but of indeterminate cause and absence of other gross or microscopic lesions (91/202, 45.04%). Considering that feline lower urinary tract disease (FLUTD) is a multifactorial disorder, it was the second most reported condition in this category (57/202; 28.21%). FLUTD was predominant in males (48/57; 84.21%) and adult (35/57; 61.40%) cats with mean age of 4.5 years. Acute renal failure, mainly due to nephrotoxicity, was responsible for 11.88% (24/202) of the deaths. Gastroduodenal ulcer accounted for 7.42% (15/202) of the cases of death were more common in males (9/15; 60%) and adult (46.66%) felines. The other causes of death in this category corresponded to less than five cases each (15/202; 7.42% (Table 2).

DISCUSSION

Of the 1,728 felines necropsied at the LAPA/UDESC, males were prevalent (46.12%), data compatible with those reported in the literature (Lacheretz et al. 2002); however, the percentage of animals that did not have the gender informed (14.52%) should be considered. In the adult age group, the proportion of necropsied males was also higher (56.96%) compared with that of females (47.79%), and one of the justifications for this difference is the greater environmental exposure of males, which leave in search of females for mating, exposing themselves to a larger number of stressors and pathogens, whereas females have periods of isolation during the puerperium (Berzins 2000). The age range including the reproductive period also explains the predominance of necropsied adults in relation to kittens and elderly. There are no studies addressing the correlation between breed and prevalence of deaths in felines; however, it is known that crossbred cats are predominant, which was confirmed by the larger number of necropsies in these animals observed in the present study (64.06%).

Infectious diseases were the most common cause of feline death in this study (15.8%). Studies conducted with dogs have also shown infectious diseases as the main cause of death (Bentubo et al. 2007, Fighera et al. 2008), but these data differed from those of previous studies on cats, in which deaths caused by trauma (Togni et al. 2018) or complications involving idiopathic urinary tract diseases were the most diagnosed (Trapp et al. 2010). Wilkie et al. (2015) assessed 252 cases of death in felines and concluded that heart disease was the most common cause of death (55%), with prevalence of hypertrophic cardiomyopathy (68%).

Adult males were the feline population most affected by infectious diseases (24.54%), with feline infectious peritonitis (FIP) as the most prevalent condition. This is in agreement with findings of previous studies, which demonstrated that a larger number of males, aged 3 months to 3 years (Oliveira et al. 2003), unneutered (Rohrbach et al. 2001) cats were affected by Coronavirus. This finding is again justified by the susceptibility of unneutered males to infectious diseases due to the search for in-estrus females and greater access to external environments, which culminate in greater contact with felines of unknown origin, facilitating the dissemination of infectious agents. Sepsis has been presented as a common condition in felines (12.04%), with pneumonia, endocarditis, pyelonephritis, pyometra, septic pancreatitis, pyothorax, peritonitis, and meningitis among its main prerequisites (Declue et al. 2011). Sepsis is defined as the systemic inflammatory reaction secondary to the action of bacteria, viruses, fungi, or parasites (Bone et al. 1992). In this study, sepsis was mainly associated with dystocia with fetal death. The occurrence of infectious diseases, particularly viral diseases in general, is also associated with the low participation in vaccination programs, fundamental in their control, which are eventually neglected by owners due mostly to socioeconomic aspects (Bentubo et al. 2007).

Cats infected with the feline leukemia virus (FeLV) are predisposed to a wide variety of diseases, especially infectious diseases, considering that there is intense immunosuppression, with a larger number of diseases diagnosed in animals with FeLV. Also, neoplasms of lymphoid origin have been largely observed in these animals (Hagiwara et al. 1997). These lymphoproliferative disorders can be classified according to

the affected sites, the morphological aspect of the neoplastic cells, and to the cytochemical and immunological markers (Rojko et al. 1989, Gabor et al. 1998). It is known that prevalence of infection with FeLV in the Santa Catarina plateau is 22.26%, and that factors such as aggressiveness contribute to seropositivity, especially in males (Biezus 2017), similarly to what was found in the present study, in which cases of lymphoma (44.93%) were more prevalent in male felines (52.69%). Lymphoma is classified as follows: multicentric - characterized by widespread involvement of structures such as the liver, spleen, lymph nodes and, in some cases, bones; mediastinal - characterized by lymphadenopathy in the mediastinum, with focal, multifocal or diffuse neoplastic infiltration in the gastrointestinal tract structures, with or without intra-abdominal lymphadenopathies; extranodal - which can affect any organ or tissue atypically, such as the central nervous system, eyeball, epidermis, kidneys, etc. (Couto 2000). In previous studies, involvement of organs in the abdominal cavity was more common; however, despite this intra-abdominal involvement, there has been predominance in structures of the gastrointestinal tract and not in the liver or spleen, characterizing lymphoma as nutritional and not as multicentric (Gabor et al. 1998), which differs from the location in the liver predominantly found in this study. The intestines, in turn, were classified only as the seventh organ most affected by the neoplastic infiltrate. Leukemia was less frequently observed than lymphoma, and also prevalent in males (54.28%).

In the category of neoplasms, mammary carcinoma (12.07%) was predominant in females (92%) with mean age of 11.7 years. It is known that carcinoma is the most common mammary neoplasia, affecting adult-to-elderly females. Quite often, the use of synthetic progestogens for suppression of estrus contributes to the emergence of benign or malignant neoplasms of the mammary gland (Amorim et al. 2004), and there is need for awareness-raising programs regarding the harm caused by this practice. In the present study, it was not possible to establish the number of females that had mammary carcinoma as a result of this practice. Squamous cell carcinoma was the fourth most prevalent neoplasm in felines (10.63%), especially in those with white coat and prolonged exposure to the ultraviolet rays of sunlight, which cause crusty and hyperemic lesions that evolve in neoplasia, and are thus locally aggressive, affecting felines with mean age of 7.3 years. This finding differs from data reported in previous studies, in which the approximate age for the occurrence of this disease was 11 years and 4 months (Ruslander et al. 1997).

Deaths resulting from trauma were only the third most frequent, in disagreement with the data obtained by Trapp et al. (2010), who classified this category as the most prevalent cause of death in felines, but in agreement with the identification of deaths caused by car accidents (67.65%) as the most common. Males were the most affected by trauma (47.06%), which may be associated with their frequent access of a larger proportion of unneutered stray male cats. A study conducted by Bentubo et al. (2007) with dogs verified that traumas were also the third most frequent cause of death. Traumatic brain injury was the most commonly described (22.06%), differing from data of previous studies, which have observed traumas in the appendicular skeleton as the most frequent (Vidane et al. 2014). Nevertheless, it should be considered that this previous study addressess trauma in live animals, because traumatic brain injury is often fatal without veterinary assistance. In addition, pets show reduced body mass and, as a consequence, large contact surface for impact, considering that an animal with traumatic brain injury is also a polytraumatized individual (Siqueira et al. 2013).

Chronic kidney disease was the most prevalent in the category of degenerative diseases (58.75%), corroborating the results of a previous study that assessed the causes of death in domestic felines aged >9 years, in which this disease was predominant, accounting for 42 deaths out of 121 animals evaluated (Manteigas et al. 2013). However, in the present study, degenerative diseases accounted for a larger number of adults (54.37%) compared with that of elderly (16.87%), although the mean age of these adult individuals (6.8 years) should be considered, closer to that of elderly. Due to lack of information in the necropsy files, it was not possible to distinguish accidental from criminal intoxications. In cases in which it was possible to identify the active principle that determined the intoxication, there was predominance of sodium fluoracetate (41.17%), a powerful rodenticide. Clinical findings associated with sodium fluoracetate poisoning include vomiting, diarrhea, hypothermia, tachypnea, hyperexcitability, mydriasis, vocalisation, and convulsive episodes, and may result in death when the condition is not reversed (Collicchio-Zuanaze 2002). Again, the most affected population comprised males (54.9%) and the adult age group (50.98%), because toxic agents are included within the risk factors for unneutered male cats that have access to the streets, especially in the case of criminal poisoning.

Feline hepatic lipidosis, the most common endocrine or metabolic disease (48.15%), is associated with accumulation of triglycerides in the hepatocytes, precluding adequate metabolism. Among the predisposing factors for its occurrence, it worth highlighting the exacerbated mobilization of fatty acids from the adipose tissue to the liver as a result of anorexia, mainly in obese animals, with greater availability of lipids for gluconeogenesis, because glucose levels in the bloodstream are reduced in anorexia (Dimski & Taboada 1995). Quite often, the primary cause of anorexia is not established, considering not only pathological, but also behavioral changes. One of the most common predisposing conditions in diabetes mellitus (37.03%) in felines is obesity. It is associated with poorly formulated diets with excessive caloric intake, leading to reversible insulin resistance and tissue resistance to glucose (Veiga 2005).

There are no epidemiological studies determining the frequency of protein-caloric malnutrition, the main one among those of nutritional origin (96%), as a cause of death. It is believed to be associated with abandonment of kittens, the predominant age group in this case (50.0%), once again evidencing the importance of the castration process in small animals, avoiding the proliferation of stray animals. Congenital anomalies are poorly described in felines, and there is difficulty in determining the prevalence of hydrocephalus and renal hypoplasia in this species due to lack of epidemiological data describing them. Regarding congenital heart anomalies, a study conducted in Sweden by Tidholm et al. (2015) demonstrated that these anomalies represent 0.2% of the total feline patients, with interventricular septal defect as the most documented, corresponding to 50% of the cases.

The literature presents no data on the prevalence of shock syndrome in felines, but it is known that it is a multifactorial event associated with deficient energy production by the cells due to inadequate blood circulation, with consequent deficient oxygen supply to the tissues, leading to cell degeneration and necrosis, organ failure, and death, if not reversed in time (Shaffran 2004). The necropsy findings include mainly pulmonary congestion and edema; however, they are nonspecific findings, and thus prevent the etiology of shock from being determined when there is no history of or concomitant lesions.

Feline lower urinary tract disease (FLUTD) was quite prevalent (32.20%) in this study. It is a multifactorial disorder often associated with the ingestion of industrialized dry diets, which favor the formation of calculi, as well as of diets rich in minerals such as magnesium, phosphates and calcium, which favor the appearance of urolithiasis, in addition to low water intake, characterized by the peculiar habits of some animals. Sedentary lifestyle and obesity may also be considered predisposing factors for FLUTD (Rosa & Quitzan 2011). FLUTD is prevalent in male (84.21%) and adult (61.40%) cats, and is associated with the anatomical conformation of the urethra of the males, which favors the installation of obstructive processes, mainly by formation of urethral plugs (Reche Junior et al. 1998). Discontinuation of urinary flow may also lead to postrenal azotemia, evolving to renal failure and death (Finco & Barsanti 1984). In acute renal failure, the third most prevalent disease in this category, decreased renal function due to factors such as nephrotoxicity is observed, as well as to deficits in the excretion of metabolites such as urea and creatinine, which acutely accumulate in the bloodstream and lead to a condition of uremia that can evolve to death (Rufato et al. 2011).

CONCLUSIONS

Infectious diseases were the main causes of death or euthanasia in domestic cats necropsied at the LAPA/UDESC in the Santa Catarina plateau region, with feline infectious peritonitis (FIP) as the most prevalent disease in adult males, followed by neoplasms, especially lymphoma and leukemia, which were also more prevalent in adult males.

Both causes of death may be associated with the way of life of these animals, exposed to a greater number of pathogens compared with females, mainly those of viral origin, reinforcing the need for vaccination and castration programs in felines.

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

REFERENCES

Amorim F.V., Souza H.J.M. & Ferreira A.M.R. 2004. Avaliação clínico-cirúrgica de nódulos mamários felinos através da citologia e histopatologia. Braz. J. Vet. Res. Anim. Sci. 41:204-205.

Bentubo H.D.L., Tomaz M.A., Bondan E.F. & Lallo M.A. 2007. Expectativa de vida e causas de morte em cães na área metropolitana de São Paulo (Brasil). Ciência Rural 37(4):1021-1026. <http://dx.doi.org/10.1590/S0103-84782007000400016>

Berzins M.A.V.D.S. 2000. Velhos, cães e gatos: interpretação de uma relação. Masters Thesis, Faculdade de Saúde Pública, Universidade de São Paulo. 162p.

Biezus G. 2017. Infecção pelos vírus da leucemia felina (FeLV) e imunodeficiência (FIV) em gatos do planalto de Santa Catarina: prevalência, fatores associados, alterações clínicas e hematológicas. Masters Thesis, Centro de Ciências Agroveterinárias, Universidade de Santa Catarina, Lages. 91p.

Bonnett B.N., Egenvall A., Hedhammar A. & Olson P. 2005. Mortality in over 350,000 insured swedish dogs from 1995-2000: I. Breed-, gender-, age- and cause-specific rates. Acta Vet. Scand. 46(3):105-120. <http://dx.doi.org/10.1186/1751-0147-46-105> <PMid:16261924>

Bone R.C., Balk R.A., Cerra F.B., Dellinger R.P., Fein A.M., Knaus W.A., Schein R. & Sibbald W.J. 1992. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. Chest 101(6):1644-1655. <PMid:1303622>

Collicchio-Zuanaze R.C. 2002. Intoxicação por Fluoroacetato de sódio: avaliações clínica, hemogasométrica, eletrocardiográfica e da eficácia do gluconato de cálcio e succinato de sódio como protocolo terapêutico. Masters Thesis, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Botucatu. 133p.

Couto C.G. 2000. Advances in the treatment of the cat with lymphoma in practice. J. Feline Med. Surg. 2(2):95-100. <http://dx.doi.org/10.1053/jfms.2000.0079> <PMid:11716600>

Declue A.E., Delgado C., Chang C. & Sharp C.R. 2011. Clinical and immunologic assessment of sepsis and the systemic inflammatory response syndrome in cats. J. Am. Vet. Med. Assoc. 238(7):890-897. <http://dx.doi.org/10.2460/javma.238.7.890> <PMid:21453177>

Dimski D.S. & Taboada J. 1995. Feline idiopathic hepatic lipidosis. Anim. Pract. 25(2):357-373. <PMid:7785168>

Fighera R.A., Souza T.M., Silva M.C., Brum J.S., Graça D.L., Kommers G.D., Irigoyen L.F. & Barros C.S.L. 2008. Causas de morte e razões para eutanásia de cães da mesorregião do centro ocidental Rio-Grandense (1965-2004). Pesq. Vet. Bras. 28(4):223-230. <http://dx.doi.org/10.1590/S0100-736X2008000400005>

Finco D.R. & Barsanti J.A. 1984. Diet-induced feline urethral obstruction. Vet. Clin. N. Am., Small Anim. Pract. 14(3):529-536. <http://dx.doi.org/10.1016/S0195-5616(84)50059-X> <PMid:6610977>

Gabor L.J., Malik R. & Canfield P.J. 1998. Clinical and anatomical features of lymphosarcoma in 118 cats. Aust. Vet. J. 76(11):725-732. <http://dx.doi.org/10.1111/j.1751-0813.1998.tb12300.x> <PMid:9862061>

Hagiwara M.K., Reche Júnior A. & Lucas S.R.R. 1997. Estudo clínico da infecção de felinos pelo vírus da leucemia felina em São Paulo. Revta Bras. Ciênc. Vet. 4:35-38.

Lacheretz A., Moreau D. & Cathelain H. 2002. Causes of death and life expectancy in carnivorous pets (I). Rev. Méd. Vét. 153:819-822.

Manteigas F., Godinho A., Almeida P. & Grande C. 2013. Causas de mortalidade em gatos com mais de nove anos: estudo retrospectivo de cem casos. Revta Lusófona Ciênc. Med. Vet. 6:47-57.

Oliveira F.N., Raffi M.B., Souza T.M. & Barros C.S.L. 2003. Peritonite infecciosa felina: 13 casos. Ciência Rural 33(5):905-911. <http://dx.doi.org/10.1590/S0103-84782003000500018>

Reche Junior A., Hagiwara M.K. & Mamizuka E. 1998. Estudo clínico da doença do trato urinário inferior em gatos domésticos de São Paulo. Braz. J. Vet. Res. Anim. Sci. 35(2):69-74. <http://dx.doi.org/10.1590/S1413-95961998000200004>

Rojko J.L., Kociba G.J., Abkowitz J.L., Hamilton K.L., Hardy Junior W.D., Ihle J.N. & O'Brien S.J. 1989. Feline lymphomas: immunological and cytochemical characterization. Cancer Res. 49(2):345-351. <PMid:2535958>

Rosa V.M. & Quitzan J.G. 2011. Avaliação restrospectiva das variáveis etiológicas e clínicas envolvidas na doença do trato urinário inferior dos felinos (DTUIF). Cesumar 13:103-110.

Rohrbach B.W., Legendre A.M., Baldwin C.A., Lein D.H., Reed W.M. & Wilson R.B. 2001. Epidemiology of feline infectious peritonitis among cats examined at veterinary medical teaching hospitals. J. Am., Vet. Med. Assoc.

218(7):1111-1115. <http://dx.doi.org/10.2460/javma.2001.218.1111> <PMid:11318361>

Rufato F.H.F., Rezende-Lago N.C.M. & De Marchi P.G.F. 2011. Insuficiência renal em cães e gatos. Revta Eletrôn. Interdisciplinar 2:167-173.

Ruslander D., Kaser-Hotz B. & Sardinas J.C. 1997. Cutaneous squamous cell carcinoma in cats. Compend. Contin. Educ. Pract. Vet. 19(10):1119-1129.

Santo A.H. 2007. Potencial epidemiológico da utilização das causas múltiplas de morte por meio de suas menções nas declarações de óbito, Brasil, 2003. Revta Panam. Salud Públ. 22:178-186.

Shaffran N. 2004. Shock overview: cardiogenic and non-cardiogenic shock syndromes. Proceedings X International Veterinary Emergency and Critical Care Symposium, San Diego, CA.

Siqueira E.G.M.S., Rahal S.C., De Araújo F.A.P. & Agostinho F.S. 2013. Trauma cranioencefálico em pequenos animais. Vet. Zootec. 20:112-123.

Tidholm A., Ljungvall I., Michal J., Haggstrom J. & Hoglund K. 2015. Congenital heart defects in cats: a retrospective study of 162 cats (1996-2013). J. Vet. Cardiol. 17(Suppl.1):215-219. <http://dx.doi.org/10.1016/j.jvc.2014.09.004> <PMid:26776580>

Togni M., Curtis A., Vargas D.P., Kommers G.D., Irigoyen L.F. & Fighera R.A. 2018. Causas de morte e razões para eutanásia em gatos na Região Central do Rio Grande do Sul (1964-2013). Pesq. Vet. Bras. 38(4):741-750. <http://dx.doi.org/10.1590/1678-5150-pvb-5075>

Trapp S.M., Iacuzio A.I., Barca Junior F.A., Kemper B., Silva L.C., Okano W., Tanaka N., Grecco F., Cunha Filho L. & Sterza F. 2010. Causas de óbito e razões para eutanásia em uma população hospitalar de cães e gatos. Braz. J. Vet. Res. Anim. Sci. 47(5):395-402. <http://dx.doi.org/10.11606/issn.1678-4456.bjvras.2010.26821>

Veiga A. 2005. Obesidade e diabetes mellitus em pequenos animais. Anais II Simpósio de Patologia Clínica Veterinária da Região Sul do Brasil, Porto Alegre, RS, p.82-91.

Vidane A.S., Elias M.Z.J., Cardoso J.M.M., Come J.A.S.S., Harun M. & Ambrósio C.E. 2014. Incidência de fraturas em cães e gatos da cidade de Maputo (Moçambique) no período de 1998-2008. Ciênc. Anim. Bras. 15(4):490-494. <http://dx.doi.org/10.1590/1089-6891v15i424279>

Wilkie L.J., Smith K. & Luis Fuentes V. 2015. Cardiac pathology findings in 252 cats presented for necropsy: a comparison of cats with unexpected death versus other deaths. J. Vet. Cardiol. 17(Suppl.1):329-340. <http://dx.doi.org/10.1016/j.jvc.2015.09.006> <PMid:26776590>

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Original Article
Wildlife Medicine

Detection of Enterobacteriaceae, antimicrobial susceptibility, and virulence genes of *Escherichia coli* in canaries (*Serinus canaria*) in northeastern Brazil [1]

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ABSTRACT.- Beleza A.J.F., Maciel W.C., Carreira A.S., Bezerra W.G.A., Carmo C.C., Havt A., Gaio F.C. & Teixeira R.S.C. 2019. **Detection of Enterobacteriaceae, antimicrobial susceptibility, and virulence genes of *Escherichia coli* in canaries (*Serinus canaria*) in northeastern Brazil.** *Pesquisa Veterinária Brasileira 39(3):201-208.* Setor de Estudos Ornitológicos, Faculdade de Veterinária, Universidade Estadual do Ceará, Av. Paranjana 1700, Fortaleza, CE 60740-903, Brazil. E-mail: jacksonxand@gmail.com

This study aimed to verify the presence of members from the Enterobacteriaceae family and determine antimicrobial susceptibility profiles of the isolates in canaries bred in northeastern Brazil; in addition, the presence of diarrheagenic *Escherichia coli* (DEC) and avian pathogenic *Escherichia coli* (APEC) was also verified in these birds. Samples were collected during an exhibition organized by the Brazilian Ornithological Federation in July 2015 in Fortaleza, Brazil. A total of 88 fecal samples were collected and submitted to pre-enrichment step using buffered peptone water, followed by enrichment with the following broths: brain-heart infusion, Rappaport-Vassiliadis, and Selenite-Cystine. Subsequently, aliquots were streaked on MacConkey, brilliant green and salmonella-shigella agar plates. Colonies were selected according to morphological characteristics and submitted to biochemical identification and antimicrobial susceptibility tests with disk-diffusion technique. *E. coli* strains were evaluated for the presence of eight DEC genes and five APEC genes through conventional polymerase chain reaction (PCR) screening. The most frequent species observed were *Pantoea agglomerans* (25%), *Serratia liquefaciens* (12.5%), and *Enterobacter aerogenes* (9.1%). A single rough strain of *Salmonella enterica* subsp. *enterica* was identified in one sample (1.1%). High resistance rates to amoxicillin (78.7%) and ampicillin (75.4%) were identified. Polymyxin B (9.8%), gentamycin (6.6%), and enrofloxacin (6.6%) were the most efficient antibiotics. The total number of multidrug-resistant strains (isolates resistant to more than three antimicrobial classes) was 23 (37.7%). Four *E. coli* strains were tested for the virulence genes, and two were positive for APEC virulence genes: one strain was positive for *iutA* and the other for *hlyF*. In conclusion, canaries in northeastern Brazil participating in exhibitions may present *Salmonella* spp., *Escherichia coli* and other enterobacteria in the intestinal microbiota with antimicrobial resistance. These results indicate that, although the *E. coli* strains recovered from canaries in this study have some virulence genes, they still do not fulfill all the requirements to be considered APEC.

INDEX TERMS: Enterobacteriaceae, antimicrobial susceptibility, virulence genes, *Escherichia coli,* canaries, *Serinus canaria*, northeastern Brazil, antibiogram, diarrheagenic, APEC, Belgian canaries, bacterioses.

RESUMO.- [**Detecção de enterobactérias, sensibilidade antimicrobiana e genes de virulência de *Escherichia coli* em canários belgas (*Serinus canaria*) da região Nordeste do Brasil.**] O objetivo deste trabalho foi verificar a presença de enterobactérias e determinar o perfil de sensibilidade aos antimicrobianos dos isolados oriundos de

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canários belgas criados em cativeiro do Nordeste do Brasil, adicionalmente verificou-se a presença de *Escherichia coli* diarreiogênicas (DEC) e *E. coli* patogênica aviária (APEC) nesses animais. A colheita das amostras ocorreu durante uma exposição de canários belgas organizada pela Federação Ornitológica do Brasil (FOB), em julho de 2015, na cidade de Fortaleza, Ceará, Brasil. Um total de 88 amostras de fezes foram coletadas e submetidas a pré-enriquecimento utilizando água peptonada, caldo de enriquecimento Brain Heart Infusion, Rappaport-Vassiliadis e Selenito-Cistina. Fez-se triagem em placas de ágar MacConkey, Verde Brilhante e ágar Salmonella Shigella. As colônias foram selecionadas e submetidas à identificação bioquímica e susceptibilidade antimicrobiana. Estirpes de *Escherichia coli* foram avaliadas quanto a presença de 8 genes de virulência de DEC e cinco de APEC por reação em cadeia da polimerase convencional (PCR). As enterobactérias encontradas com maior frequência foram *Pantoea agglomerans* (25%), *Serratia liquefaciens* (12,5%) e *Enterobacter aerogenes* (9,1%). Uma única estirpe de *Salmonella enterica* subsp. *enterica* (rugosa) esteve presente em um dos isolados (1,1%). Altos percentuais de resistência foram encontrados para dois antibióticos: amoxicilina (78,7%) e ampicilina (75,4%). Polimixina B (9,8%), gentamicina (6,8%) e enrofloxacina (6,5%) foram os antibióticos com melhor eficiência. O total de estirpes multirresistentes (a mais de três classes de antimicrobianos) foi de 23 (37,7%). Das quatro estirpes de *E. coli* isoladas, duas foram positivas para os genes de APEC, sendo uma estipe para o gene *iss* e outra para os genes *iutA* e *hlyF*. Portanto, canários belgas criados em cativeiro no Brasil que participam de exposições podem apresentar *Salmonella* spp., *Escherichia coli* e outras enterobactérias em sua microbiota intestinal com resistência antimicrobiana. Estes resultados indicam que as estirpes de *E. coli* isoladas de canário belga no presente estudo apresentam alguns, mas não todos, genes de virulência para serem caracterizadas como *E. coli* patogênica para aves (APEC).

TERMOS DE INDEXAÇÃO: Enterobactérias, sensibilidade antimicrobiana, genes de virulência, *Escherichia coli,* antibiograma, diarreiogênica, APEC, canários belgas, *Serinus canaria*, bacterioses.

INTRODUCTION

Currently, the Belgian canary (*Serinus canaria*), order Passeriformes, family Fringillidae, is one of the birds most sought by breeders worldwide, esteemed for its smooth and harmonious singing and beautiful colors, as well as for being very docile and of easy, low-cost maintenance (Mantel 2005, Arnaiz-Villena et al. 2012). The legal breeding of birds can serve as an important mechanism in environmental conservation and protection (Camargo et al. 2010); therefore, canary breeding, in addition to being a hobby, can be an activity that discourages the illegal search for wild birds.

Infections caused by bacteria of the family Enterobacteriaceae are common in birds of the Passeriformes order; however, they are considered secondary, and the presence of predisposing factors is necessary to trigger diseases in birds (Guimarães 2007). Several pathogens belonging to the Fringillidae family have been reported causing different diseases in birds (canaries, chaffinches, common linnets, goldfinches, greenfinches, red crossbills), namely, *Escherichia coli*, *Salmonella* spp., *Citrobacter* sp., *Yersinia pseudotuberculosis*, and *Klebsiella* spp. (Macwhirter 1994, Dorrestein 1997, 2009, Guimarães 2007).

Escherichia coli is a commensal bacterium commonly found in the intestinal microbiota of homeothermic animals. However, pathogenic strains of this species are capable of causing intestinal and extraintestinal diseases in humans, mammals, and birds, resulting in significant economic losses to breeders and serious public health problems (Koneman et al. 2001, Kaper et al. 2004, Croxen & Finlay 2010, Bélanger et al. 2011). Studies addressing bacterial strains that cause septicemia in humans and birds have demonstrated that the genome can show a wide variety due to the presence of plasmids, phages, and mobile elements, thus the occurrence of these elements in pathogens such as *E. coli* is common (Mokady et al. 2005). In addition, similar virulence factors have been commonly described in strains of *E. coli* isolated in humans and in avian pathogenic *E. coli* (APEC), thus demonstrating their zoonotic potential (Ewers et al. 2007, Moulin-Schouleur et al. 2007, Smith et al. 2007, Tivendale et al. 2010, Bélanger et al. 2011).

Salmonellosis is an important bacterial disease in canaries and other birds raised as pets (Harrington Junior et al. 1975, Herikstad et al. 2002). This disease is caused by bacteria of the genus *Salmonella*, a pathogen known for its zoonotic potential, which can lead to high mortality (Kanashiro et al. 2002, Soncini 2002). Infection usually develops asymptomatically, and the birds become subclinical hosts, continuously or intermittently eliminating the agent in their feces (Flammer 1999). Knowledge about the occurrence and distribution of *Salmonella* spp. in domestic and wild animals is essential to list possible hosts that may be responsible for the transmission of this agent (D'aout et al. 2001).

There are few scientific studies addressing Enterobacteriaceae in birds of the Passeriformes order bred in captivity in the literature, with most of the research focusing on the poultry industry. There is also lack of available information on the epidemiological role of canaries kept as pets regarding the epidemiology of *E. coli*, *Salmonella* spp. and other enterobacteria, and their susceptibility/antibacterial resistance profiles. In this context, this study aims to verify the presence of members of the Enterobacteriaceae family, determine the antimicrobial susceptibility profile of the isolates from Belgian canaries (*Serinus canaria*) bred in captivity in northeastern Brazil, and analyze the presence of strains of diarrheagenic *E. coli* (DEC) and avian pathogenic *E. coli* (APEC).

MATERIALS AND METHODS

Sampling. Fecal samples of Belgian canaries (*Serinus canaria*), bred for the purpose of exhibition of type or color, collected from the bottom of cages were used in this study. The canaries, which belonged to 44 breeders from several states of northeastern Brazil (Alagoas, Bahia, Ceará, Maranhão, Piauí, Paraíba, Pernambuco, Rio Grande do Norte, and Sergipe), were competing in a bird show. The event was attended by 1448 birds (1255 and 193 canaries competing for type and color, respectively) that were individually housed in cages (32cm long, 22cm wide, 21cm high). The cages were made of stainless steel, equipped with suitable perches, and easy to clean. The birds were fed a mixture of seeds (birdseed, millet, turnip) and water *ad libitum*. The bottom of the cages was coated with white sulfite paper sheets, which were removed daily along with the birds' feces and overlapped feed. Feed and water were changed and their containers were cleaned daily. Microbiological analysis

was performed using material obtained from 440 different cages of canaries participating in the exhibition. One paper sheet containing canary feces was collected from each of the cages. They were then packed in sterile plastic bags and sent in isothermal boxes containing recyclable ice to the Laboratory of Ornithological Studies, College of Veterinary Medicine (LABEO-FAVET), at the State University of Ceará (UECE) for further processing. From each white sulfite paper sheet, 2g of feces were collected for microbiological analysis. Each sample was defined as a pool of biological material (feces) from the bottom of five cages of the same breeder. Thus, a total of 88 samples were submitted to microbiological analysis, and each breeder provided sufficient material for the investigation of two samples.

Microbiological processing of Enterobacteriaceae. The methodology described in Sousa et al. (2010) was used for the isolation of enteropathogens. Upon arrival at the laboratory, the feces were processed in stages in which the temperature and incubation time of the samples in the oven were standardized at 37°C for 24 hours. In the first stage, the feces from each sample were collected using a spatula, conditioned in 10mL of 1% buffered peptone water, and then incubated. Subsequently, aliquots were transferred to enrichment broths: 1mL to Brain-heart Infusion (BHI) and Selenite-Cystine (SC) and 0.1mL to Rappaport-Vassiliadis (RV), respectively. After incubation, they were streaked on brilliant green (BG), Salmonella-Shigella (SS) and/or MacConkey (MC) agar plates, and then reincubated.

Distinct colonies were collected from the plates and inoculated into tubes containing Triple-sugar-iron (TSI) agar, Lysine iron (LIA) agar, and Sulfide-indole-motility (SIM) medium. After the incubation period, the following biochemical tests were applied for confirmation of enterobacteria: lysine decarboxylation, ornithine descarboxylation, methyl red, urea, Simmons citrate, arginine decarboxylase, malonate, Voges-Proskauer, carbohydrates fermentation, lactose, sucrose, mannitol,, arabinose, raffinose, dulcitol, adonitol, inositol, and sorbitol (Holt & Bergey 1994, Koneman et al. 2012). Samples suspected for *Salmonella* spp. were submitted to the rapid serum agglutination test using a polyvalent 'O' (somatic) antiserum. For confirmation of suspicious samples, isolates were stored in nutrient agar and sent to a reference laboratory (Fiocruz) for serotyping.

Sensitivity profile of Enterobacteriaceae (antimicrobial susceptibility test). Enterobacteriaceae isolates were submitted to the Kirby-Bauer disc diffusion method, and the zones of inhibition were read according to the standards of the Clinical and Laboratory Standards Institute-CLSI (2014). After streaking the solution (sample), disks with the antibiotics were equally distributed on a plate containing Müller-Hinton agar, and the zones of inhibition were measured after incubation at 37°C for 24h. Antibiotics of the following pharmacological classes at the following concentrations were tested: 1) Aminoglycosides (neomycin, 30µg; streptomycin, 10µg and gentamicin, 10µg); 2) Sulfonamide (sulfonamide, 300µg and sulfazotrim (sulfamethoxazole + trimethoprim), 25µg); 3) Beta lactam (ampicillin, 10µg and amoxicillin, 3µg); 4) Quinolone (nalidixic acid, 30µg); 5) Polymyxin (polimixin B, 300µg); 6) Chloramphenicol (chloramphenicol, 30µg); 7) Fluorquinolones (Enrofloxacin, 5µg); 8) Tetracycline (tetracycline, 30µg). For this test, the ATCC 25922 *Escherichia coli* strain was used as control. Multidrug resistance (MDR) was considered when the strains were resistant to at least three classes of antimicrobials (Magiorakos et al. 2012).

Detection of diarrheagenic *Escherichia coli* (DEC) and avian pathogenic *Escherichia coli* (APEC). For molecular analysis, the isolated *E. coli* strains that were maintained in nutrient agar were reactivated in BHI broth, incubated at 37°C for 24 hours, and streaked on MacConkey agar to confirm purity of strain. Two to three colonies were collected from each plate, placed in tubes containing 1mL of 0.5% Triton X-100, vortexed for 15s, and boiled for 20min at 94°C. The tubes were then centrifuged at 10.000rpm for 10min at 4°C. The supernatant containing DNA was quantified by spectrophotometry using a NanoDrop Spectrophotometer 2000 (Thermo Scientific, Wilmington, USA). For the molecular diagnosis of DEC, the DNA samples were submitted to polymerase chain reaction (PCR) screening. The presence of eight virulence genes from five pathotypes were assessed as follows: genes *stx*1 (348pb) and *stx*2 (584pb) for identification of Shiga-Toxina-producing *Escherichia coli* (STEC); *elt*B (508 pb) and *est*A (147 pb) for enterotoxigenic *E. coli* (ETEC); *eae*A (881 pb) for enteropathogenic *E. coli* (EPEC); *ipa*H (483 pb) for enteroinvasive *E. coli* (EIEC); *aat*A (630 pb) and *aai*C (215 pb) for enteroaggregative *E. coli* (EAEC) (Taniuchi et al. 2012). The strains EAEC 042, EHEC O157: H7, EIEC O124, EPEC 2348/69, and ETEC H10407 were used as positive controls for the reactions. The strains were also submitted to detection of five minimal predictors of APEC virulence genes: *iro*N (Salmochelin receptor), *iss* (serum resistant), *hly*F (toxin encoding), *omp*T (episome-encoded outer membrane protease), and *iut*A (ferric aerobactin receptor). Although there is no consensus in the scientific literature on which genes would define an APEC strain, the findings of Johnson et al. (2008) were used in this study, which showed that APEC isolates obtained from organs with lesions of birds clinically diagnosed with colibacillosis had, on average, four of the five genes considered predictive for this pathotype. DNA extraction was performed using the boiling method (Lima et al. 2013). PCR screening was performed using a GoTaqGreen kit (Promega) and 0.2uM primers in a MyCycler™ Thermal Cycler (Biorad, CA, USA) system according to the following protocol: 95°C for 15min; 40 cycles at 95°C for 30s, 57°C for 30s and 72°C for 1 min, followed by 72°C for 10min. The amplified products were submitted to agarose gel electrophoresis, stained with 2% ethidium bromide, and photographed using the ChemicDoc™ XRS 112 (Biorad, CA, USA) transilluminator system.

RESULTS

Isolated bacteria. Of the 88 fecal samples investigated, 40 were negative for Enterobacteriaceae (45.4%). The studied microorganisms tested positive in 48 cases (54.5%); however, it is worth noting that, in some of the samples, more than one bacterial species was detected, which resulted in a total of 61 isolates. *Pantoea agglomerans* was the bacterium with the highest frequency of isolation (22/61) with 25%, followed by *Serratia liquefaciens* (11/61) with 12.5%, and *Enterobacter aerogenes* (8/61) with 9.1%. The least frequently isolated pathogens were *Enterobacter cloacae* (5/61) with 5.7%, *Encherichia coli* (4/61) with 4.5%, *Hafnia alvei* (3/61) and *Cronobacter sakazakii* (3/61) with 3.4% each, *Serratia rubidae* (2/61) with 2.3%, *Salmonella enterica* subsp. *enterica* (rough) (1/61), *Shigella sonnei* (1/61), and *Klebsiella pneumoniae* (1/61) with 1.1% each (Table 1).

Antimicrobial resistance. Table 2 shows the results of the antimicrobial susceptibility test. The antibiotics that showed the best efficiency were polimixin B (9.8%), gentamicin (6.6%), and enrofloxacin (6.6%), and those to which the total bacterial isolates showed greater resistance were amoxicillin (78.7%), ampicillin (75.4%), streptomycin (45.9%), and sulfonamides (42.6%). Results of the 22 *Pantoea agglomerans* isolated strains showed that higher frequency of bacterial resistance occurred in relation to amoxicillin (n=18), ampicillin

(n=16), and sulfonamides (n=4). Among the other three most frequently occurring bacterial species (*Serratia liquefaciens, Enterobacter aerogenes,* and *Enterobacter cloacae*, respectively), high absolute frequency of amoxicillin-resistant strains was also observed. Among the four *Escherichia coli* isolated strains, the highest frequency of resistant bacteria was observed when streptomycin (n=3) was tested; for other antibiotics, resistance frequency did not exceed one occurrence. The only *Salmonella* enterica subsp. *enterica* (rough) strain isolated was resistant to amoxicillin and streptomycin. Among the 61 strains isolated in this study, only seven (11.5%) showed no resistance to any of the classes of antibiotics tested. Twenty-three strains (37.7%) showed resistance to ≥3 antibiotic classes, that is, MDR condition. Occurrence of resistance to all 12 antibiotics tested was observed in only one isolate (1.6%) (Table 3), belonging to the *Enterobacter aerogenes* species.

Detection of diarrheagenic *Escherichia coli* (DEC) and avian pathogenic *Escherichia coli* (APEC). The four *Escherichia coli* strains isolated and screened by the PCR technique tested negative for all genes characteristic of DEC pathotypes. Regarding the search of APEC genes, only two isolates were positive: one detected for the *iss* gene and another for the *iutA* and *hlyF* genes. In this search, it was verified that one isolate was presented with the *iss* gene and the other strain contained the *iutA* and *hlyF* genes; the other two cases were negative. Therefore, the APEC pathotype was not identified in this search.

Table 1. Absolute and relative frequencies of Enterobacteriaceae isolated from fecal samples of Belgian canaries (*Serinus canaria*)

Bacterium	n	%
Pantoea agglomerans	22	25
Serratia liquefaciens	11	12.5
Enterobacter aerogenes	8	9.1
Enterobacter cloacae	5	5.7
Escherichia coli	4	4.5
Hafnia alvei	3	3.4
Cronobacter sakazakii	3	3.4
Serratia rubidaea	2	2.3
Salmonella enterica subsp. *enterica* (rough)	1	1.1
Shiguella sonnei	1	1.1
Klebsiella pneumoniae	1	1.1

DISCUSSION

Results of the present study showed that Belgian canaries host different bacterial species of the family Enterobacteriaceae that have commonly been reported in several scientific studies. Conzo et al. (1998) isolated and identified *Klebsiella pneumoniae*, *Pantoae agglomerans*, *Enterobacter cloacae*, among other species of bacteria in a survey of Enterobacteriaceae conducted with Belgian canaries raised in nurseries in Naples, southern Italy. Horn et al. (2015) detected *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Pantoea agglomerans*, and *Serratia* spp. in cloacal swabs of Belgian canaries from breeders in the region of Fortaleza, Ceará state, Brazil. Giacopello et al. (2015) reported the occurrence of gram-negative bacteria such as *Escherichia coli*, *Enterobacter cloacae*, *Cronobacter sakazakii*, *Pantoea agglomerans*, among other species, in feces of canaries with signs of diseases originating from aviaries of amateur breeders in Sicily, southern Italy.

Pantoea agglomerans was the most prevalent enterobacterium in the present study. This bacterium has also been reported in other species of healthy birds kept in captivity (Santos et al. 2010). Despite being generally considered a microorganism harmless to the health of birds, in some circumstances, its presence can be harmful. Gerlach (1994) clarified that the birds are likely to be infected with this bacterium through consumption of seeds, and that feed containing a high concentration of this microorganism is considered toxic. Conzo et al. (1998) listed this pathogen as one among other isolated Enterobacteriaceae responsible for the mortality of offspring and embryos of Belgian canaries. According to Kirzinger et al. (2015), another aspect worth highlighting with respect to this bacterial species

Table 2. Absolute frequency of Enterobacteriaceae isolated from Belgian canaries (*Serinus canaria*) resistant to antibiotics

Bacterium (total number of isolates)	AMO	AMP	NAL	TET	CLO	GEN	SUL	POL	NEO	ENR	SUT	STR
Pantoea agglomerans (22)	18	16	4	1	1	2	9	1	5	1	1	8
Serratia liquefaciens (11)	10	10	5	3	2	-	5	3	5	-	2	5
Enterobacter aerogenes (8)	8	8 [IR]	5	3	3	1	4	1	3	1	4	3
Enterobacter cloacae (5)	5	4 [IR]	-	-	-	-	3	-	-	-	-	4
Escherichia coli (4)	1	1	1	1	-	-	1	-	-	1	1	3
Hafnia alvei (3)	1	1[IR]	-	-	-	1	1	-	1	-	-	1
Cronobacter sakazakii (3)	2	3	2	1	1	-	2	-	1	1	1	2
Serraria rubidaea (2)	2	2	-	-	1	-	1	1	-	-	1	1
Salmonella enterica* (1)	1	-	-	-	-	-	-	-	-	-	-	1
Shigella sonnei (1)	-	-	-	-	-	-	-	-	-	-	-	-
Klebsiella pneumoniae (1)	1	1[IR]	-	-	-	-	-	-	-	-	-	-
Total number of Enterobacteriaceae (61)	49	46	17	9	8	4	26	6	15	4	10	28

*_Salmonella enterica_ subsp. _enterica_ (rough), [IR] intrinsic resistance; AMO = Amoxicillin, AMP = ampicillin, NAL = nalidixic acid, TET = tetracycline, CLO = chloramphenicol, GEN = gentamicin, SUL = sulfonamides, POL = polymyxin B, NEO, neomycin, ENR = enrofloxacin, SUT = sulfazotrim (sulfamethoxazole + trimethoprim), STR = streptomycin.

Table 3. Multidrug-resistant (MDR) enterobacteria isolated from Belgian canaries (*Serinus canaria*)

Number of classes of antibiotics	Number of resistant strains (%)*
0	7 (11.5)
1	20 (32.8)
2	11 (18.0)
3	8 (13.1)
4	5 (8.2)
5	3 (4.9)
6	4 (6.6)
7	2 (3.3)
8	1 (1.6)

* The frequency obtained for each number of classes of antibiotics was the same when strains with and without intrinsic resistance were considered.

is that *P. agglomerans* performs important exchanges of pathogenicity-determining genetic materials in the colonization of the host with other members of the Enterobacteriaceae family, including species pathogenic to humans.

Serratia liquefaciens was the second most frequently isolated bacterium in this study. In the Passeriformes order, this bacterial species has previously been isolated from cloacal swabs of saffron finch (*Sicalis flaveola*) seized from illegal trade in the city of São Paulo, Brazil (Davies et al. 2016). In a study addressing enterobacteria in cardinals (*Paroaria dominicana* and *Paroaria coronata*) seized from illegal trade in Sao Paulo, Cunha et al. (2016) isolated this bacterial species from cloacal swabs. In both studies, the authors did not describe whether the birds were ill. Fudge (2001) reported that it is not common for this genus to cause disease, but that it can affect immunocompromised birds. In humans, this microorganism has been reported to cause popliteal artery aneurysm infection that had been previously healed in patients (Coelho et al. 2016), as well as urinary infections (Menezes et al. 2004).

Enterobacter aerogenes and *Enterobacter cloacae* are bacteria commonly isolated in clinically healthy birds; however, they may also cause primary or secondary infections in immunocompromised birds, and may act concurrently with parasites, viruses, or fungi (Fudge 2001, Joseph 2003). Gharieb et al. (2013) investigated bacteria in wild birds from various locations in Egypt and isolated *E. aerogenes* and *E. cloacae.* In humans, *E. aerogenes* and *E. cloacae* have been reported in hospitals as important opportunistic pathogens, causing sepsis in neonates (Akindolire et al. 2016, Köse et al. 2016, Boulos et al. 2017), nosocomial infection (Oliva-Menacho et al. 2016), and septicemia in health professionals (Jha et al. 2016).

In this study, only one sample tested positive for bacteria of the genus *Salmonella*, and it was not possible to identify the serotype, only of the subspecies *enterica* (rough). According to Dorrestein & Vet Pathol (2003), this pathogen can cause high mortality in canaries; however, the birds from which the samples were collected showed no symptoms of salmonellosis. In Brazil, there are no published scientific reports showing positivity for *Salmonella* sp. in fecal samples or cloacal swabs from captive Belgian canaries. In free-living birds of the Passeriformes order, Dias et al. (2014) isolated *Salmonella enterica* in saffron finch (*Sicalis flaveola*) and in chestnut-capped blackbird (*Chrysomus ruficapillus*) captured near rice fields in southern Rio Grande do Sul state, Brazil. Previous studies conducted with Belgian canaries bred in captivity in other countries have also isolated this bacterial genus, with serotype Typhimurium as the most prevalent (Harrington Junior et al. 1975, Raidal 1998, Sánchez-Cordón et al. 2007, Madadgar et al. 2009, Giacopello et al. 2015), and obtained low frequency of isolation, corroborating the findings of the present study.

In the present study, 4.5% (4/88) of the isolated samples were *Escherichia coli* strains. Similar results were found by Horn et al. (2015), who observed 3.6% positivity in samples from apparently healthy Belgian canaries, but from cloacal swabs. In contrast, other studies have reported higher frequencies of *E. coli* in the Passeriformes order. In a survey of 50 fecal samples from cages where canaries with signs of disease belonging to amateur breeders were housed in Italy, Giacopello et al. (2015) found 62.0% positivity for this microorganism. Gaio (2017) observed that cloacal swabs from wild passerines from illegal trade also showed high percentage of *E. coli* (40.8%). Therefore, it can be assumed that the environmental conditions and sanitary status to which passerines are subjected may influence the isolation rate of *E. coli*.

All four strains analyzed were negative for the DEC genes investigated, which indicates that the isolates assessed were free of eight important potential diarrheal genes in humans (Lopes et al. 2016). Some genes associated with the APEC pathotype were detected in two strains: one positive for the *iss* gene and another for the *iutA* and *hlyF* genes. Although all these detected genes showed important virulence characteristics attributed to the APEC pathotype (Lynne et al. 2006, Chouikha et al. 2008, Johnson et al. 2008), none of the *E. coli* strains could be characterized as belonging to this pathotype, because they did not have all the necessary genes. However, it is important to emphasize that, according to Oliveira et al. (2015), pathogenic strains usually have two or more predictive genes for the APEC pathotype, whereas isolates with fewer than two genes are rarely pathogenic. In contrast, Johnson et al. (2008) state the need for at least four types of predictor genes to differentiate strains with pathogenic potential from commensal *E. coli*.

In the present study, some of the tested antibiotics showed high frequency of antimicrobial resistance. Amoxicillin was the antibiotic for which the strains presented greater resistance (78.7%), followed by ampicillin (75.4%), streptomycin (45.9%), and sulfonamides (42.6%). Data relative to antimicrobial resistance in strains from Belgian canary fecal samples are quite scarce in the scientific literature, and the few existing studies show divergent results. One of the few similarities observed refers to resistance associated with ampicillin, which has very often been present as one of the antibiotics with most serious resistance problems. Giacopello et al. (2015) also observed that the highest resistance in enterobacteria analyzed in canaries in Italy occurred with respect to amoxicillin (100%), ampicillin (92.2%), and streptomycin (61.2%), and these detected rates show percentages higher than those found in the present study. Horn et al. (2015) reported percentage resistance rates of 55.7, 54.1, and 39.3% to sulfonamides,

ampicillin, and tetracycline, respectively, in canaries bred in captivity in the municipality of Fortaleza, Ceará state, Brazil.

Some aspects should be considered when analyzing the resistance results found in this study. The first refers to the fact that not all cases of resistance occur due to an acquired condition; in some cases, bacteria manifest intrinsic resistance to some antibiotics, as in the case of ampicillin associated with *Enterobacter aerogenes*, *Enterobacter cloacae*, *Hafnia alvei* and *Klebsiella pneumoniae* (CLSI 2014) pathogens, which even after subtracting the cases of resistance associated with these bacteria, still remains the antibiotic that shows the second highest resistance occurrence in relation to Enterobacteriaceae. Regarding the case of MDR (37.7%), the frequency found in this study was lower than that observed by Horn et al. (2015), who verified that 49.2% of the Enterobacteriaceae isolated were resistant to three or more of the antibiotics investigated. Nevertheless, it is worth mentioning that the analysis conducted by these researchers, as it has occurred in several other studies involving Passeriformes and birds of other species raised in captivity, did not consider, for the purpose of MDR calculations, the guidelines indicated by Magiorakos et al. (2012), in which the quantification of strains resistant to an antibiotic should be made considering the classes of antibiotics used. Regardless, the MDR frequency detected in strains isolated from canary samples in northeastern Brazil serves as an alert for breeders, because this fact can have negative consequences for animal health, considering that the occurrence of bacteria with high rates of resistant antimicrobials hinders infection treatment and contributes to increased therapeutic costs (Oliveira et al. 2012).

Currently, bacterial resistance is a public health issue. One of the most important facts that have led to the emergence of MDR strains is the excessive and inadequate use of antibiotics (Nascimento et al. 2003), either as prevention or for diseases without diagnosis. In this respect, it can be observed that veterinary guidance is not always followed or even sought by bird breeders. In addition, access to antimicrobials has been facilitated in veterinary drug stores recently. Another factor related to this matter refers to the direct contact between birds and breeders. Santos et al. (2010) states that this relationship allows the exchange of microorganisms with resistance genes.

CONCLUSIONS

Different enterobacteria were isolated in feces collected from Belgian canaries (*Serinus canaria*), and this seems to be the first report of bacteria of the genus *Salmonella* isolated from fecal samples of birds belonging to breeders from northeastern Brazil.

The high frequency of resistance that some of the isolated bacterial strains presented to some of the antimicrobials analyzed suggests the need for greater control of the use of these drugs in order to avoid possible future therapeutic difficulties in the fight against microorganisms affecting national bird breeding.

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REFERENCES

Akindolire A.E., Tongo O., Dada-Adegbola H. & Akinyinka O. 2016. Etiology of early onset septicemia among neonates at the University College Hospital, Ibadan, Nigeria. J. Infect. Develop. Ctries 10(12):1338-1344. <http://dx.doi.org/10.3855/jidc.7830> <PMid:28036314>

Arnaiz-Villena A., Areces C. & Ruiz-Del-Valle V. 2012. El origen de los canarios. Revta Ornitol. Práct. 53:3-11.

Bélanger L., Garenaux A., Harel J., Boulianne M., Nadeau E. & Dozois C.M. 2011. *Escherichia coli* form animal reservoirs as a potential source of human extraintestinal pathogenic *E. coli.* Immunol. Med. Microbiol. 62(1):1-10. <http://dx.doi.org/10.1111/j.1574-695X.2011.00797.x> <PMid:21362060>

Boulos A., Rand K., Johnson J.A., Gautier J. & Koster M. 2017. Neonatal sepsis in Haiti. J. Trop. Pediatr. 63(1):70-73. <http://dx.doi.org/10.1093/tropej/fmw077> <PMid:27765888>

Camargo C.M.J., Camargo L.J.J. & Sueiro R.F. 2010. A criação amadora de pássaros nativos e os danos ambientais à fauna do Mato Grosso do Sul. Atualidades Ornitológicas 155(3):56-61.

Conzo G., Menna L.F. & Cerrone A. 1998. Enterobacteria infection in canaries (*Serinus canaria*) related bacterial food contamination (Compania). Selezione Vet. 8-9:717-723.

Coelho A., Lobo M., Martins V., Gouveia R., Sousa P., Campos J., Augusto R., Coelho N. & Canedo A. 2016. *Serratia liquefaciens* infection of a previously excluded popliteal artery aneurysm. EJVES Short Reports 34:1-4. <http://dx.doi.org/10.1016/j.ejvssr.2016.10.002> <PMid:28856323>

Chouikha I., Bree A., Moulin-Schouleur M., Gilot P. & Germon P. 2008. Differential expression of iutA and ibeA in the early stages of infection by extra-intestinal pathogenic *E. coli.* Microbes Infect. 10(4):432-438. <http://dx.doi.org/10.1016/j.micinf.2008.01.002> <PMid:18403237>

Cunha M.P.V., Guimarães M.B., Davies Y.M., Milanelo L. & Knöbl T. 2016. Bactérias gram-negativa em cardeais (*Poroaria coronata* e *Poroaria dominicana*) apreendidos do tráfico de animais silvestres. Braz. J. Vet. Res. Anim. Sci. 53(1):107-111. <http://dx.doi.org/10.11606/issn.1678-4456.v53i1p107-111>

Croxen M.A. & Finlay B.B. 2010. Molecular mechanisms of *Escherichia coli* pathogenicity. Nat. Rev. Microbiol. 8(1):26-38. <http://dx.doi.org/10.1038/nrmicro2265> <PMid:19966814>

CLSI 2014. Performance Standards for Antimicrobial Susceptibility Testing. CLSI document M100-S24, Twenty-Fourth Informational Supplement. Clinical and Laboratory Standards Institute, Wayne.

D'aout J.Y., Maurer J. & Baley J.S. 2001. *Salmonella* species, p.141-178. In: Doyle M.P. & Beuchat L.R. (Eds), Food Microbiology: fundamentals frontiers. ASM Press, Madison.

Davies Y.M., Guimarães M.B., Milanelo L., Oliveira M.G.X., Gomes V.T.M., Azevedo N.P., Cunha M.P.V., Moreno L.Z., Romero D.C., Christ A.P.G., Sato M.I.Z., Moreno A.M., Ferreira A.J.P., Sá L.R.M. & Knöbl T. 2016. A survey on gram-negative bacteria in saffron finches (*Sicalis flaveola*) from illegal wildlife trade in Brazil. Braz. J. Vet. Res. Anim. Sci. 53(3):286-294. <http://dx.doi.org/10.11606/issn.1678-4456.bjvras.2016.109042>

Dias P.A., Wilsmann D.E., Heinen J.G., Corsini C.D., Calabuig C. & Timm C.D. 2014. Primeiro relato de *Salmonella enterica* e *Campylobacter* spp. isolados de Garibaldi (*Chrysomus ruficapillus*) e Canário-da-terra (*Sicalis flaveola*) silvestres. Revta Inst. Adolfo Lutz 73(4):368-371.

Dorrestein G.M. 1997. Passerines, p.867-885. In: Altrnan R.B., Clubb S.L., Dorrestein G.M. & Quesenberry K. (Eds), Avian Medicine and Surgery. W.B. Saunders, Philadelphia.

Dorrestein G.M. & Vet Pathol D. 2003. Diagnostic approaches and management of diseases in captive passerines. Semin. Avian Exotic Pet Med. 12(1):11-20. <http://dx.doi.org/10.1053/saep.2003.127881>

Dorrestein G.M. 2009. Bacterial and parasitic diseases of passerines. Vet. Clin. N. Am., Exotic Anim. Pract. 12(3):433-451. <http://dx.doi.org/10.1016/j.cvex.2009.07.005> <PMid:19732703>

Ewers C., Li G., Wilking H., Kiessling S., Alt K., Antáo E.M., Laturnus C., Diehl I., Glodde S., Homeier T., Böhnke U., Steinrück H., Philipp H.C. & Wieler L.H. 2007. Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: how closely related are they? Int. J. Med. Microbiol. 297(3):163-176. <http://dx.doi.org/10.1016/j.ijmm.2007.01.003> <PMid:17374506>

Fudge A.M. 2001. Diagnosis and treatment of avian bacterial diaseases. Semin. Avian Exotic Pet Med. 10(1):33-11. <http://dx.doi.org/10.1053/saep.2001.19542>

Flammer K. 1999. Zoonosis acquired from birds, p.151-156. In: Fowler M.E. & Miller R.E. (Eds), Zoo and Wild Animal Medicine: current therapy. W.B. Saunders, Philadelphia.

Gaio F.C. 2017. Isolamento, resistência e caracterização das cepas de *Escherichia coli* e *Salmonella* spp. em Psitacídeos presentes no centro de triagem de animais silvestres do Ceará. Master's Thesis, Postgraduate Program in Veterinary Science, Universidade Estadual do Ceará, Fortaleza, CE. 57p.

Gerlach H. 1994. Bacteria, p.949-983. In: Ritchie B.W., Harrinson G.J. & Harrinson L.R. (Eds), Avian Medicine: principles and application. Wingers Publishing, Florida.

Giacopello C., Foti M., Fisichella V. & Lo Piccolo F. 2015. Antibiotic-resistance patterns of Gram-negative bacterial isolates from breeder canaries (*Serinus canaria domestica*) with clinical disease. J. Exotic Pet. Med. 24(1):84-91. <http://dx.doi.org/10.1053/j.jepm.2014.12.009>

Guimarães M.B. 2007. Passeriformes, p.324-337. In: Cubas Z. S., Silva J.C.R. & Catão-Dias J.L. (Eds), Tratado de Animais Selvagens: medicina veterinária. Roca, São Paulo.

Gharieb A., Abu-El-ezz R.M. & Mohamad R.E. 2013. Prevalence of Enterobacteriacea in wild birds and humans at sharkia province; with special reference to the genetic relationship between *E. coli* and *Salmonella* isolates determined bby protein profile analysis. J. Am. Sci. 9(4):173-183.

Harrington Junior R., Blackburn B.O. & Cassidy D.R. 1975. Salmonelosis in canaries. Avian Diseases 19(4):827-829. <http://dx.doi.org/10.2307/1589197> <PMid:1106388>

Herikstad H., Motarjemi Y. & Tauxe R.V. 2002. *Salmonella* surveillance: a global survey of public health serotyping. Epidemiol. Infect. 129(1):1-8. <http://dx.doi.org/10.1017/S0950268802006842> <PMid:12211575>

Holt J. & Bergey D.H. 1994. Bergey's Manual of Determinative Bacteriology. 9th ed. William and Wilkis, Baltimore. 787p.

Horn R.V., Cardoso W.M., Lopes E.S., Teixeira R.S.C., Albuquerque Á.H., Rocha-e-Silva R.C., Machado D.N. & Bezerra W.G. 2015. Identification and antimicrobial resistance of members from the Enterobacteriaceae family isolated from canaries (*Serinus canaria*). Pesq. Vet. Bras. 35(6):552-556. <http://dx.doi.org/10.1590/S0100-736X2015000600011>

Johnson T.J., Wannemuehler Y., Doetkott C., Johnson S.J., Rosenberger S.C. & Nolan L.K. 2008. Identification of minimal predictors of avian pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. J. Clin. Microbiol. 46(12):3987-3996. <http://dx.doi.org/10.1128/JCM.00816-08> <PMid:18842938>

Joseph V. 2003. Infectious and parasitic diseases of captive passerines. Semin. Avian Exot. Pet Med. 12(1):21-28. <http://dx.doi.org/10.1053/saep.2003.127878>

Jha P., Kim C.M., Kim D.M., Chung J.H., Yoon N.R., Jha B., Kim S.W., Jang S.J., Ahn Y.J., Chung J.K. & Jeon D.Y. 2016. Transmission of *Enterobacter aerogenes* septicemia in healthcare workers. SpringerPlus 5(1):1-4. <http://dx.doi.org/10.1186/s40064-016-3011-x> <PMid:27610316>

Kanashiro A.M.I., Castro A.G.M., Cardoso A.L.S.P., Tessari E.N.C. & Tavechio A.T. 2002. Persistência de *Salmonella* sp. após antibioticoterapia em psitacídeos pertencentes a um criadouro comercial. Arqs Inst. Biológico, São Paulo, 69(2):99-101.

Kaper J.B., Nataro J.P. & Mobley H.L. 2004. Pathogenic *Escherichia coli.* Nat. Rev. Microbiol. 2(2):123-140. <http://dx.doi.org/10.1038/nrmicro818> <PMid:15040260>

Kirzinger M.W., Butz C.J. & Stavrinides J. 2015. Inheritance of *Pantoea* Type III secretion systems through both vertical and horizontal transfer. Mol. Genet. Genomics 290(6):2075-2088. <http://dx.doi.org/10.1007/s00438-015-1062-2> <PMid:25982743>

Koneman E.W., Allen S.D., Janda W.M., Schreckenberger P.C. & Winn W.C. 2001. Diagnóstico Microbiológico. 5ª ed. Guanabara Koogan, Rio de Janeiro. 1608p.

Koneman E.W., Allen S.D., Janda W.M., Procop G., Schreckenberger P. & Woods G. 2012. As Enterobacteriaceae, p.209-243. In: Koneman E.W. & Winn W.C. (Eds), Diagnóstico Microbiológico: texto e atlas colorido. Guanabara Koogan, Rio de Janeiro.

Köse Ş., Özer E., Gülay Z., Akkoçlu G., Tokgöz H., Ağuş N., Ergönül Ö. & Öztürk R. 2016. *Enterobacter cloacae* sepsis outbreak in neonatal intensive care unit due to contaminated total parenteral nutrition solution. J. Pediatr. Res. 3(2):109-112. <http://dx.doi.org/10.4274/jpr.20143>

Lima I.F.N., Quetz J.D.S., Guerrant R.L., Nataro J.P., Houpt E.R., Lima A.A.M. & Havt A. 2013. Enteroaggregative *Escherichia coli* quantification in children stool samples using quantitative PCR. Apmis 121(7):643-651. <http://dx.doi.org/10.1111/apm.12032> <PMid:23216208>

Lynne A.M., Foley S.L. & Nolan L.K. 2006. Immune response to recombinant *Escherichia coli* isso protein in poultry. Avian Dis. 50(2):273-276. <http://dx.doi.org/10.1637/7441-092105R.1> <PMid:16863080>

Lopes E.S., Maciel W.C., Teixeira R.S.C., Albuquerque Á.H., Vasconcelos R.H., Machado D.N. & Santos I.C.L. 2016. Isolamento de *Salmonella* spp. e *Escherichia coli* de psittaciformes: relevância em saúde pública. Arqs Inst. Biológico, São Paulo, 83:1-10.

Macwhirter P. 1994. Passeriformes, p.1172-1199. In: Richie B.W., Harrison G.J. & Harrison L.R. (Eds), Avian Medicine: principles and application. Wingers Publishing, Florida.

Madadgar O., Salehi T.Z., Ghafari M.M., Tamai I.A., Madani S.A. & Yahyareyat R. 2009. Study of an unusual paratyphoid epornitic in canaries (*Serinus canaria*). Avian Pathol. 38(6):437-441. <http://dx.doi.org/10.1080/03079450903349170> <PMid:19937532>

Magiorakos A.P., Srinivasan A., Carey R.B., Carmeli Y., Falagas M.E., Giske C.G., Harbarth S., Hindler J.F., Kahlmeter G., Olsson-Liljequist B., Paterson D.L., Rice L.B., Stelling J., Struelens M.J., Vatopoulos A., Weber J.T. & Monnet D.L. 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 18(3):268-281. <http://dx.doi.org/10.1111/j.1469-0691.2011.03570.x> <PMid:21793988>

Mantel M. 2005. A história do canário desde sua descoberta. Bras. Ornitol. 56:8-9.

Menezes E.A., Cezafar F.C., Andrade M.D.S.D.S., Rocha M.V.A.D.P. & Cunha F.A. 2004. Freqüência de *Serratia* sp em infecções urinárias em pacientes internados na Santa Casa de Misericórdia de Fortaleza. Revta Soc. Bras. Med. Trop. 37(1):70-71. <http://dx.doi.org/10.1590/S0037-86822004000100020> <PMid:15042191>

Mokady D., Gophna U. & Ron E.Z. 2005. Extensive gene diversity in septicemia *Escherichia coli* strains. J. Clin. Microbiol. 43(1):66-73. <http://dx.doi.org/10.1128/JCM.43.1.66-73.2005> <PMid:15634952>

Moulin-Schouleur M., Rèpèrant M., Laurent S., Brée A., Mignongrasteau S., Germon P., Rasschaert D. & Schouler C. 2007. Extraintestinal pathogenic *Escherichia coli* strains of avian and human origin: link between phylogenetic relationships and common virulence patterns. J. Clin. Microbiol. 45(10):3366-3376. <http://dx.doi.org/10.1128/JCM.00037-07> <PMid:17652485>

Nascimento A.M., Cursino L., Gonçalves-Dornelas H., Reis A., Chartone-Souza E. & Marini M.Â. 2003. Antibiotic-resistant Gram-negative bacteria in birds from the Brazilian Atlantic forest. Condor 105(2):358-361. <http://dx.doi.org/10.1650/0010-5422(2003)105[0358:AGBIBF]2.0.CO;2>

Oliva-Menacho J.E., Garcia-Hjarles M.A., Oliva-Candela J.A. & De la Cruz-Roca H.S. 2016. Contaminación con bacterias patógenas de estetoscopios del personal médico en un hospital de nivel III en Lima, Perú. Revta Med. Hered. 27(2):83-88. <http://dx.doi.org/10.20453/rmh.v27i2.2842>

Oliveira A.L., Rocha D.A., Finkler F., Moraes L.B., Barbieri N.L., Pavanelo D.B., Winkler C., Grassotti T.T., Brito K.C., Brito B.G. & Horn F. 2015. Prevalence of ColV plasmid-linked genes and in vivo pathogenicity of avian strains of *Escherichia coli.* Foodborne Pathog. Dis. 12(8):679-685. <http://dx.doi.org/10.1089/fpd.2014.1934> <PMid:26258262>

Oliveira K.W., Gomes F.C.O. & Morais P.B. 2012. Ocorrência de *Escherichia coli* multirresistentes a antimicrobianos nas principais praias do reservatório de Lajeado, TO. Eng. Amb. Pesq. Tecnol. 9(3):338-351.

Raidal S.R. 1998. Salmonellosis in two canary (*Serinus canaria*) flocks. Aust. Vet. Pract. 28(2):58-62.

Santos H.F., Flôres M.L., Lara V.M., Silva M.S., Battisti L. & Lovato L.T. 2010. Microbiota cloacal aeróbia de cracídeos cativos no Rio Grande do Sul e sua susceptibilidade a antimicrobianos. Pesq. Vet. Bras. 30(12):1077-1082. <http://dx.doi.org/10.1590/S0100-736X2010001200013>

Sánchez-Cordón P.J., Gómez-Villamandos J.C., Gutiérrez J., Sierra M.A., Pedrera M. & Bautista M.J. 2007. *Atoxoplasma* spp. infection in captive canaries (*Serinus canaria*). J. Vet. Med., Series A 54(1):23-26. <http://dx.doi.org/10.1111/j.1439-0442.2007.00909.x> <PMid:17359450>

Smith J.L., Fratamico P.M. & Gunther N.W. 2007. Extraintestinal pathogenic *Escherichia coli.* Foodborne Pathog. Dis. 4(2):134-163. <http://dx.doi.org/10.1089/fpd.2007.0087> <PMid:17600482>

Soncini R.A. 2002. Controle de *Salmonella enteritidis* na avicultura. Anais do III Simpósio Brasil Sul de Avicultura. Embrapa Suínos e Aves, Concórdia, SC. 6p.

Sousa E., Berchieri A.J., Pinto A.A., Machado R.Z., Carrasco A.O., Marciano J.A. & Werther K. 2010. Prevalence of *Salmonella* spp. antibodies to *Toxoplasma gondii*, and Newcastle disease virus in feral pigeons (*Columba livia*) in the city of Jaboticabal, Brazil. J. Zoo Wildl. Med. 41(4):603-607. <http://dx.doi.org/10.1638/2008-0166.1> <PMid:21370639>

Taniuchi M., Walters C.C., Gratz J., Maro A., Kumburu H., Serichantalergs O., Sethabutr O., Bodhidatta L., Kibiki G., Toney D.M., Berkeley L., Nataro J.P. & Houpt E.R. 2012. Development of a multiplex polymerase chain reaction assay for diarrheagenic *Escherichia coli* and *Shigella* spp. and its evaluation on colonies, culture broths, and stool. Diagn. Microbiol. Infect. Dis. 73(2):121-128. <http://dx.doi.org/10.1016/j.diagmicrobio.2012.03.008> <PMid:22541788>

Tivendale K.A., Logue C.M., Kariyawasam S., Jordan D., Hussein A., Li G., Wannemuehler Y. & Nolan L.K. 2010. Avian-pathogenic *Escherichia coli* strains are similar to neonatal meningitis *E. coli* strains and are able to cause meningitis in the rat model of human disease. Infect. Immun. 78(8):3412-3419. <http://dx.doi.org/10.1128/IAI.00347-10> <PMid:20515929>

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Original Article
Wildlife Medicine

Detection of avian metapneumovirus subtype A from wild birds in the State of São Paulo, Brazil[1]

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ABSTRACT.- Rizotto L.S., Simão R.M., Scagion G.P., Simasaki A.A., Caserta L.C., Benassi J.C., Arns C.W. & Ferreira H.L. 2019. **Detection of avian metapneumovirus subtype A from wild birds in the State of São Paulo, Brazil**. *Pesquisa Veterinária Brasileira 39(3):209-213*. Departamento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Av. Duque de Caxias Norte 225, Pirassununga, SP 13635-900, Brazil. E-mail: hlage@usp.br

The present study investigated the circulation of avian metapneumovirus (aMPV) in wild birds in Brazil. To do so, 131 samples from 366 oropharyngeal or cloacal swabs collected from 18 species of birds were tested individually or in pools by RT-PCR. Samples detected by RT-PCR were selected for DNA sequencing. Thirteen (9.9%) samples were detected by the RT-PCR targeting the N gene and four out of 13 samples were sequenced. Sequencing results showed a high identity with the aMPV subtype A. Our results confirm the circulation of the aMPV subtype A in wild birds in Brazil even five years after its last detection.

INDEX TERMS: Detection, avian metapneumovirus, subtype A, wild birds, São Paulo, Brazil, waterfowl, surveillance, aMPV, wildlife animals, birds, viroses.

RESUMO.- [**Detecção de metapneumovirus aviário subtipo A em aves silvestres no estado de São Paulo, Brasil.**] O presente estudo investigou a circulação de metapneumovírus aviário em aves silvestres no Brasil. Para tanto, 131 amostras de 366 suabes orofaringeanos ou cloacais coletados de 18 espécies de aves foram testadas individualmente ou na forma de *pools* por RT-PCR. As amostras detectadas por RT-PCR foram selecionadas para sequenciamento. Treze (9,9%) das amostras foram detectadas por RT-PCR tendo o gene N como alvo; destas, quatro foram sequenciadas com sucesso. Resultados do sequenciamento mostraram alta identidade com o aMPV de subtipo A. Nossos resultados confirmam a circulação de aMPV subtipo A em aves silvestres no Brasil mesmo cinco anos após sua última detecção.

TERMOS DE INDEXAÇÃO: Detecção, metapneumovirus aviário, subtipo A, aves silvestres, São Paulo, Brasil, aves aquáticas, epidemiologia, aMPV, animais silvestres, viroses.

INTRODUCTION

The avian metapneumovirus (aMPV; family: *Pneumoviridae*, genus: *Metapneumovirus*) (Adams et al. 2016) is divided into four subtypes (A,B,C, and D) (Juhasz & Easton 1994, Seal 1998, Bayon-Auboyer et al. 2000). These viruses can cause respiratory disease and a drop in egg production in commercial birds, such as turkeys and chickens (Jones 1996). The aMPV viruses can also infect other birds, including pheasants, guinea fowls and wild birds (Jones & Rautenschlein 2013). aMPV subtypes A and B have a worldwide distribution (Cook 2000), whereas aMPV C was isolate in USA, France, Korea and China from commercial birds (Seal 1998, Toquin et al. 1999, Lee et al. 2007, Sun et al. 2014). Finally, aMPV D was isolated in commercial turkeys in France (Bayon-Auboyer et al. 2000).

Wild birds seem to act as reservoirs or vectors of aMPV into poultry farms. aMPV introduction in a poultry farm by

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migrating wild birds has already been reported in the United Kingdom (Stuart 1989). In 2000, the occurrence of this disease in turkey flocks in the USA was correlated to the migration of wild birds (Shin et al. 2000). Since then, the aMPV subtype C has been isolated in the US and European countries in many avian species, such as wild ducks (*Anas platyrhynchos*), wild geese, wild Canada geese (*Branta canadensis*), and blue-winged teals (*Anas discors*) (Shin et al. 2000, 2002, Bennett et al. 2002, 2004, Turpin et al. 2008, Van Boheemen et al. 2012). The detection of aMPV subtype A was reported in the white-cheeked pintail (*Anas bahamensis*), orinoco goose (*Neochen jubata*), white-eyed parakeet (*Psittacara leucophthalmus),* rusty-margined guan (*Penelope superciliaris*) and pigeon (*Columba livia*) (Felippe et al. 2011). The detection of anti-aMPV antibodies have also been reported in American coots and crows, egrets, geese, wild ducks, rock pigeons and ostriches (Cadman et al. 1994, Shin et al. 2000, 2002, Turpin et al. 2008). The present study aimed to investigate the circulation of avian metapneumovirus in wild birds in State of São Paulo.

MATERIALS AND METHODS

To achieve this goal, 366 swabs (oropharyngeal or cloacal) were collected from 192 wild birds belonging to 18 species and six different orders: Anseriformes (87.5%), Psittaciformes (6.77%), Columbiformes (4.17%), Strigiformes (0.52%), Piciformes (0.52%) and Falconiformes (0.52%). These swabs were collected from 2013 to 2015 at four different locations of captive birds in the São Paulo state. Swabs were tested as follows according to bird species and location: i) grouped as pools of up to five cloacal-C or oropharyngeal-OP swabs (Spackman et al. 2013); ii) grouped as pools of up to two C and OP swabs (Araujo et al. 2014); iii) tested individually. Collected swabs were stored in 500µL Brain Heart Infusion (BHI) medium containing antibiotic and anti-mycotic (10000U/mL of penicilin, 10000µg/mL of streptomycin and 25µg/mL of amphotericin B). After collection, samples were stored in -80°C until processing. A total of 131 samples (Table 1) were tested. All swabs were collected according to international, national, and institutional guidelines for the care and use of animals (CEUA-FZEA-USP no. 2012.1.170.74.0, CEUA-FMVZ 5201050214 e CEUA-FMVZ-USP no. 2309251114) and legal approval of ICMBio-Brazil (SISBIO no. 3475-1).

The commercial live vaccines containing subtype A (Poulvac® TRT, Zoetis Industry) and B (Nemovac®, Merial Animal Health) were used as positive controls for the RT-PCR reactions. Commercial live vaccines containing Newcastle disease virus (New-Vacin La Sota, Biovet Laboratory) and an infectious bronchitis virus (Bio-Bronk-Vet, Biovet Laboratory) were used as a specified control for the RT-PCR reactions. Viral RNA was purified from 140µL in medium of swabs using a QIAamp® RNA Mini kit (Qiagen, Hilden) according to the manufacturer's instructions.

RT-PCR targeting the N gene was done using the QIAGEN® OneStep RT-PCR kit (Qiagen, Hilden) with primers previously described by Bäyon-Auboyer et al. (1999). Briefly, reactions were tested with 2.5µL of RNA and the final concentration of 1x QIAGEN OneStep RT-PCR Buffer containing 2.5mM of $MgCl_2$, 1µL of enzyme mix, 400µM of each dNTP and 0.6µM of each primer and enough water to reach the final volume of 25µL. Reactions were carried out in a C1000 Touch (Bio-Rad, Foster City). Briefly, the RT reaction was carried out at 50°C for 30min. PCR amplification was performed with an initial denaturation step at 95°C for 15min, followed by 40 cycles (95°C for 30s; 51°C for 30s; 72°C for 60s) and a final elongation step at 72°C for 5 min. PCR products (115 bp) were visualized by electrophoresis using a 2% agarose gel stained with SYBR safe (Life Technologies, Carlsbad) in TBE buffer (pH 8.0). A 100 bp DNA molecular ladder (Amresco, Solon) was used to estimate the band size.

Table 1. Tested samples according to species, number of birds, quantity and swab types (C-cloacal or OP-oropharyngeal swabs)

Species	Number of birds	C	OP	Number of tested samples (pool/individually)
Dendrocygna viduata[a]	73	73	73	37
Aix galericulata[b, c]	61	60	57	32
Dendrocygna autumnalis[a]	10	10	10	5
Aix sponsa[b, c]	8	7	8	9
Anas platyrhynchos domestica[b]	6	6	6	12
Chenonetta jubata[b]	4	4	4	4
Cairina moschata[a]	2	2	2	1
Dendrocygna bicolor[a]	1	1	1	1
Cereopsis novahollandiae[c]	1	1	1	2
Cygnus melanocoryphus[c]	1	1	1	2
Cygnus atratus[c]	1	0	1	1
Psittacara leucophthalma[c, d]	10	7	7	14
Ara chloropterus[c]	2	0	2	2
Amazona aestiva[d]	1	0	1	1
Columba livia[a]	8	8	8	4
Megascops choliba[d]	1	0	1	1
Ramphastos dicolorus[d]	1	1	0	1
Falco sparverius[d]	1	1	1	2
TOTAL	192	182	184	131

Locations and years where samples were collected: [a] Wild (migratory and resident) birds from Clube de Campo São Paulo, São Paulo/SP (2015), samples were kindly provided by Prof. Dr. Edison L. Durigon of the Laboratory of Clinical and Molecular Virology of the Institute of Biomedical Sciences (ICB-II), University of São Paulo; [b] Captive birds from Commercial Bird house, Pirassununga/SP (2014); [c] Captive birds from Municipal Ecological Park of Americana "Eng. Cid Almeida Franco", Americana/SP (2013); [d] Wild Birds from Mata Ciliar of Jundiaí (2015), were from different origins such as smuggling, injured birds. Data about those birds is usually not available and sampling was performed upon their arrival. All birds were healthy, without any clinical signs.

Positive samples detected by RT-PCR were amplified in specific pathogen free (SPF) chicken embryonated eggs (CEE). Original samples were centrifuged at 5000xg for 5min, and 100µL was inoculated in the allantoic cavity. Eggs were observed daily for mortality for seven days. Afterwards, the allantoic fluid was collected and stored at -80°C. All allantoic liquids were tested by RT-PCR after three blind passages.

DNA sequencing was done to confirm positive results using the same reactions conditions described above using 10µL of RNA in the final volume of 50µL. Amplicons were purified using the Illustra™ GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare, Buckinghamshire). The DNA sequencing reaction was sent to the CEGH-CEL facility (IB-USP) in duplicate along with each primer (5µM) and purified DNA (50 to 80mg) for DNA sequencing. Sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City) on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City).

The obtained sequences were evaluated for quality using the Sequence Scanner™ Software 2 (Applied Biosystems, Foster City) and edited by MEGA7 (Kumar et al. 2016). Afterwards, the obtained sequences and sequences available in GenBank were aligned using Clustal W (Thompson et al. 1994) Bioedit Sequence Alignment software version 7.2.5 (Hall 1999). Phylogenetic analysis and tree constructions were also performed using the MEGA7 software and deduced using the Maximum Likelihood method with a total of 1000 replications on the bootstrap.

RESULTS

The RT-PCR targeting the N gene detected 13 positives (9.9%) out of 131 tested samples. Eight samples were from Anseriformes (*Aix sponsa, Aix galericulata, Dendrocygna viduata*), three from Columbiformes (*Columba livia*), one from Falconiformes (*Falco sparverius*), and one from Psittaciformes (*Psittacara leucophthalma)*. Five (38.4%) samples were detected from OP swabs, four (30.8%) detected samples from C swabs, and the last four (30.8%) detected samples from cloacal and OP swabs together. Positive samples were detected in all locations; among those, three sampling sites had wild (migratory and resident) birds. The fourth sampling site, the commercial birdhouse in Pirassununga, had captive birds which also maintained poultry in this location.

Four (30.7%) out of 13 positive samples were successfully sequenced after the first passage in CEE, although three passages were performed. Phylogenetic analysis was performed using the four samples (two from *Aix galericulata*, one from *Dendrocygna viduata* and one from *Falco sparverius*) based on 115 nucleotides of the N gene using 46 available aMPV sequences in GenBank (Fig.1). The mean genetic distance between our samples ranged from 0.0% to 0.04%, showing a low genetic distance (0.036 to 0.049) with aMPV subtype A clustering with sequences from Italy, England, Brazil, and Wales. The genetic distances of our samples with other subgroups ranged from to 0.190 to 0.238 with subtype B, 0.218 to 0.251 with subtype D and 0.202 to 0.268 with subtype C. A tree using larger sequences was generated before including the shorter sequences to confirm the obtained tree clusters (data not shown).

DISCUSSION

Our study detected aMPV subtype A in samples from Anseriformes, Columbiformes, Falconiformes and Psittaciformes, which is in acordance with a previous study (Felippe et al. 2011). Moreover, most positive samples were obtained from wild waterfowl which also in agreement with a recent study (Jardine et al. 2018) and previous studies (Shin et al. 2000, Bennett et al. 2002, Felippe et al. 2011, Turpin et al. 2008).

Waterfowl plays an important role in the maintenance and dissemination of several commercially important viruses, such as, AIV, NDV, including aMPV (Olsen et al. 2006, Alexander 2007, Cha et al. 2013). Wild birds seem to be highly susceptible to aMPV C (Shin et al. 2000, Bennett et al. 2004, Van Boheemen et al. 2012) and they seem to be partially susceptible to aMPV A and B (Felippe et al. 2011, Gharaibeh & Shamoun 2012).

The virus isolation is a important tool to confirm the infection in birds. In our study, a low virus rate was detected after passage in eggs. These results suggest a limit replication

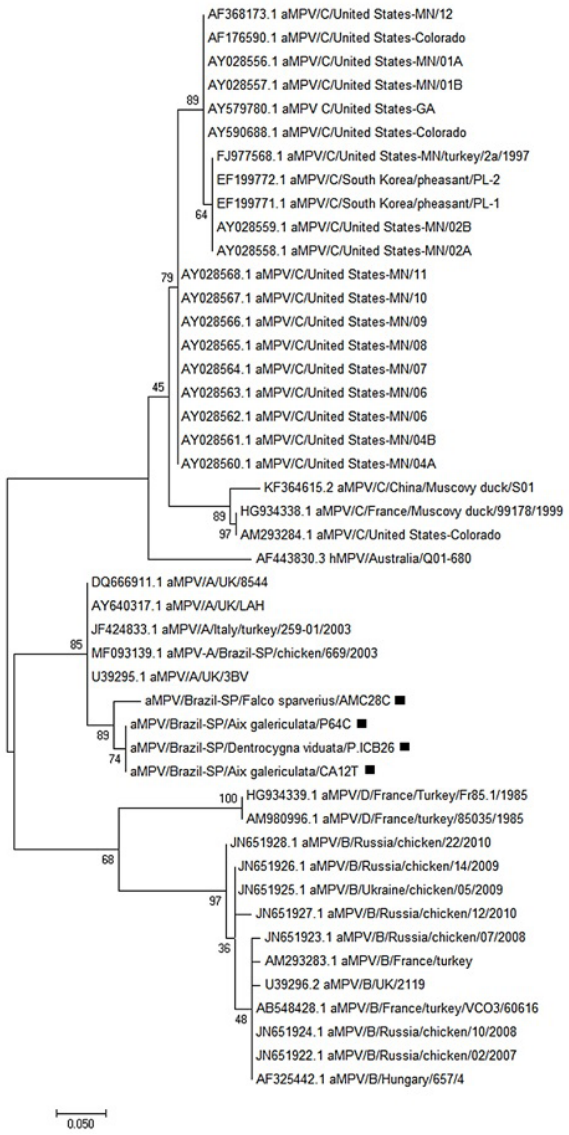

Fig.1. Phylogenetic tree of avian metapneumovirus (aMPV) samples from *Aix galericulata, Dendrocygna viduata,* and *Falco sparverius.* The evolutionary history was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). The tree with the highest log likelihood (-542.1852) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The initial tree(s) for the heuristic search was obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, followed by selection of the topology with a superior log likelihood value. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 60.7571% sites). The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. The analysis involved 46 nucleotide sequences. The codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 115 positions in the final dataset. Evolutionary analyses were conducted using the MEGA7 software (Kumar et al. 2016). The aMPVsequences originated from this study are denoted with a ■ symbol.

in wild birds and it corroborates with the low rates of virus isolation already reported. A experimental study in turkeys, which is the most susceptible specie to aMPV, reported a low virus recovery rate after the 5th day post inoculation (Cook et al. 1991). Another experimental study in pigeons showed a limit amount of virus in target tissues after infection (Catelli et al. 2012). Therefore, the limited time and replication of the virus in the tissues and excretions could explain the low rate of virus isolation from wild bird samples in chicken embryonated eggs.

CONCLUSIONS

Our study shows that aMPV subtype A continues to circulate in different wild bird species, although with very limit virus shedding, five years after the last report.

More studies are needed to investigate the role of wild birds in aMPV A epidemiology. Therefore, continuous surveillance in wild birds could be valuable in our understanding of aMPV epidemiology.

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

REFERENCES

Adams M.J., Lefkowitz E.J., King A.M., Harrach B., Harrison R.L., Knowles N.J., Kropinski A.M., Krupovic M., Kuhn J.H., Mushegian A.R., Nibert M., Sabanadzovic S., Sanfaçon H., Siddell S.G., Simmonds P., Varsani A., Zerbini F.M., Gorbalenya A.E. & Davison A.J. 2016. Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2016). Arch. Virol. 161(10):2921-2949. <http://dx.doi.org/10.1007/s00705-016-2977-6> <PMid:27424026>

Alexander D.J. 2007. An overview of the epidemiology of avian influenza. Vaccine 25(30):5637-5644. <http://dx.doi.org/10.1016/j.vaccine.2006.10.051> <PMid:17126960>

Araujo J., Azevedo Júnior S.M., Gaidet N., Hurtado R.F., Walker D., Thomazelli L.M., Ometto T., Seixas M.M.M., Rodrigues R., Galindo D.B., Silva A.C.S., Rodrigues A.M.M., Bomfim L.L., Mota M.A., Larrazábal M.E., Branco J.O., Serafini P., Neto I.S., Franks J., Webby R.J., Webster R.G. & Durigon E.L. 2014. Avian influenza virus (H11N9) in migratory shorebirds wintering in the Amazon Region, Brazil. PLoS One 9(10):e110141. <http://dx.doi.org/10.1371/journal.pone.0110141> <PMid:25329399>

Bäyon-Auboyer M.H., Jestin V., Toquin D., Cherbonnel M. & Eterradossi N. 1999. Comparison of F-, G- and N-based RT-PCR protocols with conventional virological procedures for the detection and typing of turkey rhinotracheitis virus. Arch. Virol. 144(6):1091-1109. <http://dx.doi.org/10.1007/s007050050572> <PMid:10446646>

Bäyon-Auboyer M.-H., Arnauld C., Eterradossi N. & Toquin D. 2000. Nucleotide sequences of the F, L and G protein genes of two non-A/non-B avian pneumoviruses (APV) reveal a novel APV subgroup. J. Gen. Virol. 81(Pt 11):2723-2733. <http://dx.doi.org/10.1099/0022-1317-81-11-2723> <PMid:11038385>

Bennett R.S., Mccomb B., Shin H.J., Njenga M.K., Nagaraja K.V. & Halvorson D.A. 2002. Detection of avian pneumovirus in wild Canada (*Branta canadensis*) and blue-winged teal (*Anas discors*) geese. Avian Dis. 46(4):1025-1029. <http://dx.doi.org/10.1637/0005-2086(2002)046[1025:DOAPIW]2.0.CO;2> <PMid:12495069>

Bennett R.S., Nezworski J., Velayudhan B.T., Nagaraja K.V., Zeman D.H., Dyer N., Graham T., Lauer D.C., Njenga M.K. & Halvorson D.A. 2004. Evidence of avian pneumovirus spread beyond Minnesota among wild and domestic birds in central North America. Avian Dis. 48(4):902-908. <http://dx.doi.org/10.1637/7208-051804R> <PMid:15666873>

Cadman H.F., Kelly P.J., Zhou R., Davelaar F. & Mason P.R. 1994. A serosurvey using enzyme-linked immunosorbent assay for antibodies against poultry pathogens in ostriches (*Struthio camelus*) from Zimbabwe. Avian Dis. 38(3):621-625. <http://dx.doi.org/10.2307/1592088> <PMid:7832718>

Catelli E., Lupini C., Listorti V., Marziali A., De Matteo P., Naylor C.J. & Cecchinato M. 2012. The pigeon (*Columba livia*) is not sensitive to Avian Metapneumovirus subtype B and does not play any role in virus spread in experimental conditions. VII International Symposium on Avian Corona and Pneumovirus and Complicating Pathogens, Rauischholzhausen, Germany, p.285-291.

Cha R.M., Yu Q. & Zsak L. 2013. The pathogenicity of avian metapneumovirus subtype C wild bird isolates in domestic turkeys. J. Virol. 10:1-8. <PMid:23363433>

Cook J.K. 2000. Avian pneumovirus infections of turkeys and chickens. Vet. J. 160(2):118-125. <http://dx.doi.org/10.1016/S1090-0233(00)90486-6> <PMid:10985803>

Cook J.K.A., Ellis M.M. & Huggins M.B. 1991. The pathogenesis of turkey rhinotracheitis virus in Turkey poults inoculated with the virus alone or together with two strains of bacteria. Avian Pathol. 20(1):155-166. <http://dx.doi.org/10.1080/03079459108418750> <PMid:18680008>

Felippe P.A., Silva L.H., Santos M.B., Sakata S.T. & Arns C.W. 2011. Detection of and phylogenetic studies with avian metapneumovirus recovered from feral pigeons and wild birds in Brazil. Avian Pathol. 40(5):445-452. <http://dx.doi.org/10.1080/03079457.2011.596812> <PMid:21777083>

Gharaibeh S. & Shamoun M. 2012. Avian metapneumovirus subtype B experimental infection and tissue distribution in chickens, sparrows and pigeons. Vet. Pathol. 49(4):704-709. <http://dx.doi.org/10.1177/0300985811402845> <PMid:21527377>

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

Jardine C.M., Parmley E.J., Buchanan T., Nituch L. & Ojkic D. 2018. Avian metapneumovirus subtype C in Wild Waterfowl in Ontario, Canada. Transbound Emerg. Dis. 65(4):1098-1102. <http://dx.doi.org/10.1111/tbed.12832> <PMid:29457370>

Jones R.C. 1996. Avian pneumovirus infection: questions still unanswered. Avian Pathol. 25(4):639-648. <http://dx.doi.org/10.1080/03079459608419171> <PMid:18645888>

Jones R.C. & Rautenschlein S. 2013. Avian metapneumovirus, p.125-138. In: Swayne D.E., Glisson J.R., McDougald L.R., Nolan L.K., Suarez D.L. & Nair V. (Eds), Diseases of Poultry. 13th. Ames, Iowa.

Juhasz K. & Easton A.J. 1994. Extensive sequence variation in the attachment (G) protein gene of avian pneumovirus: evidence for two distinct subgroups. J. Gen. Virol. 75(Pt 11):2873-2880. <http://dx.doi.org/10.1099/0022-1317-75-11-2873> <PMid:7964599>

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16(2):111-120. <http://dx.doi.org/10.1007/BF01731581> <PMid:7463489>

Kumar S., Stecher G. & Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33(7):1870-1874. <http://dx.doi.org/10.1093/molbev/msw054> <PMid:27004904>

Lee E.H., Song M.S., Shin J.Y., Lee Y.M., Kim C.J., Lee Y.S., Kim H. & Choi Y.K. 2007. Genetic characterization of avian metapneumovirus subtype C isolated from pheasants in a live bird market. Virus Res. 128(1/2):18-25. <http://dx.doi.org/10.1016/j.virusres.2007.03.029> <PMid:17485129>

Olsen B., Munster V.J., Wallensten A., Waldenström J., Osterhaus A.D.M.E. & Fouchier R.A. 2006. Global patterns of influenza A virus in wild birds. Nature 312(5772):384-388. <PMid:16627734>

Seal B.S. 1998. Matrix protein gene nucleotide and predicted amino acid sequence demonstrate that the first US avian pneumovirus isolate is distinct from European strains. Virus Res. 58(1/2):45-52. <http://dx.doi.org/10.1016/S0168-1702(98)00098-7> <PMid:9879761>

Shin H.J., Njenga M.K., Mccomb B., Halvorson D.A. & Nagaraja K.V. 2000. Avian pneumovirus (APV) RNA from wild and sentinel birds in the United States has genetic homology with RNA from APV isolates from domestic turkeys. J. Clin. Microbiol. 38(11):4282-4284. <PMid:11060113>

Shin H.J., Cameron K.T., Jacobs J.A., Turpin E.A., Halvorson D.A., Goyal S.M., Nagaraja K.V., Kumar M.C., Lauer D.C., Seal B.S. & Njenga M.K. 2002. Molecular epidemiology of subgroup C avian pneumoviruses isolated in the United States and comparison with subgroup A and B viruses. J. Clin. Microbiol. 40(5):1687-1693. <http://dx.doi.org/10.1128/JCM.40.5.1687-1693.2002> <PMid:11980943>

Spackman E., Pedersen J.C., Mckinley E.T. & Gelb Junior J. 2013. Optimal specimen collection and transport methods for the detection of avian influenza virus and Newcastle disease virus. BMC Vet. Res. 9(1):35. <http://dx.doi.org/10.1186/1746-6148-9-35> <PMid:23432911>

Stuart J.C. 1989. Rhinotracheitis: turkey rhinotracheitis (TRT) in Great Britain. Recent Advances in Turkey Science, 21th Poultry Science Symposium Series, London, p.217-224.

Sun S., Chen F., Cao S., Liu J., Lei W., Li G., Song Y., Lu J., Liu C., Qin J. & Li H. 2014. Isolation and characterization of a subtype C avian metapneumovirus circulating in Muscovy ducks in China. Vet. Res. 45:74. <PMid:25060776>

Thompson J.D., Higgins D.G. & Gibson T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22(22):4673-4680. <http://dx.doi.org/10.1093/nar/22.22.4673> <PMid:7984417>

Toquin D., Bayon-Auboyer M.H., Eterradossi N., Jestin V. & Morin H. 1999. Isolation of a pneumovirus from a Muscovy duck. Vet. Rec. 145(23):680. <PMid:25705778>

Turpin E.A., Stallknecht D.E., Slemons R.D., Zsak L. & Swayne D.E. 2008. Evidence of avian metapneumovirus subtype C infection of wild birds in Georgia, South Carolina, Arkansas and Ohio, USA. Avian Pathol. 37(3):343-351. <http://dx.doi.org/10.1080/03079450802068566> <PMid:18568663>

Van Boheemen S., Bestebroer T.M., Verhagen J.H., Osterhaus A.D., Pas S.D., Herfst S. & Fouchier R.A. 2012. A family-wide RT-PCR assay for detection of paramyxoviruses and application to a large-scale surveillance study. PLoS One 7(4):e34961. <http://dx.doi.org/10.1371/journal.pone.0034961> <PMid:22496880>

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Original Article
Animal Morphophysiology

Anesthetic quality and cardiovascular and respiratory effects of continuous intravenous infusion of tiletamine-zolazepam in bitches[1]

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ABSTRACT.- Pereira S.A.R.S., Henrique F.V., Medeiros L.K.G., Silva J.K.C., Goes A.B., Vaz A.F.M., Souza A.P. & Nóbrega Neto P.I. 2019. **Anesthetic quality and cardiovascular and respiratory effects of continuous intravenous infusion of tiletamine-zolazepam in bitches.** *Pesquisa Veterinária Brasileira 39(3):214-220.* Centro de Saúde e Tecnologia Rural, Universidade Federal de Campina Grande, Avenida Universitária s/n, Santa Cecília, Patos, PB 58708-110, Brazil. E-mail: sostenesarthur@bol.com.br

The objective of this study was to evaluate the quality and recovery from anesthesia promoted by the tiletamine-zolazepam (TZ) combination administered intravenously (IV) continuously in bitches pre-medicated with acepromazine. Eight cross-bred, clinically healthy bitches weighing 13.7 ±1.9kg on average were used in this study. After a food fast of 12 h and a water fast of four hours, the animals were treated with acepromazine (0.1mg/kg, intramuscular) and, after 15 minutes, anesthesia was induced with a combination of tiletamine-zolazepam (2mg/kg, IV) immediately followed by continuous IV infusion thereof at a dose of 2mg/kg/h for 60 min. The following parameters were measured in all animals immediately before administration of acepromazine (M15), immediately before anesthetic induction (M0), and at 5, 10, 20, 30, 40, 50, and 60 min after initiation of continuous infusion (M5, M10, M20, M30, M40, M50, and M60): electrocardiography (ECG), heart rate (HR), mean arterial pressure (MAP), respiratory rate (RR), body temperature (BT), and arterial hemogasometry, with the last performed only at experimental times M15, M0, M30, and M60. A subcutaneous electrical stimulator was used to evaluate the degree of analgesia. Myorelaxation and quality of anesthetic recovery were also assessed, classifying these parameters as excellent, good, and poor. Anesthetic recovery time was recorded in minutes. HR increased significantly at time M10 in relation to that at M-15, and at times M5, M10, M40, and M50 in relation to that at M0. MAP decreased significantly at M20 and M30 compared with the baseline. BT decreased significantly at M50 compared with that at M0, but no hypothermia was observed. RR showed significant reduction at M5, M10, and M20 in relation to that at M-15, and at M5 and M10 in relation to that at M0, and bradypnoea was observed during the first 20 min after anesthetic induction. Significant decreases in the PR interval at times M10, M40, and M50 were observed in relation to that at M15. Amplitude of the *R* wave showed significant decrease at M20 compared with that at M-15. In the other ECG parameters, no significant difference was observed between the times evaluated. Hemogasometric parameters and analgesia did not show significant alterations. Myorelaxation and quality of anesthetic recovery were considered excellent. Recovery time was 15.1±7.7 min for positioning of sternal decubitus and 45.5±23.1 minutes for return of ambulation. Continuous IV administration of TZ combination does not produce satisfactory analgesia and does not cause severe cardiorespiratory and hemogasometric effects in bitches pre-medicated with acepromazine.

INDEX TERMS: Anesthetic quality, cardiovascular, respiration, intravenous infusion, tiletamine-zolazepam, bitches, benzodiazepine, dissociative anesthesia, dogs.

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RESUMO.- [**Qualidade anestésica e efeitos cardiovascular e respiratória da infusão intravenosa contínua de tiletamina-zolazepam em cadelas.**] Objetivou-se avaliar a qualidade e a recuperação da anestesia promovida pela associação tiletamina-zolazepam, administrada por via intravenosa (IV) contínua, em cadelas pré-medicadas com acepromazina. Foram utilizadas oito cadelas, sem raças definidas, clinicamente sadias, pesando em média 13,7±1,9kg. Após jejum alimentar de 12 horas e hídrico de quatro horas, os animais foram medicados com acepromazina (0,1mg/kg, via intramuscular) e, após 15 minutos, a anestesia foi induzida com a associação tiletamina-zolazepam (2mg/kg, IV) seguida imediatamente pela infusão IV contínua da mesma, na dose de 2mg/kg/h, durante 60 minutos. Os parâmetros que foram mensurados em todos os animais, imediatamente antes da administração da acepromazina (M-15), imediatamente antes da indução anestésica (M0) e, aos 5, 10, 20, 30, 40, 50 e 60 minutos após o início da infusão contínua (M5, M10, M20, M30, M40, M50 e M60) foram os seguintes: eletrocardiografia (ECG), frequência cardíaca (FC), pressão arterial média (PAM), frequência respiratória (*f*), temperatura corpórea (TC) e hemogasometria arterial, esta sendo realizada apenas nos momentos M-15, M0, M30 e M60. Para avaliação do grau de analgesia foi empregado um estimulador elétrico subcutâneo. Também se avaliou o miorrelaxamento e a qualidade da recuperação anestésica, classificando estes parâmetros em: excelente, bom e ruim. O tempo de recuperação anestésica foi registrado em minutos. A FC aumentou significativamente no momento M10 em relação ao M-15, e nos momentos M5, M10, M40 e M50 em relação ao M0. A PAM diminuiu significativamente em M20 e M30 em comparação ao valor basal. A TC diminuiu significativamente em M50 em comparação ao M0, mas não foi observada hipotermia. A *f* apresentou uma redução significativa nos momentos M5, M10 e M20 em relação ao M-15, e em M5 e M10 em relação ao M0, sendo observado bradipneia durante os primeiros 20 minutos após a indução anestésica. Foram observadas diminuições significativas do intervalo PR nos momentos M10, M40 e M50, em relação ao M-15. A amplitude da onda R apresentou diminuição significativa em M20 em comparação ao M-15. Nos demais parâmetros da ECG não houve diferença significativa entre os momentos avaliados. Os parâmetros hemogasométricos e a analgesia não apresentaram alterações significativas. O miorrelaxamento e a qualidade da recuperação anestésica foram considerados excelentes. O período de recuperação foi de 15,1±7,7 minutos para posicionamento do decúbito esternal e 45,5±23,1 minutos para retorno da deambulação. A administração intravenosa contínua de tiletamina-zolazepam não produz analgesia satisfatória e não causa efeitos cardiorrespiratórios e hemogasométricos severos, em cadelas pré-tratadas com acepromazina.

TERMOS DE INDEXAÇÃO: Qualidade anestésica, cardiovascular, respiratória, infusão intravenosa, tiletamina-zolazepam, cadelas, anestesia dissociativa, benzodiazepínico, cães.

INTRODUCTION

General intravenous (IV) dissociative anesthesia can be maintained in two ways: repetitive bolus administration and continuous infusion. In both cases, initial IV administration of anesthetic and sedative drugs is required to rapidly create high plasma concentrations of the drugs and enable their rapid arrival to their respective sites of action. When option is made for maintenance of repetitive bolus administration, as drug plasma concentration decreases through the elimination processes, several re-administrations are required to maintain the desired effects; however, this may result in poor maintenance and more frequent undesirable side-effects. Continuous infusion is used to maintain plasma concentrations of one or more drugs in the continuous IV anesthesia technique, and an appropriate infusion rate is needed to maintain a given anesthetic plane, achieve distribution in all tissues, and compensate for the drug elimination processes (Duke 2013).

Among injectable dissociative anesthetics are drugs derived from phencyclidine, characterized by dissociation of the central nervous system (CNS) and causing a change in the state of consciousness. Ketamine hydrochloride (HCL) and tiletamine HCL are the most commonly used dissociative anesthetics in veterinary medicine (Berry 2017), with tiletamine HCL marketed in a fixed proportion combination with zolazepam, and widely used in dissociative anesthesia of dogs, cats, and wild animals. Addition of zolazepam aims to potentiate the effects of tiletamine HCL, promote central-acting myorelaxation, and minimize undesirable side-effects typical of dissociative anesthesia (Lin et al. 1993).

Phenothiazine derived drugs such as acepromazine show tranquilizing, sympatholytic, anxiolytic and antispasmodic action, promoting a calmer anesthetic recovery and potentiation of the anesthesia promoted by the tiletamine-zolazepam (TZ) combination (Almeida et al. 2000, Pereira 2008, Fantoni & Cortopassi 2014).

Thus, this study aimed to assess the cardiorespiratory effects and the quality and recovery from anesthesia promoted by continuous IV administration of the TZ combination in bitches pre-medicated with acepromazine.

MATERIALS AND METHODS

This study was approved by the Research Ethics Research Committee of the aforementioned Institution under protocol no. 101-2017.

Eight cross-bred, clinically healthy bitches, weighing 13.7±1.9kg on average, obtained from local breeders, who authorized their participation in the study by signing an Informed Consent Form (ICF), were used in this study. The animals were allocated, individually, in kennels of the Veterinary Hospital for 15 days aiming adaptation, where they were daily provided with fresh water and commercial feed, changed twice a day. Three days prior to the anesthetic procedure, their health status was determined based on physical examination, electrocardiography (ECG), blood count, and biochemical tests (urea, creatinine, alanine aminotransferase, and alkaline phosphatase).

Prior to anesthesia, the animals underwent a food fast of 12 h and a water fast of four hours (Henrique 2018). After that, trichotomy and antisepsis were performed on the dorsal region of the left forelimb and external face of the left ear for introduction of a 22G catheter into the cephalic vein for administration of anesthetics and of a 24G catheter into the auricular artery to measure blood pressure and collect arterial blood samples (Fig.1). Pre-anesthetic medication was performed intramuscularly (IM) with acepromazine at a dose of 0.1mg/kg and, after 15 minutes, a TZ combination bolus was administered intravenously (IV) at a dose of 2mg/kg. Immediately after the bolus administration, continuous IV infusion of these drugs was performed at a rate of 2mg/kg/h for 60 min using a linear peristaltic infusion pump. Subsequently, the animals were contained in right

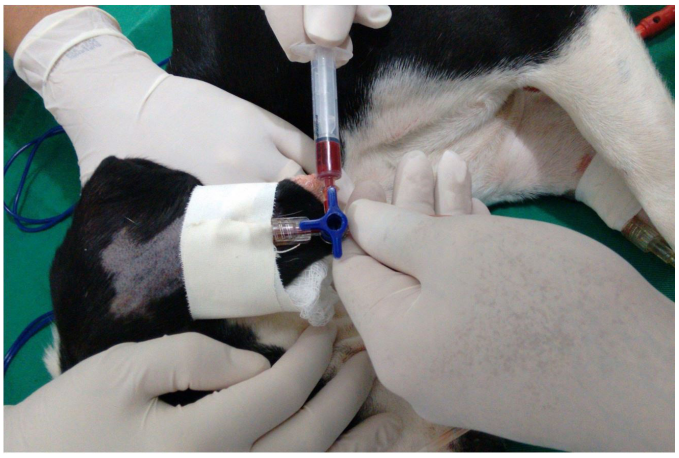

Fig.1. Catheterization of the auricular artery and connection to a 3-way tap for collection of arterial blood samples and measurement of mean arterial pressure (MAP) of bitches pre-medicated with acepromazine and submitted to continuous intravenous (IV) infusion of the tiletamine-zolazepam (TZ) combination.

lateral decubitus on an electric thermal mattress, spontaneously breathing ambient air without endotracheal intubation.

The following parameters were measured in all animals immediately before administration of acepromazine (M15), immediately before anesthetic induction (M0), and at 5, 10, 20, 30, 40, 50, and 60 min after initiation of continuous infusion (M5, M10, M20, M30, M40, M50, and M60): heart rate (HR), in beats per minute (bpm), obtained by calculation between two *R* waves, in milliseconds (ms), using a computer-based electrocardiograph machine; mean arterial pressure (MAP), in millimeters of mercury (mmHg), by connecting the catheter inserted into the left atrial artery to an aneroid sphygmomanometer by means of a cannula system filled with heparinized saline solution at a concentration of 10IU/mL, maintaining the air-solution interface at the height of sternal manubrium; respiratory rate (RR), in movements per minute (mpm), obtained through verification of respiratory movements; body temperature (BT), in Celsius degrees (°C), obtained by maintaining the sensor of the multi-parametric monitor in contact with the rectal mucosa; electrocardiography (ECG), using a computer-based ECG machine, obtaining, in milliseconds (ms) or millivolts (mV), the values referring to the duration and amplitude of the *P* wave (*P*ms and *P*mV), duration of the QRS complex (QRSms), amplitude of the *R* wave (*R*mV), duration of intervals between the *Q* and *T* waves (QTms) and between the *P* and *R* waves (PRms), in addition to search of peaked *T* waves, ST segment infra-unlevelling, abnormal electrocardiographic figures, and cardiac arrhythmias. The skin electrodes were placed in the humeral-radio-ulnar and femoro-tibial-patellar joint regions. ECG monitoring was performed throughout the anesthetic procedure and, at each experimental time (M), ECG recording was conducted for 30 s for further observation and measurement of the respective waves.

Arterial hemogasometry was also assessed, measuring the following values at experimental times M15, M0, M30, and M60: potential of hydrogen ion (pH); partial pressure of arterial oxygen (PaO_2), in mmHg; partial pressure of carbon dioxide ($PaCO_2$), in mmHg; total carbon dioxide (TCO_2) in plasma, in millimole per liter (mmol/L); arterial oxyhemoglobin saturation (SaO_2), in percentage (%); bicarbonate (HCO_3^-), in milliequivalents per liter (mEq/L); base excess (BE), in mEq/L, through collection of 0.5 mL of whole blood in dry electrolyte-balanced heparinized syringes and immediate hemogasometric analysis. All blood gas values were corrected according to body temperature, recorded at the time of sampling.

Degree of analgesia was assessed according to a methodology adapted from Figueiró et al. (2016), using an electrical stimulator connected to a pair of stainless steel needles spaced by 3cm and subcutaneously inserted into the tail ventral base. Each stimulus was applied by increasing the constant current (in mA) until a positive response was observed. Any movement of the head or limbs, occurred during stimulation, was considered a positive response. Three observers evaluated the response by direct visual observation. In case of disagreement between the observers, the response was discarded and the stimulus was repeated. Analgesia was monitored only as of the beginning of continuous infusion of the TZ combination (form times M5 to M60) considering that electrical stimulation in dogs is an alternative to the supramaximal mechanical noxious stimulation through tail-base clamp (Yamashiro et al. 2015). Thus, current intensities ≥30mA are considered supramaximal noxious stimuli for the same anatomical site in the tail (Figueiró et al. 2016)

Myorelaxation was evaluated based on the degree of extensor rigidity and resistance of the limbs to manipulation and on muscle tone; this parameter was rated as follows: excellent (score 2), when total muscle flaccidity was evident; good (score 1), when moderate maintenance of muscle tone was observed, with occurrence of discrete tremors; poor (score 0), if the animal showed tremors and stiffness, state of catalepsy, or intense movement. The quality of recovery after anesthesia was evaluated according to the following scores: excellent (score 2), when the animal rested quietly, but responsively; good (score 1), when the animal showed moderate excitement; poor (score 0), when it showed agitation, tremors and/or myoclonus (Cardoso et al. 2008). Anesthesia recovery time was recorded in minutes, comprising the time elapsed between end of continuous infusion and beginning of spontaneous ambulation, also recording the time that the animal assumed sternal decubitus. Manifestations of undesirable side-effects such as vocalization, tremors, escape behavior and/or excitement, vomiting, and defecation were verified.

Statistical analysis of the data was performed using the BioEstat 5.0 software at 5% significance level ($p<0.05$). Analysis of variance (ANOVA) was used for the repeated samples, followed by the Tukey test (parametric distribution) or the Friedman test (non-parametric distribution) to verify variation between the experimental times.

RESULTS AND DISCUSSION

Heart rate (HR) showed significant increase at experimental time M10 compared with that at M15, as well as at times M5, M10, M40, and M50 compared with that at M0 (Table 1). Possibly, the increase observed after administration of the tiletamine-zolazepam (TZ) combination was due to the sympathomimetic action of tiletamine, which increases HR, a characteristic common to dissociative agents well documented in the literature (Almeida et al. 2000, Mello & Cordeiro 2001, Valadão & Pacchini 2001, Pereira 2008, Valadão 2011, Berry 2017).

Mean arterial pressure (MAP) was significantly reduced at times M20 and M30 compared with that at M15 (Table 1). The decrease in MAP observed after administration of preanesthetic medication was probably due to the effect of acepromazine. Considering normal values of MAP from 80 to 120mmHg, this decrease led to a condition of mild hypotension, well above the threshold at which hypotension is considered severe - 60mmHg (Haskins 2017). The reduction

observed at 20 and 30 min of continuous infusion of the TZ combination was possibly potentiated by the action of zolazepam, which may induce tachycardia and decreased blood pressure, which is not observed when tiletamine is administered alone, because it increases HR and MAP (Lin et al. 1993). However, even with the use of the TZ combination, the dose of tiletamine administered could not surpass the hypotensive effect of acepromazine. Pereira (2008) performed intravenous (IV) administration of tiletamine-zolazepam (3mg/kg) in bitches pre-medicated with levomepromazine (1mg/kg) and did not observe decreased MAP; however, levomepromazine is a phenothiazine much less hypotensive than acepromazine (Fantoni & Cortopassi 2014). In addition, the values observed in the present study normalized immediately after interruption of the TZ combination infusion probably because of the shorter action duration of zolazepam compared with that of tiletamine (Berry 2017).

Table 1. Mean ±standard deviation of mean arterial pressure (MAP), in mmHg, and median ±interquartile deviation of heart rate (HR), in bpm, body temperature (BT), in °C, and respiratory rate (RR), in mpm of bitches pre-medicated with acepromazine and submitted to continuous IV infusion of the TZ combination

Experimental times	Parameters			
	MAP	HR	BT	*f*
M15	$89.5^{A} \pm 9.7$	$106.0^{AC} \pm 26.3$	$38.9^{AB} \pm 0.5$	$41.0^{A} \pm 6.5$
M0	$76.4^{AB} \pm 8.2$	$98.5^{A} \pm 10.3$	$38.5^{A} \pm 0.4$	$36.0^{AC} \pm 14.5$
M5	$75.0^{AB} \pm 6.1$	$133.5^{BC} \pm 19.5$	$38.2^{AB} \pm 1.3$	$15.0^{B} \pm 9.3$
M10	$75.0^{AB} \pm 9.2$	$130.5^{B} \pm 19.3$	$38.1^{AB} \pm 1.2$	$16.0^{B} \pm 8.8$
M20	$72.3^{B} \pm 11.7$	$124.0^{ABC} \pm 27.3$	$37.8^{AB} \pm 1.2$	$16.0^{BC} \pm 9.0$
M30	$72.0^{B} \pm 5.7$	$124.5^{ABC} \pm 49.3$	$37.7^{AB} \pm 1.3$	$22.0^{ABC} \pm 19.8$
M40	$77.3^{AB} \pm 13.7$	$121.5^{BC} \pm 53.3$	$37.7^{AB} \pm 1.6$	$28.0^{ABC} \pm 13.0$
M50	$79.3^{AB} \pm 13.9$	$145.0^{BC} \pm 43.0$	$37.6^{B} \pm 1.6$	$28.0^{ABC} \pm 14.0$
M60	$83.5^{AB} \pm 15.8$	$125.0^{ABC} \pm 49.8$	$37.7^{AB} \pm 1.5$	$23.0^{ABC} \pm 14.0$

$^{A, B, AB, AC, BC, ABC}$ Means followed by different letters in the same column differ statistically between experimental times.

A significant decrease in body temperature (BT) was observed at experimental time M50 compared with that at M0 (Table 1). The TZ combination promotes depressant effect on the temperature of dogs (Veado 2001, Mello & Cordeiro 2001), and its association with phenothiazines may potentiate depression of the thermoregulatory center (Almeida et al. 2000, Pereira 2008, Lacerda et al. 2010). However, in disagreement with the hypothermia reported by other authors, BT remained within the physiological limits (37.5-39.2°C) at all experimental times assessed (Feitosa 2014) because of the heat provided by the thermal mattress throughout the experimental period, which aimed to minimize temperature decrease. Under these conditions, the protocol employed did not interfere physiologically with BT.

Respiratory rate (RR) was significantly reduced at times M5, M10,and M20 in relation to that at M-15, and at M5 and M10 compared with that at M0 (Table 1). The mean values of RR remained below the reference values for the species, from 18 to 36mpm (Feitosa 2014), during the first 20 min after anesthetic induction. According to Valadão & Pacchini (2001), the TZ combination produces transient respiratory depression immediately after IV administration. In addition, it gives rise to an apneustic respiratory pattern characterized by deep breathing with irregular frequency and prolonged pauses (Berry 2017), similar to that observed in the present study. It is worth noting that tachypnea was observed at the mean baseline value, but an upper limit normal RR value was observed at MO, possibly, caused by the stress of pre-anesthetic manipulation.

The ECG parameters evaluated at all experimental times (Table 2) showed mean values within the reference limits for the species: *P*ms from 40 to 50ms, *P*mV up to 0.4mV, PRms from 60 to 130ms, QRSms up to 60ms, *R*mV up to 3mV, e QTms ranging from 150 to 250ms (Goodwin 2002). Therefore, the drugs under test did not alter the electrical conduction of the heart. ECG tracing did not show changes in HR during the experimental period, only confirmed a more accentuated HR increase after administration of the TZ combination. Similar results were reported by Pereira (2008) after IV administration of the TZ combination in bitches pre-medicated with levomepromazine.

Table 2. Median ±interquartile deviation of duration of the *P* wave (Pms - milliseconds) and mean ±standard deviation of amplitude the *P* wave (PmV - millivolts), duration of the PR interval (PRms - milliseconds), duration of the QRS complex (QRSms - milliseconds), amplitude of the *R* wave (*R*mV - millivolts) and of the QT interval (QTms - milliseconds) of bitches pre-medicated with acepromazine and submitted to continuous IV infusion of the TZ combination

Experimental times	Parameters					
	*P*ms	*P*mV	PRms	QRSms	*R*mV	QTms
M15	$44^{A} \pm 4$	$0.19^{A} \pm 0.04$	$105.8^{A} \pm 12.6$	$40.5^{A} \pm 3.3$	$1.01^{A} \pm 0.41$	$208.0^{A} \pm 23.8$
M0	$44^{A} \pm 4$	$0.18^{A} \pm 0.05$	$103.0^{AB} \pm 14.5$	$40.5^{A} \pm 3.3$	$0.93^{AB} \pm 0.35$	$224.5^{A} \pm 18.8$
M5	$44^{A} \pm 1$	$0.21^{A} \pm 0.07$	$92.0^{AB} \pm 7.1$	$40.0^{A} \pm 3.0$	$0.79^{AB} \pm 0.35$	$207.5^{A} \pm 22.0$
M10	$40^{A} \pm 5$	$0.21^{A} \pm 0.07$	$89.5^{B} \pm 8.3$	$40.5^{A} \pm 3.3$	$0.78^{AB} \pm 0.35$	$208.0^{A} \pm 19.6$
M20	$40^{A} \pm 1$	$0.20^{A} \pm 0.08$	$92.5^{AB} \pm 9.2$	$40.0^{A} \pm 3.0$	$0.76^{B} \pm 0.37$	$214.5^{A} \pm 21.5$
M30	$42^{A} \pm 5$	$0.22^{A} \pm 0.08$	$90.0^{AB} \pm 14.8$	$39.5^{A} \pm 3.3$	$0.86^{AB} \pm 0.38$	$204.0^{A} \pm 27.1$
M40	$44^{A} \pm 5$	$0.23^{A} \pm 0.07$	$89.0^{B} \pm 14.5$	$41.0^{A} \pm 4.1$	$0.90^{AB} \pm 0.36$	$208.5^{A} \pm 24.0$
M50	$44^{A} \pm 8$	$0.28^{A} \pm 0.12$	$89.0^{B} \pm 16.5$	$40.0^{A} \pm 3.0$	$0.89^{AB} \pm 0.33$	$208.0^{A} \pm 23.9$
M60	$42^{A} \pm 5$	$0.22^{A} \pm 0.08$	$95.0^{AB} \pm 17.9$	$41.5^{A} \pm 3.7$	$0.95^{AB} \pm 0.35$	$210.0^{A} \pm 42.7$

$^{A, B, AB}$ Means followed by different letters in the same column differ statistically between experimental times.

HR is inversely proportional to the duration of the PR interval, so that the intervals are short in tachycardia and long in bradycardia (Filippi 2011). Similar results were observed in the present study during the times of increased HR, with a significant decrease in the PR interval at times M10, M40, and M50 in relation to that at M15 (Table 2). In addition, the amplitude of the *R* wave showed a significant decrease at time M20 compared with that at M15 (Table 2); however, it is not indicative of change in ventricular activation because there is no minimum height for the *R* wave (Filippi 2011).

None of the hemogasometric parameters assessed varied significantly throughout the experiment (Table 3). Normally, atmospheric oxygen is vented in the alveoli and then diffuses through the respiratory membrane along partial pressure gradients in the plasma. Partial pressure of arterial oxygen (PaO_2) is a measure of the lung capacity to transport oxygen from the atmosphere to the blood, and its normal values for dogs spontaneously breathing ambient air vary from 80 to 110mmHg. In general, hypoxemia occurs for values of PaO_2 <80mmHg and of arterial oxyhemoglobin saturation (SaO_2) <90% (Haskins 2017). In view of that, it can be stated that the anesthetic protocol employed did not promote hypoxemia, because all means remained within the thresholds for the species (Table 3). $PaCO_2$ usually ranges from 35 to 45mmHg. $PaCO_2$ values >60mmHg may be associated with excessive respiratory acidosis and, in general, it is considered representative of hypoventilation, whereas $PaCO_2$ values <20mmHg are associated with respiratory alkalosis (Haskins 2017). In this study, considering the standard deviation, at experimental times M0 and M30, $PaCO_2$ values below normality for the species of 32.5 and 32.7mmHg were observed; however, this variable was normalized at M60, where similarly to the baseline, remained within the reference values without any evidence of respiratory acidosis or alkalosis (Table 3). It is likely that the respiratory pattern, characterized by deep breathing, and the significant decrease in BT during the experiment may have contributed to the greater elimination and lower production of carbon dioxide, respectively, at the initial times after continuous infusion (M0 and M30). In addition, a tachypneic baseline RR mean value was observed, which may temporarily induce decreased $PaCO_2$, with subsequent increase at M60.

The mean values of bicarbonate (HCO_3^-) and total carbon dioxide (TCO_2) remained within the physiological standards for canines, from 17.2 to 23.0mEq/L and 18.0 to 24.1mmol/L, respectively (Table 3). However, considering the standard deviation, the HCO_3^- values were below normality for the species (16.2mEq/L at M0 and 17mEq/L at M60). Arterial potential of hydrogen ion (pH) at experimental time M60 declined to a value smaller than the reference range for the species (7.36-7.44) (Vanova-Uhrikova et al. 2017) (Table 3). This experimental time showed coincidence between the highest and lowest $PaCO_2$ and HCO_3^- values found, respectively, and although no statistical significance was observed, the higher $PaCO_2$ value probably activated compensatory mechanisms of HCO_3^- reduction. In contrast, base excess (BE), which is also calculated from HCO_3^-, showed a mean value below that considered normal for dogs (from -5.5 to -0.9mEq/L) at M60 (Table 3), characterizing a condition of metabolic acidosis (Vanova-Uhrikova et al. 2017), which may have influenced the changes observed in pH values. These findings were similar to those reported by Pereira (2008), who observed a decrease in pH and BE, with values below normality, detecting lower mean values 45 minutes after IV administration of the TZ combination, but this was considered a disorder usually corrected without the need for alkalizing substances. Possibly, the reported changes resulted from respiratory depression caused by the drugs used and, therefore, systemic compensatory mechanisms were activated (Savvas et al. 2005).

Evaluation of the degree of analgesia showed no significant difference between the experimental times regarding the intensity of milliamperage needed to produce response to electrical stimuli (RES) (Table 4). However, the protocol used in this study did not provide sufficient analgesia to block response to the supramaximal noxious stimuli. Thus, to obtain satisfactory analgesia, association of analgesic drugs and/or local blockades with the present protocol and/or increased anesthetic maintenance dose are indicated.

Table 3. Median ±interquartile deviation of the potential of hydrogen ion (pH), partial pressure of arterial oxygen (PaO_2), in mmHg, partial pressure of carbon dioxide ($PaCO_2$), in mmHg, bicarbonate (HCO_3^-), in mEq/L, arterial oxyhemoglobin saturation (SaO_2), in %, and total carbon dioxide (TCO_2) in plasma, in mmol/L, and mean ±standard deviation of base excess (BE), in mEq/L of bitches pre-medicated with acepromazine and submitted to continuous IV infusion of the TZ combination

Parameters	Experimental times			
	M-15	M0	M30	M60
pH	$7.37^A \pm 0.04$	$7.38^A \pm 0.08$	$7.39^A \pm 0.07$	$7.27^A \pm 0.09$
PaO_2	$100.5^A \pm 8.0$	$101.0^A \pm 3.3$	$98.0^A \pm 9.0$	$100.0^A \pm 4.5$
$PaCO_2$	$40.5^A \pm 3.3$	$37.5^A \pm 5.0$	$35.5^A \pm 2.8$	$41.0^A \pm 3.0$
HCO_3^-	$21.5^A \pm 2.5$	$20.5^A \pm 4.3$	$21.5^A \pm 3.3$	$18.5^A \pm 1.5$
SaO_2	$96.0^A \pm 1.5$	$95.5^A \pm 2.5$	$96.0^A \pm 1.0$	$95.5^A \pm 2.0$
TCO_2	$22.5^A \pm 2.5$	$21.5^A \pm 4.3$	$22.5^A \pm 3.3$	$19.5^A \pm 1.5$
BE	$-3.6^A \pm 3.3$	$-3.1^A \pm 4.5$	$-1.8^A \pm 2.8$	$-7.6^A \pm 3.3$

[A] Means followed by different letters in the same column differ statistically between experimental times.

Table 4. Median ±interquartile deviation of myorelaxation (Myo), in scores, and response to electrical stimuli (RES), in milliamperes, of bitches pre-medicated with acepromazine and submitted to continuous IV infusion of the TZ combination

Experimental times	Parameters	
	RES	Myo
M0	-	$0.5^A \pm 1.0$
M5	$20.0^A \pm 4.6$	$2.0^B \pm 0.0$
M10	$20.0^A \pm 4.6$	$2.0^B \pm 0.0$
M20	$21.3^A \pm 4.4$	$2.0^B \pm 0.0$
M30	$20.0^A \pm 7.1$	$2.0^{AB} \pm 0.3$
M40	$21.9^A \pm 7.5$	$2.0^{AB} \pm 1.0$
M50	$21.3^A \pm 5.8$	$2.0^{AB} \pm 1.0$
M60	$15.6^A \pm 7.3$	$1.5^{AB} \pm 1.0$

[A, B, AB] Means followed by different letters in the same column differ statistically between experimental times.

At experimental time M0, half of the bitches showed good myorelaxant scores and half of them presented poor scores; at times M5, M10, and M20, 100% of the animals showed excellent scores; at M30, 75% had excellent scores and 25% showed good scores; at M40, 62.5% presented excellent scores and 37.5% showed good scores; at M50, 62,5, 25, 12.5% of the canines showed excellent, good and poor scores, respectively; at M60, 50, 37.5, and 12.5% had excellent, good and poor scores, respectively. Muscle relaxation increased at times M5, M10, and M20 compared with the values observed after administration of acepromazine (Table 4), which was expected after induction and anesthetic maintenance with a central acting myorelaxant - benzodiazepine zolazepam (Valadão 2011). The myorelaxation observed at these times was classified as excellent due to the deeper "sleep state" observed after anesthetic induction (Mello & Cordeiro 2001) and beginning of the anesthetic infusion; however, from time M30, excellent myorelaxation was maintained in only 50% of the animals throughout experimental period, whereas a gradual reduction was observed in the other half, and these animals would possibly need an increase in the anesthetic maintenance rate.

As of 20 minutes of infusion, tongue movements, nystagmus and salivation were observed in six, two and three bitches, respectively, which are characteristics typical of dissociative anesthesia (Valadão 2011); they disappeared 40 min after the beginning of infusion. Two animals showed movement of the pelvic limbs 50 min after infusion; these were the youngest animals in the group - aged six months to one year, and which would probably demand a higher infusion rate.

The quality scores of recovery after anesthesia were classified as excellent in seven bitches and as good in only one, which shows a slight scape behavior; even so, no changes were observed during this period. In canines, the action duration of tiletamine is longer than that of zolazepam. This means that effects typical of dissociative anesthetics are expected during recovery, including muscle stiffness, sympathetic stimulation, and emergency delirium; therefore, association with other drugs such as phenothiazines is necessary (Berry 2017). The use of acepromazine associated with the TZ combination provides prolonged anesthesia period and calmer recovery with less intense muscle tremors (Almeida et al. 2000). However, no tremors were observed during the recovery period. Anesthesia recovery time was 15.1±7.7 min for positioning of sternal decubitus and 45.5±23.1 min for return of ambulation. Mello & Cordeiro (2001) performed IV administration of 6.6mg/kg of the TZ combination in adult dogs and observed mean times of 101.6±11.4 min for positioning of sternal decubitus and 130.7±11.38 min for return of ambulation - times recorded as of intramuscular (IM) administration. If we subtract 60 min from these times, which was the infusion time in this study, these values are still greater, with 41.6 min for positioning of sternal decubitus and 70.7 min for return of ambulation, demonstrating that a bolus of 2mg/kg and a maintenance rate of 2mg/kg/h for 60 min of the TZ combination do not prolong anesthesia recovery time compared with IM administration. It is worth mentioning that, in the present study, the animals were pre-medicated with acepromazine, which interferes with anesthesia recovery time. Almeida et al. (2000) assessed the TZ combination (10mg/kg) in dogs pre-medicated or not with acepromazine (0.2mg/kg), both intravenously, and observed that, after 60 minutes, when the animals were untied from the table and placed on the floor, two of them that had not received acepromazine remained in sternal decubitus, eight were in quadruped position and, of these, two returned to ambulation; whereas of those that had received acepromazine, only one was able to remain in quadruped position while the others remained in sternal decubitus. Continuous infusion provides maintenance of constant plasma levels, prolonging the anesthesia period with faster recovery, when the anesthetics used do not have a cumulative effect, reducing the consumption of IV anesthetic agents by 25-30%, as well as presenting lower incidence of side effects (Mannarino 2002).

CONCLUSIONS

In bitches pre-medicated with acepromazine, intravenous administration of the tiletamine-zolazepam combination at a dose of 2mg/kg/h does not produce satisfactory analgesia, does not prolong anesthesia recovery time, and does not interfere severely with cardiorespiratory function.

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

REFERENCES

Almeida E.M.P., Nunes N., Fantinatti A.P., Santos P.S.P., Bolzan A.A. & Rezende M.L. 2000. Efeitos cardiorrespiratórios da associação de tiletamina/zolazepam em cães (*Canis familiaris*) pré-tratados ou não pela acepromazina. Braz. J. Vet. Res. Anim. Sci. 37(3):210-215. <http://dx.doi.org/10.1590/S1413-95962000000300007>

Berry S.H. 2017. Anestésicos injetáveis, p.829-891. In: Grimm K.A., Lamont L.A., Tranquilli W.J., Greene S.A. & Robertson S.A. (Eds), Lumb and Jones Anestesiologia e Analgesia Veterinária. 5ª ed. Roca, Rio de Janeiro.

Cardoso F.T.S., Feitosa Júnior F.S., Diniz B.L.M., Lucena L.U. & Silva Júnior J.R. 2008. Neuroleptoanalgesia associada à anestesia epidural com lidocaína e xilazina em cutias (*Dasyprocta aguti*). Acta Scient. Vet. 36(2):149-154.

Duke T. 2013. Partial intravenous anesthesia in cats and dogs. Can. Vet. J. 54(3):276-282. <PMid:23997266>

Fantoni D.T. & Cortopassi S.R.G. 2014. Medicação pré-anestésica, p.217-227. In: Fantoni D.T. & Cortopassi S.R.G. (Eds), Anestesia em Cães e Gatos. 2ª ed. Roca, São Paulo.

Feitosa F.L.F. 2014. Exame físico geral ou de rotina, p.51-68. In: Feitosa F.L.F. (Ed.), Semiologia Veterinária: a arte do diagnóstico. 6ª ed. Roca, São Paulo.

Figueiró M.R., Soares J.H.N., Ascoli F.O., Werre S. & Segura I.A.G. 2016. Isoflurane MAC determination in dogs using three intensities of constant-current electrical stimulation. Vet. Anaesth. Analg. 43(5):464-471. <http://dx.doi.org/10.1111/vaa.12341> <PMid:27531057>

Filippi L.H. 2011. O Eletrocardiograma na Medicina Veterinária. Roca, São Paulo, p.69-88.

Goodwin J.K. 2002. Eletrocardiografia, p.39-65. In: Goodwin J.K. & Tilley, L.P. (Eds), Manual de Cardiologia para Cães e Gatos. 3ª ed. Roca, São Paulo.

Haskins S.C. 2017. Monitoramento de pacientes anestesiados, p.270-353. In: Grimm K.A., Lamont L.A., Tranquilli W.J., Greene S.A. & Robertson S.A. (Eds), Lumb and Jones Anestesiologia e Analgesia Veterinária. 5ª ed. Roca, Rio de Janeiro.

Henrique F.V. 2018. Emprego da detomidina na contenção farmacológica e na anestesia intravenosa contínua com cetamina ou dextrocetamina em cadelas. Doctoral Dissertation, Universidade Federal de Campina Grande, Patos, PB. 107p.

Lacerda M.S., Sampaio R.L. & Nunes T.C. 2010. Estudo hematológico e cardiorrespiratório em cadelas anestesiadas com cetamina-s/xilazina e tiletamina/zolazepam e submetidas a ovário-histerectomia. Biosci. J. 26(6):913-918.

Lin H. C., Thurmon J.C., Benson G.J. & Tranquilli W.J. 1993. Telazol--a review of its pharmacology and use in veterinary medicine. J. Vet. Pharmacol. Ther. 16(4):383-418. <http://dx.doi.org/10.1111/j.1365-2885.1993.tb00206.x> <PMid:8126757>

Mannarino R. 2002. Determinação da taxa de infusão mínima de propofol e propofol associado a lidocaína em cães (*Cannis familiaris*). Master's Thesis, Universidade Estadual Paulista, Botucatu, SP. 128p.

Mello J.R.B. & Cordeiro M.R.O. 2001. Avaliação da associação tiletamina-zolazepam em cães de três diferentes faixas etárias. Arq. Fac. Vet. UFRGS 29(1):14-24.

Pereira T. 2008. Infusão contínua de remifentanil em cadelas pré-medicadas com levomepromazina e anestesiadas com tiletamina-zolazepam. Master's Thesis, Universidade Federal de Viçosa, Viçosa, MG. 56p.

Savvas I., Plevraki K., Raptopoulos D. & Koutinas A.F. 2005. Blood gas and acid–base status during tiletamine/zolazepam anaesthesia in dogs. Vet. Anaesth. Analg. 32(2):94-100. <http://dx.doi.org/10.1111/j.1467-2995.2004.00177.x> <PMid:15762914>

Valadão C.A.A. 2011. Anestesia dissociativa, p.73-84. In: Massone F. (Ed.), Anestesiologia Veterinária: farmacologia e técnicas. 6ª ed. Guanabara Koogan, Rio de Janeiro.

Valadão C.A.A. & Pacchini C.E. 2001. Efeitos cardiorrespiratórios da tiletamina-zolazepam em cães hipovolêmicos. Arq. Bras. Med. Vet. Zootec. 53(1):44-51. <http://dx.doi.org/10.1590/S0102-09352001000100007>

Vanova-Uhrikova I., Rauserova-Lexmaulova L., Rehakova K., Scheer P. & Doubek J. 2017. Determination of reference intervals of acid-base parameters in clinically healthy dogs. J. Vet. Emerg. Crit. Care 27(3):325-332. <http://dx.doi.org/10.1111/vec.12589> <PMid:28420042>

Veado J.C. 2001. Associação tiletamina-zolazepam na anestesia dissociativa de cães, protocolo original de utilização. Revta Bras. Saúde Prod. Anim. 1(1):19-26.

Yamashiro L.M., Floriano B.P., Pinho R.H., Wagatsuma J.T., Ferreira J.Z. & Oliva V.N.L.S. 2015. Alteração do índice biespectral antes e após estimulação elétrica ou mecânica em cães anestesiados pelo isofluorano. Ciência Rural 45(9):1648-1653. <http://dx.doi.org/10.1590/0103-8478cr20141230>

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Original Article
Animal Morphophysiology

Grayscale histogram analysis to study the echogenicity and echotexture of the walls of the common carotid arteries of horses and mules

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ABSTRACT.- Fogaça J.L., Vettorato M.C., Puoli-Filho J.N.P., Fernandes M.A.R. & Machado V.M.V. 2019. **Grayscale histogram analysis to study the echogenicity and echotexture of the walls of the common carotid arteries of equines and mules.** *Pesquisa Veterinária Brasileira 39(3):221-229.* Departamento de Reprodução Animal e Radiologia Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Rua Prof. Dr. Walter Mauricio Correra s/n, Rubião Junior, Botucatu, SP 18618-970, Brazil. E-mail: vaniamvm@fmvz.unesp.br

Visual inspection of ultrasound examination for assessment of echogenicity and echotexture of blood vessel tissues is a technique routinely used in medical practice in humans. However, simple visual inspection can lead to poor quality diagnoses and errors. The use of grayscale histogram (GSH) analysis has proved to be an efficient technique to quantify the region of interest, allowing minimization of image interpretation errors. This study aimed to evaluate and compare the echogenicity and echotexture of the walls of the common carotid arteries of healthy female horses and mules using the GSH technique and correlate them with age, body mass, and vessel diameters. B-mode ultrasound examinations were performed in the left and right common carotid arteries in three regions (cranial, middle, and caudal) in 11 horses and 11 healthy mules. The GSH of the animals showed heterogeneous walls, but did not differentiate statistically between female horses and mules. The Mean variable of the middle right, middle left and caudal right sides showed differences, more significant in the mules. On the middle right side, the Min variable was different, higher in the mules. On the middle and caudal left side, the variables Max and Mode showed higher values in the mules. For the mules, the age factor presented negative correlation with the Mean, Mode,, Mode(Count), and Mode(Count)/Count(%) variables, and the body mass factor presented negative correlation with the Mode, Mean and Max variables. For the female horses, the body mass factor showed positive correlation with the Mean and Mode variables. Echogenicity of the carotid artery walls differed between female horses and mules, whereas echotexture was heterogeneous and statistically similar among the animals. The age and body mass factors inversely influenced the echogenicity of the mules, but were not significant in the female horses, in which only the body mass factor positively influenced echogenicity.

INDEX TERMS: Grayscale histogram, echogenicity, echotexture, carotid arteries, horses, mules, histogram analysis, ultrasonography, wall of blood vessels, equines, quantitative analysis, morphology.

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RESUMO.- [**Análise por histograma em escala de cinza para estudo da ecogenicidade e ecotextura das paredes das artérias carótidas comuns de equinos e muares.**] A técnica de avaliação da ecogenicidade e ecotextura dos tecidos dos vasos sanguíneos por inspeção visual do exame de ultrassonografia, já é uma prática da rotina médica em humanos. No entanto, a simples visualização manual pode induzir à erros e diagnósticos de pouca qualidade. O uso

de análise por histograma em escala de cinza (HEC) tem se demostrado uma eficiente técnica para quantificar a região avaliada, permitindo minimizar erros de interpretação. O objetivo deste trabalho foi avaliar e comparar a ecogenicidade e ecotextura das paredes das artérias carótidas comuns das fêmeas equina e muares hígidos usando a técnica de HEC, e correlacioná-las com a idade, massa corpórea e diâmetros dos vasos. Exames de ultrassonografia modo-B foram realizados nas artérias carótidas comuns esquerda e direita em três regiões (cranial, médio e caudal) em 11 fêmeas equina e 11 muares hígidos. O HEC dos animais apresentou paredes heterogêneas, mas não diferenciaram estatisticamente entre fêmeas equinas e muares. A variável Mean do lado direito médio, esquerdo médio e caudal apresentou diferenças, sendo maiores nos muares. No lado direito médio, o Min foi diferente, sendo superior nos muares. Já para o lado esquerdo médio e caudal, as variáveis Max e Mode apresentaram valores maiores nos muares. Para os muares, o fator idade apresentou correlação negativa com Mean, Mode, Mode (Count) e Mode(Count)/Count (%) e o fator massa corpórea apresentou correlação negativa com Mode, Mean e Max. Para as fêmeas equinas o fator massa corpórea apresentou correlação positiva com Mean e Mode. A ecogenicidade das paredes das artérias carótidas diferiram entre fêmeas equina e muares, já a ecotextura foi heterogênea e semelhante estatisticamente entre os animais. A idade e a massa corpórea influenciaram inversamente na ecogenicidade dos muares, enquanto que nas fêmeas equina a idade não foi significativa, apenas a massa corpórea influenciou positivamente com a ecogenicidade.

TERMOS DE INDEXAÇÃO: Histograma, ecogenicidade, ecotextura, artérias carótidas, equinos, muares, ultrassonografia, parede dos vasos sanguíneos, equídeos, análise quantitativa, morfologia.

INTRODUCTION

Visual inspection of ultrasound (US) examination is insufficient to accurately determine the echogenicity and echotexture of the evaluated tissues. Consequently, techniques that enable quantification of the region of interest (ROI) have been created, thus allowing minimization of image interpretation errors (Maeda et al. 1998, Silva et al. 2015, Dantas et al. 2016, Mendonça 2017).

Grayscale histogram (GSH) assesses the frequency distribution in gray-levels and the quantification that forms the image of the ROI (Maeda et al. 1998, Lee et al. 2006, Vescovi et al. 2009).

GSH has been used to evaluate the echogenicity and echotexture of several organs, presenting important clinical and academic applicability. The study of a tissue shows apparent density in the two-dimensional mode (B-mode), which is altered by pathological conditions, providing the examiner with a subjective analysis of the ROI (Rosenfield et al. 1980, Queiroz & Gomes 2001, Armstrong et al. 2003).

GSH has been used in human medicine (Wohlin et al. 2009, Lee 2010, Mendonça 2017) to assess carotid artery walls in patients with atherosclerotic plaques deposited in the intima and media layers, because carotid artery intima-media complex thickening is considered a marker of early atherosclerosis, and US examination enables visual inspection of the normal-thickness vessel wall. However, there may be variation in echotexture and echogenicity not observed by the specialist during examination, and GSH may add information to diagnosis (Lind et al. 2008, Wohlin et al. 2009, Anderson et al. 2009, Lee 2010, Sarmento et al. 2014).

Deposition of atherosclerotic plaques in the carotid arteries is not as frequent in animals as it is in humans (Rosa et al. 2003, Ribeiro & Shintaku 2004, Wohlin et al. 2009). Nevertheless, there are reports of plaque deposition in dogs (Hess et al. 2003) and horses (Aguiar et al. 2014), and US examination enables the subjective evaluation of this disease; however, in situations such as those previously mentioned, just like in humans, (Baroncini et al. 2006), GSH could complement the assessment.

Mules are not considered horse, since they are the result of the crossing between horses and donkeys (Souza et al. 2013), and although these two species share common ancestry, they present significant morphological differences. Therefore, it is expected that mules showed some anatomo-physiological differences in relation to horses; however, there are few studies on equines in the literature, which produces a deficit of information (Burnhan 2002, Alsafy et al. 2008, Smith 2009). Studies including both of these species, especially addressing the common carotid arteries, are necessary, as these vessels are directly responsible for the flow of rich oxygen to the brain (Rosa et al. 2003, Chequer et al. 2006, Kobayashi & Karino 2016).

Veterinarians practicing with horses need to perfect themselves to understand the differences between them and mules. GSH in female horses and mules is still incipient, particularly in the analysis of vessel walls. This study aimed to evaluate and compare the echogenicity and echotexture of the walls of the common carotid arteries of healthy female horses and mules using the GSH technique and correlate them with age, body mass, and vessel diameters. It also sought to assess the echogenicity and echotexture of carotid artery walls between mule genders.

MATERIALS AND METHODS

This study was approved by the Ethics Committee on Animals Use (CEUA) of the School of Veterinary Medicine and Animal Science, São Paulo State University (FMVZ/Unesp) under protocol no. 0100/2017. The research was conducted at the Edgárdia farm, Botucatu campus of the FMVZ/Unesp.

Twenty-two animals belonging to the equine teaching, research, and extension area of the FMVZ/Unesp, Botucatu campus, were used in this survey. The animals were classified into two groups: 11 healthy, cross-bred, female horses with 348-486kg body mass, aged 5-25 years; 11 healthy mules (six males and five females) with 350-462kg body mass, aged 4-12 years. The mules were also divided according to gender into males and females.

For ultrasound (US) examination, the animals were driven to individual squeeze chutes by a trained guide, and their positioning was respected according to accommodation, where they all remain with the head above the line of the withers. The animals did not fast for either feed or water, and were contained manually by trained professionals during the procedures.

Prior to the US examination, isopropyl alcohol was applied at a concentration of 30% water and 70% alcohol in the region to be examined, and silicone gel was used to protect the equipment transducers. The use of alcohol eliminates the need for trichotomy, and along with the gel improves conduction of the ultrasound waves. US examination was performed using a MyLab®30 (Esaote; Italy) and linear transducers (3.0-11.0 MHz) (Esaote; Italy) to obtain B-mode

images of the female horses and mules, classifying the carotid arteries in three different regions: cranial, middle, and caudal (Fig.1).

These anatomical regions of interest (ROI) were classified as a cranial, middle and caudal measurement points. The cranial measurement point was identified as a line tangential to the condyles of the occipital bone, dorsally, and the angle of the mandible, ventrally. The middle measurement point was considered as a dorsoventral line, caudally, tangential to the articular fovea of the fourth cervical vertebra. The caudal measurement point was established as a dorsoventral line, caudally, tangential to the seventh cervical vertebra.

Owing to the difference in size and body mass between the animals, the image techniques (gain of brightness and depth) were different aiming to maximize the quality of the images in each case. After the procedures, the US images of all animals were assessed using specific software (ImageJ® - National Institutes of Health), in which the grayscale histogram (GSH) tool was applied to all images in the longitudinal plane of the right and left common carotid arteries.

For the GSH measurements, it was decided to perform the measurements on the upper wall of the vessel in the three regions (cranial, middle, and caudal) to avoid a possible acoustic reinforcement artifact. A range of 770-1000 pixels was considered for sample collection within the ROI, and values of the following image variables were obtained: Count, Mean, Max, Min, SD, Mode, and Mode(Count) (Fig.2), and subsequently inserted into a Microsoft Excel 2013 spreadsheet for statistical analysis. Echotexture was obtained according to the equation Mode(Count)/Count, and the result was then multiplied by 100 to convert the values in percentage (%). Results are more and less homogeneous as they are closer to 100% and 0%, respectively.

Statistical analysis was conducted using the SAS System 9.0 for the calculation of the mean, median, and standard deviation in each group, and the Mann-Whitney test was subsequently applied for the comparison between female horses and mules and between mule genders. Sperman's correlation was also used for comparison between the GSH variables and the factors age, body mass, and longitudinal diameter and longitudinal wall of the vessel. A significance level of 5% ($p \leq 0.05$) was adopted for all statistical analyses.

RESULTS

No statistically significant difference was observed between female horses and mules with respect to the variable Count ($P=0.0391$) on the right caudal side region, with female horses showing higher values compared with those of mules (Table 1), which means that pixel quantification was higher in this region than in the other regions.

Statistically significant difference was found between the variable Mean on the middle right side ($P=0.0270$), middle left side ($P=0.0366$), and caudal left side ($P=0.0090$), with quantification of pixels higher in mules than in female horses. This indicates that the female horses obtained means of gray-levels different from those of the mules in the previously mentioned regions, that is, more hypoechogenic. Statistically significant difference was observed for the variable Min on the middle right side ($P=0.0219$), with the mules showing higher values, that is, the female horses showed more hypoechogenic pixel quantification than the mules in this ROI. Statistically significant difference was found for the variables Max and Mode on the middle left side ($P=0.093$ for Max and $P=0.00310$ for Mode) and the caudal left side ($P=0.0140$ for Max and $P=0.0350$ for Mode), with the mules showing higher values than those of the female horses, which means that mules present greater echogenicity than female horses in these ROIs.

Joint analysis of the means of the variable Mean showed that the mules had greater echogenicity in all regions and sides evaluated compared with those of the female horses. Echotexture, obtained by the Mode(Count)/Count(%) ratio,

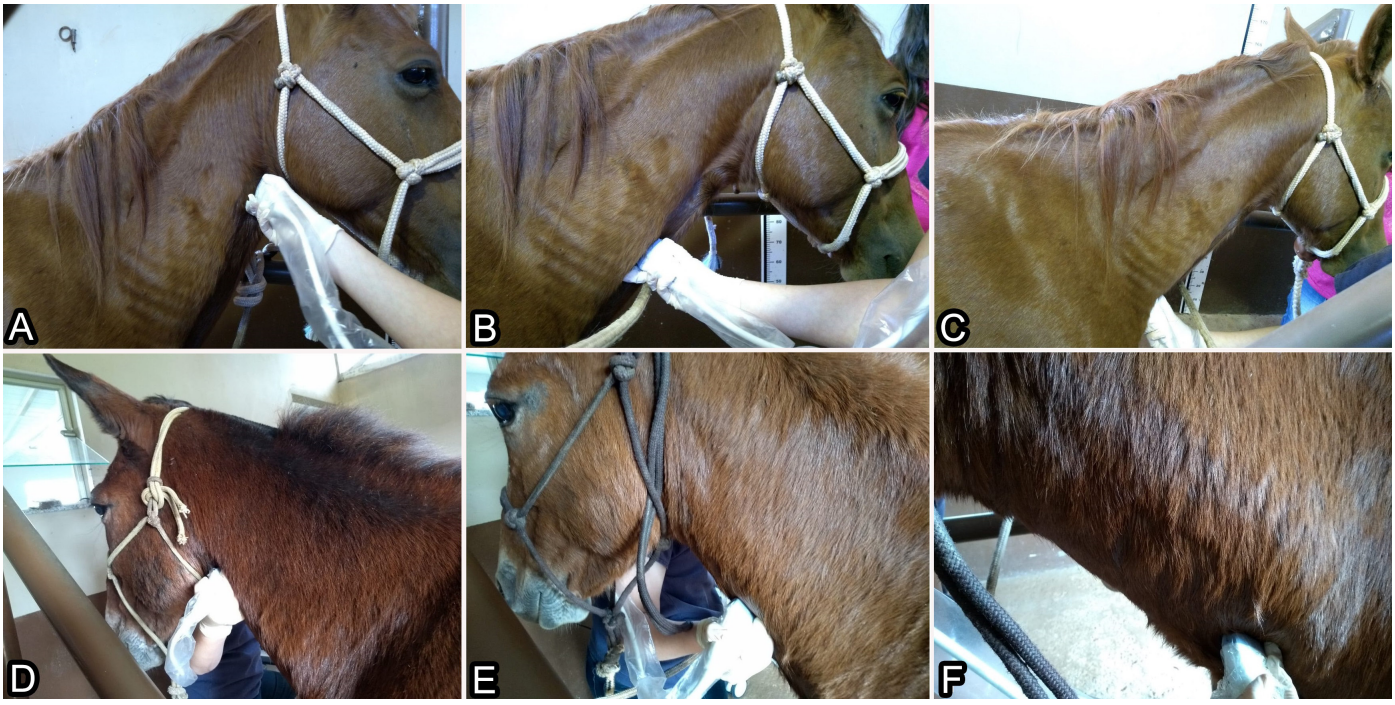

Fig.1. Longitudinal plane of the following three regions of the common carotid arteries of female horses: (**A**) cranial, (**B**) middle and (**C**) caudal; and of mules: (**D**) cranial, (**E**) middle and (**F**) caudal.

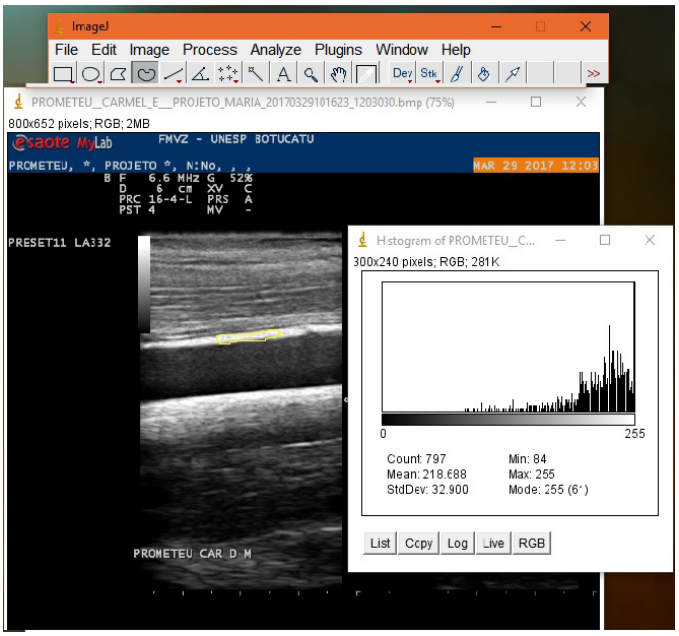

Fig.2. Ultrasound image of the left carotid artery in the cranial region of a female horse in which the following can be observed: the total of selected pixels (Count), which ranged from 770 to 1000 in each image in this study; the mean gray-level (Mean) of the selected pixels and the standard deviation (SD), where zero (0) represents a totally black (hypoechoic/hypoechogenic) pixel and 255 represents a totally white (hyperechoic/hyperechogenic) pixel; the most hyperechogenic (Max) and most hyporeogenic (Min) values observed in the sample; the mode (Mode), which represents the most frequent gray-level of the sample and, in parenthesis, the equivalent mode of pixel gray-levels [Mode(Count)].

showed heterogenic pixel quantification, as the percentages obtained were low (3-8%) in female horses and mules, although without statistically significant difference.

As for mule gender, only the variable Mode showed statistically significant difference on the cranial right side (P=0.0477), with females presenting higher pixel quantification (hyperechogenic) compared with that of males. Echotexture showed heterogenic pixel quantification, because the percentages obtained were low (2-13%) between genders, although without statistically significant difference.

Regarding the mules (Table 2), positive correlation was found between the variable Mean and the variables Min (P<0.001), Max (P<0.001), Mode (P<0.001), Mode(Count) (P<0.001), and Mode(Count)/Count(%) (P<0.001).

The variable SD showed positive correlation with the variable Max (P=0.0003) and negative correlation with the variable Min (P<0.001). The variable Min showed positive correlation with the variables Mode (P=0.0418), Mode(Count) (P=0.0002), Mode(Count)/Count(%) (P=0.0002), and Mean (P<0.001), and negative correlation with the variable SD (P<0.001).

For the variable Max, positive correlation was identified with the variables Mode (P<0.001), Mode(Count) (P=0.0160), Mode(Count)/Count(%) (P=0.0349), Mean (P<0.001), and SD (P=0.0003). The variable Mode presented positive correlation with the variables Mode(Count) (P<0.001), Mode(Count)/Count(%) (P<0.001), Mean (P<0.001), Max (P<0.001), and Min (P=0.0418).

With respect to the variable Mode(Count), positive correlation was verified with the variables Mode (P<0.001), Mode(Count)/Count(%) (P<0.001), Mean (P<0.001), Max (P=0.0160), and Min (P=0.0002). The variable Mode(Count)/Count(%) showed positive correlation with the variables Mode (P<0.001), Mode(Count) (P<0.001), Mean (P<0.001), Min (P=0.0002), and Max (P=0.0349).

As for the age factor, negative correlation was observed with the variables Mode (P=0.0004), Mode(Count) (P=0.0047), Mode(Count)/Count(%) (P=0.0034), and Mean (P=0.0011). The body mass factor negatively correlated with the variables Mean (P=0.0045), Max (P=0.0253), and Mode (P=0.0135). The factor longitudinal wall showed positive correlation with the variable Max (P=0.0072).

Regarding the female horses (Table 3), the variable Mean presented positive correlation with the variables Mode (P<0.001), Mode(Count) (P<0.001), Mode(Count)/Count(%) (P<0.001), Max (P<0.001), and Min (P<0.001).

The variable SD showed positive correlation with the variable Max (P=0.0011) and negative correlation with the variables Min (P<0.001), Count (P=0.0392), Mode(Count) (P=0.0140), and Mode(Count)/Count(%) (P=0.0152). As for the variable Min, positive correlation was found with the variables Count (P=0.0267), Mean (P<0.001), Mode (P<0.001), Mode(Count) (P=0.0038), Mode(Count)/Count(%) (P=0.0065), and Max (P=0.0181) and negative correlation was observed with the variable SD (P<0.001).

With regards to the variable Max, positive correlation was evidenced with the variables Min (P=0.0181), Mean (P<0.001), Mode (P<0.001), and SD (P=0.0011). The variable Mode showed positive correlation with the variables Mean (P<0.001), Max (P<0.001), Min (P<0.001), Mode(Count) (P<0.001), and Mode(Count)/Count(%) (P<0.001).

The variable Mode(Count) presented positive correlation with the variables Mode (P<0.001), Mode(Count)/Count(%) (P<0.001), Mean (P<0.001), and Min (P=0.0038), and negative correlation with the variable SD (P=0.0140). As for the variable Mode (Count)/Count(%), positive correlation was found with the variables Mode (P<0.001), Mode(Count) (P<0.001), Mean (P<0.001), Min (P=0.0065) and negative correlation was observed with the variable SD (P=0.0152). The body mass factor showed positive correlation with the variables Mean (P=0.0161) and Mode (P=0.0105).

DISCUSSION

In human medicine, ultrasound (US) examination has been widely used to estimate of the degree of stenosis and assess the echogenicity of atherosclerotic plaques deposited in the common carotid arteries (Sumner 1990). Physicians have relied on the echogenicity and echotexture of vessel walls to determine the soft tissue content and the amount of calcification deposited (Baroncini et al. 2006).

The US images obtained in this study sought to maintain better quality, necessitating a change in technique (gain of brightness and depth) in each case. According to Sarmento et al. (2014), this technique, even when altered, does not significantly interfere with grayscale histogram (GSH) results. According to Lima et al. (2013), the acoustic reinforcement artifact represents a certain localized increase in echo amplitude

Table 1. Mean, median and standard deviation of the variables of interest: Count, Mean, SD, Min, Max, Mode, Mode (Count), and Mode (Count)/Count (%) referring to the GSH, followed by the *p*-value of the Mann-Whitney test for the comparison between female horses and mules

Side	Region	Variables	Female horses			Mules			*p*-value
			Mean	Median	SD	Mean	Median	SD	
Right	Cranial	Count	852	866	59	875	882	67	0.5110
		Mean	156	153	63	184	192	31	0.2880
		SD	39	34	16	42	46	14	0.5112
		Min	39	22	47	68	82	47	0.1367
		Max	212	239	54	248	255	15	0.1738
		Mode	163	183	99	219	225	43	0.3167
		Mode (Count)	70	34	80	74	49	85	0.9169
		Mode (Count)/Count (%)	8	4	10	8	6	9	0.8620
	Middle	Count	880	877	61	863	837	63	0.5850
		Mean	142	147	64	201	208	24	0.0270*
		SD	45	50	11	35	35	13	0.1046
		Min	40	31	41	93	82	35	0.0219*
		Max	233	235	24	244	255	30	0.1976
		Mode	167	146	80	226	255	36	0.0807
		Mode (Count)	42	23	43	43	26	32	0.4314
		Mode (Count)/Count (%)	5	2	5	5	3	4	0.2670
	Caudal	Count	916	954	71	850	848	56	0.0391*
		Mean	156	153	34	173	175	24	0.1156
		SD	26	26	10	28	25	13	0.8457
		Min	76	80	34	93	90	29	0.1298
		Max	214	215	33	226	224	23	0.3999
		Mode	162	160	43	190	185	43	0.0959
		Mode (Count)	29	29	12	50	25	64	0.4775
		Mode (Count)/Count (%)	3	3	1	6	3	7	0.4029
Left	Cranial	Count	875	880	51	930	944	59	0.0625
		Mean	153	150	55	173	192	53	0.3892
		SD	36	40	11	39	41	10	0.6273
		Min	60	44	63	63	57	42	0.7545
		Max	224	230	32	239	255	27	0.2140
		Mode	157	160	89	195	203	76	0.3951
		Mode (Count)	59	19	102	72	32	82	0.1277
		Mode (Count)/Count (%)	7	2	11	7	3	8	0.1849
	Middle	Count	892	924	56	888	909	64	0.6049
		Mean	145	143	50	191	190	39	0.0366*
		SD	41	28	36	40	41	13	0.3358
		Min	62	59	49	70	62	43	0.5394
		Max	215	219	36	252	255	70	0.0093*
		Mode	150	151	62	216	222	53	0.0310*
		Mode (Count)	25	23	5	55	20	50	1.0000
		Mode (Count)/Count (%)	3	3	1	6	3	5	0.8968
	Caudal	Count	929	955	64	891	892	53	0.1704
		Mean	148	144	25	184	193	28	0.0090*
		SD	28	27	9	31	33	11	0.3817
		Min	69	65	25	96	88	39	0.0543
		Max	208	204	25	242	252	22	0.0140*
		Mode	158	142	46	200	206	31	0.0350*
		Mode (Count)	27	26	5	25	22	10	0.1160
		Mode (Count)/Count (%)	3	3	1	3	2	1	0.3817

* *p*-value <0.05.

Table 2. Sperman's correlation between the GSH variables: Count, Mean, SD, Min, Max, Mode, Mode (Count), Mode (Count)/Count (%) and the factors age, body mass, and longitudinal diameter and longitudinal wall of the vessels of mules

	Count	Mean	SD	Min	Max	Mode	Mode (Count)	Mode(Count)/Count (%)
Count	1.00	0.14	0.11	-0.11	0.19	0.10	0.23	0.10
	-	0.2725	0.3832	0.3985	0.1216	0.4091	0.0658	0.4276
Mean	0.14	1.00	-0.21	0.62	0.59	0.80	0.66	0.64
	0.2725	-	0.0841	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
SD	0.11	-0.21	1.00	-0.78	0.43	0.23	-0.21	-0.22
	0.3832	0.0841	-	<0.001*	0.0003*	0.0575	0.0859	0.0822
Min	-0.11	0.62	-0.78	1.00	-0.01	0.25	0.44	0.44
	0.3985	<0.001*	<.0001*	-	0.9426	0.0418*	0.0002*	0.0002*
Max	0.19	0.59	0.43	-0.01	1.00	0.71	0.30	0.26
	0.1216	<0.001*	0.0003*	0.9426	-	<.0001*	0.0160*	0.0349*
Mode	0.10	0.80	0.23	0.25	0.71	1.00	0.69	0.69
	0.4091	<0.001*	0.0575	0.0418*	<0.001*	-	<0.001*	<0.001*
Mode (Count)	0.23	0.66	-0.21	0.44	0.30	0.69	1.00	0.98
	0.0658	<0.001*	0.0859	0.0002*	0.0160*	<0.001*	-	<0.001*
Mode(Count)/Count (%)	0.10	0.64	-0.22	0.44	0.26	0.69	0.98	1.00
	0.4276	<0.001*	0.0822	0.0002*	0.0349*	<0.001*	<0.001*	-

	Count	Mean	SD	Min	Max	Mode	Mode (Count)	Mode(Count)/Count (%)
Age	-0.05	-0.41	-0.09	-0.17	-0.11	-0.44	-0.36	-0.37
	0.7302	0.0011*	0.4728	0.1839	0.3873	0.0004*	0.0047*	0.0034*
Body mass	0.00	-0.36	-0.03	-0.15	-0.29	-0.32	-0.11	-0.12
	0.9698	0.0045*	0.8140	0.2411	0.0253*	0.0135*	0.4037	0.3609
Longitudinal diameter	0.04	-0.08	-0.23	0.08	0.01	-0.26	-0.13	-0.14
	0.7943	0.5678	0.0896	0.5359	0.9264	0.0551	0.3388	0.2869
Longitudinal wall	-0.04	0.12	0.24	-0.07	0.36	0.20	-0.09	-0.09
	0.7741	0.3876	0.0690	0.6290	0.0072*	0.1415	0.5141	0.5009

* *p*-value <0.05.

that occurs subsequently to a low-attenuation structure such as liquid. In US examination, the reinforcement appears as an area of more intense clarity. In this study, measurements were performed in the upper vessel wall to avoid possible artifact. However, in most studies using GSH to address common carotid arteries in humans (Wohlin et al. 2009, Noto et al. 2012, Sarmento et al. 2014), measurements are performed in the lower (distal) wall because of the presence of atherosclerotic plaques, but it is not possible to affirm whether the reinforcement artifact significantly interferes (positively or negatively) with the GSH results.

Most GSH studies conducted with humans (Lind et al. 2008, Anderson et al. 2009, Noto et al. 2012, Sarmento et al. 2014) focus only on the evaluation of atherosclerotic plaques, and studies addressing the vessel walls of healthy patients have not been found in the surveyed literature, which shows the need for further research on this theme.

No changes were visualized in the vessels analyzed, which shows the need for a more detailed GSH study in cases of atherosclerotic plaques, considering that these alterations have become quite frequent in companion animals (Hess et al. 2003). According to Ribeiro & Shintaku (2004), the emergence of atherosclerotic plaques in humans is associated with poor nutrition. It can be assumed that the emergence of atherosclerotic plaques in animals is linked to their humanization.

Aguiar et al. (2014) described a case of atherosclerotic plaques in the common carotid artery of a 32-year-old horse with a history of heart disease, and observed an echotic mass obstructing 40% of the vessel lumen and impairing blood flow. In these situations, as well as in human medicine (Baroncini et al. 2006, Wohlin et al. 2009, Noto et al. 2012, Sarmento et al. 2014), GSH can quantitatively complement the evaluation, thus enabling spatial differentiation of distribution in gray-levels in an image or texture.

Only healthy animals were used in the present study, and according to the GSH, the vessel walls showed heterogeneous echotexture both in the mules and female horses, but without statistically significant difference between them. Regarding echogenicity, the mules showed more hyperechogenic vessel walls, which may be associated with the fact that they are anatomically and physiologically different from horses (Burnhan 2002, Alsafy et al. 2008, Smith 2009).

Results of the GSH showed statistically significant difference between mule genders only for the variable Mode on the cranial right side, with females presenting more hyperechogenic vessel walls compared with those of males. No difference between genders was observed with regard to echotexture.

Table 3. Sperman's correlation between the GSH variables: Count, Mean, SD, Min, Max, Mode, Mode (Count), Mode (Count)/Count (%) and the factors age, body mass, and longitudinal diameter and longitudinal wall of the vessels of female horses

	Count	Mean	SD	Min	Max	Mode	Mode (Count)	Mode(Count)/Count (%)
Count	1.00	0.09	-0.25	0.27	0.03	0.06	0.22	0.04
	_	0.4786	0.0392*	0.0267*	0.8287	0.6210	0.0705	0.7384
Mean	0.09	1.00	-0.03	0.64	0.82	0.94	0.47	0.46
	0.4786	_	0.7930	<.0001*	<0.001*	<0.001*	<0.001*	<0.001*
SD	-0.25	-0.03	1.00	-0.63	0.39	0.14	-0.30	-0.30
	0.0392*	0.7930	_	<0.001*	0.0011*	0.2617	0.0140*	0.0152*
Min	0.27	0.64	-0.63	1.00	0.29	0.47	0.35	0.33
	0.0267*	<0.001*	<0.001*	_	0.0181*	<0.001*	0.0038*	0.0065*
Max	0.03	0.82	0.39	0.29	1.00	0.83	0.21	0.21
	0.8287	<0.001*	0.0011*	0.0181*	_	<0.001*	0.0834	0.0905
Mode	0.06	0.94	0.14	0.47	0.83	1.00	0.49	0.49
	0.6210	<0.001*	0.2617	<0.001*	<0.001*	_	<0.001*	<0.001*
Mode (Count)	0.22	0.47	-0.30	0.35	0.21	0.49	1.00	0.97
	0.0705	<0.001*	0.0140*	0.0038*	0.0834	<0.001*	_	<0.001*
Mode(Count)/Count (%)	0.04	0.46	-0.30	0.33	0.21	0.49	0.97	1.00
	0.7384	<0.001*	0.0152*	0.0065*	0.0905	<0.001*	<0.001*	_

	Count	Mean	SD	Min	Max	Mode	Mode (Count)	Mode (Count)/Count (%)
Age	-0.02	-0.13	-0.03	0.01	-0.02	-0.16	-0.12	-0.10
	0.8972	0.3125	0.7868	0.9145	0.8442	0.2126	0.3245	0.4121
Body mass	-0.05	0.30	0.14	0.05	0.22	0.31	0.14	0.14
	0.6690	0.0161*	0.2491	0.6733	0.0774	0.0105*	0.2595	0.2533
Longitudinal diameter	0.06	0.01	0.02	0.02	-0.05	-0.04	-0.15	-0.16
	0.6812	0.9446	0.8640	0.9076	0.7026	0.7718	0.2826	0.2462
Longitudinal wall	0.01	0.17	-0.19	0.24	0.09	0.09	-0.02	0.02
	0.9664	0.2255	0.1727	0.0807	0.5378	0.5352	0.9016	0.8894

* *p*-value <0.05.

A for the mules, positive correlation was observed between the longitudinal wall factor and the variable Max, which indicates that the larger the longitudinal wall, the greater the possibility of hyperechogenic pixels; this information was not significant for female horses. In mules, negative correlation was found between the body mass factor and the variables Mean, Max, and Mode. This means that the lower the body mass of the animals, the greater the possibility of hyperechogenic pixels, which differentiates the horses, because the body mass factor showed positive correlation with the variables Mean and Mode, that is, the higher the body mass, the greater the possibility of hyperechogenic pixels.

The positive correlation between the variables Mean and Mode is quite normal, and this similarity has been described in many studies involving GSH (Santos et al. 2009, Svicero 2014), corroborating the findings of the present study, in which this similarity was observed both in the mules and female horses. It is worth highlighting that the variables Mean and Mode can be statistically different (Santos 2017), which may hinder interpretation of the GSH results.

Regarding the echotexture of the mules, it was identified that the younger the animal, the greater the homogeneity of the vessel wall; however, this affirmation was not relevant for female horses. According to Bersi et al. (2014) and Kiyota (2014), stiffening of the common carotid arteries is associated with aging. Stiffening, as well as calcification of the arteries, cause the vessel walls to present a hyperechogenic appearance on US examination, accompanied by acoustic shadow due to high acoustic impedance, which may also be associated with aging (Park et al. 1993, Saijo et al. 2006a, 2006b, Shaw et al. 2006, Kiyota 2014, Soares et al. 2015). However, in this study, the age factor in the mules had negative correlation with the variables Mode and Mode(Count)/Count(%).This means that, as the animal ages the vessel walls exhibit greater quantification of hypoechogenic pixels; this affirmation was not significant for female horses.

Due to the lack of studies in the literature addressing GSH in the vessel walls of mules and female horses, it is not possible to state whether this difference in echogenicity and echotexture occurs between these animals, which highlights the importance of further studies on GSH.

CONCLUSIONS

Echogenicity of the longitudinal walls of the common carotid arteries differed between female horses and mules.

Echotexture was heterogeneous and statistically similar among the animals.

Age and body mass inversely influenced the echogenicity of mules

The age factor did not influence the GSH variables in the equine females, whereas the body mass factor positively influenced only the echogenicity.

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

REFERENCES

Aguiar A., Dantas A., Viana G.F. & Machado V.M.V. 2014. Ateroma em artéria carótida comum de equino detectado através de exame ultrassonográfico: relato de caso. Anais IV Simpósio International de Diagnóstico por Imagem Veterinário, Belo Horizonte, MG, p.1-3. (Resumo)

Alsafy M.A.M., El-Kammar M.H. & El-Gendy S.A.A. 2008. Topographical anatomy, computed tomography and surgical approach of the guttural pouches of the donkey. J. Equine Vet. Sci. 28(4):215-222. <http://dx.doi.org/10.1016/j.jevs.2008.02.005>

Anderson J., Sundström J., Gustavsson T., Hulthe J., Elmgren A., Zilmer K., Zilmer M. & Lind L. 2009. Echogenecity of the carotid intima-media complex is related to cardiovascular risk factors, dyslipidemia, oxidative stress and inflammation: the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study. Atherosclerosis 204(2):612-618. <http://dx.doi.org/10.1016/j.atherosclerosis.2008.10.038> <PMid:19200993>

Armstrong V., Hansen W.F., Voorhis B.J.V. & Syrop C.H. 2003. Detection of cesarean scars by transvaginal ultrasound. Obstetr. Gynecol. 101(1):61-65. <PMid:12517646>

Baroncini L.A.V., Pazin Filho A., Junior L.O.M., Martins A.R., Ramos S.G., Cherri J. & Piccinato C.E. 2006. Caracterização tecidual ultra-sônica da placa carotídea vulnerável pela análise vídeodensitométrica. Revta Bras. Ecocardiografia 19(1):37-44.

Bersi M.R., Ferruzzi J., Eberth J.F., Gleason-Junior R.L. & Humphrey J.D. 2014. Consistent biomechanical phenotyping of common carotid arteries from seven genetic, pharmacological, and surgical mouse models. Anim. Biomed. Eng. 42(6):1207-1223. <http://dx.doi.org/10.1007/s10439-014-0988-6> <PMid:24590490>

Burnhan S.L. 2002. Anatomical differences of the donkey and mule. AAEP Proceed. 48(1):102-109.

Chequer G., Nascimento B.R., Navarro T.P., Falqueto E.B., Alencar M.C., Miranda M.C., Mandil A., Saad J.A., Fonseca C. & Ribeiro A.L. 2006. Espessamento médio-intimal da carótida e função endotelial na doença arterial coronariana. Arq. Bras. Cardiol. 87(2):84-90. <http://dx.doi.org/10.1590/S0066-782X2006001500004> <PMid:16951824>

Dantas A., Vettorato M.C., Oba E., Machado V.M.V. & Charlier M.G.S. 2016. Uso da técnica de histograma em escala de cinza para avaliação do desenvolvimento mamário de animais leiteiros. V Jornada Científica e Tecnológica da Faculdade de Tecnologia de Botucatu, Botucatu, SP. 6p. (Resumo)

Hess R.S., Kass P.H. & Winkle T.J. 2003. Association between diabetes mellitus, hypothyroidism or hyperadrenocorticiosm, and atherosclerosis in dogs. J. Vet. Intern. Med. 17(4):489-494. <http://dx.doi.org/10.1111/j.1939-1676.2003.tb02469.x> <PMid:12892299>

Kiyota T.A. 2014. Idade pulmonar está relacionada a alterações estruturais carotídeas em indivíduos hipertensos. Master's Thesis, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, SP. 61p.

Kobayashi N. & Karino T. 2016. Flow behavior and distribution of embolus-model particles at the terminal bifurcation of the human internal cartotid artery. World Neurosurg. 90(1):469-477. <http://dx.doi.org/10.1016/j.wneu.2016.03.012> <PMid:26987631>

Lee C.H., Choi J.W., Kim K.A., Seo T.S., Lee J.M. & Park C.M. 2006. Usefulness of standard deviation on the histogram of ultrasound as a quantitative value for hepatic parenchymal echo texture preliminary study. Ultrasound Med. Biol. 32(12):1817-1826. <http://dx.doi.org/10.1016/j.ultrasmedbio.2006.06.014>.

Lee J.J. 2010. Formação e processamento de imagens de ultrassom. Master's Thesis, Escola de Engenharia de São Carlos, Universidade de São Paulo, São Carlos, SP. 65p. <http://dx.doi.org/10.11606/D.18.2010.tde-24062010-154946>.

Lima J.L., André A. & Santos A.C. 2013. Reprodução e estudo de artefatos no ultrassom. Revta Bras. Física Med. 7(3):205-208.

Lind L., Andersson J., Rönn M., Gustavsson T., Holdfelt P., Hulthe J., Elmgren A., Zilmer K. & Zilmer M. 2008. Brachial artery intima-media thickness and echogenicity in relation to lipids and markers of oxidative stress in elderly subjects: the prospective investigation of the vasculature in Uppsala Seniors (PIVUS). Study Lipids 43(2):133-141. <http://dx.doi.org/10.1007/s11745-007-3125-6> <PMid:18004605>

Maeda K., Utsu M. & Kihaile P.E. 1998. Quantification of ultrasonographic echogenicity with grey-level histogram width: a clinical tissue characterization. Ultrasound Med. Biol. 24(2):225-234.

Mendonça J.A. 2017. O histograma de imagens coloridas permite melhor visualização de danos ecotexturais pelo ultrassom. Bras. Reumatol. 57(1):88-91. <http://dx.doi.org/10.1016/j.rbr.2014.12.016>

Noto N., Okada T., Abe Y., Miyashita M., Kanamaru H., Karasawa K., Ayusawa M., Sumitomo N. & Mugishima H. 2012. Characteristics of earlier atherosclerotic involvement in adolescent patients with Kawasaki disease and coronary artery lesions: significance of gray scale median on B-mode ultrasound. Atherosclerosis 222(1):106-109. <http://dx.doi.org/10.1016/j.atherosclerosis.2012.01.049> <PMid:22377394>

Park J.C., Siegel R.J. & Demer L.L. 1993. Effect of calcification and formalin fixation on in vitro distensibility of human femoral arteries. Am. Heart J. 125(2):344-349. <http://dx.doi.org/10.1016/0002-8703(93)90010-7> <PMid:8427126>

Queiroz J.E.R. & Gomes H.M. 2001. Introdução ao processamento digital de imagens. Revta Rita 8(1):1-31.

Ribeiro K.C. & Shintaku R.C.O. 2004. A influência dos lipídios da dieta sobre aterosclerose. ConScientiae Saúde 3(3):73-83.

Rosa E.M., Kramer C. & Castro I. 2003. Association between coronary artery atherosclerosis and the intima-media thickness of the common carotid artery measured on ultrasonography. Arq. Bras. Cardiol. 80(6):589-592, 285-288. <http://dx.doi.org/10.1590/S0066-782X2003000600002> <PMid:12856069>

Rosenfield A., Taylor T.K.J. & Jaffe C.C. 1980. Clinical applications of ultrasound tissue characterization. Radiol. Clin. N. Am. 18(1):31-58. <PMid:7378132>

Saijo Y., Hozumi N., Lee C., Nagao M., Kobayashi K., Oakada N., Tanaka N., Santos Filho E.D., Sasaki H., Tanaka M. & Yambe T. 2006a. Ultrasonic speed microscopy for imaging of coronary artery. Ultrasonics 44(1 Suppl.1):51-55. <http://dx.doi.org/10.1016/j.ultras.2006.06.050> <PMid:16844175>

Saijo Y., Tanaka A., Iwamoto T., Santos Filho E., Yoshizawa M., Hirosaka A., Kijima M., Akino Y., Hanadate Y. & Yambe T. 2006b. Intravascular two-dimensional tissue strain imaging. Ultrasonics 44(Suppl.1):147-151. <http://dx.doi.org/10.1016/j.ultras.2006.06.049> <PMid:16872653>

Santos W.A. 2017. Estudo morfológico e morfométrico por ultrassonografia modo B do ligamento nucal de equinos da raça Quarto de Milha. Master's Thesis, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Botucatu, SP. 60p.

Santos W.G., Monteiro J.N.M., Oliveira D.C., Borlini D.C., Martins-Filho S., Machado F.M., Nunes L. & Costa F. 2009. Ultrassonografia quantitativa do fígado em gatos com tirotoxicose induzida. Braz. J. Vet. Res. Anim. Sci. 46(6):438-447. <http://dx.doi.org/10.11606/S1413-95962009000600002>

Sarmento P.L.D.F.A., Plavnik F.L., Scaciota A., Lima J.O., Miranda R.B. & Ajzen A.S. 2014. Relationship between cardiovascular risk factors and the echogenicity and pattern of the carotid intima-media complex in men. São Paulo Med. J. 132(2):97-104. <http://dx.doi.org/10.1590/1516-3180.2014.1322490> <PMid:24714990>

Shaw L.J., Raggi P., Berman D.S. & Callister T.Q. 2006. Coronary artery calcium as a measure of biologic age. Atherosclerosis 188(1):112-119. <http://dx.doi.org/10.1016/j.atherosclerosis.2005.10.010> <PMid:16289071>

Silva E.G., Gonçalves M.T., Pinto S.C., Soares D.M., Oliveira R.A., Alves F.R., Araújo A.V.C. & Guerra P.C. 2015. Análise quantitativa da ecogenicidade testicular pela técnica do histograma de ovinos da baixada ocidental maranhense. Pesq. Vet. Bras. 35(3):297-303. <http://dx.doi.org/10.1590/S0100-736X2015000300014>

Smith D.C. 2009. The Book of Mules: selecting, breeding and caring for equine hybrids. The Lyons Press, Connecticut. 136p.

Soares M.Q.S., Castro R.C., Santos P.S.S., Capelozza A.L.A. & Fischer-Bullen I.R.R. 2015. Contribuição da radiografia panorâmica no diagnóstico de calcificação de ateroma de carótida: relato de caso e revisão da literatura. Revta Port. Estomatol. Med. Dentária Cirurg. Maxilofacial 56(6):127-131.

Souza L.O., Gomes L.P.B., Barbosa V.V., Medeiros G.R., Souza C.M. & Weller M. 2013. Analysis of the population of equidae in semiarid region of Paraíba. J. Biotechonol. Biodiversity 4(3):269-275.

Sumner D.S. 1990. Use of color-flow imaging technique in carotid artery disease. Surg. Clin. N. Am. 70(1):201-211. <http://dx.doi.org/10.1016/S0039-6109(16)45045-0> <PMid:2406968>

Svicero D.J. 2014. Eficácia do ultrassom na identificação de transplantes de células tronco mesenquimais em tecido muscular equino. Master's Thesis, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Botucatu, SP. 71p.

Vescovi L.A., Monteiro J.N.M., Santos W.G.D., Oliveira D.C.D., Borlini D.C. & Machado F.M. 2009. Ultrassonografia quantitativa do baço de gatos normais. Vet. Foco 7(1):4-10.

Wohlin M., Sundström J., Andrén B., Larsson A. & Lind L. 2009. An echolucent carotid artery intima-media complex is a new and independent predictor of mortality in an elderly male cohort. Atherosclerosis 205(2):486-491. <http://dx.doi.org/10.1016/j.atherosclerosis.2009.01.032> <PMid:19243779>

GUIDE FOR AUTHORS

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Exemples for References:

- Articles published in scientific journals:

Ubiali D.G., Cruz R.A., De Paula D.A., Silva M.C., Mendonça F.S., Dutra V., Nakazato L., Colodel E.M. & Pescador C.A. 2013. Pathology of nasal infection caused by Conidiobolus lamprauges and Pythium insidiosum in sheep. J. Comp. Pathol. 149(2/3):137-145.

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

(Note: The first letters of the words in the title of papers published in journals are small. It is preferable to indicate the number of the respective issue.)

- Books:

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

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

(Note: The first letter in the words of the title of books should be capital.)

- Chapters of books:

Uzal F.A., Plattner B.L. & Hostetter J.M. 2016. Alimentary system, p.1-257. In: Maxie M.G. (Ed.), Jubb, Kennedy and Palmer´s Pathology of Domestic Animals. Vol.2. 6th ed. Elsevier, St Louis, Missouri.

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

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

- Dissertations and Theses:

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

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

- Abstracts published in Events:

Massa A.T., Potter K.A. & Bradway D. 2016. Epizootic bovine abortion outbreak in Eastern Nevada cattle. Annual Meeting American College of Veterinary Pathologist (ACVP), New Orleans, Lousiana. (Abstract D-50)

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

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

(Note: Consult entire papers instead of only Abstracts)

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