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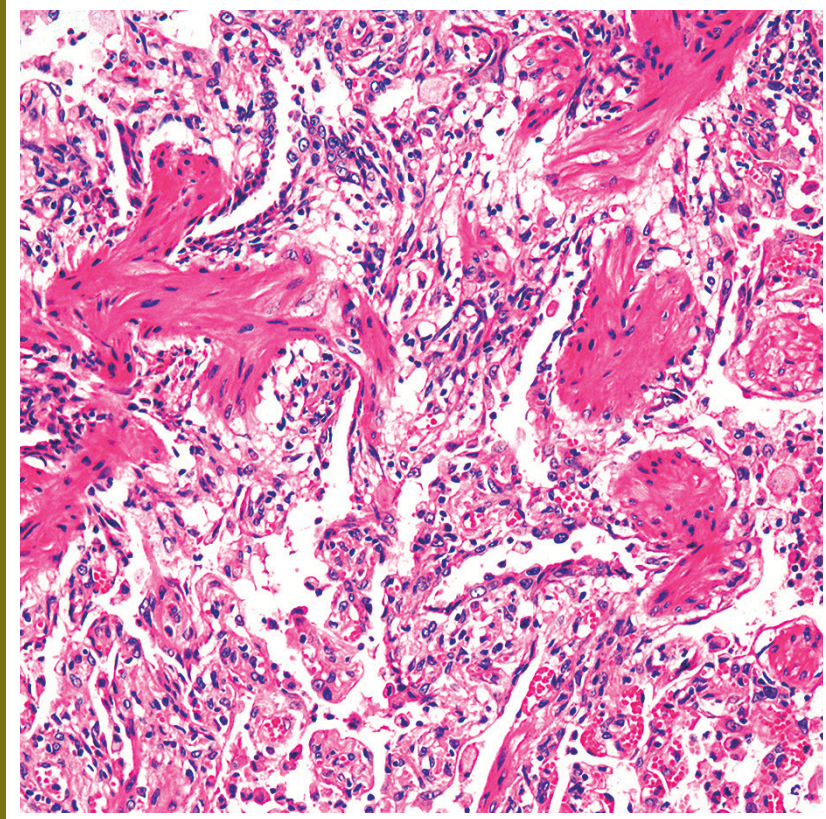

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Cover illustration: Lung with proliferation of spindle cells in the subpleural region associated with smooth muscle hypertrophy and reactive mesothelial cells in a cat with pulmonary fibrosis. HE, bar = 130µm. (Cony et al., p. 138)

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Original Article
Livestock Diseases

Ocurrence and risk factors associated with *Mycoplasma agalactiae* infection in dairy goat herds of Paraíba State, Brazil[1]

Rodrigo A.T. Matos[2]*, Sandra B. Santos[3], Renato V. Alves[4], Ednaldo J. Silva[2], Melânia L. Marinho[4], José Wilton P. Júnior[5], Rinaldo A. Mota[3] and Felicio Garino Júnior[6]

ABSTRACT.- Matos R.A.T., Santos S.B., Alves R.V., Silva E.J., Marinho M.L., Júnior J.W.P., Mota R.A. & Garino Júnior F. 2019. **Ocurrence and risk factors associated with *Mycoplasma agalactiae* infection in dairy goat herds of Paraíba State, Brazil.** *Pesquisa Veterinária Brasileira 39(2):93-98*. Graduate Program in Veterinary Medicine, Centro Universitário Cesmac, Rod. Divaldo Suruagy s/n, Marechal Deodoro, AL 57160-000, Brazil. E-mail: rodrigoatmatos@gmail.com

Mycoplasmosis is a disease that may cause severe economical losses in goat and sheep herds, and it is associated with mastitis, polyarthritis, agalactia, conjunctivitis, pneumonia and reproductive failure. The objective of this study was to determine the occurrence of *Mycoplasma agalactiae* in milk samples and investigate the main risk factors associated with infection in goats from farms of the state of Paraíba, Brazil. For *Mycoplasma agalactiae* diagnosis, 251 milk samples were submitted to DNA extraction using a commercially available *kit,* following the manufacturer's instructions and Polymerase Chain Reaction (PCR) was performed. In addition, questionnaires were applied to identify the main risk factors associated with contagious agalactia. Out of the two hundred fifty-one samples analyzed, 50 (19.9%, I.C. 15.1-25.4%) were PCR positive for *M. agalactiae*. In the risk factors analysis, some associations were observed for the following variables: size of the herd (P<0.001, OR=7.1, I.C. 2.4-20.6), replacement of farm animals (P<0.001, OR=4.7, I.C. 1.8-12.2) and participation of animals in fairs and exhibitions (P=0.029, OR=2.0, I.C.1.0-3.9). The results allowed confirming the occurrence of *Mycoplasma agalactiae* in milk samples of goats from Paraíba. Therefore, it is strictly necessary to monitor dairy goat flocks and to raise the awareness of farmers about the economic importance of the disease, since it causes severe economic losses for producers of the state. Identification of risk factors is essential for adoption of control measures and for the correction of the management factors in farms where there are animals with positive diagnosis, avoiding, so, pathogen dissemination.

INDEX TERMS: *Mycoplasma agalactiae*, infection, mycoplasmosis, polymerase chain reaction, milk, goats, caprine, bacterioses.

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RESUMO.- [Ocorrência e fatores de risco associados à infecção por *Mycoplasma agalactiae* em rebanhos caprinos leiteiros do estado da Paraíba, Brasil.] As micoplasmoses ocasionam prejuízos econômicos nas criações de ovinos e caprinos, e estão associados com quadros de mastite, poliartrite, agalaxia, conjuntivite, pneumonia e falhas reprodutivas. Objetivou-se neste estudo determinar a ocorrência de *Mycoplasma agalactiae* em amostras de leite e investigar os principais fatores de risco associados à infecção em caprinos provenientes de propriedades rurais do estado da Paraíba, Brasil. Para o diagnóstico de *Mycoplasma agalactiae*, foram analisadas 251 amostras de leite, que foram submetidas à

extração do DNA genômico usando um *kit* comercial, seguindo as recomendações do fabricante. Para diagnóstico da infecção utilizou-se a Reação em Cadeia da Polimerase (PCR). Além disso, foram aplicados questionários para identificar os principais fatores de risco associados infecção à agalaxia contagiosa. Das 251 amostras analisadas, 50 (19,9%; I.C. 15,1-25,4%) foram positivas na PCR para *M. agalactiae.* Observaram-se na análise dos fatores de risco, algumas associações para as seguintes variáveis: tamanho do rebanho (P<0,001; OR 7,1), reposição de animais da propriedade (P<0,001; OR 4,7) e participação dos animais em feiras e exposições (P= 0,029; OR 2,0). Os resultados permitiram confirmar a ocorrência do *Mycoplasma agalactiae* em amostras de leite de caprinos da Paraíba. Portanto, é necessário o monitoramento dos rebanhos caprinos leiteiros e a conscientização dos produtores rurais para a importância econômica da doença, visto que a mesma acarreta severos prejuízos econômicos para os produtores do estado. A identificação dos fatores de risco são imprescindíveis para a adoção de medidas de controle e para a correção dos fatores de manejo em propriedades que tenham animais com diagnóstico positivo, evitando assim, a disseminação do patógeno.

TERMOS DE INDEXAÇÃO: *Mycoplasma agalactiae*, infecção, micoplasmoses, reação de cadeia de polimerase, leite, caprinos, bacterioses.

INTRODUCTION

Mycoplasmosis are diseases caused by bacteria of the class *Mollicutes*, which are considered the smallest self-replicating prokaryotes without cellular walls (Chazel et al. 2010). In goats, the main mycoplasmosis are contagious caprine pleuropneumonia, CCPP (OIE 2014) and contagious agalactia of ovine and caprine animals, CAOC (OIE 2013). This disease can cause mastitis, agalactia, polyarthritis, keratoconjunctivitis and, occasionally, abortion and pneumonia (Gómez-Martín et al. 2013). The main agent of CAOC is *Mycoplasma agalactiae*, although other species, such as: *M. capricolum* subsp. *capricolum*, *M. putrefaciens* and *M. mycoides* subsp. *capri* can also cause the disease (Madanat et al. 2002, Gil et al. 2003).

Infection takes place orally, via respiratory tract or at the mammary area, and, after a period of bacteremia, it disseminates to the eyeball, mammary gland, joints, tendons, uterus and lymph nodes. Among the early signals, the most important are the birth of infeasible offspring and abortions. Transplacental infection has been described in goatlings (Azevedo et al. 2012). Infection by *M. agalactiae* spreads across the herd by direct contact with infected animals and in the environment via contaminated milk and milking equipment, ocular discharge and the hands of milkers. There are also reports of venereal transmission (Madanat et al. 2001).

CAOC is geographically distributed throughout Europe, Western Asia, USA and Northern Africa, and is endemic in most Mediterranean countries (Gil et al. 2003). In Brazil, *M. agalactiae* was isolated from sick goats in 2001, in the state of Paraiba (Azevedo et al. 2006). Reports of the disease in the states of Pernambuco and Rio Grande do Norte have been published after that (Azevedo 2005), and it has also been diagnosed in the microregion of Cariri Paraibano (Bandeira et al. 2008). However, there is neither recent study nor data available on the current situation of the disease in the State of Paraíba, nor are there are not even studies about the risk factors in Paraiba which may be associated with the infection by *M. agalactiae* in dairy goats. The aim of this research was to verify the occurrence of *M. agalactiae* in milk samples and to investigate the major risk factors associated with the infection in goats coming from farms in microregions of the State of Paraíba, in Brazil.

MATERIALS AND METHODS

Area of study and sampling. In the period from March 2016 to April 2017, 13 dairy goat farms, located in the Western and Eastern Paraíba Cariri, Western Curimataú and Eastern Seridó microregions and in the municipalities of Gurjão (07º 14' 48" S and 36º 29' 22" W), Juazeirinho (07º 04' 06" S and 36º 34' 40" W), Monteiro (07º 53' 22" S and 37º 07' 12" W), Nova Floresta (06º 27' 19" S and 36º 12' 12" W), Prata (07º 41' 27" S and 37º 04' 49" W), São Sebastião do Umbuzeiro (08º 09' 08" S and 37º 00' 37"W) and Zabelê (08º 04' 32" S and 37º 05' 54" W) were selected and visited using a convenience-oriented sampling. The selection took into account the occurrence of clinical signs suggesting infection by *Mycoplasma agalactiae*.

The size of the sample for the prevalence study was determined considering an anticipated prevalence of 10% for the infection in goats (Santos et al. 2014), which determined a minimum sampling of 138 goats, considering a level of significance of 95% and a statistical error of 5% (Thrusfield 2004).

The following formulas were used to calculate the number of animals:

$$n = 1,96^2 . P_{esp.}\left(1 - P_{esp}\right) / d^2;\ n_{ajust} = \left(N \times n\right) / \left(N + n\right)$$

Where: n = minimum number of goats to be sampled, N = total number of goats in the herd, P_{esp} = anticipated prevalence, d^2 = absolute precision.

As the criterion for inclusion of goats in the sample, all females in lactation coming from farms with animals with signals suggesting infection were selected. A total of 251 samples were collected with a safety margin at the discretion of the authors. It was not possible to standardize the number of samples and goats per farm due to the significant differences in herd sizes and to the fact that some animals were not in the lactation period.

Collection of samples. In the 13 farms, 251 samples of milk from goats of the races Alpine American, British Alpine, Anglo-Nubian, Saanen, Alpine Parda, Toggenburg and mixed-race were collected. All the farms had animals with a history of sharp decrease in the production of milk. In four farms goats with a history of reproductive disorders were observed. For the collection of samples, udders were washed with a solution of sodium hypochlorite and soaked in iodized alcohol. A pool of both udders was then made, and a sample collected from each animal in sterile Falcon-type tubes containing 50% glycerinated saline solution added with Penicillin (2.000UI/ml) was stored in freezers at -20ºC and -80ºC up to the moment of the molecular diagnosis.

Molecular diagnosis. Milk samples were aliquoted in volume of 300µl and genomic DNA was extracted using the "Wizard® Genomic DNA Purification Kit" (Promega) commercial *kit*, following the manufacturer's protocol. The quality and amount of DNA extracted were evaluated using an automatic quantifier

(Multiscan Go, ®ThermoScientific). The polymerase chain reaction (PCR) was done with the oligonucleotides described by Tola et al. (1997), *Ma* (FS1 5'-AAAGGTGCTTGAGAAATGCC-3' and FS2 5'-GTTGCAGAAGAAAGTCCAATCA-3'), which amplify a fragment of 375 base pairs of the gene 16SRNA of *Mycoplasma agalactiae*. For the reaction, 8μL of PCR Mix LGC® 2X (LGC Biotecnologia®, Code 13-11250, Cotia/SP, Brazil) were used, containing: 200mM of each dNTP; 1.5mM of $MgCl_2$; *Taq* DNA Polimerase (0.5U) in appropriate reaction concentration and buffer (Tris-HCl pH: 8.5, KCl); oligonucleotides at 30 pmol; 8μL genomic DNA and PCR water, totalizing a final volume of 25 μL of reaction. The positive reaction control was the one isolated from Pernambuco Ma62 (BrPE62), and PCR water was used as negative control. The thermal profile used was the same described by Tola et al. (1997). The reactions were carried out using a Bioer XP Thermal Cycler® thermocycler (Bioer Technology Corporation LTDA, Hangzhou, China). PCR products were stained with *Blue Green Loading Dye I*® (LGC Biotecnologia®, Code 13-15009.06, Cotia/SP, Brazil) and submitted to 1.5% agarose gel electrophoresis. Amplicons were displayed on transilluminator (®TransiluminatorLoccusBiotecnology L-Pix photodocumented).

Analysis of risk factors and statistics. For the study of risk factors, epidemiological questionnaires were applied containing objective questions to the producers about the general characteristics of farms and their productive, reproductive and sanitary management.

Data were expressed using absolute and relative frequencies. Interpretation of prevalence (high, average and low) was based on percentiles 25 and 75 of the percentages of goats which were positive in the herds. The analysis of herds evaluated the association between the history of clinical manifestations suggestive of CAOC by *Mycoplasma agalactiae* in the herds and the positive results for *Mycoplasma agalactiae* in PCR using Pearson's chi-square (X^2) statistical test (Sampaio 1998). Regarding the analysis of animal risk factors, an univariate analysis of the variables of interest was first carried out using Fisher's exact X2 test, whenever necessary, and after that a logistic regression analysis was done using as dependent variable the result obtained in PCR (positive or negative). The independent variables considered in the model were those which showed statistical significance below 0.20. This probability was stipulated so that possible risk factors of the event were not excluded from the analysis (Hosmer & Lemeshow 1989). EpiInfo™ 7 was used for statistical calculations and the level of significance adopted was 5.0%.

Ethical aspects. The research was approved by the Research Ethics Committee (CEP) of Federal University of Campina Grande (UFCG) under protocol no. 296-2015.

RESULTS AND DISCUSSION

Among the 251 samples analyzed, 50 (19.9%, I.C. 15.1-25.4%) were positive in the PCR for *Mycoplasma agalactiae*, with eight positive farms (61.5%) distributed among the following municipalities: Gurjão, Juazeirinho, Prata, São Sebastião do Umbuzeiro and Zabelê. The results per farm are shown in Table 1.

Farms A, F and M had high prevalences; the remainder showed average and low prevalence of CAOC, considering the percentiles of 25 and 75. In the distribution by municipalities, prevalences were observed in the municipalities of Gurjão (16.7%), Juazeirinho (64.3%), Prata (18.3%), São Sebastião do Umbuzeiro (40.0%) and Zabelê (12.5%). In the municipalities of Monteiro and Nova Floresta, all the goats evaluated were negative for infection by *M. agalactiae*. The results of the analysis of the association of PCR with the history of clinical signs in the herds and of the risk factors associated with infection by *Mycoplasma agalactiae* in goats are shown in Table 2 and 3.

The prevalence of CAOC by *Mycoplasma agalactiae* in this study (19.9%) was lower than that found by Azevedo et al. (2006) in Paraíba. These authors reported a prevalence of 100.0% in small ruminants after an outbreak of contagious agalactia in farms located in Paraíba, Brazil. The values were possibly different due to the occurrence of outbreaks in several farms studied by the authors. However, when compared with a study done by Bandeira et al. (2008), also in the state of Paraíba, a higher prevalence was found in goat milk than that observed by these authors, which was of 7.5% (9/120). This difference may be due to the number of animals and herds used in the survey, to the acquisition of animals from other regions of Brazil and to differences in handling between farms, since the studies were done in different farms, although some municipalities were the same. In the present study, a higher prevalence was observed in goat milk rather than that found by Alves et al.(2013) in the State of Pernambuco, which was of 3.7% (3/81). These same authors explain that the difference between the prevalence values is due to the different stages of mycoplasmosis in the herds studied. These same authors state that factors such as chronic or subclinical character of the disease and treatment of mastitis in herds with antibiotics for long periods may also have contributed to the differences between prevalences.

As for the clinical signs associated with the positive results of PCR for infection by *Mycoplasma agalactiae*, most variables presented a significant association with the diagnosis of CAOC, except for the variable regarding reproductive disorders, infertility and infertility associated with abortion (P>0.05). Although, there have been association of cases of mastitis unresponsive to treatment and polyarthritis with the diagnosis of infection by *M. agalactiae*, it is not possible

Table 1. Frequency of positive dairy goats per property

Property	N	Positivity
A	14	9 (64.3%)
B	45	12 (26.7%)
C	16	2 (12.5%)
D	11	0 (0.0%)
E	13	2 (15.4%)
F	20	7 (35.0%)
G	20	1 (5.0%)
H	26	0 (0.0%)
I	12	0 (0.0%)
J	13	0 (0.0%)
L	6	1 (16.7%)
M	40	16 (40.0%)
N	15	0 (0.0%)
Total	251	50 (19.9%)

N = number of samples collected on the property.

Table 2. Analysis of the association of the PCR results with the history of clinical manifestations in the goat herds

Variables	N	PCR	Value P[A]
		Positive	
History of mastitis that does not respond to antibiotic therapy			
Yes	40	17 (42.5%)	<0.001*
No	211	33 (15.6%)	
History of mastitis and polyarthritis at the same time			
Yes	40	17 (42.5%)	<0.001*
No	211	33 (15.6%)	
Birth history of offspring with polyarthritis			
Yes	20	10 (50.0%)	<0.001*
No	231	40 (17.3%)	
History of pneumonias, polyarthritis, conjunctivitis, sudden death			
Yes	40	17 (42.5%)	<0.001*
No	211	33 (15.6%)	
History of reproductive disorders			
Yes	85	29 (34.1%)	<0.001*
No	166	21 (12.6%)	
What reproductive disorders			
Infertility	65	19 (29.2%)	0.088
Infertility + abortion	20	10 (50.0%)	

[A] Test X2; N = total of analyzed samples, 1 different database (N = 85); * Significant association at the 5.0% level.

Table 3. Analysis of risk factors associated with *Mycoplasma agalactiae* infection

Variables	N	PCR	Value P	Regression logistic OR (I.C. 95%)	Value P
		Positive			
Racial characteristics					
Pure	226	46 (20.3%)	0.794[A]		
Mixed race	25	4 (16.0%)			
Size of the goat herd					
Up to 50 animals	65	4 (6.1%)	<0.001[B]*	-	
Between 51 and 100 animals	145	46 (31.7%)		7.1 (2.4 - 20.6)	<0.001*
Between 101 and 200 animals	15	0 (0.0%)		**	
Over 200 animals	26	0 (0.0%)		**	
Feeders and drinking troughs for young and adult goats					
Yes	60	11 (18.3%)	0.724[B]		
No	191	39 (20.4%)			
Insect control					
Yes	191	39 (20.4%)	0.724[B]		
No	60	11 (18.3%)			
Cleaning of premises					
Yes	219	43 (19.6%)	0.767[B]		
Weekly	32	7 (21.9%)			
Spare goats are owned by					
Yes	231	40 (17.3%)	<0.001[B]*		
No	20	10 (50.0%)		4.7 (1.8 - 12.2)	0.001*
Milking line					
Yes	196	34 (17.3%)	0.054[B]		
No	55	16 (29.1%)		1.9 (0.9 - 3.9)	0.056
Treatment of clinical mastitis					
Yes	80	21 (26.2%)	0.086[B]	1.7 (0.9 - 3.3)	0.088
No	171	29 (16.9%)			
Destination of diseased goats					
Slaughter	20	10 (50.0%)	0.088[B]	2.4 (0.8 - 6.7)	0.091
Antimicrobial treatment	65	19 (29.2%)			
Goats participate in fairs and/or exhibitions					
Yes	65	19 (29.2%)	0.029[B]*	2.0 (1.0 - 3.9)	0.031*
No	186	31 (16.7%)			

[A] Fisher's Exact Test, [B] Test X2, 1 different database (N = 85); N = total of samples, OR = odds ratio, I.C. = confidence interval; * Significant association at the 5.0% level, ** the OR could not be calculated.

to state that the presence of these clinical signals is decisive for the occurrence of infection by *Mycoplasma agalactiae*, although other agents may cause these same clinical signals, just as the presence of the agent in goat milk samples does not indicate the occurrence of infection. An association can often occur with other diseases such as Brucellosis, Leptospirosis and Toxoplasmosis, which exhibit similar clinical signals. Even when there is no association between the clinical signs of reproductive disease with the infection by *M. agalactiae*, an investigation should be undertaken to eliminate the possibility of infection by the agent.

In the analysis of risk factors, an association with the positive effects of PCR for infection by *Mycoplasma agalactiae* was observed for the following variables: size of the herd (P<0.001, OR=7.1, I.C. 2.4-20.6), replenishment of animals of the farm (P<0.001, OR=4.7, I.C.1.8-12.2) and participation of the animals in fairs and exhibitions (P= 0.029, OR=2.0, I.C. 1.0-3.9). As for the size of the herd, the herds which had between 51 and 100 animals showed greater chances of having positive animals than the herds with up to 50 animals (OR=7.1, I.C. 2.4-20.6). In herds in which there is acquisition of animals for breeding stock replenishment there is an increased chance of animals being positive for infection by *M. agalactiae* when compared with closed herds (OR=4.7, I.C. 1.8-12.2). This is evidence that when replenishment animals do not come from the farms there is a greater possibility of positive animals being introduced in the herds, favoring the dissemination of the agent. In a study done in Jordan, Al-Momani et al. (2008) found that the agent can be introduced by disease-carrying animals from other herds used for reproduction. These same authors reported that other factors can contribute to the occurrence of the agent in the herds, such as: inadequate cleaning of milking equipment and separation of young animals from their mothers. The participation of animals in fairs and exhibitions increased the chances of animals getting infected by *M. agalactiae* when compared with animals coming from herds which did not take part in these events (OR=2.0, I.C. 1.0-3.9). This result corroborates what was reported by Bandeira et al. (2008), who stated that mycoplasmosis outbreaks in goats in Paraíba and Pernambuco are caused by the participation of animals in fairs and exhibitions and to the introduction of animals coming from the country's Southeastern and Center-Western regions. These authors suggested that the rapid spread of *M. agalactiae* and probably of other infections is favored by the intense trade and transit of animals from the States of Paraíba, Pernambuco and Rio Grande do Norte. It should be stressed that there are few studies on the risk factors associated with the occurrence of infection by *Mycoplasma agalactiae*, and further research is needed to investigate others risk factors which may be associated with the infection by this agent.

CONCLUSIONS

The results of the study allowed the authors to confirm the occurrence of *Mycoplasma agalactiae* in milk samples from goats coming from Paraíba. However, it is necessary to monitor the dairy goat herds and to raise the awareness of rural producers about the economic relevance of this disease, since it entails severe economic losses for the producers.

The identification of risk factors is indispensable for the adoption of control measures and for the correction of the handling factors in farms which have animals with positive diagnosis, thus avoiding dissemination of the pathogen.

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

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Original Article
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Characterization of ruminal acidosis and initial phase of laminitis inducted by oligofructose in crossbred calves[1]

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ABSTRACT.- Noronha Filho A.D.F, Freitas S.L.R., Rodrigues D.F., Mendes F.F., Miguel M.P., Cunha P.H.J., Fioravanti M.C.S. & Silva L.A.F. 2019. **Characterization of ruminal acidosis and initial phase of lamnitis inducted by oligofructose in crossbred calves.** *Pesquisa Veterinária Brasileira 39(2):99-106.* Escola de Veterinária e Zootecnia, Universidade Federal de Goiás, Campus Samambaia, Avenida Esperança s/n, Goiânia, GO 74690-900, Brazil. E-mail: dionisiofnf@hotmail.com

One of the ways to study cattle laminitis is its experimental induction by supplying a large amount of high fermentation carbohydrate. The most effective protocol until now has been the use of oligofructose. The objective of this study was to evaluate clinical and histological aspects of the hoof in experimental induction of ruminal acidosis and laminitis in calves using oligofructose. Six crossbred (*Bos taurus* x *Bos indicus*) yearling calves divided into Group I (GI) and Group II (GII) were used. Animals in GI and GII received intraruminal oligofructose in doses of 13 and 17g/kg, respectively. During 28 hours the calves were clinically evaluated and 30 hours after induction, samples were taken from coronary and abaxial wall of the hoof for histologic evaluation. Were noticed signs of ruminal and metabolic acidosis like rumen distension with fluid, diarrhea, ruminal pH reduction and, at blood gas analysis, pH and bicarbonate below reference range. Lameness was not observed however, some animals had a slower gait and apathy, possibly due to metabolic acidosis, though. Histologically, typical lesions of laminitis like circulatory changes and inflammatory infiltrate in the dermis, irregularities and areas of detachment at basement membrane and morphologic changes in cells from basal epidermis were found. The protocol induced, in the first 30 hours, clinical signs of ruminal and metabolic acidosis and low grade histologic lesions in the digits. Lameness and digit pain were not observed, characterizing the prodromic phase of the disease.

INDEX TERMS: Ruminal acidosis, lamnitis inducted, oligofructose, crossbred calves, metabolic acidosis, bovine, lameness, hoof histology, cattle.

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RESUMO.- [**Caracterização da acidose ruminal e da fase inicial da laminite induzidas por oligofrutose em bezerros mestiços.**] Uma das formas de se estudar a laminite bovina é sua indução experimental por meio do fornecimento de grande quantidade de carboidrato de alta fermentação. O protocolo mais eficaz até o momento foi o uso de oligofrutose. Objetivou-se avaliar aspectos clínicos e histológicos dos dígitos de bovinos na indução experimental de acidose ruminal e laminite usando oligofrutose. Utilizaram-se seis bezerros mestiços (*Bos taurus* x *Bos indicus*) de um ano, divididos em Grupo I (GI) e Grupo II (GII). Os animais em GI e GII receberam oligofrutose por via intrarruminal nas doses de 13 e 17g/kg respectivamente. Os bovinos foram avaliados clinicamente por 28 horas e fragmentos de coroa e muralha abaxial dos dígitos foram

colhidos para histologia 30 horas após a indução. Foram identificados sinais de acidose ruminal e metabólica como distensão ruminal com líquido, diarreia e baixo pH ruminal. Os resultados de hemogasometria indicaram baixos pH e nível plasmático de bicarbonato. Os animais não apresentaram claudicação, entretanto, observaram-se apatia e marcha mais lenta, atribuídas à acidose metabólica. Histologicamente foram observadas lesões indicativas de laminite como alterações circulatórias e infiltrado inflamatório na derme, irregularidades e áreas de destacamento da membrana basal e alterações morfológicas de células da epiderme basal. O protocolo induziu, nas primeiras 30 horas, sinais de acidose ruminal e metabólica e lesões histológicas de baixa intensidade nos dígitos. Não foi observada claudicação ou sensibilidade nos dígitos, caracterizando a fase prodrômica da enfermidade.

TERMOS DE INDEXAÇÃO: Acidose ruminal, laminite induzidas, oligofrutose, bezerros mestiços, acidose metabólica, bovinos, claudicação, histologia dos dígitos.

INTRODUCTION

The growing productivity of bovine over the last decades has been accompanied by the greater occurrence of diseases associated with the production system such as indigestions and foot diseases (Greenough 2007, Radostits et al. 2007). Among the indigestions, ruminal acidosis is a fermentative imbalance caused by the ingestion of excessive amounts of rapidly fermentable carbohydrate (Nagaraja & Lechtenberg 2007). As for foot diseases, laminitis is considered one of the main diseases affecting bovine hooves. It is characterized by inflammation of the digital dermis and has ruminal acidosis as an important element of its pathogenesis (Nocek 1997, Thoefner et al. 2004). In the clinical form of laminitis, the animal shows marked lameness in all limbs (Thoefner et al. 2004, Danscher et al. 2009). In chronic cases, the hoof is elongated and with a marked concavity in the wall, defined as "slipper foot" (Greenough 2007). In the subclinical form, changes in living tissues of the hoof precede painful lesions such as sole ulcers and white-line disease by two to three months (Mendes et al. 2013).

The fermentative changes that characterize ruminal acidosis result in the production and absorption of substances that can act locally in the dermis and epidermis of the digits (Nocek 1997, Greenough 2007). Some elements generated in ruminal acidosis are indicated as the cause of laminitis such as histamine, lactic acid and endotoxins. Isolated or together, they would cause vascular lesions and degradation of the suspensory apparatus of the third phalanx within the digit (Singh et al. 1994, Nocek 1997, Concha et al. 2014). The period between the triggering factor and the appearance of the clinical signs of laminitis is called the prodromal phase, or development stage, and is studied in the equine species (Martins Filho et al. 2008). In the bovine species, the prodromal period has a varied duration (Thoefner et al. 2004, Danscher et al. 2009) because the experimental induction protocols used do not always result in specific clinical signs of clinical laminitis, such as lameness and digital sensitivity (Boosman et al. 1991a, Singh et al. 1994, Momcilovic et al. 2000). The knowledge of the changes in bovine laminitis in the development phase is important, since in addition to allowing early diagnosis, before more evident signs such as lameness, allows the adoption of more effective therapeutic measures, as occurs in the equine species, including the use of anti-inflammatory and cryotherapy (Van Eps 2010).

An important and unexplored aspect of the studies on rumen acidosis and laminitis are breed differences, with the exception of studies on experimental induction of acute acidosis (Ortolani et al. 2010). Zebu and their crosses account for a large part of the national herd, and laminitis is cited as a health problem in these animals, both for milk aptitude (Mendes et al. 2013) as for meat production (Oliveira & Millen 2014). It is known that zebu and taurine can respond differently to experimental induction of ruminal acidosis (Ortolani et al. 2010) and that they have microscopic differences in the structure of the hooves (Mendonça et al. 2003, Rabelo et al. 2015). It is assumed that these differences could also be reflected in laminitis.

As a method of study of laminitis, its induction allows the control of different variables that influence the appearance of the lesions as well as the evaluation of different aspects of the etiopathogeny (Thoefner et al. 2004, Danscher et al. 2010, Concha et al. 2014). One of the ways to try to induce laminitis is by inducing ruminal acidosis. Some protocols were not effective, others were, but failed to be replicated in other studies (Thoefner et al. 2004, Greenough 2007). A protocol that has shown more consistent results is the induction of ruminal acidosis with the use of oligofructose (Thoefner et al. 2004, Danscher et al. 2009). Oligofructose is a fructose polymer with up to ten sugar subunits and is present, among other plants, in grasses of temperate climate (Thoefner et al. 2004). In the consulted literature (Thoefner et al. 2004, Danscher et al. 2009, Concha et al. 2014), data about experimental induction of laminitis in zebu cattle, or even crosses (*Bos taurus x Bos indicus*), were not found. A study option that could help to clarify the disease at the developmental stage would be in weaned crossbred calves. One of the advantages of the study in young animals is the ease of manipulation and clinical follow-up.

The aim of the present study was to evaluate the protocol for the induction of ruminal acidosis and laminitis by employing oligofructose and to study its effects on the laminar corium in the prodromal stage of the disease using one year old crossbred bovine as an experimental model.

MATERIALS AND METHODS

The project was evaluated and approved by the Ethics Committee on the Use of Animals of the UFG (CEUA/UFG), having received protocol number 26/2013. Six crossbreed calves were used (*Bos taurus* x *Bos indicus*) with an approximate age of 12 months and an average weight of 175±22.6 kg. Three months before the start of the study, the animals were submitted to ruminostomy with implantation of ruminal cannula with 8.89cm of internal diameter (KEHL®, São Carlos, Brazil). The calves were kept in pastures of *Brachiaria decumbens* and were supplemented with Tifton 85 grass hay. In the composition of the diet, there was a mineral supplement and water supplied at will.

The crossbreed calves were divided in Group I (GI), with three animals that received oligofructose (Oligofrutose®, Viafarma®, Sao Paulo, Brazil) by intraruminal way at 13g/kg and Group II (GII), with three animals receiving oligofructose at 17g/kg in the same way. The protocol and doses adopted for administration of oligofructose were based on the literature consulted (Thoefner et al. 2004). The study, for both Group I and Group II, was divided into three phases. In stage I, during three days the calves received 10% of the calculated dose of

oligofructose per day, divided in two moments with 5% each, totaling 30%. In this period, according to the literature, clinical signs were not expected (Thoefner et al. 2004, Danscher et al. 2009), therefore, doses were considered for adaptation of the ruminal microbiota (Thoefner et al. 2004). On the fourth day, beginning of phase II, the calves received the remaining 70% at once, being considered the zero mark for the beginning of clinical alterations (Thoefner et al. 2004, Danscher et al. 2009). In both phase I and phase II oligofructose was diluted at 80% concentration in warm water. Phase III began 30 hours after phase II and consisted of harvesting hoof fragments for histological examination.

The calves were evaluated before starting phase I and during 28 hours from the beginning of phase II, when they received the oligofructose overload, 70% of the dose. Prior to phase I, physical examination and ruminal fluid were performed. The evaluations during phase II occurred every four hours, the first evaluation being titled T0, composed of general physical examination, gait evaluation and digital sensitivity, and laboratory tests, including examination of the ruminal fluid, evaluation of the globular volume and hemogasometry. In all samples, ruminal fluid samples were obtained directly from the rumen after opening of the ruminal cannula, when organoleptic and pH characteristics were evaluated (Dirksen et al. 1993).

Then, lameness and digital sensitivity analysis were performed according to methodologies cited in the literature (Sprecher et al. 1997, Thoefner et al. 2004). In the evaluation of the locomotion, calves were analyzed as they walked in a straight line on concrete floor. The evaluator was positioned laterally on the course where he assigned a score according to the severity of lameness that the animal could present (Table 1). For digital sensitivity assessment, the thoracic limb was lifted by an auxiliary and the examiner pressed the soles of both digits at different points using a hoof tester. If there was no reaction, score 1 was assigned. A discrete reaction was attributed score 2 and a marked reaction was assigned score 3 (Thoefner et al. 2004). Both the locomotion score and the digital sensitivity score were always evaluated by the same examiner. The samples destined to evaluate the globular volume were of venous blood collected in tube containing ethylenediaminetetracetic acid (EDTA). After homogenization and centrifugation, the globular volume was measured in a measuring table. The hemogasometry was done with venous blood samples collected in heparinized syringe and evaluated in bench hemogasometer (COBAS B 121®, Roche Diagnóstica, São Paulo/SP, Brasil).

After 28 hours of the start of phase II, the calves received supportive treatment with ruminal content removal and intravenous fluid therapy with lactated Ringer's solution (Lactated Ringer's, Equiplex, Aparecida de Goiânia/GO, Brazil) and sodium bicarbonate solution to 6% (Sodium Bicarbonate Solution 6%®, Prado S.A. Labboratory, Curitiba/PR, Brazil) for the treatment of metabolic acidosis. The amount of bicarbonate solution administered was calculated according to the bicarbonate deficit, a parameter evaluated in hemogasometry. After two hours with this supportive treatment phase II was closed and phase III was started, in which the animals were sedated and submitted to hoof biopsy for histological evaluation. In order to collect the samples, the bovine were sedated with 2% xylazine hydrochloride (Anasedan®, Ceva Brasil, Paulínia/SP, Brazil) at a dose of 0.2mg/kg and placed in dorsal decubitus position. To collect samples, the lateral digit of the right pelvic limb and the medial digit of the right thoracic limb were selected. The distal extremities of the locomotor limbs evaluated were initially washed with soap and water. Then, both the coronary band and the hoof wall regions were surgically prepared with topical iodopovidone and alcohol. The anesthetic block consisted of the infusion of 10mL of 2% lidocaine hydrochloride in the dorsal digital vein (Lidovet®, Bravet, Engenho Novo/RJ, Brazil).

Fragments were collected from the coronary region and the abaxial wall. For biopsy of the coronary region, a site was selected, approximately 3cm lateral to the dorsal margin of the hoof, dorsal transition region between axial to abaxial walls. Hair clipping was performed near the withdrawal site as well as application of 3mL of 2% lidocaine hydrochloride in the subcutaneous tissue around the biopsy site. Using a scalpel, forceps and scissors, a rectangular fragment of approximately 10x5mm was removed. For the biopsy of the wall region, the horn case was gradually worn with a grinder, and the wall was often complied with forceps as well as the coloring of the wall. When it became soft, by sinking lightly under pressure, the biopsy was performed by initially delimiting the scalpel fragment and then withdrawing it as a use of the Falcão-Faleiros' lamellotome (Mendes 2015).

The process began with the delimitation of three sides of a rectangle, using a scalpel blade that was introduced until reaching the phalanx. On the fourth side, not sectioned, the Falcão-Faleiros' lamelotome (Mendes 2015) was inserted perpendicularly until it reached the bone, and then directed towards the opposite cut. In this way the fragment was removed with all layers of the dermis, transition between dermis and epidermis, living layer of the epidermis and part of the worn horn layer. After removal of the fragments, iron perchloride (Friezol Estankasangue®, Pinus, Jundiaí/SP, Brazil) was applied, powdered oxytetracycline hydrochloride was sprinkled on the wounds (Terramicina Pó, Pfizer), and orthopedic cotton and bandages were applied around the hoof. The dressings continued to be changed every two days until complete healing.

The samples collected were fixed in 10% buffered formalin, routinely processed and stained by hematoxylin and eosin (HE) and periodic acid from Schiff (PAS). A same evaluator performed the histological analysis blind using a common optical microscope in the 10x objective. In HE staining, they were evaluated in the dermis, hyperemia, hemorrhage, edema and inflammatory infiltrate (Thoefner et al. 2005, Mendes et al. 2013). For these parameters, the scores 0 = absent, 1 = rare, 2 = discrete, 3 = moderate, 4 = accentuated

Table 1. Lameness score in cattle

Score	Name	Description
1	Normal	Straight back when standing in quadrupedal position and walking. Normal step.
2	Mild lameness	Straight back quadrupedal and arched when walking. Normal step.
3	Moderate lameness	Arched back when standing and walking. Shortened step of one or more members.
4	Evident lameness	Arched back when standing and walking. Locomotion changed with one step at a time or avoiding the support of a limb.
5	Severe lameness	In addition to previous signs, the calves are reluctant or have difficulty supporting one or more limbs even when standing.

Adapted from Sprecher et al. (1997).

were assigned (Mendes et al. 2013). In the epidermis cell necrosis and morphological changes were evaluated in the cell nuclei. For cell necrosis the same scores were considered as those used in the evaluation of the dermis. For the morphological alteration score 1 = approximately 50% of cells with oval nucleus perpendicular to the basement membrane and 50% of cells with round nucleus were attributed; 2 = predominance of epidermal cells with round nucleus; 3 = predominance of cells with elongated and flattened nucleus or absence of nucleus. In the PAS-stained samples, areas of irregularities and detachment of the basement membrane represented by separation between basal cells and basement membrane (Thoefner et al. 2005, Mendes et al. 2013). Scores were similarly assessed in the evaluation of the dermis, with values from 0 to 4 (Mendes et al. 2013).

For each parameter, the average of all samples of each calf was considered. In all evaluations, physical, laboratory and histological exams, the data were evaluated by descriptive statistics (Sampaio 2010).

RESULTS

Heart rate and rectal temperature did not show significant changes in none of the groups. Respiratory rate increased in both groups eight hours after induction, but decreased gradually in the following moments. Ruminal motricity was reduced in both groups reaching close to zero in the Group GII 20 hours after induction (Table 2). During the 28 hours of clinical evaluation, the calves of both groups did not present lameness or digital sensitivity. Despite this, slower gait associated with apathy was observed in some animals.

The ruminal content presented changes in all evaluated parameters (Table 3). The odor and color of the contents have changed. At first, the color was olive green and the aromatic odor. During the evaluations, the odor became acid and the coloration became milky or yellowish. The accumulation of fluid in the rumen was more pronounced in GII. In the GI the average minimum ruminal pH was 5.32 and in GII the average minimum ruminal pH was 4.6.Hemogasometry showed a reduction in blood pH, pCO2 and bicarbonate values in both groups along evaluation (Table 4). In both groups, there was a gradual increase in the values of globular volume (Table 4). It was observed that the calves of the Group GII already started the first evaluation, beginning of phase II, with values indicative of metabolic acidosis.

Regarding the biopsy of the hoof, the technique used was adequate and allowed to obtain viable samples for histological evaluations. At the collection sites there was a slight bleeding,

Table 2. Mean and standard deviation of heart rate in beats per minute (HR), respiratory rate in motions per minute (RP), ruminal movements in five minutes (MR) and rectal temperature in degrees Celsius (T°C) for crossbred calves (*Bos taurus* x *Bos indicus*) receiving 13g/kg oligofructose (GI) and 17g/kg oligofructose (GII)

		Phase I	Phase II							
			T0	T4	T8	T12	T16	T20	T24	T28
FC	GI	45.00 ± 3.00	51.00 ± 6.08	60.00 ± 13.86	58.00 ± 7.00	54.00 ± 8.89	52.00 ± 5.00	51.00 ± 9.54	51.67 ± 3.21	49.00 ± 4.58
	GII	43.00 ± 2.65	42.67 ± 7.57	51.67 ± 11.68	53.00 ± 4.36	46.00 ± 3.46	50.67 ± 13.61	60.67 ± 1.15	62.00 ± 25.53	56.00 ± 7.21
FR	GI	24.33 ± 4.73	14.33 ± 4.73	23.33 ± 7.02	30.00 ± 3.61	18.67 ± 3.51	14.67 ± 3.06	18.00 ± 5.29	11.33 ± 4.16	17.00 ± 2.65
	GII	25.30 ± 4.16	10.33 ± 2.31	23.00 ± 10.44	26.67 ± 5.77	18.00 ± 2.65	12.33 ± 5.77	11.67 ± 3.21	13.33 ± 3.06	16.33 ± 7.51
MR	GI	4.70 ± 1.53	5.33 ± 0.58	4.33 ± 0.58	3.67 ± 1.53	4.00 ± 1.73	5.00 ± 1.00	4.00 ± 1.73	4.50 ± 0.71	3.00 ± 1.73
	GII	4.00 ± 1.73	5.33 ± 0.58	2.00 ± 1.00	4.00 ± 0.00	3.33 ± 3.06	1.33 ± 0.58	0.33 ± 0.58	2.33 ± 1.15	1.33 ± 2.31
T°C	GI	38.30 ± 0.77	36.77 ± 1.04	38.37 ± 0.15	39.17 ± 0.5	38.83 ± 0.51	37.53 ± 0.7	36.97 ± 0.95	36.77 ± 1.63	38.43 ± 0.38
	GII	38.20 ± 0.26	36.47 ± 0.51	37.67 ± 0.87	38.30 ± 0.14	38.20 ± 0.2	36.80 ± 1.82	36.43 ± 0.81	35.77 ± 1.10	37.43 ± 0.51

Table 3. Means and standard deviation of ruminal pH for crossbred calves (*Bos taurus* x *Bos indicus*) receiving oligofructose (GI) 13g/kg and oligofructose (GII)

	Phase I	Phase II							
		T0	T4	T8	T12	T16	T20	T24	T28
GI	6.71 ± 0.29	6.89 ± 0.11	5.72 ± 0.49	5.32 ± 0.57	5.38 ± 1.43	5.57 ± 1.47	5.97 ± 1.41	5.88 ± 1.95	6.78 ± 0.68
GII	6.92 ± 0.02	6.69 ± 0.10	5.50 ± 0.06	5.14 ± 0.43	4.83 ± 0.36	4.70 ± 0.35	4.6 ± 0.27	5.24 ± 1.31	5.62 ± 0.86

Table 4. Means and standard deviation of blood pH values, CO2 pressure in mmHg (pCO2), bicarbonate in mmol/l (HCO3) and globular volume in% (VG) for crossbred calves (*Bos taurus* x *Bos indicus*) receiving 13g/kg oligofructose (GI) and 17g/kg oligofructose (GII)

		T0	T4	T8	T12	T16	T20	T24
pH	GI	7.38 ± 0.12	7.39 ± 0.10	7.35 ± 0.14	7.32 ± 0.19	7.26 ± 0.16	7.23 ± 0.21	7.24 ± 0.22
	GII	7.29 ± 0.08	7.32 ± 0.07	7.28 ± 0.05	7.21 ± 0.04	7.12 ± 0.03	7.08 ± 0.01	7.07 ± 0.03
PCO2	GI	41.00 ± 5.01	41.53 ± 6.27	36.57 ± 9.16	36.00 ± 7.39	37.80 ± 16.84	34.90 ± 10.7	35.23 ± 10.65
	GII	29.03 ± 9.86	28.63 ± 7.07	36.57 ± 9.83	21.50 ± 2.77	19.57 ± 3.31	18.33 ± 3.48	20.07 ± 1.68
HCO3	GI	24.83 ± 8.54	25.80 ± 8.87	21.63 ± 10.45	20.10 ± 10.14	19.23 ± 12.16	16.53 ± 9.65	17.43 ± 10.45
	GII	14.50 ± 7.81	14.90 ± 6.39	13.80 ± 6.46	8.47 ± 1.50	6.23 ± 0.65	5.33 ± 0.90	5.73 ± 0.76
VG	GI	30.33 ± 2.31	29.67 ± 2.08	30.00 ± 1.73	33.33 ± 2.89	33.33 ± 4.16	35.67 ± 5.51	36.67 ± 7.02
	GII	32.67 ± 3.51	33.00 ± 2.00	33.33 ± 3.06	33.00 ± 2.65	40.33 ± 5.69	45.00 ± 4.25	46.00 ± 3.75

which stoped by compression at the site with gauze and using haemostatic agent. On the days following the biopsy the animals did not present lameness.

In the evaluation of the dermis, alterations such as edema and inflammatory infiltrate were detected in both groups (Table 5). The edema was predominantly perivascular (Fig.1). The inflammatory infiltrate, besides also perivascular, occurred especially close to the dermis-epidermal junction (Fig.2). Hemorrhage and hyperemia were observed few times in both groups. In the epidermis, morphological changes were observed in the nuclei of the basal epidermal cells. Cellular necrosis was observed only in GII. Irregularities and detachment of the basement membrane were observed in all groups (Fig.3).

DISCUSSION

In the 28-hour phase II evaluation period, no specific signs of laminitis were observed such as lameness or digital sensitivity. There were clinical signs of ruminal acidosis and microscopic changes in the digits indicative of laminitis, characterizing this period as the prodromal phase of the disease. Other studies in which oligofructose was used indicated that the first signs of lameness started 39 hours after induction (Thoefner et al. 2004) and within 30 to 48 hours after carbohydrate administration (Danscher et al. 2009), compatible with the present study where lameness did not occur on the first day after the carbohydrate overload.

Regarding the absence of lameness on the days following the induction, there was probably a relation with the removal of all altered ruminal contents 28 hours after induction. With the emptying of the rumen, the absorption of substances potentially harmful to the digital thorium ceased. In the other studies in which the animals did not have the ruminal content removed, the permanence of the intake may have benefited the longer absorption of these substances, favoring the lameness associated with laminitis (Thoefner et al. 2004, Danscher et al. 2009). Although no lameness or digital sensitivity was observed in the evaluation period, some calves presented a slower gait associated with apathy. This effect is a result of the reduced state of consciousness that can occur in metabolic acidosis due to D-lactate observed in ruminal acidosis (Radostits et al. 2007). Probably the neurological signs associated with D-lactate acidosis are due to interference in the energetic metabolism of the brain tissue. D-lactate would impair the metabolism of pyruvate and L-lactate (Ling et al. 2012, Lorenz & Gentile 2014), important energetic substrates in neurons (Adeva-Andany et al. 2014).

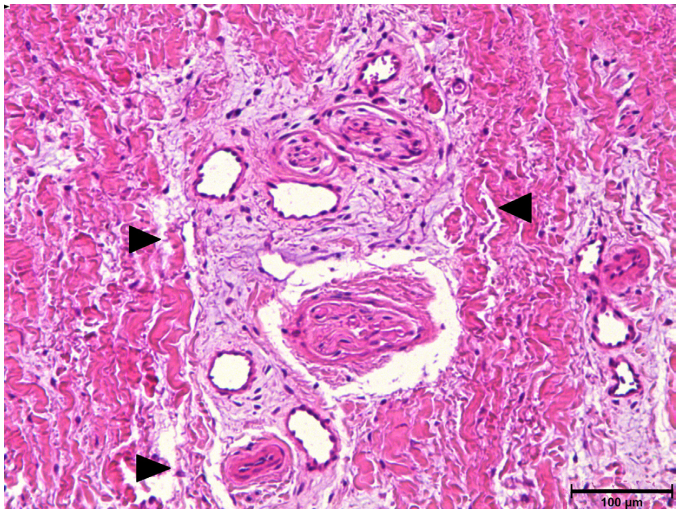

Fig.1. Fragment of the wall region of crossbred calf from group GII showing perivascular edema (arrowhead). HE, obj.10x.

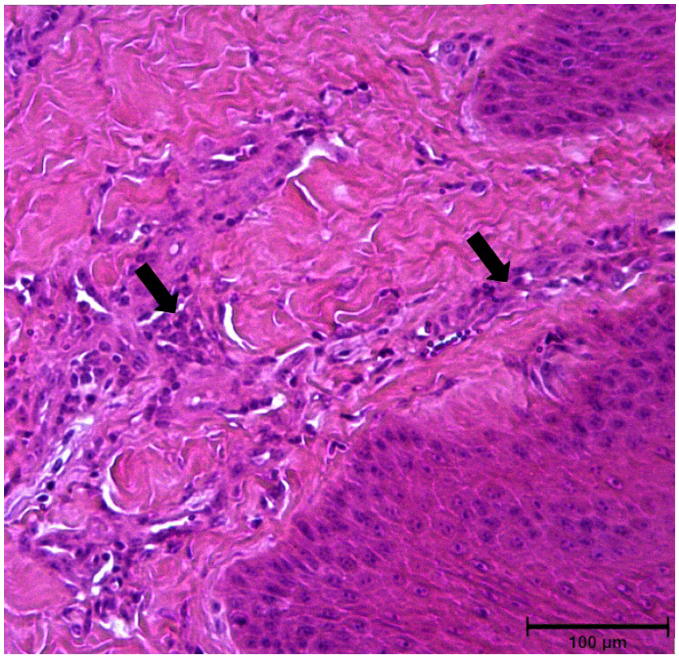

Fig.2. Fragment of the coronary region region of crossbred calf from group GI showing subepidermal inflammatory infiltrate (arrows). HE, obj.10x.

Table 5. Histological changes in samples of crown and dorsal hull wall of crossbred (*Bos taurus* x *Bos indicus*) calves receiving 13g/kg oligofructose (GI) and 17g/kg oligofructose (GII)

Histological alteration	Groups	
	GI	GII
Hyperemia	0.14 ±0.38	0.17 ±0.39
hemorrhage	0	0.33 ±0.78
Edema	1.86 ±0.38	1.75 ±0.75
Inflammatory infiltrate	1.14 ±1.07	1.25 ±0.96
Death of basal epidermal cells	0	0.5 ±0.67
Morphological alteration of basal epidermal cells	1.14±0.39	2.08 ±0.9
Morphological alteration of basement membrane	0.57 ±1.13	1.17 ±0.94

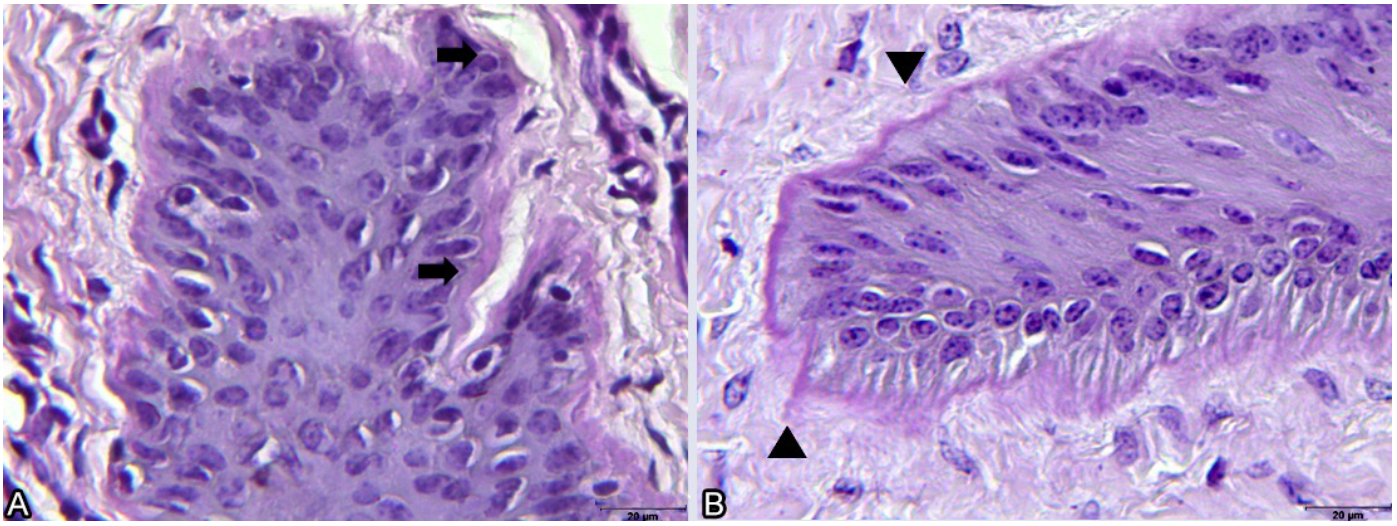

Fig.3. Photomicrographs of bovine digit fragments. (**A**) Fragment of the coronary region of calf from group GII showing a discrete multifocal detachment of the basement membrane (arrow). (**B**) Fragment of wall of calf from group GI showing irregularities of the basal membrane (arrowhead). PAS, obj.40x.

As expected (Thoefner et al. 2004, Danscher et al. 2009), after the administration of oligofructose overload, there was a marked reduction in ruminal pH, reaching levels compatible with acute acidosis in Group II and subacute acidosis in Group I. Lower values for ruminal pH in relation to the present study were reported in other studies that used oligofructose to induce ruminal acidosis. When the carbohydrate dose was 13g/kg, pH 5.0 (Thoefner et al. 2004) and 4.2 (Concha et al. 2014) were observed, while at the 17g/kg dose, pH 4.5 (Thoefner et al. 2004) and 4.3 (Danscher et al. 2009) were registered. It is believed that the highest values for pH observed were related to the average mean weight of the animals, since in other studies the mean weight of bovine was 408 kg (Thoefner et al. 2004), 375 kg (Danscher et al. 2009), and between 280 and 310kg (Concha et al. 2014). The influence of weight on the effects of ruminal acidosis was already observed in another study using oligofructose (Danscher et al. 2009) and a protocol for the induction of acidosis using sucrose (Ortolani 1995).

In addition to the reduced ruminal pH, another change observed was the decrease in ruminal motility, almost absent in GII. The reduction of ruminal motility is an expected effect of ruminal acidosis (Nagaraja & Lechtenberg 2007, Ortolani et al. 2010), even when this is established by administration of oligofructose (Thoefner et al. 2004, Danscher et al. 2009).

The group receiving the highest dose of oligofructose, 17g/kg, developed metabolic acidosis with only 30% of the total dose. It has already been demonstrated that the addition of fructose in the diet considerably increases the production of lactic acid in the rumen, when compared to the starch (Golder et al. 2012). In addition to contributing to the reduction of ruminal pH, part of this lactate is absorbed causing metabolic acidosis (Thoefner et al. 2004, Danscher et al. 2009, Concha et al. 2014). In another protocol using oligofructose, a reduction in baseline excess was observed during the three days in which the calves received 30% of the dose. However, values compatible with metabolic acidosis were not observed (Thoefner et al. 2004).

Although the calves in GII presented metabolic acidosis at the time they received most of the oligofructose dose, ruminal pH was within the reference value. It is inferred that the fermentative disorder was regularized faster than the systemic one. The same behavior was observed after administration of the remaining 70% oligofructose, elevation of ruminal pH and persistence of metabolic acidosis at the end of the evaluation period. The different responses among the studies may reflect breed aspects of the studied animals - crossbred-, since taurine and zebu may react differently to metabolic acidosis after ruminal acidosis (Ortolani et al. 2010).

The reduction of pCO2, observed in both groups, favored elevation of the blood pH to the physiological limits, being considered a compensatory mechanism (Dirksen et al. 1993, Reece 2004). Regarding respiratory frequency and pCO2, it was observed that the highest value of RF occurred eight hours after oligofructose overload, whereas the lowest values of pCO2 occurred only 20 hours later. It is believed, in this case, that the increase in respiratory rate was related to the ambient temperature. The maximum value occurred at 2:00 p.m., a warmer period on the day. The reduction of pCO_2, with minimum values 20 hours after oligofructose overload, indicated that the respiratory mechanism acted by increasing the range of respiratory movements and not necessarily by increased respiratory rate. Respiratory rate values below the reference range (Dirksen et al. 1993) were observed at various times in both groups. Decreasing values of respiratory rate over 24 hours were also observed in other research on the induction of ruminal acidosis in bovine (Ortolani et al. 2010). It is possible that this reduction in respiratory rate is due to the depression of the respiratory center due to the severe acidosis installed (Huber 1976).

In the crossbred calves of the present study, we opted for the treatment of support at a predetermined time. The time of treatment in animals submitted to experimental induction of ruminal acidosis may be based on predetermined moments or changes in hemogasometry parameters (Momcilovic et al. 2000, Thoefner et al. 2004, Danscher et al. 2009). The emptying of

the ruminal contents ceased the absorption of substances produced in greater quantity in ruminal acidosis like lactic acid, endotoxins and histamine, that could be harmful to the organism. In addition to the use of intravenous fluid therapy containing bicarbonate to correct metabolic acidosis (Radostits et al. 2007), deterioration of the clinical picture and the appearance of possible complications such as death, as reported by other authors (Thoefner et al. 2004, Danscher et al. 2009). On subsequent days, calves resumed to normal appetite and behavior, confirming the efficacy of the supportive treatment.

Regarding the hoof biopsy, the integrity of the samples obtained confirmed the efficacy of the technique used to obtain the clinical specimens (Mendes 2015). The hoof biopsy technique as performed here allowed the evaluation of changes in all layers without the need for euthanasia of the animals, which has already been done in studies on equine laminitis (Visser & Pollitt 2011). The circulatory and morphological alterations observed as in the digits are compatible with the inflammatory process triggered by the acidosis caused by the carbohydrate. Similar findings have been reported in experimental induction protocols with oligofructose (Thoefner et al. 2005), endotoxins (Boosman et al. 1991a, Singh et al. 1994) and cases of natural occurrence (Boosman et al. 1991b, Mendes et al. 2013). The predominant monocytic inflammatory infiltrate has been described in other studies (Boosman et al. 1991a, Thoefner et al. 2005, Danscher et al. 2010), which is a common finding in bovine digits after administration of oligofructose.

The histological changes scores indicated, in general, intensities from rare to discrete. In one of the studies that also performed histological evaluation of bovine receiving oligofructose (Thoefner et al. 2005), the changes were recorded only in terms of present or absent, without indication of intensity, which makes it difficult to compare with the results presented. In another study (Danscher et al. 2009), the authors used scores and compared them with control animals, which did not receive oligofructose, and the samples were collected in the phases without and with clinical signs of the disease, 24 and 72 hours post induction, respectively. In the initial phase, the only parameter where the induced calves presented significant difference in relation to the control was presence of leukocytes. In contrast, at 72 hours after induction, with evident clinical signs, a difference was observed in almost all parameters evaluated, indicating that in the initial phase of oligofructose induced laminitis the findings are still very discrete, accentuating with the appearance of the clinical signs. In the present study, it is likely that if induction were continued and a new harvest was performed after the appearance of clinical signs, higher scores for histological lesions would be observed.

The histological findings in the cases of laminitis are similar among the different studies, even considering differences of breeds, age of the animals, protocol of induction used and time of harvest (Boosman et al. 1991b, Singh et al. 1994, Thoefner et al. 2005, Danscher et al. 2010, Mendes et al. 2013). In the present study, crossbred animals (*Bos taurus* x *Bos indicus*) were used. It is possible that the same protocol of induction in zebu animals reveals a different result considering histological differences of the hoof (Mendonça et al. 2003, Rabelo et al. 2015) and metabolic differences in relation to ruminal acidosis (Ortolani et al. 2010).

The histological changes observed in the digital dermis, circulatory and inflammatory alterations, may be related to some triggers of ruminal acidosis, including histamine (Nocek 1997), lactic acid (Concha et al. 2014) and lipopolysaccharides (Boosman et al. 1991a, Zebeli & Metzler-Zebeli 2012). These components, when deposited more markedly, are responsible for changes in the microcirculation of the digit, such as vasodilation, opening of arteriovenous anastomosis, edema and thrombosis, and interfere with the migration and function of defense cells such as neutrophils (Boosman et al. 1991a, Nocek 1997, Greenough 2007, Zebeli & Metzler-Zebeli 2012). The observed basement membrane changes, areas of irregularity and detachment of the epidermis were possibly due to the action of proteases acting on components of the basal membrane, such as collagen fibers. Basal membrane alterations with activation of metalloproteinases were observed 12 hours after treatment with oligofructose in horses (Visser & Pollitt 2011, Visser & Pollitt 2012), being one of the probable mechanisms for the occurrence of the observed lesions. The morphological changes observed in the epidermal basal cells are probably due to failure of oxygen and nutrients, secondary to circulatory changes in the digits (Greenough 2007). Similar changes were observed in spontaneous and experimental cases of laminitis (Boosman et al. 1991a, Danscher et al. 2009, Mendes et al. 2013).

In parallel to the ruminal and metabolic alterations, microscopic changes were observed in the digits compatible with laminitis. The lack of lameness or digital sensitivity characterizes the period of evaluation as the prodromal stage of the disease. The results are similar to other protocols for induction of ruminal acidosis and laminitis, but have been studied only in taurine. In the prevention of laminitis associated with rumen acidosis in crossbred bovine (*Bos taurus* x *Bos indicus*), animals should be monitored not only for lameness, but also for signs of indigestion such as abdominal distension, diarrhea and apathy.

CONCLUSIONS

The intrarruminal administration of oligofructose in crossbred calves of one year as an experimental model for induction of ruminal acidosis and acute laminitis results, in the first 30 hours, in ruminal and metabolic acidosis and histological lesions with low degree of intensity in the digits.

During this period clinical signs such as lameness or digit sensitivity were not observed, characterizing the prodromal phase of laminitis.

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Original Article
Livestock Diseases

Antimicrobial susceptibility profile of historical and recent Brazilian pig isolates of *Pasteurella multocida*[1]

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ABSTRACT.- Amaral A.F., Rebelatto R., Klein C.S., Takeuti K.L., Oliveira Filho J.X., Morés N., Cardoso M.R.I. & Barcellos D.E.S.N. 2019. **Antimicrobial susceptibility profile of historical and recent Brazilian pig isolates of *Pasteurella multocida.*** *Pesquisa Veterinária Brasileira 39(2):107-111.* Department of Population Health and Pathobiology, College of Veterinary Medicine, North Caroline State University, 1060 William Moore Drive, Raleigh, NC 27607, USA. E-mail: afamaral@ncsu.edu

Pasteurella (*P.*) *multocida* is the causative agent of pneumonic pasteurellosis in swine, which is commonly associated with the final stages of enzootic pneumonia or porcine respiratory disease complex. Although this syndrome is one of the most common and important diseases of pigs, data on antimicrobial susceptibility of *P. multocida* isolates are uncommon in Brazil. Therefore, the present study was carried out to determine and to compare antimicrobial susceptibility profile of Brazilian *P. multocida* isolated from pigs with lesions of pneumonia or pleuritis during two-time periods. Historical isolates (period of 1981 to 1997; n=44) and recent isolates (period of 2011 to 2012; n=50) were used to determine the MIC of amoxicillin, enrofloxacin, florfenicol and tetracycline by microbroth dilution. Florfenicol had the lowest level of resistance for both historical and recent isolates (0% and 6%, respectively), while tetracycline had the highest (20.5% and 34%, respectively). Multi-drug resistance (MDR) to amoxicillin/florfenicol/tetracycline was observed in 6% of recent isolates. There was a significant increase ($p<0.05$) in resistance for amoxicillin and enrofloxacin in recent isolates compared with historic isolates (3.8% and 18%, respectively), most likely due to the selective pressure of antimicrobial usage to treat and prevent *P. multocida* infections. The results of this study showed an increase of isolates resistant to important drugs used in treatment of *P. multocida* infections in pigs, demonstrating the need for the implementation of rational use of antimicrobials in Brazilian swine industry.

INDEX TERMS: Antimicrobial susceptibility, Brazilian pig, *Pasteurella multocida*, minimum inhibitory concentration (MIC), microbroth dilution, resistance, swine, pigs, bacterioses.

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RESUMO.- [**Perfil de suscetibilidade a antimicrobianos de isolados históricos e contemporâneos de *Pasteurella multocida* de suínos no Brasil.**] *Pasteurella* (*P.*) *multocida* é o agente da pasteurelose pneumônica em suínos, a qual é comumente associada com o estágio final da pneumonia enzoótica suína ou complexo das doenças respiratórias dos suínos. Apesar de ser uma das doenças mais comuns e importantes na suinocultura, dados sobre suscetibilidade antimicrobiana de isolados de *P. multocida* são raros no Brasil. Dessa forma, o presente estudo foi realizado para determinar e comparar o perfil de suscetibilidade de isolados de *P. multocida* de suínos com lesões de pneumonia ou pleurite no Brasil durante dois períodos. Isolados históricos

(período de 1981 a 1997; n=44) e contemporâneos (período de 2011 a 2012; n=50) foram usados para determinar a concentração inibitória mínima (CIM) de amoxicilina, enrofloxacina, florfenicol e tetraciclina através do teste de microdiluição em caldo. Florfenicol apresentou o menor nível de resistência para ambos os isolados históricos e contemporâneos (0% e 6%, respectivamente), enquanto que tetraciclina apresentou o maior nível de resistência (20.5% e 34%, respectivamente). Resistência a múltiplos antimicrobianos (amoxicilina, florfenicol e tetraciclina) foi observada em 6% dos isolados recentes. Foi observado aumento significativo ($p<0.05$) na resistência a amoxicilina e enrofloxacina em isolados recentes comparado com isolados históricos (3.8% e 18%, respectivamente), provavelmente devido à pressão de seleção de antimicrobianos usados no tratamento e prevenção de infecções causadas por *P. multocida.* Os resultados deste trabalho demostraram o aumento de isolados resistentes a importantes drogas utilizadas no tratamento de infecções causadas por *P. multocida* em suínos, evidenciando a necessidade da implementação do uso racional de antimicrobianos na suinocultura brasileira.

TERMOS DE INDEXAÇÃO: Antimicrobianos, *Pasteurella multocida*, concentração inibitória mínima (CIM), microdiluição em caldo, resistência, suínos, bacterioses.

INTRODUCTION

Pasteurella (P.) multocida is the causative agent of pneumonic pasteurellosis in swine. It is commonly associated with the final stages of enzootic pneumonia or porcine respiratory disease complex (Register et al. 2012), wich is one of the most frequent and costly disease of pigs, since it has a negative impact on weigth gain and feed conversion (Noyes et al. 1990, Pijoan 2006).

Antimicrobials are still the first choice for prevention and control of *P. multocida* infections in swine. However, the use of antimicrobial agents can lead to increase of resistance (Schwarz et al. 2001). Monitoring antimicrobial susceptibility from *P. multocida* isolated from pigs over time provides valuable information about changes, which may be occurring in susceptibility patterns and is an important tool in effective antimicrobial therapy (Schwarz & Chaslus-Dancla 2001). Besides that, these data can serve as a decision guidance to choose the drug therapy and may help to recognize the emergence of new resistance phenotypes.

Amoxicillin, enrofloxacin, florfenicol and tetracycline are commonly used in Brazilian swine herds to prevent or to treat respiratory diseases, but data on antimicrobial susceptibility of *P. multocida* isolates are scant (Stepan et al. 1998, Borowski et al. 2002, Heres 2009, Mores et al. 2015). Moreover, to the best of our knowledge, there is no information on antimicrobial resistance profiles over time and only one study (Mores et al. 2015) about minimal inhibitory concentration (MIC) of *P. multocida* isolated in Brazil.

Therefore, this study was carried out to determine and to compare antimicrobial susceptibility profiles during two-time periods by determining the MIC of selected antimicrobial agents for a panel of *P. multocida* strains isolated from pigs with lesions of pneumonia or pleuritis in Brazil.

MATERIALS AND METHODS

A total of 94 *Pasteurella multocida* isolated from pig lesions of pneumonia or pleuritis in Brazil were tested. These isolates belonged to two groups: 1) historical isolates, wich comprised 44 isolates collected in the state of Rio Grande do Sul, Brazil, from 1981 to 1997; 2) recent isolates, wich included 50 isolates collected in six states of Brazil: Minas Gerais (21 isolates), Rio Grande do Sul (14 isolates), São Paulo (6 isolates), Santa Catarina (4 isolates), Mato Grosso (4 isolates) and Paraná (1 isolate) from 2011 to 2012. Historical isolates were preserved lyophilized and stored at 4-8°C, while recent isolates were preserved and stored in brain heart infusion (BHI) broth (Oxoid®, Cambridge, UK) containing 50% sheep blood at -70°C. Reactivation and preliminary tests for the confirmation of pure cultures of *P. multocida* were performed according to Markey et al. (2013) and Townsend et al. (2001) by microbiology and molecular assay, respectively.

MIC testing was performed by microbroth dilution method in accordance with criteria provided in the Clinical Laboratory Standards documents VET01-A4 (CLSI, 2013a) and VET-S2 (CLSI, 2013b). Isolates were tested against four antimicrobials at the listed dilutions: amoxicillin (0.0625-512µg/mL), enrofloxacin (0.00098-64µg/mL), florfenicol (0.125-32µg/mL) and tetracycline (0.0625-16µg/mL). *Staphylococcus aureus* (ATCC 29213) was used as reference strain for quality control. Prior to MIC testing, bacterial isolates were cultured on blood agar base (Oxoid®) supplemented with 5% sheep blood and incubated for 18-24h at 37°C. Each isolate was suspended in saline to obtain the 0.5 McFarland turbidity. From this suspention, 100µL was added to 9,900µL of Cation-adjusted Mueller-Hinton broth for plate inoculation. Inoculated plates were incubated for 24h at 35°C. The MIC for each isolate was determined as the lowest concentration of antimicrobial that prevented visible growth. All isolates were tested in triplicate. At least two out of three results had to be the same, if not, MIC testing was repeated. MICs were summarized and reported as susceptible (S), intermediate (I) and resistant (R) according to CLSI veterinary breakpoints (CLSI 2013b). This applies for the following antimicrobials (expressed as µg/mL in parentheses): amoxicillin (S≤0.5, I=1, R≥2), enrofloxacin (S≤0.25, I=0.5, R≥1), florfenicol (S≤2, I=4, R≥8) and tetracycline (S≤0.5, I=1, R≥2). As described in CLSI, ampicillin standards were used for interpreting data of amoxicillin.

Data were described considering values of MIC maximum, MIC minimum, MIC 50, MIC 90 and susceptibility profiles by groups of strains (historical or recent isolates). A non-parametric analysis was used to compare MIC values and susceptibility profiles between groups of strains and differences were determined using Fisher´s exact test (SAS, Version 9.4, 2012). Results were considered statistically significant if $p\leq0.05$.

RESULTS

The MICs of reference strain in each test run were within the CLSI acceptable quality control ranges. In the present study, MIC range of amoxicillin for *Staphylococcus aureus* ATCC 29213 was 1-4µg/mL. The MIC distribution, MIC range, MIC 50, MIC 90 and antimicrobial resistance profile of four antimicrobial for 94 isolates of *Pasteurella multocida* are summarized in Tables 1, 2 and 3.

Recent isolates had higher numeric MIC ranges than historical isolates for all antimicrobial tested. MIC 90 was also higher in recent isolates, except for tetracycline, that presented the same MIC 90 (4µg/mL). Florfenicol had the

Table 1. Percentage distribution (%) according to the minimum inhibitory concentration (MIC) of four antimicrobials for 44 historical (H) and 50 recent (R) *Pasteurella multocida* isolates

MIC (µg/mL)	Frequency (%) of isolates							
	AXC		ENR		FFC		TET	
	H	R	H	R	H	R	H	R
0.00098	-	-	2.3	2	-	-	-	-
0.00195	-	-	18.2	6	-	-	-	-
0.00391	-	-	52.3	10	-	-	-	-
0.00781	-	-	13.6	16	-	-	-	-
0.01562	-	-	9.1	6	-	-	-	-
0.03125	-	-	2.3	8	-	-	-	-
0.06250	0	10	2.3	10	-	-	6.8	2
0.125	4.5	26	0	6	0	2	25	18
0.25	6.8	14	0	2	9.1	8	25	14
0.5	20.5	10	0	16	81.8	68	20.5	22
1	47.7	18	0	16	6.8	12	2.3	10
2	11.4	4	0	0	2.3	4	6.8	14
4	9.1	14	0	0	0	0	13.6	16
8	0	0	0	0	0	0	0	2
16	0	0	0	0	0	4	0	2
32	0	0	0	0	0	2	-	-
64	0	0	0	2	-	-	-	-
128	0	0	-	-	-	-	-	-
256	0	0	-	-	-	-	-	-
512	0	2	-	-	-	-	-	-
>512	0	2	-	-	-	-	-	-
*P	0.0006		<0.0001		0.6696		0.4079	

AXC = amoxicillin, ENR = enrofloxacin, FFC = florfenicol, TET = tetracycline; *P = Fisher´s exact test descriptive level; differences were considered statistically significant when P≤0.05; - not tested at this antimicrobial concentration.

Table 2. Minimum inhibitory concentration (MIC) range, MIC 50 and MIC 90 of four antimicrobials for 44 historical (H) and 50 recent (R) *Pasteurella multocida* isolates

	MIC (µg/mL) of isolates							
	AMX		ENR		FFC		TET	
	H	R	H	R	H	R	H	R
MIC minimum	0.125	0.0625	0.00098	0.00098	0.25	0.125	0.0625	0.0625
MIC maximum	4	>512	0.06250	64	2	32	4	16
MIC 50	1	0.25	0.00391	0.0625	0.5	0.5	0.25	0.5
MIC 90	2	4	0.01562	1	0.5	1	4	4

AXC = amoxicillin, ENR = enrofloxacin, FFC = florfenicol, TET = tetracycline; MIC 50, MIC 90 = lowest concentration of antimicrobial agent capable of inhibiting the growth of 50% and 90% of isolates, respectively.

Table 3. Antimicrobial susceptibility profile of four antimicrobials for 44 historical (H) and 50 recent (R) *Pasteurella multocida* isolates

Susceptibility profile	Frequency (%) of isolates							
	AMX		ENR		FFC		TET	
	H	R	H	R	H	R	H	R
Resistant	18.2	22	0	18	0	6	20.5	34
Intermediate	47.7	18	0	16	0	0	2.3	10
Susceptible	34.1	60	100	66	100	94	77.3	56
*P	0.0080		<0.0001		0.2450		0.0735	

AXC = amoxicillin, ENR = enrofloxacin, FFC = florfenicol, TET = tetracycline; *P = Fisher´s exact test descriptive level; differences were considered statistically significant when P ≤0.05. Based on CLSI (2013b) standards.

lowest level of resistance for both historical and recent isolates (0% and 6%, respectively), while tetracycline had the highest (20.5% and 34%, respectively).

In historical isolates, 4.5% of the isolates showed simultaneous resistance to amoxicillin and tetracycline, while in recent isolates 16% of the isolates showed simultaneous resistance to amoxicillin/tetracycline (4%), amoxicillin/enrofloxacin (4%), and enrofloxacin/tetracycline (8%). Multi-drug resistance (MDR) to amoxicillin/florfenicol/tetracycline was observed in 6% of recent isolates, while no historical isolates presented MDR.

Susceptibility profiles of historical and recent *P. multocida* isolates were significantly different ($p<0.05$) only for amoxicillin and enrofloxacin, wich had an increase in the resistance of 3.8% and 18% in recent isolates respectively.

DISCUSSION

In Brazil, the use of antimicrobial to treat respiratory diseases is a common practice and can be reflecting in the increase of antimicrobial resistance observed overtime. Amoxicillin has been used in Brazil since 1995 and is the most commonly antimicrobial used via feed or water as a herd medication for prophylaxis of pulmonary swine pasteurellosis. Since the exposure of a bacteria to antimicrobial agents can lead to increase of resistance (Bywater 2004), the frequent use of amoxicillin may reflect on the antimicrobial susceptibility profile of *Pasteurella multocida*, as it was observed in our work an increase in level of resistance (18.2% historical vs. 22% recent isolates) and MIC90 (2μg/mL historical vs. 4μg/mL recent isolates). This result is consistent with the findings of another study that investigated the susceptible level of *P. multocida* isolates from pigs in the main swine production areas in Brazil. In that study, Mores et al. (2015) found the resistance level to amoxicillin to be 20%. On the contrary, Jong et al. (2014) observed a much lower level of resistance and MIC90 (1.3% and 0.25μg/mL respectively) in isolates collected in 11 European countries during 2002-2006.

Enrofloxacin was introduced to pig use in Brazil in 1988 and is widely used to treat pulmonary pasteurellosis. Once more, the selective pressure of the antimicrobial may explain the increase in level of resistance (0% historical vs. 18% recent isolates) and MIC90 (0.01562 vs. 1μg/mL) observed in the present study. Different from our study, Nedbalcová & Kučerová (2013) found lower level of resistance and MIC90 of enrofloxacin (1.5%; ≤0.12μg/mL, respectively) in the Czech Republic. The same author performed a further study (Nedbalcová et al. 2014) to observe the relationship between antimicrobial resistance and its sales. It was observed a logical link among consumptions and resistance patterns. Nevertheless, overall national sales data have limitations as they do not express real exposure of the animals to antimicrobials.

Florfenicol was introduced in Brazilian pig industry in 1999. In this study, this drug had the lowest level of resistance for both historical and recent isolates (0% and 6%, respectively) and a low MIC90 (0.5μg/mL and 1μg/mL respectively). This may indicate a high efficiency of the drug and/or a difficulty to develop resistance to it. Similar results were found elsewhere, such as a much larger study conducted in North America (United States and Canada) with 2.389 *Pasteurella multocida* isolates collected during 2001-2010 (Portis et al. 2013) in wich the level of florfenicol resistance was lower than 1%, while the MIC90 was 0.5μg/mL. Another study in Korea also showed a high activity of florfenicol (Shin et al. 2005). In that study, all isolates were susceptible and had a MIC ranging from 0.25 to 0.5μg/mL, and MIC90≤0.5μg/mL. According to the authors, despite florfenicol has been licensed in Korea for treatment of porcine respiratory infections in 1999, it has not been previously used.

Despite tetracycline therapeutics had a long story of use in Brazil (since 1960), its use has been decreasing since the 90´s because of high levels of resistance in Brazilian pig isolates of *Pasteurella multocida* (Stepan et al. 1998). More recently, the use has been influenced by the need to comply with export requirements of some of Brazilian pork importers requiring the ban of its use. Furthermore, since tetracycline resistance is widespread (Karriker et al. 2012) and its genes can be located in plasmid (Kehrenberg et al. 2001), resistant strains may still be present in the *P. multocida* population. This could explain the fact that no significant difference ($p>0.05$) was found in susceptibility profiles between strains isolated in different periods, with MIC90 of 4μg/mL in both historical and recent isolates. In Australia, a similar level of resistance and MIC90 (28%; 2μg/mL, respectively) against tetracycline were reported in isolates collected from 2002 to 2013 (Dayao et al. 2014).

In the present study, MDR was defined as resistance to three or more antimicrobial classes and 6% of recent isolates showed MDR. *Pasteurella multocida* isolates from pigs in Austria from 2002 to 2013 also showed MDR in 4.8% of their isolates (Dayao et al. 2014). On the other hand, our results showed that none of the historical samples presented MRD, demonstrating once more that exposure to antimicrobial agents over time can lead to increase of bacteria resistance and MDR.

Since the development of new antimicrobial agents is very expensive and time consuming, every effort must be undertaken to retain the efficacy of substances currently available for veterinary use (Boerlin & White 2013). Furthermore, it is not possible to stop resistance development, but it is possible to slow down the selection and spread of resistance (Schwarz & Chaslus-Dancla 2001). This underlines the importance of responsible use of antimicrobials when treating *P. multocida* infections and the need to create national monitoring programs as those already present in other countries like Germany (GERM-Vet), France (Résapath), Czech Republic (Control of Veterinary Biologicals and Medicaments - ISCVBM) and Europe (VetPath) to investigate quantitatively the in vitro susceptibility and assist veterinarians in the selection of the most suitable antibiotic and its correct dose.

CONCLUSION

Based on the MIC breakpoints obtained there was an increase of resistance in recent isolates for all antimicrobial tested, indicating an increase of resistance over time, most likely caused by the selective pressure of antimicrobial usage to treat and prevent *Pasteurella multocida* infections in pig farms.

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Original Article
Livestock Diseases

Efficacy of virginiamycin for the control of periodontal disease in calves[1]

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ABSTRACT.- Ramos T.N.M., Borsanelli A.C., Saraiva J.R., Vaccari J., Schweitzer C.M., Gaetti-Jjardim Jr. E. & Dutra I.S. 2019. **Efficacy of virginiamycin for the control of periodontal disease in calves.** *Pesquisa Veterinária Brasileira 39(2):112-122*. Departamento de Apoio, Produção e Saúde Animal, Faculdade de Medicina Veterinária de Araçatuba, Universidade Estadual Paulista, Rua Clóvis Pestana 793, Cx. Postal 533, Jardim Dona Amélia, Araçatuba, SP 16050-680, Brazil. E-mail: iveraldo.dutra@unesp.br

Periodontal diseases are multifactorial infectious processes caused by complexes of microorganisms, with damage to health, production, and animal welfare. The aim of the present study was to evaluate the efficacy of virginiamycin in the prevention and control of two early forms of periodontal disease: gingivitis and necrotizing gingivitis. Ten weaned calves, aged four to six months, were permanently kept in a single lot under the same rotational grazing regime in a newly reformed area of *Panicum maximum*. Five of the calves were orally administered 340mg of virginiamycin (Virginiamycin Group) daily for a period of 18 weeks, while the remaining five calves (Control Group) remained under the same food management but did not receive virginiamycin. During this period, animals underwent 18 weekly evaluations regarding periodontal health, with monitoring and recording of clinical parameters of the eight deciduous incisor teeth on the labial and lingual faces. At approximately two-week intervals, nine collections of subgingival sulcus material from five sites of the four right incisor teeth of each animal were performed and subjected to microbiological evaluation using polymerase chain reaction with primers of 25 microorganisms considered potentially pathogenic. After 1440 periodontal clinical evaluations of incisor teeth of the 10 calves, a total of 395 episodes of gingivitis were recorded, of which 267 occurred in the Control Group and 128 in the Virginiamycin Group. Similarly, 89 episodes of necrotizing gingivitis were recorded; 58 in the Control Group and 31 in the Virginiamycin Group. Comparison of between-group means found significant differences for teeth with gingivitis and necrotizing gingivitis (t test; $p<0.05$). The total number of teeth with gingivitis ($p<0.01$) and necrotizing gingivitis ($p<0.01$) in Control Group was significantly higher than that of gingivitis ($p<0.01$) and necrotizing gingivitis ($p<0.05$) in the Virginiamycin Group. There was a positive correlation between total occurrence of gingivitis and necrotizing gingivitis in the Virginiamycin Group by Pearson's test. Virginiamycin had a protective effect on treated animals compared with the Control Group (OR = 0.36: CI (95%) = 0.27-0.43). In the Control Group, *Actinomyces israelli* (4.74%), domain *Archaea* (1.58%), *Eikenella corrodens* (1.05%), *Fusobacterium nucleatum* (27.37%), class *Mollicutes* (5.26%); *Porphyromonas endodontalis* (5.26%); *Porphyromonas gulae* (0.53%),

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Prevotella buccae (6.32%), *Prevotella loescheii* (3.68%), *Prevotella nigrescens* (8.42%), *Prevotella oralis* (1.58%), *Tannerella forsythia* (0.53%), and *Treponema denticola* (4.21%) were detected at healthy sites, and gingivitis or necrotizing gingivitis samples. In the Virginiamycin Group, *A. israelli* (3.41%), domain *Archaea* (0.98%), *F. nucleatum* (9.27%), class *Mollicutes* (4.39%), *P. endodontalis* (4.39%), *P. gulae* (0.49%), *P. buccae* (8.29%), *P. loescheii* (6.83%), *P. nigrescens* (15.61%), *P. oralis* (1.46%), *Selenomonas sputigena* (0.49%), *T. forsythia* (0.49%), and *T. denticola* (2.44%) were detected. In conclusion, virginiamycin administered at a dosage of 340mg/animal/day significantly reduced the occurrence of gingivitis and necrotizing gingivitis in cattle maintained on reformed pastures, and was revealed to have action against periodontal bacterial microbiota considered to be potentially pathogenic.

INDEX TERMS: Virginiamycin, control, periodontal disease, gingivitis, necrotizing gingivitis, ruminants, cattle, microbiology, clinics.

RESUMO.- [**Eficácia da virginiamicina no controle de doença periodontal em bezerros.**] As doenças periodontais são processos infecciosos multifatoriais causados por complexos de micro-organismos, que provocam danos à saúde, produção e ao bem-estar animal. O objetivo do presente estudo foi o de avaliar a eficácia da virginiamicina na prevenção e controle de duas formas de doença periodontal; a gengivite e a gengivite necrosante. Assim, dez bezerros desmamados, com idade entre 4 e 6 meses, foram mantidos permanentemente em lote único e sob o mesmo regime de pastejo rotacionado em área reformada de *Panicum maximum.* Cinco bezerros receberam via oral 340mg de virginiamicina (Grupo Virginiamicina) diariamente, por um período de dezoito semanas, enquanto o Grupo Controle permaneceu sob o mesmo manejo alimentar, mas sem receber a virginiamicina. No período, os animais passaram por 18 avaliações semanais quanto à saúde periodontal, com monitoramento e registro dos parâmetros clínicos dos oito dentes incisivos decíduos, nas suas faces labial e lingual. Em intervalos aproximadamente quinzenais foram realizadas nove coletas de material do sulco subgengival de cinco sítios de quatro dentes incisivos direitos de cada animal para avaliação microbiológica, com o emprego da reação em cadeia da polimerase e com iniciadores de 25 micro-organismos considerados potencialmente patogênicos. Ao final das 1440 avaliações clínicas periodontais dos dentes incisivos dos dez bezerros, pôde-se registrar um total de 395 episódios de dentes com gengivite, nos quais 267 foram registrados no Grupo Controle e 128 no Grupo Virginiamicina. De forma semelhante, do total de 89 registros de gengivite necrosante, 58 foram no Grupo Controle e 31 no Grupo Virginiamicina. Na comparação entre médias dos grupos as diferenças encontradas para dentes com gengivite e gengivite necrosante foram significativas pelo teste t ($p<0,05$). Assim, o total de dentes com gengivite ($p<0,01$) e gengivite necrosante ($p<0,01$) no Grupo Controle, foi significativamente superior ao de gengivite ($p<0,01$) e gengivite necrosante ($p<0,05$) do Grupo Virginiamicina. Houve correlação positiva entre o total de ocorrência de gengivite e gengivite necrosante no Grupo Virginiamicina pelo teste de Pearson. A virginiamicina possuiu um efeito protetor nos animais tratados em comparação com o controle (OR = 0,36: IC (95%) = 0,27-0,43). Na avaliação microbiológica do Grupo Controle foram detectados nas amostras de sítios sadios, com gengivite ou com gengivite necrosante *Actinomyces israelli* (4,74%), domínio *Archaea* (1,58%), *Eikenella corrodens* (1,05%), *Fusobacterium nucleatum* (27,37%), classe *Mollicutes* (5,26%), *Porphyromonas endodontalis* (5,26%), *Porphyromonas gulae* (0,53%), *Prevotella buccae* (6,32%), *Prevotella loescheii* (3,68%), *Prevotella nigrescens* (8,42%), *Prevotella oralis* (1,58%), *Tannerella forsythia* (0,53%) e *Treponema denticola* (4,21%). Enquanto no Grupo Virginiamicina foram detectados: *A. israelli* (3,41%), domínio *Archaea* (0,98%), *F. nucleatum* (9,27%), classe *Mollicutes* (4,39%), *P. endodontalis* (4,39%), *P. gulae* (0,49%), *P. buccae* (8,29%), *P. loescheii* (6,83%), *P. nigrescens* (15,61%), *P. oralis* (1,46%), *Selenomonas sputigena* (0,49%), *T. forsythia* (0,49%) e *T. denticola* (2,44%). Em conclusão, a virginiamicina administrada na dosagem de 340mg/animal/dia reduziu significativamente a ocorrência da gengivite e gengivite necrosante em bovinos mantidos em pastos reformados e revelou ter ação frente à microbiota bacteriana periodontal considerada potencialmente patogênica.

TERMOS DE INDEXAÇÃO: Virginiamicina, controle, doença periodontal, gengivite, gengivite necrosante, ruminantes, bovinos, microbiologia, clínica.

INTRODUCTION

Periodontal diseases are a group of diseases that affect the tissues associated with protection and support of teeth. Among the reversible forms of periodontal disease are gingivitis and necrotizing gingivitis, which are caused predominantly by aggression of gingival biofilm (Konradsson et al. 2007, Kistler et al. 2013).

Untreated gingivitis can progress to periodontitis, with consequent compromise of periodontal ligaments and alveolar bone, culminating in tooth loss (Page 1986, Lyon 2005, Kinane & Bartold 2007, Herrera et al. 2014). This way, the health of the periodontium depends on the balance between bacterial composition of dental biofilm and its interaction with the host immune system (Hajishengallis 2015).

In cattle, gingivitis has been reported in calves aged 5 to 60 days old, characterized as a physiological manifestation resulting from eruption of teeth (Döbereiner et al. 1974). Additionally, necrotizing gingivitis has been described in cattle with leukocyte adhesion deficiency (Nagahata et al. 1993). However, in this animal species, little is known about gum diseases, most likely due to the difficulties in evaluating the oral cavity of the animals and by the fact that these diseases do not present evident clinical changes as seen in periodontitis. However, in humans and adolescents, these diseases have been well described (Kiran et al. 2011, Marshall et al. 2014).

In cases of gingivitis, the gingival border is noted to be intensely red and edematous, and bleeding, either spontaneous or with probing, may be observed and in more severe

cases, the gingiva may have ulcerations (Diehl & Rosychuk 1993, Lyon 2005, Riggio et al. 2011, Newman et al. 2012, Antiabong et al. 2013, Kutasi et al. 2016). In cases of necrotizing gingivitis, spontaneous bleeding or bleeding after probing as well as the presence of a layer of yellowish-white or grayish-white fibrin on the necrotic gingival border may be observed (Klokkevold 2012, Rodríguez-Pulido et al. 2016).

Periodontal diseases occur by the modification of the microbiota and constituents of the oral cavity; in humans, several modifying factors have been associated with the occurrence of gingivitis and necrotizing gingivitis, including hormonal changes and immunodeficiencies (Stamm 1986, Rowland 1999, Dufty et al. 2016). However, in cattle, modifying factors associated with the occurrence of periodontal diseases are unknown and the suspicions are related to soil management and diet (Dutra et al. 1993, Döbereiner et al. 2000).

In a recent study, oral microbiomes of healthy cattle and those with periodontitis revealed 72.1% dissimilarity; however, the diversity of bacteria found in healthy and diseased sites was similar, with a predominance of the genera *Prevotella, Fusobacterium,* and *Porphyromonas* in periodontal lesions (Borsanelli et al. 2018). Thereby, it can be evidenced that bovine oral microbiota is rich and diversified, composed of 395 genera or higher taxa and that microorganisms considered as potential periodontal pathogens are associated with the occurrence of periodontitis in these animals. These include several species of *Porphyromonas*, *Prevotella,* and *Treponema*, as well as *Fusobacterium nucleatum*, *Fusobacterium necrophorum,* and *Actinomyces naeslundii* (Dutra et al. 2000, Borsanelli et al. 2015a, 2015b, Borsanelli 2017).

In previous studies, virginiamycin has been shown to be efficient for recovery of calves with periodontitis (Tims et al. 1992), as well as periodontitis prevention (Dutra et al. 1993). In this context, the present study aimed to evaluate the efficacy of virginiamycin for the control of gingivitis and necrotizing gingivitis in cattle, in view of the need to develop strategies for the control of periodontal diseases in production animals and characteristics of the antibiotic.

MATERIALS AND METHODS

Animals. Ten male Jersey or Jersey cross calves, weaned and healthy, aged four to six months, were used. They were weighed and randomized into two groups of five animals each. The animals remained under rotational grazing and even single-plot zootechnical management in 24 paddocks (approximately 3 hectares) that had previously been reformed in order to simulate the potential representative situation for the occurrence of periodontal disease in cattle. Mixed pastures of Massai grasses (*Panicum maximum cv.* Massai) and Mombasa (*Panicum maximum cv.* Mombasa) were reformed following conventional farming practices such as soil analysis, liming, and fertilization. Water and mineral salt were supplied *ad libitum* over the total period of approximately 24 weeks of experimentation, including adaptation of the animals to the diet. Five animals (Virginiamycin Group) received daily, oral (*pour dressing*) administration of virginiamycin (340mg/day/animal) for 18 consecutive weeks while the other five (Control Group) did not receive the antibiotic.

General and periodontal clinical evaluation. At the beginning of the experiment, the animals had good body condition scores for their age and were apparently healthy. In the initial oral cavity clinical examination, they had deciduous dentition, with dental units (tooth and periodontium) normal. Weekly periodontal clinical evaluation of the incisor teeth was performed for 18 consecutive weeks, starting after four weeks of diet adaptation and management and coinciding with virginiamycin administration. For the examination, the calves were physically restraint in the trunk, and periodontal status was evaluated with the aid of a mouth opener and flashlight and recorded in individual odontograms. Although the visible clinical condition of the dental arch of the animals was examined, only the lingual and labial faces of the incisor teeth were evaluated in the present study, avoiding any means of chemical containment that could interfere in the objective of the research. The criteria for periodontal clinical examination were based on those used by Löe (1967). Gingivitis was characterized by presence of edema in the gingival border, appearance, color, spontaneous bleeding or bleeding associated with probing. For cases of necrotizing gingivitis, we sought to visualize the presence of ulcerations in the gingival margin, with or without the presence of a white-grayish/yellowish-white pseudomembrane and manipulation pain.

Collection of material for microbiological examinations. Samples were collected every two weeks throughout the study. After removal of food residues, drying of saliva, and removal of supragingival biofilm with sterile gauze, biofilm was collected from the gingival sulcus of four incisors, of the following teeth: 401 on the labial and lingual face, 402, 403, and 404. Materials from curettage (Gracey curette) or paper cone were packed into cryotubes with ultrapure water and stored at -80°C until DNA extraction.

Extraction of microbial DNA. Each sample for bacterial DNA detection in sterile ultrapure water was extracted by boiling. First, the sample was homogenized for 20 seconds; 500µl aliquots were then removed and stored in Eppendorf tubes. The samples were then boiled for 15 minutes, centrifuged at 10.36×g for 5 minutes and aliquots of 400µl. The samples were then stored at -80°C until the amplification reaction of the DNA was carried out by the polymerase chain reaction (PCR).

Detection of microorganisms by conventional PCR. Specific primers and amplification conditions were employed for the main microbial groups associated with oral biofilm gingivitis and necrotizing gingivitis in cattle and in other animal species:: *Actinomyces israelli, Actinomyces naeslundii, Archaea* domain, *Eikenella corrodens, Campylobacter spp., Fusobacterium nucleatum, Fusobacterium necrophorum,* class *Mollicutes, Parvimonas micra, Porphyromonas asaccharolytica, Porphyromonas endodontalis, Porphyromonas gingivalis, Porphyromonas gulae, Prevotella buccae, Prevotella loescheii, Prevotella intermedia, Prevotella melaninogenica, Prevotella nigrescens, Prevotella oralis, Selenomonas sputigena, Tannerella forsythia, Treponema amylovorum, Treponema denticola, Treponema maltophilum,* and *Treponema pectinovorum* (Ashimoto et al. 1996, Tran et al. 1997, Willis et al. 1999, Fouad et al. 2002, Mayanagi et al. 2004, Hardham et al. 2005, Yoshida et al. 2005, Stevenson et al. 2007, Aroutcheva et al. 2008, Gaetti-Jardim Junior et al. 2012, Nadkarni et al. 2012, Xia & Baumgartner 2003, Cogulu et al. 2008, Lopes Neto 2007). The specificity of these primers is demonstrated in the above-mentioned literature and can be evaluated throught the National Center for Biotechnology Information databases (NCBI).

Amplifications of target DNA by PCR were performed in 25µl volumes containing 1X PCR / Mg + 2 buffer (Boehring Mannheim, Indianapolis, IN, USA), 0.2µl of each dNTP (Pharmacia Biotech, Piscataway, NJ, USA), 0.5U Taq DNA polymerase (Invitrogen do Brasil, São Paulo/SP, Brazil), 0.4µl of each primer pair (Invitrogen), and 10ng template. The amplifications were performed in a thermocycler (Perkin Elmer, GeneAmp PCR System 9700, Norwalk/CT, USA) programmed at 94°C (5 minutes), 30 to 40 cycles at 94°C (30 to 60 seconds,

according to the primers used), specific annealing temperature for each primer pair, 72°C (30 to 60 seconds), followed by a 5 minute period at 72°C for the final extension of the amplified DNA strands. The PCR amplification products PCR were subjected to agarose gel electrophoresis in 1% TBE buffer (1M Tris, 0.9M boric acid, 0.01M EDTA, pH 8.4), stained with ethidium bromide (0.5mg/ml) and photographed in ultraviolet light transilluminator (UV Light Transilluminator; Eastman Kodak Co., NY, USA).

DNA samples of reference strains were used for control of detection procedures, as well as clinical samples positive for the target microorganisms (Gaetti-Jardim Junior et al. 2012). Ultrapure water was used as a negative control.

Statistical analysis. The means of the cases with gingivitis and necrotizing gingivitis were compared between the Virginiamycin Group and the Control Group using Student's t test and Pearson's test, with a significance level of $p < 0.05$. For the statistics regarding the detection of microorganisms, a comparison was made between the dichotomous variables, in which the presence of microorganisms between the Virginiamycin and Control Groups was compared; the correlation between the microorganisms, and their relation to sites with periodontal or healthy lesions, was evaluated using the chi-Square test of maximum-likelihood, with a significance level of $p<0.05$. Odds ratios (OR) and confidence intervals (CI) for the OR were calculated to verify whether the use of virginiamycin would be a protective factor against gingivitis and necrotizing gingivitis.

Research ethics commission. The experiment was approved by the Ethics Committee on Animal Use (CEUA) of the Faculty of Agrarian and Veterinary Sciences - Unesp, Campus Jaboticabal/SP (Process FCAV/Unesp No. 15.207/16).

RESULTS

At the end of 18 weeks, the animals that did not receive virginiamycin had unsatisfactory body condition scores. Furthermore, at different moments of the study, the control animals presented symptoms of diarrhea and nasal secretion, and showed an apparent increase in susceptibility to intercurrent diseases such as endoparasitoses. In contrast, among the five animals in the Virginiamycin Group, only one had episodes of diarrhea. The mean weight of calves receiving virginiamycin (188.2kg) was significantly higher ($p=0.02$) than that observed in the Control Group (123.5kg).

Periodontal evaluation of incisor teeth of the calves in the Control and Virginiamycin Groups kept in a single lot, under rotational grazing in a newly reformed area and even feeding management, revealed episodes of gingivitis and same necrotizing gingivitis, initial forms for progression of periodontitis in the animals of the two experimental groups.

During the study period, according to the weekly clinical examination of the eight deciduous teeth (labial and lingual faces), 395 episodes of teeth (dental unit) with gingivitis were recorded in the 1440 evaluations performed. Of these episodes, 267 occurred in Control Group calves and 128 in the Virginiamycin Group (Fig.1). In the same evaluation, of a total of 89 records of teeth with necrotizing gingivitis, 58 occurred in the Control Group and 31 in the Virginiamycin Group (Fig.2). The clinical manifestations of gingivitis included changes in the color of the gingival mucosa, ranging from moderate to severe red, edema, and bleeding (spontaneous or with probing). In necrotizing gingivitis, the marginal gingival epithelium presented necrotic ulcerations or epithelial areas covered with a white-gray/yellowish-white membranous layer, and bleeding (spontaneous or with probing) (Fig.3 and Fig.4).

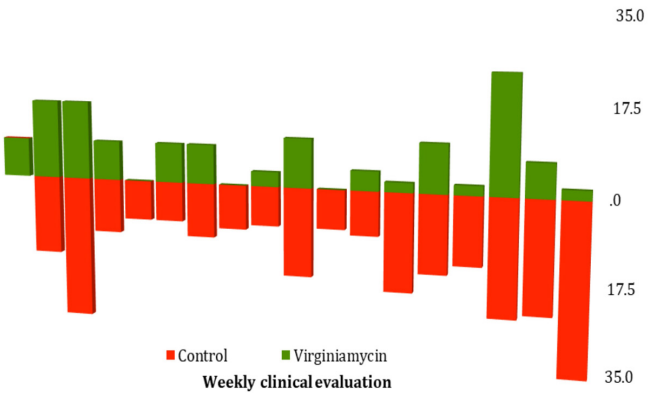

Fig.1. Number of deciduous teeth with gingivitis in calves in the Virginiamycin Group (n=5) and Control Group (n=5) after 18 evaluations in 4 and a half months, totaling 1440 evaluations and 395 episodes of gingivitis. The Virginiamycin Group had 128 episodes of gingivitis, while the Control Group presented 267 episodes. This difference was statistically significant ($p<0.01$).

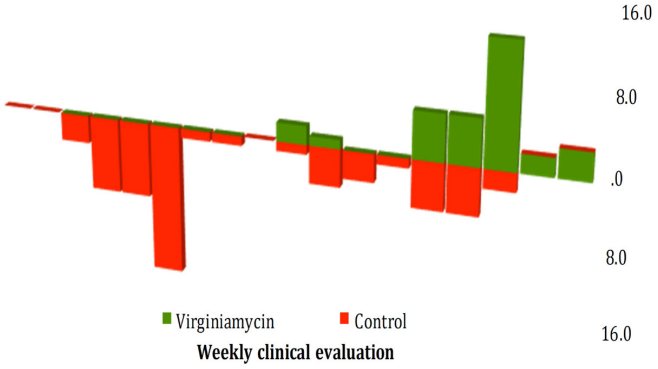

Fig.2. Number of deciduous incisor teeth with necrotizing gingivitis in calves in the Virginiamycin Group (n=5) and Control Group (n=5) after 18 clinical evaluations in 4 and a half months, totaling 1440 evaluations and 89 episodes of necrotizing gingivitis. The Virginiamycin Group had 31 episodes while the Control Group had 58 episodes. This difference was statistically significant ($P<0.05$).

In the comparison of the means of the experimental groups, the differences found for teeth (dental unit) with gingivitis and necrotizing gingivitis were significant according to the t test ($p<0.05$). Thus, the total number of teeth with gingivitis ($p<0.01$) and necrotizing gingivitis ($p<0.01$) in the Control Group was significantly higher than the total number of gingivitis ($p<0.01$) and necrotizing gingivitis ($p<0.05$) in the Virginiamycin Group. There was a positive correlation between the total occurrence of gingivitis and necrotizing gingivitis in the Virginiamycin Group according to Pearson's test. Virginiamycin was considered a protective factor against the development of gingivitis and necrotizing gingivitis (OR = 0.36; CI (95%) = 0.27-0.43).

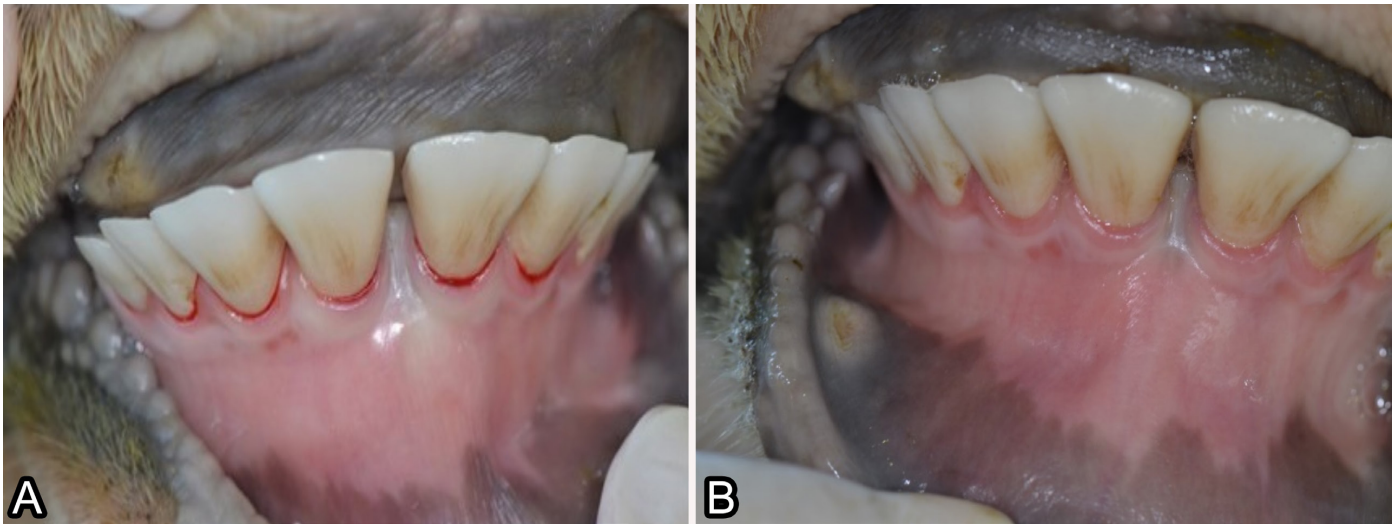

Fig.3. (**A**,**B**) Incisor teeth of calves with gingivitis, showing edema in the gingival margin of the first, second and third incisors, alteration of coloration in the second and third incisors, as well as (**A**) bleeding in first, second and third incisors after probing.

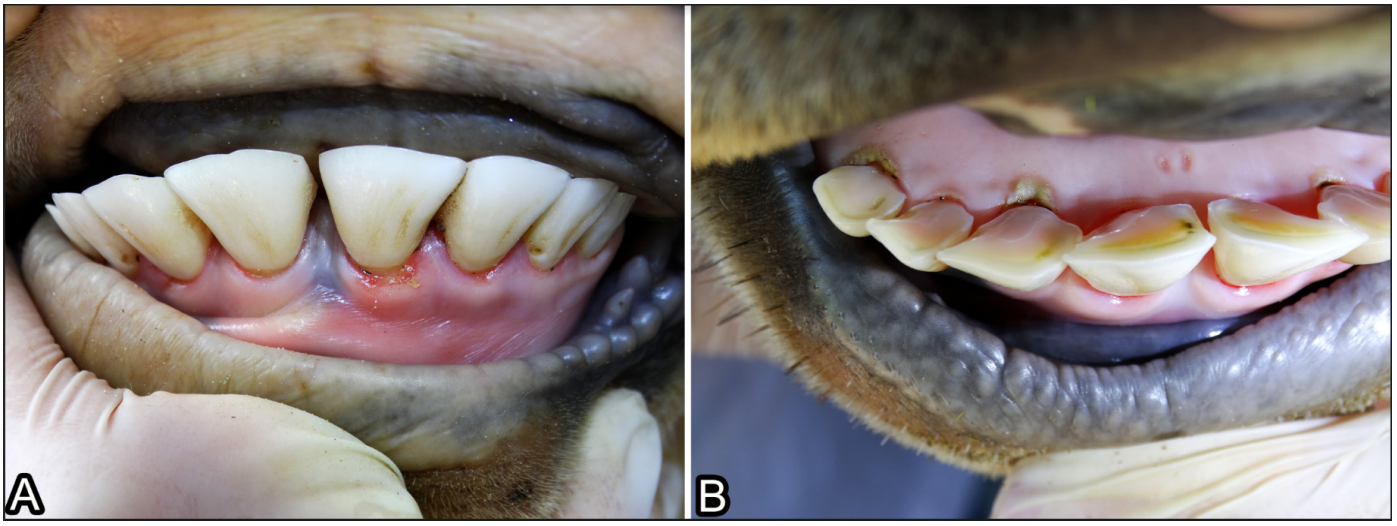

Fig.4. (**A**) Gingival edge ulceration on tooth 301 compatible with clinical signs of necrotizing gingivitis. (**B**) Deciduous incisor teeth of calves presenting necrotizing gingivitis on tooth 402 and 404 right on the lingual surface.

In the microbiological evaluation, 395 samples were evaluated by PCR (Table 1) to detect the presence of 25 microorganisms considered indicators and with pathogenic potential. The sites with the lowest number of microorganisms detected were those associated with necrotizing gingivitis (Table 2 and 3). The amount of microorganism samples at the different sites collected differed among the animals and between the groups (Fig.5). In addition, there was a difference in the types of microorganisms detected between the sample collections.

Fusobacterium nucleatum, class *Mollicutes*, *Porphyromonas endodontalis*, *Prevotella loescheii*, *Prevotella nigrescens*, *Prevotella oralis*, and *Treponema denticola* were detected in five or more collections, whereas *Actinomyces israelli*, *Archaea* domain, *Eikenella corrodens*, *Porphyromonas gulae*, *Tannerella forsythia,* and *Selenomonas sputigena* were rarer. *Prevotella buccae* was detected in all collections. *Actinomyces naeslundii*, *Campylobacter* spp., *Fusobacterium necrophorum*, *Parvimonas micra*, *Porphyromonas assacharolytica*, *P. gingivalis*, *Prevotella intermedia, P. melaninogenica, Treponema amylovorum*, *T. maltophilum,* and *T. pectinovorum* were not identified in either of the two groups. *S. sputigena* was negative in the Control Group animals and *E. corrodens* was negative in the Virginiamycin Group.

In the analysis of the variables using the chi-square test of maximum-likelihood, there was a significant difference in the frequency of detected microorganisms ($p<0.05$). The presence of *Fusobacterium nucleatum* ($p<0.01$) was more significant in Control Group, while *Prevotella nigrescens* ($p<0.04$) and other bacteria of the genus *Prevotella* ($p<0.02$) was more frequent in the Virginiamycin Group. *Prevotella loescheii* showed association with *Prevotella buccae* ($p<0.01$) and *P. nigrescens* ($p<0.01$); *P. buccae* was associated with *P. nigrescens* ($p<0.01$) and

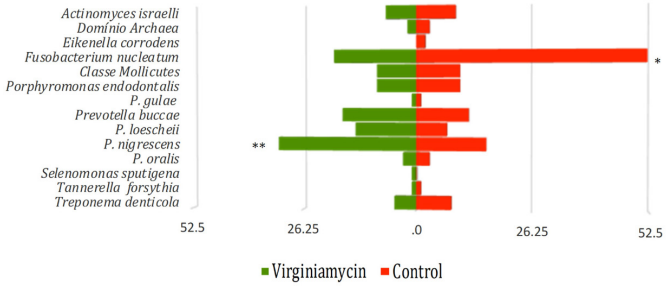

Fig.5. Number of microorganisms detected in the 395 samples collected from four deciduous teeth of each animal from the Virginiamycin Group (n=5) and Control Group (n=5). * P<0.01, ** P<0.05.

Table 1. Microorganisms detected by polymerase chain reaction (PCR) in the samples of 395 subgengival sulcus sites of 10 calves of groups Control (n=190) and Virginiamycin (n=205)

Microorganism	Control group	Virginiamycin group	*P*
	N(%)	N(%)	
Actinomyces israelli	9 (4.7)	7 (3.4)	
Archaea domain	3 (1.6)	2 (1.0)	
Eikenella corrodens	2 (1.1)	0 (0.0)	
Fusobacterium nucleatum	52 (27.4)	19 (9.3)	<0.01*
Mollicutes class	10 (5.3)	9 (4.4)	
Porphyromonas endodontalis	10 (5.3)	9 (4.4)	
P. gulae	1 (0.5)	1 (0.5)	
Prevotella buccae	12 (6.3)	17 (8.3)	
P. loescheii	7 (3.7)	14 (6.8)	
P. nigrescens	16 (8.4)	32 (15.6)	<0.05*
P. oralis	3 (1.6)	3 (1.5)	
Selenomonas Sputigena	0 (0.0)	1 (0.5)	
Tannerella forsythia	1 (0.5)	1 (0.5)	
Treponema denticola	8 (4.2)	5 (2.4)	

*Significant values of *P* by the Chi-square test of M-L.

Table 2. Microorganisms identified by PCR in four incisor healthy teeth (n=119) with gingivitis (n=63) or necrotizing gingivitis (n=8) of five calves in the Control Group

Microorganism	Healthy	Gingivitis	Necrotizing gingivitis
	N(%)	N(%)	N(%)
Actinomyces israelli	6 (5.0)	3 (4.8)	0 (0.0)
Archaea domain	3 (2.5)	0 (0.0)	0 (0.0)
Eikenella corrodens	1 (1.0)	1 (1.6)	0 (0.0)
Fusobacterium nucleatum	31 (26.1)	19 (30.2)	2 (25.0)
Mollicutes class	6 (5.0)	4 (6.4)	0 (0.0)
Porphyromonas endodontalis	9 (7.6)	1 (1.6)	0 (0.0)
P. gulae	1 (1.0)	0 (0.0)	0 (0.0)
Prevotella buccae	8 (6.7)	3 (4.8)	1 (12.5)
P. loescheii	4 (3.4)	1 (1.6)	2 (25.0)
P. nigrescens	13 (11.0)	1 (1.6)	2 (25.0)
P. oralis	1 (1.0)	2 (3.2)	0 (0.0)
Tannerella forsythia	0 (0.0)	1 (1.6)	0 (0.0)
Treponema denticola	4 (3.4)	4 (6.4)	0 (0.0)

Table 3. Microorganisms identified by PCR in samples of four healthy incisor teeth (n=156), with gingivitis (n=42) or necrotizing gingivitis (n=7) of five calves of the Virginiamycin Group

Microorganims	Healthy	Gingivitis	Necrotizing gengivitis
	N(%)	N(%)	N(%)
Actinomyces israelli	5 (3.2)	2 (4.8)	0 (0.0)
Archaea domain	1 (0.6)	0 (0.0)	1 (14.3)
Fusobacterium nucleatum	14 (8.9)	5 (12.0)	0 (0.0)
Mollicutes class	4 (2.6)	4 (9.5)	1 (14.3)
Porphyromonas endodontalis	6 (3.8)	3 (7.1)	0 (0.0)
P. gulae	0 (0.0)	1 (2.4)	0 (0.0)
Prevotella buccae	15 (9.6)	2 (4.8)	0 (0.0)
P. loescheii	13 (8.3)	1 (2.4)	0 (0.0)
P. nigrescens	25 (16.03)	7 (16.7)	0 (0.0)
P. oralis	3 (1.9)	0 (0.0)	0 (0.0)
Selenomonas sputigena	1 (0.6)	0 (0.0)	0 (0.0)
Tannerella forsythia	1 (0.6)	0 (0.0)	0 (0.0)
Treponema denticola	5 (3.2)	0 (0.0)	0 (0.0)

Archaea domain (p<0.04). Likewise, there was an association between the frequency of *P. nigrescens* and *Actinomyces israelli* (p<0.04), *E. corrodens* (p<0.01), and *Mollicutes* class (p<0.03); while *A. israelli* also showed association with *Mollicutes* class (p<0.04). Concerning the association of microorganisms with clinical signs, only *Prevotella oralis* had a level of significance with gingivitis (p<0.02).

DISCUSSION

The occurrence of gingivitis and necrotizing gingivitis in cattle kept on reformed pastures is an original description in the literature about this important complex of diseases of ruminant's oral cavity. In fact, although gingivitis and necrotizing gingivitis are precursors of necrotizing periodontitis and periodontitis, their description in farm animals is rare. In general, existing studies deal with the final event (periodontitis) that is possible to be evaluated by extensive periodontal lesions such as gingival recession, clinical loss of insertion level, and damage to animal health and welfare (Döbereiner et al. 2000, 2004, Borsanelli 2017). In this context, the present longitudinal study and clinical monitoring of deciduous teeth of calves has enabled an unprecedented documentation of a natural occurrence, in successive episodes followed by remission, of two precursor forms of periodontitis. No less important, results of virginiamycin use in periodontal disease control in a longitudinal study with clinical monitoring of the gingival condition of the incisor teeth for four consecutive months and the presence of microorganisms indicative of potentially pathogenic microbiota are also unprecedented.

Progression of gingivitis in periodontitis is associated with individual, environmental, and etiological factors (Lang et al. 2009). It is known that pasture reform, through liming and fertilization, can favor the occurrence of bovine periodontitis in previously endemic areas (Dutra et al. 2000). However, it is unknown whether this frequent association is valid for all periodontal diseases; it should be added that

retrospective analysis makes it impossible to retrieve this information in areas that have been open for at least 50years, as was the case in the present study. According to the initial purposes of the study, the retention of calves under the same diet, in a reformed pasture and in a single management batch, made it possible to simulate an epidemiological condition associated with naturally occurring periodontitis and in a supposedly uniform challenge in which the probable environmental trigger would be present. Although the occurrence of periodontitis was not clinically observed in the 1440 evaluations performed over the 18-week period, the finding of 395 episodes of gingivitis and 89 episodes of necrotizing gingivitis is unprecedented and of great significance in studies of periodontal diseases. During this period of observation, the episodes of either disease were not constant or progressive, as they are acute inflammatory diseases that are characterized by discontinuous outbreaks and are mostly transient in the affected individuals (Page 1986).

Under the present experimental conditions, it could be noted the succession of episodes and remission of gingivitis and necrotizing gingivitis in incisor teeth, loss of body condition in animals that did not receive virginiamycin and the untreated animals had a higher frequency of diarrhea and other diseases. In this context, it is possible to associate the general clinical condition of untreated animals with the frequency of the occurrence of gingival diseases. This is corroborated with the OR, which indicated that treatment with virginiamycin was associated with a favorable reduction in the occurrence of gingivitis and necrotizing gingivitis.

Virginiamycin is a streptogramin marketed as a growth promoter at a dose of 340mg/head/day, according to Brazilian legislation (Brasil 2003). The product acts on ruminal bacteria sensitive to this class, allowing a better rumen feeding (Araújo et al. 2016). In the present study, the use of virginiamycin at this same dosage for one of the groups probably contributed to improvement of the periodontal condition, favoring prehension, chewing, rumination, and improvement in animal performance in addition to promoting weight gain.

According to Holmstrup & Westergaard (2008), individuals with successive episodes of gingivitis are more susceptible to the development of necrotizing gingivitis; this is a very common clinical condition in children and causes severe pain in the oral cavity. Necrotizing gingivitis is associated with individuals with immunodeficiency (Williams et al. 1990), and in Holstein cows with a genetic defect in leukocyte adhesion activity (Nagahata et al. 1993). In the present study, it was observed that the animals with several episodes of gingivitis presented episodes of necrotizing gingivitis later (Fig.1 and Fig.2), consistent with the aforementioned information. As this was not the main objective of the study, it is not possible to associate necrotizing gingivitis with any changes in immunological factors of the two groups of animals.

The presence of altered color, edema, and bleeding, both spontaneously and with gingival sulcus examination, observed in animals with gingivitis in both groups, parallels observations of gingivitis in small animals (Gorrel et al. 2008). Sheep with necrotizing gingivitis present necrotic ulcerations covered by a pseudomembrane (Salisbury et al. 1953), similar to what was observed in the present study.

It is worth noting that the succession of episodes of these two forms of periodontal disease precursors in cattle is paralleled throughout the literature with respect to this group of diseases in humans and other animals (Kinane & Bartold 2007, Marshall et al. 2014). In an objective and conclusive manner, it is possible to attribute a lower frequency of episodes of these two periodontal diseases in calves treated with virginiamycin. The difference between the two experimental groups was significant, with significant benefit to the group that ingested 340mg of virginiamycin daily for the study period. Tims et al. (1992) previously reported the effect of the antibiotic on the recovery of animals with the aggressive form of periodontitis even when they continued under the same epidemiological condition that triggered the outbreak of the disease.

As multifactorial infectious diseases, periodontal diseases are non-linear processes, external to the organism but associated with the bacterial biofilm adhered to the tooth and planktonic microbiota (Socransky & Haffajee 2005, Borsanelli et al. 2018). In this approach, the action of virginiamycin probably has been in the promotion of bacterial homeostasis favorable to the maintenance of periodontal health or in the prevention of dysbiosis, which causes the onset of periodontal diseases.

In periodontal diseases, gingival biofilm plays an essential role in its etiology (Löe 1994). In the calves included in the present study, there was practically no biofilm accumulation or dental calculus visible in the incisors.

A widely evidenced concept in the literature is that each tooth can have its own microbial complex, which, when analyzed, show differences in their constitution among teeth, such as supragingival and in the constitution and formation of the subgingival biofilm (Haffajee et al. 2009, Teles et al. 2012). The importance of conducting research at different collection sites, in other words, in each subgingival groove of the labial incisor teeth, was evident when analyzing the frequency and presence of these microorganisms (Table 1-3). In the comparisons between the collections and in the collections by animals, it was observed that a microorganism detected at one site would not necessarily be detected elsewhere, indicating that each one may have a specific biofilm at the time of collection.

In cattle with periodontitis, the disease does not occur without the presence of certain microorganisms that are considered normal constituents of the oral microbiota (Dutra & Döbereiner 2001, Borsanelli et al. 2018). Thus, in animals with gingivitis and necrotizing gingivitis, *A. israelli*, *E. corrodens*, *F. nucleatum*, *P. buccae*, *P. loescheii*, *P. nigrescens*, *P. oralis*, *P. endodontalis*, *T. forsythia,* and *T. denticola* were detected, which were also previously identified in healthy or in periodontal pockets in cattle (Borsanelli 2017). It is interesting to note that the role of bacteria in periodontal infections must meet Sokransky's postulate, since they are not conventional exogenous infectious diseases, but diseases of an endogenous nature, associated with the microbial biofilm, so that the interaction between the different taxa is important for the development of these diseases (Socransky & Haffajee 2010). In this sense, a statistical correlation of co-occurrence was observed between the different members of the genus *Prevotella*, which are part of the so-called "orange complex", associated with the first manifestations of inflammatory

periodontal diseases, in both gingivitis and necrotizing gingivitis (Socransky et al. 1998, Larsen & Fiehn 2017).

Porphyromonas spp., *Tannerella* spp., *Campylobacter* spp., *Eikenella* spp., *Parvimonas* spp., *Treponema* spp., and *Selenomonas* spp. were detected in monkeys with gingivitis, but in greater quantity in monkeys with periodontitis and periodontal abscesses (Gaetti-Jardim Junior et al. 2012). Thus, the use of amplification of the target DNA by conventional PCR in the present study made it possible to detect several microorganisms considered potentially pathogenic. However, it is a qualitative test, which requires caution in interpreting the results, especially in the face of endogenous disease or dysbiosis. On the other hand, Gram-positive bacteria, and some Gram-negative bacteria such as *F. nucleatum* and spirochetes, detected in sites with gingivitis and necrotizing gingivitis, can be associated as agents involved in the progression of these diseases in cattle. This is similar to observations in humans with these diseases (Harvey 2017).

In the present study, there was a trend in the frequency of occurrence of microorganisms detected in necrotizing gingivitis. In the Control Group, *F. nucleatum*, *P. buccae*, *P. loescheii,* and *P.nigrescens* were detected, while in the Virginiamycin Group, the *Archaea* domain and *Mollicutes* classes were observed. In the samples of animals with necrotizing gingivitis, no spirochetes of the genus *Treponema* were detected, which diverges from studies in humans, in which this genus has a recognized role (Herrera et al. 2014). *T. denticola* was identified in samples from healthy or gingivitis sites, similar to that observed in the oral microbiota of healthy adult bovines (Borsanelli 2017).

In ruminants and other animals it is known that *Porphyromonas* spp. is present in the periodontal pockets of animals with periodontitis (Senhorinho et al. 2011, Borsanelli et al. 2015b, Agostinho 2017, Campello 2017). Based on the results of the present study, it can be stated that besides being related to periodontitis, *P. endodontalis* and *P. gulae* are present in samples from healthy calves and calves with gingivitis.

Mollicutes class and *Archaea* domain, in humans and animals, are present in healthy patients with gingivitis, necrotizing gingivitis, or periodontitis (Yamabe et al. 2008, Faveri et al. 2011, Griffen et al. 2012, Chen et al. 2015, Harris et al. 2015). In cattle with periodontitis, *Mollicutes* class is more often associated with the disease (Borsanelli et al. 2018). In the calves in the present study, class *Mollicutes* was detected in sites with gingivitis and necrotizing gingivitis, and it can be noted that this microorganism was present in the different phases of periodontal diseases. *Archaea* domain to date has not been reported in this species; this is the first study to detect *Archaea* domain in healthy sites of both groups and in calves with necrotizing gingivitis that ingested virginiamycin. This difference indicates that the promoter leads to dysbiosis, favoring the growth of these microrganisms.

Prevotella nigrescens is associated with the development of necrotizing gingivitis and periodontitis in humans (Loesche et al. 1985, Stingu et al. 2013) and was also identified in healthy sites and periodontal pockets of bovines (Borsanelli et al. 2015a). In the present study, the frequency of *P. nigrescens* was significant, being detected in greater number in treated animals. This genus has evidence of resistance to this class of streptogramin, which would explain the higher number of positive samples in the group that took the antibiotic (Chung et al. 2002). Another factor that makes the detection of the genus *Prevotella* important in this work is due to the greater number of samples detected at the same time as the increase of cases of necrotizing gingivitis in the Virginiamycin Group, suggesting that in the treated animals this bacterium possibly has some function in evolution of necrotizing gingivitis.

Among the microorganisms associated with gingivitis, *F. nucleatum* presents great importance, since it is one of the organisms responsible for the inflammatory process, the beginning of the development of periodontitis, and the recruitment of other periodontopathogens. Although *F. nucleatum* is commonly found in microbiological studies of periodontal diseases, it is also detected in healthy periodontal sites, as well as in sites with gingivitis and periodontitis in humans and animals (Kolenbrander 2000, Kolenbrander et al. 2002, Senhorinho et al. 2011, Signat et al. 2011, Harris et al. 2015). Species of *Fusobacterium* genus are susceptible to the action of virginiamycin (Araújo et al. 2016), which is related to the results of this present study, in which the highest number of positive samples were in the Control Group (Fig.3 and Fig.4). Consistent with these events, more episodes of gingivitis and necrotizing gingivitis were observed in the Control Group animals.

Antibiotic treatment causes changes in the structure of the microbial community (Antiabong et al. 2013), which reflects the condition between health and disease. In the calves, it was possible to visualize the difference caused by the treatment between the groups and the presence of certain microorganisms in the analyzed sites. This is consistent with observations of the bacterial composition of humans with periodontitis treated with amoxicillin and metronidazole, in which individuals with a good response to treatment had reduced periodontopathogens such as *P. endodontalis*, *Prevotella* spp., and *Fusobacterium* spp. (Colombo et al. 2012).

In a previous study, Tims et al. (1992) reported the benefit of virginiamycin in the recovery of cattle kept in an endemic area. In the present study, the results show the benefit of the use of the antibiotic in the control of periodontal diseases, which are considered complex, polymicrobial, and dependent on relationships among the bacterial, host, and environment communities. Employed in growth promoter dose in this study, virginiamycin was effective in controlling two precursor forms of periodontitis, gingivitis and necrotizing gingivitis, and it can be considered a form of protection against the development of periodontal disease. In this context, it represents an excellent alternative to the control of periodontal diseases in bovines, especially as it does not leave residues in the meat and does not pose any risks to public health (Menzies-Gow & Young 2011, Bessegatto et al. 2017).

CONCLUSION

Virginiamycin administered at a dosage of 340mg/animal/day reduced significantly the occurrence and was effective in control and prevention of periodontal diseases (gingivitis and necrotizing gingivitis) in calves kept in reformed pastures and with a potentially periodontal pathogenic bacterial microbiota.

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Original Article
Livestock Diseases

Spontaneous poisoning by *Ricinus communis* leaves (Euphorbiaceae) in goats[1]

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ABSTRACT.- Brito L.B., Riet-Correa F., Almeida V.M., Silva Filho G.B., Chaves H.A.S., Braga T.C., Evêncio-Neto J. & Mendonça F.S. 2019. **Spontaneous poisoning by *Ricinus communis* leaves (Euphorbiaceae) in goats.** *Pesquisa Veterinária Brasileira 39(2):123-128.* Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. E-mail: fabio.mendonca@pq.cnpq.br

The aim of this study was to report the clinical and pathological aspects of an outbreak of poisoning by the ingestion of *Ricinus communis* leaves in a herd of goats at Pernambuco, northeastern Brazil. Within 3-5 hours after ingesting the sprouts and young shrubs of the plant, twenty Toggenburg female goats and two adults crossbred wethers presented acute neurological clinical signs, which were initially characterized by decreased locomotor activity that later evolved to severe ataxia, depression, incoordination and staggering gait. Four goat that died spontaneously were necropsied. Gross lesions were unspecific and consisted in focal areas of lungs edema, petechial hemorrhages in the epicardium and congestion and enlargement of liver. The contents of the rumen, reticulum and omasum were dry and contained leaves of the plant. Histologically there were no lesions in the CNS. In the liver, the main lesion consisted in cytoplasmic vacuolization and necrosis of hepatocytes. Eighteen goats recovered after a supportive therapy with activated charcoal, glycated isotonic solution, dexamethasone and vitamin B12. There is no specific therapy for poisoning by *R. communis*, however supportive and symptomatic treatments are recommended and should be based on the clinical signs.

INDEX TERMS: Plant poisoning, *Ricinus communis* leaves, Euphorbiaceae, ataxia, depression, ruminants, goats, toxicoses.

RESUMO.- [**Intoxicação espontânea por folhas de *Ricinus communis* (Euphorbiaceae) em caprinos.**] O objetivo deste estudo foi relatar os aspectos clínicos e patológicos de um surto de intoxicação pelas folhas de *Ricinus communis* em um rebanho de caprinos em Pernambuco, Nordeste do Brasil. Três a cinco horas após a ingestão dos brotos e arbustos jovens da planta, vinte cabras da raça Toggenburg e dois machos mestiços apresentaram quadro clínico neurológico agudo caracterizado principalmente pela diminuição da atividade locomotora, grave ataxia, depressão, incoordenação e marcha cambaleante. Quatro caprinos morreram espontaneamente e foram necropsiados. Macroscopicamente, as lesões eram inespecíficas e consistiam em áreas focais de edema pulmonar, hemorragias petequiais epicárdicas e aumento do volume e congestão do fígado. Os conteúdos do rumem, retículo e omaso eram ressecados e continham folhas da planta. Histologicamente, não foram observadas lesões no SNC. No fígado, havia vacuolização citoplasmática e necrose de hepatócitos. Dezoito caprinos se recuperaram após receberem terapia de suporte com carvão ativado, soro glicosado, dexametasona e vitamina B12. Não existe terapêutica

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especifica para a intoxicação pelas folhas de *R. Communis*. Os tratamentos sintomáticos e de suporte são recomendados e devem basear-se nos sinais clínicos.

TERMOS DE INDEXAÇÃO: Intoxicação por plantas, *Ricinus communis*, Euphorbiaceae, ataxia, depressão, ruminantes, mamona, caprinos, toxicoses.

INTRODUCTION

Ricinus communis L. (Euphorbiaceae), an upright shrub until 3-4m of high, commonly called castor bean, palma Christi or wonder tree (Worbs et al. 2011, Tokarnia et al. 2012, Albuquerque et al. 2014) is well known for centuries as a toxic plant to both humans and animals (Worbs et al. 2011, Tokarnia et al. 2012). Despite its toxicity, the plant is widely used to produce a variety of products for different purposes: medical, as a laxative or for treatment of infections and inflammation (Poli et al. 2007); industrial, fertilizing and biofuel/biodiesel production (Berman et al. 2011, Lima et al. 2011) and animal feeding with detoxified press cake (Gowda et al. 2009, Diniz et al. 2011).

Ricinus communis contains a complex mixture of toxic substances including the type II ribosome-inactivating ricin, the *R. communis* haemagglutinin and the alkaloid ricinine. Furthermore, other compounds like fatty acids, flavonoids and saponins have been found to exhibit deleterious effects on bacteria, virus, fungi, invertebrates and other animals (Upasani et al. 2003, Bigi et al. 2004, Assis Júnior et al. 2011, Worbs et al. 2011).

In humans, most poisonings are accidental due to consuming unprocessed seeds of *R. communis* (De Paepe et al. 2005, Nishiyama et al. 2005, Lucas 2008, Al-Tamimi & Hegazi 2008). In animals, most poisonings occur after ingestion of the processed products, although poisoning associated with the consumption of fresh seeds and leaves may also occur (Aslani et al. 2007, Albuquerque et al. 2014, Bianchi et al. 2018). The ingestion of leaves and pericarp causes nervous clinical signs while the ingestion of fruits containing seedscauses digestive disease in ruminants (Albuquerque et al. 2014, Riet-Correa et al. 2017). In Brazil, *R. communis* is often mentioned by farmers as the cause of death, especially for cattle, and was mentioned as an important toxic plant in different epidemiological surveys from the semiarid regions of Pernambuco and Paraíba in the northeastern of the country (Silva et al. 2006, Assis et al. 2009, Mello et al. 2010, Riet-Correa et al. 2017). In cattle, spontaneous poisoning is always associated with intense hunger and, historically, poisonings have occurred on those years of prolonged drought (Albuquerque et al. 2014, Riet-Correa et al. 2017). Data reported until this date were mainly from experimental studies in cattle, sheep and rabbits (Armién et al. 1996, Brito & Tokarnia 1997, Tokarnia & Döbereiner 1997) and spontaneous cases of poisoning in goats have not been described.

This study aimed to describe the clinical and pathological aspects of a spontaneous poisoning by *Ricinus communis* leaves in a goat's herd at Pernambuco, northeastern Brazil.

MATERIALS AND METHODS

Epidemiological and clinical data of *Ricinus communis* poisoning in goats were obtained during technical visits in the municipality of Limoeiro, Pernambuco, in northeastern Brazil.

Twenty Toggenburg female goats and two adults' crossbred wethers (castrated males) presented an acute neurological clinical picture. Two female goats and two wethers were selected for a systematized nervous clinical exam performed according to Riet Correa et al. (2002). Their general condition, appetite, color of mucous membranes, rectal temperature, heart and respiratory rates, form of abdomen, and rumen/reticulum motility were recorded. Rumen fluids were also collected to perform laboratory examinations. Examination of rumen fluid was performed according to Dirksen (1993) and Miranda Neto et al. (2005). The pH of the rumen fluid samples was measured at the time of sampling using pH indicator strips. The color, odor, appearance, sedimentation-flotation, reduction of methylene blue and protozoa activity were analyzed. Density, motility, live-dead ratio and predominance of protozoa were evaluated by direct microscopy (Dehority 1993).

To perform biochemical and blood count tests, blood samples from each goat were collected using a vacuum system through puncture of the jugular vein. These samples were stored in two 10mL tubes, one with the anticoagulant ethylenediaminetetraacetate acid (EDTA) in a 10% aqueous solution and the other without. The serum was separated by centrifugation at 2.500rpm for 10 minutes and maintained at -20°C until analysis. The biochemical tests were performed using a kinetic process with commercial enzyme kits for aspartate aminotransferase (AST), creatine phosphokinase (CPK), gamma glutamyl transferase (GGT), urea, creatinine and bilirubin (Lopes et al. 2007).

As treatment, it was administered a supportive therapy with orally activated charcoal in a dose of 2g/kg every 6 hours for 24 hours, glycated isotonic solution 5% (20mL/kg/h) for three days, a single dose of 1.00mg/kg/IM of dexamethasone and 0.07mg/kg/IM of vitamin B12 every two days totalizing three administrations.

Four goats were necropsied after spontaneous death. Samples of the CNS were obtained from the cerebrum, brainstem, cerebellum, diencephalon and spinal cord. Moreover, fragments of liver, kidney, heart, lung, spleen, rumen, reticulum, omasum, abomasum and intestines were collected, fixed in 10% formalin, processed routinely, stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS) and examined microscopically.

RESULTS

The outbreak of poisoning occurred in a herd of Toggenburg goats from a farm aiming milk production and sale of selected breeding animals. A total of 120 goats composing the herd were raised in a semi-extensive management and few crossbred wethers were also raised. The farm contained feedlots composed by buffel grass (*Cenchrus ciliaris*) to the goat's graze in the morning and in the afternoon they were removed to stalls to receive fresh water, mineral salt for goats, chopped elephant grass (*Pennisetum purpureum*) and commercial ration. The farm was located at the Middle Capibaribe Region, municipality of Limoeiro, in the state of Pernambuco, Brazil, in a semi-arid region known as the drought polygon. The rainfall do not exceed 295mm in the rainy season and 25mm in the dry season, however, presents a drought period less severe than the one on the semiarid region, due to the proximity to the coast.

On the day of poisonings, some sprouts and young shrubs of *Ricinus communis* present in the paddock were cut and forgotten by employees on the grazing area. After this, a herd composed by twenty Toggenburg female goats, averaging 4 years of age, and two adults crossbred wethers ate all the withered leaves found on the area and presented acute clinical signs of poisoning. The pasture area was inspected and sprouts of *R. communis* with evidence of being consumed were observed (Fig.1). The goats did not consumed fruits because the shrubs were young and were not in fructification period.

Clinical signs with different levels of intensity developed within 3-5 h after the goats were moved to the area where the plant was left. All goats in this lot were poisoned and more severely affected goats presented dehydration, congestion of episcleral vessels, tachycardia, dyspnea, decreased free locomotor activity until severe ataxia (when standing the goats remained with the limbs crossed) (Fig.2A,B), sialorrhea, depression, constant chewing movements, lateral deviation of head and neck (Fig.2C,D), incoordination, staggering gait, abnormal postures and mild bloat.

Ruminal movements were present but were hypotonic. The main changes observed in ruminal fluid were decreased of methylene blue activity, and slight reduction of the density and motility of rumen microfauna. Hematological abnormalities were mild and consisted of hemoconcentration, slightly increased values of total plasma protein and leukocytosis with neutrophilia in the examined goats. No changes in plasma fibrinogen or serum levels of AST, CPK, GGT, urea, creatinine or bilirubin were identified.

After supportive therapy, eighteen goats recovered totally between 12 to 24 hours after the end of treatment. In two female goats and two wethers the signs progressed to severe depression, sternal recumbency, lateral recumbency and death within 24 hours. Gross lesions in these goats consisted mainly in extensive focal areas of edema on right and left cranial lungs, petechial hemorrhages in the epicardium, congestion and enlargement of liver with a full distended gallbladder and prominent vessels on its surface and the cortical region of the kidneys was pale to yellowish. The contents of the rumen, reticulum and omasum were dry and contained leaves of the plant. In the CNS there was mainly congestion of the leptomeninges blood vessels. These lesions were more pronounced in one goat but were present in all the necropsied goats.

No gross or histologically lesions were found in the central nervous system. In the liver, a diffuse PAS-negative vacuolation of hepatocytes was the main lesion observed. In this case, the hepatocytes had a central nucleus rounded by one, two or three small vesicles into the cytoplasm or a confluent single vesicle. Single necrosis of hepatocytes was also observed and sometimes this lesion was focally extensive. Congestion, edema of Disse's spaces and swollen hepatocytes were also observed diffusely. In the lungs, the main lesion was alveolar edema.

DISCUSSION AND CONCLUSION

In this outbreak the history of consumption of *Ricinus communis* and the observation of partially digested leaves in the rumen content were an important factor for the diagnosis of poisoning, because the clinical picture, gross macroscopy and histological lesions are nonspecific (Worbs et al. 2011). Other poisonings like that caused by urea and cyanogenic plants must be considered as differential diagnosis when animals present sialorrhea, muscular tremors, staggering gait and a quick death (Tokarnia et al. 2012).

The clinical picture reported in urea poisoning also include nystagmus, incoordination and pushing against obstacles. These signs arise due to the high concentration of ammonia in the brain and causes neuronal degeneration and spongy degeneration of the neuropils (Visek 1984). While in the poisoning by cyanogenic plants, cyanide inhibits the

Fig.1. The pasture area was inspected and sprouts of *Ricinus communis* with evidence of being consumed were observed.

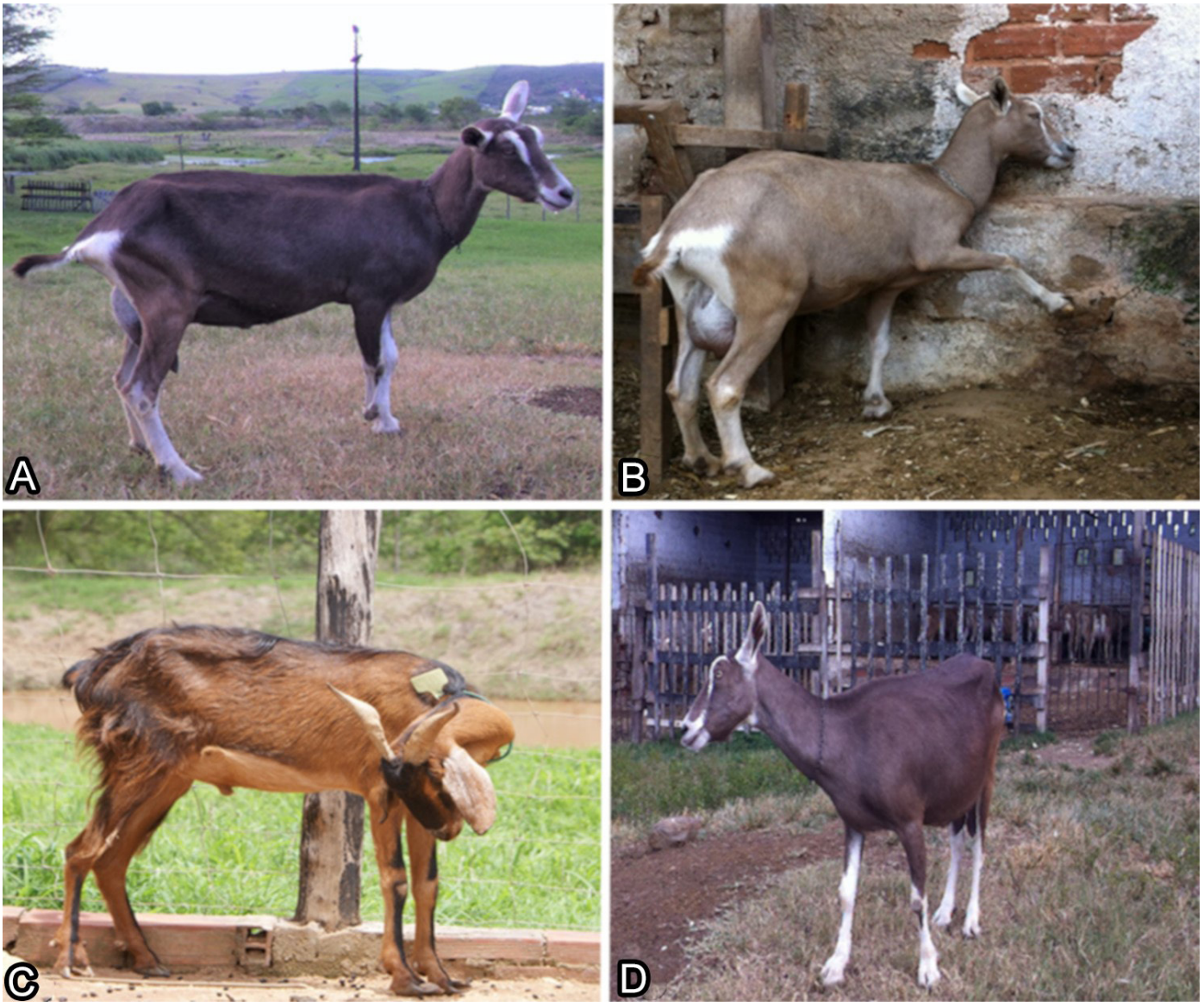

Fig.2. All goats in this lot were poisoned and more severely affected goats presented (**A**,**B**) dehydration, congestion of episcleral vessels, tachycardia, dyspnea, decreased free locomotor activity until severe ataxia (when standing the goats remained with the limbs crossed); (**C**,**D**) sialorrhea, depression, constant chewing movements, lateral deviation of head and neck, incoordination, staggering gait, abnormal postures and mild bloat.

action of metal-containing enzymes, especially cytochrome oxidase, resulting in the interruption of ATP production in mitochondria (Câmara et al. 2014). This mechanism is associated to cyanotic mucosa, nystagmus, head and eyelid tremors, incoordination followed by falls, lateral recumbency, opisthotonos and death. In Brazil, mainly in the northeastern region, most important cyanogenic plants reported causing outbreaks of poisoning were *Anadenanthera colubrina* var. *cebil* (*Piptadenia macrocarpa*), *Cnidoscolus quercifolias* (*C. phyllacanthus*), *Manihot carthaginensis* var *glaziovii*, *Passiflora foetida*, *Piptadenia viriflora*, *Sorghum halepense* and *S. bicolor* (Tokarnia et al. 2012, Câmara et al. 2014).

In cattle, the intense hunger is the main condition responsible for the ingestion of *Ricinus communis* and the acute poisonings (Albuquerque et al. 2014). The intense hunger seems not be the unique condition that facilitates the poisoning by *R. communis* leaves because in this outbreak, the goats were raised with enough pasture, not overcrowded and they had an adequate provision of water, mineral salt and commercial ration. The same situation was reported in sheep that ingested pruning waste containing *R. communis* (Bianchi et al. 2018). In another hand, withered leaves could concentrate more toxins and may be more palatable to ruminants (Tokarnia et al. 2012). In this situation, the presence of withered leaves in the pasture after the pruning could be a factor that facilitates the ingestion and the poisoning by the *R. communis* leaves in goats.

Doses of 10g/kg of fresh leaves of *R. communis* causes severe clinical picture in cattle and the lethal dose is 20g/kg; but this dose must be ingested in a short period of time to

cause death (Tokarnia et al. 1975, Döbereiner et al. 1981). For sheep, the lethal dose of fresh leaves is 30g/kg, but in goats this dose and doses until 40g/kg just caused mild clinical signs. For goats the lethal dose of fresh leaves was not established (Bezerra & Brito 1995). The main clinical signs observed in goats poisoned by *R. communis* consisted of a neurologic disease, with decreased free locomotor activity and severe ataxia, since they only consumed the leaves, which contains ricinine, an alkaloid that has typical CNS activity (Khafagy et al. 1983, Ferraz et al. 1999, Ohishi et al. 2014).

In a study with poisoned sheep by *Ricinus communis*, the clinical signs consisted in some neurological and digestive clinical picture and gross lesions consisted mainly in a severe gastroenteritis, cardiac haemorrhage and necrosis, hepatic necrosis and acute tubular necrosis in kidneys (Aslani et al. 2007). But these sheep ingested both leaves and seeds and because this, showed gross lesions mainly compatible by ricin toxicosis (Worbs et al. 2011, Tokarnia et al. 2012, Albuquerque et al. 2014). All other studies of poisoning by *R. communis* leaves in ruminants, including this report in goats, revealed no gross or microscopic lesions in CNS (Tokarnia et al. 1975, 2012, Döbereiner et al. 1981, Bezerra & Brito 1995, Bianchi et al. 2018). But the clinical picture consisted of ataxia, depression, chewing movements, lateral deviation of head and neck, incoordination and staggering gait are probably caused by biochemical alterations in the cerebrum, cerebellum and brainstem (Riet-Correa et al. 2002) because in poisoned mice by ricinine, it was suggested that an increased release of glutamine in the cerebral cortex can be implicated in the genesis of the neurological clinical picture (Ferraz et al. 2002).

Ricinus communis is often cited by farmers as a cause of death, especially in cattle in northeastern Brazil and spontaneous poisoning is always associated with intense hunger (Albuquerque et al. 2014). In goats, the ingestion of leaves seems to be an unusual event, thus the poisoning is not common and occurred mainly due to accidental ingestion of pruning waste, as reported previously with other species of poisonous plants such as *Nerium oleander* and *Kalanchoe blossfeldiana* (Soto-Blanco et al. 2006, Mendonça et al. 2018). There is no specific therapy for poisoning by *R. communis* and for this reason, supportive and symptomatic treatment in all species is recommended (Albretsen et al. 2000, Albretsen 2003, Doan 2004) and should be based on the clinical signs. In years of severe drought in the semiarid northeastern region, prophylaxis consists of eradication of the plant or keeping the ruminants away from areas where there is a severe propagation of *R. communis* (Albretsen et al. 2000, Albretsen 2003, Doan 2004, Tokarnia et al. 2012).

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Original Article
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Seroepidemiological study of feline coronavirus (FCoV) infection in domiciled cats from Botucatu, São Paulo, Brazil[1]

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ABSTRACT.- Almeida A.C.S., Galdino M.V. & Araújo Jr. J.P. 2019. **Seroepidemiological study of feline coronavirus (FCoV) infection in domiciled cats from Botucatu, São Paulo, Brazil.** *Pesquisa Veterinária Brasileira 39(2):129-133.* Laboratório de Virologia, Departamento de Microbiologia e Imunologia, Instituto de Biotecnologia, Universidade Estadual Paulista, Alameda das Tecomarias s/n, Chácara Capão Bonito, Botucatu, SP 18607-440, Brazil. E-mail: arianicristina@yahoo.com.br

Feline coronavirus (FCoV) is responsible for causing one of the most important infectious diseases of domestic and wild felids, the feline infectious peritonitis (FIP), which is an immune-mediated, systemic, progressive and fatal disease. FCoV is highly contagious, and infection is common in domestic feline populations worldwide. The present study aimed to determine the seropositivity of FCoV infection and its associated epidemiological variables (risk factors) in domiciled cats in Botucatu, São Paulo, Brazil. Whole blood samples (0.5-1mL) were collected from 151 cats, and sera were extracted by centrifugation. These sera were tested by an commercial enzyme-linked immunosorbent assay (ELISA) for the detection of IgG anti-FCoV antibodies. The assessed risk factors were age range, breed, gender, reproductive status, outdoor access and rearing mode (living alone or in a group). The seropositivity was 64.2% (97/151). There was no statistical significance for risk factors related to breed, gender or rearing mode. There were significant differences in seropositivity (p-values ≤0.05) for age range (p=0.0157), reproductive status (p=0.0074) and outdoor access (p=0.0001). This study verified a wide dissemination of FCoV in the studied population, with a higher than expected seropositivity for indoor cats. Among the risk factors, age range, reproductive status and outdoor access presented statistically significant differences, thus helping to establish an epidemiological profile of this population.

INDEX TERMS: Seroepidemiology, feline coronavirus, FCoV, domiciled cats, São Paulo, Brazil, cats, viroses.

RESUMO.- [**Estudo soroepidemiológico da infecção pelo coronavírus felino (FCOV) em gatos domiciliados de Botucatu, São Paulo, Brasil.**] O coronavírus felino (FCoV) é responsável por causar uma das mais importantes doenças infecciosas que acometem os felinos domésticos e selvagens, a peritonite infecciosa felina (PIF), que é uma enfermidade imunomediada, sistêmica, progressiva e fatal. O FCoV é altamente contagioso e a infecção é comum nas populações de felinos domésticos por todo o mundo. O presente estudo objetivou determinar a soropositividade da infecção pelo FCoV e correlacionar variáveis epidemiológicas (fatores de risco) de gatos domiciliados de Botucatu, São Paulo, Brasil. Amostras de sangue total (0,5 a 1mL) foram colhidas de 151 gatos e os soros foram obtidos após centrifugação. Estes soros foram testados por um teste commercial de ELISA para detecção de anticorpos IgG anti-FCoV. Os fatores de risco avaliados foram faixa etária, raça, gênero, condição reprodutiva, acesso à rua e modo de criação (viver solitário ou em grupo). Observou-se uma soropositividade de 64,2% (97/151). Não houve significância estatística para os fatores de risco relacionados à raça, gênero e modo de criação. Houve significância estatística quanto a soropositividade (p-values ≤0,05) para os fatores de risco faixa etária (p=0,0157), condição reprodutiva (p=0,0074) e acesso à rua (p=0,0001). Através do presente estudo verificou-se que o FCoV está

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amplamente disseminado na população estudada, onde a soropositividade encontrada foi maior do que a esperada para gatos domiciliados. Dentre os fatores de risco, faixa etária, condição reprodutiva e acesso à rua apresentaram diferenças estatisticamente significativas, contribuindo assim, para se estabelecer um perfil epidemiológico desta população.

TERMOS DE INDEXAÇÃO: Soroepidemiológico, coronavírus felino, FCoV, gatos domiciliados, São Paulo, Brasil, felinos, viroses.

INTRODUCTION

The feline coronavirus (FCoV) belongs to the order *Nidovirales*, family Coronaviridae, subfamily Coronavirinae, genus *Alphacoronavirus* and species *Alphacoronavirus 1* (ICTV 2017). It is an enveloped virus containing single-stranded RNA and positive polarity (Sparkes 2006, Pratelli 2008).

FCoV infection is widely distributed in domestic cats and sometimes observed in wild cats (Hoskins & Loar 1993, Foley et al. 1997). FCoV remains a habitual pathogen in cat groups because of chronic carriers that make up approximately 20% of the population within heavily populated areas (Hartmann 2005). Antibodies are present in approximately 80-90% of cats living in shelters and 30-50% of domiciled cats (Addie & Jarrett 2006, Brown et al. 2009). Overall, FCoV is a highly contagious virus, transmitted through the fecal-oral route, which usually causes a mild intestinal infection (Addie & Jarrett 2006, Pedersen 2009).

FCoV causes one of the most important infectious diseases affecting domestic and wild cats, feline infectious peritonitis (FIP), which is an immune-mediated, systemic, progressive and fatal disease (Addie & Jarrett 2006). FIP was discovered in the 1960s and has been reported worldwide ever since (Pedersen 2009, Le Poder 2011). There is evidence to suggest that the causative agent of FIP is a FCoV mutation called feline infectious peritonitis virus (FIPV), and its benign counterpart is feline enteric coronavirus (FECV). Both viruses are indistinguishable from one another in terms of their physical and antigenic properties (Addie & Jarrett 2006, Norsworthy 2006, Cornelissen et al. 2007).

The occurrence of FIP is most common in young cats between three months and three years of age (Addie & Jarrett 2006). However, cats older than 10 years may develop FIP as they experience a decline in immune response typical of old age. FIP is more frequent in environments with a high feline concentration, where higher rates of viral infection and dissemination of FIPV variants exposes animals to significant infective doses (Hoskins & Loar 1993, Foley et al. 1997). Approximately 5-10% of seropositive cats may show signs of sickness and, consequently, die from FIP (Addie & Jarrett 2006). Clinical signs of FIP can be variable, because many organs can be involved, as the liver, kidneys, pancreas, eyes and the central nervous system. The PIF can present itself in two forms, the first being the "wet" or effusive form (more common), characterized by with effusions in the abdomen, thorax, and/or pericardium (Hartmann 2005). A second form of the disease is called "dry" or non-effusive (there is no into effusions body cavities), characterized by the presence of granulomas in organs (Pedersen 2009).

Investigations into the seroprevalence of FCoV infection and other viral agents important to feline medicine, such as feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV), contribute to controlling these agents by identifying risk factors and addressing strategies for infection prevention (Little et al. 2009, Westman et al. 2016). In Brazil, relatively few cases of cats exposed to or infected by FCoV are investigated in labs, except for some cases in certain animal shelters with high sanitary standards. As a general rule, domiciled cats are only investigated in the laboratory if they manifest clinical signs.

Studies describing FCoV seropositivity of domiciled cats are scarce in Brazil. Therefore, regional and national studies of seroepidemiology are necessary to identify the main risk factors of FCoV infection in the household feline population of Brazil. The present study aimed to determine the seropositivity of FCoV infection and the correlated epidemiological risk factors in domiciled cats in Botucatu, São Paulo, Brazil.

MATERIALS AND METHODS

Ethics statement. This work was submitted and approved by the ethics committee (CEUA) of Unesp, Botucatu, with approval protocol 51/2014 (registration number).

Animals and samples. The samples (n=151) were randomly collected. The cats lived in several neighborhoods around Botucatu's urban zone (22°53'09"S, 48°26'42"0), located in the South-Central region, in the State of São Paulo. The source of the samples was through personal contacts, veterinary clinics the city and municipal kennel (from cats that were taken for free neutering). The samples were collected from 52 houses, each one having from one to eleven cats, all cats being part of the research. The State of São Paulo houses a population of around 947.539 domestic cats, and the city of Botucatu houses 3684 animals, claiming 0.4% total (Pasteur Institute 2016). The number of samples was calculated having in mind the number of cats in Botucatu in 2016, based on estimated prevalence of 90% (literature worldwide data about FCoV seropositivity, ever since there isn't national results available) with a margin of error allowable error of 5% and confidence level of 95%. The sample calculation resulted in 134 samples, however were collected 151.

Blood samples (0.5 to 1mL) were collected aseptically by cephalic or jugular vein puncture and stored in a siliconized glass tube containing clot activator gel (Vacutainer®, Becton Dickinson) to obtain serum. Then, samples were centrifuged at 4000g for 10 minutes, and the sera were stored in 1.5mL microtubules free of nucleases (Axygen®) and frozen at -20°C until they were used. Individual data for each animal, such as age range (kitten, junior, prime/mature, senior/geriatric), breed, gender, reproductive status (whole/castrated), environment (outdoor access or confined), and rearing mode (in group/solitary) were recorded on an epidemiological card.

Serological test. Sera were tested using the ImmunoComb FCoV kit® (FIP) (Biogal Galed Labs, Acs. Ltd.) following manufacturer recommendations. The ImmunoComb test is a modification of ELISA test, based on immunoassay tenet on solid phase (DOT-ELISA). The test is able to determinate a semi-quantitative measure of the FCoV antibody titer present in whole blood, plasma, serum, effusion or cerebrospinal fluid (Bell et al. 2006b). The antibodies levels are determined according to the intensity of the test color result. Thus, the absence of color or a light gray color indicates negative or low level of antibodies. Higher levels of antibodies are indicated by darker color results. The results were scanned by Combo Scan software to classify specimens as seropositive or seronegative. These analyses were performed by Laboratory of Virology at Unesp, IBTEC, Botucatu, São Paulo, Brazil.

Statistical analysis of data. The data were analyzed with Statistical Analysis System software (SAS 9.3) and Microsoft Office Excel 2007.

All variables were described using descriptive statistical methods and expressed in frequency and percentage. Logistic regression models were used to verify the existence of significant differences in seropositivity (0 = seronegative, 1 = seropositive) between groups of each variable, with a statistical significance level of p≤0.05. The difference was considered statistically significant when 1 was not included within the 95% confidence interval.

RESULTS

The study revealed a seropositivity of 64.2% (97/151). The descriptive statistics of all variables are shown in Table 1. In total were sampled 151 animals from 52 different houses, where 40.3% (21/52) had only one cat; 28.8% (15/52) had 2 cats; 5.7% (3/52) had 3 cats; 7.7% (4/52) had 4 cats; 1.9% (1/52) had 5 cats; 5.7% (3/52) had 7 cats; 3.8% (2/52) had 9 cats; 3.8% (2/52) had 10 cats and 1.9% (1/52) had 11 cats. The risk factor analysed, were found meaningful statistics differences for the age range variable (p=0.0157), reproductive status (p=0.0074) and outdoor access (p=0.0001) (Table 2), where three variable combined helps to explain the seropositive phenomenon on the researched population (p-values ≤0.05) (Table 3).

Were found meaningful statistics differences when different categories of age range, reproductive status and street access were compared (Table 4). Prime/mature animals are 4.5 times more likely to be seropositive when compared to kittens, and prime/mature+senior/geriatric (analyzed in group) are 6.7 times more likely than kittens+junior animals. The chance of seropositivity for whole animals is greater (2.76 times) than for castrated animals. Animals without outdoor access are 4 times more likely to be seropositive than those that have outdoor access (Table 5).

Table 1. Frequencies of seroprevalence classifications for age range, breed, gender, reproductive status, outdoor access and rearing mode (solitary or group)

Variables	Positive	Negative	Total
Age range			
Kitten: 1 to 12 months	36	34	70 (46.3%)
Junior: >1 to 3 years	20	09	29 (19.2%)
Prime/mature: >3 to 8 years	25	07	32 (21.1%)
Senior/geriatric: >8 years	16	04	20 (13.2%)
Breed			
Mongrel cat	63	48	111 (73.5%)
Persian	29	4	33 (21.8%)
Exotic	02	0	2 (1.3%)
Siamese	02	02	4 (2.6%)
Maine coon	01	0	1 (0.6%)
Gender			
Male	45	33	78 (51.6%)
Female	52	21	73 (48.3%)
Reproductive status			
Whole	31	07	38 (25.1%)
Castrated	66	47	113 (74.8%)
Outdoor access			
Yes	22	32	54 (35.7%)
No	75	22	97 (64.2%)
Rearing mode			
Solitary	8	5	13 (8.6%)
Group	89	49	138 (91.4%)

DISCUSSION

This study revealed that FCoV infection is widely disseminated in the assessed feline population, with a seropositivity of 64.2%. The presence of antibodies, which normally varies from 30-50%, is higher than expected for domiciled cats, according to global data in the literature (Addie & Jarrett 2006, Brown et al. 2009, Pedersen 2009). There are no data available from studies conducted in Brazil.

Regarding the age groups, the study sample had a large number of kittens (1 to 12 months), representing 46.3% of the total animals sampled. Age is considered an important risk factor for the development of PIF (Hartmann 2005, Horzinek et al. 2008). Cats may become infected by FCoV in all age ranges, but the highest risk of developing FIP is for cats from three months to three years old (kitten and junior). Senior/geriatric cats older than 10 years are also considered high-risk animals due to the decline of their immune system (Rohrbach et al. 2001, Addie & Jarrett 2006). Statistical analysis demonstrated that prime/mature animals are more likely to be seropositive than kittens. When analyzed in groups (prime/mature+senior/geriatric and kitten+juniors), the prime/mature+senior/geriatric group is more likely to have anti-FCoV antibodies. In another seroprevalence study, Akkan & Karaca (2009) also found greater seropositivity in adult and elderly individuals. These animals, possibly due to their age, have a greater chance of coming into contact with the virus and producing antibodies, though this may occur in any age range.

Moreover, 73.5% of the specimens were "mixed breed" cats (MBC). No significant differences were found related to the breeds we analyzed, but this could be due to the low number of animals sampled from certain types. All cat breeds can become infected with FCoV and develop FIP. However, some purebred cats seem to have a genetic predisposition to systemically manifest the disease (Horzinek et al. 2008).

Table 2. Statistical significance (p-value) for each variable

Variables	p-value [a] (logistic regression)
Age range	0.0157
Breed	1.0000
Gender	0.0818
Reproductive status	0.0074
Outdoor access	0.0001
Rearing mode	0.8325

[a] Significant p-values ≤0.05.

Table 3. Logistic regression of the combined statistically significant variables

Variables	Degrees of freedom	Chi-square statistic	p-value [a]
Age range	3	10.42	0.0153
Reproductive status	1	4.39	0.0361
Outdoor access	1	12.49	0.0004

[a] Significant p-values ≤ 0.05

Table 4. Statistical significance of the differences between several categories, including age, range, reproductive status and outdoor access

Comparison	Degrees of freedom	Chi-square statistic	p-value [a]
Kitten x Junior	1	2.35	0.1256
Junior x Prime/mature	1	1.46	0.2275
Prime/mature x Senior/geriatric	1	0.23	0.6318
Prime/mature x Kitten	1	8.89	0.0029*
Senior/geriatric x Kitten	1	3.44	0.0635
Senior/geriatric x Junior	1	0.29	0.5902
(Kit. + Sr./geriat.) x (Jr. + Prime/mat.)	1	1.50	0.2201
(Prime/mat. + Sr./geriat.) x (Kit. + Jr.)	1	4.62	0.0317*
Reproductive status (castrated or whole)	1	4.39	0.0361*
Outdoor access (yes or no)	1	12.49	0.0004*

[a] Significant p-values ≤ 0.05

Table 5. Estimates and confidence intervals (95%) for the odds ratio

Variable	Variable	Estimate	L. L.[a]	S. L.[b]
Whole	Castrated	2.7624	1.0276	7.4254#
No outdoor access	Outdoor access	4.0044	1.8293	8.7653#
Junior	Kitten	2.1357	0.7951	3.1482
Prime/mature	Kitten	4.5367	1.5854	12.9817#
Senior/geriatric	Kitten	3.1562	0.8785	11.3395
Prime/mature	Junior	2.1242	0.6196	7.2766
Senior/geriatric	Junior	1.4780	0.3518	6.2090
Prime/mature	Senior/geriatric	1.4372	0.3294	6.2703
Prime/mat. + Sr./geriat.	Kitten + Junior	6.7050	1.1300	39.7866#

[a] L.L = Lower limit, [b] S.L. = superior limit; the interval confidences (95%) without value 1.

Abyssinus, Bengal, Burmese, Himalayan, Ragdoll, Rexes, Burmese, Exotic Shorthair, Manx, Persian, Russian Blue and Siamese are some of the breeds especially prone to developing the disease (Bell et al. 2006a, Pesteanu-Somogyi et al. 2006, Horzinek et al. 2008). The increased prevalence in these purebred cats may be due to a concentration of hereditary risk factors caused by inbreeding (Foley & Pedersen 1996).

Related the gender of the sampled animals, 51.6% were males and 48.3% females. There was no statistically significant difference in seropositivity between the gender groups. These results corroborate the findings of Bell et al. (2006a). Some studies point out a greater predisposition for FIP in male cats (Robison et al. 1971, Rohrbach et al. 2001, Pesteanu-Somogyi et al. 2006). For the reproductive condition variable, 74.8% of the individuals were castrated and 58.4% of the castrated individuals were seropositive. In whole animals, seropositivity was 81.5%. Statistical analyses showed that whole animals were 2.7 times more likely to be seropositive than castrated animals. Other authors describe a greater risk of developing the sickness in whole cats (Robison et al. 1971, Rohrbach et al. 2001, Pesteanu-Somogyi et al. 2006, Worthing et al. 2012). Male and whole indoor cats easily go out, being subjected to a higher stress from fights disputing territory or females. This may become them more vulnerable to PIF, also increasing the contact with a innumerous variety of FCoV strains.

Concerning rearing mode, 91.4% of the animals lived in groups of 2 to 10 cats. Environments with multiple cats appear to be at greater risk for the development of the disease, because the infection prevalence is higher in houses with more than one cohabitant (Addie & Jarrett 2006). However, no significant differences were found for this risk factor despite most cats in the studied population cohabitating with others. There were statistically significant differences linked to outdoor access. Animals kept inside were 4 times more likely to be seropositive compared to those with outdoor access. The cat's creation in closed environments has contributed to increase the exposure to a great quantity of infectious agents, specially when created in groups. The confinement has brought changes for the specie's hygiene habbits, wich before used to burry it's stools and nowadays use shared sandboxes. The cat's main way to eliminate FCoV is through the stools, and the sandboxes has made theses cats get more contact with theses stools, making easier the acute infectious and consecutive cycles of reinfections, with prolonged increasements of seropositivety and the risk of developing PIF. Suitable waste management (cleaning and disinfection, not overcrowding single spaces) by their owners is fundamental for PIF prevention.

CONCLUSIONS

This seroepidemiological study demonstrated that FCoV is widely disseminated in the studied cat population. Seropositivity was higher than expected for domiciled cats relative to data from other parts of the world.

The statistically significant differences found in risk factors, such as age range, reproductive condition and outdoor access, help to create an epidemiological profile of this population.

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Original Article
Small Animals Disease

Clinical and pathological aspects of idiopathic pulmonary fibrosis in cats[1]

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ABSTRACT.- Cony F.G., Argenta F.F., Heck L.C., Moreira L.F., Costa F.V.A., Sonne L. & Pavarini S.P. 2019 **Clinical and pathological aspects of idiopathic pulmonary fibrosis in cats.** *Pesquisa Veterinária Brasileira 39(2):134-141.* Setor de Patologia Veterinária, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves 9090, Agronomia, Porto Alegre, RS 91540-000, Brazil. E-mail: saulo.pavarini@ufrgs.br

Interstitial lung diseases are a group of diffuse parenchymal lung diseases that include interstitial lung fibrosis. The aim of this study is to characterize the clinical and pathological findings of idiopathic pulmonary fibrosis in three cats and to investigate possible etiological agents through bacteriological and mycological exams and immunohistochemistry. All three cats were female and aged from 10 to 14 years old, they presented with a clinical history of weight loss and dyspnea. The radiographic changes were similar in all cats and included increased pulmonary radiopacity with a mixed bronchointerstitial pattern progressing to an alveolar pattern. Two cats died during lung biopsy procedures. At necropsy, the lesions were limited to the pulmonary parenchyma and were firm, hypocrepitant with a multinodular appearance on the pleural surface; they failed to completely collapse when the thorax was opened. In the pleural region, there were multifocal star-shaped scarring lesions, with parenchymal retraction. Microscopically, all three cats had multifocal-to-coalescing fibrosis, type II pneumocyte hyperplasia, hypertrophy or hyperplasia of the smooth muscle tissue of terminal bronchioles and an accumulation of macrophages within the alveolar spaces. There was no growth on bacteriological or mycological cultures, and the immunohistochemical evaluations for the presence of viral etiological agents (FIV, FeLV, FCoV, FCV and FHV-1) were also negative.

INDEX TERMS: Clinics, pathology, idiopathic pulmonary fibrosis, interstitial lung disease, dyspnea, immunohistochemistry, type II pneumocytes, cats.

RESUMO.- [**Aspectos clínicos e patológicos em felinos com fibrose pulmonar idiopática.**] As enfermidades pulmonares intersticiais são um grupo de doenças difusas do parênquima pulmonar, nas quais a fibrose pulmonar está incluída. O objetivo deste trabalho é caracterizar os achados clínicos e patológicos da fibrose pulmonar idiopática em três gatas, e avaliar possíveis agentes etiológicos através dos exames bacteriológicos, micológicos e imuno-histoquímicos. As três gatas tinham entre 10 e 14 anos de idade e histórico clínico de emagrecimento e dispneia. As alterações radiográficas observadas foram similares, com aumento de radiopacidade difuso dos campos pulmonares de padrão misto broncointersticial e eventualmente alveolar. Dois felinos morreram durante procedimento de biópsia pulmonar. No exame de necropsia as lesões eram exclusivas no parênquima pulmonar os quais estavam firmes, hipocreptantes, com aspecto levemente multinodular em superfície pleural e não colapsaram após a abertura da cavidade torácica. Em região pleural havia lesões cicatriciais de aspecto estrelar multifocais, com retração do parênquima. Microscopicamente, todos os gatos apresentaram fibrose multifocal a coalescente, hiperplasia dos pneumócitos do tipo II e hiperplasia e hipertrofia do músculo liso de bronquíolos terminais e acúmulo de macrófagos no interior de espaços alveolares. Não houve crescimento nas culturas bacteriana e micológica, e os exames de imuno-histoquímica

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para avaliação de possíveis agentes virais (FIV, FeLV, FCoV, FCV e FHV-1) foram negativos em todos os felinos.

TERMOS DE INDEXAÇÃO: Clínica, patologia, doenças pulmonares intersticiais, dispneia, imuno-histoquímica, pneumócitos tipo II, felinos.

INTRODUCTION

Interstitial lung diseases compose a group of diffuse diseases of the pulmonary parenchyma that include pulmonary fibrosis (Cushley et al. 1999). It is a common pulmonary disease in humans, and recently it has been seen in domestic felines (Selman et al. 2010). It is suggested that in humans, the onset of the condition is related to continuous pulmonary injuries associated with a genetic predisposition. The pathogenesis of the disease in felines is not yet well elucidated; however, the microscopic characteristics of type II pneumocytes seen in cats with the condition are similar to the form of this disease in humans (Thomas et al. 2002).

The disease affects adult cats that are an average of eight years old, and there is no sex or breed predilection (Evola et al. 2014). Due to the progressive nature and the absence of specific treatment, the condition has an unfavorable prognosis, and the definitive diagnosis is usually made *post mortem* based on anatomopathological findings (Cohn et al. 2004). The aim of this study is to characterize the clinical and pathological findings of the idiopathic pulmonary fibrosis in three cats and to investigate possible etiological agents through bacteriological and mycological exams and immunohistochemistry (IHC).

MATERIALS AND METHODS

The three cats that were included in the study (Cats 1, 2 and 3) were evaluated at the Veterinary Clinical Hospital of the Federal University of Rio Grande do Sul (HCV-UFRGS) and had a pathological diagnosis of idiopathic pulmonary fibrosis made between 2016 and 2017 at the Veterinary Pathology Department of the Federal University of Rio Grande do Sul (SPV-UFRGS). All patients were from the metropolitan area of Porto Alegre, Rio Grande do Sul, Brazil.

The cats were necropsied and fragments from various organs were collected, fixed in 10% formalin, routinely processed for histology and stained with hematoxylin and eosin (HE). Additionally, lung fragments were stained with Masson's trichrome (MT), according to the protocol described by the Armed Forces Institute of Pathology (Mc Elroy 1992). Lung samples were kept refrigerated and were submitted to bacteriological and mycological examinations. A sample of lung was inoculated in 5% sheep blood Mueller Hinton Agar and in MacConkey Agar. The sample was aerobically incubated at 37°C for 72 hours. Lung samples were seeded in Sabouraud glucose agar with chloramphenicol and cycloheximide followed by incubation at 26°C for seven days.

IHC analysis was performed by the peroxidase-labeled antibody method (MACH 4, Universal HRP-Polymer, Biocare Medical) to evaluate lung sections for feline immunodeficiency virus (FIV), feline leukemia virus (FeLV), feline herpesvirus type 1 (FHV-1), feline calicivirus (FCV), feline coronavirus (FCoV), vimentin, pancytokeratin, smooth muscle actin and lysozyme. IHC was also performed on bone marrow sections for to evaluate them for the presence of FIV and FeLV. Table 1 shows the immunohistochemical antibodies and protocols used. IHC positive controls included samples of skin (smooth muscle actin, vimentin and pancytokeratin) and previously tissues for FIV, FeLV, FHV-1, FCV, FCoV and lysozyme (Rolim et al. 2016). Negative controls consisted of tissue samples incubated with phosphate buffered saline (PBS) instead of primary antibody.

RESULTS

Clinical findings

Animal number 1 was a 10-year-old female spayed mixed breed cat that presented with a complaint of respiratory distress and anorexia for two days. On physical exam, the patient was moderately dehydrated and mixed restrictive dyspnea, without any abnormalities on cardiopulmonary auscultation. Blood samples were collected for hematology and biochemical evaluation (Table 2), and chest radiography showed a moderate diffuse increase in radiopacity in the lung fields and a mixed bronchointerstitial to alveolar pattern, with a predominant bronchial pattern (Fig.1A). The patient was discharged home

Table 1. Antibodies and immunohistochemical protocols used in cats with idiopathic pulmonary fibrosis

Antibody/code	Clone	Antigenic recovery	Dilution	Chromogen
Vimentin[a] (18-002)	Monoclonal (v9)	3 min/125°C, plus citrate, pH 6.0	1:200	DAB[b]
Pancytokeratin[b] (M3515)	Monoclonal (AE1/AE3)	3 min/125°C, plus citrate, pH 6.0	1:80	DAB[b]
Alpha smooth muscle actin[b] (M0851)	Monoclonal (1A4)	3 min/125°C, plus citrate, pH 6.0	1:80	DAB[b]
Lysozyme[b] (A0099)	Polyclonal	3 min/125°C, plus citrate, pH 6.0	1:80	DAB[b]
FIV[b] (MCA 2278)	Monoclonal (PAK32C1)	40 min/96°C, 0.01 M, plus citrate, pH 6.0	1:100	Permanent red[b]
FeLV[b] (MCA 1897)	Monoclonal (C11D8)	40 min/96°C, 0.01 M, plus citrate, pH 6.0	1:500	Permanent red[b]
FHV-1[c] (FHV7-5)	Monoclonal (CM1)	40 min/96°C, 0.01 M, plus citrate, pH 6.0	1:100	AEC[b]
FCV[c] (FCV2-16)	Monoclonal (FCV143)	40 min/96°C, 0.01 M, plus citrate, pH 6.0	1:50	AEC[b]
FCoV[d] (MCA 2194)	Monoclonal (F1PV370)	40 min/96°C, 0.01 M, plus citrate, pH 6.0	1:300	AEC[d]

Acquisition sources: [a] Zymed, [b] Dako, [c] Custom Monoclonals, [d] Serotec; AEC = 3-amino-9-ethylcarbazole, DAB = 3,3'-diaminobenzidine.

Table 2. Hemogram results and biochemical evaluation of three cats with idiopathic pulmonary fibrosis

Exams	Cat number 1	Cat number 2	Cat number 3	Reference
Hemoglobin (g/dL)	12.6	11.9	13.2	8 - 15
Hematocrit (%)	40	33.2	40	24 - 45
Total leukocytes (/L)	11.700	11.500	19.300	5.000 - 19.500
Segmented neutrophils (/L)	7.722	9.775	16.019	2.500 - 12.500
Albumin (g/L)	30	----	33	21 - 33
ALT* (U/L)	327	<10	2	<83
Creatinine (mg/dL)	1.0	1.4	0.78	0.8 - 1.8

*ALT= Alanine transaminase.

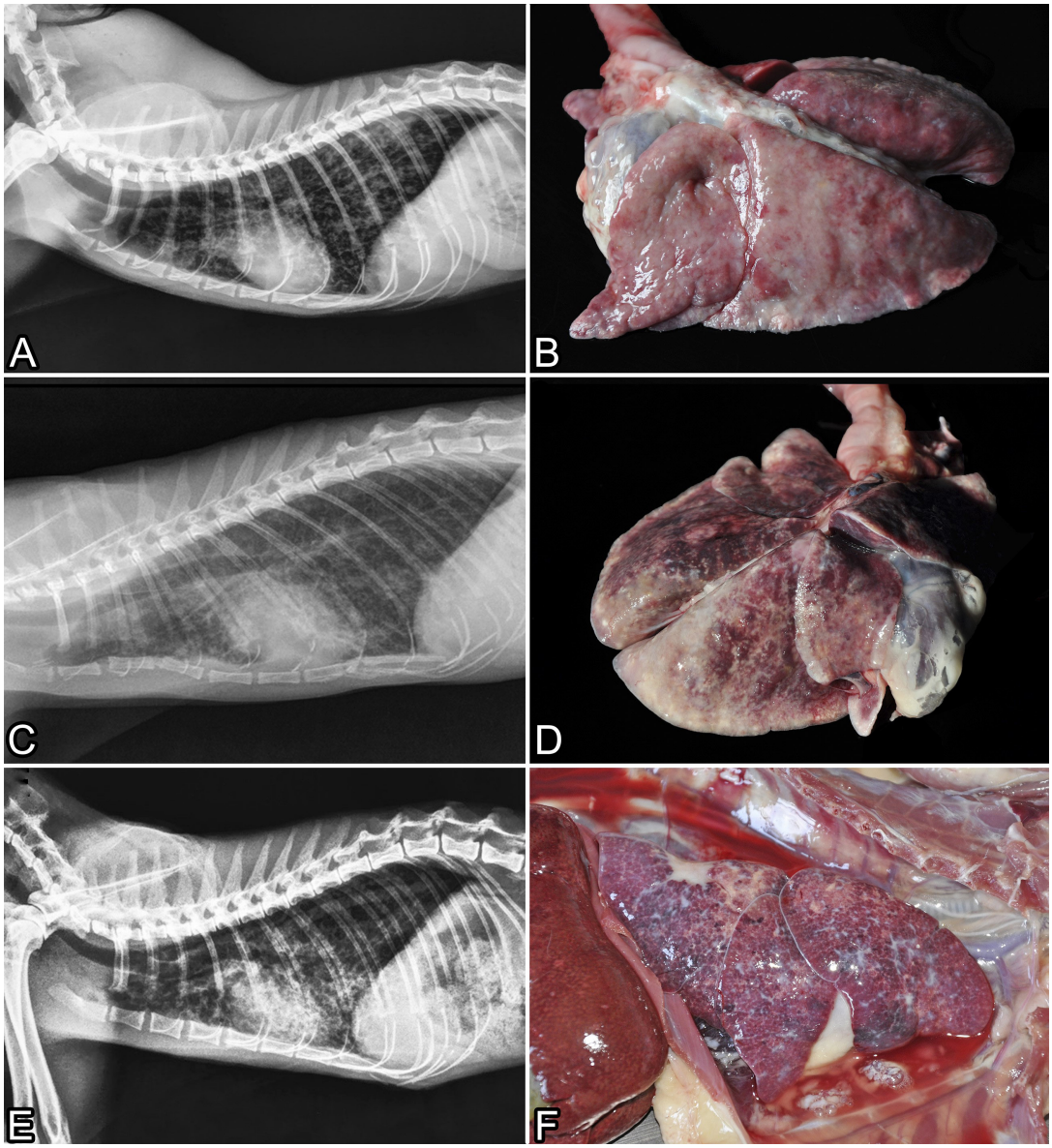

Fig.1. Radiographic and macroscopic findings in 3 cats with idiopathic pulmonary fibrosis. (**A**) Chest radiography from Cat 1 showing a moderate diffuse increase in radiopacity in the lung fields and a mixed bronchointerstitial to alveolar pattern, with a predominant bronchial pattern. (**B**) Macroscopic features of the lungs of Cat 1 with idiopathic pulmonary fibrosis. The lungs show multifocal-to-coalescing whitish areas and were firm with a slightly nodular appearance on the pleural surface. (**C**) Chest radiography from Cat 2 with a moderate diffuse increase in radiopacity in the lung fields and a mixed bronchointerstitial to alveolar pattern, with a predominant bronchial pattern. (**D**) Macroscopic features of the lungs of Cat 2 with idiopathic pulmonary fibrosis, similar to Cat 1. (**E**) Chest radiography from Cat 3 showing a severe diffuse increase in radiopacity in the lung fields and a mixed bronchointerstitial to alveolar pattern. (**F**) Macroscopic features of the lungs of Cat 3. The thoracic cavity had serosanguinous effusion, and the lungs showed multifocal-to-coalescing whitish areas and were firm with a multifocal slightly nodular appearance on the pleural surface.

with prescriptions of analgesics, corticosteroids and protective medications in addition to force-feeding. Fourteen days after the first consultation, the patient returned presenting with the same symptoms. She was referred a fine-needle aspirate of the lung for cytological evaluation. The patient was administered Zoletil and methadone as preanesthetic medications and propofol for induction, and maintenance anesthesia was provided with inhaled isoflurane. During the procedure, fluid therapy rate of 5ml/kg/h was maintained. The patient was placed in lateral recumbency and, after clipping and antisepsis with 2% chlorhexidine, a 25G x 7/8 needleattached to a 10mL syringe was inserted into the tenth dorsal intercostal space, according to previous planning after thoracic radiography was performed. Aspiration was performed with repeated movements and negative pressure. On cytological analysis, the sample had low cellularity and was composed of rare well-differentiated spindle cells and yielded an inconclusive result. After the fine-needle aspiration, the patient decompensated and progressed to death.

Animal number 2 was a 14-year-old female spayed mixed breed cat. She presented with inappetence, weight loss and mild respiratory distress. The patient had been treated with two antimicrobials with no clinical improvement. On physical examination, she was tachypneic with slight bilateral pulmonary crackles auscultated during cardiopulmonary auscultation. Blood samples were collected for hematology and biochemical analysis (Table 2), and chest radiography revealed a moderate diffuse increase in radiopacity in the lung fields with a mixed bronchointerstitial to alveolar pattern, with a predominant bronchial pattern (Fig.1C). She was referred for pulmonary biopsy by thoracoscopy; she was administered Zoletil and methadone as preanesthetic medications and propofol for induction, and maintenance anesthesia was provided with inhaled isoflurane. During the procedure, a fluid therapy rate of 5ml/kg/h was maintained. Analgesics and corticosteroids were administered after the procedure. The patient died within less than 24 hours after the surgical procedure.

Animal number 3 was a 10-year-old female spayed mixed breed cat. She presented with a complaint of dyspnea, sneezing for two days and weight loss. On physical examination, she was hypothermic (35°C) and moderately dehydrated, and had a low body condition score, mixed dyspnea, pulmonary crackles auscultated during cardiopulmonary auscultation. Chest radiography demonstrated a severe diffuse increase in radiopacity in the lung fields and a mixed bronchointerstitial to alveolar pattern (Fig.1E). Blood samples were collected for hematology and biochemical analysis (Table 2). The patient was referred for hospitalization and treatment with bronchodilators, corticosteroids, analgesics, oxygentherapy and nebulization with physiological saline solution. The cat remained hypothermic and dyspneic for two days and progressed to death.

Pathological and immunohistochemical findings

At necropsy, in all three cats, the lungs had whitish areas and were firm with a slightly nodular appearance on the pleural surface; they also failed to completely collapse when the thorax was opened. In the pleural region, there were multifocal star-shaped scarring lesions, with parenchymal retraction (Fig.1A-C). When the lung was cut, whitish multifocal areas were observed, and the parenchyma exhibited low crepitation. No significant changes were observed in other organs. Cats 2 and 3 had mild to moderate pleural effusion (hydrothorax) (Fig.1F).

Microscopically, the pulmonary parenchyma exhibited marked proliferation of fibrous connective tissue that was distributed in a multifocal-to-coalescing pattern, with more pronounced areas in the subpleural region (Fig. 2A). There was marked hypertrophy of the terminal bronchiolar musculature and marked multifocal proliferation of type II pneumocytes (Fig.2B,C), rarely forming syncytial cells. Occasionally, there was a marked infiltration of macrophages with a broad, sometimes foamy, cytoplasm and cellular debris within the alveoli, as well as discrete multifocal interstitial lymphocytic inflammatory infiltrates, moderate congestion and alveolar edema. In the pleural region, a proliferation of mesothelial cells was observed, especially in areas with marked subpleural fibrosis. Within the bronchi, moderate multifocal accumulation of mucinous material and cellular debris were observed. Multifocal areas of alveolar rupture (alveolar emphysema) that formed large voids were observed. On MT staining, the fibrous connective tissue proliferation stained was marked blue (Fig.2D), and the remainder of the pulmonary parenchyma, stained red.

On IHC, the proliferative spindle cells were remarkably reactive for vimentin (Fig.3A) and actin, which was also observed in the hyperplastic smooth muscle (Fig.3B). On IHC for pancytokeratin, there was an intense proliferation of type II pneumocytes around the alveoli (Fig.3C), and on IHC for lysozyme, an accumulation of alveolar macrophages was observed (Fig.3D).

There was no growth on bacteriological or mycological cultures, and immunohistochemical evaluations for the presence of viral etiological agents (FIV, FeLV, FCoV, FCV and FHV-1) was also negative.

DISCUSSION

The diagnosis of idiopathic pulmonary fibrosis in the patients in this study was based on clinical, radiographic, pathological and immunohistochemical findings. All three cats were females ranging in age from 10 to 14 years old, which is the common age group of cats affected by this disease (Cohn et al. 2004). As in humans, cats with pulmonary fibrosis are older, reflecting the insidious pathogenesis of the disease (Cohn et al. 2004). Because the number of included cats was small, it was not possible to determine sex or breed predilection; however, the three patients in this study were females, as previously described in several other studies (Le Boedec et al. 2014, Evola et al. 2014, Pereira et al. 2016).

These cats had similar clinical signs, characterized by anorexia, lethargy and respiratory distress with acute progression, similar to the previously reported clinical signs (Cohn et al. 2004). Although this disease is characterized as a chronic condition, the clinical course of patients with idiopathic pulmonary fibrosis may be short due to the ability of these animals to mask pulmonary clinical signs (Cohn et al. 2004). The radiographic changes were similar in the cats in this study, with adiffuse increase in radiopacity lung fields and a mixed bronchointerstitial pattern progressing to an alveolar pattern; however, some authors have stated that diffuse and heterogeneous lesions pattern are atypical in cats with fibrosis (Le Boedec et al. 2014). However, the imaging features of this disease are variable (Cohn et al. 2004, Evola et al. 2014) and may include a predominantly diffuse bronchointerstitial

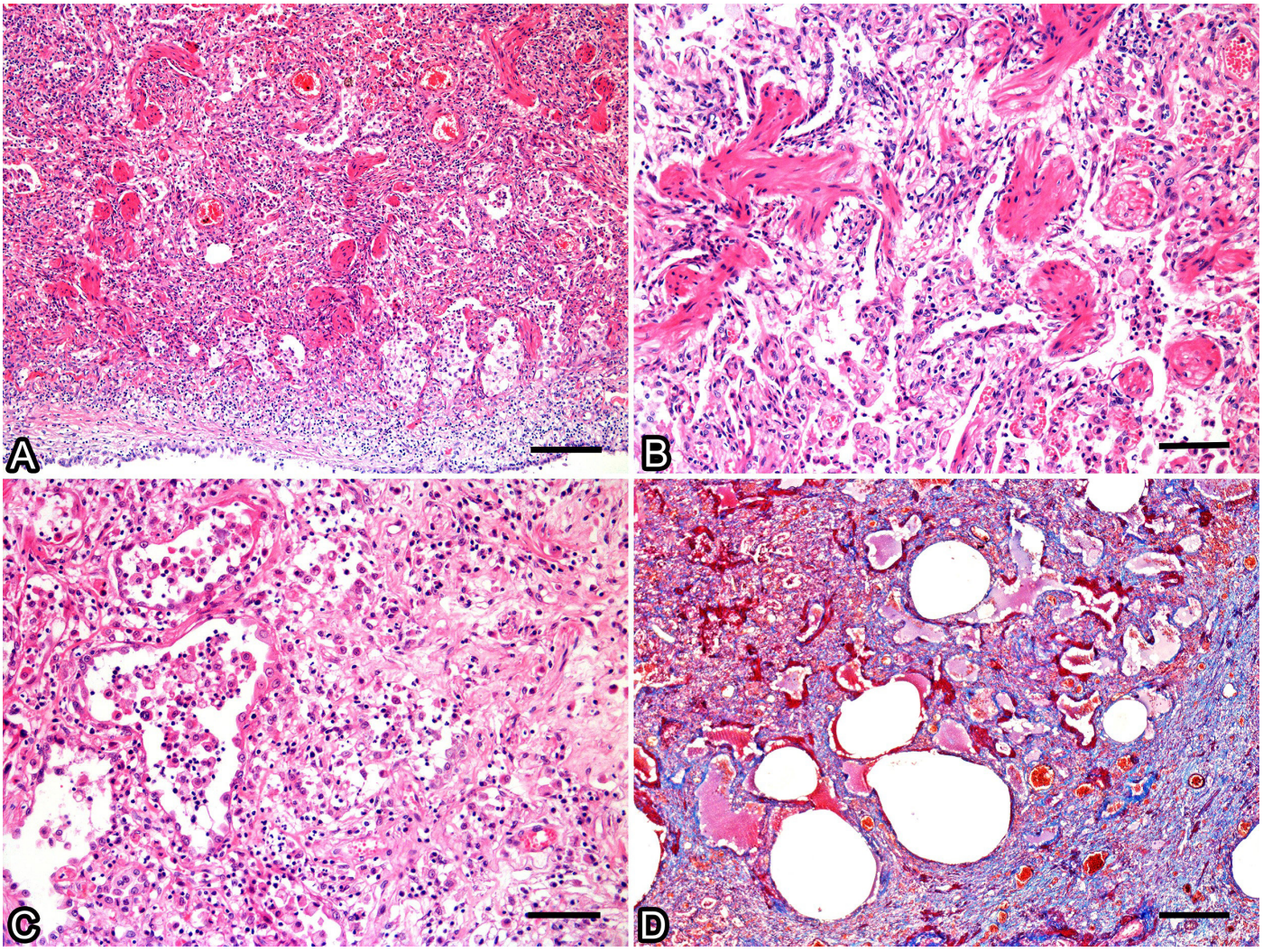

Fig.2. Histological features of idiopathic pulmonary fibrosis in felines. (**A**) Pulmonary parenchyma showing marked proliferation of spindle cells, especially in the subpleural region, associated with smooth muscle hypertrophy and reactive mesothelial cells. HE, bar = 250µm. (**B**) Marked hypertrophy of the terminal bronchiolar musculature and marked multifocal proliferation of type II pneumocytes. HE, bar = 130µm. (**C**) Marked multifocal proliferation of type II pneumocytes, infiltration of macrophages with a broad, sometimes foamy, cytoplasm and cellular debris within the alveoli, as well as proliferation of fibrous connective tissue. HE, bar = 130µm. (**D**) Pulmonary parenchyma showing a marked proliferation of fibrous connective tissue, distributed in a multifocal-to-coalescing form. MT, bar = 500µm.

to alveolar pattern, as seen in this study. Pleural effusion, pulmonary nodules and mineralization are rarely observed (Evola et al. 2014); however, in the present study, two cats had mild to moderate pleural effusion. In terms of blood test results, there are no significant hematological alterations in both cats and humans (Cohn et al. 2004).

The clinical presentation of this disease is similar to other pulmonary diseases in felines and due to the variation in radiographic findings and the absence of hematological and biochemical alterations, the clinical diagnosis is challenging (Cohn et al. 2004). Procedures, such as bronchoscopy, were not useful for diagnosis in a previous study (Cohn et al. 2004). Pulmonary aspiration may result in low cellularity samples because fibroblasts are juxtaposed cells, as observed in Cat 1. To achieve a definitive diagnosis histopathological evaluation of the lung is necessary (Cohn et al. 2004, Pereira et al. 2016). However, because the treatment of the disease is limited, patients usually die after such an invasive diagnostic procedure, as occurred in two patients in this study (Cohn et al. 2004, Le Boedec et al. 2014). Oxygen therapy, bronchodilators, antimicrobials, diuretics and immunosuppressive drugs are used with no success or with a very short therapeutic response. Usually, the disease progresses very fast, leading to worsening respiratory conditions despite therapeutic attempts, as in the cases reported (Cohn et al. 2004, Evola et al. 2014).

The cats in this study were domesticated and healthy; they did not have a historic of previous pulmonary disease. Based on IHC examinations, it was confirmed that these cats were negative for FIV, FeLV, FHV-1, FCV and FCoV, and there was no significant growth on bacteriological or mycological cultures, excluding the possibility of these infectious agents in the disease. As in humans, the pathogenesis of the disease

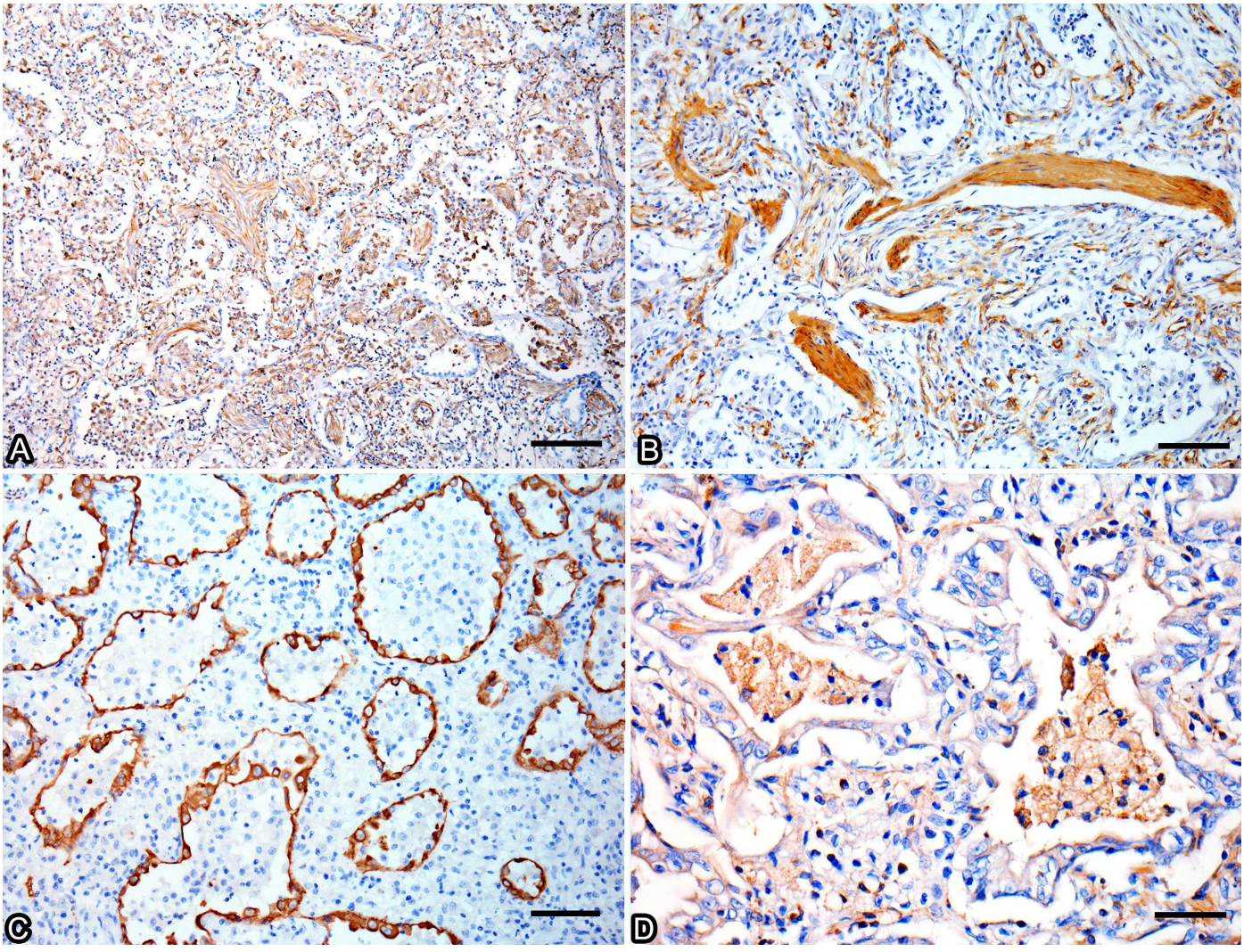

Fig.3. Immunohistochemical evaluation of idiopathic pulmonary fibrosis in felines. (**A**) Intense intracytoplasmic diffuse staining of mesenchymal cells for vimentin. IHC and DAB, bar = 210µm. (**B**) Moderate and diffuse intracytoplasmic staining for smooth muscle actin. IHC and DAB, bar = 120µm. (**C**) Proliferation of type II pneumocytes around heavily immunoreactive alveoli for pancytokeratin. IHC and DAB, bar = 100µm. (**D**) Alveolar macrophages reactive for lysozyme. IHC and DAB, bar = 60µm.

in domestic felines has not been well elucidated. It has been suggested that, in humans, the onset of the condition is associated with pulmonary injury (Gross & Hunninghake 2001). Latent viral infection, particularly herpes viral infections, also has been reported as possible causes of pulmonary fibrosis (Gross & Hunninghake 2001). The same has been suggested for cats; however, the animals in this study were negative for the FHV-1 infection and were not previously diagnosed with any pulmonary parenchymal diseases that could have caused injury, such as feline asthma, pneumonia or neoplasia. Moreover, chemotherapy with nitrosourea for the treatment of intestinal lymphoma in felines has also been indicated as a cause of pulmonary fibrosis (Skorupski et al. 2008); however, none of the cats in this study had been treated with this medication. The microscopic and structural characteristics of type II pneumocytes observed in cats with the condition are similar to the familial form of this disease in humans, which occurs because of a defect in the gene encoding the surfactant protein C, suggesting a genetic component to the development of this disease (Van Moorsel et al. 2010).

Pulmonary fibrosis may be observed in others domestic animals, such as in West Highland White Terriers dogs. These may present with a chronic progressive interstitial fibrosing lung disease of unknown etiology, with poor prognosis and no response to treatment (Heikkilä et al. 2011). The main finding of this condition is a diffuse interstitial fibrosis of the lung (Heikkilä et al. 2011), which is similar to that seen in cats affected by the same condition. Moreover, pulmonary fibrosis may be caused by specific etiological agents, such as the equine herpesvirus-5 (Panziera et al. 2014), popularly called equine multinodular pulmonary fibrosis, that is a chronic infectious disease characterized by interstitial fibrosis of the lung in horses (Williams et al. 2007). In humans, it is known that the inhalation of silica and asbestos may result in the development of fibrotic nodules (Oberdorster 1996). Interstitial fibrosis with concentric collagen deposition may occur in the lung of animals subsequently to chronic

pulmonary hypertension, a condition caused by increased pulmonary vascular resistance or venous congestion (Caswell & Williams 2016). Moreover, additional histological changes of chronic pulmonary hypertension include intimal and medial hypertrophy in the pulmonary arterioles, as well as alveolar hemosiderophages (Caswell & Williams 2016).

Macroscopically, the lungs were firm with diffusely distributed lesions in the pulmonary parenchyma (Cohn et al. 2004). The lesions were whitish and interspersed with areas of reddish color (mottled appearance). Other diseases may have similar appearance, such as chronic fungal or bacterial pneumonia and neoplasia (Le Boedec et al. 2014). There is a known association between pulmonary fibrosis and pulmonary carcinoma (Aubry et al. 2002). There are several theories that have explained this association, including the progression of epithelial hyperplasia to neoplasia and the induction of carcinogenesis by chronic inflammation (Bouros et al. 2002).

The main microscopic lesion seen in cats with this disease is pulmonary fibrosis, as well as smooth muscle hyperplasia and type II pneumocyte proliferation (Cohn et al. 2004). In all cases in this work, there was intense IHC staining for smooth muscle actin in proliferative spindle cells, suggesting the presence of myofibroblasts in the lesion. In most organs, tissue injury activates local fibrocytes that differentiate into contractile myofibroblasts, characterized by the expression of smooth muscle actin (Masseno et al. 2010). IHC for pancytokeratin showed a marked proliferation of type II pneumocytes around the alveoli. It is known that type II pneumocytes proliferate in order to repair injured tissue. Pneumocytes hinder gas exchange because these cells are cuboidal. This cuboidal form may no longer be observed on microscopy five to seven days after a mild injury, but it can remain present for a long period, in cases in which the injurious stimulus continues or in the presence of interstitial fibrosis (Caswell & Williams 2016). All of these findings suggest that pulmonary fibrosis in cats may be related to scarring from lung injury, whether the injury is ongoing or not. Although it is a routine in cat clinics to care for dyspneic cats, patients often die acutely without a diagnosis. Thus, it is necessary to consider pulmonary fibrosis as a differential diagnosis in cats with dyspnea, to increase the understanding of the pathogenesis of this disease and to determine an adequate prognosis and effective therapy in the future.

CONCLUSIONS

The animals were domesticated females ranging in age from 10 to 14 years old. The main clinical signs observed were mixed restrictive dyspnea, mild dehydration, and anorexia, and the clinical course lasted less than one month. The main radiographic findings were a mixed bronchointerstitial to alveolar pattern and a moderate diffuse increase in radiopacity in the lung fields.

Macroscopically, the lungs were firm with whitish areas and a slightly nodular appearance on the pleural surface, and in all cases, star-shaped scarring lesions with parenchymal retraction were present. Microscopically, the main findings were pulmonary fibrosis, type II pneumocyte proliferation, and hypertrophy or hyperplasia of smooth muscle tissue. On IHC, it was confirmed that the cats were negative for the main viral agents of the species.

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

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Original Article
Small Animals Diseases

Sedative and electrocardiographic effects of low dose dexmedetomidine in healthy cats[1]

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ABSTRACT.- Carvalho E.R, Champion T., Vilani R.G.O.C., Freitas G.C., Ambrosini F., Silva G.A., Gonçalves K.S. & Fischborn J.C.J. 2019. **Sedative and electrocardiographic effects of low dose dexmedetomidine in healthy cats.** *Pesquisa Veterinária Brasileira 39(2):142-171.* Universidade Federal da Fronteira Sul, Rodovia BR-182 Km 466, Avenida Edmundo Gaievski 1000, Cx. Postal 253, Realeza, PR 85770-000, Brazil. E-mail: beth_rcarvalho@hotmail.com

In feline veterinary practice sedation is often needed to perform diagnostic or minimally invasive procedures, minimize stress, and facilitate handling. The mortality rate of cats undergoing sedation is significantly higher than dogs, so it is fundamental that the sedatives provide good cardiovascular stability. Dexmedetomidine (DEX) is an α2-adrenergic receptor agonist utilized in cats to provide sedation and analgesia, although studies have been utilized high doses, and markedly hemodynamic impairments were reported. The aim of this study was to prospectively investigate how the sedative and electrocardiographic effects of a low dose of DEX performing in cats. Eleven healthy cats were recruited; baseline sedative score, systolic arterial pressure, electrocardiography, and vasovagal tonus index (VVTI) were assessed, and repeated after ten minutes of DEX 5μg/kg intramuscularly (IM). A smooth sedation was noticed, and emesis and sialorrhea were common adverse effects, observed on average seven minutes after IM injection. Furthermore, electrocardiographic effects of a low dose of DEX mainly include decreases on heart rate, and increases on T-wave amplitude. The augmentation on VVTI and appearance of respiratory sinus arrhythmia, as well as sinus bradycardia in some cats, suggesting that DEX enhances parasympathetic tonus in healthy cats, and therefore will be best avoid in patients at risk for bradycardia.

INDEX TERMS: Sedative, electrocardiographic effects, dexmedetomidine, healthy cats, α2-agonist, bradycardia, feline, sedation, T-wave, cats.

RESUMO.- [**Efeitos sedativos e eletrocardiográficos da baixa dose de dexmedetomidina em gatos saudáveis.**] Na rotina clínica da medicina veterinária felina a sedação é frequentemente requerida para realização de procedimentos diagnósticos ou minimamente invasivos, para minimizar o estresse e facilitar o manuseio dos pacientes. A taxa de mortalidade de gatos submetidos à sedação é mais elevada do que em cães, por esse motivo, é fundamental que os sedativos confiram estabilidade hemodinâmica. A dexmedetomidina (DEX) é um α2-agonista utilizado em felinos para promover sedação e analgesia, porém os estudos têm utilizado doses elevadas, e com isso prejuízos hemodinâmicos importantes foram relatados. O objetivo desta investigação foi avaliar os efeitos sedativos e eletrocardiográficos da baixa dose de DEX em gatos. Para tal, onze felinos saudáveis foram recrutados, foram obtidos valores basais para escore de sedação, pressão arterial sistólica e eletrocardiografia, além do índice de tônus vaso vagal (ITVV). Após dez minutos da aplicação intramuscular (IM) de DEX 5μg/kg todos os exames foram repetidos. Após a DEX, sedação suave foi detectada, e a êmese e sialorreia foram efeitos adversos comuns, observados em média 7 minutos após a injeção IM. Ademais, os principais efeitos eletrocardiográficos foram redução na frequência cardíaca e aumento na amplitude da onda T. O ITVV mais elevado e surgimento de arritmia sinusal respiratória, bem como bradicardia sinusal em alguns gatos, sugerem que a

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DEX eleva o tônus parassimpático, e por esse motivo deve ser utilizada com cautela em pacientes com predisposição à bradicardia.

TERMOS DE INDEXAÇÃO: Sedativos, eletrocardiografia, dexmedetomidina, gatos saudáveis, α2-agonista, bradicardia, felinos, sedação, onda T.

INTRODUCTION

In feline veterinary practice sedation is often needed to perform diagnostic or minimally invasive procedures, minimize stress, and facilitate handling. An ideal sedation protocol allows for quick and smooth decreased responsiveness while maintaining cardiopulmonary function and providing quiet recovery (Cremer & Riccó 2017). The mortality rate of cats undergoing sedation is significantly higher than dogs (0.12% vs 0.07%), and one of limitations is due to sedation monitoring of physiologic variables is usually more limited/underestimated than under general anesthesia, so it is important that the sedatives provide good cardiovascular stability (Brodbelt et al. 2008).

Sedation and analgesia are prominent effects of central α2-adrenergic receptor activation, and these effects have been reported with agents such as xylazine, medetomidine, and romifidin in cats (Granholm et al. 2006). Dexmedetomidine (DEX) is an highly selective α2-adrenergic receptor agonist, the active enantiomer of racemic medetomidine, that induces dose-dependent sedation, analgesia and muscle relaxation in cats (Ansah et al. 1998). However, studies reporting sedative effects of this drug in cats mainly utilized high doses of DEX (10- 75μg/kg), and indeed marked decreases in heart rate, cardiac output, and transient changes in blood pressure were noticed (Ansah et al. 1998, Selmi et al. 2003, Granholm et al. 2006), moreover the electrocardiographic effects of DEX has not been completely characterized in this species.

The electrocardiography (ECG) is a widely used exam in veterinary medicine, mainly to detect/exclude arrhythmias, as part of pre-anesthetic evaluation, and/or cardiac monitoring in patients under intensive care unit, or during general anesthesia. In cats, additional clinical applications include assessment of cardiac dimensions, for which an ECG is a poor substitute for an echocardiogram (Pellegrino et al. 2016), and monitoring of extracardiac disturbances that could possible lead to cardiac impairments, such as hyperkalemia in cats with urethral obstruction, oliguric/anuric renal failure, reperfusion injury (Schaer 1977, Côté 2010, Garcia de Carellan Mateo et al. 2015), or hypoxemia (Boyden 1992). The aim of this study was to prospectively investigate how the sedative and electrocardiographic effects of a low dose of DEX performing in healthy cats.

MATERIALS AND METHODS

Study design and ethics statement. This was a prospective cohort study, performed with the ethical approval of the Federal University of Fronteira Sul Committee for Animal Experimentation (protocol number 23205.004198/2017-56).

Animals. Eleven client-owned adult domestic shorthair male cats were recruited for the study. Cats were considered healthy based on clinical examination, routine hematology, systolic arterial pressure (SAP), electrocardiography (ECG), and echocardiography - in order to exclude heart diseases. These values were within published reference intervals for complete blood count (Feldman et al. 2000), SAP (Brown et al. 2007), ECG (Tilley & Burtinick 1999), and echocardiography (Boon 2011). Animals were fasted for 12 hours, but had free access to water until 2 hours prior to sedation.

Baseline sedative score. On the day of the experiment each cat was weighed, a physical examination was performed and they had their hair clipped on right and left thoracic limbs palmar faces. Cats were acclimated to cardiology exam room during 30 minutes before measurements. The baseline sedative score was assessed by a single and experienced anesthesiologist, using a subjective scoring criteria proposed by Granholm et al. 2006 to evaluate sedation on cats treated with DEX or medetomidine. This scoring criteria ranges from zero to twelve, where the biggest score corresponds to deeper sedation, and takes into account spontaneous posture, response to noise, muscle tone of jaw and tongue, as well as pedal withdrawal response to pinching of a digit or interdigital web.

Baseline SAP measurement. After evaluation of sedative score, the animal was gently positioned on right lateral recumbence, a cuff size corresponding to 30-40% of the distal radius diameter was utilized (Brown et al. 2007), and SAP was obtained with a vascular Doppler (Medmega, Franca, Brazil) attached to a sphygmomanometer. Five consecutive measurements were made, minimum and maximum values were excluded and a mean of the other three was recorded.

Baseline electrocardiographic assessment. Posteriorly, also on right lateral recumbence (Harvey et al. 2005), a six leads ECG (ECGPC TEB, Tecnologia Eletrônica Brasileira, São Paulo, Brazil) was recorded over two-minutes. In order to obtain the bipolar leads I, II and III, as well as increased unipolar leads aVR, aVL and aVF, the right (red) and left (yellow) thoracic electrodes were fixed above the olecranon region, and the right (black) and left (green) pelvic electrodes above the patellar ligaments (Tilley 1992), alcohol 70% was instilled among skin and electrodes to improve electric recipiency. The register speedy was adjusted to 50mm/s, and calibration of 1mV=1cm. All measurements were made in triplicate, on lead II, by a single and experienced observer, as follows: predominant heart rhythm, heart rate (HR), P-wave width and amplitude, PR interval, QRS complex width, R-wave amplitude, QT interval, T-wave polarity and width, and ST leveling and morphology, according previously described (Tilley & Burtinick 1999). The rate-corrected QT interval (QTc) was calculated from the following equation (Van de Water et al. 1989): $QTc=QT–0.087(RR-1000)$. Furthermore, the ECG tracings were reviewed to presence of arrhythmias (atrioventricular blocks of second and/or third degrees, ventricular ectopic beats, atrial premature contractions, junctional P-waves or junctional ectopic beats), as described elsewhere (Tilley 1992, Tilley & Burtinick 1999). Also on lead II, the first 20 consecutive R-R intervals in which cardiac rhythm was of sinus origin were used to calculate vasovagal tonus index (VVTI) for each patient. The index was obtained by calculating the natural logarithm of the variance of the 20 measured R-R intervals, as described by the equation VVTI=NL[VAR(R-R1 - R-R20)], where NL: natural logarithm, VAR: variance (Häggström et al. 1996).

Sedation. After all baseline measurements previously described, cats received 5μg/kg of DEX (Dexdormitor, Zoetis) intramuscularly (IM) and were housed in cages on cardiology exam room during ten minutes. Adverse effects as emesis and sialorrhea were recorded. Ten minutes apart from IM injection, the sedation score was accessed, followed by SAP and ECG recording, as described above.

Statistical analysis. All analyses were performed using the software GraphPad Prism (Version 5.0 - San Diego/CA, USA). The D'Agostino and Pearson omnibus normality test was used to investigate data distribution. Comparisons between baseline measurements and

post-sedation were accomplished by either Mann-Whitney test or Student's t-test, according to distribution. Associations between qualitative variables were analyzed with Fisher' exact test. Correlation among HR and VVTI was accomplished by Pearson test. Statistical significance was defined as P<0.05.

RESULTS

Baseline mean sedation score was 0 (ranging from 0 to 0, minimum and maximum), and 3 (ranging from 0 to 8, minimum and maximum) post sedation (P=0.0022). None of animals achieved lateral recumbency after low dose of DEX. The baseline SAP was 118±9mmHg, and 130±14mmHg after sedation (P=0.0421). Six cats (55%) exhibited sialorrhea and emesis after 3 to 15 minutes of DEX injection, between then, two animals (33%) had a second episode of vomiting 5 to 8 minutes apart.

Descriptive statistics of electrocardiographic assessment in healthy cats submitted to sedation with dexmedetomine is shown in Table 1. The HR was considered different among moments (P=0.0028), being significantly slower after DEX. The T-wave amplitude increased after sedation (P=0.0236), although no cat presented a T-wave>0.3 mV. Concerning the T-wave polarity, two patients (18%) changed the polarity after DEX (one cat had a biphasic T-wave that turned only positive after sedation, and another had a negative T-wave that turned positive), but no statistical difference was detected (P=0.4762). No changes on ST segment were seen after DEX.

At baseline all animals presented sinus rhythm, while after DEX four cats (36%) presented respiratory sinus arrhythmia (RSA), defined as a naturally occurring variation of R-R interval bigger than 20% during breathing cycle (Tilley & Burtinick 1999), and three animals (27%) became bradycardic (88-94bpm), defined as HR<100 bpm (Tilley & Burtinick 1999). Figure 1 illustrates a RSA detected post sedation.

After DEX, the VVTI was considered bigger than it was at baseline (P=0.0433), as shown in Figure 2. All the other electrocardiographic measurements were found to be similar between moments. VVTI and HR were not considered correlated (P=0.0564; R = -0.47; 95% of confidence interval = -0.76 to 0.01).

Table 1. Descriptive statistics of electrocardiographic assessment in healthy cats submitted to sedation with dexmedetomidine (DEX) 5µg/kg IM. Parametric data are shown as mean ± standard deviation, while non-parametric variables are represented as median (interquartile range)

Measurement	P	Baseline	After DEX
Heart rate (bpm)	0.0028	187 (132-201)	96 (89-126)
P (ms)	0.9439	47 (43-48)	47 (43-49)
PR (ms)	0.1013	72 ± 9	79 ± 6
QRS (ms)	0.6147	47 (43-55)	47 (43-48)
QT (ms)	0.1869	183 ± 25	200 ± 29
QTc (ms)	0.9616	239 ± 25	238 ± 22
P (mV)	0.3756	0.13 (0.08-0.14)	0.09 (0.09-0.12)
R (mV)	0.1496	0.48 ± 0.17	0.56 ± 0.15
T (mV)	0.0236	0.10 (0.07-0.14)	0.18 (0.12-0.25)
VVTI	0.0433	2.68 ± 0.60	3.34 ± 0.86

VVTI = Vasovagal tonus index.

DISCUSSION

This study showed that a low dose of DEX on pre-medication produces a smooth sedation in cats (average 3 points), when a subjective scoring criterion graduated from zero to twelve was utilized. None of animals achieved lateral recumbency, and physical restrainment to perform ECG after sedation was laborious in three cats (two scored zero, and another scored one point). Limited data are available on the sedative effects with low doses of DEX alone in cats, most of them are reports with the labeled dose, ranging from 20 to 40µg/kg (Johard et al. 2018, Martin-Flores et al. 2018). In another study, Selmi et al. 2003 reported a satisfactory sedation and lateral recumbence in all cats after DEX 10µg/kg IM. Previously studies suggesting that sedation occurs in a dose related manner (Ansah et al. 1998, Johard et al. 2018, Martin-Flores et al. 2018), and associations with opioids (Johard et al. 2018) and/or ketamine (Cremer & Riccó 2017) promote more intense sedative effects in cats.

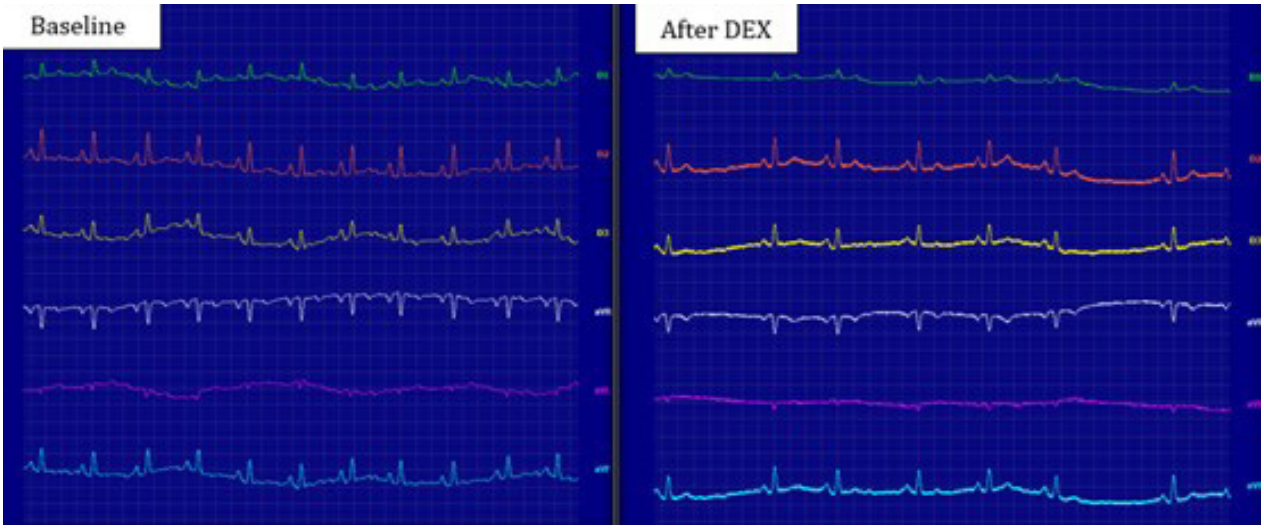

Fig.1. Heart rhythm of a cat from this study. At baseline it was recorded a sinus rhythm and heart rate of 187bpm, after ten minutes of dexmedetomidine 5µg/kg IM it was noticed a respiratory sinus arrhythmia (RSA) and heart rate ranging from 108-139bpm. The RSA is not considered a physiological heart rhythm in cats. Electrocardiographic tracings were both recorded at 50mm/s and 2N amplitude.

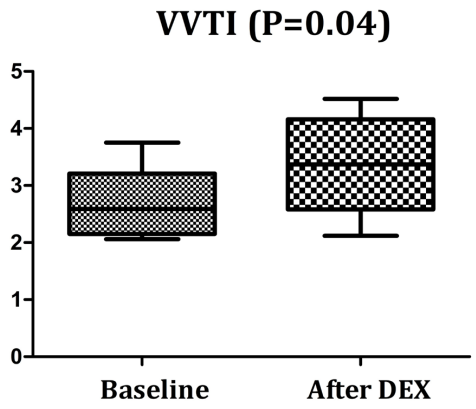

Fig.2. Box plot depicting the medians, interquartile ranges and amplitude of vasovagal tonus index (VVTI) in healthy cats at baseline and after being submitted to sedation with dexmedetomidine 5µg/kg IM.

The incidence of emesis on this investigation was considerable higher than the 7% anteriorly reported with high dose (40µg/kg IM) administration (Granholm et al. 2006). Similarly, Selmi et al. 2003 did not noticed emetic events after 10µg/kg IM in healthy cats. However, another group of researchers showed that 78% of cats sedated with DEX (4 µg/kg) plus buprenorphine (20µg/kg) IM vomited at 0 to 13 minutes post-injection (Santos et al. 2011). The fasting period was 12 hours on both ours and the above-mentioned experiments.

When DEX binds to α2-adrenergic receptor on the vascular smooth muscle, systemic vascular resistance increases (Ruffolo Junior 1985, Duka et al. 2000) ultimately leading to increases on systemic arterial blood pressure (Bloor et al. 1992, Martin-Flores et al. 2018). After pre-medication, SAP significantly increased (130±14mmHg) from baseline (118±9 mmHg). Overall, this smooth rise is well tolerated in healthy animals, and SAP <150mmHg is considered by the American College of Veterinary Internal Medicine - *Guidelines for the identification, evaluation, and management of systemic hypertension in dogs and cats* - as minimal risk of future target organ damage (Brown et al. 2007). Although, it seems reasonable that caution should be taken in cats already hypertensive, as frequently observed in hyperthyroidism (Stiles et al. 1994) and chronic kidney disease (Stiles et al. 1994, Sander et al. 1998), despite no studies have been addressed to the use of DEX in such individuals, at author's knowledge.

The vasopressor action of DEX increases arterial and pulmonary pressures, leading to reflex bradycardia (Devcic et al. 1994, McSweeney et al. 2012). According to our findings, the concomitant decrease in HR and increases in SAP after DEX might suggest a reflex phenomenon in cats. Moreover, it has been reported that this drug decreases sympathetic nervous system tone and increases parasympathetic nervous system activity within the central nervous system, decreasing both GABAergic and glycinergic inhibitory input to cardiac vagal neurons, which may contribute to the bradycardia (Sharp et al. 2014).

The VVTI is an useful time domain indicator of heart rate variability obtained from the standard ECG, being mainly influenced by the parasympathetic tone, as recognized in previous studies (Häggström et al. 1996, Pereira et al. 2008, Kocabaş et al. 2009, Brüler et al. 2017, Pecceu et al. 2017). Indeed, the lower HR and the higher VVTI seen after DEX in this study are indicative of increased parasympathetic tonus. In addition, the RSA noticed in some cats after sedation is another evidence that a parasympathetic activation was markedly present (Wardlaw 1985). The RAS is a regular irregular rhythm, considered a physiologic heart rhythm in dogs (Tilley 1992, Tilley & Burtinick 1999) and healthy human beings (Cooke 1998, Sturgeon et al. 2014). In cats at the clinical setting, RAS is not normally seen, and is usually considered pathologic (Rishniw & Bruskiewicz 1996), once normal rhythms in healthy subjects include sinus rhythm and sinus tachycardia (>240bpm) due to handling excitement (Tilley & Burtinick 1999). However, some studies have indicated that healthy cats in their home environment (Hanås et al. 2009) or under general anesthesia (Lewis et al. 2013) commonly have periods of RSA.

It was already well characterized that DEX significantly depressed sinus and atrioventricular nodal function in human pediatric patients (Hammer et al. 2008). However, it was shown in previously studies that DEX did not have a direct effect on ventricular or atrial refractoriness, and spontaneous atrioventricular block was not reported in patients with normal baseline atrioventricular nodal conduction (Hammer et al. 2008, Chrysostomou et al. 2010, Char et al. 2013). Similarly, it was found no difference between PR interval at baseline and post low dose of DEX, also all measurements were within reference values to cats (PR interval: 50-90 ms) (Tilley & Burtinick 1999). This finding differ from those reported with xylazine in dogs (Klide et al. 1975, Haskins et al. 1986), and romifidine administration in horses (Clarke et al. 1991, Freeman et al. 2002), once these two less selective α2-adrenergic receptor agonists promoted second degree atrioventricular block in such species.

On ECG, the T-wave represents rapid ventricular repolarization (i.e. phase 3) of the ventricular action potential (Issa et al. 2009). During phase 3, there is closure of the calcium channels, while the potassium channels remain open, resulting in rapid loss of positive charge from the cardiomyocytes and restoration of the resting membrane potential (Issa et al. 2009). As such, the configuration of the T wave is dependent on the spatial-temporal characteristics of ventricular repolarization (Lin et al. 2013). The T-wave in cats can be positive, negative, or biphasic (Tilley & Burtinick 1999). A relation between low dose of DEX in cats and direct effects of such drug over ventricular repolarization could be anticipated, although measurements remained within reference values (T wave <0.3mV). Furthermore, more studies should be addressed to investigate if this possible effect of DEX is indeed related to ventricular repolarization or not.

CONCLUSIONS

A low dose of dexmedetomidine (5µg/kg IM) alone produces a smooth sedation in cats, and handling to minimally invasive procedures could be difficult in non-collaborative animals. Emesis and sialorrhea are common adverse effects, observed on average seven minutes after intramuscularly (IM) injection. Even a low dose of DEX increases systolic arterial pressure in healthy cats, although nonehypertensive episodes were recorded.

Furthermore, electrocardiographic effects of a low dose of DEX mainly include decreases on heart rate, and increases on T-wave amplitude. The augmentation on vasovagal tonus index and appearing of respiratory sinus arrhythmia, as well as sinus bradycardia in some cats, suggesting that DEX enhances parasympathetic tonus in healthy cats, and therefore will be best avoid in patients at risk for bradycardia.

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

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Original Article
Wildlife Medicine

Risk of exposure of farms and subsistence nurseries to contact with wild boar in southern Mato Grosso do Sul[1]

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ABSTRACT.- Braz P.H., Oliveira M.R., Silva V.S., Tomas W.M., Juliano R.S., Moreira T.A., Zimmermann N.P. & Pellegrin A.O. 2019. **Risk of exposure of farms and subsistence nurseries to contact with wild boar in southern Mato Grosso do Sul.** *Pesquisa Veterinária Brasileira 39(2):148-154*. Universidade Federal de Mato Grosso do Sul, Cx. Postal 549, Campo Grande, MS 79070-900, Brazil. E-mail: pauloh.braz@hotmail.com

With the advancement of wild boar distribution in the rural environment, its impacts are not limited to health in the pig sector, but the requirements for monitoring and control of the species are requirements laid down by the OIE for the recognition of classical swine fever free zone status. The construction of ecological models of favorability or suitability for the occurrence of pest species are necessary tools for the decision making on priority areas of management aiming at risk management. This work aims to map the level of suitability for the occurrence of wild boar in the southern state of Mato Grosso do Sul, as well as to identify the main risk variables for contact with the wild boar and evaluate the biosecurity measures adopted by commercial farms integrated in the south of the State of Mato Grosso do Sul. To evaluate the risk potential of wild boar for commercial and subsistence swine farming in southern Mato Grosso do Sul, a model of environmental suitability was constructed for this species in the swine producing region. This model considered different environmental strata, being the selection of the layers considered the physiological and behavioral characteristics of the species. In parallel, interviews were carried out in a sample of commercial farms integrating the region to survey the perception of the presence of the invasive species and the biosafety measures adopted. The results of this work indicate that the risk of contact among wild boars and animals reared in closed production systems may be high in the study area and only establishment of appropriate biosecurity measures that consider the characteristics and habits of the boar may prevent the intrusion of this species and contact with domestic swine. The built model can be considered of high reliability and it is recommended to apply it to other areas of the state, being a useful tool for the productive sector, environmental agencies and decision makers.

INDEX TERMS: Exposure farms, nurseries, wild boar, Mato Grosso do Sul, landscape epidemiology, biosegurity, pig production system.

RESUMO.- [**Risco de exposição de granjas e criatórios de subsistência ao contato com javalis no sul do Mato Grosso do Sul.**] Com o avanço da distribuição do javali no ambiente rural, seus impactos não se restringem somente a sanidade suidea, embora as exigências quanto ao monitoramento e controle da espécie sejam exigências previstas pela OIE, para o reconhecimento do status de zona livre de peste suína clássica. A construção de modelos ecológicos de favorabilidade ou adequabilidade para a ocorrência de espécies-praga são ferramentas necessárias para as tomadas de decisão sobre áreas prioritárias de manejo visando gestão de risco. Este trabalho objetiva mapear o nível de adequabilidade para

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a ocorrência de javalis no sul do Estado de Mato Grosso do Sul, bem como levantar as principais variáveis de risco para o contato com o javali asselvajado e avaliar as medidas de biosseguridade adotadas por granjas comerciais integradas no sul do Estado do Mato Grosso do Sul. Para avaliar o potencial de risco exercido pelos javalis para a suinocultura comercial e de subsistência nesta região foi construído um modelo de adequabilidade ambiental para essa espécie na região produtora de suínos. Esse modelo considerou diferentes estratos ambientais, sendo que para a seleção das camadas consideram-se características fisiológicas e comportamentais da espécie. Em paralelo, entrevistas foram realizadas em uma amostragem de granjas comerciais de integração da região para levantamento da percepção quanto a presença da espécie invasora e as medidas de biossegurança adotadas. Os resultados desse trabalho indicam que o risco de contato entre javalis de vida livre e os animais criados em sistemas de produção fechados pode ser alto na área de estudo e somente estabelecimento de medidas de biosseguridade apropriadas, que considerem as características e hábitos do javali poderá impedir a intrusão dessa espécie e o contato com os suínos domésticos. O modelo construído pode ser considerado de elevada confiabilidade e recomenda-se a sua aplicação para as outras áreas do estado, sendo uma ferramenta útil para o setor produtivo, os órgãos ambientais e os tomadores de decisão.

TERMOS DE INDEXAÇÃO: Exposição de granjas, criatórios, javalis, Mato Grosso do Sul, epidemiologia espacial, biosseguridade, suíno cultura.

INTRODUCTION

Brazil occupies the fourth place in the global ranking of production and exportation of swine meat and represents 7.8% of the amount of swine meat exported around the world in the year of 2015, with an exportation of 555 thousand tons (ABPA 2017). Meanwhile, the state of Mato Grosso do Sul (MS), Brazil, slaughtered 1.4 million swine in 2015, reaching 127.1 thousand tons of meat, an increase of 7.1% over the previous year, according to data of the Federation of Agriculture and Livestock of Mato Grosso do Sul (Federação da Agricultura e Pecuária de Mato Grosso do Sul - Semagro 2016).

The main threats to the guarantee of access to the international market are the sanitary issues of production, which is why the production chain of swine farming has been guided by its actions to comply with the standards established by the World Organization for Animal Health (OIE). One of the main goals of this sector is the establishment and maintenance of the status of free zone of Classical Swine Fever (CSF), according to the sanitary code of the terrestrial animals, followed by the National Suidae Health Program (Programa Nacional de Sanidade Suídea - PNSS) (Salgado et al. 2015, Zanella et al. 2016). The PNSS provides biosecurity norms and control of some endemic diseases for Certified Suidae Breeder Farms (Granjas de Reprodutores de Suídeos Certificados - GRSC), while in other types of farms only some diseases listed by the OIE are predicted to control.

Biosecurity is defined as the implementation of measures that reduce the risk of introduction and spread of etiological agents in a herd or flock. The main elements of biosecurity are segregation, where barriers to limiting opportunities of entry of infected animals or contaminated materials into a healthy herd are created and maintained (FAO/OIE/WB 2010).

Normative Instruction (IN) No. 25 (Brasil 2016) of July 19th, 2016 declares that the animals of the species *Sus scrofa,* that includes boar and wild boars in free life, are considered swine, being necessary for the State that wishes to export to be considered free of classical swine fever.

Wild boar, original from Eurasia, is an exotic species in the Brazilian territory and, in recent years, has shown an uncontrolled population increase (Salvador & Fernandez 2014). Exotic species considered invasive that have the capacity to move long distances are considered to be a major threat to local biodiversity and generate great impacts on agricultural activities (Wittenberg & Cock 2001). Wild boars affect the dynamic of the soil and of water bodies, they feed on birds and from eggs of species that nest in the soil, prey on seeds, and alter the regenerative ability of forests (Lowe et al. 2000).

There are data that demonstrate the distribution of this species worldwide (Mitchel-Jones et al. 1999, Afonin et al. 2008, IUCN 2012), although the given geographic informations are limited, mainly coming from data ceded by hunters (Servanty et al. 2011). The wild boars use reforested areas as refuge, especially in places where the weather is warm or very cold. High temperatures favor the propagation of the species, which facilitates the search for different sources of protein and carbohydrates to maintain their energy (Bieber & Ruf 2005, Melis et al. 2006).

The construction of ecological models of favorability or suitability for the occurrence of pest species are necessary tools for decision making on priority areas for management aiming at risk management.

This study aimed to map the level of suitability for the occurrence of wild boars in the South of the Mato Grosso do Sul state, to identify the main risk variables for the contact with the wild boar, and to evaluate the biosecurity measures adopted by commercial farms integrated in this region.

MATERIALS AND METHODS

Study area.14 commercial swine farms were visited, located in an area of 37767.025 Km^2 (Fig.1) in the South of Mato Grosso do Sul state, in the municipalities of Rio Brilhante, Itaporã, Dourados, Vicentina, Jateí, Glória de Dourados, and Ivinhema, all belonging to an integrated production system. This sample corresponds to 100% of the commercial farms of the region. The Southern region of the state was chosen because it is an area known for the presence of wild boars and is the region with the highest concentration of swine farms in the state. Among the farms, 9 were piglet production units (PPU), 2 termination unit farms (TU), 2 nurseries (FN) and a complete cycle farm (CC). At the same time, 32 settlements for swine production for subsistence were selected by sortition in the Southern region of Mato Grosso do Sul, 16 settlements in the municipality of Deodápolis, 4 in Angélica, 9 in Rio Brilhante, and 3 in Nova Alvorada do Sul.

Interviews. The collection of information occurred through semi-structured interviews, from January 2017 to July 2017. The issues aimed at raising the biosecurity measures adopted in the production units and the perception of those responsible in relation to the risk represented by the wild boar such as: the presence of wild boar in the vicinity of the farm; occurrence of wild boar invasion on the farm and contact with these animals; proximity to forested areas and agriculture area; possible contact amongdomestic swine

Fig.1. Location of the study area in the south of Mato Grosso do Sul.

with wild boars, practices for contact prevention; and knowledge related to biosecurity. In the interview the denomination wild boar was used to indicate pure animals and all their hybrids, wild boars in free life and the so-called *javaporco*, domesticated swine crossed with wild boar or domesticated swine crossed with *javaporcos*, as described by PNSS.

Survey of farms' biosafety measures. The farms' biosafety measures were recorded by photographic images, in accordance with the standards for certification of suidae breeding farms, in the Annex to Normative Instruction SDA No. 19 of February 15, 2002. The biosecurity measures of the swine farms visited were documented by photographic images, especially those related to the prevention of entry of the wild boar in the facilities: quality of isolation of the farm - fences and quality of isolation of the farm - green belt by photographic images; images of the fences of swine to evaluate the biosecurity measures and the practices used to prevent contact amongwild boars and their hybrids with domestic swine .

Environmental suitability model for the wild boar. The soil cover classes used were selected considering the physiological and behavioral characteristics of the species. Six classes were selected: forest environments, wetlands, water bodies, corn crop, sugarcane plantations and settlements (Table 1), where subsistence farms were also considered (Braz 2017). In order to evaluate the potential risk of wild boars on commercial swine farms, different environmental strata were selected (Table 1) and the ranking was established according to Bosch et al. (2016), using expert opinion assessments on the suitability of wild boar habitat for different classes of soil cover (Table 2). Forest areas are considered to be at greater risk, followed by tree areas mixed with lawn areas, wetlands, plantation areas, and finally, urban areas.

The maize and sugarcane crops, as well as settlements, were acquired from the SISLA system (sisla.imasul.ms.gov.br), from the Mato Grosso do Sul government. The other classes were generated from the Supervised Classification technique, in the Spring 5.5 program.

Table 1. Level of relevance of soil cover classes for wild boars (*Sus scrofa*) used in the construction of environmental suitability models for the species in Southern Mato Grosso do Sul, Brazil

Coverage classes	Relevance level (%)
Forest environments	20
Wet areas	20
Water bodies	20
Corn crop	15
Cane plantation	15
Settlement	10

Table 2. Weight attributed to distance ranges from soil cover classes used to model suitability for wild boar (*Sus scrofa*) in Southern Mato Grosso do Sul, Brazil

Distance zones	Weight
0 km	100
From 0.01 to 1.00 km	80
From 1.01 to 2.00 km	60
From 2.01 to 3.00 km	40
From 3.01 to 4.00 km	20
Above 5 km	5

In the classification were used images of the satellite Sentinel 2, with spatial resolution of 10 meters, dated from July 2017, obtained from the website of the United States Geological Survey (USGS).

The environmental layers used in the modeling corresponded to the distance zones of each of the soil cover classes, using the tool "Euclidean Distance" of the program Arcgis, version 10.1. The distance zones were from 0 km, 0.01 to 1.00 km, from 1.01 to 2.00 km, from 2.01 to 3.00 km, from 3.01- km to 4.00 km 4.01 to 5.00km and ≥5.00 km of distance from each one of the classes of soil cover, corresponding

to the environmental layers used in the modeling. The limit of 5 km corresponds to the maximum distance that these animals can travel throughout their life (Salvador & Fernandez 2014). Classes that were available throughout the year had a greater weight (forests, water and wetlands) followed by temporary resources (corn and sugarcane plantations) as well as settlements - included by the risk of attracting wild boars due to the lack of biosecurity (Braz 2017) and the presence of females of reproductive age, characteristics of subsistence creation. Weights corresponding to the relevance of each soil cover class (Table 1) as well as the different distance ranges of these classes (Table 2) made up the environmental layers used in the modeling of environmental suitability for the wild boar. For the elaboration of the environmental suitability map, the Arcgis weighted overlay tool was used. This tool requires the assignment of a level of influence of each class and weights for each of these distance ranges (Table 1 and 2).

Risk of contact. The analysis of the risk of contact among farms and subsistence farms with wild boars was based on the environmental suitability model for this species. Each property was plotted on the map of environmental suitability and buffers were defined around them, centralized in the exact location of breeding sites/farms. These zones were defined as 1, 2 and 5 km radius, within which the coverage percentage of each adequacy level (low, medium, high, very high) was calculated and considered as a risk indicator (Table 3).

Table 3. Average and standard deviation (± SD) of the percentage of coverage of the different levels of environmental suitability for the wild boar (*Sus scrofa)* in areas of 1, 2, and 5 kilometers radius around commercial farms and subsistence farms in Southern Mato Grosso do Sul, Brazil

	Subsistence breeding	
Levels of suitability	% Average ± SD	% Average ± SD
1km		
Low	0	0
Medium	28.3 ± 4.6	44.5 ± 25.3
High	82.1 ± 3.7	78.6 ± 10.1
Very high	44.4 ± 17.3	16.7 ± 12.6
2 km		
Low	0	0
Medium	20.9 ± 3.51	28.9 ± 14.4
High	81.9 ± 3.25	77.2 ± 7.7
Very high	46.7 ± 8.8	17.4 ± 7.9
5 km		
Low	0	0
Medium	26.2 ± 4.2	17.0 ± 6.4
High	70.4 ± 3.8	76.3 ± 4.9
Very high	18.6 ± 18.7	19.7 ± 6.4

RESULTS AND DISCUSSION

In Brazil, establishment biosafety standards are only for Certified Suidae Breeder Farms (Granjas de Reprodutores de Suídeos Certificados - GRSC). This is highly relevant since in situations of high environmental suitability for wild boars, farms with low biosecurity become susceptible to contact among the wild species and domestic swine. For Bellini et al. (2016) it is of fundamental importance the physical isolation of the production establishments, preventing an infected free-living animal from having contact with the animals of the herd. As the state of Mato Grosso do Sul is currently in areas free of classical swine fever, according to the OIE, the maintenance of this status is a concern that must be considered by both producers and public authorities.

For sanitary risk management, it is essential to know the areas with the greatest potential for occurrence of wild boar, which can be accessed through maps of environmental suitability for this species, especially when it is known that this species already has a wide distribution in the region. The proximity of the property to the different levels of environmental suitability for the wild boar can be an indicator of contact risk. The results of this study show that, on average, most of the areas up to 1 km in the vicinity of subsistence nurseries analyzed are constituted by areas of high (82.1% ± 3.7) and very high (44.4% ± 17.3) environmental suitability for wild boar; areas up to 1km in the vicinity of commercial farms are composed of zones of high (78.6% ± 10.1) and very high (16.7% ± 12.6) environmental suitability for the species (Table 3). This condition does not change substantially at greater distances, such as 2 and 5 km from commercial farms and subsistence farms (Table 3). The results suggest an exposure to the risk of contact with free-living wild boars (Fig.2), which also implies sanitary risk, considering the movement capacity of these animals (Salvador & Fernandez 2014).

In this study, only 22% of those interviewed reported seeing the wild boar close to commercial farms (Table 4), and all reports of wild boar sightings were within 1 km of areas of high environmental suitability for the species, according to the built model. On the other hand, 93% of sightings occurred within the area of high suitability. These

Table 4. Main responses attributed by the interviewees regarding the wild boar and the areas surrounding the commercial farms in the South of Mato Grosso do Sul, Brazil

Questionnaire	GRSC*		Complete cycle		PPU*		Nursery		Termination		Total	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Is the area surrounding the farm forested?	1 (100%)	0 (0%)	1 (100%)	0 (0%)	10 (90.9%)	1 (9.2%)	0 (0%)	2 (100%)	1 (33.4%)	2 (66.6%)	13 (72.2%)	5 (27.8%)
Have you seen wild boars or *javaporcos* near the farm?	0 (0%)	1 (100%)	0 (0%)	1 (100%)	4 (36.4%)	7 (63.6%)	0 (0%)	2 (100%)	0 (0%)	3 (100%)	4 (22.2%)	14 (77.8%)
Are there any supplementary methods for wild boar control?	0 (0%)	1 (100%)	0 (0%)	1 (100%)	1 (9.2%)	10 (90.9%)	0 (0%)	2 (100%)	1 (33.4%)	2 (66.6%)	2 (11.1%)	16 (88.9%)
Do the surrounding properties produce any kind of culture?	1 (100%)	0 (0%)	1 (100%)	0 (0%)	11 (100%)	0 (0%)	2 (100%)	0 (0%)	3 (100%)	0 (0%)	18 (100%)	0 (0%)

*GRSC = Certified Suidae Breeder Farms, PPU = piglet production unit.

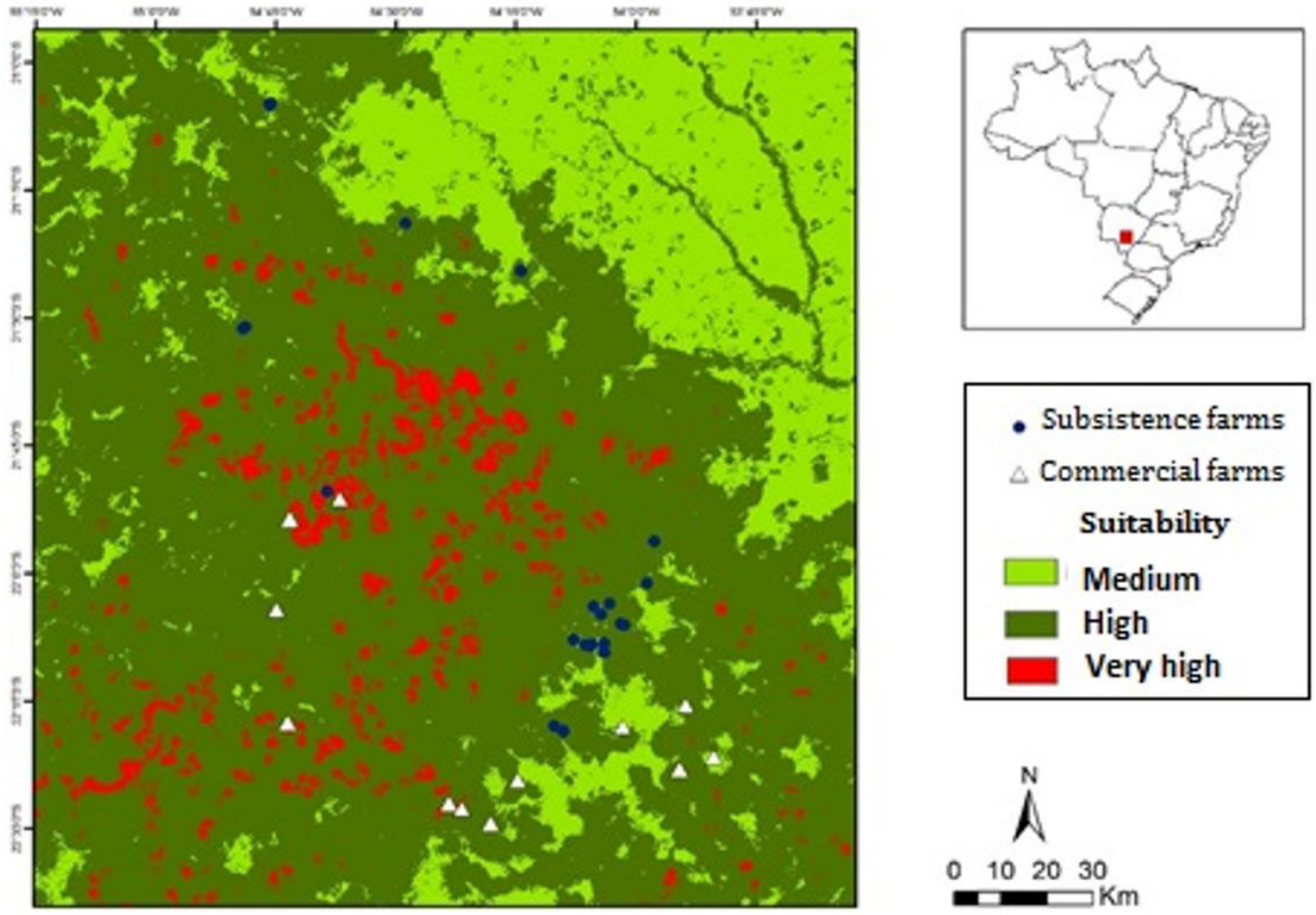

Fig.2. Environmental suitability model for wild boar (*Sus scrofa*) in Southern Mato Grosso do Sul, Brazil.

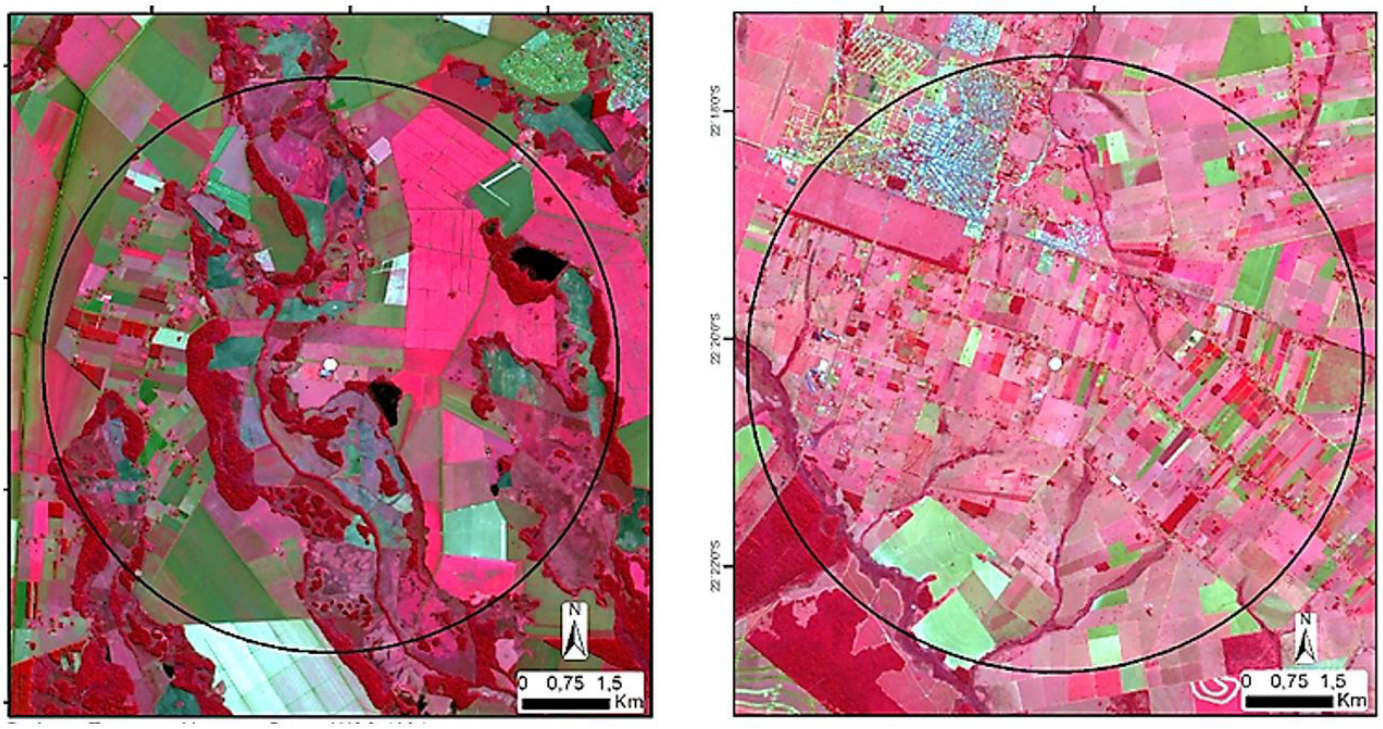

Fig.3. Biosecurity (physical barrier) of one of the swine farms visited in Southern Mato Grosso do Sul, Brazil.

qualitative results suggest that the model constructed based on habitat requirements of the species in this study can be considered plausible.

All the commercial farms visited in the study area adopted physical biosecurity structures, consisting of resistant fencing fences and masonry (Fig.3), thus ensuring the maintenance of separate internal and external compartments. In the conducted interviews there was no record of wild boar in the internal areas of commercial farms, indicating that this biosecurity measure has been effective. For Bellini et al. (2016) it is of fundamental importance the physical isolation of the production establishments, preventing an infected free-living animal from having contact with the animals of the herd. Still in relation to biosafety in commercial farms, 88.9% of the respondents stated that they use what is regulated by Normative Instruction/SDA No. 19, which consists of a peripheral fence with a single entrance and a hygiene and disinfection system for the admission of people and vehicles; distance from the nearest uncertified swine production unit or swine slaughterhouse; density of swine herds within a radius of 3.5 km; number of farm supplying swine for replacement purposes; distance from the road carrying swine; quality of the farm's insulation relative to desirable characteristics; quality of the isolation of the farm relative to the existence of green belt and its desirable characteristics, among others.

Of the total of 14 farms interviewed, 72.2% answered to maintain an environment of the forested farm with eucalyptus as a barrier and security. On the other hand, all the farms around the farms were destined to animal or vegetable production (Table 4), with maize, soybean and sugarcane being the most relevant in the study area. Agricultural regions are of great importance for the survival of wild boars, as they are areas where there is plenty of food throughout the year (Brook & Van Beest 2014), ensuring the persistence and support of these landscapes for the population of the species. In addition, forested areas provide shelter for the wild boar, where thermal regulation occurs during warm seasons, and serves as a hiding place and refuge. However, wetlands are places where animals find water to be ingested, as well as thermal comfort (Higginbotham 2013, Michel et al. 2017).

In general, the conditions of cover and use of the soil of areas of greater suitability for the wild boar, in comparison with the ones of lesser suitability, are quite clear (Fig.4). At first, areas with lower forest cover and higher human density in the study area have lower habitat conditions for the conservation of wild boar populations. However, in these areas are the majority of subsistence farms that do not adopt biosecurity measures, thus facilitating the occurrence of intrusion of wild boars and crosses with domestic swine (Braz 2017).

For Brook & Van Beest (2014), a fundamental flaw so far in the management of wild boar control has been the fact that the damage caused by this animal is seen as a biological problem, leaving aside the important social aspects of the wild boar problem. There are intense efforts to publicize the problems and impacts caused by wild boars in crops and deforestation, which leads to loss of natural resources. However, social factors such as loss of production and risk of zoonotic diseases are scarcely reported (Walker et al. 2004). The situation described in this paper indicates that a substantial and adequate effort needs to be applied to control populations of wild boars and their free-living hybrids. This need arises from high exposure to sanitary risks, economic damages and environmental impacts, due to the predominance of situations favorable to the species in the study area.

Fig.4. (**A**) Coverage and land use in areas of 5 Km radius in regions of high environmental suitability, and (**B**) average suitability for wild boar (*Sus scrofa*) in Southern Mato Grosso do Sul, Brazil. Satellite image Sentinel 2; false color composition RGB-458.

Likewise, subsistence creations in areas where there is a wild boar present a 63% greater risk of contact with the wild boar, which responds to the sexual attraction of sows in heat, as already demonstrated in Switzerland and the United States. Another aggravating factor is the possibility of swine escape due to the absence of adequate physical barriers in the property and return to a feral or free-living condition, increasing the *javaporco* population in the region by crossing with the wild boar (Wu et al. 2012). Considering that commercial swine farming is concentrated in the southern region of the state, the potential risk of an increase in the population of *javaporco* should be subject to constant monitoring so that its management can be undertaken based on technical data.

The population control of wild boar is quite difficult, due to the high growth rate under favorable habitat conditions (Bieber & Ruf 2005), but may also be expensive. In addition, the effort required for effective population control or its reduction has been reported as large even in regions where hunting is legalized and systematic. Population models developed for Texas indicate that an annual extraction of 66% may be enough to keep the population stable. Densities can range from over 40 animals/km^2 in wet areas to 1 animal/km^2 in drier savannas, going to 4 animals/km^2 in wet areas with freshwater and 3.1/km^2 in tropical wet regions of Queensland, Australia. In Texas, the overall density of wild boar varies from 0.47 to 1.0 animal/km^2, although these estimates may be higher depending on habitat quality Based on the percentage of areas of high and very high suitability estimated for the studied region in Southern Mato Grosso do Sul, it is estimated that this area may be home to approximately 12,000 wild boars in a scenario of 0.5 animals/km^2, while in a more pessimistic scenario of 2.0 animals/km^2, this population can surpass 50,000 wild boars and their hybrids in free life.

CONCLUSIONS

The constructed model can be considered probable and can be improved with our modeling, validation and extension approaches to other areas of the state. This type of tool can be very useful for the productive sector, environmental agencies and decision makers.

The results of this work indicate that the risk of contact among wild boars and domestic swine is high, and the establishment of strategies for control, monitoring and mitigation of environmental impacts, as well as appropriate biosecurity measures, are essential to deal with this problem that came to stay.

The effectiveness of these measures will depend on the consideration of habitat characteristics, biology and behavior of the species, as well as the establishment of adequate governance to address the magnitude of the problem in its economic, health, social and environmental dimensions.

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Original Article
Morphophysiology

Response of vital functions, Apgar and cortisol in the prognosis of vigor against neonatal factors of lambs[1]

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ABSTRACT.- Fagundes G.B., Nascimento D.M., Santiago M.R., Neves C.A., Silva C.M.G., Oba E., Arrivabene M. & Cavalcante T.V. 2019. **Response of vital functions, Apgar and cortisol in the prognosis of vigor against neonatal factors of lambs.** *Pesquisa Veterinária Brasileira 39(2):155-162*. Universidade Federal do Piauí, Campus Professora Cinobelina Elvas, Rod. Municipal Bom Jesus Viana Km 1, Planalto Horizonte, Bom Jesus, PI 64900-000, Brazil. E-mail: glauciatuante@hotmail.com

The management of lambs during the neonatal period has been studied in several researches due to the vital and hormonal organic adaptations undergone by the calf after birth. However, gender, number of pups and type of delivery play an important role in understanding neonatal vigor. The study of these groups with the monitoring of clinical evolution and cortisol metabolism becomes an indispensable subsidy for a better understanding of this neonatal phase, aiming to minimize the losses generated. The objective of this study was to evaluate the influence of gender, number of pups and type of delivery in the prognosis of neonatal vigor of lambs through clinical and cortisol diagnosis. Thirty crossbred Santa Inês lambs with Dorper in the neonatal phase were divided into three groups: male and female, number of pups (single and twin) and type of delivery (eutocic and dystocic). In each group, clinical evaluation of heart and respiratory rate, rectal temperature, Apgar score and weight were performed; and with the exception of cortisol, all evaluations were performed at fifteen and sixty minutes, as well as at twelve and twenty-four hours. In addition, blood samples were collected for cortisol dosage obtained in two moments at fifteen and sixty minutes using the radioimmunoassay technique. Among the three experimental groups related to lamb vigor, the heart rate was the only one that showed lower mean values ($P<0.05$) at twenty-four hours in the male group 90.00±20.20bpm, twins 96.44±20.02bpm and eutocic 93.25±18.11bpm. Differences in respiratory rate values were observed in the eutocic group (64.00±14.75mpm) at twenty-four hours. In the group of males there was a significant reduction in body temperature during the evaluation moments ($P<0.05$). Lambs from the group of twins showed lower body weight during the evaluations. At both times the analysis of serum cortisol was less than at sixty minutes. It was concluded that soon after the birth

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there were marked changes in the physiological parameters and weight of Santa Inês lambs, but were not enough to cause negative effects on the vigor of the neonates, indicating the occurrence of effective neonatal adaptation capacity in this species.

INDEX TERMS: Vital functions, Apgar score, cortisol, prognosis of vigor, neonatal, lambs, newborns, type of birth, sex, birth, time period moments.

RESUMO.- [**Resposta das funções vitais, de Apgar e cortisol no prognóstico de vigor frente a fatores neonatais de cordeiros**.] O manejo dos cordeiros durante o período neonatal tem sido objeto de estudo em diversas pesquisas devido às adaptações orgânicas vitais e hormonais sofridas pela cria após o parto. Todavia, o gênero, número de filhotes e o tipo de parto parecem desempenhar um papel importante para melhor compreensão do vigor neonatal. Além disso, o estudo destes grupos com o acompanhamento da evolução clínica e do metabolismo do cortisol torna-se um subsidio indispensável para melhor compreensão dessa fase neonatal, visando minimizar as perdas geradas. Dessa forma, o objetivo do presente trabalho foi avaliar a influência do gênero, número de filhotes e tipo de parto na apresentação do vigor neonatal dos cordeiros através do diagnostico clinico e de cortisol. Foram utilizados trinta cordeiros mestiços da raça Santa Inês com Dorper em fase neonatal divididos em três grupos: gênero (macho e fêmea), número de filhotes (único e gemelar) e tipo de parto (eutócico e distócico). Em cada grupo, foi realizada a avaliação clínica da frequência cardíaca e respiratória, temperatura retal, escore Apgar e peso; e com a exceção do cortisol, todas as avaliações foram realizadas aos quinze e sessenta minutos, como também às doze e vinte e quatro horas. Adicionalmente, procedeu-se com a coleta de amostras de sangue total para dosagem de cortisol obtida em dois momentos aos quinze e sessenta minutos através da técnica de radioimunoensaio. Dentre os três grupos experimentais relacionados com vigor dos cordeiros, a frequência cardíaca foi a única que evidenciou menores médias (P<0,05) às vinte e quatro horas no grupo dos machos 90,00±20,20bpm, gêmeos 96,44±20,02bpm e eutócicos 93,25±18,11bpm. Observou-se no grupo eutócico diferenças nos valores da frequência respiratória de 64,00±14,75mpm às vinte e quatro horas. No grupo dos machos houve redução significativa na temperatura corpórea durante os momentos de avaliação (P<0,05). Cordeiros do grupo de gêmeos demonstraram menor peso corpóreo durante as avaliações. Em ambos momentos a análise do cortisol sérico demonstrou se menor aos sessenta minutos. Pôde se concluir que logo após o parto ocorreram alterações marcantes nos parâmetros fisiológicos e peso de cordeiros Santa Inês, porém não foram suficientes para causar efeitos negativos sobre o vigor dos neonatos, indicando a ocorrência de efetiva capacidade de adaptação neonatal nesta espécie.

TERMOS DE INDEXAÇÃO: Funções vitais, Apgar escore, cortisol, prognóstico de vigor, neonatal, cordeiros, ovinos, recém-nascidos, aspectos fisiológicos.

INTRODUCTION

The number of lambs sold per sheep affects profitability favorably (Lôbo et al. 2011), mainly because the sale of lambs has increased in the last decades (Morel et al. 2008). The production of more lambs followed by greater mortality is unacceptable, both from the economic point of view and from the animal and veterinary standpoints (Nowak 1996, Everett-Hincks et al. 2005). At this stage it is convenient to resort to clinical intervention, to minimize or avoid future negative consequences for the health and vigor of these animals. To end this financial loss and minimize mortality, veterinarians carry on specific resuscitation procedures and supportive care (Ravary-Plumioën 2009). To achieve high performance levels in lamb breeding, attention to the lamb from birth (Kenyon & Blair 2014) up to its first 24 hours of life is needed, as this is the most important period for the newborn pup.

The newborn's adaptive development period is considered critical and crucial, since fast physiological adjustments are needed that are important for the lamb's health, which is intrinsically related to the organic immaturity of compensatory and regulatory multisystems, especially due to the animal's new status of vulnerability vis-à-vis the environment, plus the difficulty of rendering clinical assistance, especially under extensive management conditions. This is a period seen as critical to newborn pups, as profound physiological, nutritional and environmental adjustments are required, due to the replacement of the uterine medium by the extrauterine environment. This period is dramatic and dangerous, since the immaturity of organic systems may be decisive for organ failure or dysfunction leading to death, a situation that calls for great care in the identification of risk factors.

The general death rate quoted in the literature about Santa Inês lambs is of 15.1% (Girão et al. 1998), and constitutes a challenge for the production of small ruminants. Although reports of lamb mortality rates at the fetal-neonatal transition stage in the Northeast are scarce, it is believed that its economic impact in the region is high and, thus, a deficient or absent clinical assistance/support may lead to an increase in perinatal mortality, limiting the production and survival of lambs. At birth and in subsequent days of extrauterine life, thermoregulatory, cardiovascular, respiratory and homeostatic mechanisms complete the maturation of the newborn (Chniter et al. 2013). This adaptive process depends on the activation of the hypothalamic-pituitary-adrenal axis, mediated by cortisol, which is a strong metabolic stimulator (Wood 1999).

The little research aimed at investigating the vital parameters contemplates lambs at the adult stage, and there has been no advancement in the neonatology of lambs at any particular age bracket. This emphatically justifies this research, which could be usually employed in the clinical routine and, along with the evaluations associated with the Apgar score and the determination of weight, would allow for an early diagnosis of low neonatal adaptation capacity, with positive implications for the newborn's health. The use of neonatal vitality scores can guide the choice of suitable therapeutic measures to

ensure and increase the newborns' survival (Lourenço & Machado 2013).

This study aimed to evaluate the influence of gender, number of pups and type of delivery in the presentation of the neonatal vigor of lambs through clinical and cortisol diagnosis.

MATERIALS AND METHODS

The experiment was carried out at Fazenda Santa Tereza, in the village of Barra da Ininga, located 100 km from the county of Matões, in the state of Maranhão (MA), in the period between December 2016 and May 2017. The farm's geographical coordinates are: latitude 5°28'47" S, longitude 43°0'95" W and altitude 102m. The experiment was approved by the Ethics Committee under protocol no. 034/2015.

Thirty sheep of the Santa Inês breed aged from 1.5 to 4 years were used, after being dewormed and vaccinated against enterotoxaemia. The animals were submitted to a hormonal estrus synchronization protocol. Intravaginal sponges containing medroxyprogesterone acetate[1] were inserted at a random phase of the estrus cycle (day 0) and kept there for 14 days, after which the sponges were removed and 300 international units (IU) of equine chorionic gonadotrophic[2] (eCG) were administered intramuscularly. The type of conception adopted was natural impregnation using four (n=4) Dorper sheep. After 45 days of the treatment and impregnation, the study proceeded with 19 pregnant females, diagnosed by ultrasound (ALOKA model).

The pregnancy time of females was between 146 and 157 days. During the whole pregnancy period the sheep were handled extensively during the day in pastures with Tanzania grass (*Panicum maximum* cv. *Tanzania*) and the native pasture typical of the region, gathered in the afternoon and fed a ration concentrate containing soy meal, corn, urea, salt and water at will. Close to delivery of the pups the sheep were handled in the pen, remaining under supervision of the researcher at the place of parturition. After birth, time was allowed for removal of fetal wraps and cleansing of the airways as well as for the establishment of the postpartum maternal-fetal bond. All lambs had their navels cured with a 10% iodine solution and were breast-fed by their mothers, avoiding further stress which would result from separation.

After birth, the newborn (n=30) were broken down into groups according to criteria checked at birth, such as gender (male and female), number of pups (one or twins) and type of delivery (euthymic and dystocic) evaluated at different moments. In the pen, each group of newborns was evaluated using the heart and respiratory rates, rectal temperature, Apgar score and weight parameters, checked at M 15 and M 60min and at M 12 and M 24 hours. The blood samplings for ascertainment of the cortisol hormone took place at M 15 and M 60min. A chronometer was used in the neonatal evaluation for 60-second measurements to check heart rate (HR) in beats per minute (bpm) using a stethoscope, and respiratory frequency (RF) was checked and measured in movements per minute (mpm) through the observation of movements of the thorax and abdomen (flank). A digital clinical thermometer was inserted in the rectum until the stabilization of rectal temperature in degrees "Celsius" (°C) was reached. Weighing (kg) was done using a cloth ribbon to suspend the lamb from the portable electronic balance using a hook.

To obtain the Apgar score modified for sheep, points were attributed to the following items: evaluation of head movement with cold water, response to the eye-palpebral and interdigital reflex, breathing type and mucosal staining, with scores going from zero to two. Each item was classified by assigning 0, 1 or 2 points, and the final result (the sum from 0-10) was considered as the Apgar score.

Scoring is interpreted as follows: seven to eight points represent good vitality; four to six characterize average vitality; and zero to three is considered a low-vitality score (depressed).

The lambs' jugular veins were punctured at M 15min and, later, at M 60min, to obtain the serum and for the determination of cortisol using the radioimmunoassay technique. This was done at the Laboratory of Endocrinology of the Department of Animal Reproduction and Veterinary Radiology (Laboratório de Endocrinologia do Departamento de Reprodução Animal e Radiologia Veterinária) of Faculdade de Medicina Veterinária e Zootecnia of Universidade Estadual Paulista (Unesp/SP) of the Botucatu Campus, using an IM1841 solid cortisol commercial kit from Beckman Coulter, following the manufacturer's recommendations, and the reading was carried out by radioimmunoassay gamma counter (Perkin Elmer 1470, automatic gamma counter).

To minimize individual variation in all evaluations, animals were kept preferably in a stationary position and in repose, and measurements were carried out by a single person at a station and by a single examiner.

Data were tested for normality and homogeneity of variances using the Shapiro-Wilk and Bartlett tests, respectively; afterwards, the figures were subjected to analysis of variance with time-repeated measurements (ANOVA). Parameters which showed an abnormal (parametric) distribution were compared at the points in time in each group using the Tukey test, and between groups at each point using the t-Student test, at the 5% probability level, respectively.

As for the parameters which did not present a normal distribution (non-parametric), these were compared at the points in each group using the Kruskal-Wallis test, and between groups at each point using the Mann & Whitney test at the 5% probability level, respectively. The mucosal staining and Apgar score which showed non-parametric distribution were compared between groups at each point in time (moment, M) using Fisher's exact test. The cortisol concentrations were evaluated at 15 and 60 minutes after delivery, and compared using the t-Student test at the 5% probability level.

RESULTS AND DISCUSSION

All lambs presented a significant drop ($P<0.05$) in their heart rates (HR) at the points in time evaluated in each group according to gender, number of pups and types of delivery (Table 1). This fact was also evidenced by (Piccione et al. 2007) in lambs analyzed up to 30 days after birth; the existence of an inversely proportional ratio between age and heart rate should also be noted. The values found for these groups contrast with the reference values set for the species reported by (Bovino et al. 2014), who observed increases in the animals' HR in the moments mentioned, between 175±33bpm and 189±20bpm, at 15 minutes and 24 hours, respectively. Compared to that of adult sheep, the heart rate of newborns is rapid and relatively unstable (Koether et al. 2016); it is under the control of the autonomic system, which action derives only from the sympathetic system (Ulian et al. 2014).

As for the gender (male and female), the heart rate was significantly lower ($P<0.05$) in males (Table 1). This contrasted with the reports by (Koether et al. 2016), who did not observe influence of gender on the electrocardiogram of lambs of the Bergamácia breed. However, an increase was reported due to physical maturation, especially at 24 hours; this increase shows that this distinct method can interfere in heart rate

[1] Progespon®, Syntex. Buenos Aires, Argentina.

[2] Novormon®, Syntex. Buenos Aires, Argentina.

Table 1. Averages ($\bar{x}$) and standard deviations (S) of heart rate (HR) values in mixed-race lambs evaluated as a function of gender, number of pups and types of delivery. Matões/MA, 2017

Variable	Moments	n	$\bar{x}$ ± S	n	$\bar{x}$ ± S
			Gender		
			Male		Female
	M 15min	14	129.55 ± 15.42aA	14	139.43 ± 21.22aA
	M 60min	14	112.86 ± 21.06abA	14	123.54 ± 13.77abA
	M 12 hours	14	95.65 ± 16.47bcA	14	108.19 ± 21.12bA
	M 24 hours	14	90.00 ± 20.20cB	14	108.64 ± 17.58bA
			No. of pups		
		n	Single	n	Twins
	M 15min	9	140.00 ± 22.45aA	19	131.88 ± 16.98aA
	M 60min	9	131.11 ± 12.29aA	19	112.08 ± 17.65bB
HR (bpm)	M 12 hours	9	106.67 ± 16.25bA	19	99.67 ± 21.10bA
	M 24 hours	9	105.33 ± 22.54bA	19	96.44 ± 20.02bA
			Type of delivery		
		n	Euthymic	n	Dystocic
	M 15min	16	129.00 ± 19.79aA	19	141.81 ± 15.45aA
	M 60min	16	116.25 ± 16.30abA	19	120.79 ± 21.11bA
	M 12 hours	16	103.50 ± 23.64bcA	19	99.82 ± 13.33cA
	M 24 hours	16	93.25 ± 18.11cA	19	107.41 ± 22.28bcA

[aABbc] Averages followed by different small letters in the same column differ between themselves by the Tukey test; averages followed by different capital letters in the same column differ between themselves by the t-Student test, respectively, at the 5% probability level.

results. Comparing heart rate values (Table 1) in the group classified by number of pups (single and twin), a difference was found only at 60 minutes, with a lower heart frequency in twin pups. Giannetto et al. (2017) observed greater HR values in twins due to the smaller heart size of the Comisana lambs. This has not been demonstrated in the present study, but the differences in breed and locality may have contributed to the increase in heart rates.

However, analyzing the group classified according to type of delivery (euthymic and dystocic) compared at each point in time, no significant difference can be observed in heart rate values (Table 1). Investigating the euthymic and dystocic types of delivery in calves, Gasparelli et al. (2009) observed a significant drop in HR at 24 hours, with average values of 132.20±18.74 and 116.72±17.68bpm, respectively. According to the authors, due to the action of catecholamines derived from the stress of delivery, there is an increase in heart rate at this stage which is physiological, followed by a gradual drop in HR over time. These results were higher than those reported in the present study, as a consequence of the species evaluated.

There was no significant difference in heart rate (HR) related to gender, number of pups and types of delivery at each point evaluated (Table 2). However, there was a statistical difference at the points in the case of lambs born in euthymic deliveries, in which there was a decrease along the first 24 hours (64±14.65mpm). In the present study the HR of lambs recorded at 24 hours was below the reference values described by Bovino et al. (2014), who reported an increase in the HR of mixed-race Suffolk/Texel lambs, which averaged 75±18mpm at that moment. It is likely that the differences between lamb breeds have interfered in the HR results. The irregular activity of the respiratory system in the neonatal period was reported by Piccione et al. (2007), and can be related to the fact that the adaptation of the lambs' respiratory system is different in the two types of delivery, with direct implications for the desirable post-delivery vigor. Hilaire & Duron (1999) and Vestweber & Rieß (1997) report the occurrence of alternate states of bradipnea and tachypnea until eupnea sets in, a period in which breathing frequency is at first irregular. Linke et al. (2013) emphasized the need for a minimum period of 14 days for the pulmonary units to be integrated into the gas exchange.

As for rectal temperature (RT), the study found that there were significant differences only between genders at 24 hours after birth and in the group classified according to the number of pups (single and twin) at each point in time (Table 3), with rectal temperature being lower in twins. There was no significant difference between the types of delivery in the first 24 hours of life. It deserves notice that the RT kept constant and within a very narrow range (38-39°C) at those points. Ball et al. (2010) proposed normothermic reference temperatures of around 38-39°C for neonates of the sheep species. These results support the performance of sheep in relation to the homeostasis mechanism (Piccione et al. 2007), which requires for the most part changes in the energy metabolism (Greenwood et al. 2002), achieved through the 17% increase in the generation of heat (Dwyer et al. 2016) for the guarantee of vigor (Matheson et al. 2012) directed to the pup's search for position, search and suck of udder (Abdul-Rahman & Bernard 2017) and post-birth survival (Dwyer et al. 2005). Thus, the behavioral development of neonates may be related to their vigor in the ability to achieve thermoregulation.

In the analysis of the body weight of lambs, significant differences were observed only in the group classified

Table 2. Averages ($\bar{x}$) and standard deviations (S) of respiratory frequency (RF) values in mixed-race lambs evaluated as a function of gender, number of pups and types of delivery. Matões/MA, 2017

Variable	Moments	n	$\bar{x}$ ± S	n	$\bar{x}$ ± S
			Gender		
			Male		Female
	M 15min	14	79.43 ± 15.20	14	81.14 ± 16.93
	M 60min	14	67.14 ± 13.69	14	74.57 ± 13.46
	M 12 hours	14	65.14 ± 19.93	14	74.86 ± 16.56
	M 24 hours	14	66.29 ± 23.57	14	74.29 ± 19.00
			No. of pups		
		n	Single	n	Twins
	M 15min	9	82.22 ± 20.41	19	79.37 ± 13.68
	M 60min	9	71.11 ± 13.97	19	70.74 ± 14.18
RF (mpm)	M 12 hours	9	74.22 ± 19.30	19	68.00 ± 18.52
	M 24 hours	9	76.44 ± 20.04	19	64.37 ± 21.92
			Type of delivery		
		n	Euthymic	n	Dystocic
	M 15min	16	81.25 ± 18.86aA	12	79.00 ± 11.20aA
	M 60min	16	73.25 ± 15.44abA	12	67.67 ± 11.24aA
	M 12 hours	16	69.22 ± 17.86abA	12	71.33 ± 20.38aA
	M 24 hours	16	64.00 ± 14.75bA	12	78.67 ± 26.33aA

mpm = Movements per minute; [aAb] averages followed by small letters differ between themselves in the column by the Tukey test; averages followed by capital letters differ between themselves in the line by the t-Student test, at the 5% probability level.

Table 3. Averages ($\bar{x}$) and standard deviations (S) of rectal temperature (RT) values in mixed-race lambs evaluated as a function of gender, number of pups and types of delivery. Matões/MA, 2017

Variable	Moments	n	$\bar{x}$ ± S	n	$\bar{x}$ ± S
			Gender		
			Male		Female
	M 15min	14	38.64 ± 0.71aA	14	39.07 ± 0.71aA
	M 60min	14	38.76 ± 0.68aA	14	39.04 ± 0.56aA
	M 12 hours	14	38.99 ± 0.35aA	14	39.01 ± 0.43aA
	M 24 hours	14	38.97 ± 0.40aB	14	39.36 ± 0.32aA
			No. of pups		
		n	Single	n	Twins
	M 15min	9	39.32 ± 0.24aA	19	38.63 ± 0.71aB
	M 60min	9	39.26 ± 0.30aA	19	38.73 ± 0.56aB
RT (ºC)	M 12 hours	9	39.23 ± 0.22aA	19	38.88 ± 0.43aB
	M 24 hours	9	39.41 ± 0.23aA	19	39.05 ± 0.32aB
			Type of delivery		
		n	Euthymic	n	Dystocic
	M 15min	16	38.88 ± 0.76	12	38.83 ± 0.72
	M 60min	16	39.04 ± 0.52	12	38.70 ± 0.72
	M 12 hours	16	38.97 ± 0.34	12	39.03 ± 0.45
	M 24 hours	16	39.13 ± 0.38	12	39.22 ± 0.45

[AaB] Averages followed by small letters differ between themselves in the column by the Kruskal-Wallis test; averages followed by capital letters differ between themselves in the line by the Mann & Whitney test, at the 5% probability level.

according to number of pups within each time period evaluated (Table 4). Lambs from twin deliveries had a lower weight average from 15 minutes to 24 hours after birth. In the present study it can be observed that there is no difference related to gender and type of delivery. These results contradict the values mentioned by Fazio et al. (2016), who did not find weight differences in the neonatal period in lambs born in twin deliveries (3.20±0.13 kg) and single deliveries (3.87±0.34 kg). Nobrega et al. (2005) mention that the temperatures of the semi-arid region favor the survival of pups, but the inadequate nutritional level of the matrixes in the final third of pregnancy contributes to the occurrence of starvation/hypothermia as a consequence of the low weight at birth. There are episodes of slower intrauterine growth rates

in the maternal environment (Ergaz et al. 2005) as well as of smaller muscle mass (McCoard et al. 1997). Probably the way to minimize the occurrence of starvation/hypothermia and low weight in lambs is the nutrition of matrixes in the final stage of pregnancy.

When evaluating the Apgar score percentages of lambs, no significant difference was observed (P>0.05) between the groups (gender, number of pups and type of delivery) at the time of evaluations, which shows the importance of an early determination of the Apgar score, immediately after birth, in all groups. The lambs were generally healthy and exhibited from good to moderate vitality, regardless of the groups analyzed.

A low Apgar score is particularly related to neonatal depression, which is caused by the various neonatal adjustments due to parturition, and may be similarly combined with other equally important factors such as gender, number of pups and type of delivery during the neonatal period. In view of these factors, a significant alteration in the Apgar score may take place in these pups, which should be analyzed to detect neonates with greater propensity to lower Apgar scores and requiring intervention for their reanimation. Thus, further studies need to be undertaken to gather knowledge on the occurrence of different Apgar score values associated with these factors during the neonatal period, especially at birth, as well as on the influence of the self-efficacy of the method in clinical situations requiring the emergency identification of high-risk lambs.

Among the 30 samples of serum used for cortisol dosage, only one was eliminated due to the fact that the result was zero at the points in time. As shown in Figure 1, cortisol levels were influenced by the moment (P<0.01), and a greater hormone concentration of (457,76µg/ml) was found at M 15min, followed by a continuous drop of circulating cortisol at M 60min (178µg/ml). This was probably due to the fast metabolization of cortisol at the points in time (M 15 and 60min), the effective adaptation to stress and regulation of basal metabolism. According to Ferguson & Warner (2008), cortisol is related to the adaptive metabolism of stress and to the basal level of metabolic regulation. The average cortisol serum concentration was higher than that found by Gasparelli et al. (2009) when these authors evaluated the level of the hormone in calves at birth, which was of 9.85± .31µg/dL and 9.02±2.83 µg/dL, and at 24 hours, 3.45±2.11µg/dL and 4.70±4.02µg/dL, for euthymic and dystocic deliveries respectively. These differences are due

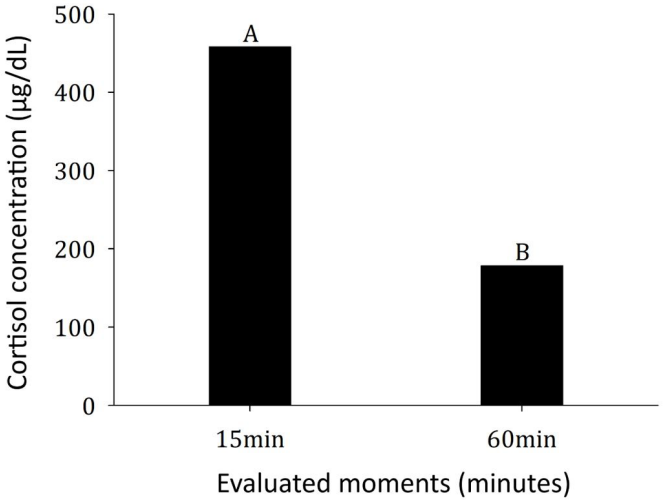

Fig.1. Cortisol levels (µg/dL) at each point in time:15 minutes and 60 minutes.

Table 4. Averages ($\bar{x}$) and standard deviations (S) of weight values in mixed-race lambs evaluated as a function of gender, number of pups and types of delivery. Matões/MA, 2017

Variable	Moments	n	$\bar{x}$ ± S	n	$\bar{x}$ ± S
			Gender		
			Male		Female
	M 15min	14	2.85 ± 0.84	14	2.87 ± 0.65
	M 60min	14	2.89 ± 0.89	14	2.88 ± 0.68
	M 12 hours	14	2.81 ± 0.92	14	2.87 ± 0.75
	M 24 hours	14	2.81 ± 0.88	14	2.88 ± 0.78
			No. of pups		
		n	Single	n	Twins
	M 15min	9	3.37 ± 0.76aA	19	2.62 ± 0.62aB
	M 60min	9	3.43 ± 0.80aA	19	2.62 ± 0.64aB
Weight (kg)	M 12 hours	9	3.42 ± 0.83aA	19	2.57 ± 0.69aB
	M 24 hours	9	3.40 ± 0.85aA	19	2.59 ± 0.67aB
			Type of delivery		
		n	Euthymic	n	Dystocic
	M 15min	16	2.71 ± 0.64	12	3.07 ± 0.83
	M 60min	16	2.75 ± 0.72	12	3.06 ± 0.86
	M 12 hours	16	2.71 ± 0.75	12	3.02 ± 0.92
	M 24 hours	16	2.73 ± 0.72	12	3.01 ± 0.94

[AaB] Averages followed by small letters differ between themselves in the column by the Kruskal-Wallis test; averages followed by capital letters differ between themselves in the line by the Mann & Whitney test, at the 5% probability level.

to the species. However, the cortisol serum concentration followed the same trend at the points in time.

CONCLUSIONS

Marked changes in the physiological parameters and weight of Santa Inês lambs took place immediately after birth, but these changes were not sufficient to cause negative effects on the vigor of neonates, which indicates the effective capacity for neonate adaptation in this species.

These findings reinforce the need of further studies in this area for better understanding of neonatal vigor, which can serve as a diagnostic tool for the health management of newborn lambs, provided that they are adjusted for that stage.

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

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