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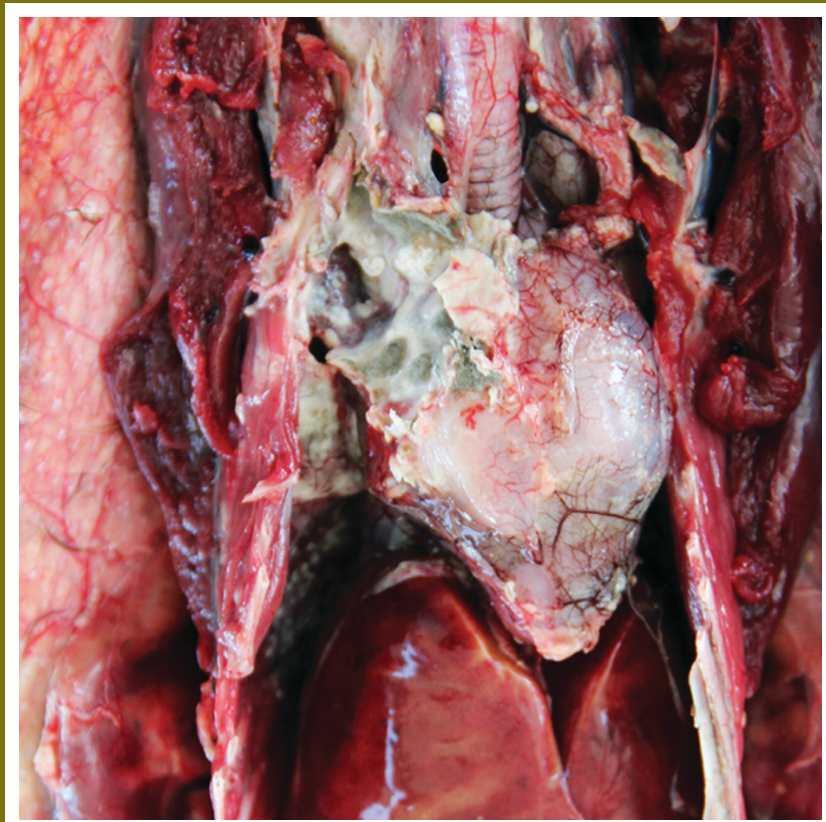
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Cover illustration: Aircacculitis of cervical air sac with white to green exudate typical of aspergillosis in a neotropical cormorant (*Phalacrocorax brasilianus*). (Echenique et al., p. 126)



Epidemiological analysis of bovine tuberculosis in the southern region of Rio Grande do Sul from 2000 to 2015¹

Taina S. Alberti^{2*} , Fabio Raphael P. Bruhn³, Rosimeri Zamboni² ,
Fabiano R. Venancio⁴ , Haide Valeska Scheid² , Margarida B. Raffi³,
Ana Lucia Schild⁵ and Eliza Simone V. Sallis³

ABSTRACT.- Alberti T.S., Bruhn F.R.P., Zamboni R., Venancio F.R., Scheid H.V., Raffi M.B., Schild A.L. & Sallis E.S.V. 2020. **Epidemiological analysis of bovine tuberculosis in the southern region of Rio Grande do Sul from 2000 to 2015.** *Pesquisa Veterinária Brasileira* 40(2):77-81. Departamento de Patologia Animal, Universidade Federal de Pelotas, Campus Universitário s/n, Capão do Leão, RS 96010-900, Brazil. E-mail: taina_alberti@yahoo.com

Bovine tuberculosis (BTB) is an infectious and zoonotic disease with socioeconomic importance, responsible for economic losses in livestock due to the decrease in animal productivity, mortality, bovine carcass condemnation and restriction on exportations. The objective of the present study was to evaluate the epidemiological aspects of this disease through a descriptive analysis and time series of cases of bovine tuberculosis diagnosed at the *Laboratório Regional de Diagnóstico* (LRD) of the *Faculdade de Medicina Veterinária, Universidade Federal de Pelotas* (UFPe) from 2000 to 2015. A survey was carried out in the LRD database of cases diagnosed as tuberculosis in cattle during this period. The incidence rate of BTB [(cases/population) x 100000] was calculated. Qualitative variables (sex, age, race, origin, type of material and presumptive diagnosis) were analyzed in a descriptive way, aiming to establish the profile of affected animals, characterize the municipalities in which the disease is originated and also fix the main referral organs for diagnosis. The time series analysis was performed using the Gretl statistical software 1.9.12 (GNU Regression, Econometric and Time-series Library). To verify the existence of the stationary distribution, the test of Dickey-Fuller was used, considering a value of $p < 0.05$. During this period, 331 cases of BTB were diagnosed in the LRD. The present study identified that the predominant profile of the bovines affected by tuberculosis was female bovine aged more than two years old and crossbreed; the main organ sent for histopathological examination was lymph nodes. The microregion of Pelotas presented the highest incidence of the disease. There was also a tendency of a decrease in the incidence rate of this disease over the years, absence of seasonal influence in the occurrence of the disease and no cases of epidemics occurred from 2002 to 2015 in the area covered by the LRD-UFPe.

INDEX TERMS: Epidemiology, bovine tuberculosis, Rio Grande do Sul, *Mycobacterium* spp., cattle, tuberculosis, *post mortem* inspection, histopathological examination.

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RESUMO.- [Análise epidemiológica da tuberculose bovina na região sul do Rio Grande do Sul no período de 2000 a 2015.] Tuberculose bovina é uma enfermidade infectocontagiosa e zoonótica, de importância socioeconômica, responsável por prejuízos econômicos na pecuária em decorrência da diminuição na produtividade animal, mortalidades, condenações de carcaças e restrições nas exportações. O objetivo do presente estudo foi avaliar os aspectos epidemiológicos, por meio de uma análise descritiva e de séries temporais, dos casos de tuberculose bovina diagnosticados no Laboratório Regional

de Diagnóstico da Faculdade de Veterinária, Universidade Federal de Pelotas (LRD-UFPel), no período de 2000 a 2015. Foi realizado um levantamento no banco de dados do LRD dos casos diagnosticados como tuberculose em bovinos, entre os anos de 2000 e 2015. Foi calculada a taxa de incidência da tuberculose bovina $[(\text{casos/população}) \times 100000]$. As variáveis qualitativas (sexo, idade, raça, procedência, tipo de material e diagnóstico presuntivo) foram analisadas de forma descritiva, tendo como finalidade estabelecer o perfil dos animais acometidos, caracterizar os municípios de origem, e estabelecer os principais órgãos remetidos para diagnóstico. A análise de série temporal foi feita através do software estatístico Gretl 1.9.12 (GNU Regression, Econometric and Time-series Library) e para verificar a existência da estacionariedade utilizou-se o teste de Dickey-Fuller aumentado, considerando um valor de $p < 0,05$. Durante este período foram diagnosticados 331 casos de tuberculose bovina no LRD. O presente estudo permitiu concluir que o perfil predominante dos bovinos acometidos por tuberculose foi de fêmeas, com mais de dois anos de idade e mestiças; o principal órgão encaminhado para exame histopatológico foram linfonodos e a microrregião de Pelotas apresentou a maior incidência da doença. Foi constatada, ainda, uma tendência de queda na taxa de incidência da doença ao longo dos anos analisados, ausência de influência sazonal na ocorrência da enfermidade e, não foram registrados casos de epidemias nos anos de 2002 a 2015, na área de abrangência do LRD-UFPel.

TERMOS DE INDEXAÇÃO: Epidemiologia, tuberculose bovina, Rio Grande do Sul, *Mycobacterium* spp., bovinos, inspeção *post mortem*, série temporal, zoonose.

INTRODUCTION

Bovine tuberculosis (BTB) is an infectious and zoonotic disease caused by *Mycobacterium bovis*, a highly pathogenic bacterium for cattle and humans (López 2013). The disease is characterized by the progressive development of nodular lesions, called “tubers”, in any organ or tissue (Riet-Correa & Garcia 2007). In livestock, this disease is responsible for significant economic losses due to the decrease in animal productivity and owing to interfere in the trade in animals and related products (Perez et al. 2002, Rocha et al. 2012).

BTB is a common disease that poses a public health risk, mainly in developing countries (Humblet et al. 2009). It is estimated that 5% to 10% of human tuberculosis cases are caused by *M. bovis* (Prasad et al. 2005, Schiller et al. 2011, Rocha et al. 2012). Consumption of raw milk and dairy products, produced without thermal processing, as well as direct contact with infected animals, constitute the primary forms of transmission of this bacterium to humans. The inspection service represents an essential tool for the control of this zoonosis (Pinto 2003).

The distribution of BTB across the national territory varies from the different production systems to the sanitary and technological practices employed in each region (Kantor & Ritacco 2006, Furlanetto et al. 2012). The prevalence of the disease in Brazil is higher in dairy farms, seeing that latest technologies are applied (Acha & Szyfres 2003, Ferreira Neto et al. 2016). The state of Espírito Santo and the northern regions of São Paulo, southern Minas Gerais, and southern Goiás have the highest BTB prevalence rates in the country, as

they concentrate the largest dairy farming (Ferreira Neto et al. 2016). The prevalence of *M. bovis* infecting cattle herds in the state of Rio Grande do Sul (RS) is 2.8%. The highest rates in this state are also observed in the main milk producing regions (metropolitan area 5.2%, north region 3.9%, and mountain range 3.3%) (Queiroz et al. 2016). Due to the great socioeconomic impact of this disease, the objective of the present study was to carry out an epidemiological analysis of BTB cases, as well as evaluating the temporal distribution of this disease diagnosed at LRD of the *Faculdade de Veterinária* (FV-UFPel) from 2000 to 2015.

MATERIALS AND METHODS

A survey was carried out in the LRD-UFPel database regarding cases of BTB diagnosed from 2000 to 2015. Necropsy protocols and materials submitted for diagnosis were reviewed. Information regarding breed, sex, and age of animals, as well as presumptive diagnoses, type of material referred for diagnosis, the origin of animals, and month/year of disease occurrence were recorded in Excel spreadsheets and subsequently evaluated. The profile of the affected animals was established through a descriptive statistical analysis of the qualitative variables. In addition, the municipalities of disease occurrence were characterized, it was also checked the efficiency of *post mortem* examination in identifying macroscopic lesions and the main organs referred for histopathological examination. Incidence rates $[(\text{cases/population}) \times 100000]$ were calculated by municipality, month and year; after time series analysis from 2002 to 2015.

Time series. Data were analyzed by evaluating the distribution of BTB cases over time, in order to evaluate possible changes in the behavior of the disease over the years, especially about the incidence rate of tuberculosis and the sensitivity of the information system. LRD-UFPel. The temporal statistical analyses were done by using the Gretl 1.9.12 statistical software (GNU Regression, Econometric and Time-series Library), and an analysis of the existence of the trend and seasonality components was performed by verifying the series and their respective correlograms. Once verified the existence of these components, the series were differentiated and subsequently statistically tested for detecting analysis of stationarity, through increased Dickey-Fuller tests (Dickey & Fuller 1979), considering a value of $p < 0.05$ (Moretton & Tolo 2006, Alberti et al. 2018). Once analysis of stationarity was observed in the differentiated series, the presence or absence of the trend and seasonality components in the monthly series considered was inferred. Ordinary Least Squares (OLS) model calculations were performed to estimate the statistically significant trend ($p < 0.05$), and the coefficient of determination (R^2) was calculated based on indicators time-series over time.

In addition, control diagrams were constructed based on the distribution of cases from 2002 to 2015, following the methodology of geometric mean, more appropriate when observing the asymmetric distribution of cases over the years, as described by Bortman (1999). The logarithmic transformation ($\log_{10}$) of the original data was performed. Regarding the transformed values, the geometric mean and 95% confidence intervals (95% CI) were calculated, which are used as the lower and epidemic endemic limits in the endemic curve (Bortman 1999).

RESULTS

From 2000 to 2015, 392 samples were sent to the LRD-UFPel with a presumptive diagnosis of tuberculosis, including 305 cases confirmed. In the same period, 23 samples were sent to the

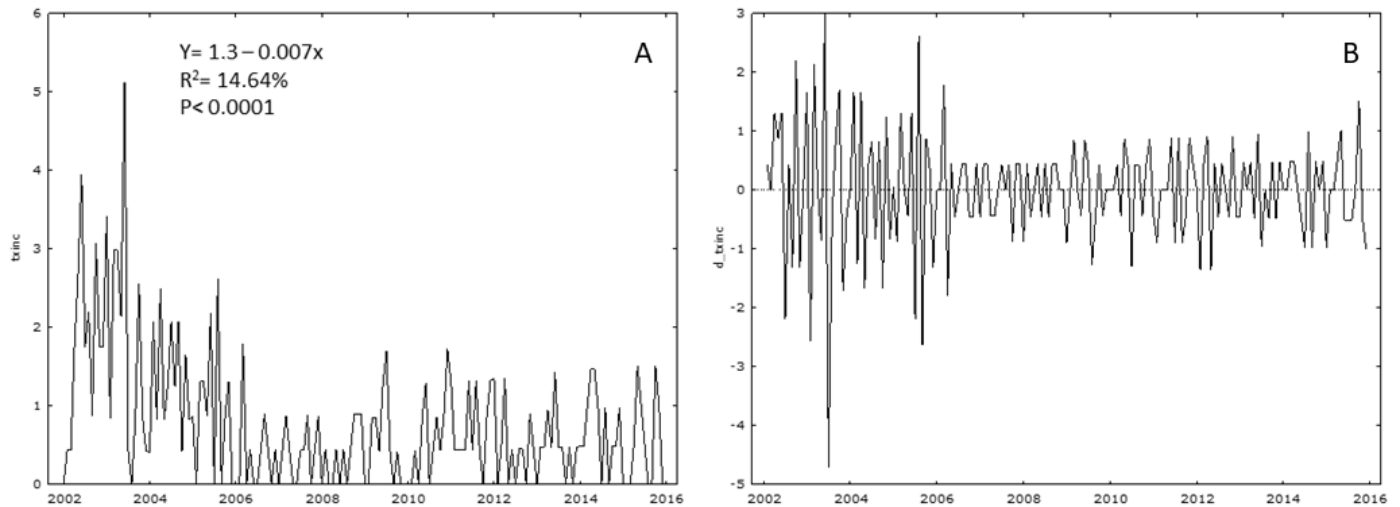


Fig.1 (A-B) Time series and equation of the trend line of the incidence rate of bovine tuberculosis (BTB) (cases/100000 cattle), in the area of “Laboratório Regional de Diagnóstico” (LRD) from the “Faculdade de Veterinária” (FV), “Universidade Federal de Pelotas” (UFPeL), Rio Grande do Sul, from 2002 to 2015. (A) Original time series showing a downward trend in the number of cases diagnosed as tuberculosis in cattle in the LRD-FV-UFPeL. (B) Differentiated time series (trend free), showing a higher occurrence of tuberculosis from 2002 to 2006.

laboratory without presumptive diagnosis of tuberculosis, and after, another three samples with suspected bovine actinobacillosis, cysticercosis and leukosis were also diagnosed as tuberculosis, consisting of a total of 331 diagnoses of BTB during this period. Considering the 314/331 (94.86%) cases of BTB, the samples were sent by the municipal inspection service of Pelotas/RS, which is responsible for inspecting the slaughtering of seven refrigerators in this municipality resulting in 1098 cattle/month. These establishments receive cattle from the municipalities of the micro-region of Pelotas. Field veterinarians referred another 17/331 (5.13%) cases from different municipalities in the southern region of RS.

Regarding animal profile, 251/331 (75.83%) animals were older than two years, and 7/331 (2.11%) younger. Female bovines represented 175/331 (52.87%) animals and males 88/331 (26.58%). In 73/331 (22.05%) and 68/331 (20.54%) of the cases, neither age nor gender were not informed. The breed was informed in 178/331 (53.77%) cases, with predominance of crossbred cattle, mainly with dairy breeds (69/331, 20.84%), followed by Holstein (46/331, 13.89%) and Jersey animals (13/331, 3.92%). Among the samples referred for diagnosis, lymph nodes were the most frequent organ sent to analysis (228/331, 68.89%). In 86/331 (25.98%) cases, together with lymph nodes, organ fragments, such as lung and liver, could be obtained; in 15/331 (4.53%) corpse, and in 2/331 (0.6%) the data was not informed. The main municipalities, where the most animals could be found, were Pelotas (45.32%), followed by Rio Grande (11.78%), São Lourenço do Sul (6.95%), Piratini (6.04%), Canguçu (5.74%) and Capão do Leão (5.45%).

To assess the time series of the incidence of BTB from 2002 to 2015, there was a downward trend in the number of cases of this disease ($p < 0.05$) and absence of seasonality ($p < 0.05$) (Fig.1A). The trend graph showed a higher number of cases from 2002 to 2006, with a sharp drop in the occurrence of this disease from 2003 to 2004 (Fig.1B). After comparing the monthly incidence rates obtained over the years concerning the epidemic limit observed in the endemic curve (Fig.2), it

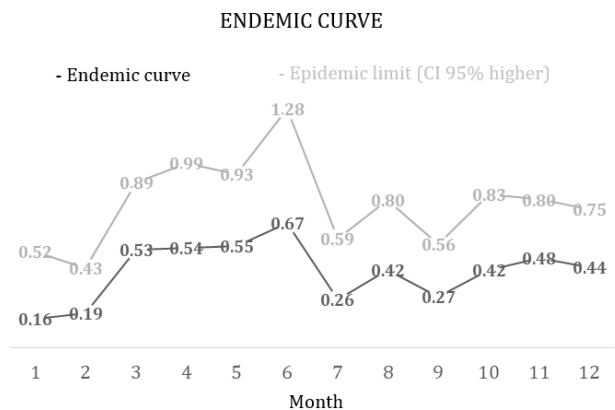


Fig.2. Endemic curve of the incidence rate of bovine tuberculosis (BTB) (cases/100000 cattle), in the area of “Laboratório Regional de Diagnóstico” (LRD) from the “Faculdade de Veterinária” (FV), “Universidade Federal de Pelotas” (UFPeL) from 2002 to 2015.

was possible to assess that there were no epidemics of BTB in the LRD region, indicating any incidence regarding the epidemic limit proposed by the curve.

DISCUSSION

In RS, 57.48% of the cattle herd was classified as beef, and 43.11% as dairy animals, and this population consists mainly of female bovines over 36 months (38.95%) (Silva et al. 2014). The predominant profile of the bovines was adult females aged older than two years, crossbred, followed by dairy animals (Holstein and Jersey), and this result corroborated these findings with those observed by Grisi Filho et al. (2011). In a study conducted in the state of Mato do Grosso, the disease was also more frequent in animals older than two years (97.6%). However, lesions were more prevalent in male bovines (76.2%) than in female bovines

(23.8%) (Furlanetto et al. 2012), and this fact may be related to the larger population of beef cattle in this state, including an elevate number of male bovine slaughter. According to Humblet et al. (2009), the sex and breed of cattle affected by tuberculosis varied according to the rearing system adopted in each region, and these are not considered risk factors in the occurrence of the disease. However, age is a significant risk factor, as it is directly related to the development of this pathology. Tuberculosis is a disease that affects mainly adult animals, due to its chronic character and higher risk of exposure to the agent over the years (Acha & Szyfres 2003), which justifies the result of this study, since female bovine animals have a longer productive life longer, staying longer in the properties. The prevalence of tuberculosis is higher in dairy than in beef cattle, due to larger agglomeration of animals in dairy farms, which favors the transmission of the disease; on the other hand, dairy cattle remain in the farms (Acha & Szyfres 2003, Grisi Filho et al. 2011).

In the present study, regarding the 228/331 cases of BTB, the lymph node was the only organ referred to the laboratory, and in 86/331 cases besides lymph nodes, fragments of other organs, such as lung and liver, were obtained. Souza et al. (2014) evaluated the slaughtering of 140 cattle and among animals, it could be noted that 78 animals presented some type of macroscopic lesion suggestive of tuberculosis infection, and in 38 cases, the lesions occurred exclusively in the mediastinal lymph nodes, 22 only in the liver, 11 in the lung and seven carcasses presented concomitant lesions in lymph nodes and liver and/or lung. The significant role of lymph nodes in fighting infections is related to the main pathway of mycobacterial infection (respiratory tract). The most frequent lesions occurred in the head, neck, and thoracic cavity lymph nodes (França et al. 2013).

In this study, the presumptive diagnosis of tuberculosis was confirmed in 77.8% of cases, a result that is similar to that found by Andrade et al. (1991). In 94.86% of the diagnoses, the samples were sent to seven municipal inspection refrigerators, located in Pelotas. These samples were sent to the LRD-UFPEL when the macroscopic lesion did not present typical features of tuberculosis. Slaughterhouses are considered "epidemiological sentinels" in disease control (Baptista et al. 2004), but regarding tuberculosis, only 47% of lesions are macroscopically detected during *post mortem* inspection (Corner 1994), due to different stages of the disease and no time for inspection (Souza et al. 2014). The histopathological examination has high sensitivity and specificity in the diagnosis of tuberculosis, being a reliable and fast tool that can help *post mortem* examination in the diagnosis (Pinto et al. 2004, França et al. 2013), furthermore, it is important to note the differentiation of other diseases detected in macroscopic lesions, such as actinobacillosis (Andrade et al. 1991, Uzal et al. 2016).

According to data from the *Ministério da Agricultura, Pecuária e Irrigação* (MAPA), Pelotas presented the highest positive number of bovines in tuberculin tests in 2015, including 463 positive results for bovines and an estimated prevalence of 1.35% (SEAPI 2016). In this study, the incidence of BTB in the LRD-UFPEL was evaluated, and a higher rate (81.28%) was observed in the municipalities of the micro-region of Pelotas (Pelotas, Rio Grande, São Lourenço do Sul, Piratini, Canguçu, and Capão do Leão). However, this result may be related to the geographic location of the LRD-UFPEL, which facilitates

the referral of samples for testing by veterinarians of this region, and to the partnership with the municipal inspection service of Pelotas-RS.

According to Ferreira Neto et al. (2016), there was a significant increase (52%) in the number of BTB in 2015 when compared to 2014 in Brazil. Over the analyzed period, there was a downward trend in the frequency of BTB cases diagnosed in the LRD-UFPEL. This downward trend in the number of cases in the LRD-UFPEL may be related to an efficiency of the municipal inspection service in recognizing the lesions found during the slaughter. In most cases, a macroscopic diagnosis is made, reducing the total of samples sent to the laboratory for histopathological examination. Another factor that may have influenced this downward trend was the implementation of the "Programa Nacional de Controle e Erradicação da Brucelose e da Tuberculose Animal" (PNCEBT) in 2001, which provided an improvement in the health status of the cattle.

In a study conducted in a slaughterhouse in Nigeria, tuberculosis showed a cyclical behavior (seasonality), with greater occurrence in July and August, differing from the result found in the present study. The authors related this seasonality to stressful factors that occur in this period (off-season) and the increase in the number of religious slaughters (Okeke et al. 2016). The absence of seasonal influence on the occurrence of tuberculosis, observed in the present study, can be explained by the variables, such as: incubation period of the microbiological agent and chronic character of the disease. Thus, the transmission cycle of disease is uncertain to detect, seeing that the disease is diagnosed, in most cases, during the slaughter of the animals. The incidence rate of BTB, according to diagnoses made on the base of LRD-UFPEL, did not show an epidemic behavior in this region; since the frequency of the cases remained within the estimated safety zone for the diagnosis same. In the evaluation of the endemic curve, it was also observed that the epidemic limit determined for June is higher and lower in July.

CONCLUSIONS

The prevalence of tuberculosis diagnosed in the LRD-UFPEL from 2000 to 2015 decreased significantly and the profile of affected animals is female bovine, aged over 36 months of age, and dairy cattle, especially crossbred.

Macroscopic identification of lesions during slaughter is efficient since 77.8% of suspected *post mortem* examination cases were histologically confirmed as tuberculosis.

During the study period, there were no seasonal and epidemic cycles of the disease in the region.

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

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Microbiological and molecular detection of *Mycoplasma bovis* in milk samples from bovine clinical mastitis¹

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ABSTRACT.- Salina A., Timenetsky J., Barbosa M.S., Azevedo C.M. & Langoni H. 2020. **Microbiological and molecular detection of *Mycoplasma bovis* in milk samples from bovine clinical mastitis.** *Pesquisa Veterinária Brasileira* 40(2):82-87. Departamento de Higiene Veterinária e Saúde Pública, Universidade Estadual Paulista, Rua Doutor Walter Maurício Correa s/n, Distrito de Rubião Júnior, Botucatu, SP 18618-681, Brazil. E-mail: hlangoni@fmvz.unesp.br

The genus *Mycoplasma* includes more than 200 bacterial species that cause disease in animals. It is responsible for causing mastitis in bovines and may be related to other manifestations, such as arthritis and pneumonia in calves and heifers. The present study aimed to detect *Mycoplasma bovis* isolated from milk samples of bovine clinical mastitis, and to compare the isolation rates in two culture media: Hayflick and SP4. An initial screening was performed in order to detect the presence of the class Mollicutes in 1166 milk samples from clinical mastitis by the conventional Polymerase Chain Reaction (PCR) technique. According to the 1166 milk samples evaluated, 8.6% (100/1166) were positive to class Mollicutes. Regarding molecular analyses, 1.1% (13/1166) of conventional PCR for positive *M. bovis* was obtained and 0.9% (11/1166) in real-time PCR. The results of the microbiological culture of the 100 samples previously screened demonstrated that 6% (6/100) of colony growth have been developed when using the Hayflick medium, and 11% (11/100) when using the SP4 medium (including the positive on Hayflick medium). Concerning the 11 isolates obtained in the microbiological culture, conventional PCR confirmed *M. bovis* in nine of them, and two cultures were negative. In the phylogenetic analysis of the isolates, all of them were grouped in *M. bovis* and *M. agalactiae* clusters. The results confirmed the importance of the presence of *M. bovis* in the etiology of bovine clinical mastitis and reinforced the need for further studies to elucidate other *Mycoplasma* species that may be involved in bovine clinical mastitis in Brazil.

INDEX TERMS: Microbiology, molecular detection, *Mycoplasma bovis*, milk, bovine mastitis, mycoplasmosis, PCR, Mollicutes, cattle.

RESUMO.- [Detecção microbiológica e molecular de *Mycoplasma bovis* em amostras de leite oriundas de mastite clínica bovina.] O gênero *Mycoplasma* inclui mais de 200 espécies que causam doenças nos animais. É responsável

por quadros de mastite em bovinos, podendo também estar relacionado à outras manifestações como artrite e pneumonia em bezerros e novilhas. O presente estudo objetivou a detecção de *Mycoplasma bovis* isolados a partir de amostras de leite de mastite clínica bovina, bem como, a comparação da taxa de isolamento em dois meios de cultura: Hayflick e SP4. Para efeito de triagem amostral, foram avaliadas quanto à presença da classe Mollicutes 1166 amostras de leite de casos de mastite clínica pela técnica de PCR convencional. Das 1166 amostras de leite avaliadas, 8,6% (100/1166) foram positivas à classe. Nas análises moleculares, obteve-se 1,1% (13/1166) de positividade para *Mycoplasma bovis* na PCR convencional e 0,9% (11/1166) na PCR em tempo real. Os resultados do cultivo microbiológico das 100 amostras triadas previamente demonstraram 6% (6/100) de crescimento

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de colônias ao se utilizar o meio Hayflick e 11% (11/100) ao se utilizar o meio SP4 (incluindo as positivas ao primeiro). A partir dos 11 isolados obtidos no cultivo microbiológico, a PCR convencional confirmou *Mycoplasma bovis* em nove deles, e dois foram negativos para o agente. Na análise filogenética dos isolados, todos agruparam no cluster *Mycoplasma bovis* e *Mycoplasma agalactiae*. Frente aos resultados, ressalta-se a importância da presença de *Mycoplasma bovis* na etiologia da mastite clínica bovina e reforça a necessidade de estudos mais aprofundados para a elucidação de outras espécies de micoplasmas que possam estar envolvidas na mastite bovina.

TERMOS DE INDEXAÇÃO: Detecção molecular, microbiologia, *Mycoplasma bovis*, leite, mastite bovina, micoplasmose, PCR, Mollicutes, bovinos.

INTRODUCTION

Mycoplasma bovis is an important agent responsible for occasional respiratory problems, clinical mastitis, and sharp drop in milk production, especially in large herds (Aebi et al. 2012). On the other hand, regarding small herds with few numbers of animals, the manifestations resulting from the presence of *M. bovis* mainly occur in the form of arthritis and pneumonia in calves and heifers (Pfützner & Sachse 1996).

More than 25 species belonging to the class Mollicutes cause mastitis and other clinical manifestations in dairy cattle (cows and heifers), such as: *M. californicum*; *M. alkaescens*; *M. arginini*; *M. bovirhinis*; *M. bovigenitalium*; *M. canadense*; *M. dispar*; *M. bovirhinis* (Higuchi et al. 2013) and *M. bovis* (Aebi et al. 2012, Nicholas et al. 2016, Al-Farha et al. 2017). In Brazil, the first report of the disease in dairy cattle was from Mettifofo et al. (1996). So far, in Brazil, few studies have been conducted to elucidate the occurrence of *Mycoplasma* infection in the mammary glands in dairy herds. Junqueira et al. (2017) reported the occurrence of *M. bovis* (3%) in samples of bovine clinical mastitis. Manzi et al. (2018), in a study regarding the occurrence of *M. bovis* in milk expansion tanks, obtained 1.4% for the same species.

The prevalence of *M. bovis* in the herds differs between studies. Al-Farha et al. (2017) obtained 76.7% of positive milk samples for *Mycoplasma* spp., and 6.2% of them, *M. bovis* was detected. In a study conducted in herds in Japan, the prevalence of *M. bovis* was higher than in other species obtained in that study, representing 59.7% of the isolates (Higuchi et al. 2013). In southeastern France, the prevalence of clinical mastitis by this agent is less than 1% (Arcangioli et al. 2011).

The identification of mycoplasmas in milk occurs through microbiological culture, considered the gold standard for detection of infections by this agent. Generally speaking, the technique is simple, but specific enrichment culture media are required for the different species involved in the etiology of mastitis (Brown et al. 2010), sometimes making the diagnosis laborious.

Molecular techniques are widely used to identify mycoplasma species involved in mastitis (Baird et al. 1999, Aebi et al. 2012, Higuchi et al. 2013). Among them, Polymerase Chain Reaction (PCR) is the most used then PCR allows rapid and sensitive result when compared to microbiological culture, furthermore, also has the possibility of identifying different species, depending on the use of different species primers.

Considering the importance of mastitis, its negative impact on the milk supply chain and the occurrence of *M. bovis* in dairy cattle reported in the national and international literature, the present study aimed to detect this pathogen by two techniques molecular: conventional and real-time PCR, as well as comparing the detection rate of the agent in two culture media, Hayflick and SP4.

MATERIALS AND E METHODS

Samples. A total of 1166 milk samples of bovine clinical mastitis originated from nine dairy farms in the region were evaluated in the area of dairy basin ABCW (Arapoti, Batavo, Castro, Witmarsum), – in the state of Paraná (PR) and property located in Sao Pedro, in the state of São Paulo (SP), identified from 'A' to 'J'. All properties have highly technified bovine production system of bovine milk, being the only type of production. The evaluated milk samples were sent to the Núcleo de Pesquisa em Mastites – Universidade Estadual Paulista (Nupemas-Unesp, Botucatu) for the diagnosis of *Mycoplasma* spp. in the herds, therefore being considered as convenience samples.

DNA extraction. After receiving milk samples, 1mL aliquots were kept in microtubes for bacterial DNA extraction. For sample screening, DNA extraction was performed following the thermolysis method described by Fan et al. (1995). An additional DNA purification step consists of adding 10% of the recovered volume of 3M sodium acetate solution (molar concentration = 3). Then 2.5x of the recovered volume of 100% ice-cold (4°C) ethanol was added, and the material was kept at -20°C overnight (Kazemiha et al. 2009).

Conventional PCR for Mollicutes and *Mycoplasma bovis*. Incubation was performed in a Thermal Cycler Mastercycler Gradient (Eppendorf). The reaction was prepared as follows: 12.5µL GoTaq® Green Master Mix (Promega), 1µL of each primer, 7.5µL ultra-pure water (Gibco®) and 3µL of extracted DNA to a final volume of 25µL. Initially, class Mollicutes of DNA amplification was performed, and once positive, *M. bovis* DNA amplification was performed using the specific primers listed in Table 1. For all reactions, a reference strain *M. bovis* Donetta (ATCC 25523) was used as a positive control. Visualization of the amplified material was assessed by an electrophoretic run on 1.5% agarose gel plus 0.06µL/mL Nancy (Sigma-Aldrich®). The gel was visualized in UV light transilluminator and the image captured by an electronic documentat system.

Real-time PCR for detection of *Mycoplasma bovis*. Taqman® real-time PCR was performed on equipment Applied Biosystems Step One™ (Applied Biosystems, Life Technologies Corporation). The real-time PCR reaction was prepared as follows: 12.5µL TaqMan® Universal PCR Master Mix (Applied Biosystems), 10µM of each primer, 10µM TaqMan® probe, 20ng or 1µL extracted DNA, ultra-pure distilled water (Gibco®) to a final volume of 25µL. The primers amplify a gene fragment *uvrC* with a 112 base pair product and show specificity for *M. bovis* using GenBank (Clothier et al. 2010) and are described in Table 1. To determine the sensitivity of the PCR reaction, serial dilutions of pure culture *M. bovis* Donetta were prepared. All reactions were performed in duplicate in 96-well plates, sealed with optical seal film.

Modified Hayflick and SP4 insulation. For isolation of *Mycoplasma* species, 10µL of each PCR-positive milk sample was cultured for Mollicutes in supplemented Hayflick solid medium as described by Whitford et al. (1994) plus 0.01% thallium acetate (González & Wilson 2003), as well as SP4 medium, according to Tully (1995). Incubation was performed in a 5% microaerophilia environment in a CO₂ greenhouse, observing microbial isolation three, five, seven, and 10 days after incubation. Microbial growth was monitored by the stereoscopic magnifying glass (Model ZEISS SteREO Discovery.V8), at 3x magnification; positive results are those with colonies with

Table 1. Oligonucleotide sequences used in conventional and real-time PCR reactions for the detection of *Mycoplasma bovis* in bovine clinical mastitis in milk samples

	Oligonucleotide sequence	Amplicon	Gene	Reference
MGSO	5' TGC ACC ATC TGT CAC TCT GTT AAC CT 3'	270pb	16S rRNA	Van Kuppeveld et al. (1992)
GPO-3	5' GGG AGC AAA CAG GAT TAG ATA CCC T 3'			
MBOF	5' CCT TTT AGA TTG GGA TAG CGG ATG 3'	360pb	16S rRNA	González et al. (1995)
MBOF	5' CCG TCA AGG TAG CAT CAT TTC CTA T 3'			
Mbov F2024	5' TCT AAT TTT TTC ATC ATC GCT AAT GC 3'	112pb	<i>uvrC</i>	Clothier et al. (2010)
Mbov R2135	5' TCA GGC CTT TGC TAC AAT GAA C 3'			
Mbov <i>uvrC</i>	FAM - AAC TGC ATC ATA TCA CAT ACT - MGB			

characteristic “egg-fried” aspect (Pretto et al. 2001). Once positive, a colony was removed along with the culture medium and inoculated into broth Pleuropneumonia-like-organisms (PPLO) plus 1% arginine for growth promotion. The isolates were identified by PCR to detect *M. bovis*.

16S rRNA gene sequencing of isolates. The sense and antisense sequences obtained as electropherograms were visualized in Chromas software version 2.6.5 (Technelysium Pty Ltd 1998-2018[®]), and manually checked for edge removal and identification of errors or discrepancies. Next, the sequences were submitted to BLASTn¹ in order to confirm the identity of the products obtained in PCR. They were then submitted to multiple alignments by the method ClustalW in the MEGA X program (Kumar et al. 2018), along with 13 other sequences from eight different mycoplasma species, including species whose isolation from bovine dairy cattle were reported in the literature (Gioia et al. 2016). The sequence of the *M. bovis* Donetta strain, used as a positive control in PCR assays, was also included.

The phylogenetic reconstruction was also performed in the MEGA X program by the Maximum Likelihood method, using the replacement model Kimura 2-parameter, assuming that some sites were evolutionarily invariable ([+ I], 66.24%) (Kimura 1980). This replacement model was defined as the most appropriate to the database, determined by the model selection procedure in MEGA X, and the one with the lowest score under the Bayesian Information Criterion (BIC). The consensus tree was inferred from 1000 Bootstrap replicates (Felsenstein 1985). The first trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms in an array of estimated peer distances using the Maximum Composite Likelihood (MCL), then selecting the topology with superior log likelihood value.

RESULTS

Molecular analysis

Regarding the 1,166 milk samples analyzed from clinical mastitis cases, 8.6% (100/1166) were positive in molecular detection for class Mollicutes. By investigating the presence of *M. bovis* in the 100 positive samples for class Mollicutes, 1.1% (13/1166) positive results for conventional PCR were obtained, while real-time PCR was 0.9% (11/1166). The positive results at least presented in one of the molecular analyzes for detection of *M. bovis* was 1.4% (16/1166) in five of the ten properties evaluated.

Considering the positive result for *M. bovis*, in any of the techniques used in this study, either molecular or microbiological

analysis, 1.5% (17/1166) of positive samples for this agent was obtained.

Three samples were negative to conventional PCR for *M. bovis*, however, positive samples were detected in real-time PCR. Eight samples were positive in both molecular tests. Five samples were negative for real-time PCR and positive for conventional PCR.

Considering that convenience samples were chosen among a total of 10 properties, with at least one positive milk sample for *M. bovis*, 50% (5/10 - A, B, C, D, and F) of the properties were positive for *M. bovis* when evaluated by molecular techniques.

Sequencing analysis

Concerning to the amplified product of the isolates and sequencing, the isolates were similarly grouped for *M. bovis*, confirming the results obtained in PCR from the analysis of clinical mastitis milk. Two negative isolates for conventional PCR in *M. bovis* were also grouped with similarity to the same agent in the phylogenetic analysis.

Microbiological analysis

The 100 PCR-positive milk samples for class Mollicutes were grown in modified Hayflick medium, and SP4 supplemented medium. Isolation of 6% (6/100) for the genus *Mycoplasma* were obtained from Hayflick, and 11% (11/100) from SP4, and all Hayflick positive samples were also SP4 positive. SP4 culture medium has been shown to be 18.8 times higher for isolation of the genus *Mycoplasma* (P=0.0253) when compared to isolation of *Mycoplasma* in Hayflick medium (95% IC=8.01-44.05).

One sample was negative in both molecular tests for *M. bovis* detection originated from the clinical mastitis in milk samples. However, there was a growth of colonies in the microbiological culture. The isolate obtained after cultivation was confirmed as *M. bovis* in conventional PCR technique.

Three analyzed clinical mastitis in milk samples were positive for all tests performed: conventional and real-time PCR, Hayflick medium, and SP4 medium.

DISCUSSION

Molecular analysis

The *M. bovis* research on bovine milk samples is widely performed due to the higher prevalence and importance of this pathogen in the etiology of mastitis, reported in previous studies (Aebi et al. 2012, Higuchi et al. 2013, Gioia et al. 2016). The detection of 1.4% of this species obtained in the

¹ Nucleotide Basic Local Alignment Search Tool (BLAST), National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine, Bethesda, Maryland, USA. Available at <http://www.ncbi.nlm.nih.gov/BLAST>

present study is lower than that reported by other authors (Higuchi et al. 2013, Lysnyansky et al. 2016).

It is important to note that no studies are referred to the etiology of *Mycoplasma* spp. infection in the mammary glands in Brazil, this way being difficult to compare the occurrence of the most prevalent species of this agent in the country. The literature provides data related only to the occurrence of *M. bovis* in dairy herds. In milk expansion tanks, Manzi et al. (2018) obtained a 1.4% prevalence of *M. bovis*, in 67 tank samples analyzed. Junqueira et al. (2017) analyzed milk samples from clinical mastitis and reported the occurrence of 3% of *M. bovis* detected by conventional PCR.

On the other hand, in other countries, the occurrence of other *Mycoplasma* spp. is widely studied. In a study conducted in Japan, Higuchi et al. (2013) obtained 89% (184/208) of fourth breasts infected with only one species of mycoplasma, being: *M. bovis* the most prevalent, followed by *M. alkalescense*, *M. californicum*, *M. arginini*, *M. canadense*, *M. bovigenitalium* and *M. adleri*. Furthermore, it is noted that some animals were coinfecting with more than one species of *Mycoplasma*.

In New York State, United States of America (USA), the prevalence of *M. bovis* was reported to be highest among other *Mycoplasma* spp.. Gioia et al. (2016) described the occurrence of the following species of class Mollicutes, responsible for causing mastitis: 78% of *M. bovis*; 17% of *M. alkalescens*; 11% of *Acholeplasma laidlawii*; 10% of *M. bovigenitalium*; 10% of *M. canadense*; 3% of *M. californicum*; 2% of *M. arginini*; 2% of *Acholeplasma oculi* and 1% *M. bovirhinis*.

PCR is a molecular tool widely used in research involving a variety of samples, including milk samples. One of the obstacles to this technique is the presence of Taq polymerase inhibitors that may trigger false negative reactions. These inhibitors may be from the sample itself or introduced during some reaction step. Milk samples naturally have calcium and fat in their composition, which may inhibit PCR depending on the concentrations (Schrader et al. 2012). Powell et al. (1994) could find in milk, a natural Taq polymerase inhibitor, in other words, plasmin, a component capable of inhibiting the PCR reaction.

It is suggested the presence of some PCR inhibitor in the milk of one of the evaluated samples. The referred clinical sample was decisive in the detection of class Mollicutes; on the other hand, had a negative impact on both molecular techniques for the detection of *M. bovis*. However, it was positive in microbiological culture for that species. This fact suggests a false negative result for *M. bovis* from milk analysis. The possibility of a low concentration of DNA from *M. bovis* in the clinical sample may be related to this finding. Real-time PCR amplifies specific regions of the bacterial genome from minimal amounts of DNA (Valasek & Repa 2005), and real-time PCR is more sensitive than conventional PCR. However, this sensitivity depends directly on the quality of genomic DNA. Templeton et al. (2003) compared diagnostic techniques for the detection of *M. pneumoniae* and concluded that real-time PCR is more sensitive, specific, and superior to conventional PCR in detecting the pathogen in samples from adult patients with infection of lower respiratory tract. The present study did not evaluate the sensitivity between the two techniques in the detection of *M. bovis*.

The thermolysis method is a “non-onerous” and simple option, but it could be detected results with lower DNA quality

DNA than commercial kits, with proteins or other PCR inhibitors. However, it is a feasible technique for diagnosing *Mycoplasma* spp. in milk, with excellent results obtained and already standardized. The group decided to perform the extraction by this method, followed by a protocol of DNA purification to obtain a better quality analytical material, taking into account the costs and practicality of the method. For conventional PCR, it was possible to use thermolysis method with satisfactory results in detecting *M. canadense* and *M. californicum* from isolates of milk samples (Tamiozzo et al. 2014).

In the present study, the efficiency of extracted DNA was not evaluated for the presence of inhibitors or cell lysis, and also the thermolysis protocols were not compared with other data, so it could not be affirmed if false-negative results were resulting from poor DNA quality, by considering the DNA extraction used.

The differentiation of *M. agalactiae* and *M. bovis* species from the sequence of their 16s rRNA is difficult to detect due to their high similarity between their 16s rRNA (Königsson et al. 2002, Bashiruddin et al. 2005). Among the isolates obtained in the present study, two of them were opposite to conventional PCR for positive *M. bovis* from bovine clinical mastitis in milk sample for the same agent. In sequencing analysis, the results from the isolates were grouped in cluster that includes the species *M. bovis* and *M. agalactiae*, due to the high similarity between them.

Given the positive molecular results for *M. bovis* obtained from clinical mastitis in milk samples, it is suggested that two species, even without the amplification of the isolate, would be responsible for their epidemiology in animals. However, it is suggested to use other *primers* and further studies to investigate variations or mutations that may be occurring in these isolates to confirm this theory (Lysnyansky et al. 2008).

The epidemiology of the two species, *M. bovis* and *M. agalactiae* in animals, are distinct. The first occurs in cattle, mainly causing mastitis, pneumonia, and arthritis (Pfützner & Sachse 1996), and the second, in small ruminants, responsible for contagious agalactia (González et al. 1995). There are reports of the incidence of *M. agalactiae* in bulls with the involvement of ocular, cerebral, and auditory canal (Catania et al. 2016), however, regarding mastitis, there are no reports so far.

The properties evaluated in the present study use high technology for the production of bovine milk and no goat or sheep rearing; therefore, *M. agalactiae* bovine mastitis is unlikely to occur in this study.

Microbiological analysis

The difficulty in isolating *Mycoplasma* spp. in culture medium is known due to the characteristics related to the different species. To growth the detection of other species, two culture media were used in the present study, but there was no growth of different species of *M. bovis*.

Modified Hayflick medium is recommended for the isolation of *M. bovis* in milk samples (Pfützner & Sachse 1996) and successfully used by several authors (Pretto et al. 2001, Cai et al. 2005, Riekerink et al. 2006). The use of more than one culture medium may increase the isolation rate of *Mycoplasma* spp., as observed by Lai et al. (1986), when comparing Chalquest and Hayflick's means for detecting *M. pulmonis*, with 92% and 66% bacterial isolation, respectively.

Tortorelli et al. (2017) obtained only positive isolation on Hayflick medium in 32 sections of bovine respiratory disease, stressing a low percentage when compared to other studies. The authors also observed that the result of bacterial isolation is often not associated with PCR in a positive way, comprising negative molecular and positive culture results. This result could also be observed in the present study.

The samples used in this study were satisfactory, i.e., referred for diagnosis of *Mycoplasma* spp. in different herds. All samples were obtained frozen and kept at freezing temperature for a period of up to one month to be processed. Freezing causes a decrease in the number of viable microorganisms that cannot be recovered in microbiological cultivation (Parker et al. 2018). The low isolation rate obtained in the present study may be associated with freezing or thawing of milk samples, viable microbial load reduction, and the possibility of false-negative results in microbiological culture.

The occurrence of negative results to the microbiological culture associated with concomitant positive PCR results is reported in the literature (Férandon et al. 2011). Positive results in microbiological cultivation may vary between different studies. The use of more than one type of culture medium can influence the results positively since it is known that mycoplasma species present different mechanisms of bacterial growth. Férandon et al. (2011) obtained 29.4% (45/153) of positive results in the microbiological culture, superior to that obtained in the present study, which was 11%, and may be related to the quality and cell viability of the samples analyzed.

The high percentage of negative samples in the culture can be mainly due to the possibility of cellular unfeasibility. However, the culture medium was not specific for all *Mycoplasma* spp., considering that only *M. bovis* was identified in PCR. In addition to these aspects, the possibility of other pathogens present in the samples should be considered, seeing that may occur microbial competition, thus hinder the isolation of *Mycoplasma* spp., considering the longer time required for isolation.

It is expected that the PCR results may outperform microbiological culture, whereas detecting degenerate DNA from non-viable *mycoplasmas* or negative cultures may contain a low concentration of detectable DNA in PCR. This finding was observed in the present study, with 89% of negative samples in microbiological culture.

CONCLUSION

It was possible to confirm the presence of *M. bovis* by using genetic sequencing. The use of SP4 culture medium increased the detection rate of viable mycoplasmas when compared to Hayflick's medium, and its use is satisfactory for the microbiological diagnosis of *M. mastitis*.

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

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Pathogenic potential of *Brucella ovis* field isolates with different genotypic profile and protection provided by the vaccine strain *B. ovis* $\Delta abcBA$ against *B. ovis* field isolates in mice¹

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ABSTRACT.- Carvalho T.P., Ribeiro N.Q., Mol J.P.S., Costa F.B., Eckstein C., Paula N.F., Paixão T.A. & Santos R.L. 2020. **Pathogenic potential of *Brucella ovis* field isolates with different genotypic profile and protection provided by the vaccine strain *B. ovis* $\Delta abcBA$ against *B. ovis* field isolates in mice.** *Pesquisa Veterinária Brasileira* 40(2):88-96. Departamento de Clínica e Cirurgia Veterinária, Escola de Veterinária, Universidade Federal de Minas Gerais, Campus Pampulha, Av. Pres. Antônio Carlos 6627, Belo Horizonte, MG 31270-901, Brazil. E-mail: rls@ufmg.br

Brucella ovis causes economic and reproductive losses in sheep herds. The goal of this study was to characterize infection with *B. ovis* field isolates in a murine model, and to evaluate protection induced by the candidate vaccine strain *B. ovis* $\Delta abcBA$ in mice challenged with these field isolates. *B. ovis* field strains were able to colonize and cause lesions in the liver and spleen of infected mice. After an initial screening, two strains were selected for further characterization (*B. ovis* 94 AV and *B. ovis* 266 L). Both strains had *in vitro* growth kinetics that was similar to that of the reference strain *B. ovis* ATCC 25840. Vaccination with *B. ovis* $\Delta abcBA$ encapsulated with 1% alginate was protective against the challenge with field strains, with the following protection indexes: 0.751, 1.736, and 2.746, for mice challenged with *B. ovis* ATCC25840, *B. ovis* 94 AV, and *B. ovis* 266 L, respectively. In conclusion, these results demonstrated that *B. ovis* field strains were capable of infecting and inducing lesions in experimentally infected mice. The attenuated vaccine strain *B. ovis* $\Delta abcBA$ induced protection in mice challenged with different *B. ovis* field isolates, resulting in higher protection indexes against more pathogenic strains.

INDEX TERMS: Pathogeny, *Brucella ovis*, isolates, genotypic profile, protection, vaccine strain $\Delta abcBA$, mice, immunization, field isolated strains, brucellosis.

RESUMO- [Vacina viva atenuada *Brucella ovis* $\Delta abcBA$ encapsulada protege camundongos frente a desafios de *B. ovis* isoladas de campo.] *Brucella ovis* é responsável por perdas econômicas e reprodutivas em rebanhos ovinos. O objetivo deste trabalho foi caracterizar a infecção com as cepas isoladas de campo de *B. ovis* em modelo murino e avaliar a eficiência vacinal da mutante *B. ovis* $\Delta abcAB$

para proteção contra desafio com as cepas isoladas de campo. Foram utilizadas sete cepas isoladas de campo foram capazes de colonizar e provocar lesões no fígado e no baço de camundongos após sete dias pós-infecção. Após triagem, duas cepas foram selecionadas para a melhor caracterização (*B. ovis* 94 AV and *B. ovis* 266L). Ambas apresentaram crescimento em placa de cultivo semelhante ao da cepa de referência *B. ovis* ATCC 25840. A vacinação com a cepa de *Brucella ovis* $\Delta abcBA$ encapsulada com alginato a 1% foi capaz de proteger camundongos desafiados com as cepas isoladas de campo, com os seguintes índices de proteção: 0,751, 1,736 e 2,746, para camundongos desafiados com *B. ovis* ATCC 25840, *B. ovis* 94 AV e *B. ovis* 266 L, respectivamente. Estes resultados demonstraram que as cepas isoladas de campo de *B. ovis* são capazes de infectar e induzir lesão em camundongos experimentalmente infectados. O uso da cepa

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mutante atenuada *B. ovis* $\Delta abcBA$ para vacinação de fêmeas C57BL/6 desafiados com diferentes cepas de *B. ovis* induziu proteção nos camundongos desafiados com diferentes cepas de *B. ovis*. Deste modo, mostrando-se eficiente na proteção das cepas de campo de *B. ovis*.

TERMOS DE INDEXAÇÃO: Vacina viva, *Brucella ovis*, $\Delta abcBA$, camundongos, imunização, brucelose ovina, mutante encapsulada, cepas isoladas de campo.

INTRODUCTION

Brucellosis is a group of infectious diseases caused by facultative, intracellular, Gram-negative coccobacillary bacteria of the genus *Brucella* that affects domestic and wild animals and causes zoonotic infections in man (Olsen et al. 2011). *B. ovis* is considered a non-zoonotic species, and it is responsible for economic and reproductive losses in sheep herds (Poester et al. 2013).

Brucellosis in rams is clinically characterized by unilateral or bilateral granulomatous epididymitis and seminal vesiculitis. These changes result in poor sperm quality with increased defects of the tail of the spermatozoa, presence of inflammatory cells in the ejaculate, and consequent subfertility or infertility (Carvalho Júnior et al. 2012, OIE 2015). In ewes, the disease is usually asymptomatic, but endometritis and, more rarely, abortions, stillbirths, and weak offsprings may be observed (Grilló et al. 1999).

Currently, the vaccine available in some countries for *B. ovis* prevention is the Rev-1 strain, a live attenuated *Brucella melitensis* vaccine (Ridler & West 2011). However, the Rev-1 strain can interfere with serological tests, it can induce abortions when administered to pregnant animals, and it is capable of infecting and causing disease in humans (Blasco & Díaz 1993, Blasco 1997).

Brucella spp., as well as other intracellular bacteria has several strategies to achieve a safe replication niche within the host cell (Gorvel & Moreno 2002). Intracellular survival of *Brucella* spp. requires a functional *virB*-encoded type IV secretion system (T4SS). *Brucella* strains lacking a functional T4SS cannot evade degradation in lysosomes so they do not replicate or survive within the host cell (Celli et al. 2003). A previous study demonstrated that a *B. ovis* specific ABC transporter is required for *B. ovis* survival *in vivo* and evasion from phagosome/lysosome fusion (Silva et al. 2011b, Macedo et al. 2015). Additionally, *B. ovis*-specific ABC transporter is required for normal expression of the *virB*-encoded T4SS since in the absence of this ABC transporter there is a post-transcriptional impairment of expression of *virB*-encoded proteins (Silva et al. 2014). Indeed, *B. ovis* mutant strains lacking a functional *B. ovis*-specific ABC transporter (Silva et al. 2011b) or the *virB*-encoded T4SS (Sá et al. 2012) have similar phenotypes.

ABC transporters have various substrates including polyamines (Igarashi et al. 2001), peptides (Detmers et al. 2001), and amino acids (Hosie & Poole 2001, Danese et al. 2004). *Brucella* spp. genome encodes several ABC transporters, whereas *B. ovis* has 29 pseudogene-forming mutations in coding sequences for ABC-like carrier systems, so *B. ovis* cannot transport some substances such as polyamines, erythritol, and glycine (Jenner et al. 2009). A *B. ovis*-specific genomic island (Tsolis et al. 2009), named BOPI-1 for *B. ovis* pathogenicity island 1 (Silva et al. 2011b), encodes an ABC transporter that

is essential for pathogenesis since the *B. ovis* $\Delta abcBA$ strain is strongly attenuated *in vitro* and *in vivo* so this genomic island has been named BOPI-1 for *B. ovis* pathogenicity island 1 (Silva et al. 2011b). However, the substrates of this particular ABC transporter are still unknown (Silva et al. 2014). In spite of its attenuation, *B. ovis* $\Delta abcBA$ triggers humoral and cellular immune responses in rams that are indistinguishable from those triggered by the wild-type parental strain (Silva et al. 2013). Therefore, *B. ovis* $\Delta abcBA$ has been tested as an experimental candidate vaccine strain and provided protection in a mouse model of infection (Silva et al. 2015a). Furthermore, when tested in the natural host, this vaccine strain prevented any clinical sign of disease, macro- and microscopic lesions, and induced sterile immunity in experimentally challenged rams (Silva et al. 2015b).

There is relatively low genetic variability within the genus *Brucella*, which has even supported the proposition of a monospecific genus (Verger et al. 1985). However, there are striking differences in host specificity and pathogenicity among different *Brucella* species (Chain et al. 2005). Therefore, the Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA) has been used as a tool for genetic and epidemiologic characterization of *Brucella* spp. (Whatmore 2009). The analysis of fourteen *B. ovis* Brazilian field isolates demonstrated some degree of genetic diversity (Dorneles et al. 2014). Considering the molecular differences identified by MLVA-16 among *B. ovis* field isolates, the aim of this study was to characterize field isolates of *B. ovis* in a murine model of infection and to evaluate the efficiency of the *B. ovis* $\Delta abcBA$ vaccine strain to protect mice challenged with *B. ovis* field isolates.

MATERIALS AND METHODS

Bacterial strains. As detailed in Table 1, this study included seven field *Brucella ovis* strains isolated from semen of naturally infected rams, which have been previously genotypically characterized by MLVA-16 (Dorneles et al. 2014), the reference wild-type strain *B. ovis* ATCC 25840, and the candidate vaccine strain *B. ovis* $\Delta abcBA$ (Silva et al. 2011b, 2015a, 2015b). Bacteria were grown on the tryptic soy agar (TSA) plates supplemented with 1% hemoglobin, for 3 days at 37°C with 5% CO₂. For the vaccine strain (*B. ovis* $\Delta abcBA$), TSA was supplemented with 1% hemoglobin and 100 µg/mL of kanamycin. Bacteria were suspended in phosphate-buffered saline (PBS) (pH 7.4) and bacterial concentration was estimated by spectrophotometry (Smart Spec, Bio-Rad, Hercules, CA) at the optical density of 600nm (OD 600).

Animals. The experimental protocol used in this study has been approved by the Animal Experimentation Ethics Committee at the Universidade Federal de Minas Gerais (CEUA-UFMG protocols 41/2018 and 107/2015). Mice were maintained in cages under controlled temperature and humidity (25°C, 70%), fed commercial feed and water *ad libitum*. Mice were intraperitoneally infected with 1 x 10⁶ colony forming units (CFU) of *B. ovis* suspended in 100µL of sterile PBS. Euthanasia was performed at 1, 7, or 30 days post-infection (dpi).

***In vitro* growth of *Brucella ovis* ATCC 25840 and field isolated strains.** *In vitro* growth of *B. ovis* ATCC 25840 and field isolates (94 AV and 266L) was evaluated on solid media as follows: bacterial suspensions were prepared in PBS to a concentration of 10³ CFU/mL. 100µL of each suspension were then plated on TSA medium with 1% hemoglobin and without antibiotics. Plates were incubated at 37°C in 5% CO₂ and at 0, 12, 24, 48, 72, 96, and 120 hours post-inoculation colonies were harvested and suspended in either 1mL (0 to 48h)

Table 1. *Brucella* strains used in this study

Strain	City	Country	Year of isolation
<i>B. ovis</i> ATCC 25840	—	Australia	1960
<i>B. ovis</i> $\Delta abcBA$	—	—	2011
<i>B. ovis</i> 94 AV	Livramento/MS	Brazil	1995
<i>B. ovis</i> 266 L	Livramento/MS	Brazil	1995
<i>B. ovis</i> 0204	Uruguaiana/MS	Brazil	1997
<i>B. ovis</i> 286 L	Livramento/MS	Brazil	1995
<i>B. ovis</i> 252 L	Livramento/MS	Brazil	1995
<i>B. ovis</i> 100 V	Livramento/MS	Brazil	1995
<i>B. ovis</i> 203 L	Livramento/MS	Brazil	1995

or 2mL (72 to 120h) of sterile PBS. Bacterial suspensions were serially diluted (10-fold dilutions) and plated on TSA plus 1% hemoglobin by using the drop plate method. The experiment was performed in duplicate, and the total numbers of CFU per milliliter were determined at each time point.

***In vitro* infection of RAW 264.7 murine macrophages.**

The murine macrophage cell line RAW 264.7 was cultured in RPMI medium (Gibco; Invitrogen) supplemented with 10% fetal bovine serum (FBS). Cells were seeded in 96-well culture plates (5×10^5 macrophages/well) and incubated at 37°C with 5% CO₂. Macrophages were infected with the *B. ovis* ATCC 25840 or field isolates (*B. ovis* 94 AV or 266 L) at a multiplicity of infection (MOI) of 100. Plates were centrifuged at $1,000 \times g$ for 5min at 15°C and incubated at 37°C for 30min. Macrophages were washed once with sterile PBS and then incubated at 37°C for 1h with RPMI solution supplemented with 10% FBS and 50µg/mL of gentamicin (Invitrogen, São Paulo, Brazil). Next, each well was washed once with sterile PBS, and macrophages were lysed with sterile distilled water for 20min at 0, 4, 24, and 48hpi. Intracellular bacteria recovered from lysed macrophages were serially diluted (10-fold dilutions) in PBS and plated in duplicate on TSA medium with 1% hemoglobin for 3-6 days of incubation at 37°C with 5% CO₂ for CFU counting. Two independent experiments were performed in triplicates.

Vaccine experiments. Encapsulation of the *B. ovis* $\Delta abcBA$ vaccine strain was performed as previously described (Silva et al. 2015a). Briefly, 2×10^{10} CFU of *B. ovis* $\Delta abcBA$ were resuspended in 2mL of 1% alginate solution (Sigma-Aldrich) and dripped in 10mL of polymerization solution (0.5 mM CaCl₂), using a 0.23mm x 4mm needle, followed by homogenization for 15 minutes. Capsules were washed twice with 10mM MOPS solution with 0.85% NaCl (pH 7.4) for 5min. Capsules were then shaken with 0.05% alginate solution for 5min. The encapsulated vaccine strain was subcutaneously inoculated in fifteen female C57BL/6 mice with a final dose of 1×10^8 CFU per mouse. Other fifteen mice were inoculated with sterile PBS by the same route. The size of the capsules has previously been described by Silva et al. (2015a). Four weeks after immunization, mice were intraperitoneally challenged with 10^6 CFU of wild-type *B. ovis* (ATCC 25840) or field strains (*B. ovis* 94 AV and 266 L). Two weeks later, mice were euthanized, and samples of liver and spleen were aseptically collected, weighed, and homogenized in 2mL of sterile PBS. Serial 10-fold dilutions of the homogenates were plated for the CFU counting. Briefly, organs were homogenized in sterile PBS and plated on the TSA plates with 1% hemoglobin. Bacterial colonies were counted at 3-6 days after plating.

Histopathology. Liver, spleen, superficial cervical lymph node, and the subcutaneous site of vaccination were sampled, fixed by immersion in 10% buffered formalin for 24 hours, and embedded

in paraffin. Four µm tissue sections were stained with hematoxylin and eosin. Lesions (inflammation and necrosis) were scored from 0 to 3, being 0-absent, 1-mild, 2-moderate, and 3-severe, with a total score ranging from 0 to 6.

Statistical analysis. Statistical analyses were performed using the Graph Pad Prism version 5.0 software. CFU values were logarithmically transformed prior to analysis of variance (ANOVA). Means were compared by the Tukey's test. Histopathological scores were compared using the non-parametric Mann-Whitney test.

RESULTS

***Brucella ovis* field isolates were capable of infecting mice**

Infectivity of field isolates was assessed in BALB/c mice (n=3 per group) that were inoculated with 10^6 CFU of each *Brucella ovis* strain (100 V, 203 L, 266 L, 204, 286 L, 252 L, and 94 AV). At 7 days post-infection, strains 94 AV and 252 L had higher numbers of CFU/g in the spleen when compared to the other strains ($p < 0.05$), with differences of more than one log of CFU (Fig.1A). In the liver, *B. ovis* 94 AV was recovered in higher numbers when compared to other strains (Fig.1B). The spleen and liver (Fig.1C,D) from all infected mice had multifocal microgranulomas characterized by a histiocytic and neutrophilic inflammatory infiltrate with epithelioid macrophages. There were no significant differences in histopathology scores between different strains (data not shown). These results indicate that all *B. ovis* field isolates were capable of colonizing and cause lesions in the liver and spleen of BALB/c mice.

***Brucella ovis* field isolates had variable growth kinetics in RAW 264.7 cells**

Considering our initial results (Fig.1), two strains were selected for further characterization: *B. ovis* 94 AV, which was recovered in higher numbers in the liver and spleen; and *B. ovis* 266 L, which had a phenotype similar to the other isolates. Initially, *in vitro* growth of *B. ovis* field isolates was compared to that of the reference strain *B. ovis* ATCC 25840 in TSA medium with 1% hemoglobin. Both field isolates had *in vitro* growth curves similar to the reference stain. All strains had an exponential growth phase between 24 and 72h of incubation at 37°C with 5% CO₂, and then entered the stationary growth phase (Fig.2A). The kinetics of intracellular growth of these strains was then assessed by infecting RAW 264.7 murine macrophage cells. At 0 and 4 hours after infection, significantly higher CFU numbers of *B. ovis* ATCC 25840 were recovered when compared to the field isolates ($p < 0.05$), with more than

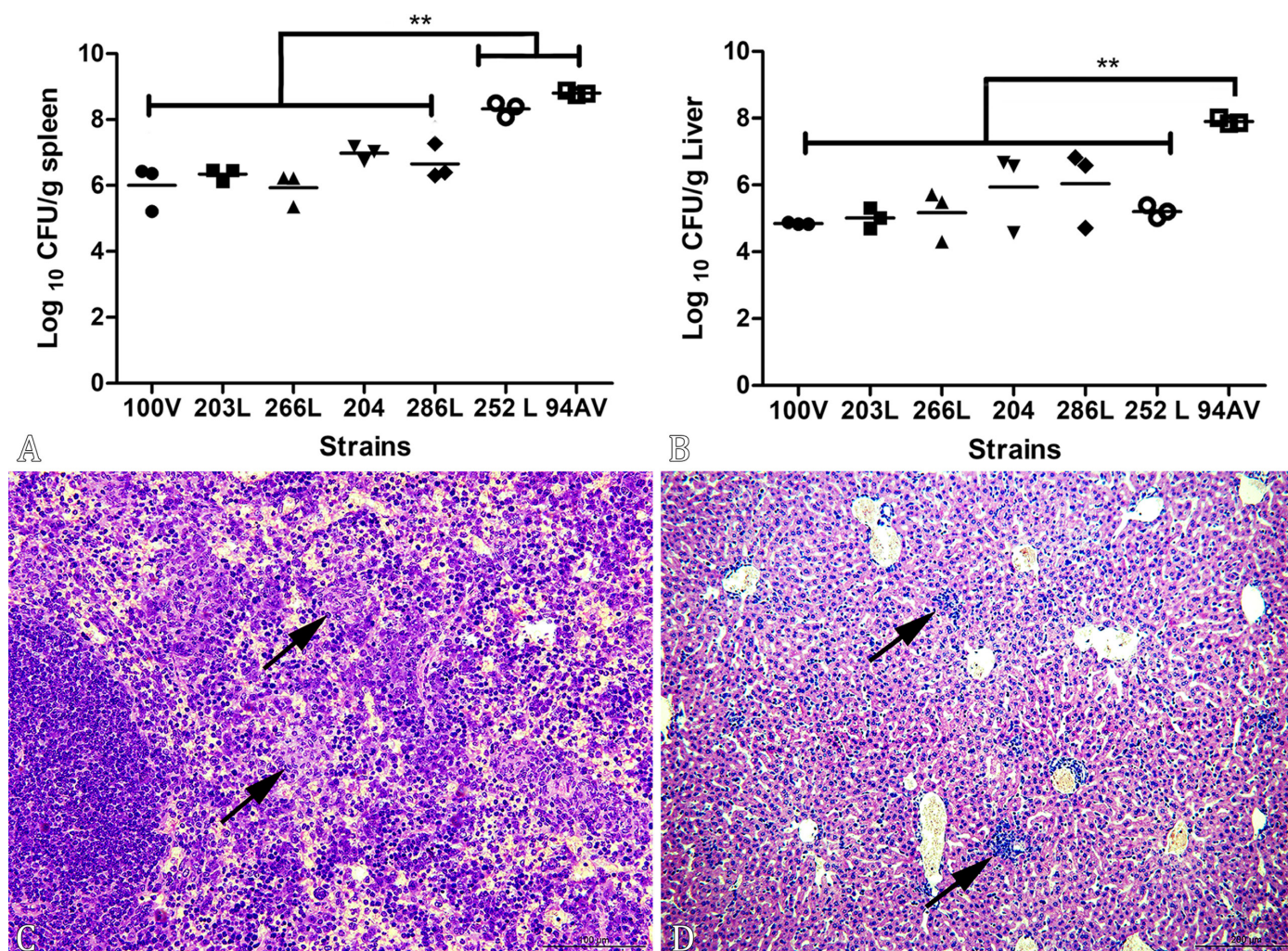


Fig.1. Experimental infection of BALB/c mice with different field isolated strains of *Brucella ovis*. Mice were infected with 1×10^6 CFU/mouse from different field strains (100 V, 203, 266 L, 204, 252 L, 286 L, and 94 AV). Each data point represents the number of CFU/g recovered from the (A) spleen and (B) liver of each mouse at 7dpi. The line in each group indicates the mean. All data were logarithmically transformed prior to ANOVA, and the means were compared by the Tukey's test. Statistically significant differences are indicated by asterisks (** $p < 0.01$). All infected mice developed microgranulomas (arrows) in the (C) spleen and (D) liver. (C,D) HE, bar = 100 μm, bar = 200 μm.

one log difference at time 0, indicating that the reference strain *B. ovis* ATCC 25840 had higher levels of internalization in RAW cells when compared to field strains (Fig.2B). At 24 hours after inoculation, the opposite was observed with significantly higher CFU numbers of field isolates recovered from the intracellular compartment of macrophages (approximately one log difference) when compared to the reference strain ($p < 0.05$), demonstrating that the reference strain underwent a decrease in its intracellular population before it started growing intracellularly, whereas the field isolates, although less invasive, grew steadily from the beginning of the time course. At 48 hours after inoculation, both field isolates were recovered in higher numbers when compared to the previous time points indicating they were all able to survive and grow intracellularly in RAW cells (Fig.2B). These results clearly demonstrated a different kinetics of internalization and intracellular survival between the reference strain and

field isolates. Although less invasive, field isolates were able to start multiplying intracellularly at very early time points, when compared to the reference strain, which had an initial decline before start multiplying within macrophages (Fig.2B).

Colonization of spleen and liver of mice infected with *Brucella ovis* field isolates

Considering the differences in intracellular growth, we investigated the kinetics of infection of *B. ovis* 94 AV and 266 L in the mouse model. BALB/c mice ($n=5$ per group) were intraperitoneally infected with 10^6 CFU/mice of the reference strain *B. ovis* ATCC 25840 or the two field isolates. Mice were sampled at 1, 7, and 30dpi. At 1dpi, bacterial loads in the spleen and liver were significantly higher (nearly 2 log difference) in mice infected with the reference strain ($p < 0.05$), when compared to both field isolates (94 AV and 266 L). At 7dpi, all strains had similar bacterial loads in the spleen

(Fig.3A), whereas *B. ovis* 266 L was recovered in lower numbers from the liver when compared to the reference strain (Fig.3B). At 30dpi, mice challenged with *B. ovis* 266 L had higher bacterial loads in the spleen and liver ($p<0.05$) when compared to mice infected with the other field isolate (94 AV) and the reference strain (Fig.3A,B).

Histological changes were similar in the liver and spleen from mice infected with different strains. At 1dpi, there were no inflammatory changes, whereas at 7 and 30dpi there were moderate multifocal microgranulomas in the liver and spleen. There were no significant differences in histopathology scores attributed to histological lesions in the spleens and livers from mice infected with different strains.

Immunization with encapsulated *Brucella ovis* $\Delta abcBA$ induces protection of experimentally challenged mice with field isolates

Previous studies demonstrated that the attenuated mutant strain *B. ovis* $\Delta abcBA$ induces protection in mice and in rams (Silva et al. 2015a, 2015b). Here we assessed whether vaccination with *B. ovis* $\Delta abcBA$ protects mice challenged with field isolated *B. ovis* strains, which is relevant since all previous

studies evaluated protection against the reference strain, and in here we demonstrated differences in the kinetics of intracellular and *in vivo* infection and growth when comparing field isolates with the reference strain. As expected, immunized mice had significant reduction in bacterial loads in the liver and spleen when compared to non-immunized mice ($p<0.001$). Protection indexes are described in Table 2. Interestingly, protection indexes were higher in mice challenged with field strains, particularly mice challenged with *B. ovis* 266 L, with reductions in splenic bacterial loads close to 1, 2 or 3 logs of CFU in mice challenged with *B. ovis* ATCC 25840, *B. ovis* 94 AV, or *B. ovis* 266 L, respectively.

Bacterial colonization in the liver was also significantly lower in vaccinated mice, with decreases in bacterial loads in the range of 0.5, 1, and 1 for mice challenged with *B. ovis* ATCC 25840, *B. ovis* 94 AV, and *B. ovis* 266 L, respectively.

Immunized mice challenged with different *B. ovis* strains did not develop splenomegaly, while non-immunized mice developed evident splenomegaly after infection. In the liver of all non-immunized mice, there were multifocal coalescent firm white nodular lesions of approximately 0.1 to 0.4cm in diameter. In contrast, immunized mice developed less

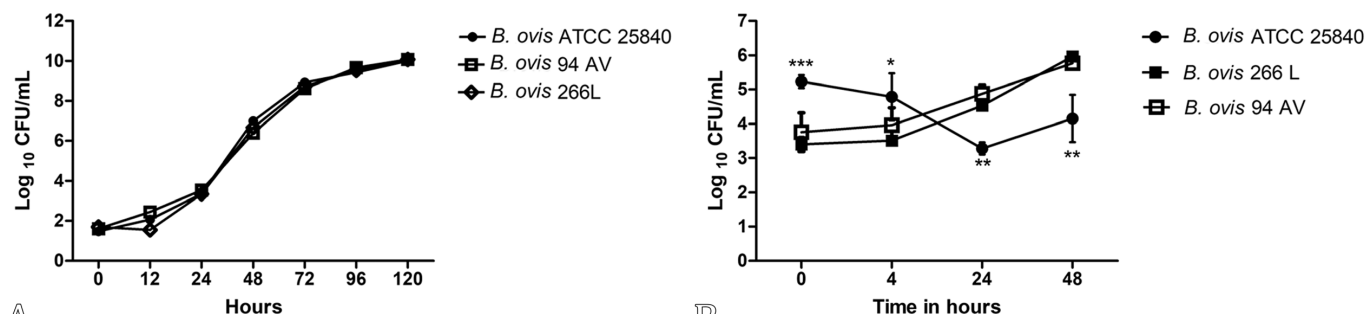


Fig.2. (A) *In vitro* growth curve of different *Brucella ovis* strains. *B. ovis* ATCC 25840, 94 AV, and 266 L were grown on TSA plates with 1% hemoglobin at 37°C with 5% CO₂. (B) *In vitro* infection of murine macrophage cell line RAW 264.7 (MOI 100) with the reference *B. ovis* strain (ATCC 25840) or field isolates (94 AV and 266 L). Macrophages were grown in 96-well plates and infected with *B. ovis* strains with a MOI of 1:100. Intracellular bacteria were recovered at 0, 4, 24, and 48hpi. Time zero represents the number of CFUs of bacteria after 1 hour of incubation with gentamycin. Data points represent the mean and standard deviation of two independent experiments performed in triplicates. Data were logarithmically transformed prior to ANOVA, and means were compared by the Tukey's test. Statistically significant differences are indicated by asterisks (* $p<0.05$; ** $p<0.01$).

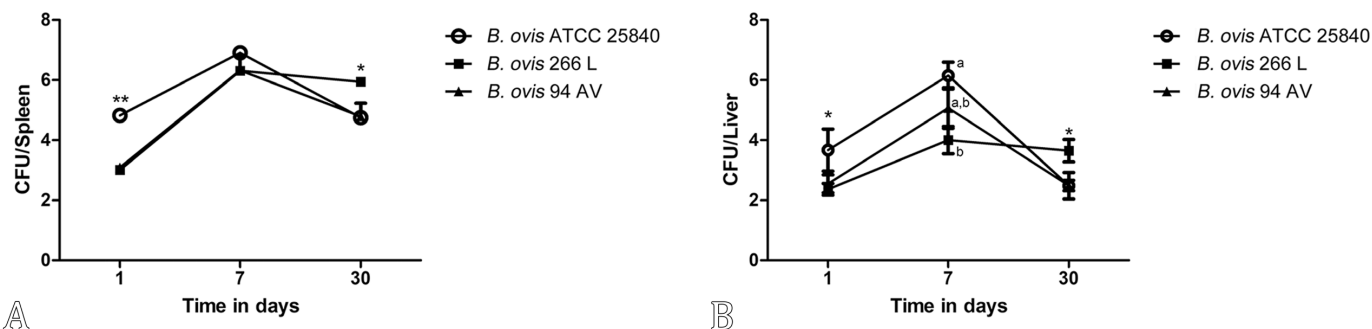


Fig.3. Kinetics of *Brucella ovis* infection in BALB/c mice. Mice were intraperitoneally infected with 1×10^6 CFU of *B. ovis* ATCC 25840, 94 AV, or 266 L. Samples of (A) spleen and (B) liver were collected for bacterial counts at 1, 7, and 30dpi. Each data point represents the mean and standard deviation ($n=5$). Data were logarithmically transformed prior to ANOVA, and means were compared by the Tukey's test. Statistically significant differences are indicated by asterisks (* $p<0.05$; ** $p<0.01$). Differences between the groups in the liver at 7dpi are indicated by different letters ($p<0.05$).

severe lesions (Fig.4). Histologically, non-immunized mice had a moderate to severe, multifocal, inflammatory infiltrate composed of epithelioid macrophages and neutrophils with mild accumulation of fibrin in the marginal zone and red pulp in the spleen, characterizing a moderate to severe, multifocal pyogranulomatous splenitis. Immunized mice had milder similar microscopic lesions. Histopathology scores were

significantly lower in immunized mice when compared to non-immunized controls ($p < 0.01$). Histological changes in the liver of nonimmunized mice were characterized by a mild to moderate, multifocal, randomly distributed, inflammatory infiltrate composed of epithelioid macrophages, neutrophils, and lymphocytes, associated with moderate multifocal necrosis and thrombosis. Immunized mice developed only a few mild

Table 2. Protection indexes induced by *Brucella ovis* $\Delta abcBA$ encapsulated with alginate in C57BL/6 mice experimentally challenged with different strains of *B. ovis*

Challenge strain (1×10^6 per mouse)	CFU/spleen immunized mice	CFU/spleen non-immunized mice	Protection index
<i>B. ovis</i> ATCC 25840	4.668 ± 0.383	5.419 ± 0.219	0.751*
<i>B. ovis</i> 266 L	3.786 ± 0.276	6.532 ± 0.649	2.746**
<i>B. ovis</i> 94 AV	4.846 ± 0.599	6.596 ± 0.355	1.736**

* Statistically significant difference ($p < 0.05$), ** statistically significant difference ($p < 0.01$)

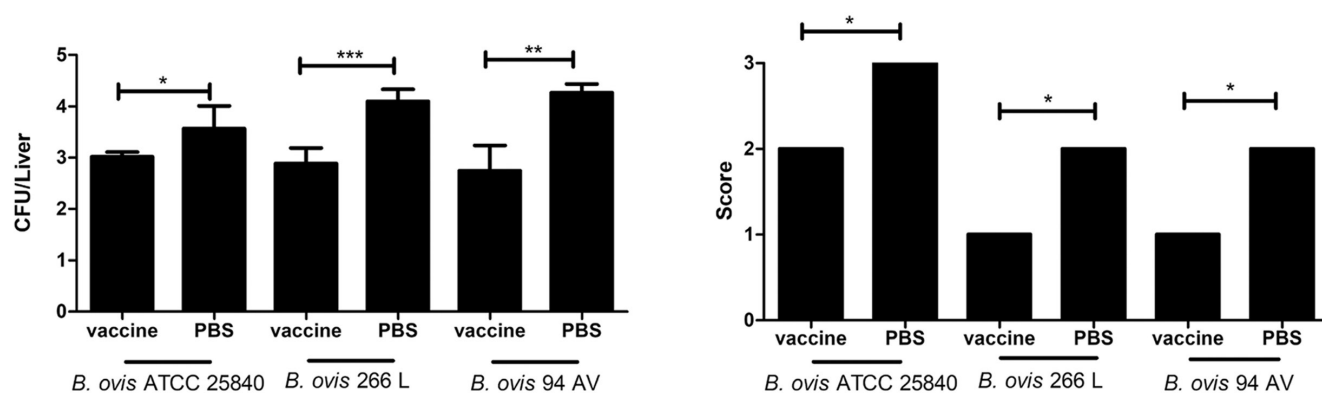


Fig.4. Protection induced by the vaccine strain *Brucella ovis* $\Delta abcBA$ encapsulated with alginate in C57BL/6 mice experimentally challenged with different *B. ovis* strains. (A) Number of *B. ovis* CFU recovered from the liver. Each column represents the mean and standard deviation ($n=5$). Raw data were logarithmically transformed prior to ANOVA, and means were compared by Tukey's test. Significant differences between bacterial strains are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; * $p < 0.001$). (B) Score for lesions in the liver of mice. Means were compared by the Kruskal-Wallis nonparametric test. Representative histological changes in the liver of (C) non-immunized mice with extensive microgranulomas associated with necrosis; or (D) immunized mice with very mild changes. Mice challenged with *B. ovis* 266 L. (C,D) HE, bar = 100 μm.**

microgranulomas in the liver (Fig.4). Histopathology scores for hepatic lesions in groups immunized with encapsulated *B. ovis* $\Delta abcBA$ were significantly lower when compared to non-immunized mice ($p < 0.01$) regardless of the challenge strain (Fig.4).

Vaccination sites were initially swollen, but this change regressed significantly until the day of euthanasia. Histopathologically, there were small granulomas at the site of vaccination (data not shown).

DISCUSSION

This study characterized *in vivo* and *in vitro* behavior of *Brucella ovis* field isolates. There were clear differences in pathogenic potential among *B. ovis* field isolates based on intracellular growth as well as *in vivo* infection in the mouse model. This is a relevant finding considering the fact that *Brucella* spp. have little genetic variability (Tsolis 2002). Interestingly, protection indexes induced by the candidate vaccine strain *B. ovis* $\Delta abcBA$ were higher for strains with higher virulence. These results indicate that the vaccine strain protects against different strains of *B. ovis*, and protection is even more evident against more pathogenic strains, demonstrating a robust immunogenicity of this experimental vaccine formulation.

Variable pathogenicity among field isolates should not be considered unexpected since *Brucella*, like other bacteria, is able to undergo spontaneous mutations or metabolic adaptations depending on the environmental conditions to which it is exposed, including temperature, humidity, host cell defense, and intracellular environment. Minimal genomic mutations may result in major phenotypic changes affecting survival and virulence of bacteria. For instance, the vaccine strain *B. abortus* S19, isolated in 1923 from the milk of a Jersey cow (Buck 1930), that after being accidentally left out at room temperature for one year spontaneously developed an attenuated phenotype (Graves 1943). Importantly, there were no previous studies comparing the pathogenicity of *B. ovis* strains with different genotypes based on MLVA-16 (Dorneles et al. 2014).

All seven *B. ovis* strains included in this study were directly isolated from the semen of naturally infected rams (Dorneles et al. 2014), indicating that these rams likely had clinical changes and were sources of infection to other sheep within their herds (Burgess 1982). Although there is no information regarding clinical signs associated with these isolates, MLVA16 demonstrated different genotypes (Dorneles et al. 2014). Therefore, in order to assess possible differences in pathogenic potential of these strains, we used the mouse, which has been extensively employed as an infection model for *Brucella* spp. (Silva et al. 2011a) being a suitable model for *B. ovis* infection (Silva et al. 2011b). In this study, mice infected with 10^6 CFU of *B. ovis* field isolates (100 V, 203 L, 266 L, 204, 286 L, 252 L, and 94 AV) became experimentally infected. All strains were capable of causing lesions in the liver and spleen at 7dpi, although there were significant differences in their ability to multiply intracellularly and colonize and survive *in vivo*.

B. ovis strains included in this study were isolated directly from the natural host (Dorneles et al. 2014), where the bacteria face harsh intracellular conditions including exposure to reactive oxygen species, low pH, and low nutrient levels. *Brucella* spp. can adapt to the intracellular environment upon activation of

expression of certain virulence factors (Kohler et al. 2002). Different strains of a given *Brucella* species may exhibit different intracellular kinetics (Harmon et al. 1988, Kohler et al. 2002, Silva et al. 2014). This may explain the differences in intracellular and *in vivo* survival and multiplication observed in this study, whereas *in vitro* growth on solid medium was remarkably similar among these isolates. Adaptation and attenuation of *Brucella* reference strains handled frequently in the laboratory conditions to *in vitro* and *in vivo* models is described (Bosseray 1991, Grilló et al. 2012). Although it may warrant different phenotype of reference strain from field isolates, it does not explain *in vivo* difference between field isolates.

BALB/c and C57BL/6 are suitable models for *B. ovis* infection since they develop a systemic infection that results in lesions in the liver and spleen. BALB/c mice are more susceptible to *B. ovis* than C57BL/6 mice, and under experimental conditions, mice do not develop *B. ovis*-induced genital lesions as observed in the natural host, which makes the mouse a useful model of infection although they do not mimic the natural disease (Silva et al. 2011b). In general, the mouse model is useful for comparing different strains. In this study, strains 94 AV and 266 L were able to colonize the spleen and liver and persist for up to 30 dpi. Virulent *Brucella* strains are capable of colonizing the liver and spleen of mice and persist for a long period (Silva et al. 2011b, Grilló et al. 2012). Our results indicate that the two field isolates tested were fully virulent since they were capable to establish systemic infection and persist in the mouse, and survive intracellularly in cultured macrophages. Importantly, the field strain *B. ovis* 266 L had the best fitness both intracellularly in cultured macrophages as well as *in vivo* in mice.

Recent studies have demonstrated that the candidate vaccine strain *B. ovis* $\Delta abcBA$ induces a protection in mice (Silva et al. 2015a), while it promotes sterile immunity in experimentally challenged rams (Silva et al. 2015b). In mice, higher protection indexes were induced by the vaccine strain *B. ovis* $\Delta abcBA$ in C57BL/6 mice when compared to BALB/c mice (Silva et al. 2015a). This study demonstrated that the vaccine strain *B. ovis* $\Delta abcBA$ also provided protection for mice challenged with different field isolates. Interestingly, the highest protection index was observed in the group challenged with strain 266 L, which had the best adaptation to intracellular survival in cultured macrophages and *in vivo* colonization and persistence. Importantly, vaccinal protection was not restricted to lower colonization by the virulent strain, but also by prevention of lesions since histopathology scores were lower in the vaccinated mice. These results are quite encouraging since the vaccine strain performed even better when vaccinated mice were challenged with more pathogenic field strains, which likely activate virulence factors more efficiently for adaptation to the host (Kohler et al. 2002). These results also support the dogma in brucellosis vaccinology that live vaccines are more efficient compared to the other vaccine categories (Carvalho et al. 2016). Considering the efficiency of this vaccine strain under experimental conditions, and the absence of a commercially available and specific *B. ovis* vaccine, this vaccinal protocol may potentially be an efficient tool for preventing reproductive losses caused by *B. ovis* (Carvalho Júnior et al. 2012, Poester et al. 2013). Furthermore, unlike the *B. melitensis* Rev1 vaccine strain used in several

countries, *B. ovis* $\Delta abcBA$ does not have zoonotic potential, thus eliminating the occupational risks due to accidental human vaccine exposure (Xavier et al. 2009).

CONCLUSION

In conclusion, there were significant differences in pathogenicity among *Brucella ovis* field isolates. Importantly, the *B. ovis* $\Delta abcBA$ vaccine strain induced protection against field isolates with protections indexes that were higher for mice challenged with more pathogenic strains.

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Influence of organic chromium supplementation on the performance of beef calves undergoing weaning-related stress¹

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ABSTRACT. - Sousa I.K.F., Sousa R.S., Mori C.S., Morini A.C., Neves K.A.L., Minervino A.H.H. & Ortolani E.L. 2020. **Influence of organic chromium supplementation on the performance of beef calves undergoing weaning-related stress.** *Pesquisa Veterinária Brasileira* 40(2)97-101. Departamento de Clínica Médica, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Butantã, São Paulo, SP 05508-270, Brazil. E-mail: ortolani@usp.br

Dietary chromium supplementation before, during, and after weaning was conducted to evaluate the hypothesis that chromium supplementation could reduce weaning-induced cortisol release in beef calves. We examined the effects of chromium supplementation in 150 crossbred calves (male and female) between five and six months of age. The calves were randomly divided by sex and breed into two equal homogeneous groups (n=75). One group was used as the control, and the other experimental group received supplementation with 0.9mg of chromium carbon-amino-phospho-chelate per 100kg BW. The chromium supplement was mixed with mineral salt for the consumption of 0.1% of BW, and the supplement was administered via creep feeding 60 days before and 60 days after forced weaning. Calves were weighed, and their blood and urine samples were obtained at four time-points: T0 (60 days before weaning), T1 (at weaning), T2 (48 hours after weaning), and T3 (60 days after weaning). Blood samples were used to determine chromium, cortisol, total protein, and albumin concentrations, and urine samples were used to determine urinary creatinine and chromium levels. Cumulative weight gain was higher in calves supplemented with chromium before weaning and during the experiment (P<0.05). In addition, weaning-related stress caused an increase in chromium excretion in the urine, and chromium supplementation reduced stress, which resulted in lower cortisol and total protein levels during weaning.

INDEX TERMS: Organic chromium, supplementation, beef calves, weaning stress, cattle, performance, stress, mineral supplementation, chromium.

RESUMO. - [Influência da suplementação com cromo orgânico no desempenho de bezerros de corte submetidos ao estresse da desmama.] O estudo foi realizado para avaliar a hipótese de que a suplementação dietética com cromo antes, durante e após a desmama possa diminuir a concentração de cortisol causado por este processo em bezerros de corte. Para tal, foram utilizados 150 bezerros mestiços, machos e fêmeas, entre cinco e seis meses de

idade. Os animais foram divididos randomicamente por sexo e grupo genético em dois grupos homogêneos (n=75), um mantido como controle e outro suplementado com 0,9mg de carboaminofosfoquelato de cromo/100 kg PV misturado a um sal proteinado para ser consumido na base de 0,1% do PV via *creep feeding*, no decorrer de 60 dias antes e 60 dias após à desmama forçada. Os animais foram pesados e foram coletadas amostras sanguíneas e urinárias no M0 (60 dias antes da desmama), M1 (desmama), M2 (48 horas após a desmama) e M3 (60 dias após à desmama) para determinação de cromo, cortisol, proteína total e albumina no sangue e da concentração urinária de creatinina e cromo. O ganho acumulado de peso foi superior nos bezerros suplementados com cromo antes da desmama e no decorrer de todo o experimento (P<0,05). A suplementação com cromo reduziu

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os teores de cortisol e de proteína total durante a desmama. O estresse da desmama provocou aumento da excreção de cromo pela urina.

TERMOS DE INDEXAÇÃO: Suplementação, cromo orgânico, bezerros de corte, estresse da desmama, bovinos, desempenho, estresse, suplementação mineral, desmama.

INTRODUCTION

During weaning, calves are exposed to several stressful factors, including separation from the mother, limited udder access, milk deprivation, and changes to their physical and social environment (Newberry & Swanson 2008, Weary et al. 2008). In addition to the modification of normal physiological mechanisms in cattle, stress can lead to increases in disease susceptibility (Enriquez et al. 2011), plasmatic cortisol levels (Lay et al. 1998), and concentrations of acute phase proteins (Carroll et al. 2009).

When exposed to severe stress conditions, rats and humans exhibit significantly increased urinary chromium excretion (Anderson 1987). Although strong evidence suggests that this phenomenon also occurs in cattle, the hypothesis still needs to be tested. To date, there are no established chromium requirements for ruminants; however, previous studies have shown that chromium requirements increase under stressful conditions (Underwood & Suttle 1999). The creep feeding system provides specific mineral supplementation to calves, including chromium (Cr) supplementation (Burton et al. 1994, Wright et al. 1995, Gudev et al. 2007, Yanchev et al. 2008), in addition to favoring contact with solid food prior to weaning progressively reduced the nutritional and social dependency of calves (Weary et al. 2008, Enriquez et al. 2011).

Several studies have also shown that chromium supplementation under stressful conditions exerted a positive effect on productive performance and overall health status. In addition, it also helped to improve the immune response, and this was observed following vaccination in cattle (Faldyna et al. 2003, Pechova & Pavlata 2007, Montemor & Marçal 2009, Kumar et al. 2013). Therefore, increased interest in using chromium as a dietary supplement for beef calves is justified by the possible stimulatory effect on their growth rates and immune responses (Chang & Mowat 1992, Moonsie-Shageer & Mowat 1993, Depew et al. 1998, Kumar et al. 2013).

The present study aimed to verify the effect of dietary chromium supplementation on cattle performance during pre- and post-weaning periods, and it also sought to evaluate the relationship between weaning-induced stress and urinary chromium excretion.

MATERIALS AND METHODS

A total of 150 Nelore crossbred calves were analyzed in this study, and the calves were randomly divided by sex, genetic group, and weight into two experimental groups (75 nursing calves in each group). During the 120-day experiment, calves were kept in paddocks containing *Brachiaria brizantha* and *Panicum maximum* grass, and they were given access to creep feeding troughs. The calves were weighed at the beginning of the experiment T0 (60 days before weaning), T1 (at weaning) and T3 (60 days after weaning). All weighing was conducted after 16 hours of fasting.

The control group received a protein-salt supplement, which included 20% raw protein and an increased amount of essential macro- and microelements as compared to chromium. The treatment group received the same diet, with a 30mg chromium carbon-amino-phospho-chelate (as Cr⁺³)/kg dry mass (DM) supplement. In both groups, each supplement was offered so that the average daily consumption per calf was 0.1% of the body weight (BW) (adjusted based on the animal's weight). The protein salt was formulated with 63% corn bran and 37% soy bran, to which 30% mineral supplement was added. This provided 1.17-2.45mg Cr/animal/day to the smallest and largest calves, respectively. Moreover, this amount met the Nutritional Requirement Criteria (NRC 2000) for beef cattle, which state that the chromium concentration should range between 0.6 and 0.75mg Cr/animal/day. Grass samples were collected from paddocks throughout the experiment to determine the chromium levels, which varied from 0.05 to 0.12ppm (average of 0.08ppm.).

The weighing was conducted in corrals, which separated calves from the rest of the cattle. The calves were separated into two different compartments based on the experimental group, and each animal was provided a social space of 10m². During the 48-hr weaning period, water and protein salt were provided *ad libitum*. Weaned calves, separated from their mothers, were then returned to the paddocks until the end of the experiment (60 days after weaning).

During all time points (T0, T1, T2 (48 hours after weaning), and T3), blood samples were collected from calves in tubes without anticoagulants (BD Vacutainer®, New Jersey, EUA). Analyses of serum samples were conducted to determine concentrations of total protein, albumin, cortisol, and chromium. Biochemical analyses were carried out in a Daytona RX (Randox®, Antrin, UK) automatic biochemical analyzer, using specific kits (Randox®, Antrin, UK) for each variable. The determination of cortisol concentrations was performed using a chemiluminescent assay kit (Siemens®, Munich, Germany) in an immunoassay analyzer (Immulite 1000, Siemens®, Munich, Germany).

Urine samples were collected at the four time-points during the experiment, after manual stimulation of the vulva or foreskin. The sample were centrifuged and the supernatant stored in microtubes and frozen at -20°C until analysis. Samples were analyzed to determine chromium and creatinine (Cr_t) concentrations. The urine creatinine were determined in equipment Daytona RX (Randox®, Antrin, UK), using kit Randox®. The resulting calculations and the metabolic weight (live weight^{0.75}) of each animal were used to calculate the chromium urinary secretion rate (CrUSR), which corresponds to the secreted amount of chromium in the urine (CrU) (corrected by urinary Cr_t and metabolic weight). This value was calculated following the recommendations of Fleming et al. (1991) and Soares (2004).

Serum and urine also samples were used to determine chromium concentration via digestion with chromium-free nitric acid and perchloric acid solutions (4:1 v/v), which were analyzed using optical spectrophotometry by plasma emission (Varian®, Saint Claire, USA) (Minervino et al. 2018).

Statistical analyses were performed with the aid of the SAS statistical software (SAS 2009). The normal distribution of the data was assessed by the Shapiro-Wilk test, and data that followed a parametric distribution were subjected to an analysis of variance using the PROC MIXED procedure for repeated measurements over time. The effects of treatment, time, and the interaction between treatment and time were examined for each variable. Data that did not follow a parametric distribution were subjected to the Mann-Whitney test, and median values were reported. The correlation and determination coefficients were calculated to determine the correlation between two variables. A P-value equal to or lower than 5% was considered significant for all statistical tests.

RESULTS AND DISCUSSION

Compared to control animals, chromium-supplemented calves exhibited greater accumulated weight gain 120 days after the beginning of the experiment ($P=0.037$), and an average gain of 6.5kg was observed in treated animals. This effect of chromium on the accumulated weight gain was verified during the weaning period (T0-T1, $P=0.006$), and there was no significant difference between groups when the weight gain during the post-weaning period (T1-T3) was considered (Table 1).

The relationship between chromium and animal production is controversial, because different studies indicated that no relationship exists between chromium supplementation and increased body weight in ruminants (Chang & Mowat 1992, Zanetti et al. 2003, Pechova & Pavlata 2007, Yanchev et al. 2008, Kumar et al. 2013). However, the methodology and analysis employed in the present study are likely factors that contributed to the positive results obtained. For instance, most studies assessed BW data and daily weight gain, which are typically heterogeneous because of the individual characteristics of animals. In the present study, the accumulated weight gain was defined as the amount (in kg) that each animal gained during a given period, and the use of this parameter negated the effects associated with the variability of BW. In addition, the large number of animals employed in this study and the use of timed artificial insemination (TAI) generated animal consignments resulted in greater homogeneity between groups.

In contrast to most studies, which did not correlate chromium supplementation to enhanced productive performance, this study provided the mineral to calves before the occurrence of an acute stress event (60 days prior). The factors associated with increased accumulated weight gain in supplemented animals were associated with lower stress, reduced fighting, and frequent visits to the trough. Among animals with limited stress, the benefits of chromium supplementation would not correspond to enhanced productive gain (Zanetti et al. 2003).

The mean chromium serum concentrations in supplemented and non-supplemented animals are shown in Table 3. Chromium levels in serum were expected to significantly increase in response to supplementation, but there were no differences between the treated and control groups. Despite the fact that excessive chromium levels in the blood correlate to toxic levels, few cattle studies have assessed chromium levels in the blood, which may vary based on the amount of chromium in the food (e.g., 57.7-1,769.0nM) (Pechova & Pavlata 2007, Tomza-Marciniak et al. 2011). The chromium levels in blood values found in this study were within the indicated interval and were non-toxic. The results of a recent study indicated that the concentration of chromium in serum significantly increased when calves were supplemented with a diet containing high levels of chromium ($\geq 4\text{mg/day}$) (Kumar et al. 2013). In the present study, calves ingested an estimated maximum of 2.5mg Cr/day, which did not interfere with the

Table 1. Accumulated weight gain (kg) of the calves during the experiment

Time point	Control group		Treatment group		P
	Mean	SD	Mean	SD	
T1-T0	54.5 ^B	13.9	60.1 ^A	11.7	0.006
T3-T1	14.9	8.0	15.8	5.2	0.464
T3-T0	68.1 ^B	17.3	74.6 ^A	14.7	0.037

^{A,B} Uppercase letters in the column indicate differences between groups, and lowercase letters indicate differences between time points.

Table 2. Urinary chromium excretion rate (nM) in control and treated calves assessed at different time points

Time point	Control group	Treatment group	P
	Median	Median	
T0	1.93 ^b	2.14 ^b	0.5725
T1	14.00 ^a	10.93 ^{a*}	0.3254
T2	9.24 ^a	19.28 ^{a*}	0.0735
P	0.0235	0.0218	

^{a,b} Lowercase letters indicate differences between time points based on the Mann-Whitney test.

Table 3. Average concentration and standard deviation of serum and urinary chromium (Cr, nM), cortisol (nM), and total protein (Pt, g/dL) in control and treated calves assessed at different time points

Variable	Group	Time point			
		T0	T1	T2	T3
Serum Cr (nM)	Control	257.3 ± 90.2	216.9 ± 49.9	-	257.3 ± 94.1
	Treatment	222.7 ± 78.7	222.5 ± 57.6	-	266.9 ± 113.3
Urinary Cr (nM)	Control	192.2 ± 123.4 ^c	238.1 ± 92.9 ^c	443.1 ± 148.2 ^a	316.8 ± 147.2 ^b
	Treatment	228.2 ± 104.2 ^c	185.6 ± 91.6 ^c	379.0 ± 131.7 ^a	333.1 ± 142.4 ^b
Cortisol (nM)	Control	64.03 ± 21.25 ^b	110.4 ± 41.44 ^a	76.45 ± 28.98 ^{Ab}	98.25 ± 36.43 ^a
	Treatment	66.79 ± 3.03 ^b	99.63 ± 4.69 ^a	63.73 ± 3.03 ^{Bb}	106.53 ± 5.79 ^a
Pt (g/dL)	Control	69.0 ± 4.2 ^c	70.4 ± 4.1 ^{Aa}	69.5 ± 4.7 ^b	69.6 ± 5.3 ^{Bb}
	Treatment	69.2 ± 6.6 ^b	68.8 ± 4.5 ^{Bb}	68.3 ± 4.3 ^b	71.4 ± 5.0 ^{Aa}

^{A,B} Uppercase letters in the column indicate differences between groups, ^{a,b,c} lowercase letters indicate differences between time points.

serum concentration. Furthermore, the results of this study did not corroborate the conclusion proposed by Pechova & Pavlata (2007), which stated that serum concentrations were poor indicators of chromium status in normal cattle.

Several studies in humans have shown that various forms of stress led to increased urinary chromium excretion that could be up to 10 times higher than normal (Pechova & Pavlata 2007). However, there are no studies in ruminants that report increased urinary chromium excretion under stressful conditions. The results of this study show an increase in the urinary chromium excretion rate at peak stress levels (T2) relative to the T0 time point, and these levels ranged from 380% to 800% in the control and supplemented groups, respectively (Table 2). By examining this variable, it was possible to determine that dietary chromium supplementation tended to increase this index ($P=0.0791$) (Table 2) relative to the control group, which suggests that chromium is lost from its organic reserves in calves under stressful conditions.

When creatinine levels were not used to appropriately correct the urinary chromium levels, no differences in urinary chromium levels were detected between the supplemented and the control groups (Table 3). However, the levels varied in relation to the time points in that levels were higher in T2 as compared to T3, and both values were higher when compared to T0 ($P<0.05$) for the supplemented and control groups. These results highlighted the fact that the excretion rate was more effective and accurate when assessing urinary chromium levels, following proper adjustment with urinary creatinine data. In fact, many animals under stress become more dehydrated and urinate less, which results in concentrated urine that could mask the results.

Lower levels of cortisol were detected in animals supplemented with chromium at T2 relative to controls, and the time point analysis within each group indicated that cortisol was greater at T1 and T3 relative to the values obtained at T0 and T2 (Table 3). This result suggested that under stressful conditions, chromium diminished excessive cortisol excretion, which causes metabolic disorders in animals. Similar results were previously described in a study where chromium was added to cattle diets (Chang & Mowat 1992, Mowat et al. 1993, Yanchev et al. 2008, Kumar et al. 2013).

Relative to the control group, lower and higher concentrations of total protein were detected in the supplemented group at T1 and T3, respectively. Within the control group, higher protein concentrations were found at T1, followed by T2 and T3, and these concentrations were greater than those detected at T0. In the supplemented group, the highest protein level was detected at T3 (Table 3). The analysis did not detect a positive correlation ($r=0.196$) between albumin and serum protein levels in the control group at T1. Considering that the serum protein level at this time point was higher than levels at the other time points for this group, we might speculate that the protein increase was triggered by the presence of acute phase proteins. This hypothesis is based on the fact that weaning-mediated stress causes an immediate increase in the level of acute phase proteins in blood; however, this aspect was not addressed in this study (Wright et al. 1995, Carroll et al. 2009).

The supplemented group displayed a greater concentration of total protein and serum albumin as compared to the control group at T3 (Table 3). An analysis of these variables at T3

indicated a positive correlation ($r=0.611$), which suggested that increased total protein at this time point was due to increased albumin levels that indicated protein synthesis in the organism (Kaneko et al. 2008).

CONCLUSIONS

The chromium supplementation promoted greater accumulated weight gain in calves before weaning and during the experiment, reduced levels of cortisol and serum protein and diminished urinary chromium excretion rates during weaning were detected.

Continuous and prolonged supplementation of organic chromium in beef calves reduced weaning-related stress.

Ethics Committee.- This study was approved by the Ethics Committee in Animal Experimentation of the "Faculdade de Medicina Veterinária e Zootecnia" of the "Universidade de São Paulo" (CEUA-FMVZ-USP) under protocol number 2429/2011.

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Antifungal susceptibility profile of *Aspergillus fumigatus* isolates from avian lungs¹

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ABSTRACT. Spanemberg A., Ravazzolo A.P., Denardi L.B., Hartz S.A., Santurio J.M., Driemeier D. & Ferreiro L. 2020. **Antifungal susceptibility profile of *Aspergillus fumigatus* isolates from avian lungs.** *Pesquisa Veterinária Brasileira* 40(2):102-106. Setor de Micologia, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil. E-mail: spanemberg.ad@gmail.com

Susceptibility testing is essential to inform the correct management of *Aspergillus* infections. In this study we present antifungal susceptibility profile of *A. fumigatus* isolates recovered from lungs of birds with and without aspergillosis. Fifty three isolates were tested for their antifungal susceptibility to voriconazole (VRC), itraconazole (ITZ), amphotericin (AMB) and caspofungin (CSP) using the M38-A2 broth microdilution reference method. Five isolates were resistant to more than one antifungal drug (CSP + AMB, VRC + ITZ and AMB + ITZ). Fifteen (28%) isolates with susceptible increased exposure (I) to ITZ were sensible to VRC. Resistance to AMB (>2µg/mL) was observed in only four isolates. Eleven (21%) *A. fumigatus* present resistance to ITZ (13%) and VRC (8%). Fungal isolation from respiratory samples has been regarded as being of limited usefulness in the *ante mortem* diagnosis of aspergillosis in birds. However, the results suggest that the detection and antifungal susceptibility profile may be helpful for monitoring of therapy for avian species and where antifungal resistance might be emerging and what conditions are associated to the event.

INDEX TERMS: *Aspergillus fumigatus*, antifungal, susceptibility, isolates, birds, lungs.

RESUMO. [Perfil de suscetibilidade antifúngica de isolados de *Aspergillus fumigatus* provenientes de pulmões de aves.] Os testes de suscetibilidade são essenciais para informar o correto manejo das infecções por *Aspergillus*. Neste estudo apresentamos o perfil antifúngico de isolados de *A. fumigatus* provenientes de pulmões de aves com e sem aspergilose. Cinquenta e três isolados foram testados quanto à suscetibilidade antifúngica ao voriconazol (VRC),

itraconazol (ITZ), anfotericina B (AMB) e caspofungina (CSP) pelo método de referência de microdiluição do caldo M38-A2. Cinco isolados foram resistentes a mais de um antifúngico (CSP + AMB, VRC + ITZ e AMB + ITZ). Quinze (28%) isolados suscetíveis - com exposição aumentada (I) ao ITZ foram sensíveis ao VRC. A resistência ao AMB (>2µg/mL) foi observada em apenas quatro isolados. Onze (21%) *A. fumigatus* apresentaram resistência a ITZ (13%) e VRC (8%). O isolamento de fungos de amostras respiratórias tem sido considerado de utilidade limitada no diagnóstico *ante mortem* de aspergilose em aves. No entanto, os resultados sugerem que a detecção e o perfil de suscetibilidade a antifúngicos podem ser úteis para o monitoramento da terapia de espécies aviárias, assim como a emergência da resistência antifúngica e quais condições podem estar associadas ao evento.

TERMOS DE INDEXAÇÃO: *Aspergillus fumigatus*, antifúngicos, suscetibilidade, isolados, aves, pulmões.

INTRODUCTION

Aspergillosis is a fungal infection that causes high economic losses in poultry production. *Aspergillus* belonging to section *Fumigati* is the main causative agent of aspergillosis in birds,

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it is commonly found in bed, soil and decomposing organic matter or in feed. Birds become infected through aspiration in large numbers of conidia (Dagenais & Keller 2009, Serrano et al. 2011). *Aspergillus fumigatus* may be present in the lungs of healthy birds and/or may be causing bird's diseases. The avian respiratory tract is the most affected, because small conidia easily reaches to the lungs and air sacs of the birds and from the lungs can produce a systemic infection (Fedde 1998, Beernaert et al. 2009).

Three antifungal classes are available for the treatment of aspergillosis, polyenes, azoles and echinocandins. Azoles inhibit the synthesis of ergosterol causing damage to the fungal cell membrane and currently, are the first line of treatment of *Aspergillus* infections with voriconazole (VRC) the choice treatment for invasive aspergillosis in humans (Walsh et al. 2008). At the same time, a large number of *A. fumigatus* isolates resistant to azoles recovered from environmental and patients have been reported in many countries of the world (Diaz-Guerra et al. 2003, Mellado et al. 2004, Snelders et al. 2009). Although not much reported yet, resistance to azoles has already been documented in *Aspergillus* infections in birds (Beernaert et al. 2009).

Echinocandins (caspofungin, micafungin, and anidulafungin) are the most recent class of antifungal agents that have action against *Aspergillus*, they act by inhibiting the synthesis of glucans, essential components for the fungal cell wall. Only caspofungin (CSP) is licensed for the treatment of aspergillosis, while micafungin and anidulafungin, although good *in vitro* action, still require dose adjustments *in vivo*. Echinocandin resistance is still considered as unusual (Walsh et al. 2008, Pfaller et al. 2009), although some studies show variable susceptibility to caspofungin were observed *in vitro* against *A. fumigatus* species complex (Barrs et al. 2013, Pelaez et al. 2013). Also, other authors have reported differences of *Aspergillus* spp. susceptibility to echinocandins (Denardi et al. 2018).

Amphotericin B (AMB) has been used for more than 40 years in aspergillosis treatment and also targets the fungal cell membrane. Its less toxic lipid formulations have been used in cases of aspergillosis refractory to azole antifungals (Linden 2003). Resistance to AMB is a rare phenomenon in *A. fumigatus*, however *A. terreus* is intrinsically resistant and *A. flavus* has reduced sensitivity to this antifungal (Gonçalves et al. 2016).

Susceptibility testing is essential to inform the correct management of *Aspergillus* infections in humans and animals. In this study we present antifungal susceptibility profile of *A. fumigatus* isolates recovered from lungs of birds with and without aspergillosis.

MATERIALS AND METHODS

***Aspergillus* isolates and molecular identification.** A total of 53 *A. fumigatus* isolates from lungs of health broilers (n=34) and with aspergillosis (n=19) were included in the study. The isolates were recovered from lung samples from 2010 to 2016 after *post-mortem* examination of birds. They were deposited in the culture collection of the "Laboratório de Pesquisas Micológicas", "Faculdade de Veterinária" (FaVet), "Universidade Federal do Rio Grande do Sul" (UFRGS), and maintained by periodic sub-culture. The study (Project no. 26640) was approved by the Research Committee of Faculty of Veterinary-UFRGS. All isolates were identified as *A. fumigatus* by macro and micromorphology and molecular methods. The Qiagen DNeasy® plant mini DNA extraction kit (Qiagen, Hilden, Germany) protocol

was used to extract DNA from the *Aspergillus* conidia according to manufacturer instructions. Molecular identification was performed using specific primers for identification of *Aspergillus* section *Fumigati* and *A. fumigatus* (Spanemberg et al. 2016). Multiplex PCR amplification was performed a total volume of 25µL containing 1µL of DNA extract, 12.5µL Qiagen Taq PCR master mix (Qiagen, Hilden, Germany) and 0.5µL of each primer (for a 0.2µM final concentration of each primer). The cycling parameters was as follow: pre-incubation at 94°C for 15min, 35 cycles of denaturation at 94°C for 30s, annealing at 69°C for 90s, extension at 72°C for 1min, and a final extension step of 10min at 72°C. Samples were analyzed by electrophoresis through 2% agarose gels. Gels were stained with ethidium bromide and DNA was visualized under UV. The electrophoretic profile with three bands (105, 198 and 313bp) was similar in all tested strains of *A. fumigatus*. The reference *A. fumigatus* strain ATCC 46645 was identified according to standard conditions yielding a final electrophoretic profile corresponding to *A. fumigatus* (Serrano et al. 2011). PCR product was purified using PuriLink™ PCR Purification Kit (Invitrogen), and sequencing to confirm the identity.

Drug susceptibility assays in *A. fumigatus*. Isolates were tested for their antifungal susceptibility to ITZ, VRC, AMB and CSP using the M38-A2 broth microdilution reference method (CLSI 2008). All antifungal drugs were obtained as standard powders. ITZ and AMB were obtained from Sigma-Aldrich (São Paulo/SP, Brazil) and CSP (Merck Sharp and Dohme, São Paulo/SP, Brazil) and VCZ (Laboratórios Pfizer Ltda, São Paulo/SP, Brazil) from their respective manufacturers. Stock solutions of all antifungal drugs were prepared in dimethyl sulfoxide (DMSO) and storage at -70°C. The final antifungal concentrations tested ranged from 0.031 to 16µg/mL for azoles and AMB, and from 0.001 to 0.500µg/mL for CAS.

The inoculum was prepared from cultures grown for 48-72h at 37°C in Potato Dextrose Agar (PDA). Saline solution (2mL) with 0.1% Tween 20 was added to tubes counting the *A. fumigatus* colonies and the conidia were carefully collected with a sterile loop. The suspensions remained standing for 2 to 5min for sedimentation of the hyphae, after the conidial suspension was transferred to another sterile tube. Then, the suspensions were adjusted in spectrophotometer at $\lambda=530\text{nm}$ and absorbance of 0.09 to 0.13. To obtain a final working inoculum concentration of 0.4 to 5×10^4 cells/mL, a 1:50 dilution was made in RPMI. A total of 100µL of the inoculum was placed into the microdilution wells containing the different concentrations of each antifungal drug. The plates were incubated at 35°C. The MEC (minimal effective concentration, resulting in abnormal, short, and branched hyphae) readings were taken after 24h to CAS and the MIC (minimal inhibitory concentration, 100% of inhibition) readings at 48h for azoles and AMB. *A. fumigatus* was classified as susceptible - standard doing regimen (S), susceptible - increased exposure (I) and resistant (R) following the breakpoints proposed by EUCAST table v. 9.0 and Arendrup et al. (2019): ITZ and VRC $\leq 1\mu\text{g/mL}$ (S), $2\mu\text{g/mL}$ (I) and $>2\mu\text{g/mL}$ (R); AMB $\leq 1\mu\text{g/mL}$ (S) and $>2\mu\text{g/mL}$ (R); and CSP $\geq 0.5\mu\text{g/mL}$ (R).

RESULTS

All isolates were confirmed as *Aspergillus fumigatus stricto sensu* following the amplification of β -tub and rodA gene fragments and gel electrophoresis. The results of *in vitro* susceptibilities tests are show in Table 1, Figure 1 and Figure 2. Five isolates were resistant to more than one antifungal drug (CSP + AMB, VRC + ITZ and AMB + ITZ). Fifteen (28%) isolates with susceptible increased exposure to ITZ were sensible to VRC. Resistance to AMB ($>2\mu\text{g/mL}$) was observed in only four isolates.

Table 1. Antifungal susceptibility profile of *Aspergillus fumigatus* isolates from avian lungs

<i>A. fumigatus</i> isolates	Antifungal	MIC Range ^a	MIC ₅₀ ^b	MIC ₉₀ ^c
			µg/mL	
Aspergillosis cases n=19 (36%)	Itraconazole	0.5 - 32.0	1.0	2.0
	Voriconazole	0.5 - 8.0	0.5	0.5
	Amphotericin	0.5 - 4.0	1.0	2.0
	Caspofungin	0.003 - 0.125	0.06	0.125
<i>Aspergillus</i> lung colonization n=34 (64%)	Itraconazole	0.5 - 32.0	1.0	16.0
	Voriconazole	0.5 - 32.0	0.5	0.5
	Amphotericin	0.5 - 8.0	1.0	2.0
	Caspofungin	0.03 - 0.5	0.06	0.125

^a Variation of MIC values (number of dilution variation) for every antifungal, ^b minimal inhibitory concentration required for inhibit the growth of 50% of the strains, ^c minimal inhibitory concentration required for inhibit the growth of 90% of the strains

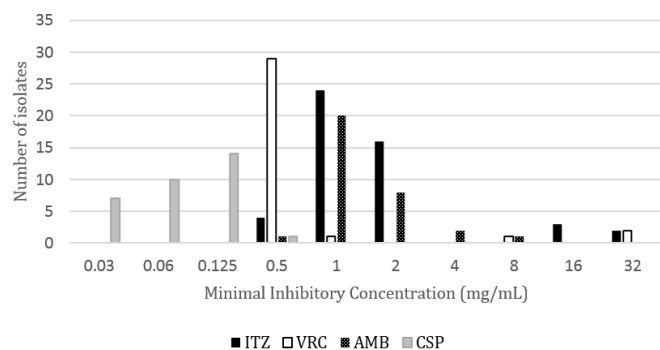


Fig.1. Antifungal susceptibility profile of *Aspergillus fumigatus* isolates (n=34) from avian lungs with fungal colonization. Itraconazole (ITZ), voriconazole (VCR), amphotericin B (AMB), caspofungin (CSP). *A. fumigatus* was classified as susceptible - standard doing regimen (S), susceptible-increased exposure (I) and resistant (R) following the breakpoints: ITZ and VRC $\leq 1\mu\text{g/mL}$ (S), $2\mu\text{g/mL}$ (I) and $>2\mu\text{g/mL}$ (R); AMB $\leq 1\mu\text{g/mL}$ (S) and $>2\mu\text{g/mL}$ (R); and CSP $\geq 0.5\mu\text{g/mL}$ (R).

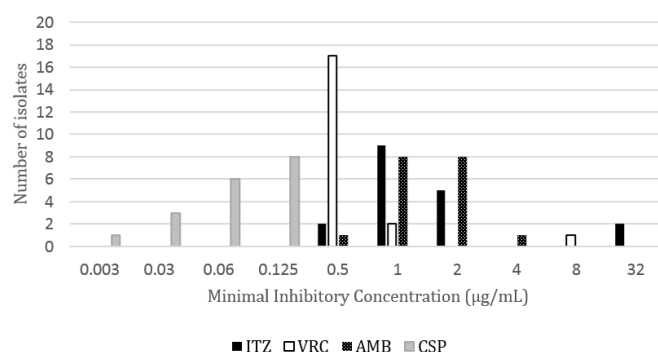


Fig.2. Antifungal susceptibility profile of *Aspergillus fumigatus* isolates (n=19) from avian lungs with aspergillosis. Itraconazole (ITZ), voriconazole (VCR), amphotericin B (AMB), caspofungin (CSP). *A. fumigatus* was classified as susceptible - standard doing regimen (S), susceptible-increased exposure (I) and resistant (R) following the breakpoints: ITZ and VRC $\leq 1\mu\text{g/mL}$ (S), $2\mu\text{g/mL}$ (I) and $>2\mu\text{g/mL}$ (R); AMB $\leq 1\mu\text{g/mL}$ (S) and $>2\mu\text{g/mL}$ (R); and CSP $\geq 0.5\mu\text{g/mL}$ (R).

DISCUSSION

Aspergillus is found worldwide causing infections in humans and animals and is considered as one of the major respiratory pathogens in birds (Arné et al. 2011), being frequently reported in the recent Brazilian veterinary literature (Spanemberg et al. 2012, Silva Filho et al. 2015, Echenique et al. 2016). All isolates in this study were *A. fumigatus* obtained from healthy birds and ones with aspergillosis (without previous antifungal therapy). Similar results were observed by other authors who demonstrated the dominant association of *Aspergillus fumigatus stricto sensu* in avian mycoses (Sabino et al. 2019). Papers about antifungal susceptibility of *A. fumigatus* from avian lung samples are scarce. Molecular identification is very important to understand the evolution of resistance pattern of *A. fumigatus stricto sensu*, because the species of section *Fumigati* may present distinct susceptibility profiles (Snelders et al. 2009). In addition, the significant increase in the use of antifungal agents, both for human treatment and use of azole fungicides in agricultural crop protection has influenced the emergence of resistant clinical isolates,

particularly to the triazoles and echinocandins (Gonçalves 2017).

Resistance to AMB (MIC $>2\mu\text{g/mL}$) was observed in four (7%) isolates. This finding is in disagreement with results reported in the literature. In a study conducted by Silvanose et al. (2006), for 16 *A. fumigatus*, 5 (31%) isolates showed resistance (MIC of AMB $>2.0\mu\text{g/mL}$). Ziołkowska et al. (2014) also showed resistance for 60 (70.6%) *A. fumigatus* isolates (MIC range of AMB for 4 to $16\mu\text{g/mL}$). The low degree of resistance to amphotericin B found in the study can be explained by the lack of use of this drug as prophylactic measure in poultry farming. In relationship to isolates obtained from humans, *A. fumigatus* is rarely resistant to this antifungal unlike *Aspergillus nidulans* and *Aspergillus terreus* (Newton et al. 2016). In domestic as well as in captive birds (non-poultry species), several management strategies against aspergillosis have been suggested, and the nebulization with AMB is used in breeding establishments to prophylactically (Orosz 2000, Rochette et al. 2003).

Our isolates showed most overall resistance to ITZ. Eleven (21%) *A. fumigatus* present resistance to ITZ (13%) and VRC (8%), being five isolates above MIC $>16\mu\text{g/mL}$ for both

antifungals. Azole resistance might be developed through the use of azole compounds in the environment and specifically in the case of birds; conditions that favor the development of fungi in confinement buildings expose commercial poultry to a higher risk of inhaling conidia of *A. fumigatus* during the farming period. Under these conditions, birds could inhale resistant conidia and subsequently develop azole-resistant disease or have fungal colonization in respiratory tract (Arné et al. 2011, Spanamberg et al. 2013). The use of fungistatic agents (sprayed, fogged, or nebulized to treat surfaces or indoor) like thiabendazole, nystatin, or copper sulfate contributes to decreased fungal contamination of beddings. Enilconazole is available in special formulations for decontamination of the poultry houses (Seyedmousavi et al. 2015).

Aspergillus species are present in a wide variety of substrates and environments as saprophytic, prevalently found in soil, decaying vegetation and compost. Furthermore, azoles are commonly used for plant protection as well as material preservation. The present study detected two azole-resistant *A. fumigatus* isolated from birds without aspergillosis, suggesting that resistance might be developed in the environmental conditions, which can favors the appearance of strains cross-resistant to medical triazoles used for treatment of human aspergillosis (Serfling et al. 2007).

CONCLUSION

Fungal isolation from respiratory samples has been regarded as being of limited usefulness in the *ante mortem* diagnosis of aspergillosis in birds. However, the results suggest that the identification and antifungal susceptibility profile may be helpful for monitoring of therapy for avian species and where antifungal resistance might be emerging and what conditions are associated to the event.

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Evaluation of the euthanasia of seropositive dogs for canine visceral leishmaniasis as the only method of controlling the disease in the enzootic area in the Midwestern Minas Gerais¹

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ABSTRACT- Vaz T.P., Gama-Melo M.O., Quaresma P.F., Gontijo C.M.F., Santos G., Barbosa F.S. & Fontes G. 2020. **Evaluation of the euthanasia of seropositive dogs visceral canine leishmaniasis as the only method of controlling the disease in the enzootic area in the Midwestern Minas Gerais.** *Pesquisa Veterinária Brasileira* 40(2):107-112. Universidade Federal de São João Del Rei, Rua Sebastião Gonçalves Coelho 400, Chanadour, Campus Centro Oeste, Divinópolis, MG 35501-296, Brazil. E-mail: gfontes@ufsj.edu.br

Human Visceral Leishmaniasis (VL) is expanding, and it is distributed in the five geographic regions of Brazil, with the Northeast being the region that presents the most significant number of registered cases. The main urban reservoir of the etiological agent of VL is the domestic dog, and it is known that canine cases precede human cases. One of the control actions of VL, recommended by the Ministry of Health, is performing the euthanasia of seropositive dogs. In 2013, in the municipality of Iguatama/MG, the first canine serological survey was carried out in the city, with a prevalence of 8.3% of seropositive dogs for *Leishmania infantum*. Of the seropositive animals for Canine Visceral Leishmaniasis (CVL) in this survey, 84% were euthanized, and 16% died within two years after diagnosis. No other control measures for CVL were taken in the municipality. The objectives of the present study were to determine the current prevalence of seropositive dogs for CVL in the municipality of Iguatama and to observe the impact of euthanasia of seropositive dogs to VL as the only control measure performed in this municipality, which is considered an enzootic area of the disease. For this, a new canine epidemiological survey was carried out in the municipality of Iguatama, following the guidelines of the Manual of Surveillance and Control of Visceral Leishmaniasis. Blood samples were collected from 270 dogs and tested by DPP® immunochromatographic test and by Enzyme-Linked Immunosorbent Assay (ELISA). The animals that presented inconclusive results had new blood samples collected and analyzed. The only animal that showed a positive result in DPP® and undetermined in the ELISA, in the first evaluation, became seropositive four months later, so this study reinforces the recommendation of the Ministry of Health to reassess dogs that present inconclusive results for CVL. From a total of 270 samples, 21 (7.8%) were reagents in both tests. Thus, the prevalence of seropositive dogs for CVL, in 2017, in the urban area of the municipality of Iguatama was 7.8%. The Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) molecular technique confirmed infection by *L. infantum* in the ten dogs sampled with CVL-positive serology.

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The comparison between the current prevalence (after euthanasia) with that found in 2013 (8.3%), there was no significant difference ($p=0.764$). The prevalence of CVL in the urban area of the municipality of Iguatama is still high, making it possible to presume that the euthanasia of seropositive dogs for CVL, and the absence of monitoring or other interventions measures, did not contribute to decrease the transmission of the parasite and was not sufficient for the control of CVL in the municipality of Iguatama, an area considered as non-endemic to CVL until 2013.

INDEX TERMS: Evaluation, euthanasia, seropositive, dogs, canine visceral leishmaniasis, control, enzootic area, Midwest, Minas Gerais, Brazil, *Leishmania infantum*, parasitic.

RESUMO.- [Avaliação da eutanásia de cães soro reagentes para leishmaniose visceral canina como único método de controle da doença em área enzoótica no Centro Oeste de Minas Gerais.]

A Leishmaniose visceral humana (LV) está em franca expansão e distribuída nas cinco regiões geográficas do Brasil, o Nordeste sendo a região em que há o maior número de casos registrados. O principal reservatório urbano do agente etiológico da LV é o cão doméstico e sabe-se que casos caninos antecedem o aparecimento de casos humanos. Uma das ações de controle da LV, preconizada pelo Ministério da Saúde, é a eutanásia dos cães soro reagentes. Em 2013, no município de Iguatama/MG, foi realizado o primeiro inquérito sorológico canino na cidade, sendo constatada uma prevalência de 8,3% de cães soro reagentes para *Leishmania infantum*. Dos animais com sorologia positiva para Leishmaniose visceral canina (LVC), neste inquérito, 84% foram eutanasiados e 16% foram a óbito em, no máximo, dois anos após o diagnóstico. Nenhuma outra medida de controle para LVC foi realizada no município. Os objetivos deste estudo foram determinar a prevalência atual de cães soro reagentes para LVC no município de Iguatama e observar o impacto das eutanásias de cães soro reagentes para LV como única medida de controle realizada neste município, considerado área enzoótica para a doença. Para isso, foi realizado um novo inquérito epidemiológico canino no município de Iguatama, seguindo as normas do Manual de Vigilância e Controle da Leishmaniose Visceral. Foram colhidas amostras sanguíneas de 270 cães que foram examinadas pelo teste imunocromatográfico DPP® e pelo ensaio imunoenzimático (ELISA). Os animais que apresentaram resultados inconclusivos tiveram novas amostras de sangue coletadas e analisadas. O único animal que apresentou resultado positivo no DPP® e indeterminado no ELISA, na primeira avaliação, soro converteu quatro meses depois, o que reforça a recomendação do Ministério da Saúde de reavaliar os cães que apresentam resultado inconclusivo para LVC. Do total das 270 amostras, 21 foram reagentes nos dois testes. Portanto, a prevalência de cães soro reagentes para LVC, em 2017, na área urbana do município de Iguatama foi igual a 7,8%. A infecção pela espécie *Leishmania infantum* foi confirmada através da técnica molecular de PCR-RFLP em 10 cães amostrados com sorologia positiva para LVC. A comparação entre a prevalência atual (depois das eutanásias) com a encontrada em 2013 (8,3%), não mostrou diferença significativa ($p=0,764$). Uma vez que a soro prevalência de LVC na área urbana do município de Iguatama ainda é alta, pode-se supor que a eutanásia dos cães soro reagentes para LVC, somada à ausência de monitoramento e de outras medidas de intervenção, não contribuiu para a diminuição da transmissão do parasito, e não foi suficiente para o controle

de LVC no município de Iguatama, uma área considerada, até 2013, indene para LVC.

TERMOS DE INDEXAÇÃO: Avaliação, eutanásia, cães soro reagentes, leishmaniose visceral canina, método de controle, área enzoótica, *Leishmania infantum*, caninos, Centro Oeste, Minas Gerais, Brasil, parasitoses.

INTRODUCTION

Visceral leishmaniasis (VL) is part of a group of diseases considered neglected by the World Health Organization (WHO), with an estimated 200,000 to 400,000 new human cases per year worldwide (Alvar et al. 2012, WHO 2018). The disease has high mortality when untreated, and there is a higher risk for late-diagnosed cases, inadequately treated cases, and immunosuppressed or drug-resistant individuals (Marzochi et al. 2014, WHO 2015). Transmission occurs through the bite of females of phlebotomine insects infected by protozoan species *Leishmania infantum* (WHO 2015).

In Brazil, the domestic dog (*Canis familiaris*) is the main reservoir of the etiologic agent of VL in urban areas (Gontijo & Melo 2004, Dantas-Torres 2006), being the most prevalent canine infection than the human one (Brasil 2014). Canine visceral leishmaniasis (CVL) cases precede human cases (Bevilacqua et al. 2001, Brasil 2014, Teixeira-Neto et al. 2014). Infection among dogs spreads rapidly in the presence of excellent conditions for transmission, such as the high density of vector insects and susceptible dogs, and the seroprevalence of dogs with VL is considerably higher than the prevalence of sick animals (Baneth et al. 2008). The asymptomatic and oligosymptomatic dogs are also capable of infecting the insect vectors of the etiologic agent of VL (Michalsky et al. 2007, Marcondes & Rossi 2013).

Prior knowledge of the infection in dogs serves as a warning for the beginning of control actions to prevent the occurrence of human cases. Control methods for VL in Brazil are explicit in the Visceral Leishmaniasis Control Program (VLCP), and among them is the euthanasia of seropositive dogs for CVL (Brasil 2014). Oppositions against this action and the debate about the affective and economic value of dogs have gained more and more strength, supported by studies suggesting more effective control measures (Costa 2011, Werneck et al. 2014, Shimozako et al. 2017).

The municipality of Iguatama, located in the Midwest region of the state of Minas Gerais (MG), recorded its first autochthonous case of CLV in 2013 (Faria et al. 2017) and has not yet reported any human cases of VL (DATASUS-SINAN-MS 2018). In 2013, the State Department of Health of Minas Gerais conducted an entomological survey in the city and found

specimens of insects of the species *Lutzomyia longipalpis*. In the same year, a canine sample epidemiological survey was carried out and found a prevalence of 8.3% of CVL in the urban area of the city, and after conducting the survey, 84% of the serum reagent dogs found were euthanized (Faria 2014) and 16% died within two years of diagnosis (Vaz 2018). No other control measures for VL were adopted in the municipality.

Given the difficulties in controlling VL, it is crucial to analyze the control methods employed, mainly from the euthanasia of seropositive dogs, which is the most practiced control action today. The objective of this study was to reevaluate the seroprevalence of CVL in Iguatama/MG, and to observe the impact of euthanasia of VL-seropositive dogs as the only control measure performed in this municipality, considered an enzootic area for the disease.

MATERIALS AND METHODS

The study was approved by the Animal Use Ethics Committee of *Universidade Federal de São João Del Rei* (UFSJ) (protocol no. 28/2016). It is about a cross-sectional study by stratified sampling, held from April to August 2017 in the urban area of the municipality of Iguatama, the first city bathed by the São Francisco river, located in the Midwest of Minas Gerais. Its territorial area is 628.2 km² and is limited to the municipalities of Arcos, Doresópolis, Luz, and Bambuí (IBGE 2016). In 2016, according to the census conducted by the *Instituto Brasileiro de Geografia e Estatística* (IBGE), the total population (urban and rural) of Iguatama was estimated at 8,182 inhabitants (IBGE 2016). According to data from the Geographic Reference System (GRS) of the Unified Health System (UHS), the urban population of Iguatama in March 2017 was 6,765 inhabitants.

To estimate the size of the canine population in the municipality of Iguatama/MG, data from the anti-rabies vaccination campaign in the city of 2017 were used, which determine a canine population of approximately 13.5% of the human population. Based on this, it was possible to estimate that the dog population in the urban area is about 913 animals. It is estimated that in the urban area of the city, there is approximately one dog for 7.4 people. Knowing that the vaccination coverage of the rabies campaign is approximately 90% of the animals, the number of dogs in the urban area of the municipality of Iguatama to be considered in the study goes from an estimated 913 to approximately 1,000 dogs.

A canine sample survey was conducted to determine the current prevalence of CVL in the municipality, as recommended by the Visceral Leishmaniasis Surveillance and Control Manual for municipalities with a canine population of over 500 animals (Brasil 2014). The table 1 from the Visceral Leishmaniasis Surveillance and Control Manual (Brasil 2014) was used to calculate the sample size to be obtained, according to the estimated dog population and the expected CVL prevalence considering a significance level of 5% (Brasil 2014).

Knowing the estimated number of dogs in the urban area, approximately 1,000 animals, and the previous prevalence of CVL found in the city was 8.3% (Faria 2014), a total of 159 dogs should be sampled in the municipality of Iguatama, according to the table in the Visceral Leishmaniasis Surveillance and Control Manual (Brasil 2014). However, following the epidemiological survey for CVL conducted in 2013, 84% of seropositive dogs

were euthanized (Faria 2014). Whereas euthanasia of serum dogs reagents for CVL is one of the disease control measures (Brasil 2014), it would be expected that the prevalence of CVL in the municipality had decreased after 2013. It was estimated for the current study a prevalence of 3.1% to 4% of CVL in the municipality of Iguatama, and considering the canine population maintained around 1,000 animals, the sample used in the study for 270 dogs (Brasil 2014).

Neighborhoods stratified the sample and worked by conglomerates that corresponded to the blocks. From each block, 50% of the properties were worked, with a maximum of two dogs per property, to ensure a better distribution of the sample. In April 2017, the 270 dogs were examined, from which 4mL of cephalic or jugular vein blood was collected. The collected blood was immediately deposited in a tube containing a clot activator and identified with the dog's name and dog number in the sample. The samples were centrifuged at 3,000g for 10 minutes to separate the serum, which was transferred to 1.5mL microtubes identified with the dog number in the sample and stored at -20°C until the serological tests were performed. Also, bone marrow aliquots were collected from 10 dogs with CVL-positive serology included in the sample for molecular diagnostic methods. The dogs also underwent a clinical evaluation by the veterinarian responsible for the study.

The *Ministério da Saúde* (MS) determines two immunologic tests for the diagnosis of CVL: Dual Path Platform (DPP®) immunochromatographic test as screening and confirmation by the ELISA enzyme immunoassay. In this study, all 270 samples were tested by DPP® and ELISA, and both tests were performed at the *Laboratório de Parasitologia* of UFSJ, which is accredited with the *Fundação Ezequiel Dias* as a regional reference for the diagnosis of CVL in the Midwest of Minas Gerais State. To conclude both tests, kits produced by Biomanguinhos/Fiocruz were used, following the manufacturer's recommendations.

Animals that showed inconclusive results (DPP® reagent and undetermined ELISA or DPP® nonreactive and reagent or undetermined ELISA results) had new blood samples collected and analyzed four months after the first collection. The reevaluation of these dogs followed the same protocol as the first evaluation.

Aliquots of 300µL of bone marrow from dogs with positive serology for CVL were used as a DNA source for molecular tests. The Internal Transcribed Spacer-1 (ITS-1) PCR-RFLP technique (Schonian et al. 2003) was employed to identify the *Leishmania* species responsible for the infection of the ten dogs sampled with CVL-positive serology.

Statistical calculations were performed using the OpenEpi software, version 3.01, and the Yates-corrected Chi-square test, which was used to compare prevalence.

RESULTS

Of the 270 dogs evaluated, 20 were reagents for CVL in both serological tests, and 11 showed inconclusive results. After a reassessment of 11 inconclusive serums, only one of them, the only one that showed reagent results in DPP® and undetermined in ELISA, became reagent in both tests. Thus, the seroprevalence of CVL in the municipality of Iguatama in 2017 was 7.8%, e.g., 21 of the 270 dogs that were seropositive for CVL.

Most CVL seropositive dogs (58%) were up to four years old. Most of them were symptomatic 17/21 (81%), and the most frequently observed clinical signs were: onychogryphosis (48%), weight loss (38%), and hypocolored mucosa (38%).

There was no significant difference between the CVL seroprevalence found in the urban area in the municipality of Iguatama in the first survey conducted in 2013 (8.3%) (Faria 2014), with the CVL seroprevalence found in 2017, four years after euthanasia (7.8%) ($p=0.764$).

The ITS-1 PCR-RFLP results showed that all ten dogs presented positive PCR by analysis of bone marrow material, being infected by *L. infantum*, corroborating the presence of a large number of CVL-seropositive animals in the municipality of Iguatama.

DISCUSSION

Maintaining high serum CVL prevalence in the municipality of Iguatama, as found in this study, serves as a warning to health professionals and local authorities about the need for more efficient CVL control measures in the municipality. The prevalence of CVL in Iguatama today is similar to that found in the Paraíba semi-arid region of northeastern Brazil (Silva et al. 2016), a geographical region with the highest prevalence of Human Visceral Leishmaniasis (HVL) and CVL in the country (Bavia et al. 2005).

Cases of CVL are known to precede human cases of the disease (Bevilacqua et al. 2001, Brasil 2014, Teixeira-Neto et al. 2014). Florianópolis, in the state of Santa Catarina (SC), recorded its first autochthonous case of CVL in 2012 (Figueiredo et al. 2012), and in 2017 the first two autochthonous cases of the disease in humans (SMSF 2017, SBMT 2017), showing the previous occurrence of cases of the disease in dogs. It is also important to note the short time elapsed between autochthonous cases of CVL and HVL. The municipality of Iguatama has not yet reported any cases of HVL but is vulnerable, and human cases and deaths from the disease may be occurring that were not correctly diagnosed, and therefore, not reported.

Given the importance of dogs as a reservoir of *L. infantum* in the urban environment, and the recommendation to perform euthanasia of seropositive dogs for VL, the correct diagnosis of canine infection becomes crucial.

The current study confirms the recommendation of the MS to reassess dogs with reagent results in DPP® and undetermined in ELISA, therefore, the only dog in the sample that presented this result, serum converted four months after the first evaluation. Lopes et al. (2017) observed that the serological protocol has a low detection capacity of *L. infantum* when compared with the molecular method (PCR) yet has good ability to identify infected animals correctly. In fact, in this study, we were able to confirm *L. infantum* infection in a sample of ten dogs out of a total of 21 CVL positive serum animals in both serological methods employed. This result is significant to reinforce that most serum reagent dogs are indeed infected with *L. infantum* therefore, they are reservoir hosts that act as a source of infection for the sand fly vectors, contributing to the maintenance of the parasite in the studied area.

Most of the seropositive dogs were symptomatic, which is consistent with the findings of Mendonça et al. (2017). DPP® test sensitivity is known to be higher in symptomatic dogs (Grimaldi Junior et al. 2012). This characteristic of the

test may explain that most of the CVL-seropositive dogs found in the municipality of Iguatama are symptomatic. In symptomatic dogs, onychogryphosis, weight loss, and mucosa were more frequently observed, findings consistent with Silva et al. (2017).

The absence of a significant difference between the prevalence of CVL in Iguatama in 2013 and 2017, together with the lack of monitoring and other control measures in the municipality, suggests that the euthanasia of seropositive dogs did not contribute to reducing the prevalence of CVL in the county. A study conducted by Werneck et al. (2014) in Northeastern Brazil, reported from low to moderate the effectiveness of euthanasia of dogs with CVL as a control measure of VL, and an urgent need for revision of the Brazilian Program for Control of the disease.

In a systematic review commissioned by the Pan American Health Organization (PAHO) to evaluate VL control actions it is concluded that there is no firm evidence of a significant impact of the interventions analyzed on VL transmission, and although, canine euthanasia is the least acceptable intervention from the community point of view and has low efficiency due to the replacement of dogs eliminated by susceptible dogs (Romero & Boelaert 2010). Shimozako et al. (2017) when assessing the impact of five VL control measures on the human and canine population, taking into account epidemiological control and the cost-effectiveness observed that the use of insecticide collar in dogs presented the best association between disease control and cost-effectiveness, and should be the first choice among VL control strategies. Regarding the euthanasia of VL-seropositive dogs as a disease control measure, Shimozako et al. (2017) observed that when dog mortality is intensified, canine population renewal also occurs.

The mean age of the dogs sampled (four years) was equal to the time interval between the two canine surveys performed, and most VL reagent dogs in this study were up to four years old. These dogs may have been replacing the dog's serum eliminated reagents. According to Oliveira & Araújo (2003), the immediate replacement of dogs with eliminated CVL, performed by the human population, compromises the efficiency of euthanasia in seropositive dogs for CVL as a disease control measure.

Nunes et al. (2008) in a study conducted in an endemic area for CVL in Brazil found that about 40% of dogs were replaced with an average time of four months, and at nine months of follow-up of the replacement dogs, 42% of them were already serum reagents for CVL. The euthanasia of dogs with CVL seems to influence the structure of the canine population rather than its size, leading to the increase in the young portion of the canine population that may also have severe epidemiological consequences on other diseases, such as rabies, because these animals may not be vaccinated (Andrade et al. 2007).

Educational actions directed to the population should accompany euthanasia of serum dogs reacting to CVL. In endemic areas, responsible tenure programs may be more useful for VL control than canine population control means (Andrade et al. 2007). In the municipality of Iguatama, no educational actions were carried out at any time, which may have impaired the effectiveness of euthanasia as a method of controlling CVL.

CONCLUSIONS

The prevalence of CVL in the municipality of Iguatama is still high and has not changed since the first canine survey conducted in 2013, which led to the euthanasia of seropositive dogs for CVL.

It may be suggested that the euthanasia of seropositive dogs, with the lack of monitoring and other control measures, did not contribute to a decrease in CVL seroprevalence in the municipality of Iguatama.

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Thrombin generation and thromboelastometry tests in dogs with chronic kidney disease¹

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ABSTRACT.- Gonçalves D.S., Geraldês S.S., Duarte R.C.F., Carvalho M.G., Guimarães-Okamoto P.T.C. & Takahira R.K. 2020. **Thrombin generation and thromboelastometry tests in dogs with chronic kidney disease: A comparative analysis.** *Pesquisa Veterinária Brasileira* 40(2):113-120. Departamento de Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Botucatu, SP. E-mail: danielesilvano@gmail.com

Patients with chronic kidney disease (CKD) have paradoxical hemostatic potential because they have bleeding episodes but are also prone to thrombosis. Few studies have evaluated blood viscoelastic properties in dogs with kidney disease; on the other hand, hypercoagulability has been observed in these patients. It is also emphasized that the platelet function and its participation in this process have not yet been fully understood. The objective of this study was to evaluate and compare the Thrombin Generation Test (TGT) and also viscoelastic properties of the blood measured by thromboelastometry (TEM) in dogs with proteinuria in CKD. Twenty healthy dogs (Control Group) and 19 dogs with CKD in stage III or IV, classified according to International Renal Interest Society - IRIS, were selected, and the reference test of urine protein:creatinine ratio (UPCR) should be greater than one (CKD group). Blood samples for TEM, thrombin generation, Prothrombin Time (PT), activated Partial Thromboplastin Time (aPTT), and fibrinogen concentration was collected at a single time for both groups after inclusion criteria being confirmed. Statistical analysis was performed according to the distribution of variables at 5% significance level. Differences were observed between healthy dogs and those with proteinuria in CKD noted in TEM. The TGT was unable to differentiate between sick and healthy groups. However, when the nephropathy was stratified, increases in TTP and peak thrombin concentration by TGT were observed in females and dogs over 30 days of diagnosis of CKD. Both tests signaled a discrete state of hypercoagulability. In fact, TEM is more sensitive to detect hypercoagulability in dogs with CKD. However, the TGT has potential clinical application by allowing long-term sample storage.

INDEX TERMS: Comparative analysis, thrombin generation, thromboelastometry, dogs, proteinuria in chronic kidney disease, chronic kidney disease, hemostasis, thrombosis, hemorrhage.

RESUMO.- [Testes de geração de trombina e tromboelastometria em cães com doença renal crônica.]

Os pacientes com doença renal crônica (DRC) apresentam um

potencial hemostático paradoxal, pois apresentam episódios de sangramento, mas também são propensos à trombose. Poucos estudos avaliaram as propriedades viscoelásticas sanguíneas em cães com doenças renais, entretanto, a hipercoagulabilidade já foi observada nestes pacientes. Ressalta-se ainda que a função plaquetária e sua participação neste processo ainda não foram totalmente esclarecidas. O objetivo foi avaliar e comparar o teste de geração de trombina (TGT) e as propriedades viscoelásticas sanguíneas medidas pela tromboelastometria (TEM) em cães com DRC proteinúrica. Foram selecionados 20 cães saudáveis (grupo controle) e 19 cães com DRC em estágios III ou IV classificados segundo o IRIS e a relação proteína/creatinina urinária maior que um

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(grupo DRC). As amostras de sangue para a realização da tromboelastometria (TEM), geração de trombina, tempo de protrombina (TP), tempo de tromboplastina parcial ativada (TTPA) e concentração de fibrinogênio foram colhidas em momento único para ambos os grupos após os critérios de inclusão confirmados. A análise estatística foi realizada de acordo com a distribuição das variáveis, ao nível de 5% de significância. Foi observada diferença entre os cães saudáveis e os com DRC proteinúrica observados na TEM. O teste de geração de trombina não foi capaz de diferenciar os grupos doente e saudável. Entretanto, quando os nefropatas foram analisados de forma estratificada, foram observados aumentos do ETP e da concentração máxima de trombina (*peak*) pelo TGT em fêmeas e em cães com mais de 30 dias de diagnóstico da DRC. Ambos os testes sinalizando para um discreto estado de hipercoagulabilidade. A tromboelastometria é mais sensível para detectar a hipercoagulabilidade em cães com DRC. Entretanto, o teste de geração de trombina tem melhor aplicabilidade por permitir o armazenamento da amostra em longo prazo.

TERMOS DE INDEXAÇÃO: Análise comparativa, testes de geração, trombina, tromboelastometria, cães, doença renal crônica proteinúrica, geração de trombina, hemostasia, trombose, hemorragia.

INTRODUCTION

It has been noted that patients with chronic kidney disease (CKD) present paradoxical hemostatic potential, with increased rates of bleeding, and unfortunately these patients are prone to thrombosis (Eberst & Berkowitz 1994, Lutz et al. 2014). Increased bleeding might be mainly caused by platelet dysfunction, even as additional contributions from changes in the coagulation cascade, nitric oxide, and fibrinolysis, and also anemia (Mischke & Schulze 2004, Sohal et al. 2006, Marques et al. 2010). However, the mechanisms favoring the prothrombotic state are unclear.

Few studies have been evaluated blood viscoelastic properties and hypercoagulability in dogs with kidney disease (Falco et al. 2013, Lennon et al. 2013). Platelet function (Gonçalves 2016, Dudley et al. 2017) and its participation in hypercoagulability have not yet been fully understood. In addition, there are no reports on thrombin generation potential associated with dogs with CKD. However, in humans, changes in thrombin generation in CKD patients were not observed (Trappenburg et al. 2012).

The Thrombin Generation Test (TGT) consists of continuous assessment of plasma thrombin generation after the addition of activators, such as tissue factor (TF) (Hemker et al. 2003). TGT can measure the potential of a total thrombin generation and have advantage over conventional coagulometric methods. Conventional tests, such as Prothrombin Time (PT) and activated Partial Thromboplastin Time (aPTT), are not capable of detecting hemostatic changes; therefore, they cannot determine the thrombin potential. It is also known that only 5% of the thrombin which is generated can contribute to convert fibrinogen into fibrin, thus leading to the final stages of the PT and APTT reactions (Lecut et al. 2015). Due to this property, thrombin levels are underestimated by these screening tests. Thrombin has been widely studied, and a new model for cell surface-based coagulation cascade has

been proposed, in which thrombin is the primary regulator (Smith 2009).

TEM is a viscoelastic hemostatic assay widely used in clinical settings and being closer to *in vivo* coagulation characteristics when compared with conventional coagulation tests. It consists of measurement and recording of entire coagulation process, from the beginning of platelet-fibrin interaction, platelet aggregation, and clot formation, to its eventual lysis, being able to detect blood hypocoagulability with hemorrhagic risk and hypercoagulability with thrombotic risk (Kol & Borjesson 2010). The state of hypercoagulability has already been characterized in dogs by shortening clotting time (CT), increasing maximum clot firmness (MCF) and alpha angle (α angle) (Hanel et al. 2014).

Clinical studies are needed to better characterize the hemostatic profile in dogs with CKD, given to the prevalence of CKD from 0.37% to 3.74% in diverse dog populations (Polzin, 2011, O'Neill et al. 2013). In addition to complications such as anemia, secondary hyperparathyroidism, uremic gastritis, and hypertension (Polzin 2011), CKD may be accompanied by hemostatic changes, which undoubtedly aggravates the disease.

Due to the gap in the hemostatic profile in dogs with CKD, the present study aimed to investigate hemostatic changes in these dogs with proteinuria in CKD, employing tests that allow the global evaluation of hemostasis, in order to establish a similar hemostatic profile, using both methodologies.

MATERIALS AND METHODS

Experimental design. A cross-sectional observational study was performed on dogs treated at *Faculdade de Medicina Veterinária e Zootecnia (FMVZ) Universidade Estadual Paulista (Unesp)* in Veterinary Hospital routine from March 2014 to February 2016, after being approved by the Animal Use Ethics Committee (protocol 33/2014).

Animals. 19 dogs with CKD in stages III and IV were selected, and classified according to IRIS (2018). Inclusion criteria were renal azotemia (serum creatinine >2mg/dL), urinary protein/creatinine ratio (PRC) >1.0, and characteristic ultrasound findings (e.g. small and irregular kidneys, loss of corticomedullary renal definition). Excluded criteria from this group were animals with neoplasia, concomitant infectious diseases, congestive heart failure, liver disease, nephrolithiasis, and animals in shock or another disease that would mask and/or influence the results of hemostatic tests. Animals that used erythropoietin or drugs that interfered with the hemostatic process were also excluded from the study.

The animals with CKD were subclassified according to age (up to 5 years and >5 years), sex, proteinuria degree (RPC up to 2.5 and >2.5), anemia degree (hemoglobin up to 8g/dL and >8g/dL) and disease history (up to 30 days and >30 days) for assessing comparative analysis of thrombogram and TEM. The subclassifications were determined according to the distribution of the sample population.

In the same period, 20 healthy dogs from voluntary owners were analyzed. Blood count selected healthy animals, by assessing their biochemical profiles (urea, creatinine, protein and fractions, Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), Gamma-glutamyl transferase (GGT) and cholesterol and fractions), urinalysis (including PRC), including those animals with results within the reference limits (Kaneko et al. 2008, Rizzi et al. 2010), and absence of previous disease.

Sample collection and processing. Blood collection was performed at a single time, preferably by jugular venipuncture using 10mL syringe (20G needle), immediately transferred to tubes with EDTA K3¹ and 3.2% sodium citrate⁴ (according to manufacturer's instructions). Urine samples were collected by catheterization or cystocentesis and immediately analyzed. The blood count was performed in a blood counter² immediately after collection. Plasma samples were centrifuged at 1700g for 10 minutes and then kept at -80°C until the time of analysis.

Thrombin generation. The thrombogram was performed on a fluorimeter³ by Calibrated Automated Thrombogram (CAT) method developed by Hemker et al. (2003) using standard reagents⁴, and according to the manufacturer's instructions. In short, 80µL of each sample was placed into the wells of a 96-well microplate, and then 20µL of the coagulation activation triggering reagent (PPP-Low or PPP-High Tissue Factor) was added. For each plasma tested, a well containing the same plasma volume was made, but replacing the coagulation triggering agent with 20µL of the thrombin bound (Thrombin calibrator-α2 macroglobulin). Thus, the microplate was incubated for 10 minutes at 37°C, and then 20µL of the trigger agent with calcium chloride and the fluorogenic agent (FluCa[®] kit) were automatically dispensed into each well by the fluorimeter. Thrombin generation was evaluated using the wavelengths of 390nm and 460nm to detect fluorescence for one hour. Data were collected from Thrombinoscope BV software⁵ and included lagtime (corresponding to CT), peak (maximum thrombin concentration), time to peak (representing time to maximum thrombin generated), and Endogenous Thrombin Potential (ETP) which represents the amount of thrombin formed within 60 minutes.

Thromboelastometry. Three channels were used simultaneously to perform TEM. 300µL of recalcified citrated whole blood were used and 20µL of 0.2 mol/L calcium chloride (startem[®]), and coagulation activation was performed separately in each channel using extem reagent[®] (20µL, tissue thromboplastin). The following variables were evaluated on the ROTEM Delta[®] apparatus maintained at 37°C for two hours: CT: latency until initial fibrin formation; α angle: fibrin accumulation and cross-linking velocity (fibrinogen level); and MCF: measure of peak clot strength/resistance.

Statistical analysis. The sample size calculation⁷ (n) was performed considering a statistical power of 80%. The Shapiro-Wilk test was used to assess the normality of the data. For comparison between groups, the Mann-Whitney U test was applied. Kruskal-Wallis test was used to compare the median between groups and Dunn's test was used to adjust the *p* values resulting from multiple comparisons. Results were expressed as median, second, and third quartiles. Analyses were performed with GraphPad Prism version 5.01 at the 5% significance level.

RESULTS

Nineteen dogs diagnosed with proteinuria in CKD were analyzed in this study, eleven females and eight males. The average age was eight years (8±3 years). The average weight of the dogs was 20 (±11.7) kg. The evaluated races were: Dalmatian (1), Weimaraner (1), Yorkshire (1), Rottweiler (1), German Shepherd (1), Boxer (1), and mixed breed (13).

The healthy group consisted of 20 dogs, ten females and ten males, with an average age of five years (5 ± 2.5 years). The breeds evaluated were: Border Collie (1), Dachshund (1), Fox Terrier (1), Labrador (6), Lhasa Apso (1), Pitbull (4), Poodle (1), Redhillier (1), Shitzu (1), Yorkshire (1) and mixed breed (2). According to the results of this group, reference values were obtained for TGT and TEM, assuming a confidence interval of 95%.

The proteinuria in CKD group in healthy animals presented anemia [median [2nd and 3rd quartiles] Ht: 27% [22-30] P<0.001, Hb: 8.4g/dL [7.2-9.6] P<0.001], between normocytic (MCV: 70fL [68-73], P=0.17), normochromic anemia (CHCM: 33 32-33], P<0.001) and platelets (276x10³ [205-358] within reference values (Rizzi et al. 2010) (P=0.80). The CKD group had a higher leukocyte count (11x10³/µL, P<0.05) compared to the healthy group, but both groups were within reference limits (Rizzi et al. 2010). Eleven (11/19) dogs were classified in stage III CKD and 8/19 in stage IV CKD, according to IRIS (2018) guidelines. Albumin levels were significantly reduced in the CKD group (2.3g/dL, P<0.001) compared to the healthy group. PRC ranged from 1.4 to 12.4 in the proteinuria in CKD group, with 16/19 animals presenting PCU>1.6. The results obtained by thrombin generation analysis are shown in Figure 1-4 and Table 1. In TEM (Fig.5-8, Table 2), animals up to 20kg presented increased values of MCF and α angle. The other subclassifications did not present statistically significant differences.

⁷ Statstodo Trading Pty Ltd., Hong Kong, China.

Table 1. Thrombin Generation Test (TGT) of dogs with proteinuria with CKD, the dogs were classified according to disease duration

Reference range*	Lagtime (minutes)	ETP (nM/min)	Peak (nM)	ttpeak (minutes)
	2.1-2.5	213-284	66-88	4.3-5.0
Up to 30 days (n=6)	2.3 (1.9-2.4)	268 (181-379)	84 (48-114)	4.3 (4.3-4.8)
≥30 days (n=13)	2.2 (2.0-2.7)	302 (174-383)	89 (53-148)	4.0 (4.0-4.2)
P	0.93	0.82	0.82	0.05

* IC 95% of control group; ETP = endogenous thrombin potential, ttpeak = time to peak; Values expressed as median, first (1q) and third quartiles (3q).

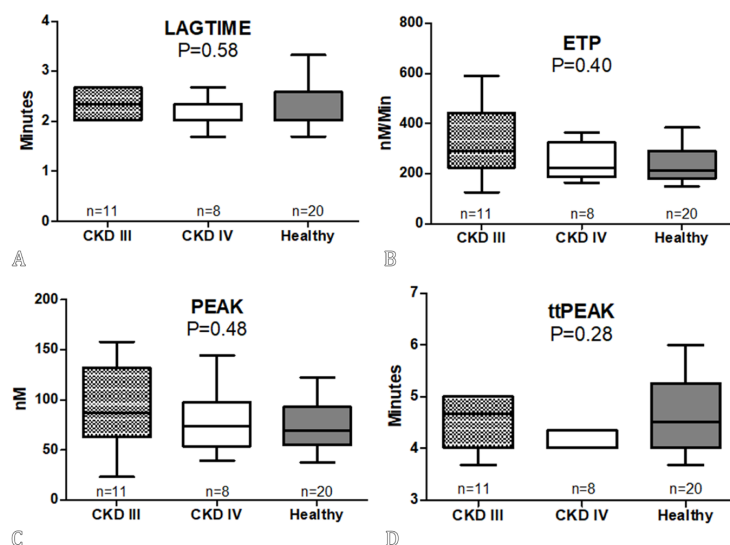


Fig.1. Thrombin Generation Test (TGT) of healthy dogs with proteinuria in CKD in stages III and IV. ETP = Endogenous Thrombin Potential.

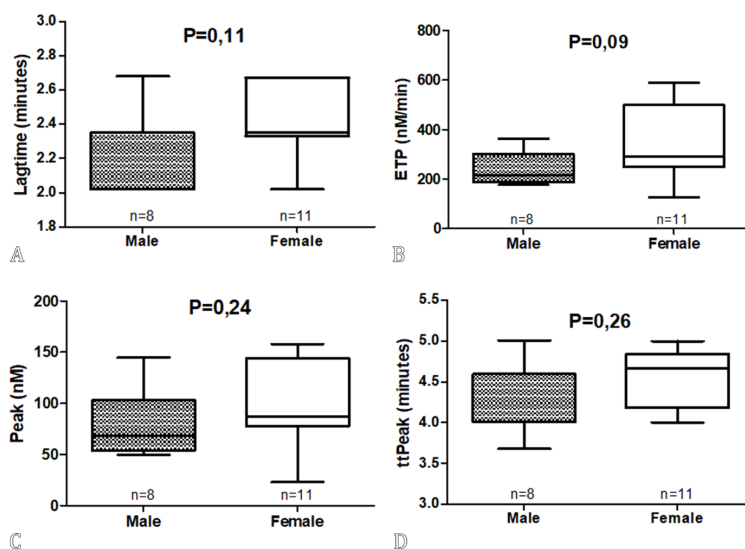


Fig.2. Thrombin Generation Test (TGT) of dogs with proteinuria in CKD, classified by sex. ETP = Endogenous Thrombin Potential.

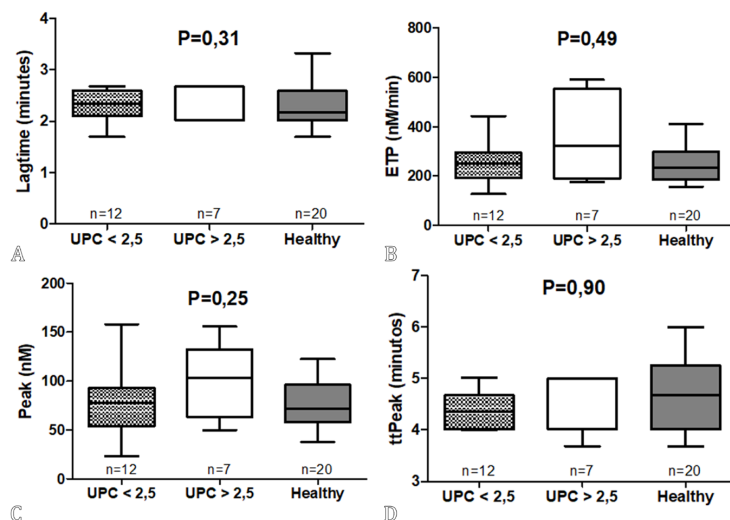


Fig.3. Thrombin Generation Test (TGT) of dogs with proteinuria in CKD, classified according to the degree of proteinuria. ETP = Endogenous Thrombin Potential, UCPR: Urine Protein:Creatinine Ratio.

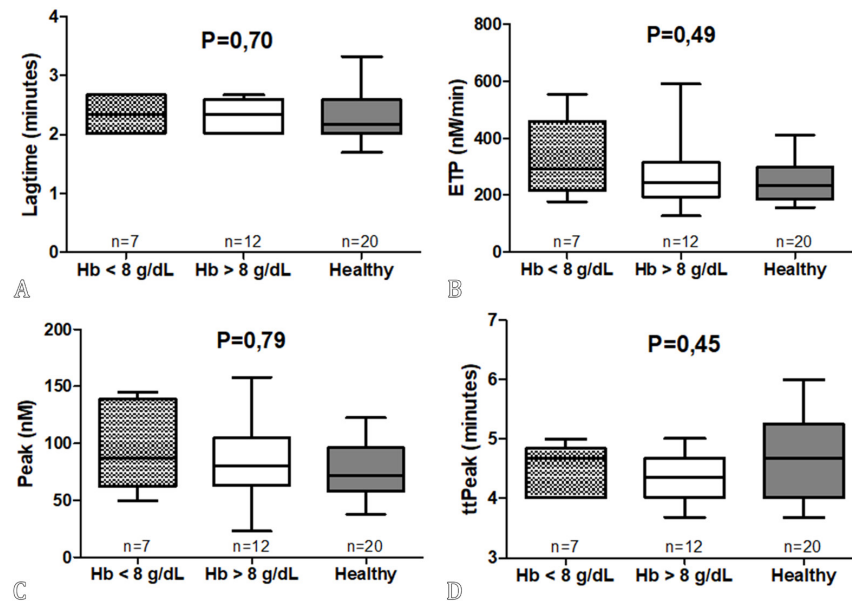


Fig.4. Thrombin Generation Test (TGT) of dogs with proteinuria in CKD, the dogs were classified according to the degree of anemia. ETP = Endogenous Thrombin Potential.

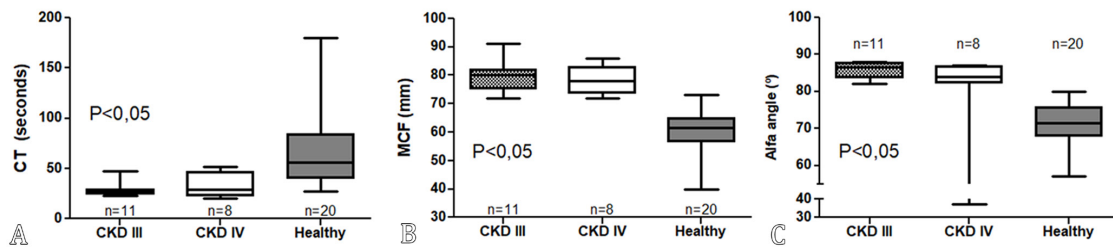


Fig.5. Thromboelastometry (TEM) of healthy dogs with proteinuria in CKD in stages III and IV. EXTEM® = tissue factor activation, CT = Coagulation Time, MCF = Maximum Clot Firmness.

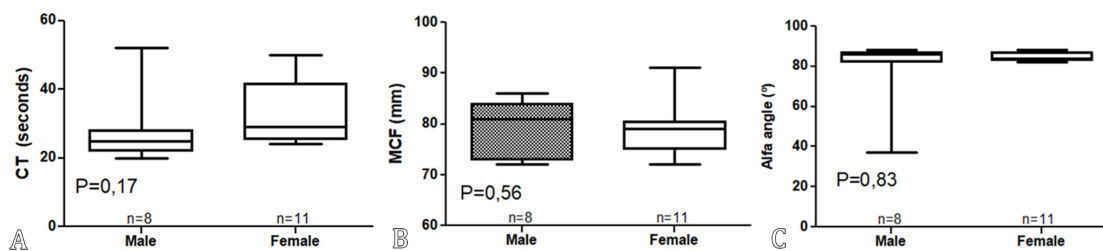


Fig.6. Thromboelastometry (TEM) of dogs with proteinuria in CKD, the dogs were classified by gender. EXTEM® = tissue factor activation, CT = Coagulation Time, MCF = Maximum Clot Firmness.

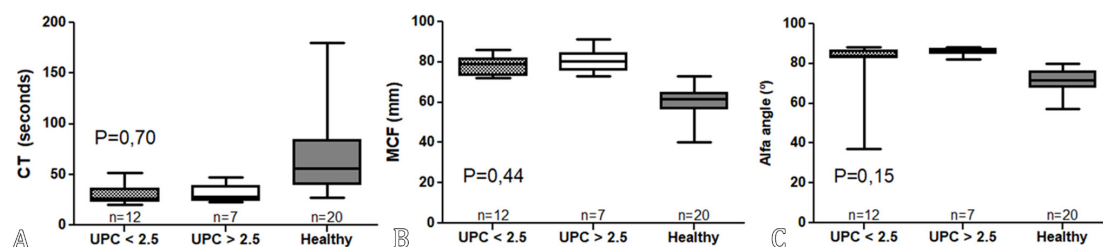


Fig.7. Thromboelastometry (TEM) of dogs with proteinuria in CKD, the dogs were classified according to the degree of proteinuria. EXTEM® = tissue factor activation, CT = Coagulation Time, MCF = Maximum Clot Firmness, UCPR: Urine Protein:Creatinine Ratio.

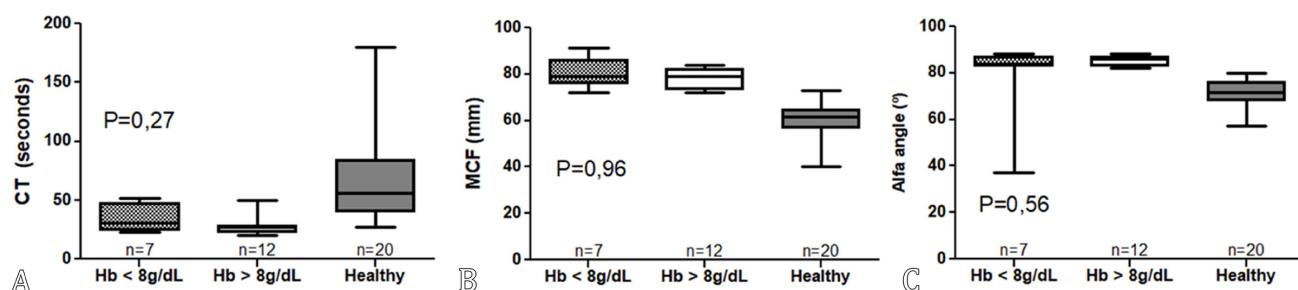


Fig.8. Thromboelastometry (TEM) of dogs with proteinuria in CKD, the dogs were classified according to the degree of anemia. EXTEM® = tissue factor activation, CT = Coagulation Time, MCF = Maximum Clot Firmness; Hb = hemoglobin.

Table 2. Thromboelastometry (TEM) of dogs with proteinuria in CKD, the dogs were classified according to disease duration

EXTEM®	CT	MCF	α angle
Reference range*	48-83	57-64	69-74
Up to 30 days (n=6)	28 (24-48)	79 (72-84)	85 (84-87)
≥30 days (n=13)	27 (25-44)	76 (73-81)	82 (59-85)
p	0.78	0.78	0.08

* IC 95% of control group; EXTEM® = Tissue factor activation, CT = Clotting Time, MCF = Maximum Clot Firmness; Values expressed as median, first (1q) and third quartiles (3q).

DISCUSSION

This study aimed to understand hemostatic changes in dogs with CKD in stages III and IV by using two global assessment tests for hemostasis, including TGT and TEM. In addition, there are no reports of thrombin generation potential in dogs with CKD.

Considering all TGT parameters, endogenous thrombin potential (ETP) seems to be the most widely used, with the best correlation to the clinical phenotype (Lipets & Ataullakhanov 2015). However, according to our results, no TGT parameter performed with low concentration tissue factor (1pM) was able to discriminate between healthy group and dogs with CKD (Table 1). Although not significant, it is important to characterize that dogs with CKD in stage III had higher ETP ($P=0.40$) and peak ($P=0.48$) values. The findings of TGT are probably explained by the association of natural anticoagulant reduction factors by proteinuria in CKD, as well as hyperfibrinogenemia in these patients.

However, when several variables stratified sick animals, some findings should be discussed. Thus, females presented a tendency to increase ETP ($P=0.09$) when compared to males; this may be partly explained by hormonal factors that may contribute to increased thrombin generation. Concerning women who use oral contraceptives, it could be observed among them a potential thrombin generation (Brummel-Ziedins et al. 2005), and it can be inferred that different hormones may influence the hemostatic profile.

Patients presenting severe anemia (hemoglobin <8g/dL) had higher ETP than the others, despite the $P=0.49$ value. In humans, ETP increases hematocrit levels due to higher exposure of membrane phospholipids (Horne et al. 2006). In fact, the increase in ETP observed in this study can be understood by the higher platelet exposure. Normocytic

and normochromic anemia in CKD are proportional to the degree of erythropoietin deficiency, while the most significant endothelial lesions owing to worsening renal disease occur (Polzin 2011). Thus, on such lesions, there would be activation of platelets and hemostatic mechanism, being the main activation surface for thrombin generation (Whelihan & Mann 2013). In addition, decreased competence of the natural anticoagulation system (due to proteinuria), it may be factors favoring a higher generation of thrombin. Blood viscosity is independent of erythrocyte volume (Brooks et al. 2014), and even with the presence of anemia, hypercoagulability was characterized in patients with CKD.

Proteinuria could be present in all patients with CKD, however, animals with more intense protein loss ($RPC>2.5$) had higher thrombin generation potential ($P=0.49$) (Table 2). Although there is no significant difference, this increase can be explained, at least partially, by the occurrence of significant renal injury, which would facilitate the loss of antithrombin in the urine (Prihirunkit et al. 2011). Protein S (PS) is also a natural anticoagulant, and its urinary loss also occurs in CKD. Only 40% of PS is known to perform anticoagulant function (free PS), while the remainder circulates in C4bBP-bound protein (C4b-binding protein), which increases in inflammatory processes such as CKD. The inflammatory process contributes to the reduction of free PS (anticoagulant) and higher formation of the PS-C4bBP complex, contributing more to thrombin generation (Prihirunkit et al. 2011), and consequent hypercoagulability state.

According to the time course of disease, it was observed in the present study that dogs with >30 days of diagnosis had lower tt_{peak} ($P=0.05$) compared to those with recent diagnoses, this result suggests a faster speed to reach reaction peak rate. This could be related to a slight, though not significant,

increase in thrombin generation, justified by the worsening of the disease over time.

Overweight and/or obesity have already been associated as a predictor of thrombotic events in humans (Beijers et al. 2010). Regarding the weight variable, in the present study, the body scores of the animals were not included - a limiting factor for interpretation by this classification. Considering the different breeds included in the study and that SRD dogs have different sizes, we cannot attribute any findings to the variable weight.

When MET results were compared between groups, significant differences could be observed for CT, MCF and α angle parameters (Fig 7); these results indicate better differences between sick and healthy dogs. TEM seems to be more efficient for detecting hemostatic changes than TGT in the studied groups. Assuming that TEM is sensitive to changes in fibrinogen levels and TGT not, it could be assumed that levels of this coagulant protein were probably higher in CKD animals, which is compatible with the pathophysiology of CKD (Polzin 2011, O'Neill et al. 2013). Blood figurative elements regarding the process of hemostatic alterations in dogs with CKD, which would contribute to tissue factor exposure enhancing the activation of the coagulation cascade and consequent fibrin formation, cannot be excluded.

When animals with CKD were stratified by age, sex, proteinuria degree, and anemia degree, such variables were not able to influence the hemostatic profile revealed by TEM. This fact may be explained by the limited sample size that was not able to show small differences among subgroups.

Despite the limitations of information in the patients' clinical files, patients' history allows us to evaluate how long the CKD patient has been suffering from pathophysiological changes of this disease. According to our TEM data, there was a tendency ($P=0.08$) to increase α angle as a function of shorter disease duration, as well as in dogs up to 20kg ($P=0.02$). Although α angle is not specific, it may be signaling increased platelet function, fibrinogen concentration and/or coagulation factors (Kol & Borjesson 2010). Furthermore, recent diagnosis of the disease and shorter treatment time could reflect more severe hemostatic changes due to the treatment period and patient compensation.

In fact, TEM was able to detect increased MCF and α angle in dogs with CKD when compared to healthy dogs. Such observation may have significant clinical relevance regarding the antithrombotic therapeutic protocol. However, more robust studies are needed to understand hemostatic changes in dogs with CKD.

A complete analysis of the data revealed that TEM was able to discriminate between healthy and CKD dogs. However, when there was stratification of the sample originated from diseased dogs, the convergence of results was observed regarding the two methods used to evaluate hemostasis. Thus, it should be emphasized that both TGT and TEM showed hemostatic changes, suggesting hypercoagulability up to 30 days of diagnosis as well as in those dogs under 20kg, especially in TEM.

Given the multifactorial context of hemostatic changes in CKD, further clinical studies are needed to understand and determine the predictive values of global hemostatic tests in thrombotic or hemorrhagic risk of CKD. Such knowledge could revert to appropriate prophylactic therapeutic actions

in such patients contributing to a significant quality of life and more prolonged survival of these patients.

Thromboelastometry may be considered an effective tool for hemostatic evaluation, but its applicability is restricted to intensive care centers and hospitals, due to simplify sample processing time. In contrast, the thrombin generation test allows storage of citrated plasma samples for later analysis, allowing their performance in laboratories. However, one technique does not replace another one: both are important.

CONCLUSION

Thromboelastometry is more sensitive to detect hypercoagulability in dogs with CKD, however, the thrombin generation test is applicable for allowing long-term sample storage.

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

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Diseases of wild birds in southern Rio Grande do Sul, Brazil¹

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ABSTRACT.- Echenique J.V.Z., Soares M.P., Albano A.P.N., Bandarra P.M. & Schild A.L. 2020. **Diseases of wild birds in southern Rio Grande do Sul, Brazil.** *Pesquisa Veterinária Brasileira* 40(2):121-128. Laboratório Regional de Diagnóstico, Faculdade de Veterinária, Universidade Federal de Pelotas, Campus Capão do Leão, Pelotas, RS 96010-900, Brazil. E-mail: alschild@terra.com.br

Necropsy protocols of the “Laboratório Regional de Diagnóstico” of “Faculdade de Veterinária” of the “Universidade Federal de Pelotas” were reviewed, ranging the period from 2000 to 2018. Three hundred eighty one necropsies, 25 refrigerated and/or formaline fixed organs, and seven biopsies were received, representing 413 samples. Most of these materials were sent by the “Núcleo de Reabilitação da Fauna Silvestre” of “Universidade Federal de Pelotas” (NURFS-CETAS-UFPEL) and were from municipalities within the range area of LRD-UFPEL influence. Of the 413 cases 55 (13.31%) corresponded to metabolic/nutritional diseases; 50 (12.10%) to trauma; 35 (8.47%) to bacterial diseases/toxi-infections; 30 (7.26%) to parasitic diseases; 28 (6.77%) to fungal diseases; four (0.97%) to viral diseases and 17 (4.11%) to other diseases. Cases where it was not possible to determine the etiology, were in severe autolysis or were inconclusive totaled 194 (46.97%). Metabolic/nutritional diseases and traumatic injuries were the main cause of death in wild birds, being Passeriformes the most affected order.

INDEX TERMS: Wild birds, spillover, wildlife animals, epidemiology.

RESUMO.- [Doenças de aves silvestres no sul do Rio Grande do Sul.] Foi realizado um estudo retrospectivo dos diagnósticos de causas de morte e de lesões em aves silvestres na região Sul do Rio Grande do Sul de 2000 a 2018. Foram revisados os protocolos de necropsia e materiais de aves silvestres encaminhados ao Laboratório Regional de Diagnóstico da Faculdade de Veterinária da Universidade Federal de Pelotas no período. Foram recebidos 381 cadáveres para necropsia, 25 órgãos refrigerados e/ou em formol e 7 biopsias, totalizando 413 materiais. A maioria desses materiais foi remetida pelo Núcleo de Reabilitação da Fauna Silvestre

da Universidade Federal de Pelotas (NURFS-CETAS-UFPEL) e provenientes de municípios da área de influência do LRD-UFPEL. Dos 413 casos 55 (13,31%) corresponderam a doenças metabólicas/nutricionais; 50 (12,10%) a traumas; 35 (8,47%) a doenças bacterianas/toxi-infecções; 30 (7,26%) a doenças parasitárias; 28 (6,77%) doenças fúngicas; 4 (0,97%) doenças virais e 17 (4,12%) outras doenças que não se encaixavam nas categorias. Ainda em nos casos em que não foi possível determinar a etiologia, apresentaram autólise acentuada ou foram inconclusivos somaram 194 (46,97%). As doenças metabólicas/nutricionais e lesões traumáticas foram as principais causas de morte de aves silvestres, sendo a ordem mais afetada a Passeriformes.

TERMOS DE INDEXAÇÃO: Aves silvestres, *spillover*, animais selvagens, epidemiologia.

INTRODUCTION

Brazil has the largest number of bird species in the world, many of which is endemic and threatened or in risk of extinction (Mittermeier et al. 2005, Marinha Militar 2016, Machado et al. 2008). In this scenario, Brazil is one of the

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main collection sites and leading routes of wildlife illegal trade (Oliveira 2003). This illegal trade moves around US \$ 6 billion and extracts from nature more than four million birds, being one of the main threats to many species survival. In addition to illegal trade, habitat loss, illegal hunting and capture are other causes of bird population decline, causing an ecological imbalance in ecosystems (Redford 1992). This imbalance may facilitate pathogen transmission of an adapted population (reservoir) to a new host population (naïve) in an epidemiologic process called spill-over (Frölich et al. 2002). This phenomenon can be observed when wild birds approach urban centers and can be infected with agents that are not described for the species.

Infectious or parasitic diseases affect wild birds often, mainly because of bare transport conditions of illegal trade. Consequently, 90% of captured birds do not reach their final destination (Redford 1992, RENTAS 2001, Wilson-Wilde 2010). Cachexia and trauma injuries caused by this transportation can predispose the occurrence of infectious/parasitic diseases. In a study conducted in the state of São Paulo with apprehended birds from illegal trade, the main diseases were infectious and parasitic (Godoy & Matushima 2010).

The aims of this paper were to identify the main diseases that occur in wildlife birds in southernmost region of Rio Grande do Sul State, highlighting those diagnostics with zoonotic/spillover potential.

MATERIALS AND METHODS

A retrospective study of diseases of wild birds diagnosed in the “Laboratório Regional de Diagnóstico” of “Faculdade de Veterinária” of the “Universidade Federal de Pelotas” (LRD-UFPe) was carried out. The study period comprehended January 2000 until November 2018. Epidemiological data were retrieved from the necropsy protocols and information as species, sex and age were recorded. The birds were classified according to the age group in nestling, juvenile, and adult; and categorized into a higher taxon (Order) in order to group species with the same characteristics. The gross and microscopic changes observed in necropsies or biopsies/remitted organs, results of microbiological/mycological culture, and parasitology techniques were also reviewed. The diagnoses were grouped into different categories according to the nature of the etiological agent, in metabolic/nutritional diseases, traumas, parasitic diseases, bacterial diseases, fungal diseases, viral diseases, and other diseases. In cases which there were concomitant diseases, the disease responsible for the bird's death was considered. The cases that did not fit into any of the mentioned etiologies were classified as indeterminate etiology. The inconclusive diagnoses, samples in autolysis and without bacterial growth were also included in this study. In this study, domestic birds considered as pests (House Sparrows and Domestic Pigeons), non-traditional farmed species (Turkeys, Rheas and Quails), exotic or ornamental birds and/or kept as pets (Canaries, Cockatiels and Parakeets), and Penguins (Sphenisformes) were excluded.

RESULTS

From January 2000 to November 2018, 381 necropsy cadavers, 25 refrigerated and/or formalin fixed organs, and seven biopsies of wild birds, totaling 413 samples, were submitted to LRD. Most of these materials were sent by the “Núcleo de Reabilitação da Fauna Silvestre” of the “Universidade Federal

de Pelotas” (NURFS-CETAS-UFPe) and was from the LRD's influence area municipalities.

Of the 413 cases, 55 (13.31%) corresponded to metabolic/nutritional diseases; 50 (12.10%) to trauma; 35 (8.47%) bacterial diseases/toxi-infections; 30 (7.26%) parasitic diseases; 28 (6.77%) fungal diseases; 4 (0.97%) to viral diseases, and 17 (4.11%) other diseases. The inconclusive or severe autolytic cases accounted for 194 (46.97%) individuals. The bird's species were informed in 413 cases and it was possible to group them in 21 orders, being 219 (53.02%) Passeriformes (song birds); 44 (10.65%) Psittaciformes (parrots and monk parakeet); 37 (8.95%) Strigiformes (owls); 25 (6.05%) Anseriformes (waterfowls); 14 (3.39%) Charadriiformes (scavenger birds); 11 (2.66%) Pelecaniformes (herons); 9 (2.18%) Columbiformes (wild pigeons); 9 (2.18%) Gruiformes (common gallinules); 7 (1.69%) Accipitriformes (hawks); 7 (1.69%) Piciformes (woodpeckers); 6 (1.45%) Falconiformes (falcons); 5 (1.21%) Suliformes (cormorants); 4 (0.97%) Coraciiformes (kingfishers); 4 (0.97%) Rheiformes (rheas); 3 (0.73%) Tinamiformes (tinamous); 2 (0.48%) Ciconiiformes (storks); 2 (0.48%) Procellariiformes (albatrosses and petrels); 2 (0.48%) Cuculiformes (cuckoos); 2 (0.48%) Podicipediformes (grebes); 1 (0.24%) Apodiformes (hummingbirds); 1 (0.24%) Caprimulgiformes (pauques) and in one case (0.24%) it was not possible to determine the order.

In 243 (58.84%) cases the birds were classified as adults; 37 (8.95%) in juveniles, and 22 (5.32%) in nestlings. In 112 (27.11%) this information was not available in the necropsy protocols. The most frequent diseases of each etiology were distributed according to the order and age. This data is shown in Tables 1-3. The traumatism cases were classified according to the order and age group (Fig.1).

Less frequently, fungal diseases totaled 28 diagnoses, with 25 cases of aspergillosis. This disease was observed mainly in Anseriformes, Acipitriformes, Charadriiformes, Gruiformes, Podicipediformes, Strigiformes, Suliformes, and Passeriformes adult birds. The lesions were observed in air sacs and lungs,

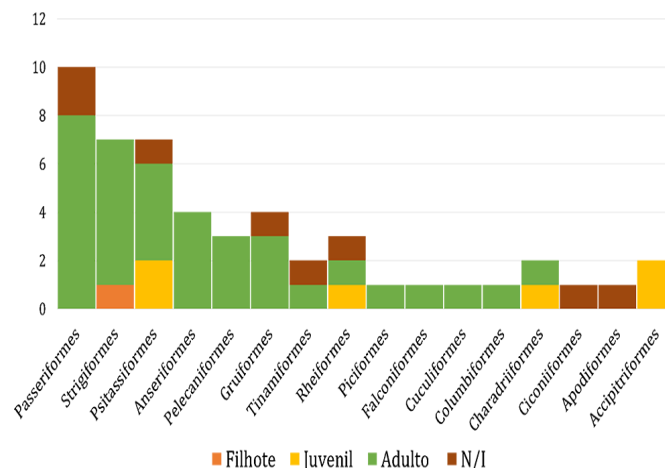


Fig.1. Distribution of traumatic lesions (n=50), according to order and category, diagnosed in wild birds at the “Laboratório Regional de Diagnóstico” of the “Faculdade de Veterinária” of the “Universidade Federal de Pelotas” from January 2000 to November 2018.

Table 1. Metabolic/nutritional diseases (n=55) distribution according to order and age category in wild birds submitted to the “Laboratório Regional de Diagnóstico” of the “Faculdade de Veterinária” of the “Universidade Federal de Pelotas” from January 2000 to November 2018

Order	Cachexia/ Age category	Hepatic steatosis/ Age category	Microvesicular cardiac degeneration*/ Age category	Capture myopathy/ Age category	Vitamin A deficiency/ Age category	Egg retention/ Age category
Anseriformes	5/1A, 3N, 1J	1/1F	-	-	-	-
Caprimulgiformes	1/A	-	-	-	-	-
Coraciiformes	1/1 Ni	-	-	-	-	-
Gruiformes	2/2A	-	-	-	-	-
Passeriformes	17/13 A, 1J, 1N, 3Ni	9/7A, 1N, 1Ni	6/5A, 1 Ni	-	1/1 A	-
Podicipediformes	1/1 Ni	-	-	-	-	-
Psittaciformes	3/1A, 1J, 1Ni	-	-	-	-	1/1 A
Strigiformes	3/2A, 1J	-	-	-	-	-
Charadriiformes	-	2/ 2F	-	-	-	-
Rheiformes	-	-	-	1/1A	-	-
Suliformes	-	-	-	1/1A	-	-
TOTAL	33 (60%)	12 (21.81%)	6 (10.90%)	2 (3.63%)	1(1.81%)	1(1.81%)

Category: N = nestling, J = juvenile, A = adult, Ni = not informed; * Lesions were observed only in cardiomyocytes, mainly in the papillary muscles of the left ventricle without skeletal muscle necrosis.

Table 2. Bacterial diseases (n=35) distribution according to order and age category in wild birds submitted to the “Laboratório Regional de Diagnóstico” of the “Faculdade de Veterinária” of the “Universidade Federal de Pelotas” from January 2000 to November 2018

	Colibacillosis/ Age category	Sepsis/ Age category	Botulism/ Age category	Pododermatitis/ Age category	Salmonellosis/ Age category	Micobacteriosis/ Age category
Accipitriformes	-	1/1N	-	-	-	-
Anseriformes	1/1Ni	1/1Ni	2/2Ni	-	-	-
Charadriiformes	-	1/1Ni	1/1A	-	-	-
Coraciiformes	2/1N, 1Ni	-	-	-	-	-
Passeriformes	4/2A, Ni	7/1A, 1N, 4Ni	-	1/1 A	-	-
Psittaciformes	8/2A, 1J, 5Ni	3/2N, 1J	-	-	1/1 Ni	-
Strigiformes	1/1A	-	-	-	-	1/1 Ni
TOTAL	16 (45.71%)	13 (27.14%)	2 (5.71%)	1 (2.94%)	1(2.94%)	1(2.94%)

Category: N = nestling, J = juvenile, A = adult, Ni = not informed.

Table 3. Parasitic diseases (n=30) distribution according to order and age category in wild birds submitted to the “Laboratório Regional de Diagnóstico” of the “Faculdade de Veterinária” of the “Universidade Federal de Pelotas” from January 2000 to November 2018

	Trichomoniasis/ Age category	Coccidiosis/ Age category	Singamosis/ Age category	Trematode hepatitis/ Age category	Hytrichis sp. parasitism/ Age category	gastrintestinal parasitosis/ Age category	Filariosis/ Age category	Nematode Hepatitis/ Age category	Trichomoniasis Aspergillosis/ Age category
Passeriformes	-	8/7A, 1 Ni	3/3 A	-	-	1/1 Ni	1/1 Ni	1/1 Ni	-
Strigiformes	5/4A, 1J	-	-	2/2A	-	-	-	-	-
Falconiformes	3/1N, 2J	-	-	-	-	-	-	-	1/1J
Columbiformes	2/1A, 1J	-	-	-	-	-	-	-	-
Pelecaniformes	-	-	-	-	2/2A	-	-	-	-
Accipitriformes	-	-	-	-	-	-	-	-	-
Psittaciformes	-	-	-	-	-	1/1A	-	-	-
TOTAL	10 (33.33%)	8 (26.66%)	3 (10%)	2(6.45%)	2(6.66%)	2 (6.66%)	1(3.33%)	1(3.33%)	1(3.33%)

Category: N = nestling, J = juvenile, A = adult, Ni= not informed.

and in one case, the bird also presented avian pox skin lesions. Another important fungal disease observed in that period was oral candidiasis, being diagnosed in three cases.

Regarding the viral diseases the poxvirus was observed in four opportunities and in all cases only the integument was affected. This disease was observed in Psittaciformes (1), Passeriformes (1), and Strigiformes (2). The age category were adult (2), juvenile (1) and in one case was not informed. The diagnostics that did not fit in any category included: avian leucosis (4) captive maladaptation (1), deforming arthritis (1), xanthoma (1), skin squamous cell carcinoma (1), hepatic cirrhosis (1), non-specific granuloma (1), congenital malformation (1), and others (6). These cases summed up 17 individuals of different avian Orders.

DISCUSSION

Metabolic diseases were the main cause of death in wild birds from southernmost Brazil and Passeriformes being the most frequent Order. Song birds are very addressed by wildlife trade due to its high commercial value and high profit rate (RENTAS 2001). Additionally, this reflects the reality of rehabilitation centers where this is the main Order submitted in these facilities (De Freitas et al. 2015).

Cachexia was the most frequent diagnostic with the widest Orders range. The majority of birds were from NURFS, which suggests an inability of those animals to adapt in captivity after their illegal capture in nature. The second major metabolic cause of death was hepatic steatosis. This disease can also be related to illegal trafficking and poor housing conditions. The main affected order was Passeriformes, mainly *Paroaria coronata* (red crested cardinal) (Fig.2). Those birds when submitted to NURFS were seized from illegal trade and were in poor sanitary conditions without an appropriated diet (diet rich in sunflower seeds and often with mold) during its previous captivity. Despite offering the proper diet, the animals came to death. This framework suggests that those birds may have been in prolonged fasting. In domestic felids, food deprivation can cause severe hepatic steatosis (Valtolina

& Favier 2017) and it is believed that the same disease onset could have happened in these cases.

The sudden death without cachexia occurred exclusively in Passeriformes. All these cases presented a history of stress episodes (apprehension by the ambiental patrol) consisting with capture (exertional) myopathy. In these cases, microscopically there was severe microvesicular cardiac degeneration, more marked within the papillary muscles in the left ventricle. This lesion can be observed in some cases of capture myopathy (Friend & Thomas 1999). However, in classic cases it is noted segmented muscular necrosis of muscular fibers in great skeletal muscle masses (mainly pectoral and inferior limbs) (Friend & Thomas 1999). In the present cases, skeletal muscle and renal lesions were not observed, which leads to believe that Passeriformes cases had an extremely acute clinical course. All those animals underwent a stressful event what can release endogenous catecholamines by sympathetic nerve endings and adrenal medulla. The prolonged exposure to high concentrations may result in coronary spasm, arrhythmias, contractile dysfunction, and cell damage which lead to myocardial necrosis (Dhalla et al. 1992). Based on this mechanism, it can produce solely cardiac lesions in these songbirds without the skeletal lesions commonly seen in other species (Spraker et al. 1987, Tully Junior et al. 1996).

The second major cause of death was trauma, unlike shown in other surveys (Sharma et al. 2014, Montesdeoca et al. 2017). This may have happened because trauma diagnosis is not difficult to the clinician and the cadavers are not submitted to diagnostic laboratories. It is also necessary to consider the high number of Passeriformes presented with trauma, which is related to poor transport conditions of illegal trade (RENTAS 2001). In cases involving Strigiformes (owls)(Fig.3), the trauma injuries were probably results of accidental run over by vehicles that transited at night. In southernmost Brazil, human depredation is also a reality due to the superstitious feature (bad luck) that owls have amongst rural populations. In the Psittaciformes (parrots and monk parakeets) cases of this study, the traumas occurred mainly in nestlings and could be related to the collection of these specimens being sold and used as pet birds.



Fig.2. *Paroaria coronata* (red crested cardinal) adults, pectoral muscle. Marked atrophy of pectoral muscles of both cardinals, severe onychogriphosis and feces accumulation around the cloaca.



Fig.3. *Bubo virginianus* (great horned owl), adult, humerus. Body of humerus exposed oblique fracture in its medial third associated with edema and hemorrhage.

Regarding the bacterial diseases, avian colibacillosis was the most frequent disease. The diagnosis was based on macroscopic and histological lesions with bacterial isolation. Colibacillosis is caused by avian pathogenic *Escherichia coli* (APEC) and it has a wide range of systemic and localized lesions (Barnes & Gross 1997). In this study, this disease occurred more in adult Psitaciformes and Passeriformes. This is an opportunistic disease and can affect a large number of animals in rehabilitation centers, mainly because the high density of birds, which can lead to immunosuppression by stress. (Valtolina & Favier 2017). As well as colibacillosis, different opportunistic microorganisms can cause sepsis (Chadfield et al. 2005, Seymour et al. 2016). Bacterial diseases were the second most frequent cause of death in wild birds and were observed predominantly in nestlings. This age group is more exposed to ruthless weather condition when they are away of parental care. This can predispose to bacterial infections and consequent septicemia (Benskin et al. 2009). Furthermore, some authors have observed that the high concentration of organochlorines (OGC) in tissues of northern Baltic Sea nestlings seagulls found dead can enhance the septicemia risk (Hario et al. 2004). OGCs decrease the ability of the immune system to respond correctly to infections (Hario et al. 2004, Morrissey et al. 2014). It should be emphasized that the southernmost region of RS, where most of those birds of this work came from, is a well-developed agricultural region with large rice and soybeans crops. Various pesticides are often used in these plantations, including organochlorines, which remain in the environment and birds can bioaccumulate these substances in their bodies over the years (Flores et al. 2004). The sepsis in adult birds was associated with trauma. These lesions may permit the entry of pathogenic microorganisms such as *Staphylococcus* sp. (Huckabee 2000).

Two cases of botulism were observed in birds with aquatic and scavenger habits (Rocke & Bollinger 2008). The diagnosis was based on the clinical signs, absence of gross and histologic lesions, and bioassay in mice. These birds could work as sentinels for botulism, since the high unusual mortality indicates contamination sources for other domestic species.

Salmonellosis and mycobacteriosis, despite being diagnosed only in one case each, highlighted the spillover risk in southernmost Brazil. It has been mentioned that the proximity between species can facilitate the transmission of these diseases (Converse 2008, Daoust & Prescott 2007). In cases of salmonellosis (Turquoise-fronted amazon - *Amazona aestiva*) and mycobacteriosis (great horned owl - *Bubo virginianus*), the animals had close contact with people and other domestic birds.

In relation to parasitic diseases, trichomoniasis was more frequently observed affecting mainly birds of prey and less frequently wild pigeons (Fig.4A). This disease is a great limitation for conservation of wild birds (Bunbury et al. 2007, Forzán et al. 2010), since domestic pigeons are the main reservoirs of *Trichomonas gallinae* (Amin et al. 2014.).

Birds of prey can be contaminated by domestic dove predation. In addition, those birds can contaminate waterer and grains in feeders, which can lead to transmission to wild birds (Purple & Gerhold 2015). Regarding this, it is suggested that wild-doves sharing waterers with domestic pigeons (*Columbia livia*) can be contaminated and transmit the disease to their predators who are extremely sensitive to trichomoniasis. In this context, birds of prey could contaminate themselves as they approached urban centers to prey domestic doves.

Coccidiosis affected exclusively adult red crested cardinals, suggesting a susceptibility of this species. Godoy & Matushima (2010) analyzed birds from illegal trafficking in São Paulo state and observed that coccidiosis was one of the main causes of death in that population. Passeriformes of this present study were from illegal trafficking, which may have caused a stressful condition and triggered the disease. This disease has declined over the years following the adoption of deworming protocols upon birds' arrival at NURFS.

Singamosis (Fig.4B) was diagnosed only in song birds that stayed at the "Núcleo de Reabilitação da Fauna Silvestre" (NURFS-CETAS-UFPEL) and were in a soft release enclosure (Clarke et al. 2002). This disease is mainly diagnosed in Galliformes (Pheasants) (Gethings 2018) and is not commonly observed in Passeriformes. Other parasitic diseases were diagnosed in only in a few cases.

Regarding fungal diseases, aspergillosis was the main diagnosis in this etiology (Fig.5). The disease was observed in adult birds and was generally associated with other conditions or trauma injuries. Immunosuppressive factors are determinants for the disease occurrence (Carrasco et al. 2001) and it has been observed that aspergillosis cases, and other mycoses, are limiting factors for wild birds conservation (Pitarch et al. 2017). In addition, cases of oral candidiasis were observed only in Passeriformes and Columbiformes, both adult and nestling. In nestlings of scavenger birds in Spain, oral candidiasis is related to the accidental ingestion of residual antibiotics in bovine carcasses (Pitarch et al. 2017). It has been mentioned that in adults, candidiasis is usually associated with other diseases (Hubbard et al. 1985). In this survey, this condition was not observed in any of the adult bird's cases.

The most important viral disease was avian pox and it was observed in juvenile birds of three different orders. Avian pox summed four cases only, being of minor importance for wild birds' conservation in southern Brazil. On the other hand, this disease was very frequent in songbirds from São Paulo state (Godoy & Matushima 2010) and the most important skin disease in wild turkeys of the United States (Elsmo et al. 2016). The leukosis cases happened in adult Passeriformes and did not have epidemiological importance. This fact is supported by a five years survey held in England. One-thousand-two-hundred-thirty-four cadavers from a zoo were examined and the disease was observed only in six occasions (Wadsworth et al. 1981).



Fig.4. (A) *Carcara plancus* (southern crested caracara), juvenile, oral cavity, oral trichomoniasis. Multiple coalescent yellow tan nodules affecting 1/3 of oral cavity. There is also velvet green plaques covering the tongue. (B) *Saltator similis* (green saltator), adult, trachea, singamiasis. Multiple reddened areas when opened revealed parasites.

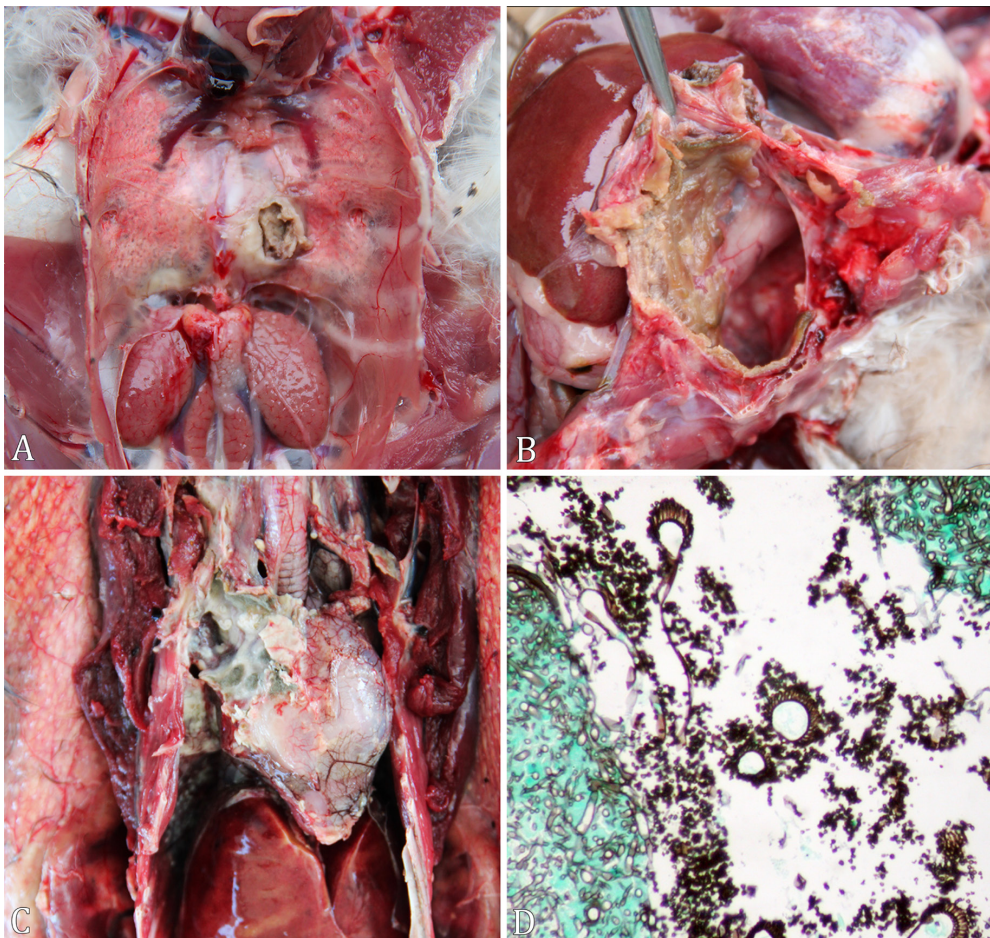


Fig.5. (A) *Tyto furcata* (common barn owl), juvenile, lung, pulmonary aspergillosis. Two tan nodules within the pulmonary parenchyma. (B) *Buteo gallusmeridionalis* (savanna hawk), juvenile, abdominal air sac, aspergillosis. Marked left abdominal air sac with wall thickening of yellowish caseous material. (C) *Phalacrocorax brasilianus* (neotropical cormorant), adult, cervical air sac, aspergillosis. Presence of velvet white to green fungal *Aspergillus* spp. colonies. (D) *Phalacrocorax brasilianus* (neotropical cormorant), adult, cervical air sac, aspergillosis. Gomori Methenamine-Silver (GMS) reveals septate hyphae with dichotomous branching, conidiophores and multiple free conidia. GMS, obj.40x.

CONCLUSIONS

This study demonstrated that metabolic diseases and trauma lesions are the main cause of death of wild birds in southernmost region of Brazil and Passeriformes were the most affected order.

Infectious and parasitic diseases are also important causes of wild bird mortality. Spillover in wild birds within this area may have a limited importance, since diseases caused by shared agents both by domestic and wild animals were diagnosed at low frequency.

Another important fact highlighted by this survey was that illegal trafficking was a determinant factor for a wide range of disease manifestations.

More studies are needed in the area in other wild animals' classes to better understand the possibilities of spillover occurrence.

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

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Companion psittacine birds as reservoir of gentamicin and vancomycin-resistant *Enterococcus* spp.¹

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Enterococcus are recognized worldwide as significant nosocomial agents that have been continuously evolving to adapt to different niches and acquire resistance to several antibiotic classes. Vancomycin and gentamicin-resistant strains of *E. faecalis* and *E. faecium* have been associated with nosocomial human infections. Some epidemiological studies suggest the participation of pets as reservoirs of vancomycin and gentamicin-resistant *Enterococcus* strains. However, the role of companion birds as reservoirs of these strains has been poorly studied. In this study, 126 psittacine birds were evaluated and 26.9% carried *Enterococcus* spp., including the species *E. faecalis*, *E. faecium*, *E. hirae*, *E. phoeniculicola*, *E. gallinarum* and *E. casseliflavus*. The antibiotic resistance profile showed four high-level gentamicin-resistance (HLGR) strains. In addition, two strains presented intermediate levels of vancomycin resistance. Resistant strains were isolated from fecal and oropharynx samples of sick and clinically healthy birds, suggesting that psittacine birds may act as reservoirs of HLGR *Enterococcus* spp. However, sick birds appear to be more implicated in the enterococci transmission than healthy birds.

INDEX TERMS: Psittacine birds, gentamicin, vancomycin, resistance, *Enterococcus* spp., avian diseases, antibiotic resistance, parrots, birds, wildlife animals, zoonosis.

RESUMO.- [Psitacídeos de companhia como reservatórios de *Enterococcus* spp. gentamicina e vancomicina-resistentes.]

Enterococcus são reconhecidos mundialmente como significantes agentes nosocomiais, que têm continuamente se adaptado a diferentes nichos e adquirido resistência a várias classes de antibióticos. Cepas de *E. faecalis* e *E. faecium* vancomicina e gentamicina-resistentes têm sido associadas a infecções nosocomiais em humanos. Alguns estudos epidemiológicos sugerem a participação de aves como reservatórios de cepas de *Enterococcus* vancomicina e gentamicina-resistentes. Entretanto, a relação das aves de companhia como reservatórios destas cepas

tem sido pouco estudada. Neste estudo, 126 psitacídeos foram avaliados, e 26,9% destes eram portadores de *Enterococcus* spp., incluindo as espécies *E. faecalis*, *E. faecium*, *E. hirae*, *E. phoeniculicola*, *E. gallinarum* e *E. casseliflavus*. O perfil de resistência antibiótica mostrou quatro cepas com alto nível de resistência a gentamicina (ANRG). Além de duas cepas com nível intermediário de resistência a vancomicina. As cepas resistentes foram isoladas de amostras fecais e de orofaringe de aves doentes e clinicamente saudáveis, sugerindo que psitacídeos podem estar atuando como reservatórios para *Enterococcus* spp. com ANRG. Contudo, Aves doentes parecem estar mais relacionadas à transmissão de enterococcus, do que aves saudáveis.

TERMOS DE INDEXAÇÃO: Psitacídeos, *Enterococcus* spp., gentamicina, vancomicina, doenças aviárias, resistência antimicrobiana, psitacídeos, pássaros, animais selvagens, zoonoses.

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INTRODUCTION

Enterococcus infections associated with healthcare settings emerged in the 1970s and 1980s as a leading cause of antibiotic-resistant infections such as bacteraemia and urinary tract infection (UTIs) (Gilmore et al. 2013). These bacteria generally present low virulence potential as evidenced by their presence in enteric microbiota of healthy humans and animals (Arias & Murray et al. 2012, Hammerum 2012, Gilmore et al. 2013). Despite this, the importance of *Enterococcus* infections is highlighted by the intrinsic resistance to first-line antimicrobial agents (e.g. low-level resistance to β -lactams and aminoglycosides) and the capacity to acquire resistance to several other antimicrobial agents, including last resort antibiotics (glycopeptide) (van den Bogaard & Stobberingh, 2000, Hammerum et al. 2010).

Starting in 1979, the frequent finding of high-level gentamicin resistance (HLGR) in isolates from clinical conditions led to further challenges for the treatment of life-threatening enterococcal infections. The only new effective antibiotic available at that time was developed in the 1990s in the form of vancomycin. Nevertheless, throughout the last decades there has been a rising prevalence of vancomycin-resistant *Enterococcus* (VRE) (Woodford et al. 1998). VRE were reported as the second most common cause of human nosocomial infections in the USA, being responsible for up to approximately 25,000 deaths each year (McKinnell et al. 2012). However, the prevalence of VRE in South America is still relatively low, although the clonality of the strains follows the pattern described in other parts of the world with trends to become more disseminated (Panesso et al. 2010).

Several studies have been conducted concerning the epidemiology of antimicrobial resistance between production animals and humans, and the role of growth promoters in livestock resulting in drug-resistant strains which might have zoonotic potential (Hammerum 2012). In the domestic environment, pets such as dogs and cats are reported as potential reservoirs for resistant microorganisms to humans (Guardabassi et al. 2004, Gülhan et al. 2006, Iseppi et al. 2015). It has been shown that resistant bacteria that colonize the intestinal tract of healthy pets could represent a risk to humans as well as transmitting their resistance genes located on mobile genetic elements to the human intestinal microbiota. Similarly, companion pets can get colonized by resistant bacteria after human contact (Simjee et al. 2002, De Leener et al. 2005).

Free-ranging wild birds have also been identified as possible reservoirs for vancomycin and gentamicin antimicrobial-resistant enterococci isolates (Oravcova et al. 2013, Roberts et al. 2016). Recently, Freitas et al. (2018) reported the identification of captive blue-fronted-parrot (*Amazona aestiva*) as potential zoonotic sources of multidrug-resistant *Enterococcus* spp. in two wild animal screening centers. Parrots are popular pets throughout the world. Their close contact with humans in captivity may lead to the transmission of several microorganisms with zoonotic potential (Boseret et al. 2013). We aimed to address this question by identifying enterococci species isolated from psittacine birds maintained as companion pets, both from clinical cases and asymptomatic individuals, establishing the phenotypic antibiotic resistance profile to gentamicin and vancomycin.

MATERIALS AND METHODS

Ethics statement. This study was approved by Ethics Committee of “Universidade de São Paulo” and authorized for scientific purposes (5174111215).

Samples. A total of 126 captive psittacine pet birds belonging to 13 species from the state of São Paulo (Brazil) were sampled in this study (Table 1). The sampled birds included native and exotic adult psittacine kept as companion pets. The age and gender were undetermined, as well the history of antimicrobial therapeutic use and the contact with other species. The collection of birds were grouped into apparently healthy animals (n=109) and sick birds (with respiratory disease, n=17). Cloacal and oropharynx swabs were collected from asymptomatic individuals and sick birds.

Isolation and identification of *Enterococcus*. The samples were collected with sterile swabs and inoculated in BHI broth (Brain heart-infusion) and incubated at 37°C for 18 hours. Standard culture procedures were performed using Columbia agar with 5% sheep blood and Chromagar VRE culture media (OXOID™, Basingstoke, UK). Isolated colonies were screened according to Gram stain morphology and identified through MALDI-TOF MS technique (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry); the acquired spectra were loaded in MALDI BioTyper™ 3.0 software (Bruker Daltonik) and compared to the manufacturer database accepting scores ≥ 2.0 for species identification. Also, the software Graph Pad was employed to compare the health and sick groups by Fisher's Exact Test 84 (with 95% confidence interval and $p < 0.05$).

Phenotypic resistance profile. The phenotypic resistance profile was determined using the disc diffusion technique for high-concentration gentamicin (120 μ g) and vancomycin (30 μ g), following the recommendations of the Clinical Laboratory Standards Institute (CLSI 2019).

Table 1. Species of *Enterococcus* spp. isolated from psittacine birds kept as pet in São Paulo, Brazil, 2018

Psittacine birds	<i>Amazona Aestiva</i> n=80	<i>Amazona amazonica</i> n=7	<i>Anodorhynchus hyacinthinus</i> n=3	<i>Amazona xanthops</i> n=1	<i>Nymphicus hollandicus</i> n=25	<i>Pionus maximiliani</i> n=1	Total of <i>Enterococcus</i> spp. strains
<i>E. faecalis</i>	9	1	2	1	2		15
<i>E. hirae</i>	10	2					12
<i>E. faecium</i>	4					1	5
<i>E. phoeniculicola</i>		2					2
<i>E. galinarum</i>	1						1
<i>E. casseliflavus</i>	1						1
TOTAL	25	5	2	1	2	1	36

Enterococcus spp. were not isolated in the following species of birds: *Amazona rhodocorytha* (n=1), *Psittacus erithacus* (n=1), *Alisterus scapularis* (n=1), *Psittacula krameria* (n=1), *Eclerus roratus* (n=1), *Cacatuidae* (n=3) and *Loriinae* (n=1).

RESULTS

Enterococci isolates

Enterococci isolates (36 in total) were obtained from 34 birds (26.9%). Isolates were obtained statistically more frequently from the 17 birds presenting respiratory disease (70.58%) than from the 109 healthy birds (22.01%) (p values 0.0001).

Enterococci species

Enterococcus faecalis was the most frequent specie identified (15/36), followed by *E. hirae* (12/36), *E. faecalis* (5/36), *E. phoeniculicola*, (2/36), *E. gallinarum* (1/36), and *E. casseliflavus* (1/36), (Table 1). In clinically healthy birds *E. hirae* was the most prevalent species (11/24), followed by *E. faecalis* (9/24), *E. faecium* (3/24) and *E. phoeniculicola* (1/24). However, among birds with respiratory disease the species *E. faecalis* and *E. faecium* were more prevalent, with 6/12 and 2/12 strains, respectively. The species *E. gallinarum* (1/12), *E. casseliflavus* (1/12) *E. hirae* (1/12) and *E. phoeniculicola* (1/12) were also detected in sick birds.

Antibiogram results

The antibiogram results are presented in Table 2. The analysis of the 36 *Enterococcus* spp. isolates revealed that 4 strains presented high-level gentamicin resistance (HLGR). Two HLGR strains were identified as *E. faecalis* and the other two as *E. faecium*. In addition, resistant strains were identified in both healthy and sick birds.

A total of two isolates of *E. faecalis* and *E. hirae* showed intermediate levels of resistance to vancomycin (Table 2). The intrinsic resistance of *E. gallinarum* was disregarded. No concomitant resistance among gentamicin high level and vancomycin was found among these isolates.

DISCUSSION

The results of our study showed that 26.9% of 126 sampled birds presented *Enterococcus* spp. However, while the healthy birds presented a frequency of 22.01% of *Enterococcus* spp., the sick birds presented 70.58%. The species identified were *E. faecalis*, *E. hirae*, *E. faecium*, *E. phoeniculicola*, *E. gallinarum* and *E. casseliflavus* (Table 1). In healthy birds, the most frequently isolated species in our study was *E. hirae* (10.09%). *E. hirae* has been described as microbiota of asymptomatic parrots as well as associated with disease in parrots; however, it seems to be rarely isolated from humans (Devriese et al. 1995, Freitas et al. 2018). The most prevalent species among symptomatic individuals in our study were *E. faecalis* and *E. faecium* (Table 1). In avian species, it has been shown that *E. faecium* can be a cause of septicemia and that *Enterococcus faecalis* can also be associated with amyloid arthropathy (Shivaprasad 2014). Here, we report a frequency of 35.2% for

E. faecalis and 11.7% for *E. faecium* in psittacine birds with respiratory disease, and 8.25% for *E. faecalis* and 2.75% for *E. faecium* from asymptomatic birds.

It is now suggested that *E. faecium* from animal origin are less likely to be transferred to humans, but they may still represent a risk due to the transfer of resistance genes to virulent enterococci while *E. faecalis* is a more capable zoonotic agent with the same clones being recovered from human and animal sources (Hammerum et al. 2010). *E. faecalis* has also been strongly suggested as a zoonotic agent that can cause urinary-tract infections in humans either by having close contact working with pig, poultry or by ingestion of pork or poultry meat (Larsen et al. 2010, Poulsen et al. 2012, Abat et al. 2016).

De Leener et al. (2005) studied enterococci isolated from dogs and cats and determined that HLGR was present in rectal swab samples besides resistance to several other antibiotics (De Leener et al. 2005). The authors concluded that the enterococci present in the intestinal microbiota of pets might act as a reservoir of resistance genes for humans and animals alike (De Leener et al. 2005). In dogs, Simjee et al. (2002) identified a high prevalence of HLGR *E. faecium* (54%) and a sole vancomycin-resistant *E. faecium* strain (Simjee et al. 2002).

Data on the occurrence and epidemiology of HLGR in captive psittacine pet birds are very scarce. Freitas et al. (2018) reported a survey with 88 captive blue-fronted parrots (*Amazona aestiva*) and highlight the high proportion (48%) of multidrug-resistant enterococci, but only one strain of *E. faecalis* were classified as HLGR (Freitas et al. 2018). Our results showed that four strains (*E. faecalis*/*E. faecium*) were classified as HLGR (Table 2). HLGR is frequently found in association with the bifunctional aminoglycoside modifying enzyme AAC6'-APH2" (Hammerum et al. 2010, Arias & Murray 2012). The combined use of gentamicin with β -lactam antibiotics or glycopeptides is of high importance for treating severe enterococci infections in humans. Still, this synergistic effect is lost if high-level aminoglycoside resistance is present. In the present study, we report 11.1% of the *Enterococcus* isolates presented resistance to high level gentamicin (120 μ g), which is of concern regarding the zoonotic potential of these isolates. Aminoglycosides are often used in specific conditions of pet birds, in particularly infections of the respiratory tract that are difficult to eradicate. Therefore, it is imperative to carefully consider this type of resistance when deciding to prescribe aminoglycosides treatments in companion birds (van den Bogaard & Stobberingh 2000, Arias & Murray 2012).

The hypothesis that animals could also be sources of zoonotic VRE has been explored in several studies (Woodford et al. 1998, Stobberingh et al. 1999, van den Bogaard & Stobberingh, 2000, Gülhan et al. 2006, Arias & Murray 2012, Simjee et al. 2002). Concerns for its use as a feed additive in livestock

Table 2. Antimicrobial resistance of *Enterococcus* spp. isolated from pet parrots in São Paulo, Brazil, 2018

Birds	<i>Enterococcus</i> species	High level gentamicin	Vancomycin
<i>Amazona amazonica</i>	<i>E. hirae</i>		Intermediary
<i>Anodorhynchus hyacinthinus</i>	<i>E. faecalis</i>	Resistant	
<i>A. hyacinthinus</i>	<i>E. faecalis</i>		Intermediary
<i>Nymphicus hollandicus</i>	<i>E. faecalis</i>	Resistant	
<i>N. hollandicus</i>	<i>E. faecium</i>	Resistant	
<i>Pionus maximiliani</i>	<i>E. faecium</i>	Resistant	

leading to cross-resistance between avoparcin and an analogue vancomycin resulted in the ban of its use in agriculture. These measures were organized by the European Union and followed by several countries around the world, including Brazil, after the first report of VRE in 1998 (Hammerum et al. 2010). Unlike several countries around the world, the USA has never allowed the use of avoparcin in livestock and the observed resistance to vancomycin in that country was in the past related almost exclusively to hospital settings (Guardabassi et al. 2004). However, a shift has been noticed and several studies in the USA have also shown the presence of VRE in migrating and synanthropic birds as well as in pet dogs and cats. These reports suggest that the acquisition of resistant enterococci in non-livestock sources might be linked to human sources either as environmental pollution or due to direct close contact (Guardabassi et al. 2004, Gülhan et al. 2006, Roberts et al. 2016). Another possible cause for the presence of pets carrying vancomycin resistance is the co-selection of VRE due to the use of other antimicrobials such as tetracyclines, macrolides and aminoglycosides. Indeed, VRE isolates in pets are also frequently found showing resistance to these antibiotics classes (Guardabassi et al. 2004). In the current study, we have not identified VRE, but one strain of *E. faecalis* and one of *E. hirae* presented intermediate resistance to this antibiotic (Table 2).

There are no other studies at present showing the contribution of pet birds as reservoirs for resistant *Enterococcus* spp. and this work shows the possible role that these animals may have as important, yet underrated, infection sources in the domestic environment.

CONCLUSIONS

We describe in this study that 26% of companion birds were colonized by *Enterococcus* spp., with *E. faecalis* and *E. faecium* being most frequently detected in sick birds.

High levels of gentamicin resistance isolates (4/36) were detected in sick and apparently healthy birds. Intermediary resistance to vancomycin was found among two isolates (2/36).

The data here presented show the potential that pet birds have as possible reservoirs for resistant gentamicin *Enterococcus* spp., which is of grave concern regarding the maintenance of these birds as companion animals, although the risks regarding the capacity to transmit these isolates and/or of their mobile genetic material remains to be further evaluated.

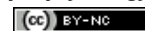
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Uterine secretory activity and histology of prepubertal female dogs in an animal model of partial ablation of endometrial glands development¹

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ABSTRACT- Martins B.B., Teixeira N.S., Teixeira S.A.M.V., Giannotti J.G., Nunes L.C., Freitas P.M.C., Machado-Neves M. & Luz M.R. 2020. **Uterine secretory activity and histology of prepubertal female dogs in an animal model of partial ablation of endometrial glands development.** *Pesquisa Veterinária Brasileira* 40(2):134-140. Setor de Reprodução Animal, Departamento de Clínica e Cirurgia Veterinárias, Escola de Veterinária, Universidade Federal de Minas Gerais, Campus Pampulha, Av. Antonio Carlos 6627, Cx. Postal 567, Belo Horizonte, MG 31270-901, Brazil. E-mail: luzmr@uol.com.br

Inadequate exposure of the female reproductive system to steroids in uterine developmental periods can partially inhibit the development of endometrial glands in dogs. However, the effects of steroids on the formed glands functionality remain unknown, as well as the possible occurrence of endometrial fibrosis. This study aimed to evaluate the secretory activity of endometrial glands in prepubertal female dogs submitted to a protocol of partial ablation of the uterine adenogenesis. Sixteen females of non-specific breed were distributed into two groups; MPA (n=8), females that received applications of medroxyprogesterone acetate every 3 weeks; and C (n=8) untreated control females. Ovariohysterectomy was performed in all animals at the age of 6 months and evaluated the uterine horns by histological and histochemistry exams. The secretion intensity (degrees 1-4) was evaluated using periodic acid-Schiff (PAS) and alcian blue (AB) pH 2.5. Histological evaluation was performed using Masson's trichrome and toluidine blue. Only degree 1 and 2 marks for PAS were observed in both groups, with no difference of uterine secretion intensity between the groups regarding the degrees found. However, the MPA group revealed higher intensity of uterine secretion compared to group C ($p < 0.05$). Staining with AB pH 2.5 also revealed only degree 1 and 2 marks in both groups, with no statistically significance between them. Masson's trichrome staining revealed no marks in the periglandular region in both groups. A higher amount of mast cells was observed in the myometrial region of the uterus in both groups. Prepubertal female dogs with partial ablation of the uterine adenogenesis present minimal uterine secretory activity, absence of periglandular fibrosis and increased presence of mast cells in the myometrium compared to endometrium.

INDEX TERMS: Uterus, secretion, histology, prepubertal, dogs, partial ablation, endometrial glands, development, medroxyprogesterone acetate, glandular ablation, bitch, morphology.

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RESUMO.- [Atividade secretória uterina e histologia de cadelas pré-púberes em um modelo animal de ablação parcial do desenvolvimento das glândulas uterinas.]

A exposição inadequada do sistema reprodutor feminino a esteróides em períodos do desenvolvimento uterino pode inibir parcialmente o desenvolvimento das glândulas endometriais em cães. Entretanto, não se conhece os efeitos dos esteróides sobre a funcionalidade das glândulas formadas, bem como a possível ocorrência de fibrose endometrial. Objetivou-se avaliar a atividade secretória das glândulas endometriais de cadelas pré-púberes submetidas a protocolo de ablação parcial da adenogênese uterina. Foram utilizadas 16 fêmeas, sem-raça-definida, distribuídas nos grupos MPA (n=8), fêmeas que receberam aplicações de acetato de medroxiprogesterona a cada 3 semanas, e C (n=8), fêmeas controle não tratadas. Aos seis meses de idade, foi realizada ovariectomia em todos os animais, e avaliados os cornos uterinos pelo exame histológico e de histoquímica. Para avaliar a intensidade de secreção (graus 1-4), foram utilizadas periodic acid-Schiff e alcian blue (AB) pH 2,5. Para a avaliação histológica foram utilizados tricrômico de Masson e azul de toluidina. Apenas marcações graus 1 e 2 foram observadas para PAS em ambos os grupos, sem diferença na intensidade de secreção uterina entre grupos com relação aos graus encontrados. Entretanto, o grupo MPA apresentou maior intensidade de secreção uterina em relação ao grupo C ($p<0,05$). Com relação ao AB pH 2,5, em ambos os grupos também foram encontradas apenas marcações de graus 1 e 2, sem diferença estatística entre grupos. Não foram observadas marcações para a coloração de tricrômico de Masson na região periglandular, em ambos os grupos. Foi observada maior quantidade de mastócitos presentes no útero na região do miométrio, em ambos os grupos. Conclui-se que cadelas pré-púberes com ablação parcial da adenogênese uterina apresentam mínima atividade secretória uterina, ausência de fibrose periglandular e maior presença de mastócitos no miométrio em relação ao endométrio.

TERMOS DE INDEXAÇÃO: Útero, secreção, histologia, cadelas, pré-púberes, modelo animal, ablação parcial, desenvolvimento, glândula uterina, acetato de medroxiprogesterona, ablação glandular, cadela, morfologia.

INTRODUCTION

Endometrial glands are structures present in the uterus of all female mammals. Their function is to synthesize, secrete and transport a complex mixture of substances necessary for the embryonic development and maintenance before placentation (Gray et al. 2001). Mucus is a secretion composed mainly of mucin, as well as ions, water and antimicrobial compounds, secreted by several cells and epithelial glands, specialized in maintaining the mucous surface humid (Pluta et al. 2011). It has an important role in the reproductive function and defense of the reproductive system. It undergoes biochemical and physical changes according to the plasma concentration of estrogens and progesterone (Wolf et al. 1978).

Several animal species that received systemic steroids in the first weeks or months of life present reduced reproductive efficiency or even sterility (Gray et al. 2001), which could represent an applicable method to dog population control. Sheep exposed to progestins in the prepubertal period presented ablation of the endometrial glands formation, exhibiting a phenotype called "uterine gland-knockout" (UGKO) (Bartol et al. 1997). In bovines, newborn females treated with

implants of progesterone and estradiol benzoate, from birth to puberty, presented with reduced myometrial and endometrial area, in addition to partial ablation of the endometrial glands' development, represented by different degrees of endometrial gland density decrease (Bartol et al. 1995).

In canines, the development of endometrial glands is a post-natal event that usually occurs between birth and the 60th day of life (Cooke et al. 2012). Similarly, to the described for bovines (Bartol et al. 1995), female dogs treated with medroxyprogesterone acetate from birth to six months of age presented with partial ablation of the endometrial glands development (Teixeira et al. 2018). However, there is no description in the literature on the secretory activity of the endometrial glands formed in animals with partial ablation.

This study aimed to evaluate the secretory activity of endometrial glands formed in female dogs submitted to serial applications of medroxyprogesterone acetate from birth to six months of age.

MATERIALS AND METHODS

Ethics statement. All procedures were performed in accordance with the principles from the Brazilian Colleges of Animal Experiments (COBEA), and at the end of the experiments all animals were donated for adoption.

Animal houses and study design. Sixteen female puppies, with no defined breed, from 5 different litters were used. A total of three or four puppies from each litter were used. Each newborn was randomly assigned to one of two groups on the day of birth: Medroxyprogesterone acetate (MPA) group (Promone-E, Pfizer, Brazil) (n=8), which received subcutaneous (SC) application of MPA (10mg/kg) in intervals of three weeks, since birth to 6 months of age, for induction of partial ablation of uterine adenogenesis (Teixeira et al. 2018); and Control Group (C) (n=8), composed by animals that received application of NaCl 0.9% solution, similarly to the MPA group. Animals were kept in collective kennels with access to water and food *ad libitum*.

Uterine sampling. Samples were collected by ovariectomy from both uterine horns of all female dogs, 6 months after the beginning of experiments. Female dogs were submitted to 12 hours solid fasting and 6 hours liquid fasting before OHS. The anesthetic protocol used included 0.1mg/kg/IM acepromazine (Acepran 0.2%; Vetnil, Brazil), 5.0mg/kg/IV propofol (Propovan; Cristália, Brazil). After induction, animals were kept with inhaled anesthesia using isoflurane (Forane, Baxter, USA).

Histology and histochemistry. Fragments of 0.5cm of the uterine horns medial third were fixed in 10% buffered paraformaldehyde and histologically processed to paraffin inclusion (Luna 1968).

Paraffin blocks containing the fragments were cut in microtome (EDGE SL-200) to obtain histological sections of 3µm thickness. These were placed in histological slides and stained with different dyes, according to the following description.

Histochemical description was performed using periodic acid-Schiff (PAS) test (Tolosa et al. 2003) to characterize the organ and glands for secretion of neutral polysaccharides (mucins); and alcian blue (AB) pH 2.5 (Bancroft & Cook 1994), to verify the presence of acidic polysaccharides (mucins).

Histological evaluation of the uterine wall used Masson's trichrome (Luna 1968) to show collagen and muscular fibers; and toluidine blue (Tolosa et al. 2003) to identify metachromatic cells (mast cells).

Histological slides were incubated in periodic acid 0.5% for 25 minutes and washed with distilled water. They were then kept in cuvette covered with aluminum foil at room temperature, immersed in reactive of Schiff for 15 minutes, and then washed in running water

for 5 minutes, and in distilled water for 1 minute. Counter-staining was performed with hematoxylin for 30 seconds, followed by a quick wash in running water (Tolosa et al. 2003).

Slides were first washed in 3% glacial acetic acid solution for 5 minutes and incubated in 1% AB, pH 2.5, in 3% glacial acetic acid solution for 40 minutes at 40°C. The material was washed in the same buffer solution, and then washed in distilled water. Counter-staining was performed with fast red 0.5% for 2 minutes and, later, the slides were washed with distilled water (Bancroft & Cook 1994).

Slides remained submersed in Bouin solution, on 60°C oven for one hour, followed by cooling in room temperature. This was followed by washing in running water for 15 minutes and quickly rinsed in distilled water. After this step, the slides were positioned in a staining support and the dyes were applied in the following order: ferrous hematoxylin for 5 minutes, washed in running water for 5 minutes and then distilled water; fuchsin acid with ponceau for 10 minutes, washed in running water for 1 minute and then distilled water; phosphomolybdic acid 5% for 5 minutes until the cuts were whitening, washed in running water and quickly in distilled water; aniline blue 0.5% for 5 minutes, washed in running water and quickly in distilled water, acetic acid for 1 minute (Luna 1968).

Histological slides remained 2 minutes in toluidine blue 1% and were washed in running water (Tolosa et al. 2003).

Three uterine horn cuts were selected for histochemical techniques with PAS and AB pH 2.5; these were submitted to evaluation in light microscopy to identify the presence or absence of staining by the chemicals determined by each technique, using optical microscope

(Olympus BX 50) with 20x and 40x objectives. When staining was detected, the secretion analysis was evaluated according to the following classification: degree 1 - absence of staining of the secretory cells; degree 2 - slight staining of the secretory cells, or minimal amounts in the lumen; degree 3 - moderate staining of the secretory cells, or moderate amounts in the lumen; degree 4 - intense staining of the secretory cells, or intense amounts in the lumen.

The histological evaluation with Masson's trichrome was performed in light microscopy and identified the presence or absence of staining. The presence of mast cells in the uterus was identified by the characteristic metachromatic staining of the secretory granules, stained by toluidine blue. Endometrium and myometrium were used as the tissue compartments to evaluate the distribution of mast cells. Five fields per histological sample were used to determine the numeric distribution of mast cells/mm².

Statistics. The analysis of the intensity of different staining was performed using the non-parametric test of Wilcoxon in independent samples. Data analysis was performed using R (R Development Core Team 2011). Significance level of $p \leq 0.05$ was adopted.

RESULTS AND DISCUSSION

Glandular fibrosis

There were no stainings for Masson's trichrome in the periglandular region in both groups, which shows that the chronic treatment with MPA did not cause periglandular fibrosis in the female dogs' uterus (Fig.1).

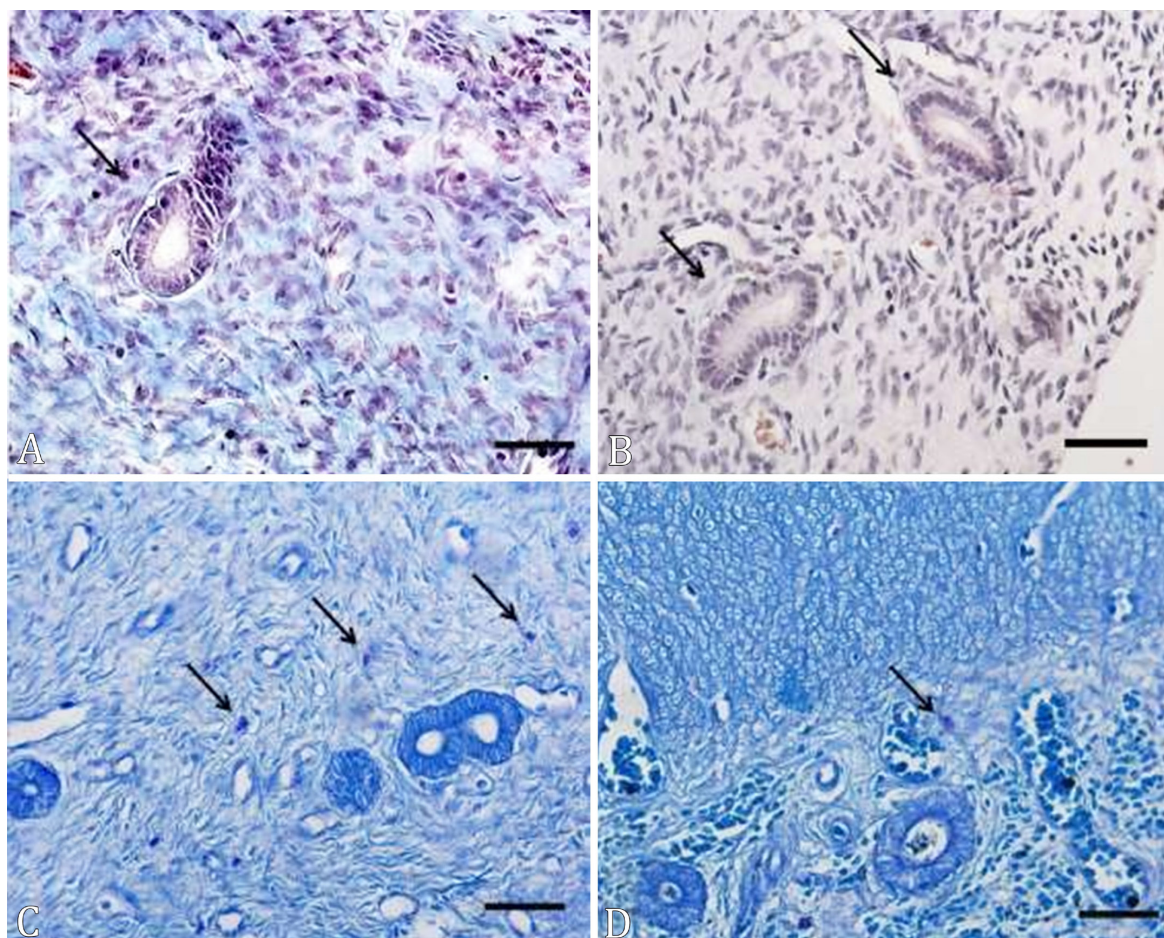


Fig.1. (A-D) Cross-sections of the uterus of prepubertal female dogs treated with medroxyprogesterone acetate, from birth to 6 months of age. (A) Group C, (B) group MPA; mast cells (arrows). MT, bar = 20μm.

Fibrosis is one of the main elements of tissue reaction; therefore, it is important to determine its conformation, localization and composition in order to evaluate how the endometrium is affected. In these lesions, the collagen deposition occurs more commonly around the glands, or associated to the basal membrane (Kenney & Doig 1986). This alteration compromises the integrity and function of the endometrial glands, which are structures necessary since the pre-implantation period until the complete placental development. Fibrotic glands have irregular epithelium, in addition to changes to the glandular secretion (Walter et al. 2001). High doses of medroxyprogesterone acetate or progesterone or repeated or prolonged exposure in adult female dogs lead to endometrial cystic hyperplasia, with proliferation of endometrial glands and cystic dilation of the same, accumulating fluid in the uterine lumen; fibrosis is a frequent finding (Dow 1959). Thus, the absence of periglandular fibrosis in the present study indicates that the chronic treatment with MPA in prepubertal female dogs possibly do not compromise the fertility of these animals in the adult life regarding the presence or absence of fibrosis. Canine endometrium goes through two phases of development and differentiation; one at the end of anestrus, which is associated with increase of circulating estrogens; and other in the middle of estrus and first week of diestrus, which is associated with estrogens and to luteal progesterone (Galabova et al. 2003). However, according to Van Cruchten et al. (2004), the patterns of endometrial proliferation in cyclical female dogs and its relationship to the concentration of steroid receptors remain unknown. Possibly, this relationship is also unknown in prepubertal female dogs, even though prepubertal female dogs show expression of receptors for progesterone and estradiol in their uterus (Cooke et al. 2012).

Endometrial cystic hiperplasia

Low vessel density could be subjectively and macroscopically observed in the endometrium of the bitches immediately after ovariectomy. This pattern of vascularization was similar in all experimental bitches. In addition, none of the uterus examined presented any luminal content.

No female dog, in both groups, showed endometrial cystic hyperplasia (ECH) and presence of content in the uterus lumen, regardless of the 6 months long chronic treatment with MPA. These results differ from the found by Dhaliwal et al. (1999), and Kooistra et al. (1997), who verified the occurrence of ECH in adult female dogs treated for 5 or 13 weeks with the same protocol used in the present study. In cyclic adult female dogs, ECH is an abnormal finding; however frequent in the uterus, being more frequent in older dogs, whose uterus was exposed to repeated and chronic progesterone stimuli by the occurrence of several estrous cycles (De Bosschere et al. 2001). The absence of ECH in female dogs in this study is probably due to the immaturity of the dog's reproductive system, as they were still growing and developing, not presenting endometrial glands totally developed and responsive to progestins, although they followed by long-term progesterone treatment with high doses of MPA and at short intervals, and also because at this stage they already have receptor for progesterone and estradiol in the uterus (Cooke et al. 2012). Another possibility is that the absence of endometrial cystic hyperplasia could have been due to the lack of estradiol priming (Dhaliwal et al. 1999), since the animals were prepubertal.

Mast cells

A higher amount of mast cells in the myometrial region of the uterus was observed in each group compared to the endometrial region (Table 1). However, there was a difference between the groups regarding the amount of mast cells in the myometrium, but not endometrium. In addition, the number of mast cells in the uterus was higher in the animals of the MPA group compared to the C group (Table 1, Fig.1). The mast cells found in the endometrium were observed in the stroma adjacent to the endometrial glands, but they were not present in the epithelium. These were found in the interior of the myometrium, between muscle bundles and around the blood vessels.

Other researchers have reported the presence of mast cells in the uterus of mammals (Brandon & Evans 1983, Drudy et al. 1991). Similarly, to the observed in this study, studies have observed a smaller amount of mast cells in the endometrium when compared to the myometrium of rats and goats (Karaca et al. 2007, 2009). Moreover, Milne et al. (2001) showed that progestogen treated human myometrium has higher number of mast cells when compared to the endometrium, but the authors did not observe significant difference in the amount of mast cells 24 and 48 hours after removing the progestogen.

A study showed that the presence of mast cells in the uterus of female dogs is significantly related to the plasma concentration of progesterone and with the different phases of the estrous cycle (Goericke-Pesch et al. 2010). In addition, some researchers also observed an increased number of mast cells during the progestational phase of the cycle and a reduction due to the action of estrogens (Maraspin & Bo 1971). In fact, among the known functions of the mast cells present in the uterus, is the high release of histamine during the period of embryonic implantation (Bath & Parshad 2000). This occurs in a period of the reproductive cycle in which the plasma concentrations are high, justifying the high amount of mast cells observed in the female dog of our study. Even though they were prepubertal dogs, their uterus was impregnated by MPA, which has a long half-life of 24 hours, and strong progestational activity (Romagnoli & Sontas 2010).

Uterine secretion

Both groups had only staining of degree 1 (absent) and 2 (slight) for PAS, and there was no difference of intensity of the uterine secretion between the groups at the degree found (Fig.2). However, the MPA group revealed higher intensity of uterine secretion (degree 2) compared to group C ($p \leq 0.05$). Staining with AB pH 2.5 also revealed only degree 1 and 2 marks in

Table 1. Distribution of mast cells/mm² in the myometrium and endometrium of prepubertal female dogs, treated with medroxyprogesterone acetate from birth to six months of age, by toluidine blue staining

Group	Myometrium	Endometrium	Uterus
MPA	2.5 aA	0.2 bA	3.2 A
C	1.4 aB	0.0 bA	1.6 B

a,b = Medians followed by lower case letters distinct in the same row differ between uterus regions by the Wilcoxon test ($p < 0.05$), A,B = medians followed by capital letters distinct in the same column differ between groups by the Wilcoxon test ($p < 0.05$).

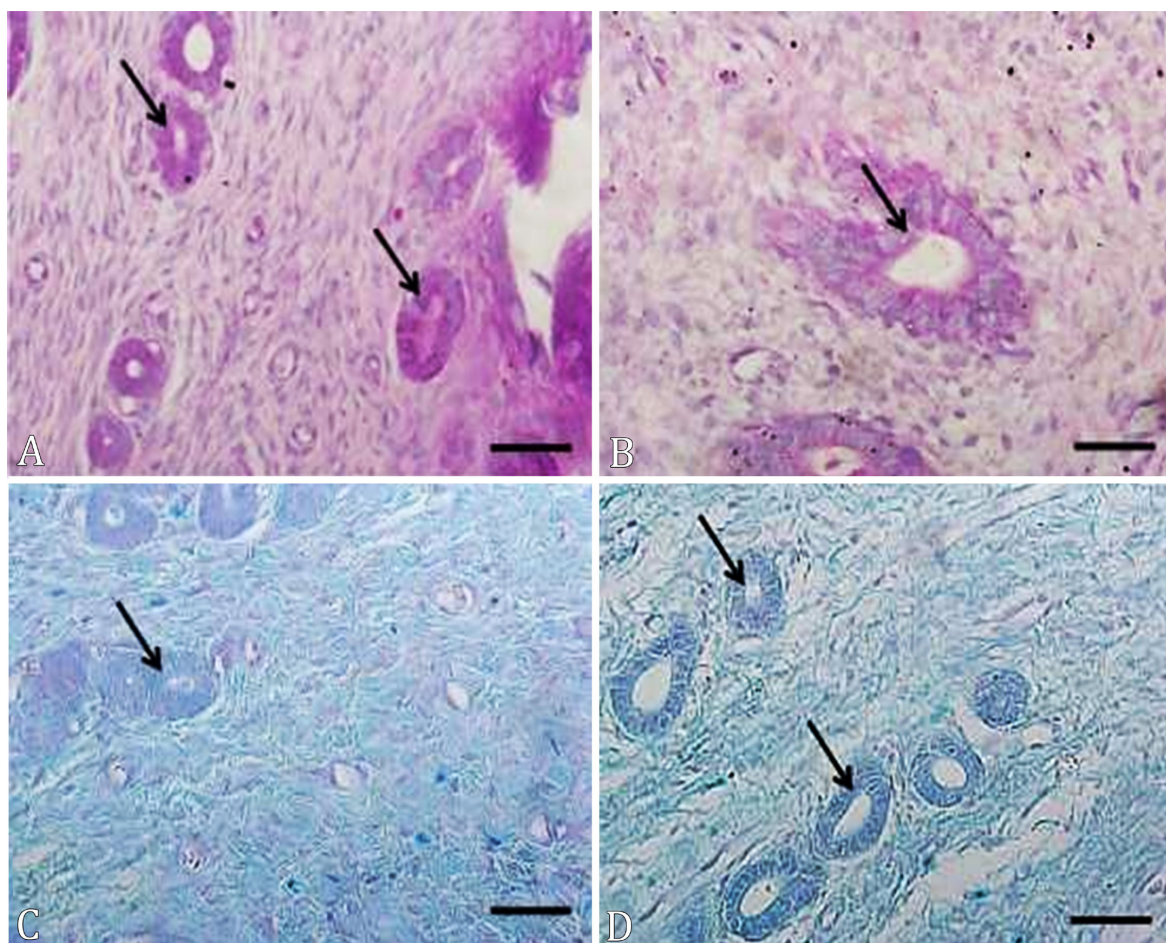


Fig.2. (A-D) Photomicrographs of the cross-sections of the uterus of prepubertal female dogs' treated with medroxyprogesterone acetate, from birth to 6 months of age. (A,C) MPA group, (B,D) group C. Arrows indicate the intensity of secretion in the endometrial glands. (A-B) PAS, bar = 20μm. (C-D) AB pH 2.5, bar = 20μm.

Table 2. Staining intensity medians (degrees 1 and 2) in the uterus of prepubertal female dogs treated with medroxyprogesterone acetate from birth to six months of age, by PAS and AB pH 2.5 staining

Group		PAS		AB pH 2.5	
Degree 1	Degree 2	Degree 1	Degree 2	Degree 1	Degree 2
MPA	0.0 Aa	2.0 Ab	0.5 Aa	1.0 Aa	2.0 Aa
C	0.5 Aa	1.0 Aa	0.0 Aa	0.0 Aa	0.0 Aa

a,b = Medians followed by lower case letters distinct in the column differ among themselves by the Wilcoxon test ($p < 0.05$), A,B = medians followed by lower case letters distinct in the column differ among themselves by the Wilcoxon test ($p < 0.05$).

both groups, with no statistically significance between them regarding the uterine secretion ($p \leq 0.05$) (Table 2, Fig.2).

Physiologically, under the progesterone stimuli, the endometrium undergoes hypertrophy due to the increase in number and size of its glands, which increase the secretory activity, leading to the accumulation of sterile fluid in the uterus lumen. These responses to hormonal actions can be exacerbated with the use of exogenous progesterone (De Bosschere et al. 2001, Pretzer 2008), such as medroxyprogesterone acetate, a progestogen with actions like progesterone. Female dogs from group MPA, after 24 months of treatment, showed higher intensity of uterine secretion (degree 2) by PAS, when compared to group C ($p < 0.05$); probably due to the response of the progesterone receptors present in the endometrial glands (Cooke et al. 2012). Even

after long-term treatment with MPA, progesterone receptors possibly escaped the down-regulation caused by the excess of circulating hormone, as described by De Bosschere et al. (2002a, 2002b) for adult female dogs. On the other hand, the absence of degrees 3 and 4 in the uterine secretion can be justified by the animals' age, prepubertal, and at the beginning of body development. Dogs from both groups were equally stained by AB pH 2.5.

In contrast, the low intensities of uterine secretion (only degrees 1 and 2) could reflect a picture of subfertility or even sterility in the adult female, in which the total ablation of development of the glands could be explored as an efficient method of population control of dogs. Thus, new protocols for glandular ablation and function impairment should be investigated.

CONCLUSION

The long-term exogenous progesterone treatment of prepubertal bitches in a canine model of partial ablation of endometrial glands did not cause periglandular fibrosis or endometrial cystic hyperplasia, increased presence of mast cells in the myometrium compared to endometrium, and also the uterine secretion, even in low degrees. If these findings could reflect alterations in the bitch's fertility remains to be elucidated.

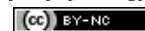
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Conflict of interest statement.- The authors declare that there is no conflict of interest.

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Reproductive biology, morphology, and morphometry of ovaries and oviducts of *Trachemys scripta elegans* in Brazilian Cerrado¹

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ABSTRACT. Gradela A., Pires I.C., Matos M.H.T., Corrêa F.M., Faria M.D., Silva J.S., Torres L.R.C. & Milanelo L. 2020. **Reproductive biology, morphology, and morphometry of ovaries and oviducts of *Trachemys scripta elegans* in Brazilian Cerrado.** *Pesquisa Veterinária Brasileira* 40(2):141-154. Laboratório de Anatomia dos Animais Domésticos e Silvestres, Colegiado de Medicina Veterinária, Fundação Universidade Federal do Vale do São Francisco, Rodovia 407 Km 12, Lote 543, Projeto Nilo Coelho C1, Petrolina, PE 56300-000, Brazil. E-mail: agradela@hotmail.com

Although *Trachemys scripta elegans* is an exotic species popular as a pet in Brazil, studies on reproductive biology and capacity are non-existent in the Brazilian Cerrado. This study analyzed ovarian and oviduct characteristics and the egg production capacity of *T. scripta elegans* grown in this biome. The findings will associate with the size of the specimens and the sexual maturity, aiming at comparisons with native and exotic populations, as well as interspecific and contributing to the understanding of its impact on the invaded ecosystems and the establishment of eradication programs. Thus, 39 females had evaluated the body biometry and the morphology and morphometry of the ovaries and oviducts. G2 (N=20): with Class I (>5-10mm) follicles, with Class I and Class II (>10-fold) follicles, 25mm) and G3 (N=9) with Class I, Class II and Class III (>25mm) follicles. Analysis of variance, Scott-Knott's test, and Pearson's correlation analysis showed that there was no significant difference between the groups in body biometry; in the mean gonadosomatic index and gonadal morphometry, only the width of the oviducts in the right antimer and the mass and width in the left antimer were higher in G3, the only one that presented eggs. There was positive and harmonic development between body mass, carapace, and plastron, and gonadal growth occurred concomitantly with body growth, indicating a higher reproductive potential and a positive relationship between the size of the litter and the female litter. The gonadosomatic index proved to be an excellent reproductive indicator, and the ovarian evaluation was a better indicator of sexual maturity than the maximum carapace length. Ovaries were irregular structures, without delimitation between the cortical and medullary regions and filled with vitelogenic follicles of different diameters, atresic follicles, and corpora lutea, which reflected the ovarian complexity of the species and the presence of follicular hierarchy. In the scarce stroma, two germinative beds were observed per ovary and the presence of gaps very close to the follicles and associated with the blood vessels. Analysis of gonadal tissue revealed three types of oocytes according to cytoplasmic characteristics: homogeneous, vesicular or vesicular in the cortex with apparent granules. Oviducts were functional and separated, joining only in the final portion to form the cloaca and subdivided into infundibulum, tuba, isthmus, uterus, and vagina. The structure of the uterine tube was composed of serosa, muscular and mucous, which was full of glands. The presence of eggs in the oviducts indicated

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that the specimens can reproduce in the Brazilian Cerrado. This study provides necessary and relevant information on the reproductive biology and capacity of *T. scripta elegans* in the Brazilian Cerrado and can contribute to the understanding of its impact on the invaded ecosystems and the establishment of eradication programs. The extraction of females with capacity can reduce the annual reproductive yield of the species and decrease its effect on local biodiversity.

INDEX TERMS: Reproduction, biology, morphology, morphometry, ovaries, oviducts, *Trachemys scripta elegans*, Brazilian Cerrado, Testudines, uterine tube, ovary, follicles, *corpus luteum*.

RESUMO.- [Biologia reprodutiva, morfologia e morfometriade ovários e ovidutos de *Trachemys scripta elegans* no Cerrado Brasileiro.]

Embora *Trachemys scripta elegans* seja uma espécie exótica popular como animal de estimação no Brasil, estudos sobre biologia e capacidade reprodutivas são inexistentes no Cerrado brasileiro. Este estudo analisou características ovarianas e do oviduto e a capacidade de produção de ovos em *T. scripta elegans* criadas neste bioma, correlacionando estes achados ao tamanho dos espécimes e a maturidade sexual, visando comparações com populações nativas e exóticas, bem como interespecíficas e contribuir para a compreensão de seu impacto nos ecossistemas invadidos e com o estabelecimento de programas de erradicação. Assim, 39 fêmeas tiveram avaliadas a biometria corporal e a morfologia e morfometria dos ovários e ovidutos. De acordo com o tamanho dos folículos ovarianos as fêmeas foram separadas em G1 (N= 10): com folículos Classe I (>5-10 mm), G2 (N= 20): com folículos Classe I e Classe II (>10-25 mm) e G3 (N= 9) com folículos Classe I, Classe II e Classe III (>25 mm). À análise de variância, teste de Scott-Knott e à análise de correlação de Pearson verificou-se que não houve diferença significativa entre os grupos na biometria corporal; no índice gonadossomático médio e na morfometria gonadal, apenas a largura dos ovidutos no antímero direito e a massa e a largura no antímero esquerdo foram maiores no G3, o único que apresentou ovos. Houve desenvolvimento positivo e harmônico entre massa corporal, carapaça e plastrão e o crescimento gonadal ocorreu concomitante ao crescimento corporal, indicando maior potencial reprodutivo e relação positiva entre o tamanho da ninhada de ovos e o da fêmea. O índice gonadossomático mostrou-se um bom indicador reprodutivo e a avaliação ovariana um melhor indicador da maturidade sexual que o comprimento máximo da carapaça. Ovários foram estruturas irregulares, sem delimitação entre a região cortical e medular e repletos de folículos vitelogênicos de diferentes diâmetros, folículos atresícos e corpos lúteos, que refletiram a complexidade ovariana da espécie e a presença de hierarquia folicular. No estroma escasso foram observados dois leitos germinativos por ovário e a presença de lacunas muito próximas aos folículos e associadas aos vasos sanguíneos. A análise do tecido gonadal revelou três tipos de oócitos de acordo com as características do citoplasma: homogêneo, vesicular ou vesicular no córtex com grânulos aparentes. Ovidutos eram funcionais e separados, unindo-se apenas na porção final para formar a cloaca e subdividiu-se em infundíbulo, tuba uterina, istmo, útero e vagina. A estrutura da tuba uterina era constituída de serosa, muscular e mucosa, a qual era repleta de glândulas. A presença de ovos nos ovidutos indicou que os espécimes podem se reproduzir no cerrado brasileiro. Este estudo fornece informações básicas e relevantes da biologia e capacidade reprodutivas de *T. scripta elegans* no Cerrado

brasileiro e pode contribuir com a compreensão de seu impacto nos ecossistemas invadidos e com o estabelecimento de programas de erradicação, uma vez que a extração de fêmeas com capacidade reprodutiva pode contribuir com a diminuição do rendimento reprodutivo anual da espécie e diminuir seu efeito sobre a biodiversidade local.

TERMOS DE INDEXAÇÃO: Biologia, reprodução, morfologia, morfometriade, ovários, ovidutos, *Trachemys scripta elegans*, Cerrado Brasileiro, Testudines, tuba uterina, ovário, folículos, corpo lúteo.

INTRODUCTION

The introduction of exotic species is one of the reasons for the extinction of half of the other species of turtles (TCF 2002), representing a severe threat to the maintenance of biodiversity (Strayer et al. 2006, Ricciardi 2007, Ficetola et al. 2009). The impact of the species on the native biodiversity has been observed with *Trachemys scripta elegans* (Wied, 1839), an underwater turtle from North America (Ernst & Barbour 1989), very popular as pet worldwide (Telecky 2001, Crescente et al. 2014). Its popularity is due to small size, simple breeding requirements, and affordable price. However, when adult, they may acquire a considerable size (up to 30 cm carapace length) and can survive for up to 50 years in captivity (Teillac-Deschamps et al. 2008), which may result in their abandonment by breeders (Lever 2003, Martinez-Silvestre et al. 2003, Teillac-Deschamps et al. 2008). As it presents earlier maturity and higher fecundity than most other species when establishing themselves in new habitats, they compete with native chelonians (Perez-Santigosa et al., 2008) and threaten local biodiversity (Fonseca 2001, Buhlmann et al. 2009). In some areas where they are introduced, *T. scripta elegans* are unable to reproduce due to low temperatures or limited precipitation (Bringsøe 2001, Ficetola et al. 2009), with few reports of naturalized populations (Pleguezuelos 2002, Lever 2003, Cadi et al. 2004, Ramsay et al. 2007, Kikillus et al. 2010).

In Quelonians the studies about morphology of the ovary and uterine tubes (O'Malley 2005, Machado Junior et al. 2006, Hildebrand & Goslow 2006, Cabral et al. 2011, Silva et al. 2011, Chaves et al. 2012, Firmiano et al. 2012), particularly the biometrics of the somatometric and intern. The available studies, mostly, focus on the description of breeding behavior and nesting (Firmiano et al. 2012) and the few existing ones emphasize the morphofunctional aspects of gonads in females (Ferreira & Dolder 2003, Ceriani & Wyneken 2008, Peixoto et al. 2012, Souza et al. 2014). However, the study of reproductive organs and ovarian follicular development and egg production are essential to understanding the adaptive and evolutionary aspects involved in their differentiation, as well as the establishment of strategies for species management,

conservation and eradication (Newman et al. 2003, Pessoa et al. 2008, Pérez-Bermúdez et al. 2012).

In marine turtles, morphophysiological alterations of the reproductive tract are observed as a function of age and the reproductive and non-reproductive season. A pair of ovaries composes the reproductive system; a pair of oviducts; suspensory ligaments (mesovary, mesosalpinx, and mesotubaria) and clitoris (Wyneken 2001). The oviduct begins in the region adjacent to the ovary and extends to the sewer, presenting five regions: infundibulum, tubal uterine or magno, isthmus, uterus, and vagina (gross muscle region) (Girling 2002). Ovulated follicles form the luteal bodies that, after ceasing the progesterone production, become albicans, similarly to domestic mammals (Hafez & Hafez 2004). Larger corpora lutea are usually younger than the smaller ones and indicative of ovulation (Wyneken 2001).

This study aimed to describe the reproductive biology of *T. scripta elegans* bred in the Brazilian Cerrado through gonadal and oviduct analysis, and of the presence of eggs in the oviducts, correlating these findings with the size of the specimens and sexual maturity, and adaptation to the biome, aiming at comparisons with native, exotic and populations, as well as interspecific populations. In this form we hope, we shall contribute to the understanding of the impact of this species on the invaded ecosystems and the establishment of eradication programs.

MATERIALS AND METHODS

Animals. Adult females of *Trachemys scripta elegans* (Wied, 1839, Testudines order) (N=39) received by the “Centro de Triagem de Animais Silvestres” (CETAS) of the “Parque Ecológico do Tietê”, Guarulhos/SP (23°29'23.15" S and 46°31'10.90" W) were euthanized using the combination of xylazine hydrochloride (40 mg kg⁻¹) and ketamine (60 mg kg⁻¹) administered intramuscularly and perfusion of propofol (50 mg kg⁻¹) in the vertebral canal through the atlantooccipital joint. Afterward, they were frozen and transported (Licenses IBAMA no. 136/2011 and no. 048/2012) in Styrofoam boxes with dry ice to the Laboratory of Anatomy of Domestic and Wild Animals (LAADS) of the “Universidade Federal do Vale do São Francisco” (Univasf) in Petrolina, state of Pernambuco (9°23'34" S and 40°30'28" W) for study. SISBIO/IBAMA (protocol # 38601-1) and the Ethics Committee on Animal Use (CEUA) of Univasf (protocol number 0002/160412; 0003/160412; 0004/160412) approved this study.

Body biometry. After body thawing, the body mass (BM) was evaluated using the millimeter precision analytical balance (Bioprecisa®, Labmais Ltda., Curitiba/PR, Brazil), the maximum length (CL) and width (CW) of the carapace; the maximum length (PL) and width (PW) of the plastron using a tape measure (in cm) according to Malvasio et al. (1999). Post-cloacal length (PoCL in cm) was analyzed with the aid of a millimeter precision caliper (Mitotoyo®).

Biometrics of ovaries, ovarian follicles and oviducts. After incision of the bridge with hammer and chisel and removal of the plastron the general disposition of the internal organs was observed and the intestines, liver, and stomach were removed with the help of thin tipped scissors for visualization of the ovaries and oviducts. Ovaries and oviducts were removed from the coelomic cavity. The right (R) and left (L) ovaries had the mass (ROM and LOM, in grams) evaluated using KERN analytical balance and the length (ROL and LOL, cm), width (ROW e LOW) and follicle diameter (mm) with the aid of a millimetric precision pachymeter. The follicles were

classified into three types according to the diameter: Class I (>5-10mm); Class II (>10-25mm) and Class III (>25mm) and quantified. Follicles of 0 to 4 mm were excluded from the count because they were generally pre-vitellogenic and provided little useful information on ovarian activity (Kuchling & Bradshaw 1993). Based on the follicular type present in the ovaries, the females were divided into three groups: G1 (N=10): Class I follicles; G2 (N=20): Class I and Class II follicles; and G3 (N=9): Class I, Class II and Class III follicles. The presence of atresic follicles and corpora lutea was also recorded, but not quantified.

The right (R) and left (L) oviducts had the mass (ROvM and LOvM, in grams) evaluated using KERN analytical balance and the length (ROvL and LOvL, in cm), width (ROvW e LOvW, in cm) and thickness (ROvT and LOvT, in cm) with the aid of a millimetric precision pachymeter.

For the quantitative analysis of the reproductive activity, the gonadosomatic index (GSI) was calculated using the formula: $GSI = TMg/BM \times 100$, where TMg is the total mass of the gonads and BM is the body mass of each female. The right oviduct (ROv) and left oviduct (LOv) had their mass (M, in grams); length (L, in cm), width (W, in cm) and thickness (T, in cm) evaluated as described above and the number of eggs estimated.

Sexual maturity evaluation. Sexual maturity was established following the criteria of Limpus & Limpus (2003). Females were considered pre-pubescent immatures when they had ovaries without expanded stroma, vitellogenic follicles, corpus luteum or albicans, and white oviducts. They were considered immature pubescent when they had ovaries with small expansion, developing follicles, but without the presence of corpus luteum or albicans. Mature females displayed expanded stroma, vascularized and vitellogenic follicles (0.5-3.0mm in diameter), the presence of corpus luteum and albicans, distended and sinuous oviducts with or without the presence of eggs.

Morphology of reproductive organs and ovarian follicles. Fragments of the right ovary and oviduct were fixed by buffered immersion in 10% buffered formalin for 24 hours, dehydrated in increasing alcohol (70%, 80%, 90%, and 100%), clarified in xylol and embedded in paraffin. The five µm sections were stained with hematoxylin-eosin (HE) and evaluated under light microscopy for morphological description. The oviduct fragment was collected from the largest region, which corresponds to the uterine tube between the area between the infundibulum and the isthmus region.

Evaluation of the oocyte development stage. The oocyte development stage was established according to the methodology proposed by Rosa (2005). The initial stage (Ho) was characterized by homogeneity of the cytoplasm, which, although enlarged, did not display any synthesis of reserve material. The second stage (Vc) by the presence of vacuoles in the cytoplasm, which had no uniform distribution or evident granules. The third stage (Vp) by the presence of vacuoles and apparent granules in the cytoplasm cortex, and the fourth stage (Gr) by the presence of granular oocytes and increased cytoplasm.

Statistical analysis. Confidence intervals were constructed for the sample mean ($\alpha=0.05$), and the variables were submitted to analysis of variance and Scott-Knott's test afterward. The observed variables were also submitted to Pearson correlation analysis using the model residues. For the variables related to the total number of eggs produced per group, correspondence analysis was performed to evaluate the association between oocyte size and quantity produced. All analyzes were performed using software R.

RESULTS

Analysis of body, ovarian, oviductal and follicular biometrics

The variables that presented a significant difference were the mass, width, and thickness of the right oviduct and the width and thickness of the left tube (Table 1).

Three size classes were defined, each with an interval of five centimeters, according to the CL (Fig.1). In all three groups, the majority of females presented from 17.00 to 21.00cm of CL, with the lowest female G1=14.00cm, G2=12.40cm, and G3=16.70cm. The largest female had, respectively in each group, 23.80cm, 22.80cm, and 22.90cm.

Correlation analysis indicated a significant correlation between BM and CL, CW, PL, PW and LOL; between CL and CW, PL, PW and PoCL; between CW and PL; between PL and PW and LOL; between ROM and ROL; between LOM and LOL and ROvW; between ROvM and LOvM; between ROvW and ROvT, LOvW and LOvT; between ROvT and LOvW and LOvT and between LOvW and LOvT (Fig.2) in all groups studied.

G2 had a higher total number of follicles ($P<0.05$) than G1 and G3 in the right antimer and G1 in the left and greater total numbers ($P<0.05$) than the G1 and G3 groups (Table 2).

Correspondence analysis indicated that there is an association between the class of follicles and the size of the follicles produced, independent of the ovary side. In other words, Class I follicles were associated with greater follicle size of less than 10mm, Class II follicles associated with greater follicle production from 10mm to 25mm, and Class III follicles associated with higher production of larger follicles than 25mm (Fig.3). Although the higher Classes follicles produced larger sized follicles, these larger sized follicles occurred in smaller quantities than the smaller ones.

Assessment of sexual maturity

All females were considered sexually mature because they had an ovarian stroma that was vascularized and had vitellogenic follicles (0.3-3.0mm in diameter), corpora lutea and albicans, and distended and sinuous oviducts with or without eggs.

Morphology of the reproductive organs and ovarian follicles

The reproductive apparatus was composed by a pair of ovaries and oviducts located dorsally within the coelomates cavity, caudally to the pleuroperitoneal cavity and suspended

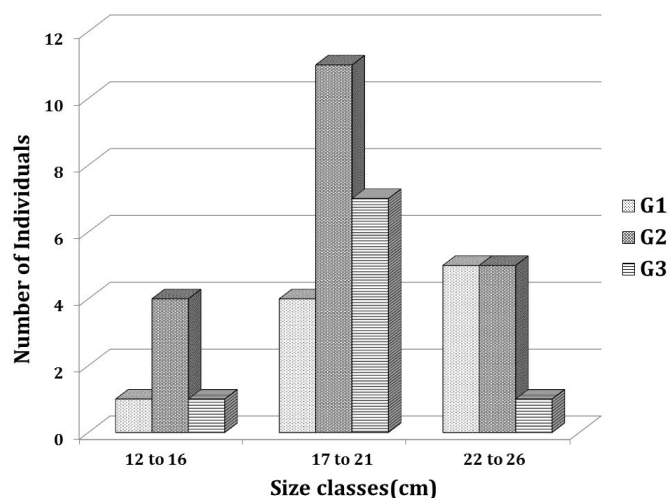


Fig.1. Distribution of size classes according to the maximum carapace length of *Trachemys scripta elegans* from the Wild Animals Triage Center (CETAS) of the "Parque Ecológico do Tietê", Guarulhos/SP.

Table 1. Averages $\pm$ standard error for the analyzed variables among the different groups of *Trachemys scripta elegans* from the Wild Animals Triage Center (CETAS) of the Tietê Ecological Park, Guarulhos/SP

Variable	Groups		
	G1	G2	G3
Corporal mass (g)	1319.5 $\pm$ 213.8	1124.9 $\pm$ 114.9	1164.6 $\pm$ 189.4
Maximum carapace length (cm)	21.3 $\pm$ 1.5	19.6 $\pm$ 1.1	19.6 $\pm$ 1.1
Maximum carapace width (cm)	16.2 $\pm$ 1.1	15.3 $\pm$ 1.0	14.5 $\pm$ 2.2
Maximum plastron length (cm)	19.4 $\pm$ 1.5	18.3 $\pm$ 0.8	19.0 $\pm$ 1.2
Maximum plastron width (cm)	12.1 $\pm$ 1.2	11.6 $\pm$ 0.6	11.7 $\pm$ 0.7
Post-cloacal length (cm)	2.8 $\pm$ 0.2	2.7 $\pm$ 0.3	2.6 $\pm$ 0.3
Height (cm)	7.8 $\pm$ 1.0	7.6 $\pm$ 0.6	7.5 $\pm$ 0.9
Right ovary mass (g)	7.5 $\pm$ 3.1	13.0 $\pm$ 3.0	15.8 $\pm$ 5.9
Right ovary length (cm)	12.7 $\pm$ 1.8	12.3 $\pm$ 1.9	12.6 $\pm$ 3.7
Left ovary mass (g)	10.0 $\pm$ 4.8	18.6 $\pm$ 3.6	19.2 $\pm$ 5.1
Left ovary length (cm)	14.3 $\pm$ 2.3	14.6 $\pm$ 1.8	14.5 $\pm$ 2.5
Right oviduct mass (g)	29.0 $\pm$ 4.5 ^b	29.9 $\pm$ 4.0 ^b	40.4 $\pm$ 1.9 ^a
Right oviduct length (cm)	0.11 $\pm$ 0.01	0.15 $\pm$ 0.02	0.15 $\pm$ 0.06
Right oviduct width (cm)	7.6 $\pm$ 2.0 ^b	7.6 $\pm$ 1.2 ^b	28.7 $\pm$ 7.9 ^a
Right oviduct thickness (cm)	7.7 $\pm$ 2.0 ^b	8.3 $\pm$ 1.5 ^b	30.6 $\pm$ 10.2 ^a
Left oviduct mass (g)	31.0 $\pm$ 3.8	34.3 $\pm$ 2.5	41.0 $\pm$ 3.3
Left oviduct length (cm)	0.13 $\pm$ 0.03	0.15 $\pm$ 0.02	0.4 $\pm$ 0.4
Left oviduct width (cm)	7.4 $\pm$ 1.6 ^b	9.3 $\pm$ 1.8 ^b	28.6 $\pm$ 12.6 ^a
Left oviduct thickness (cm)	7.5 $\pm$ 1.7 ^b	8.9 $\pm$ 1.9 ^b	30.7 $\pm$ 14.2 ^a
Gonadosomatic index (%)	8.33	15.23	14.67

^{a,b} Averages followed by the same lowercase letter on the same line did not differ significantly ($P<0.05$) by the Scott-Knott test.

Table 2. Number of class I follicles (5-10mm), Class II (>10-25mm) and Class III (>25mm) follicles in *Trachemys scripta elegans* from the Wild Animals Triage Center (CETAS) of the Tietê Ecological Park, Guarulhos/SP, 2012

Group	Class I	Class II	Class III	Total number by antimer	Total number by group
Right ovary					
G1	49 ^a	0 ^a	0 ^a	49 ^a	113 ^a
G2	92 ^a	111 ^b	0 ^a	203 ^b	436 ^b
G3	22 ^b	31 ^c	25 ^b	78 ^a	209 ^a
Total	163	142	25	330	758
Left ovary					
G1	64 ^a	0 ^a	0 ^a	64 ^a	-----
G2	110 ^a	123 ^b	0 ^a	233 ^b	-----
G3	57 ^a	41 ^c	33 ^b	131 ^b	-----
Total	231	164	33	428	

^{a,b,c} Values in the same column differ by the t-test ($p < 0.05$); G1 = with Class I follicles (5-10mm), G2 = with Class I and Class II follicles (>10-25mm), G3 = with Class I, Class II and Class III follicles (>25mm).

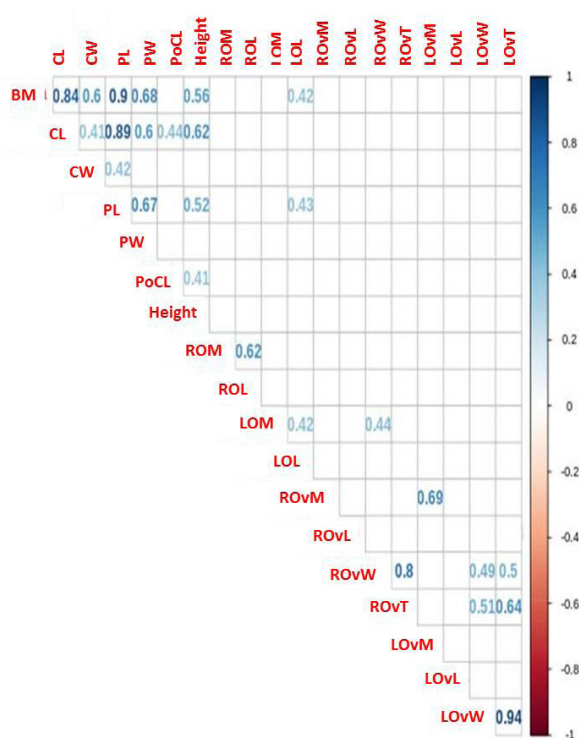


Fig.2. Significant Pearson correlation ($P < 0.05$). BM = body mass, CL = maximum length of carapace, CW = maximum width of the carapace, PL = maximum length of the plastron, PW = maximum width of the plastron, PoCL = post-cloacal length, ROM = right ovary mass, ROL = right ovary length, LOM = left ovary mass, LOL = left ovary length, ROvM = right oviduct mass, ROvL = right oviduct length, ROvW = right oviduct width, ROvT = right oviduct thickness, LOvM = left oviduct mass, LOvL = left oviduct length, LOvW = left oviduct width, LOvT = left oviduct thickness.

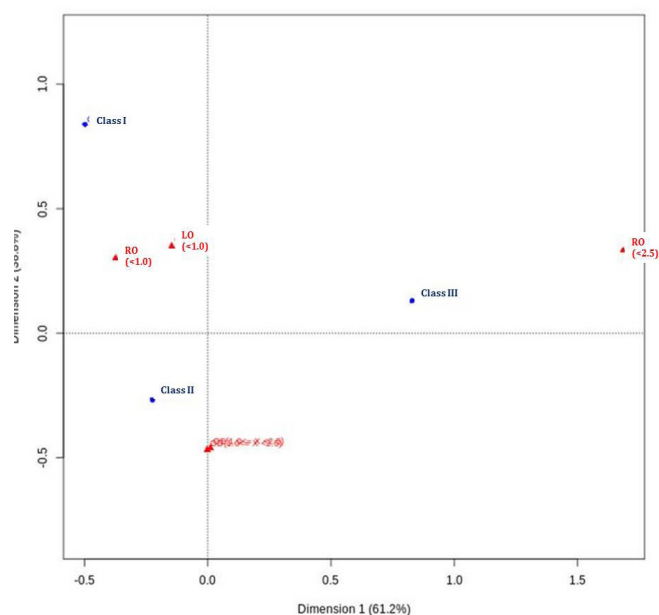


Fig.3. Correspondence analysis for association between follicle class and size of oocytes produced.

up to 30mm; as well as atresic follicles and *corpora lutea*. The oviducts were also irregular, tubular-shaped structures with the presence of lumen (Fig.4A,B). In the G3 females, eggs were observed in the oviducts, which had a varied number and diameter (Fig.4C), whose mean number was 6.44 ± 0.69 , varying from 3 to 10 eggs per female.

Histologically, the ovary had a poorly defined cortical and medullary region and stroma consisting of blood vessels, smooth muscle tissue, connective tissue, gaps, and collagen fibers. The gaps were located very close to the vitellogenic follicles and were associated with the blood vessels (Fig.5). Two ovary germ beds were observed, randomly distributed in the cortex, surrounded by connective tissue and containing ovogonies, I oocytes and primordial follicles positioned in the ovarian stroma near the highly vascularized regions, primary and secondary follicles. The primordial follicles occurred in groups of four or five and the primary scattered stroma, consisting of a single layer of epithelial cells encased in connective tissue. The secondary and tertiary follicles had

to the ceiling of the cavity by the mesovary and mesoviduct. The ovaries were irregular structures, with the appearance of a grape bunch, arranged asymmetrically within the coelomic cavity, the left ovary is more cranial than the right ovary. The oviducts extended from the cranial end of the ovary to its insertion in the lateral wall of the cloaca, locating dorsally in each body antimer. The ovarian stroma was very small and had vitellogenic follicles at various stages of development, reaching diameters

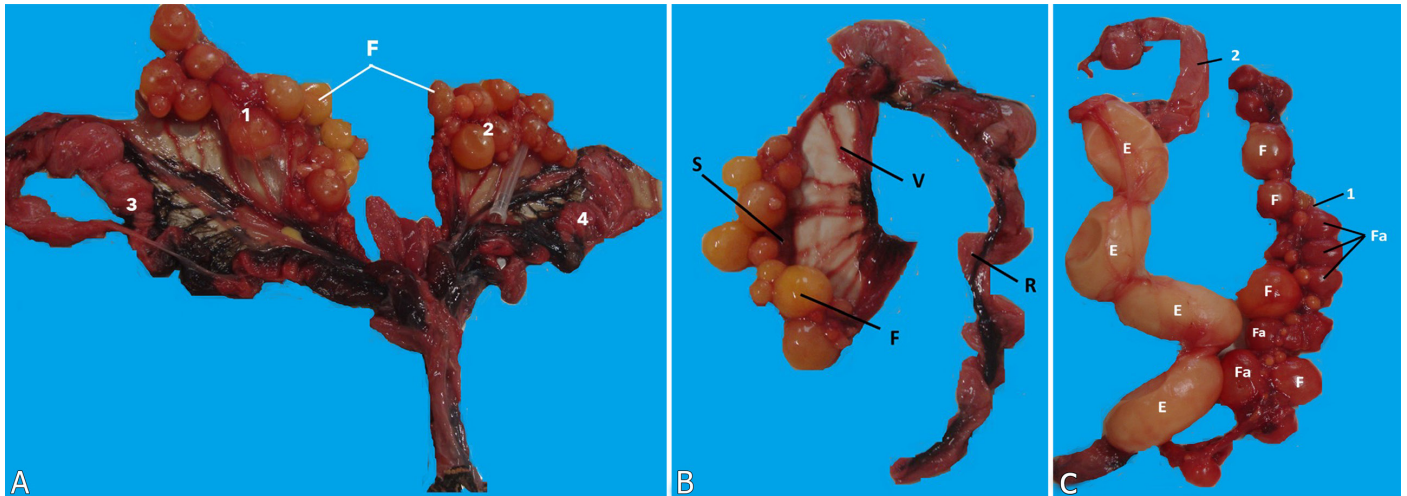


Fig.4. Representative photographs of the reproductive organs of *Trachemys scripta elegans* from the Wild Animals Triage Center (CETAS) of the "Parque Ecológico do Tietê", Guarulhos/SP. (A) Left (1) and right ovaries (2) filled with follicles (F) and left (3) and right oviducts (4). (B) Ovary with vitellogenic follicles (F) of different sizes on a richly vascularized (V) stroma (S) and the right oviduct (R). (C) Vitellogenic (F) and atresic follicles (Fa) in the ovary (1) and oviduct (2) with diameter increased by the presence of eggs (E).

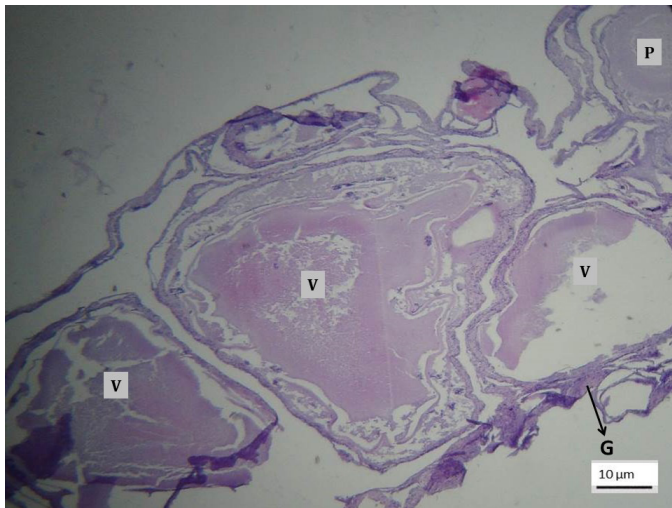


Fig.5. Photomicrograph of *Trachemys scripta elegans* adult ovary from the Wild Animals Triage Center (CETAS) of the Tietê Ecological Park, Guarulhos/SP. Observe the poorly defined cortical and medullar regions with the presence of pre-vitellogenic (P) and vitellogenic (V) follicles adjacent to the gaps (G).

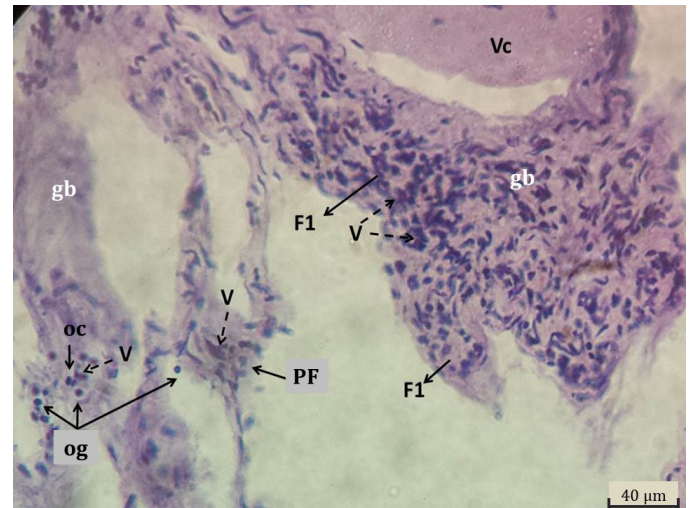


Fig.6. Photomicrograph of *Trachemys scripta elegans* adult ovary from the Wild Animals Triage Center (CETAS) of the "Parque Ecológico do Tietê", Guarulhos/SP. In this picture we shall observe the germinative beds (gb) with ovogonies (og), oocytes I (oc) positioned in the ovarian stroma near the highly vascularized regions (V); primordial follicles (PF); primary follicles (F1) and also a tertiary follicle in the second stage of development (Vc) characterized by the presence of vacuoles in the cytoplasm, with no uniform distribution or evident granules.

a higher number of layers, than the primary. Tertiary follicles were wider and with more defined follicular layers (Fig.6).

Tertiary follicles presented the wall composed of four cell layers, being from the outer to the inner surface: outer theca, inner theca, granular layer with perivitelline membrane and radiate zone with the vitelline membrane (Fig.7).

The ovarian stroma also displayed corpora lutea, whose color was translucent white, and atretic follicles at different stages of regression, which differed from the developing (yellow) follicles by their pink or reddish-brown coloration (Fig.8).

The oviducts were subdivided into five anatomical regions: infundibulum, uterine tube (magnum), isthmus (transition region), uterus and vagina. The infundibulum was the most cranial region and presented an ostium in the portion near

the cranial end of the ovary. This portion of the oviduct is responsible for capturing the oocyte released at the time of ovulation. The uterine or major tuba was the largest segment and attached the non-glandular segment to the glandular segment represented by the isthmus. The uterus was responsible for the production of the eggshell, and the vagina, the final portion of the oviduct, was attached to the cloaca and was the thickest segment (Fig.9A). Histologically the uterine tube had a serous layer, which enveloped the entire region externally and was composed by a thin layer of loose connective tissue covered by a layer of pavement epithelium.

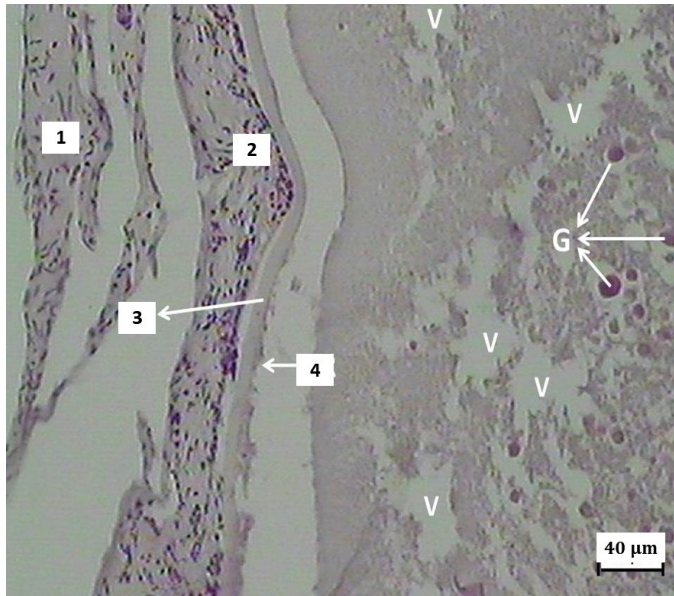


Fig.7. Photomicrograph of the tertiary follicle wall of an oocyte in the third stage (Vp) of *Trachemys scripta elegans* development from the Wild Animals Triage Center (CETAS) of the "Parque Ecológico do Tietê", Guarulhos/SP. Outer theca layer (1), inner theca layer (2), granular layer with the perivitelline membrane (3), radial zone with vitelline membrane (4) and presence of vacuoles (V) and apparent granules (G) in the oocyte cytoplasm.

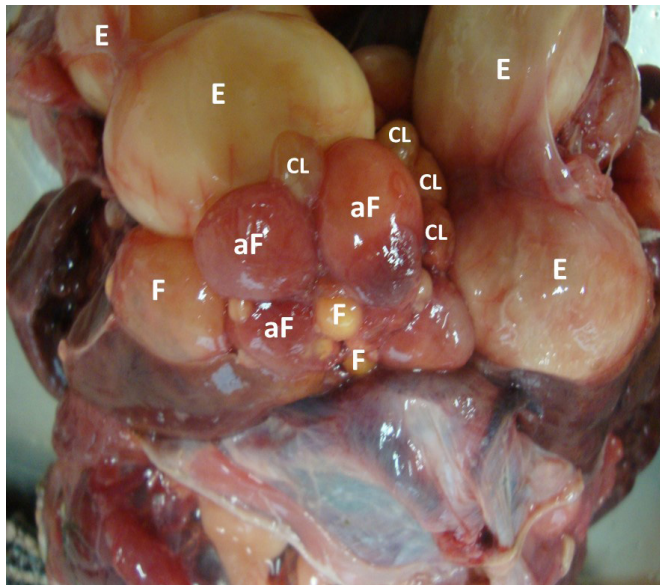


Fig.8. Right ovary, and both right and left oviducts of *Trachemys scripta elegans* from the Wild Animals Triage Center (CETAS) of the "Parque Ecológico do Tietê", Guarulhos/SP. Vitellogenic follicles at different stages of development (F), atretic follicles (aF), corpora lutea (CL) and eggs (E) in oviducts.

The muscular layer was composed of longitudinally arranged smooth muscle fibers and a well-developed mucosal layer, with a cylindrical pseudostratified epithelium with ciliary, mucus-secreting and basal cells, and *lamina propria* of loose connective tissue, with the presence of vessels and numerous tubular glands (Fig.9B).

Evaluation of the stage of oocyte development

In the gonadal tissue, there were oocytes at the following stage of development: initial stage (Ho) (Fig.10A), second stage (Vc) (Fig.10-B) and third stage (Vp) (Fig.10C,D). Follicles in the fourth stage (Gr) were not visible.

DISCUSSION

There are just a few studies dealing on the biology and capacity reproductive of turtles involving gonadal analysis and egg production. Some of them are restricted to the natural environment and with low number of specimen of adult females (Martinez-Silvestre 1997, Lutz et al. 2003, Rosa 2009, Chaves et al. 2012) or newly hatched individuals (Malvasio et al. 1999, Lutz et al. 2003, Wyneken et al. 2007, Ceriani & Wyneken 2008, Malvasio et al. 2011). Few other studies analyze adult females in an unnatural environment (Bertolero & Canicio 2000, Capalleras & Carretero 2000, Cadi et al. 2004, Vieira & Costa 2006). Given the deficiency of information about this species outside its natural habitat, the present study described for the first time the ovarian and oviductal characteristics of *Trachemys scripta elegans* bred in the Brazilian Cerrado correlating these findings with the size of the specimens, sexual maturity, and reproductive capacity.

The absence of significant difference in body biometry between groups (Table 1) suggested that all females were in the same stage of body development, with most females exhibiting from 17.00 to 21.00cm maximum carapace length (Fig.1). Average body mass values did not differ from Perez-Santigosa et al. (2008) and Gradela et al. (2017a), but were slightly higher than Vieira & Costa (2006); lower than those of Gradela et al. (2017b) and well above those of Taniguchi et al. (2017). The maximum carapace length did not differ from Perez-Santigosa et al. (2008) and Gradela et al. (2017a, 2017b), but was slightly higher than described by Vieira & Costa (2006) and lower than Taniguchi et al. (2017). In all groups, there was a positive and harmonic development among mass, carapace, and plastron, which were positively correlated. These data corroborate with the results of Gradela et al. (2017b) and diverge from Gradela et al. (2017a) who observed development uniformity only between body mass and carapace length and width of the plastron, and between the carapace and plastron lengths.

In *T. scripta* females the sexual maturity is influenced mainly by body size and not by age (Gibbons & Greene 1990), so that females mature with plastron length from 16.00 to 17.00cm (Perez-Santigosa et al. 2008) and of 16.70cm in the subspecies *T. scripta elegans* (Tucker & Moll 1997) when they reach three to four years of age (Pupins 2007). Studies performed in Southern Spain describe that some females reach sexual maturity at the length of 15.95cm and age of three years, displaying similar or even higher growth rates than those in the native habitats (Perez-Santigosa et al. 2008). Our study highlights a similar situation, as the smallest female had a maximum carapace length of 12.4 cm.

Some papers suggest that only carapace length is considered as indicative of sexual maturity. This orientation shall be deemed inappropriate: among populations and even within a population there is a difference in the minimum size of the individuals at the beginning of reproduction (Limpus et al. 1994). Duarte et al. (2011) point out that the stages of maturation of females are associated with the yolk deposition in ovarian

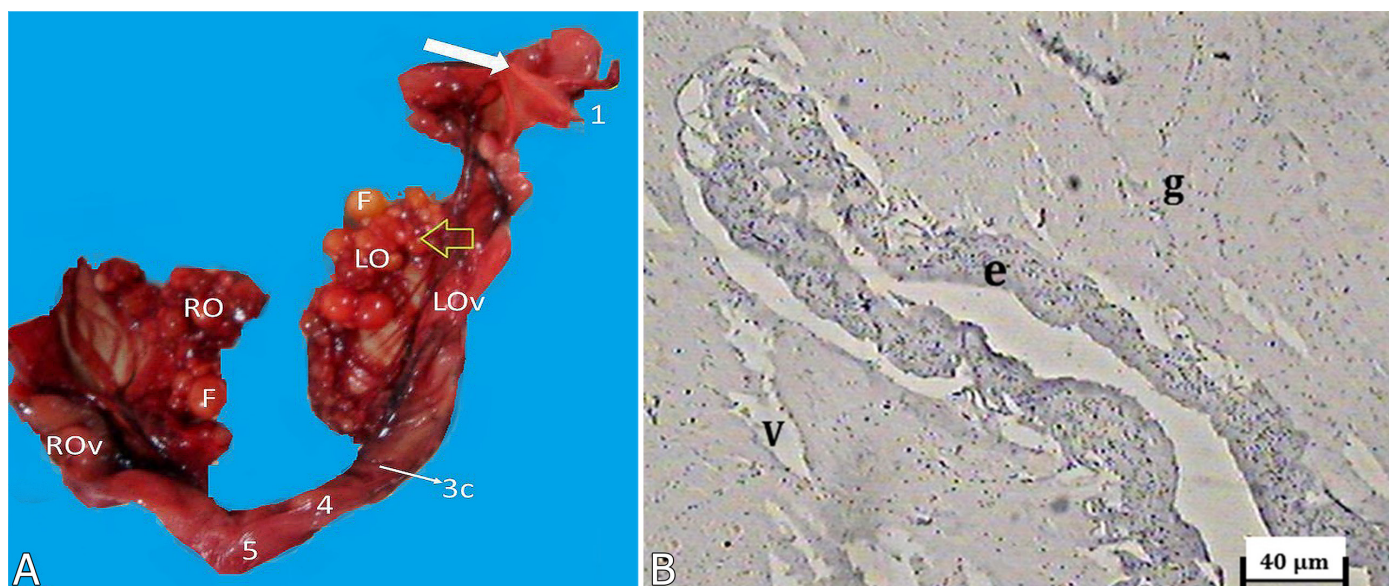


Fig.9. Oviduct of *Trachemys scripta elegans* from the Wild Animals Triage Center (CETAS) of the “Parque Ecológico do Tietê”, Guarulhos/SP. (A) Right (RO) and left (LO) ovaries filled with follicles (F); right (ROv) and left oviducts (LOv) and oviduct regions: infundibulum (1) with infundibularostium (broad arrow, 2); isthmus (3), uterus (4) and vagina (5). (B) Histological section of the uterine tube showing the mucosa layer consisting of pseudostratified cylindrical epithelium (e), tubular glands (g) and vessels (v). HE, obj.40x.

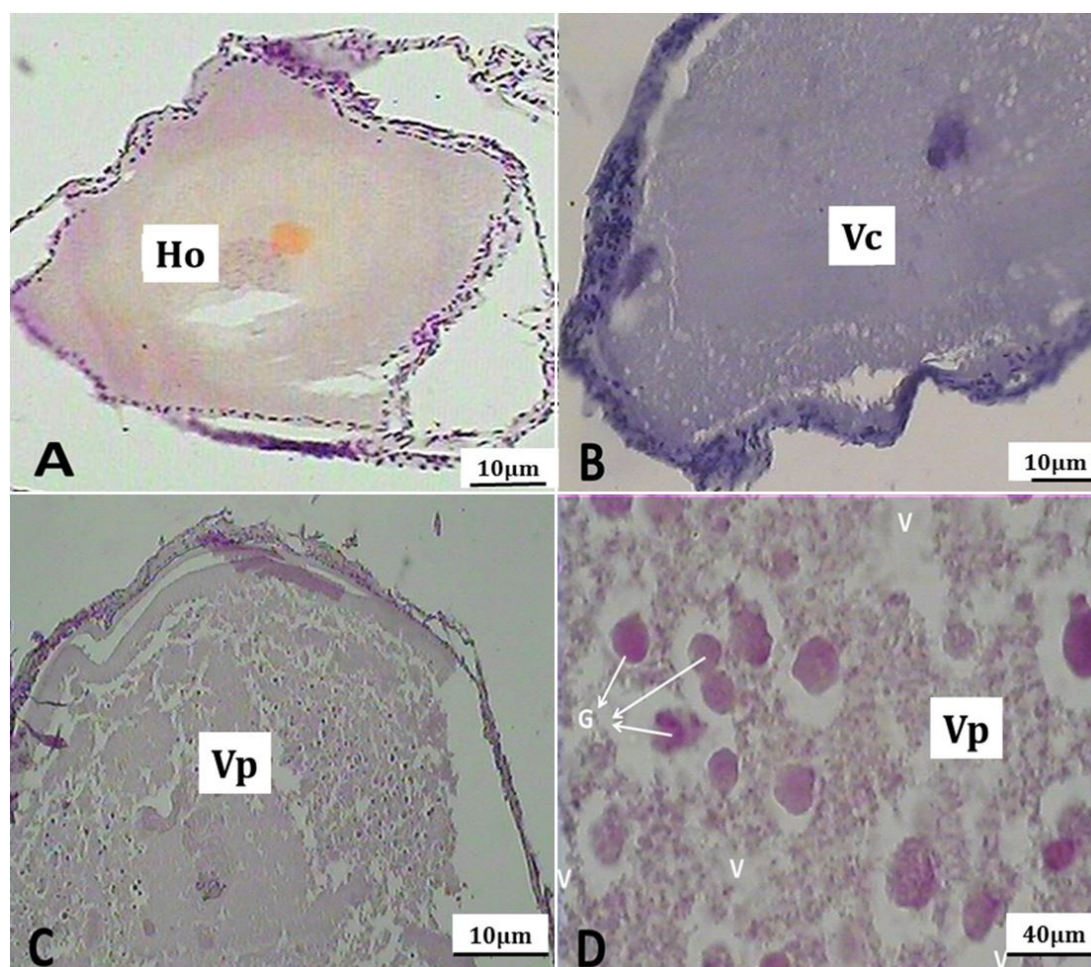


Fig.10. Photomicrography of *Trachemys scripta elegans* oocytes from the Wild Animals Triage Center (CETAS) of the “Parque Ecológico do Tietê”, Guarulhos/SP. (A) Oocyte in the initial stage (Ho) presenting homogeneous cytoplasm. (B) Oocyte in the second stage (Vc) with the presence of vacuoles (V) in the cytoplasm. (C,D) Oocyte in the third stage (Vp) with the presence of vacuoles (V) and apparent granules (G) in the cytoplasm.

follicles. In reptiles, turtles (Machado Júnior et al. 2006) and birds (Dyce et al. 2004) the yolk serves as a nutritive matter to the embryo throughout its development within the egg and its production and secretion are stimulated by estrogen (Rosanova et al. 2002), which is released after puberty and acts locally in the ovarian cortex and medulla promoting the differentiation of gonads (Pieau & Dorizzi 2004) and oviducts (Owens & Morris 1985). This study associates the analysis of the length of the carapace with the presence of vitellogenic follicles, atretic follicles, corpora lutea and corpus albicans in the ovaries and distended and sinuous oviducts with or without the presence of eggs as indicative of sexual maturity (Ernst 1971, Wyneken 2001, Limpus & Limpus 2003, Castro 2006, Rossi et al. 2006, Delgado 2006, Chaves et al. 2012). In this paper, the results showed that all females were sexually mature regardless of the maximum length of the carapace.

The reproductive status of a population can be estimated by analyzing the gonadal status of females (Delgado 2006). In this sense, the gonadosomatic index (GSI) has been used as an indication of the functional status of ovaries in fish (Querol et al. 2002, Costa et al. 2005, Zeyl et al. 2013), because it usually increases with the increase of the gonadal stage (Nascimento et al. 2012, Zeyl et al. 2013), and the highest values observed in the mature stage (Nascimento et al. 2012), as in the females in this study that has shown active vitellogenesis (Querol et al. 2002). Therefore, the gonadosomatic index proved adequate as an indicator of the reproductive period of *T. scripta elegans*, whose mean values were significantly higher than those of *Kinosternon scorpioides* (Chaves et al. 2012).

A pair of ovaries and oviducts represented the female genital organs of *T. scripta elegans*, attached to the ceiling of the celoma cavity, respectively, by the mesovarium and mesoviduct, as described in other species of turtles (Wyneken 2001, Machado Júnior et al. 2006, Chaves et al. 2012, Pérez-Bermúdez et al. 2012, Silva et al. 2017) and reptiles (Storer et al. 2000), which differ from birds that have only developed left oviduct (Dyce et al. 2004). The ovaries presented a topographic layout, appearance, and presence of yellow-colored follicles at different stages of development similar to those of sea turtles (Wyneken 2001); *K. scorpioides* (Machado Júnior et al. 2006, Chaves et al. 2012), *Chelonia mydas* (Kondak 2012) and *Eretmochelys imbricata* (Pérez-Bermúdez et al. 2012). The asymmetric position of the ovaries, caused by differences in volume and quantity of follicles, corroborated with Chaves et al. (2012) disagreeing with the symmetry observed by Storer et al. (2000) and Machado Júnior et al. (2006).

No significant difference was observed in the ovarian mass between the studied groups. The total number of follicles did not differ statistically between the right (330) and left (428) antimeres. This finding was in opposition to that described in *K. scorpioides*. In this species the right ovary was larger than the left due to the higher number of vitellogenic follicles (Chaves et al. 2012). The average mass of right and left ovaries in the G1 groups (7.46 and 9.99g, respectively); G2 (13.01 and 18.57g) and G3 (14.58 and 14.45g) were higher than those of *K. scorpioides* (6.25±4.23g and 2.27±1.42g, respectively) (Chaves et al. 2012), while Velasquez & Vogt (2011) observed in *Peltocephalus dumerilianus* values ranging from 1.31 to 48.25g and 1.21 to 45.66g, respectively. In *T. scripta elegans* the presence of enlarged or pre-ovulatory follicles, eggs or distended oviducts, suggest recent oviposition (Perez-Santigosa et al. 2008) and, after the

oviposition period, no eggs or follicles larger than 21mm, but only follicles of 7 to 13mm, and in a small proportion of females, also a few pre-ovulatory follicles of 14-20mm, corroborating with those observed in groups G1 and G2, respectively.

The significant relationship between female size (body mass and plastron length) and left ovary length suggested that the gonadal growth was concomitant with the body growth, and supported the existence of a positive relationship between brood size and female size as described in other species (Ceriani & Wyneken 2008, Delgado 2006, Velasquez & Vogt 2011, Failla et al. 2018), but different from sea turtles (Hamann et al. 2003) and *C. mydas* juveniles Rosa (2005, 2009).

The presence of follicles of different diameters (<5mm up to >25mm), as well as corpus luteum and eggs in the oviducts, highlighted a similarity of follicular development among specimen of the same species (Perez-Santigosa et al. 2008) and other species (Ramo 1982, Velasquez & Vogt 2011) and reflected the ovarian complexity of the analyzed females, which ranged from the beginning of the period of ovarian maturation to the presence of eggs and corpus luteum. These findings denote the presence of ovarian follicular hierarchy (Velasquez & Vogt 2011) characterized by the presence of follicles at all maturation stages, including also not-vitellogenic follicles (Etches & Petitte 1990). This hierarchy can be accepted as a reproductive strategy to keep the highest possible number of follicles available and thus reduce the probability of losses caused by excessive and effective ovulation, making a possible rapid ovarian recovery and ensuring maximum reproduction of females at each station. In chelonians, these characteristics may increase as a function of the size of the female (Velasquez & Vogt 2011), as observed in G2, which presented a significant number of follicles (Table 2).

The follicular development in reptiles divides into two phases: pre-vitellogenic and vitellogenic, which is characterized by the accumulation of yolk inside the oocyte. Pre-vitellogenic follicles are small and white. As they enter the vitellogenic stage become yellow and large (Jacobson 2007). It should be noted that, although follicles of 5-6 mm become vitellogenic, and those of 16-18mm, pre-ovulatory, in most animals some vitellogenic follicles do not reach pre-ovulatory size and, in some cases, those of pre-ovulatory size do not ovulate by becoming atretic (Kuchling & Bradshaw 1993), as observed in this study, because although the larger Classes produced larger follicles, they occurred in a smaller number, since some became atretic. These follicles differed from vitellogenic follicles by pink or reddish-brown coloration (Fig.8), similar to that described in *Chelodina rugosa* and *Elseya dentata* (Kennett 1999).

In *T. scripta elegans* small and large follicles were also distributed throughout the ovary, with neither elongation of the largest cranial nor the concentration of the smaller and immature in the posterior third of the ovary as described in marine turtles (Wyneken 2001, Flint et al. 2009). On the other hand, larger follicles (> 25 mm) occurred in post-ovulatory females, that is, G3, agreeing with other species (Dobie 1971, Iverson 1979) since a new wave of ovulation usually occurs 36 h after a oviposition (Wibbels et al. 1990). Therefore, in *T. scripta elegans* the presence of follicles of different sizes in the ovaries suggests that at least up to four clutches annually may be produced (Perez-Santigosa et al. 2008).

The presence of increased cytoplasm in the oocytes indicates their development phase, with the follicles more developed being those with cytoplasmic vacuoles or granules, which characterize the nutrient reserve in the cell and indicate the onset of vitellogenesis (Kondak 2012). In this study, the analysis of the gonadal tissue highlighted three stages of oocyte development according to the structure of the cytoplasm: homogeneous (Ho), vesicular (Vc) and vesicular in the cortex with apparent granules (Vp). Our observations agreed with Kondak (2012) disagreeing with Rosa (2005) who also observed the fourth stage (Gr) characterized by the presence of granular oocytes and increased cytoplasm.

After complete maturation of the oocytes and before mating, a group of mature follicles is ovulated together (Mateus 2014). The oocytes are picked up by the fimbriae of the region of the oviduct infundibulum from where they pass into the non-glandular segment, and enter the uterine tube (magno), where they remain for about three days and are covered by a layer of albumin. Later on, the oocytes go to the glandular region and, after secretion of proteins and membrane carbohydrates (corium) and a matrix of aragonite, they become eggs. After six to seven days, these eggs undergo calcification and are deposited in the vagina, where they remain for several days until they go to the cloaca and are spawned (Wyneken 2001, McArthur 2008). Concomitantly to ovulation, the cells of the follicle wall hypertrophy and the corpus luteum develops (Mateus 2014). This structure resembles, in some aspects, those of some species of mammals (Guillette Junior et al. 1995) and has as their key function, the preservation of the pregnancy or of the eggs which are developing inside the oviducts (Gemmell 1995). For this reason, the higher activity of the corpus luteum coincides with the formation of external membranes in the oviducts (Altland 1951), which explains their regression shortly after (Velasquez & Vogt 2011) or up to three months after spawning in the genera *Pseudemys* and *Graptemys* (Webb 1961). In *T. scripta elegans* corpus luteum were visualized in females with eggs inside the oviducts and were distinguished from the follicles by their translucent white coloration (Fig.8) as in sea turtles (Owens 1980). According to these authors, corpus luteum produces and secretes progesterone in response to the luteinizing hormone, similar to mammals and birds (Hafez & Hafez 2004), and this has the function of stimulating the production of albumin. During the reproductive season, the corpora lutea regress and become albicans bodies (Wyneken 2001, Jacobson 2007) corroborating findings in mammals and birds (Hafez & Hafez 2004).

Microscopically reduced ovarian stroma and non-differentiation between the cortical and medullary regions agreed with and *K. scorpoides* (Chaves et al. 2012), diverging from mammals, presenting these easily recognizable regions (Hafez & Hafez 2004) and birds, which have the most discrete and diffuse medullary region (Banks 1992). In contrast to mammals, where the vascularization is restricted to the medullary region (Hafez & Hafez 2004), in *T. scripta elegans*, the vascularization of the ovary is widespread throughout the stroma, which is composed of pre-vitellogenic follicles, vitellogenic, blood vessels, gaps, germinal beds, connective tissue and smooth muscle as in other Testudines (Chaves et al. 2012, Pérez-Bermúdez et al. 2012).

The observation of two germinative beds per ovary corroborates with similar observations performed in other species of reptiles, *Pseudemys scripta elegans*, *Coturnix coturnix japonica* (Callebaut et al. 1997) and lizards (Radder & Shine 2007) and diverging from *E. imbricata* (Pérez-Bermúdez et al. 2012), *C. mydas* and *C. caretta* (Aitken et al. 1976) that have multiple beds. The arrangement of the primordial follicles in germ beds, in groups of four or five, differed from ostrich, in which they usually occur alone (Madekurozwa & Kimaro 2006). Morphology and distribution of primary, secondary and tertiary follicles were similar to *K. scorpoides* (Chaves et al. 2012), with the primary follicles being close to blood vessels and some dispersed in the stroma, while in *E. imbricata* these are distributed randomly in the cortex (Pérez-Bermúdez et al. 2012). The constitution of the wall of the tertiary follicles, formed by external theca, internal theca, granular layer with perivitelline membrane and radial zone with vitelline membrane did not diverge from *K. scorpoides* (Chaves et al. 2012); snakes (Almeida-Santos 2005) and eels (Parizzi et al. 2007).

According to Callebaut et al. (1997), in *T. scripta elegans* gaps are smaller and histochemically more homogeneous than in *E. imbricata* (Pérez-Bermúdez et al. 2012) and *Pelodiscus sinensis* (Nainan et al. 2009), which have the most developed and histochemically polymorphic lacunar system. In common with these species, the gaps in *T. scripta elegans* were also located very close to the follicles and were associated with blood vessels. This positioning seems to be related to its function, since they represent space for the follicular expansion during the maturation (Pérez-Bermúdez et al. 2012), besides being escape route for the increase of the blood pressure in the ovary (Callebaut 1988) and have paper in follicular nutrition (Chen et al. 1998).

There are few studies on reptiles addressing the structure and function of the oviduct, particularly in turtles. *T. scripta elegans*, like most reptiles, exhibits both functional and separate oviducts, having the only junction in their final portion to form a cloaca (Fox 1977), diverging from birds that only have the left oviduct developed (Dyce et al. 2004). The topography of the oviducts, beginning near the cranial end of the ovaries and extending laterally to the insertion in the cloaca, agreed with previous reports (Wyneken 2001, Machado Júnior et al. 2006, Silva et al. 2017) and the anatomical division in five regions: infundibulum, uterine tube, isthmus, uterus, and vagina, agreed with other reptiles (Girling 2002), turtles (Alkindi et al. 2006, Machado Júnior et al. 2006, Firmiano et al. 2012, Silva et al. 2017) and birds (Girling 2002, Parizzi 2006), but diverged from lizards (Guillette Junior et al. 1989, Girling et al. 1997), snakes (Perkins & Palmer 1996), and geckos (Sever et al. 2000). In these species the oviducts are subdivided into three or four regions.

Similar to *P. geoffroanus* (Firmiano et al. 2012); reptiles (Sever et al. 2000, Nogueira et al. 2011) and mammals (Junqueira & Carneiro 2008), *T. scripta elegans* presented the histological structure of the uterine tube constructed of serosa, muscular and mucosa. The uterine tube region was similar to *P. geoffroanus* (Firmiano et al. 2012) and the abundance of glands in the mucosal layer, which are responsible for the production of albumin (Firmiano et al. 2012), agreed with other species of turtles and crocodilians (Aitken et al. 1976, Palmer & Guillette Junior 1988, Girling 2002, Firmiano et al. 2012).

In *T. scripta elegans* out of its natural distribution there is little information about its reproductive capacity (Cadi et al. 2004), and egg deposition has been observed in countries such as Spain (Bertolero & Canicio 2000, Capalleras & Carretero 2000, Bringsøe 2001, Pleguezuelos 2004), France (Cadi et al. 2004), Italy (Ferri & Soccini 2003, Crescente et al. 2014) and Taiwan (Chen & Lue 1998) and now also demonstrated for the first time in Brazil. According to Vieira & Costa (2006), although the population of *T. scripta elegans* studied in Brasília-DF was in reproductive condition, no cut rituals, nests or other signs of reproduction were observed, indicating that it was stagnant and its growth was due to the release of the individuals by the visitors of the Park. Rossi et al. (2006), although they observed the opening of pits, also did not observe the oviposition of eggs in the *T. scripta elegans* raised in captivity in São Paulo (SP). This finding is important because evidence of breeding in introduced species is critical to understanding their impact on invaded ecosystems (Cadi et al. 2004). In a natural environment, *T. scripta elegans* can lay up to six clutches each year, with 2 to 30 eggs each and a variable incubation period of 59 to 112 days (Ernst et al. 1994, Bringsøe 2006). However, in an unnatural environment the egg production can vary from 2 to 11 (Cadi et al. 2004), 1 to 16 (Crescente et al. 2014); 1 to 19 (Taniguchi et al. 2017) or in an average of 11.5 eggs (Perez-Santigosa et al. 2008) as in this study, being lower than that of North American specimens producing 12.7 to 15.1 eggs (Tucker et al. 1998) or, on average, 10.5 (Ernst & Lovich 2009). In this study there was no significant difference in the number of eggs among the oviducts, agreeing with Velasquez & Vogt (2011) and disagreeing with Legler (1960), as well as no significant difference in egg size, as in other species of chelonians (Tucker & Janzen 1998). The presence of eggs in G3 oviducts was responsible for the greater width of this organ in the right antimer and the greater mass and width in the left antimer (Table 1).

CONCLUSIONS

In *Trachemys scripta elegans* the sexual maturity should be established based on ovarian evaluation and not on maximum carapace length. The structure of the ovary resembles that of other species of oviparous reptiles and can be used for phylogenetic morphological comparisons. In addition, the gonadal-somatic index is an adequate indicator of the reproductive period; there is a relationship between body and gonadal growth, and therefore between brood size and size of female. Ovaries with pre and vitellogenic follicles of different diameters, luteal bodies and the presence of eggs in the oviducts reflect the ovarian complexity of this species and denote a follicular hierarchy.

The results of the present study described the characteristics of the ovaries, oviducts and uterine tubes of *T. scripta elegans* created in the Brazilian Cerrado demonstrating that reproduction occurs in this environment. Through this knowledge, essential and relevant information is available to understand their impact on the invaded ecosystems and to establish eradication programs, since the extraction of adult females associated with the knowledge of their reproductive capacity can contribute to the reduction of the annual reproductive yield of this species and diminish its effect on local biodiversity.

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

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

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

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

Notem: para evitar a separação em duas linhas, os numerais devem ser apresentados junto com suas unidades, ou seja, sem espaçamento, por exemplo: 100ppm, 10mm, 50cm, 18x10cm, (P<0,05), 15h. A abreviação de número é “n^o” e não “n°”; grau Celsius é “°C” e não “aC”.

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

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

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

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

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

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

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

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

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

3. Apresentação das Figuras:

- As figuras devem ser salvas em 300dpi, arquivo TIF.
- Enviar cada figura separadamente.
- Identificar as figuras em ordem conforme a menção no texto.
- As figuras solitárias devem ter seus arquivos identificados como (Fig.1, Fig.2 ...)
- As figuras que serão destinadas a formar uma prancha devem ter seus arquivos identificados como (Fig.1A, Fig.1B ...). As pranchas devem ser compostas por múltiplas subfiguras. Imagens destinadas a uma prancha devem ser de mesmo tamanho.
- Para micrografias usar, de preferência, barras de escala para indicar o aumento; apresentar na legenda sempre o método de coloração e a objetiva, p. ex.: HE, obj.40x.
- As legendas de figuras devem conter inicialmente o que se observa na imagem, seguida das informações adicionais (Formato típico da legenda: Fig.1. (**A**) Descrição da imagem. Diagnóstico, órgão ou tecido, espécie animal, número do caso. Método de coloração e objetiva.).
- As legendas de figuras devem ser apresentadas junto com o texto do artigo, após as Referências.

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

Exemplos de Referências

➤ Artigos publicados em periódicos:

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

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

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

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

➤ Livros:

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

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

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

➤ Capítulos de livros:

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

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

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

➤ Dissertações e Teses:

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

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

➤ Resumos publicados em eventos:

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

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

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

GUIDE FOR AUTHORS

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

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

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

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

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

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

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

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

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

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

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

The author rights of the accepted papers are preserved.

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

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

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

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

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

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

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

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

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

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

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

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

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

m) **Conflict of interest or none** should be mentioned.

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

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

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

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

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

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

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

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

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

The abbreviation for number is "n°" and not "nº"; for degree Celsius "°C" and not "°C".

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

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

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

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

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

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

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

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

3. Figure presentation:

a) Save images at 300 dpi, TIF files.

b) Send each figure separately.

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

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

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

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

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

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

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

Exemples for References:

➤ Articles published in scientific journals:

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

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

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

➤ Books:

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

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

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

➤ Chapters of books:

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

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

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

➤ Dissertations and Theses:

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

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

➤ Abstracts published in Events:

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

Em 15 de agosto de 2019

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

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

(Note: Consult entire papers instead of only Abstracts)

LIVESTOCK DISEASES

- Epidemiological analysis of bovine tuberculosis in the southern region of Rio Grande do Sul from 2000 to 2015** [Análise epidemiológica da tuberculose bovina na região sul do Rio Grande do Sul no período de 2000 a 2015.]. Alberti T.S., Bruhn F.R.P., Zamboni R., Venancio F.R., Scheid H.V., Raffi M.B., Schild A.L. & Sallis E.S.V. 77-81
- Microbiological and molecular detection of *Mycoplasma bovis* in milk samples from bovine clinical mastitis** [Detecção microbiológica e molecular de *Mycoplasma bovis* em amostras de leite oriundas de mastite clínica bovina.]. Salina A., Timenetsky J., Barbosa M.S., Azevedo C.M. & Langoni H. 82-87
- Pathogenic potential of *Brucella ovis* field isolates with different genotypic profile and protection provided by the vaccine strain *B. ovis* $\Delta abcBA$ against *B. ovis* field isolates in mice** [Vacina viva atenuada *Brucella ovis* $\Delta abcBA$ encapsulada protege camundongos frente a desafios de *B. ovis* isoladas de campo.]. Carvalho T.P., Ribeiro N.Q., Mol J.P.S., Costa F.B., Eckstein C., Paula N.F., Paixão T.A. & Santos R.L. 88-96
- Influence of organic chromium supplementation on the performance of beef calves undergoing weaning-related stress** [Influência da suplementação com cromo orgânico no desempenho de bezerros de corte submetidos ao estresse da desmama.]. Sousa I.K.F., Sousa R.S., Mori C.S., Morini A.C., Neves K.A.L., Minervino A.H.H. & Ortolani E.L. 97-101
- Antifungal susceptibility profile of *Aspergillus fumigatus* isolates from avian lungs** [Perfil de suscetibilidade antifúngica de isolados de *Aspergillus fumigatus* provenientes de pulmões de aves.]. Spanemberg A., Ravazzolo A.P., Denardi L.B., Hartz S.A., Santurio J.M., Driemeier D. & Ferreira L. 102-106

SMALL ANIMALS DISEASES

- Evaluation of the euthanasia of seropositive dogs for canine visceral leishmaniasis as the only method of controlling the disease in the enzootic area in the Midwestern Minas Gerais** [Avaliação da eutanásia de cães soro reagentes para leishmaniose visceral canina como único método de controle da doença em área enzoótica no Centro Oeste de Minas Gerais.]. Vaz T.P., Gama-Melo M.O., Quaresma P.F., Gontijo C.M.F., Santos G., Barbosa F.S. & Fontes G. 107-112
- Thrombin generation and thromboelastometry tests in dogs with chronic kidney disease** [Testes de geração de trombina e tromboelastometria em cães com doença renal crônica.]. Gonçalves D.S., Geraldes S.S., Duarte R.C.F., Carvalho M.G., Guimarães-Okamoto P.T.C. & Takahira R.K. 113-120

WILDLIFE MEDICINE

- Diseases of wild birds in southern Rio Grande do Sul, Brazil** [Doenças de aves silvestres no sul do Rio Grande do Sul.]. Echenique J.V.Z., Soares M.P., Albano A.P.N., Bandarra P.M. & Schild A.L. 121-128
- Companion psittacine birds as reservoir of gentamicin and vancomycin-resistant *Enterococcus* spp.** [Psitacídeos de companhia como reservatórios de *Enterococcus* spp. gentamicina e vancomicina-resistentes.]. Cabral B.G., Davies Y.M., Menão M.C., Saidenberg A.B.S., Gomes V.T.M., Moreno L.Z., Sato M.I.Z., Moreno A.M. & Knöbl T. 129-133

ANIMAL MORPHOPHYSIOLOGY

- Uterine secretory activity and histology of prepubertal female dogs in an animal model of partial ablation of endometrial glands development** [Atividade secretória uterina e histologia de cadelas pré-púberes em um modelo animal de ablação parcial do desenvolvimento das glândulas uterinas.]. Martins B.B., Teixeira N.S., Teixeira S.A.M.V., Giannotti J.G., Nunes L.C., Freitas P.M.C., Machado-Neves M. & Luz M.R. 134-140
- Reproductive biology, morphology, and morphometry of ovaries and oviducts of *Trachemys scripta elegans* in Brazilian Cerrado** [Biologia reprodutiva, morfologia e morfometria de ovários e ovidutos de *Trachemys scripta elegans* no Cerrado Brasileiro.]. Gradela A., Pires I.C., Matos M.H.T., Corrêa F.M., Faria M.D., Silva J.S., Torres L.R.C. & Milanelo L. 141-154