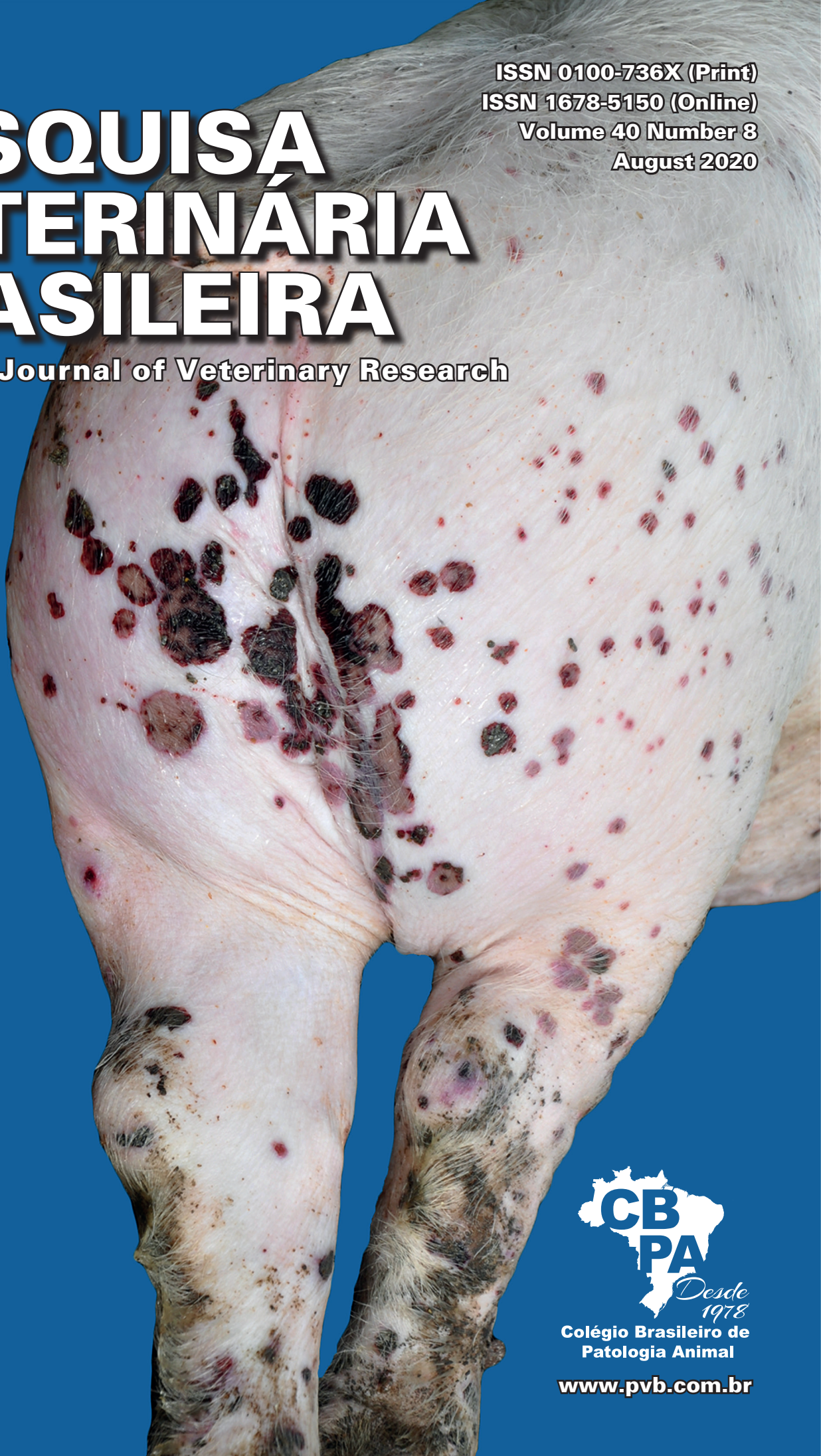
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Cover illustration: Dark red multifocal macules and papules in the skin of a swine with porcine dermatitis and nephropathy syndrome. (Pereira P.R. et al., p.582)

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Review Article
Livestock Diseases
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Causes of death in feedlot beef cattle and their control: a brief review[1]

Pablo Estima-Silva[2], Haide Valeska Scheid[2] and Ana Lucia Schild[3]*

ABSTRACT.- Estima-Silva P., Scheid H.V. & Schild A.L. 2020. **Causes of death in feedlot beef cattle and their control: a brief review.** *Pesquisa Veterinária Brasileira 40(8):571-578*. Laboratório Regional de Diagnóstico, Faculdade de Veterinária, Universidade Federal de Pelotas, Campus Universitário, Pelotas, RS 96010-900, Brazil. E-mail: alschild@terra.com.br

This review reports the leading causes of death in feedlot beef cattle. It describes economic losses resulting from these deaths and suggests control alternatives. Diseases associated with the respiratory and digestive systems were the most frequently observed. In different geographical areas, the importance of each one might vary. Outbreaks of diseases such as botulism occur occasionally and can cause important economic losses. Cattle tick fever can cause significant losses in zones of enzootic tick instability. Technical assistance and sanitary and food management are critical for the best productivity in feedlot cattle.

INDEX TERMS: Feedlot, cattle, morbidity, mortality, pneumonia, acidosis, bloat.

RESUMO.- [**Causas de morte em bovinos de corte confinados e seu controle: uma breve revisão.**] Esta revisão discute as principais causas de morte em bovinos de corte em confinamento. Descreve as perdas econômicas resultantes dessas mortes e sugere alternativas de controle. As doenças associadas aos sistemas respiratório e digestivo foram as mais frequentemente observadas. Em diferentes áreas geográficas, a importância de cada uma pode variar. Surtos de doenças como o botulismo ocorrem ocasionalmente e podem causar importantes perdas econômicas. A tristeza parasitária bovina pode causar perdas significativas em zonas de instabilidade enzoótica do carrapato. A assistência técnica e um bom gerenciamento sanitário e alimentar são essenciais para a melhor produtividade em bovinos de corte confinados.

TERMOS DE INDEXAÇÃO: Confinamento, bovinos, morbidade, mortalidade, pneumonia, acidose, timpanismo.

INTRODUCTION

A feedlot is a system for raising a significant number of cattle with high-cost control in restricted areas, feeding them with commercial rations or rations produced in the premises (Barbieri et al. 2016). The main objective of the feedlot is to obtain finished cattle for slaughter throughout the year, including those periods of forage scarcity, and depending to a minimum on pastures and favorable climatic conditions. Besides, feedlots are used for stock and quarantine beef cattle before the export of livestock (Bailone 2019), and for raise and hold breeding bulls (Malafaia et al. 2016).

The first feedlot on record dates from 1876 in the United States, when a large number of cattle were fed simultaneously on grain in a crop farm close to slaughterhouses. Thee feedlot practice was then an alternative to extensive livestock farming, which would be too distant from slaughterhouses and, thus, too expensive (Hubbs 2010). Subsequently, in the 1950s and 1960s, feedlots emerged as a result of new technologies and improved grain production with more productive crops (Hubbs 2010). Currently, most of the finishing of cattle for slaughter in the United States occurs in feedlots (Edwards 2010) since farmers raise more than 75% of calves for meat production in this system (Grandin 2016). In Australia, the feedlot industry started in the 1960s and has kept on expanding until today, having increased from 750,000 feedlots (comprising 3.4% of the country's total cattle herd) in the 1990s to 2.8 million (12%) in 2015 (Mayberry et al. 2019).

In Brazil, the first known beef cattle feedlot to exist was created in 1961 in the city of Ourinhos, state of São Paulo, as an alternative for finishing cattle during the dry season (USP-ESALQ 2019). Since the 1970s, there has been an exponential increase in the Brazilian bovine population. In the same period, there was also an expansion of agricultural areas facilitated by new technologies that allowed plowing and sowing of large plots of land in a short period. This took

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over extensive areas for crops leaving increasingly lesser space for livestock. The search for an alternative resulted win a steep increase in the number of cattle raised in the feedlot system (Rezende 2010), and since 1980, feedlots became a real alternative to cattle raising (Malafaia et al. 2016). In 2018, 12.6% of the 44.23 million cattle officially slaughtered across the country were finished at feedlots (ABIEC 2019).

Traditionally, in the State of Rio Grande do Sul (RS), in the southern region of Brazil, cattle ranching has always been associated with large extensions of land. Cattle were raised grazing on native or cultivated pastures or rice or soybean crop leftovers. However, in recent years, the number of feedlots with different purposes has increased in the region (Estima-Silva et al. 2020).

With the advancement of feedlot systems in the world, cattle diseases became an increasing concern for producers and veterinarians. A review (Kelly & Janzen 1986) on diseases of feedlot cattle has shown that cattle morbidity in these systems can reach 69%, ranging from 15-45%. Regarding mortality, the rate reached up to 15%, with most reports mentioning 1-5% mortality. The same authors observed that the peak of disease occurred in the first three weeks of admission to the feedlots, and the leading conditions were those affecting the respiratory system (Kelly & Janzen 1986). In Brazil, in a feedlot in the Southeast region, morbidity rates of 7.05% and mortality of 0.64% have been reported (Baptista et al. 2017). In Minas Gerais, the morbidity of 7.76% and mortality of 1.5% were observed (Martins 2016). In the southern region of RS, mortality rates of 0.11% and 1.33% were found in feedlots for the export of live animals and for finishing cattle, respectively (Estima-Silva et al. 2020).

This paper aimed to review the leading causes of death of feedlot beef cattle. It was also our intention to demonstrate the economic impact that they cause in the livestock industry and how to assist veterinary practitioners and farmers, offering solutions to reduce financial losses in their farming systems.

POTENTIAL CAUSES OF MORTALITY IN FEEDLOT BEEF CATTLE

Feedlot cattle are more susceptible to infections respiratory diseases such as pneumonia and non-communicable diseases of the alimentary tract such as lactic acidosis and bloat (Smith 1998, Borges & Afonso 2007). There are reports of occasional outbreaks of other conditions affecting cattle in feedlots with significant losses, such as botulism in Rio Grande do Sul (Maboni et al. 2010) and the Midwest (Soares et al. 2018, Guizelini et al. 2019) regions and tick fever in RS (Estima-Silva et al. 2020).

Pneumonias

Respiratory diseases (Fig.1) are the leading morbid conditions in feedlot cattle in the United States (Sanderson et al. 2008, Edwards 2010, Wilson et al. 2017), Australia (Cusack 2004), Canada (Church & Radostits 1981, Smith 1998), and Brazil (Malafaia et al. 2016, Baptista et al. 2017). These diseases impact the meat industry, with direct losses due to deaths, treatment, and human resources and indirect losses associated with low animal development, increased permanence of cattle in the feedlot, and lower carcass yield (Smith 1998, Wilson et al. 2017). Despite advances in management and new treatment protocols and vaccines, the cumulative incidence of bovine respiratory disease (BRD) in feedlot cattle has not decreased over the past 30-40 years (Edwards 2010).

The causes of BRD are complex and associated with several viruses, namely bovine respiratory syncytial virus (BRSV), bovine herpesvirus-1 (IBR), bovine viral diarrhea virus (BVDV) and parainfluenza virus type 3 (PIV-3). Such viruses establish a favorable environment for the colonization and replication of pathogenic bacteria such as Pasteurella multocida, Mannheimia haemolytica, Streptococcus pneumoniae, Mycoplasma bovis, and Histophilus somni, resulting in pneumonia (Urban-Chmiel & Grooms 2012). These diseases represent 45% of the causes of death in feedlot cattle in the United States (Edwards 2010, Cernicchiaro et a.l. 2012, Avra et al. 2017) and approximately 75% of the total morbidity (Edwards 2010). In Australia, the mortality rate reaches 50% (Hay et al. 2016). In one study the Midwest region of Brazil (Malafaia et al. 2016), 45% of the diagnoses of diseases in feedlot cattle were BRD, and in one feedlot in the Southeast (Baptista et al. 2017), 87% of the total diagnoses were pneumonia and mortality from this disease was 20%. In the Southern region of RS, over 20 years, BRD represented 11% of all illnesses that causing death in cattle from several different feedlot systems (Estima-Silva et al. 2020).

The economic impact of pneumonia on feedlot cattle. Costs associated with respiratory diseases in feedlot beef cattle in the United States is estimated at 4 billion dollars a year, including, in addition to deaths, costs of treatment, prevention, and loss of productivity (Cernicchiaro et al. 2012). In the USA, in a study on treatment failure of these diseases (Avra et al. 2017), the authors describe an increase of approximately 85% in treatment costs over 12 years, that is, it was US $ 12.59 per bovine in 1999 and $ US 23.60 in 2011. In a study carried out in Brazil (Malafaia et al. 2016), the economic impact with treatment for BRD was, on average, $ US 21.70 per cattle with losses on average of 13.4kg body weight per animal. In another study in cattle feedlots in the Southeast region of Brazil (Baptista et al. 2017), treatment



Fig.1. Thorax cavity from a feedlot steer that died from pneumonia. There are dark areas of consolidation affecting all pulmonary lobes. Adjacent areas show checkers pattern with intercalated dark and pale areas.

costs were US $ 26.62 per animal and losses due to mortality were US $ 682.40 per animal; the calculation included the treatment, and the human resources cost added to the value of the animal. The relevance of respiratory diseases and their economic impact on the management of feedlot cattle has led to the development of programs seeking to minimize the costs in these types of cattle raising systems (Edwards 2010).

Control alternatives for pneumonia in feedlot cattle. The first 45 days of arrival at the feedlot are critical to the occurrence of BRD since this is a period of stress caused by weaning, the shipment of cattle to the feedlot, nutritional changes, and handling of animals shortly after arrival (Smith 1998, Edwards 2010). To reduce the losses, the administration of antibiotics to cattle on arrival at feedlots (metaphylaxis) have been advised, regardless of whether these animals show any clinical signs of illness (Catry et al. 2008, Edwards 2010, Nickell & White 2010, Rezende 2010). This way of preventing pneumonia in feedlot cattle is deemed efficient considering the drop in rates of morbidity and mortality due to BRD and the increase in weight gain of treated animals, which makes the practice economically viable (Catry et al. 2008, Edwards 2010, Nickell & White 2010, Rezende 2010). The use of a metaphylaxis program shortly after the arrival of cattle to the feedlot reduced the occurrence of BRD by 50% and the mortality from this disease by 25% (Urban-Chmiel & Grooms 2012). Rezende (2010), in an experiment performed in feedlots in the Midwest region of Brazil, observed that a single application of florfenicol to a group of cattle at their entrance to the feedlot was effective in reducing BRD morbidity by 54%. Also, an increase in weight gain occurred, indicating this treatment as economically viable when BRD morbidity rates are higher than 2.7%. However, the use of antibiotics is not free of secondary problems. Antibiotic residues in the meat and inducement of resistance to the active ingredient are threats that linger on (Moreno & Lanusse 2017).

Vaccines can be used to minimize the occurrence of BRD in feedlot cattle. Currently, vaccines against the different viral and bacterial agents involved in BRD are widely available in Brazil. Proper use of these vaccines reduces the risk of disease (Urban-Chmiel & Grooms 2012). In a study on BRD mortality in feedlot cattle, those who were not vaccinated were 11.95 times more likely to die from BRD than those who were (Estima-Silva et al. 2020). Mortality was significantly higher in non-vaccinated finishing feedlot cattle in comparison to in younger, more susceptible animals vaccinated at arrival at the feedlot (Estima-Silva et al. 2020). However, the stress experienced by cattle during the first days at the feedlot can compromise the efficiency of the immune response to the vaccine (Edwards 2010, Urban-Chmiel & Grooms 2012). Two vaccinations performed 60 and 30 days before the entrance to the feedlot is more efficient than a single dose vaccination on arrival at the feedlot. In the latter case, cattle were 2.5 times more likely to die from BRD than those of animals on the arrival at the feedlot or 14 days after the entry did not significantly influence the occurrence of the disease (Richeson et al. 2008). In general, vaccine protocols show controversial results concerning BRD morbidity and mortality in confined cattle (Edwards 2010).

The increase in the efficiency of the BRD vaccine has been evident when it is performed during pre-conditioning programs. This pre-conditioning consists in performing surgical procedures, such as dehorning and castration, treating the animals with anthelmintics, and adapting them to feed in troughs and drinking fountains approximately 30-45 days before entering the feedlot. This is a strategy used in the United States that aims to reduce the stress of cattle due to the sudden change of feed and surgical procedures necessary on arrival at the feedlot. These animals have greater sale values, but, at the end, they are more profitable since the morbidity and mortality due to BRD and other diseases decrease (Urban-Chmiel & Groons 2012).

BRD is a multifactorial disease complex of great importance in feedlot systems in general. Several viral and bacterial agents are involved in the pathogenesis of BRD. Consequently, BRD requires the use of different strategies for its proper control and reduction of economic losses. Several studies report the optimal time for vaccination or the use of metaphylaxis, or even adopting pre-conditioning programs that can make a difference in the morbidity and mortality rates for BRD. This pre-conditioning can also be significant for animal welfare, since the cattle to a new feeding system in the period before confinement, can avoid the stress produced by all these practices on arrival at the feedlot (Mota & Marçal 2019).

Digestive tract disorders

Acidosis is one of the most common gastrointestinal disorders of feedlot cattle since grain feeding became a widespread practice (Nagaraja & Lechtenberg 2007, Snyder & Credille 2017). In Brazilian feedlot cattle, ruminal acidosis is second only to pneumonia as the leading cause of morbidity and mortality (Malafaia et al. 2016). In one study, done in feedlot cattle for finishing or export, acidosis was the leading cause of death, surpassing pneumonia (Estima-Silva et al. 2020).

Ruminal acidosis results from the consumption of high amounts of rapidly fermentable carbohydrates (CH). (Galyean & Rivera 2003, Nagaraja & Lechtenberg 2007). Ruminal bacteria respond to the increased availability of fermentable substrates by increasing growth rates and fermentative activities, that is, the introduction of this highly fermentable CH in the diet leads to a reduction in fibrolytic bacteria, the rapid growth of amylolytic bacteria and a decrease in ruminal pH (Bevans et al. 2005). Although many ruminal bacteria can use starch, the explosive growth of Streptococcus bovis, in response to the availability of fermentable CH, is observed only in situations where the ruminant is not adapted to the grain or during the period of intensification of feeding (Nagaraja & Lechtenberg 2007). Acute acidosis (Fig.2), which causes mortality, is due to excessive CH intake when animals are not adapted to such a diet, when there is a change of diet after fasting or when an already adapted bovine abruptly ingests high amounts of highly fermentable carbohydrate (Borges & Afonso 2007). In acute acidosis lactate and volatile fatty acids accumulate, resulting in an increased osmotically ruminal content. Lactate also causes damage to the epithelial lining of ruminal mucosa. Those changes together lead to extensive extravasation of liquid into the rumen resulting in marked dehydration that may be fatal (Owens et al. 1988). Ruminal acidosis can, therefore, be defined as a ruminal fermentation disorder characterized by below the normal ruminal pH, reflecting an imbalance between microbial production, microbial utilization, and ruminal absorption of volatile fatty acids (Hernández et al. 2014).

Another frequent digestive disorder in intensively bred cattle is frothy bloat (Fig.3) resulting from the fermentation of grain feed, with excessive production of gases, mainly carbon dioxide and methane that mix with the rumen content forming the foam (Meyer & Bryant 2017, Abdisa 2018). Physiologically, these gases are produced by the action of bacteria and rumen protozoa on fermentable foods. The gases are expelled by absorption through the rumen wall, passing to the next stomach compartment or by eructation through the esophagus, which is the main route of expulsion (Meyer & Bryant 2017, Abdisa 2018). The foam pathologically accumulated in bloat is due to a bacterial mucopolysaccharide that originates in the bacterial wall or in its cytoplasm. The increased production of this mucopolysaccharide, due to the increase in ruminal bacteria, turns the ruminal fluid thick and viscous, making it difficult to separate it from the gas in the rumen content resulting in foam (Borges & Afonso 2007).

Reports on morbidity and mortality rates of digestive disorders in feedlots cattle are variable. In the USA, it is estimated that approximately 4.4% of feedlot cattle are diagnosed with digestive disorders (USDA 2011). The same report states that 14-42% of deaths in feedlot cattle are due to these problems, making digestive disorders (Smith 1998, Nagaraja & Lechtenberg 2007, Snyder & Credille 2017), which would make these disorders the second most important cause of mortality in feedlot cattle. Others report mortality rates from digestive disorders as 19.5%-28.4% of all diseases diagnosed in American feedlot cattle (Meyer & Bryant 2017). These latter authors found 96.3% of cases of bloat and 3.7% of cases of ruminal acidosis.

In Brazil, rates of 31%-32.7% were found among all observed diseases in feedlot finishing cattle and breeding bulls (Malafaia et al. 2016). In the South of Rio Grande do Sul, digestive diseases represented 57.3% among all diseases that caused the death of cattle in different feedlot systems over 20 years. Of those deaths, 52.2% resulted from acidosis and 45.8% from bloat (Estima-Silva et al. 2020).

Control alternatives to digestive disorders. The acute course of digestive disorders is challenging to timely diagnosis and treatment (Meyer & Bryant 2017). Ruminal acidosis and bloat are complex and overlapping disorders, which makes it virtually impossible to establish a single management practice in their control (Cheng et al. 1998, Meyer & Bryant 2017). The control of acidosis clearly depends on proper nutritional management and adaptation of animals to a high CH diet; the disorder can, however, occur even when cattle are gradually adapted to a grain diet (Bevans et al. 2005). The adaptation consists of decreasing the concentration of roughage and gradually increasing the concentrate in the diet. To carry out the adjustment, the history of the animals, whenever available, should be considered. Cattle previously raised on pasture with no exposure to concentrated feed are more susceptible to changes in ruminal pH and, therefore, more vulnerable to acidosis and bloat. Adaptation to the concentrate should last a minimum of 3-4 weeks; s cattle adapted for shorter periods remain more susceptible to digestive disorders (Nagaraja & Lechtenberg 2007, Hernández et al. 2014, Meyer & Bryant 2017).

Basically, the percentage of fiber in the feed at the beginning of the adaptation is 45% to 55% (Meyer & Bryant 2017). Studies have shown that the provision of highly concentrated diets *ad libitum* during the first weeks of adaptation results in a drastic reduction in food intake (Schwartzkopf-Genswein et al. 2003, Brown et al. 2006, Meyer & Bryant 2017). A comparative study between rapid versus gradual adaptation of feedlot heifers to a diet that increased from 40% to 90% in concentrate is reported (Bevans et al. 2005). In both types of adaptation, concentration went from 40% to 905% of dry matter. In fast adaptation, the percentage of concentrate went from 40 to 90% in 3 days with an intermediate phase of 65% of concentrate for three days. In the gradual transition, the concentrate levels increased by 48.3%, 56.7%, 65.0%, 73.3%, and 81.7% every five days until reaching 90%. The authors concluded that the majority of heifers were able to adapt quickly, requiring few steps of gradual increase in the diet. However, ideal adaptation should favor those most vulnerable individuals to prevent outbreaks.

The association of concentrate with roughage is therefore essential to avoid morbidity from digestive diseases in feedlot cattle since fiber is necessary to stimulate ruminal movements and promote saliva production. Roughage dietetic levels



Fig.2. Rumen from a feedlot steer that died from acute ruminal lactic acidosis. The ruminal content is abundant, liquid, and yellow-green.



Fig.3. Rumen from a feedlot steer that died from frothy bloat resulting from grain overload. Ruminal content is yellow and frothy.

decreased over the past few years due to the difficulty in storage, handling, and mixing with the concentrate. Currently, roughage corresponds to a maximum of 8% of dry matter, less than that 10%-15 % used 20 years ago (Nagaraja & Lechtenberg



Fig.4. Stool score indicating the balance between concentrate and roughage in the diet of feedlot cattle. The score ranges from 1 (excess protein or starch) to 5 (poor digestion of forage. Score 3 represents the balance in the composition of the diet (Adapted from Vigne et al. 2019). (**A**) Score 1: liquid stools; diarrheic. (**B**) Score 2: semi-liquid stools. (**C**) Score 3: pasty stools. (**D**) Score 4: Light and moderately dry stools, with concentric rings and 3-4cm layers. (**E**) Score 5: dry and hard stools.

2007). In many cases, despite the use of roughage associated with the concentrate, digestive disorders are still a significant cause of death in feedlots operations in RS (Estima-Silva et al. 2020).in feedlots operations in RS (Estima-Silva et al. 2020).

The strategy to decrease the morbidity rates of bloat in feedlot operations includes, besides the association of concentrate with roughage, the choice of the cereal to be fed. Wheat has a higher fermentation rate than corn, sorghum, and barley; thus, bloat tends to be more frequent in feedlot cattle fed wheat-based diets (Cheng et al. 1998).

The processing of cereal entering the diet of feedlot cattle also plays a part in the occurrence of both acidosis and bloat. The more finely ground the starch, the more facilitate its digestion by microbial enzymes, leading o accelerated production of organic acids and mucopolysaccharides, consequently lowering the pH and increased viscosity of rumen content (Cheng et al. 1998, Meyer & Bryant 2017).

There is a parameter called "stool score", which consists of evaluating the stool on a scale of 1-5. Score 1 represents diarrheic stools indicating excess protein or starch; score 5 indicates dry stools denoting inadequate digestion of forage by ruminal microrganisms and scarcity of degradable protein in the diet (Fig.4). This parameter aids in assessing the balance of concentrate and roughage administered to cattle maintaining the weight gain and minimizing the occurrence of digestive disorders. The ideal score is 3 (Schwartzkopf-Genswein et al. 2003, Ferreira et al. 2013, Meyer & Bryant 2017).

Trough management asseses the consumption of an ideal amount of the diet administered to animals (Schwartzkopf-Genswein et al. 2003, Meyer & Bryant 2017). This practice takes it account the daily leftover of feed in the troughs daily, observed at the same period of the day, namely Feedbunk Scoring System (Table 1). Scores correspond to 0, for a clean trough and 4 for an intact trough. Score 1 denotes the ideal diet that will avoid acidosis and maintain weight gain (Meyer & Bryant 2017).

Another popular practice of controlling digestive disorders in feedlot cattle is the use of food additives such as ionophores, mainly monensin. Those substances will affect lactic acid-producing gram-negative bacteria are effective in controlling acute acidosis and promoting significant food efficiency (Meyer & Bryant 2017). Ionophores also prevent the occurrence of bloat. Cases of bloat in feedlot cattle were decreased by 64% and 92% respectively by monensin and lasalocid (Bartley et al. 1983).

Other additives have been suggested to prevent digestive diseases in feedlot cattle, such as baking soda, mineral oil, and probiotics whose, but their results are controversial (Meyer & Bryant 2017). Some authors, however, consider these additives to be good tools to control the digestive diseases of feedlot cattle (Hernández et al. 2014, Valente et al. 2017).

Other precautions are suggested to prevent digestive diseases that cause death in feedlot cattle. Weight of livestock entering the feedlot should be uniform as possible to avoid the dominance of larger animals and monitoring animals to observe behavior and early clinical signs that might suggest bloat. When more than 3% of the animals in the flock have ruminal distension, action must be taken immediately to prevent an outbreak of bloat (Hernández et al. 2014).

Table 1. Feedbunk scoring system

Score	Description
0	No feed remaining in bunk
0.5	Scattered feed remaining. Most of the bottom of the bunk exposed
1	Thin, uniform layer of feed remaining. About one corn kernel deep
2	25 to 50% of feed remaining
3	More than 50% of feed remaining. Crown is thoroughly disturbed
4	Feed is virtually untouched. Crown of feed still noticeable

Source: Pritchard R.H. (1993).

Other diseases that cause mortality in feedlot cattle

In feedlot beef cattle, conditions such as botulism (Maboni et al. 2010, Soares et al. 2018, Guizelini et al. 2019, Le Maréchal et al. 2019) occasionally cause economic losses in feedlot beef cattle operations. The study of an outbreak of botulism in the Midwest region with morbidity and mortality of 0.4% and a financial loss of more than US $ 13,000.00 is described (Curci et al. 2013). In another outbreak in the same region (Guizelini et al. 2019), a total of 1090 out of 1700 feedlot steers died due to the ingestion of corn silage contaminated with Clostridium botulinum neurotoxin type C (Fig.5). Vaccine can be effective. Still, vaccinating cattle after the outbreak started, it is not the best practice (Curci et al. 2013).

The elimination of the source of contamination of the botulinic toxin is an adequate measure (Soares et al. 2018). The storage and periodic cleaning of the food and water storage place and troughs minimize the risks of botulism outbreaks and are also part of animal welfare (Mota & Marçal 2019).

Bovine tick fever is observed mainly in feedlot cattle, the southern region of Rio Grande do Sul with mortality of up to 2.5% (Estima-Silva et al. 2020). The disease is especially important in areas of enzootic instability for the tick that results in outbreaks with considerable losses (Farias 2007). Although the feedlot environment is not ideal for the vector,



Fig.5. Botulism. Several feedlot steers are affected in a massive outbreak in Mato Grosso do Sul. A total of 1090 out of 1700 feedlot steers died due to the ingestion of corn silage contaminated with Clostridium botulinum neurotoxin type C (Courtesy of Dr. Tessie Möck, Ulmer Straße 14 89180 Berghülen, Germany).

many cattle arrive as during the incubation period of the disease (Estima-Silva et al. 2020). In these cases, chemoprophylaxis with anaplasmicidal and babesicidal drugs should be considered, which, in addition to demonstrating efficiency in controlling the disease (Estima-Silva et al. 2020), increase the weight gain of confined cattle (Silva et al. 2015).

CONCLUSIONS

The primary diseases affecting feedlot cattle are those associated with the respiratory and the digestive systems.

Technical assistance and proper sanitary and food management are critical in raising cattle in confinement for meat production, the export of live animals, or breeding.

Also, practitioners should consider endemic diseases in each region when facing mortality outbreaks in feedlots.

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Primary skin diseases and cutaneous manifestations of systemic diseases in swine[1]

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ABSTRACT.- Pereira P.R., Bianchi R.M., Hammerschmitt M.E., Cruz R.A.S., Hesse K.L., Sonne L., Pavarini S.P. & Driemeier D. 2020. **Primary skin diseases and cutaneous manifestations of systemic diseases in swine**. *Pesquisa Veterinária Brasileira 40(8):579-588*. Setor de Patologia Veterinária, Departamento de Patologia Clínica Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Prédio 42505, Porto Alegre, RS 91540-000, Brazil. E-mail: paula.rpereira@hotmail.com

Skin diseases in pigs can negatively impact the production. They cause losses related to the death of the affected pigs, to the cost with the treatment, growth retardation and condemnations in the slaughterhouses. This study was developed to determine the frequency and describe the histopathological findings of skin diseases in pigs in different age groups through a retrospective study from 2006 to 2018. A total of 154 conclusive cases were analyzed, including skin restricted diseases (allergic dermatitis, exudative epidermitis, vesicular dermatitis, pityriasis rosea, swinepox, follicular cyst, papilloma and scrotal hemangioma) or skin lesions secondary to systemic diseases (erysipelas, porcine dermatitis and nephropathy syndrome [PDNS], bacterial septicemia and multiple hemorrhages without definite cause). The skin lesions were classified as bacterial (46.1%), viral (26.6%), allergic (12.3%), neoplastic (1.3%) and others (13.6%). Swine erysipelas was the most frequent diagnosis (47/154), followed by PDNS (23/154), allergic dermatitis (19/154) and exudative epidermitis (15/154). Vesicular dermatitis (9/154), pityriasis rosea (9/154), septicemia with cutaneous manifestations (9/154), swinepox (9/154) and multiple hemorrhages without definite cause (7/154) were also observed. Follicular cyst (3/154), hyperkeratosis without definite cause (2/154), papilloma (1/154), and scrotal hemangioma (1/154) were less frequently described. Of the conclusive diagnosis, age was reported in 138 cases, with the highest frequency of skin lesions observed at the inspection process during slaughter (56/138).

INDEX TERMS: Swine, dermatitis, pathology, skin lesions, pigs, Brazil.

RESUMO.- [**Doenças primárias da pele e manifestações cutâneas de doenças sistêmicas em suínos.**] As doenças de pele em suínos podem impactar negativamente a produção. Estas causam perdas relacionadas à morte dos acometidos, a custo com tratamentos, atraso no crescimento e condenações nos frigoríficos. Este trabalho foi desenvolvido para determinar a frequência e descrever os achados histopatológicos das doenças de pele em suínos nas diferentes faixas etárias, através de um estudo retrospectivo no período de 2006 a 2018. Foram analisados 154 casos conclusivos, incluindo as doenças restritas a pele (dermatites alérgicas, epidermite exsudativa, dermatite vesicular, pitiríase rósea, varíola suína, cisto folicular, papiloma e hemangioma escrotal) e as secundárias a doenças sistêmicas (erisipela, síndrome dermatite nefropatia suína [SDNS], septicemia bacteriana e hemorragias múltiplas de causa não determinada). Estas foram classificadas em bacterianas (46,1%), virais (26,6%), alérgicas (12,3%), neoplásicas (1,3%) e outras (13,6%). A erisipela suína foi a enfermidade mais diagnosticada (47/154), seguida por SDNS (23/154), dermatite alérgica (19/154) e epidermite exsudativa (15/154). Observamos ainda dermatite

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vesicular (9/154), pitiríase rósea (9/154), septicemia bacteriana com manifestações cutâneas (9/154), varíola suína (9/154) e hemorragias múltiplas de causa não determinada (7/154). Em menor número, cisto folicular (3/154), hiperqueratose sem causa definida (2/154), papiloma (1/154) e hemangioma escrotal (1/154). Dos casos conclusivos, a idade foi informada em 138 casos, sendo a maior frequência das lesões de pele observadas na linha de inspeção, durante o abate (56/138).

TERMOS DE INDEXAÇÃO: Dermatite, patologia, lesões de pele, suínos, Brasil.

INTRODUCTION

Swine production in Brazil ranks fourth in the world for pork production and export (USDA 2019). It is considered that the Brazilian pig herd has a good sanitary condition, proven by high productivity, with rates similar to those of other countries where pig farming is also developed. Even so, the rapid and efficient diagnosis of the most varied pathologies must be prioritized (Ciacci-Zanella et al. 2016). Skin diseases in pigs can negatively impact production, because they generate treatment costs, decrease the growth rate and, sometimes, can cause the death of the animal (Turton 2001, Bender et al. 2011). Swine skin is an important by-product of refrigeration industries, as it contributes to improving the quality of some products, as well as the amount of collagen (Schilling et al. 2003). Therefore, in addition to the losses mentioned in the production, the condemning and/or impossibility of utilization of carcasses of pigs with skin lesions can also cause important economic losses (Doster 1995, Bender et al. 2011).

A variety of diseases affect the skin of pigs and can be infectious (bacterial, viral, mycotic and parasitic), or non-infectious (environmental, nutritional, hereditary and neoplastic) (Torrison & Cameron 2019). These diseases may involve only the skin or be cutaneous manifestations of a systemic disease (Torrison & Cameron 2019). However, studies characterizing histologically the skin lesions in swine, whether primary or secondary, as well as establishing the age affected are scarce in the literature. Thus, the objective of this study was to determine the frequency and describe the histopathological findings of skin diseases in pigs in different age groups, diagnosed from January 2006 to December 2018 in southern Brazil.

MATERIALS AND METHODS

From January 2006 to December 2018, the files of the "Setor de Patologia Veterinária" of the "Universidade Federal do Rio Grande do Sul" (SPV-UFRGS) were reviewed, and cases of pigs with skin lesions submitted for histological analysis were select. Samples of a scientific experiment or submitted to other tests that did not include histological analysis were excluded from the research. The selected cases were from pig farms and slaughterhouses. The protocols were reviewed, and the information regarding age, history, anatomopathological descriptions and final diagnosis were analyzed and compiled. Pigs were classified into five stages of production, according to Machado (2014), in maternity (1-21 days), nursery (22-61 days), growth (62-120 days), finishing (121-160 days) and adults (over 161 days). Samples from the slaughterhouses were categorized as slaughter. Gross lesions of the main diseases were standardized according to the data provided in the histories and, to represent them, figures from the SPV-UFRGS database were used. The archived paraffin-embedded blocks were searched for making histological slides that were stained by hematoxylin and eosin technique, for later histological description. In all cases, the diagnoses were based only on the histological lesions observed, without the use of complementary exams. The study included cases in which the lesion was restricted to the skin (primary) or a cutaneous manifestation to systemic diseases (secondary). In the latter case, other organs were also analyzed when subjected together the skin. As an inclusion criterion for cases of septicemia, it was considered the anatomopathological description of erythema or cutaneous cyanosis, especially in the extremities (Torrison & Cameron 2019), and foci of necrosis in multiple organs associated with bacterial thromboembolism (Yaeger 1995). The cutaneous diseases were classified as bacterial, viral, allergic, neoplastic and "other" (when it was not possible to insert in one of the previous categories).

RESULTS

During the period of this study, 7.782 pig tissue samples were analyzed by the laboratory, of which 167 (2.1%) corresponded to skin lesions, which included both diseases restricted to the skin and systemic diseases with cutaneous manifestations. Of the 167 cases, seven (4.2%) were inconclusive and six (3.5%) unsuitable for histopathological evaluation, due to scalding and dehairing resulting from the slaughter process.

Allergic dermatitis, exudative epidermitis, vesicular dermatitis, pityriasis rosea, swinepox, follicular cyst, papilloma, and scrotal hemangioma were the primary skin lesions diagnosed. While swine erysipelas, porcine dermatitis and nephropathy syndrome (PDNS), bacterial septicemia and multiple hemorrhages without definite cause were the main skin lesions secondary to systemic diseases.

When considering conclusive diagnoses, bacterial diseases accounted for 46.1% (71/154) of cases, and included swine erysipelas, exudative epidermitis and bacterial septicemia with cutaneous manifestations. Viral infections were also frequent (26.6%, 41/154), and represented by cases of PDNS, swinepox and vesicular dermatitis. Allergic diseases accounted for 12.3% (19/154) and corresponded to cases in which the final diagnosis was suggestive of dermatitis allergic to scabies or insect bites. Neoplastic category was the less frequent (1.3%, 2/154) and represented by papilloma and scrotal hemangioma. In the category "others", 13.6% (21/154) of the cases were included, with diagnoses of pityriasis rosea, multiple hemorrhages without definite cause, follicular cyst and hyperkeratosis without definite cause. The frequencies of the main skin diseases diagnosed in this study are shown in Figure 1. Less frequent diagnoses were follicular cyst (1.9%), hyperkeratosis without a defined cause (1.3%), papilloma and scrotal hemangioma (0.6% each).

The age or production stage of the pigs was reported in 138 of the 154 conclusive diagnoses, with greater occurrence in the slaughter process (56/138). The occurrence of the main skin diseases by pig production stage is described in Table 1. Of the cases of follicular cysts, 2/3 and 1/3 were of the slaughter and adult categories, respectively. The cases of hyperkeratosis without a definite cause had no age information. Papilloma was observed in a slaughter pig and scrotal hemangioma in an adult pig.

Swine erysipelas caused by *Erysipelothrix* spp. was the skin disease most frequently diagnosed (47/154). Grossly,

the disease was characterized by reddish multifocal lesions, with raised edges and a characteristic diamond shape (Fig.2A). Histologically, multifocal vasculitis was observed in the dermis and subcutaneous tissue, characterized by predominantly neutrophilic inflammatory infiltrate, surrounding and intermingling the blood vessels wall, associated with fibrinoid vascular degeneration and necrosis. There were also multifocal areas of necrosis in the dermis and subcutaneous tissue associated with fibrin deposition, as well as multifocal thrombosis and neutrophilic infiltrate in sweat gland ducts (Fig.2B and 2C).

The PDNS caused by porcine circovirus type 2 (PVC2) corresponded to 23/154 cases, of which 52.2% were diagnosed in the first two years of the study, 2006 and 2007. On the skin, dark red, multifocal macules and papules have been described, which sometimes coalesce to form erythematous plaques, predominantly in the pelvic limbs, ventral and perineal region (Fig.2D). Microscopically, the lesions were characterized by dermal vasculitis, associated with inflammatory infiltrate of neutrophils, lymphocytes, plasma cells and macrophages, as well as fibrinoid vascular degeneration, sometimes with multifocal hemorrhage (Fig.2E). There was also focal ulceration of the epidermis in two cases. In the kidneys, there was a marked accumulation of fibrin and neutrophils in glomeruli and in the urinary space (fibrinosupurative glomerulitis), in addition to glomerulosclerosis and membranoproliferative glomerulonephritis. There were also marked tubular ectasia, hyaline cylinders and mononuclear interstitial nephritis (Fig.2F). In 21/23 cases, granulomatous lymphadenitis was also observed, characterized by inflammatory infiltrate of macrophages and occasionally multinucleated giant cells in the nodal parenchyma.

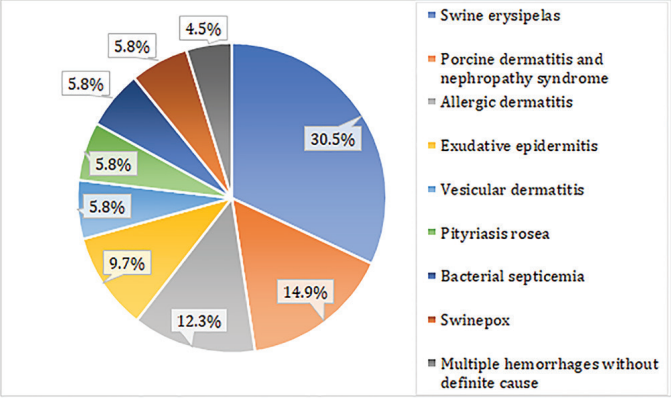


Fig.1. Frequency of the main skin diseases in swine diagnosed by the "Setor de Patologia Veterinária" of UFRGS from 2006 to 2018.

Allergic dermatitis corresponded to 19/154 cases, and were composed by small rounded, red and raised spots (Fig.3A). Histologically, they were characterized by a multifocal eosinophilic dermatitis. The inflammatory infiltrate of eosinophils, lymphocytes and plasma cells was often observed associated with weakly basophilic and myxomatous material (edema) and, sometimes, with perivascular distribution (Fig.3B). In three cases, multifocal ulceration of the epidermis was also observed, and in none of the 19 cases, mites were seen. The 15/154 cases of exudative epidermitis were characterized by marked greasy exudate and multifocal formation of crusts (Fig.3C). Microscopically, they consisted of focal areas of extensive ulceration of the epidermis, and crusted formations with marked deposition of fibrin, neutrophilic inflammatory infiltrate and coccoid bacterial aggregates. The inflammatory infiltrate also extended to the dermis and was associated with multifocal thrombosis (Fig.3D).

The cases of vesicular dermatitis (9/154) were observed only in 2015. The disease was characterized by vesicular and ulcerative lesions in the snout, hooves and coronary band (Fig.4A). It was noted that in eight cases the vesicles had ruptured and, histologically, they were composed by discrete multifocal areas of necrosis of keratinocytes, associated with infiltrate of intact and degenerate neutrophils, fibrin deposition and coccoid bacterial aggregates. In the adjacent epidermis hydropic degeneration and moderate multifocal hyperkeratosis were also observed in keratinocytes (Fig.4B). In the case where the vesicle remained intact, there was a cystic formation covered by keratinized epithelium.

Pityriasis accounted for 9/154 of the cases, initially observed as papules on the abdomen and medial face of the pelvic limbs, evolving into circular lesions outlined by a raised red border (Fig.4C). Histologically, the lesions were characterized by marked hyperplasia of keratinocytes, which formed digitiform projections towards the dermis, multifocal intracorneal pustules, in addition to marked multifocal

Table 1. Occurrence of the main skin diseases in swine at different stages of production and slaughter histopathologically diagnosed by the "Setor de Patologia Veterinária" of UFRGS during the period from 2006 to 2018

Disease	Stages of production						
	Maternity	Nursery	Growth	Finishing	Slaughter	Adults	NI*
Swine erysipelas	-	2	-	1	43	-	1
PDNS**	-	3	9	6	2	2	1
Allergic dermatitis	1	-	2	5	8	2	1
Exudative epidermitis	3	9	-	-	-	1	2
Vesicular dermatitis	9	-	-	-	-	-	-
Pityriasis rosea	-	-	9	-	-	-	-
Bacterial septicemia	5	2	2	-	-	-	-
Swinepox	-	-	-	-	-	-	9
Multiple hemorrhages	6	1	-	-	-	-	-
TOTAL	24	17	22	12	53	5	14

*NI = not informed, **PDNS = porcine dermatitis and nephropathy syndrome.

inflammatory infiltrate of neutrophils, lymphocytes and macrophages in the dermis, sometimes, with perivascular distribution (Fig.4D).

In cases of bacterial septicemia (9/154), multifocal hemorrhages were observed in the dermis and subcutaneous tissue. In other organs there were multifocal thrombosis

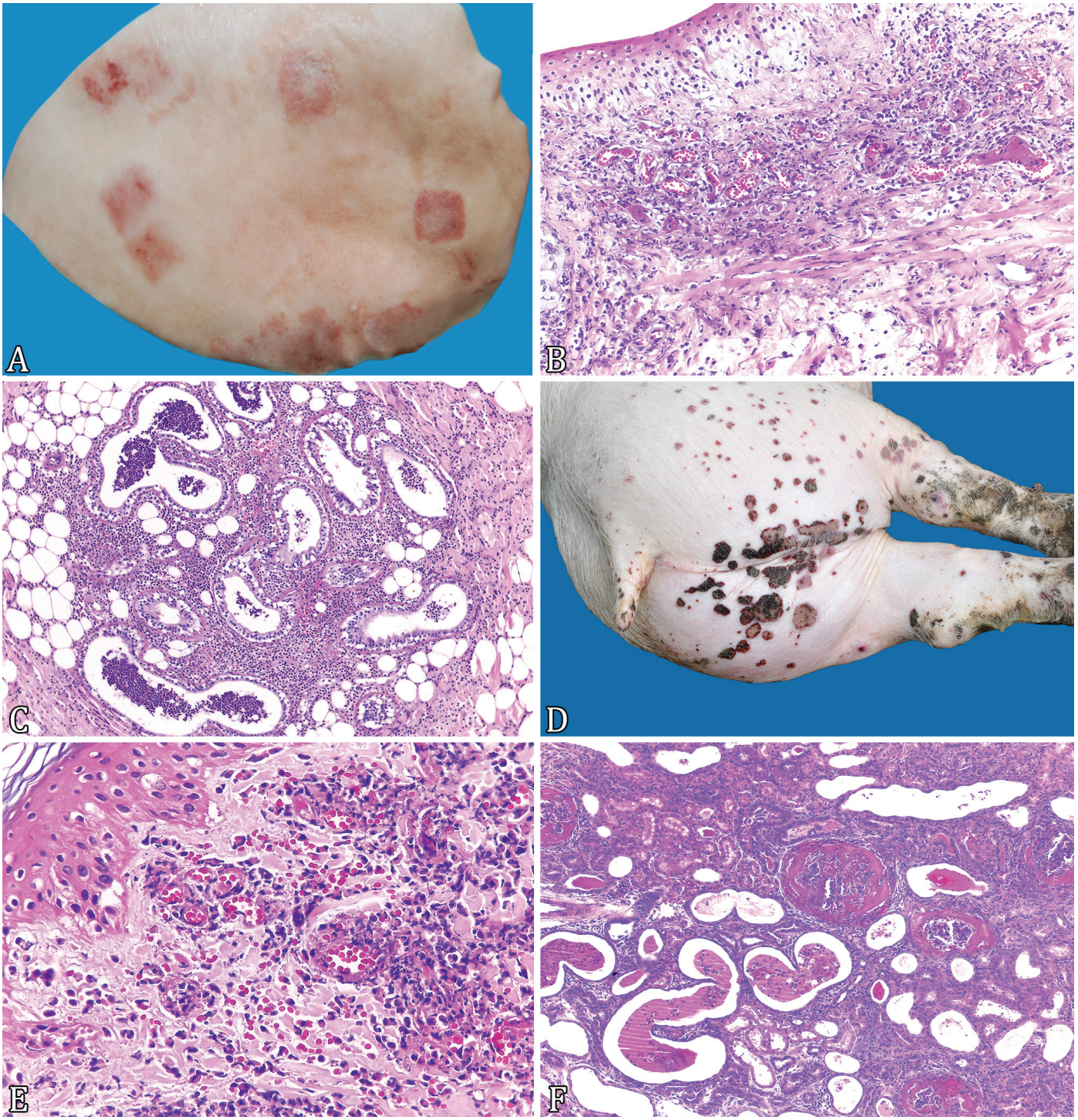


Fig.2. (**A**) Swine erysipelas grossly with reddish multifocal lesions, with raised edges and a characteristic diamond shape. (**B**) Swine erysipelas. Histologically, multifocal areas of necrosis were observed in the dermis and subcutaneous tissue, also multifocal vasculitis, fibrinoid vascular degeneration and thrombosis. HE, obj.20x. (**C**) Swine erysipelas. Marked inflammatory infiltrate surrounding and invading the sweat glands. HE, obj.10x. (**D**) Porcine dermatitis and nephropathy syndrome (PDNS). Gross appearance of the skin, dark red multifocal macules and papules in the pelvic limbs and perineal region. (**E**) PDNS. Microscopically, dermal vasculitis with mixed inflammatory infiltrate and fibrinoid vascular degeneration, also multifocal hemorrhage. HE, obj.40x. (**F**) PDNS. In the kidneys, there was a marked fibrinosupurative glomerulitis. HE, obj.10x.

and necrosis, sometimes associated with fibrin deposition, neutrophilic infiltrate and bacterial aggregates. Swinepox, also diagnosed in 9/154 cases, was initially characterized by macules that evolved to vesicles and pustules, with subsequent formation of crusts (Fig.4E). Histologically, the lesions were composed by moderate multifocal acanthosis of the epidermis with occasional vacuolization of spinous stratum cells. Sometimes intracytoplasmic eosinophilic inclusion bodies, of approximately 3µm, were also observed in keratinocytes (Fig.4F). Intracorneal pustules and necrosis of epidermis and dermis were also evidenced. Edema in the superficial dermis with infiltration of eosinophils and mast cells, compatible with allergic dermatitis was observed in eight cases. In the 7/154 cases of multiple hemorrhages without a defined cause, there was only hemorrhage and multifocal congestion in multiple organs, without associated inflammatory infiltrated or necrotic lesions.

DISCUSSION

Skin diseases can be restricted (primary) or be cutaneous manifestations of systemic (secondary) diseases. As primary, we can mention pityriasis rosea, vesicular diseases and swinepox, while secondary to erysipelas, classical swine fever (PSC) and PDNS (Torrison & Cameron 2019). In our study, as primary skin lesions, we observed cases of allergic dermatitis, exudative epidermitis, vesicular dermatitis, pityriasis rosea, swinepox, follicular cyst, papilloma and scrotal hemangioma. While erysipelas, PDNS, bacterial septicemia and multiple hemorrhages without a defined cause were secondary. These results confirm the wide variety of skin lesions that can affect pigs. Bacterial skin diseases, including primary and secondary diseases, accounted for 46.1% of the cases in this study. Similar to that described by other authors, bacterial dermatitis is considered common, unlike fungal diseases that

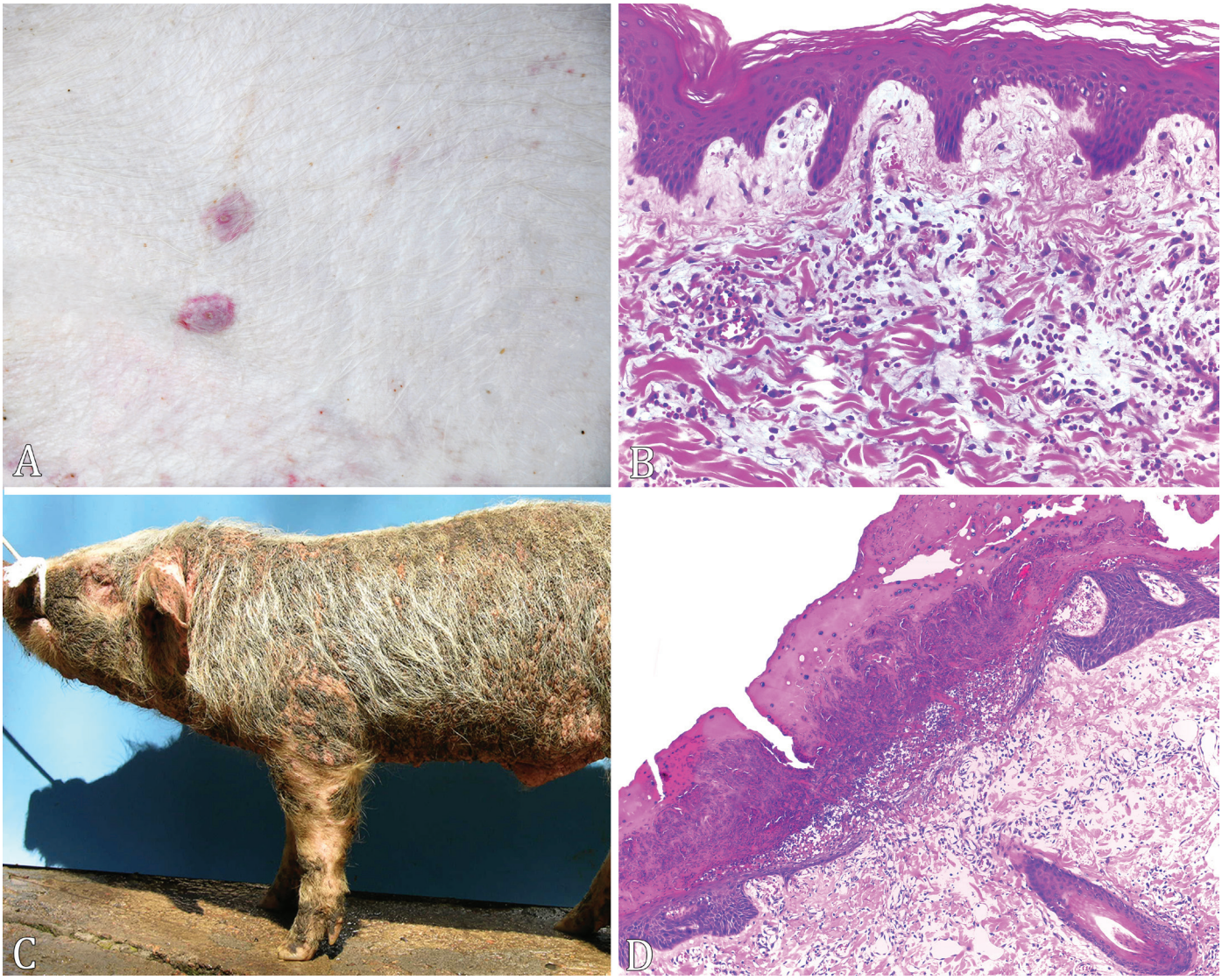


Fig.3. (**A**) Allergic dermatitis. Small round, red and raised spots grossly. (**B**) Allergic dermatitis. Multifocal eosinophilic dermatitis, sometimes perivascular, associated with edema. HE, obj.20x. (**C**) Exudative epidermitis. Marked greasy exudate and formation of crusts. (**D**) Exudative epidermitis. Microscopically observed ulceration of the epidermis, and crusted formations with marked deposition of fibrin, neutrophilic inflammatory infiltrate and coccoid bacterial aggregates. HE, obj.10x.

are extremely rare in pigs (Jackson & Cockcroft 2007) and were not observed in this research.

Swine erysipelas was the main diagnosis observed, and can affect pigs of all ages, however, there is a greater susceptibility for pigs between two and 12 months of age (Wood 1999). In our study, most of the cases were from pigs subjected to slaughter and can be justified by the worsening of the disease due to transport stress (Schwartz 2002). In the present study, histopathological diagnoses were analyzed, of which 30.5% corresponded to swine erysipelas. Most studies related to erysipelas include evaluations of carcasses in refrigerators and bacteriological examinations, and present a wide variation, with frequencies of 0.41% (Piva Filho et al. 2011) and 84.3% (Bender et al. 2011). Brum et al. (2013), when studying swine infectious and parasitic diseases, diagnosed erysipelas in 1.4% of cases. Histological findings observed in this study were similar to those described by other authors (Barman et al. 2016, Mauldin & Peters-Kennedy 2016), which were mainly characterized by vasculitis, degeneration and fibrinoid vascular necrosis, as well as multifocal necrosis in the dermis and subcutaneous tissue associated with fibrin deposition, thrombosis and neutrophilic inflammatory infiltrate in sweat gland ducts.

Exudative epidermitis corresponded to 9.7% of the diagnoses in this study, similar to that observed by other authors, with 5.4% (Brum et al. 2013) and 6.25% (Coelho et al. 2017). This disease occurs worldwide, and the main etiological agent involved is *Staphylococcus hyicus* (Frana & Hau 2019), although some studies are related to a strain VA654 of *Staphylococcus chromogenes* (Andresen et al. 2005). In our study, the affected pigs were in the maternity and nursery, as described by Frana & Hau (2019) in which pigs aged three to 32 days are the most affected. We observed a case in an adult pig, but it is known that finishing animals and adults are rarely affected (Barcellos et al. 2012). As described by other authors (Andresen et al. 2005, Frana & Hau 2019) we observed ulcerative epidermitis with crust formation, marked fibrin deposition, neutrophilic inflammatory infiltrate and coccoid bacteria, as well as multifocal thrombosis in the dermis.

In the nine (5.8%) cases of septicemia, complementary exams were not performed to establish the etiological agent. However, many agents may be involved in cases of septicemia, such as *Erysipelothrix rhusiopathiae*, *Salmonella Choleraesuis*, *Actinobacillus suis*, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, *Streptococcus suis*, *Pasteurella multocida* tipo A and *Escherichia coli* (Miniats et al. 1989, Sanford et al. 1990, Yaeger 1995, Del'Arco et al. 2008, Oliveira Filho et al. 2015, Torrison & Cameron 2019). As to the stage of production, five pigs were from the maternity, two from the nursery and two from the growth. Some of the agents that can cause septicemia mainly affect pigs in these stages, ranging from 21 days to four months of age in cases of salmonellosis by *Salmonella choleraesuis* (Torrison & Cameron 2019), two to 28 days in cases of *A. suis* (Sanford et al. 1990) and *E. coli* in newborn pigs (Yaeger 1995).

Viral diseases represented here by PDNS, swinepox and vesicular dermatitis, accounted for 26.6% of cases. PDNS was the second most frequent diagnosis, with half of the cases in the years 2006 and 2007, the lowest number observed in the following years is explained by the commercialization of vaccines from 2008 (Ciacci-Zanella et al. 2016). We observed 14.9% cases of PDNS, rates higher than those observed by Segalés et al. (1998), which reports a low frequency, usually between 0.05 and 0.5%, and Corrêa et al. (2006), in Rio Grande do Sul, which described 4.1% of PNDS cases associated with suspected necropsies of circovirus. However, the frequency of our study is very similar to that observed in the United Kingdom and other countries that had a frequency ranging from 0.25 to 20% (Gresham et al. 2000). In our study, most cases were observed in growth and finishing, agreeing, in part, with other authors, who report that this syndrome is more often described in nursery, growth and finishing pigs (Drolet et al. 1999, Gresham et al. 2000). PDNS is believed to be a type III hypersensitivity (Helie et al. 1995) and the microscopic findings observed in the kidneys, skin and lymph nodes in the cases were similar to that described in the literature, differing only in two cases, in which there was ulceration of the epidermis (Rosell et al. 2000, Wellenberg et al. 2004, Cadar 2009).

Vesicular dermatitis was associated with 5.8% of cases, a lesion that can be seen in cases of foot-and-mouth disease, swine vesicular disease, vesicular stomatitis, vesicular exanthema (Torrison & Cameron 2019), vesicular disease associated with Senecavirus A (SVA) (Segalés et al. 2017) and swine parvovirus (Kresse et al. 1985). We had information that they were negative for foot-and-mouth disease, and although there was no confirmatory examination for SVA, the involvement of this agent in our cases is suggested. These occurred in the year 2015, it was in this year and in 2014, that outbreaks occurred in Brazil of a vesicular disease of which the SVA was detected by Polymerase chain reaction (Vannucci et al. 2015, Leme et al. 2016). The affected pigs were all from the maternity, agreeing with data from the SVA outbreaks that occurred in Brazil, with the death of piglets in the first week of life (Vannucci et al. 2015, Leme et al. 2016). During the first half of 2015, of the 61 cases of swine diseases reported to the Official Veterinary Service of Rio Grande do Sul, 14.7% corresponded to idiopathic vesicular disease (Campos et al. 2016). In eight cases, the vesicles were ruptured and fibrinonecrotic dermatitis associated with bacterial colonies was observed in these areas. These histological lesions were similar to those described in the literature (Vannucci et al. 2015, Segalés et al. 2017). Segalés et al. (2017) report that ulcers begin to heal in seven days, and epithelial regeneration is usually complete within two weeks.

Swinepox corresponded to 5.8% of our diagnoses, in a study on swine diseases, it corresponded to 0.2% of the infectious and parasitic diseases diagnosed (Brum et al. 2013). This disease is caused by a poxvirus, has a wide distribution and outbreaks have already been described in São Paulo (Bersano et al. 2003, Medaglia et al. 2011), Tocantins (Bersano et al. 2003), in the Brazilian northeast (Olinda et al. 2016), Italy (Mariano et al. 2015) and India (Mech et al. 2018). The histological aspects were similar to those described by Olinda et al. (2016), of the nine cases, in eight, viral infection was associated with histological lesions suggestive of allergic dermatitis, suggesting the theory that *Haematopinus suis* lice and flies may be mechanical vectors for the transmission of the swinepox virus (Torrison & Cameron 2019).

Allergic dermatitis represented 12.3% of diagnoses, these generate economic losses due to reduced growth rate, reduced feed efficiency and loss of carcass value at slaughter

(Kessler et al. 2003, Brewer & Greve 2019). Most of the cases corresponded to finishing and slaughter pigs, similarly to what reported by Cole (1990) in cases of hypersensitivity to insect bites in slaughter pigs and for Pedroso-de-Paiva et al. (2003) with lesions indicative of scabies in the slaughter line. Microscopy showed eosinophilic dermatitis, predominantly perivascular, in addition to edema and ulceration of the epidermis in three cases (Cole 1990, Mauldin & Peters-Kennedy 2016).

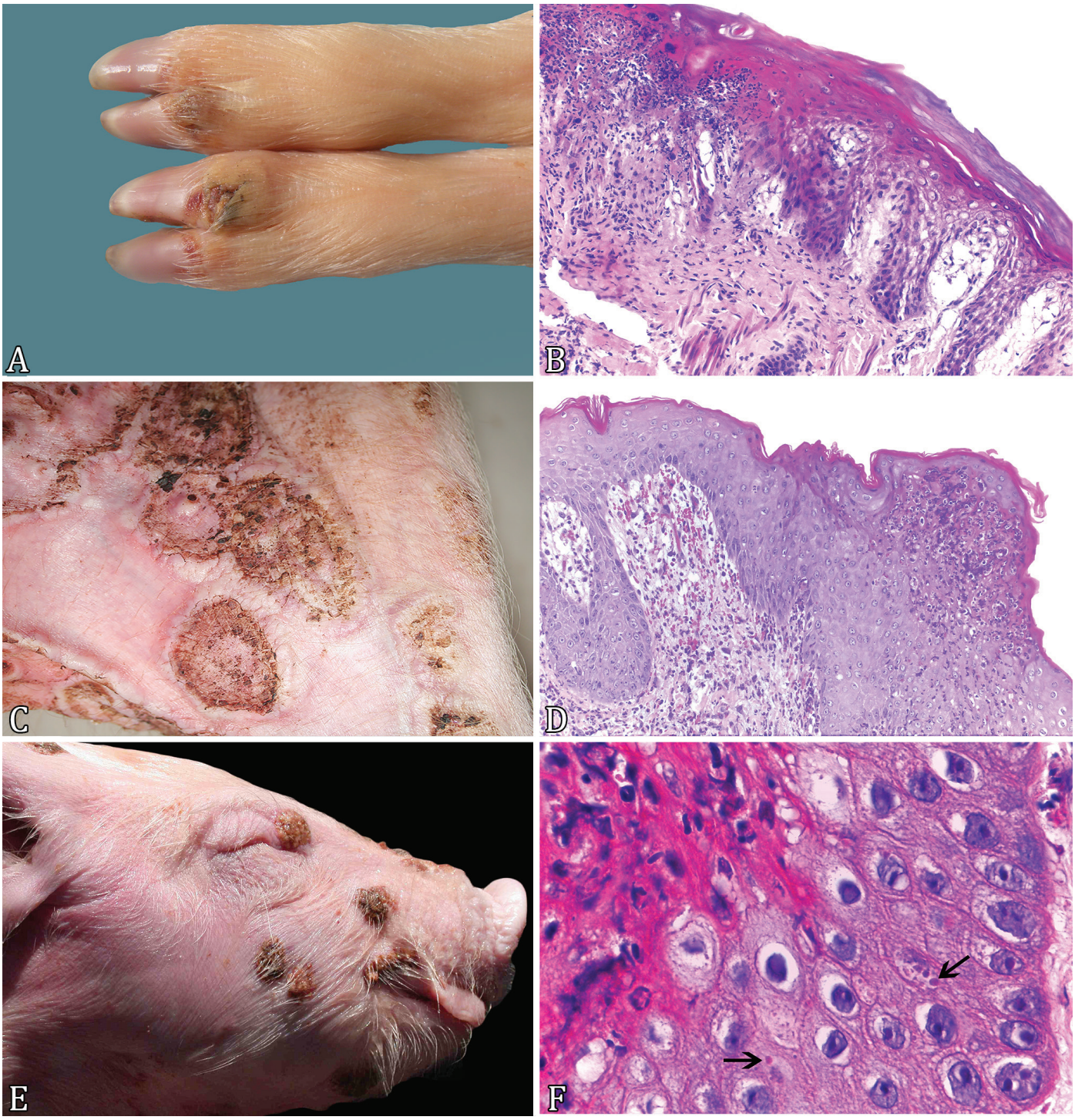


Fig.4. (**A**) Vesicular dermatitis. Ulcerative lesions in the coronary band in the hoof of a piglet. (**B**) Vesicular dermatitis. In the left corner of the figure, discrete focal necrosis of the epidermis with infiltrate neutrophils and fibrin. Adjacent epidermis hydropic degeneration in keratinocytes. HE, obj.20x. (**C**) Pityriasis rosea. Circular lesions outlined by a raised border on the abdomen and medial face of the pelvic limbs in pig. (**D**) Pityriasis rosea. Histologically, marked hyperplasia of keratinocytes and multifocal intracorneal pustules. In the dermis mixed multifocal inflammatory infiltrate. HE, obj.20x. (**E**) Swinepox. Vesicles, pustules and crusts on the face and ears. (**F**) Swinepox. Arrows show intracytoplasmic eosinophilic inclusion bodies of approximately 3µm in the cytoplasm of keratinocytes. HE, obj.100x.

In histological sections no mites were observed, however it does not mean that any of these cases cannot be sarcoptic mange, as the agent may not be detected in histology (Brewer & Greve 2019).

Neoplastic skin diseases represented a low number of diagnoses (1.3%), which was expected, since neoplasms in pigs are rare (Brum et al. 2015, Torrison & Cameron 2019). Papilloma occurred in a slaughter pig, with no information on the anatomical location, but it can affect growing pigs, especially around the neck, along the back and in the ears (Torrison & Cameron 2019). Hemangioma was observed in the scrotum of an adult pig, corroborating with Rech et al. (2013) who described that papillomas and hemangiomas can be seen in the scrotum of old necks and are considered lesions with no clinical significance. Papilloma and scrotal hemangioma represented 0.6% each of the total skin lesions diagnosed. Brum et al. (2015), in a study on swine neoplasms, diagnosed 2/37 (5.4%) cases of papilloma.

In the category "others", skin diseases were added that could not be inserted with bacterial, viral, allergic or neoplastic. Pityriasis rosea corresponded to 5.8% of diagnoses and affected animals in growth, as described by Kimura & Doi (2004), different from Altrock & Höltig (2013), who mention affecting nursery pigs. The cause of this disease is still unknown, but some authors believe that it is hereditary (Torrison & Cameron 2019), others cite *Scopulariopsis brevicaulis* as a possible agent (Purchio et al. 1980). Kimura & Doi (2004) describe three pigs and even using different methods of analysis (microbiological, histopathological, hematological and blood biochemistry) have not been able to elucidate the cause of this pathology. Histologically the lesions observed were similar to those described in the literature (Kimura & Doi 2004, Mauldin & Peters-Kennedy 2016).

In cases of multiple hemorrhages (4.5%) and hyperkeratosis (1.3%), it was not possible to confirm the diagnosis based on the information (history and anatomopathological findings) of the reports. Multiple hemorrhages in pigs can be seen in cases of coumarin poisoning (Amaral et al. 2015), purple piglet thrombocytopenia (Forster 2007), prolonged treatment with sulfas, lead poisoning, zinc phosphate, ricin, aflatoxins and ophidian accident (Sobestiansky et al. 2012). In cases of multiple hemorrhages, histology showed only hemorrhage and severe congestion. Hyperkeratosis can be observed in metabolic disorders related to nutrition, such as deficiencies of vitamin A, zinc and fatty acids, or with the formation of local calluses due to trauma associated with pressure and friction (Sobestiansky et al. 1989, Altrock & Höltig 2013, Torrison & Cameron 2019). During the performance of this retrospective study, we observed that some skin diseases, which are known to occur in swine farms, such as injuries from fights, sunburn and imperfect epitheliogenesis, were not diagnosed. We believe that the recognition of these and other injuries by employees and veterinarians of swine producing units, prevents them from being sent to the laboratory for analysis, therefore interfering with the diagnoses of this study.

CONCLUSIONS

This retrospective study demonstrated that the diagnosed skin diseases were more frequent in slaughter pigs and most of the bacterial origin.

The three main diagnoses were swine erysipelas, PDNS and allergic dermatitis. Followed by cases of exudative epidermitis, vesicular dermatitis, pityriasis rosea, bacterial septicemia with cutaneous manifestations, swinepox and multiple hemorrhages without a defined cause. Skin neoplasms in pigs were rare.

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Original Article
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Adverse effects of foot-and-mouth disease vaccine in dairy cattle[1]

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ABSTRACT.- Robattini J.A., Kumer R.M. Velho G.S., Buttelli M.M. Soares A.C., Corbellini L.G. & Dalto A.G.C. 2020. **Adverse effects of foot-and-mouth disease vaccine in dairy cattle**. *Pesquisa Veterinária Brasileira 40(8):589-592*. Setor de Grandes Ruminantes, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil. *Corresponding author: andre.dalto@ufrgs.br

Foot-and-mouth disease represents an important barrier to the international commerce of animal products, potentially associated with significant economic losses. The systematic vaccination of bovines and buffaloes was fundamental for the eradication of this disease; however, the use of vaccines can lead to reactions at the application site. The objectives of this study were to evaluate the effects of the vaccination protocol to the production of dairy cows and to observe the occurrence of vaccinal reactions in the animals. At one property located in the municipality of Salvador do Sul, Rio Grande do Sul, 270 dairy cows were vaccinated against foot-and-mouth disease in May 2019. The vaccine was administered via a subcutaneous application using disposable syringes and needles for each animal. Inspection of the animals was performed before and 20 days after the vaccination to verify the presence of reactions to the vaccine. The study's sample was set by convenience, including 203 lactating animals with or without bovine somatotropin (BST) administration during the data collection period, which was limited to 20 days before and 20 days after the vaccination. Milk production data was obtained through SmartDairy® HerdMetrix™ software, tabulated in electronic spreadsheets using Microsoft Excel® and processed using the program SAS®, considering a 5% significance level for mixed model statistical analysis. A total of 160 animals (78.82%) presented local lesions at the application site, even when the recommended vaccination practices were followed, suggesting that the high reaction power was provoked by the vaccinal components. In regards to milk production, a statistically significant ($p<0.05$) decrease of 0.30kg of milk per animal/day was observed in the average daily production in the 20 days post-vaccination. These results demonstrate the local and systemic effects caused by the foot-and-mouth disease vaccine, evidenced by reduced levels of milk production and the occurrence of vaccine reactions, implying significant economic losses.

INDEX TERMS: Adverse effects, foot-and-mouth disease, vaccine, dairy cattle, *Aphthovirus*, cattle diseases, eradication, vaccine reaction, milk production.

RESUMO.- [**Efeitos adversos da vacina contra febre aftosa em bovinos de leite.**] A febre aftosa representa uma importante barreira no comércio internacional de produtos de origem animal, podendo acarretar em significativas perdas econômicas. A vacinação sistemática de bovinos e bubalinos foi fundamental para a erradicação da doença. No entanto, a utilização de vacinas pode causar reações no local da aplicação. O objetivo deste trabalho foi avaliar os efeitos da vacina em bovinos leiteiros e observar a incidência de reações vacinais no local de aplicação. O estudo foi realizado numa propriedade leiteira do município de Salvador do Sul, Rio Grande do Sul, onde foram vacinados 270 bovinos

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contra febre aftosa no mês de maio de 2019. A vacina foi administrada por via subcutânea, com seringas e agulhas descartáveis para cada animal. Foi realizada inspeção dos animais antes da vacinação e 21 dias após a vacinação, para verificar a presença de reações vacinais. A amostra foi definida por conveniência, incluindo 203 vacas em lactação com ou sem administração de somatotropina bovina (BST) durante o período de coleta de dados, que l foi de 20 dias antes e 20 dias após a vacinação. Estes dados de produção de leite foram obtidos através do software SmartDairy® HerdMetrix™, tabulados em planilhas eletrônicas do Microsoft Excel® e processados usando o programa SAS®, considerando 5% de nível de significância para uma análise estatística modelo misto. Foi observado que 160 (78,82%) vacas apresentaram lesões no local de aplicação, mesmo quando a aplicação era realizada de acordo com as boas práticas de vacinação, o que indica o alto poder de reação provocada pelos componentes da vacina. Em relação à produção de leite, observou-se uma redução significativa (p<0,05) na produção média diária de 0,30kg de leite por animal/dia nos 21 dias após a vacinação. Esses resultados demonstram os efeitos locais e sistêmicos provocados pela vacina da febre aftosa, evidenciados pela redução na produção de leite e pela incidência de reações vacinais, o que implica em significativas perdas econômicas.

TERMOS DE INDEXAÇÃO: Efeitos adversos, vacina, febre aftosa, bovinos de leite, *Aphtovirus*, doenças de bovinos, erradicação, reação vacinal, produção de leite.

INTRODUCTION

Foot-and-mouth disease (FMD) is a viral disease caused by the *Aphthovirus*, which affects all cloven-footed animals (Constable et al. 2017). It is a highly contagious disease that requires compulsory notification and represents an essential barrier to the international commerce of animal products, possibly leading to significant economic losses (IFAH 2012).

Currently, FMD is considered eradicated in Brazil and is recognized by the OIE as a "FMD-free zone" with vaccination since 2018 (MAPA 2018a). The systematic immunization of cattle and buffalo against FMD was fundamental in its eradication and for the change to its sanitary status in Brazil. Vaccination strategies are described in the Brazilian National Program for the Eradication of Foot-and-Mouth Disease (PNEFA), and the vaccination calendar varies by state and by region, being mandatory in both bovine and buffalo of all ages in all of the national territory, with the exception of the state of Santa Catarina, which has been recognized as an FMD-free zone without vaccination since 2007 (MAPA 2007). However, FMD vaccine use can cause reactions such as abscesses at the application site, which can be related to incorrect administration and contamination at the time of vaccination (De Souza et al. 2009). Additionally, the occurrence of exacerbated reactions to the vaccine has also been related to its adjuvant, a substance called saponin (CNA 2017). In an attempt to reduce these reactions, in 2018, the dose for the FMD vaccine was lowered from 5ml to 2ml and saponin was removed from its composition (MAPA 2018b).

Previous studies have shown that these reactions can cause important economic losses due to the lowering of carcass income in beef cattle (Moro et al. 2001, França Filho et al. 2006, Leal et al. 2014). Meanwhile, in dairy cattle, no articles with proven methodology have shown the influence of FMD vaccination on milk production.

The objectives of this article were to evaluate the effects of the FMD vaccination on the milk production of high-production dairy cattle and to assess the incidence of vaccine-caused reactions at the administration site.

MATERIALS AND METHODS

A total of 270 lactating cows with an average production of 25kg of milk/cow/day were kept in a dairy farm located in the municipality of Salvador do Sul (29°26'16" S, 51°30'39" W). The cows were confined in a *Free Stall* system and milked twice daily. According to the vaccination calendar against FMD developed by the "Ministério da Agricultura Pecuária e Abastecimento", all of the cows on the farm were vaccinated in May. Vaccines were acquired and stored at 2-8ºC until administration and were applied subcutaneously at the post-scapular region using individual disposable 3ml syringes and hypodermic disposable 40x1.2mm needles for each animal.

Prior to vaccination, each animal was inspected in order to certify that there were no previous lesions present. A second inspection was conducted 20 days post-vaccination to detect the presence, dimensions, and amount of vaccinal reactions if any.

Bovine somatotropin (BST) was administered to the cows at 11-dayintervals starting at the 100th lactation day; thus cows that did not receive BST doses homogeneously during the observational period were excluded from the study. The study sample was therefore set by convenience, composed of 203 lactating animals with or without the administration of bovine somatotropin (BST) during the data collection period, a sample comprised of 139 (68.47%) Holstein cows and 64 (31.53%) Jersey cows.

For the sake of standardization, cows were sorted according to the lactation period, into either early phase (<100 lactating days), medium phase (100 to 200 lactating days), or late phase (>200 lactating days). For each cow, the momentum of vaccination was classified as "B", i.e., 20 days before, and "A", 20 days after vaccination. Individual milk production of the 203 cows was obtained from 20 days before FMD vaccination until 20 days after vaccination, through use of the SmartDairy® HerdMetrix™ software, which stores data from the Boumatic® milking machine. Data were tabulated in electronic Microsoft Excel® spreadsheets for better visualization of the herd's productive patterns and processed using the program SAS Studio® considering a 5% significance level for mixed model statistical analysis. The mixed model was fit to assess the effect of vaccination ('before' and 'after' vaccination; fixed effect) on the milk production (dependent variable) adjusted for lactation period ('early', medium', and 'late' phase) and BST usage. The model accounted for the repeated measure for each subject, using data from 203 cows with 40 observation each (n=8,120 observations). The procedure "PROC MIXED" with an 'unstructured' covariance was used. Interactions between vaccine and BST and vaccine and lactation phase were tested. The estimated marginal mean of milk production was extracted from the model using the "lsmeans" command of the SAS Studio. To analyze racial influence, the chi-square test was performed.

The current research was approved by the "Comissão de Ética no Uso de Animais" of "Universidade Federal do Rio Grande do Sul", Protocol number 23078.017735/2017-21 in a meeting held on October 25th, 2017 in Porto Alegre, Rio Grande do Sul, Brazil.

RESULTS

Of the 203 cows in this study, none had previous lesions at the vaccination site administration. At 20 days post-vaccination, 160 cows (78.82%) presented a local vaccinal reaction. Concerning breed characteristics, 83% of Holstein cows and 77% of Jersey cows presented reactions to the vaccine in the form of rounded, firm lesions, ranging from 10cm to 25cm (Fig.1).

The estimated marginal milk production before and after vaccination, adjusted for covariates (i.e., lactation period and BST) was 19.16kg (SE=0.95) and 18.86kg (SE=0.30), respectively. That means an estimated mean difference of 0.30kg of milk per animal/day to the average daily production 20 days post vaccination. This difference in milk production was statistically significant (p=0.035). Interactions between the factors vaccine/BST and vaccine/lactation phase were not significant.

DISCUSSION

Even with the altered vaccine composition, which aimed to reduce vaccinal reactions (MAPA 2018b), as well as the methodology which followed good practices and vaccination protocols, a meaningful number of vaccinal reactions (78.82%) was still found. This result differs from Moro et al. (2001), who observed only a 29.9% incidence of vaccinal reactions in culled bovine carcasses, and from Leal et al. (2014), who found reactions to the foot-and-mouth vaccine in 1,815 out of 5,000 culled bovines in a slaughterhouse (36.3%). On the other hand, an important consideration is that these studies evaluated vaccinal reactions at culling, which could have resulted in a wider time gap between vaccination and lesion evaluation, potentially leading to a misevaluation due to lesion regression. Nevertheless, the present study demonstrates that in cases of vaccine administration followed by immediate evaluation, the majority of immunized animals presented a vaccinal reaction. George et al. (1995) proved that by administering the same vaccine against clostridiosis at several different dosages, the lowest dose (2ml) resulted in less vaccinal reactions compared to the highest dose (5ml), yet similar results were not achieved in the present study since most of the herd showed reactions even with the dose reduction. Another important factor that must be considered is the route of application of the vaccine, as our study was carried out subcutaneously and according to Lima et al. (2014) it was found that the animals submitted to the application of the vaccine by the subcutaneous route showed a greater post-vaccine reaction than the animals subjected to the intramuscular route.



Fig.1. Rounded, firm vaccine reaction of approximately 25cm in Holstein cattle 21 days after immunization against foot-and-mouth disease.

The use of different disposable needles for each animal seeks to rule out contamination as a cause for vaccinal reactions (De Souza et al. 2009). Even though the reason for the occurrence of reactions cannot be determined, it is likely related to the composition of the vaccine itself: saponin was removed from the composition, but the oily adjuvant was sustained, which, according to Tizard (2002) can lead to granuloma formations at the administration site through a chronic inflammatory response. These granulomas can be either sterile or should there be contamination at administration, infected abscesses.

Both Jersey and Holstein cows presented a large number of vaccinal reactions; therefore no significant statistical difference between breeds was found in regards to vaccinal reaction prevalence, suggesting that the occurrence of these lesions does not follow any breed predisposition. Aside from the exacerbated local effects, systemic effects from the vaccination were also observed, highlighted by the significant decrease of milk production. This result should not be expected in an inactivated vaccine production such as the FMD, since these usually exhibit reactions restricted to the administration site. The possibility of systemic reactions related to the pyrogenic effect of the antigen and adjuvants has been previously mentioned in the literature, especially in oily emulsions and saponins (Martinod 1995).

In agreement with the dairy production results, Yeruham et al. (2001) observed that animals that presented vaccinal reactions to the FMD vaccine showed reduced levels of milk production in comparison with animals not presenting any vaccinal reaction eight days after vaccination. Conversely, Santos et al. (2019) found no influence of the FMD vaccination on milk production in the nine days following vaccination. Therefore, due to the scarcity of literature on the subject and the divergence of results, the current study included a wider observational period and the consideration of the influence of other factors, such as the use of BST, breed and lactation phase.

Interactions of the vaccine/BST and vaccine/lactation phase were not significant, showing that the average milk reduction seen after vaccination was similar regardless

of hormonal administration or lactation phase. Such an observation is imperative in order to validate the findings disregarding external variables, such as the lactation phase and BST administration, since these are factors that could impact the average milk production or interfere in the analysis of this study.

The economic impact on milk production of the farm of this study can be calculated by a loss of 0.30kg/milk/day/cow in 203 cows studied, represented on average a total reduction of 1,295kg of milk in the 20 days post-vaccination. As the price of milk at the time of this study was 1.60 Brazilian reais, such a decrease implied an economic loss of R$ 2,072.00 Brazilian reais during the observed period.

CONCLUSIONS

Adverse reactions of FMD vaccination occur at but is not restricted to the vaccination sites.

There are also adverse systemic reactions associated with significant losses in milk production.

Local reactions are granulomas or pyogranulomas which may influence the occurrence of systemic reactions.

Adverse reactions may occur despite good practices in vaccination.

Work presented the direct negative economic impact.

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

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Original Article
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Co-infection by *Neopora caninum* and bovine viral diarrhea virus in cattle from Rio Grande do Sul, Brazil, destined to exportation[1]

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ABSTRACT.- Alves M.E.M., Fernandes F.D., Monteiro F.L., Braunig P., Cargnelutti J.F., Flores E.F., Weiblen R. & Vogel F.S.F. 2020. **Co-infection by *Neospora caninum* and bovine viral diarrhea virus in cattle from Rio Grande do Sul, Brazil, destined to exportation.** *Pesquisa Veterinária Brasileira 40(8):593-597*. Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Avenida Roraima 1000, Santa Maria, RS 97105-900, Brazil. E-mail: fagnermedvet@gmail.com

Reproductive tests in cattle are of great economic importance, given the impact it can have on the production system and may be caused by agents. *Neospora caninum* and Bovine Viral Diarrhea virus (BVDV) are considered of great importance as reproductive and should be considered responsible for keeping animals persistently infected. The present study included 479 calf serum samples for export in the state of Rio Grande do Sul (RS). All samples were screened for BVDV by an ELISA antigen. BVDV antigen-positive ELISA samples were isolated from BVDV in cell culture. An indirect immunofluorescence (IFT) technique was used to detect anti-*N. caninum* antibodies. Of the 479 export-treated serum samples, 361 were positive for BVDV antigens by ELISA and/or viral isolation test (361/479-75.36%), and 109 IFT-positive samples for *N. caninum* (109/479-22.75%). Despite detection of antibodies anti-*N. caninum* did not differ statistically between naturally infected BVDV and non-BVDV infected animals suggesting that there is no interference of BVDV infection on infection or detection rate of animals with *N. caninum*, positive animals in viral isolation and high DO in BVDV-Ag ELISA. may present active disease and consequent immunosuppression, contributing to a potential reactivation of *N. caninum*.

INDEX TERMS: Coinfection, *Neospora caninum*, bovine viral diarrhea virus, cattle, Rio Grande do Sul, Brazil, exportation, BVDV.

RESUMO.- [**Coinfecção por *Neospora caninum* e vírus da diarreia viral bovina em bovinos do Rio Grande do Sul, Brasil, destinados à exportação.**] Testes reprodutivos em bovinos são de grande importância econômica, dado o impacto que podem ter no sistema de produção e podem ser causados por agentes. O *Neospora caninum* e o vírus da Diarreia Viral Bovina (BVDV) são considerados de grande importância como reprodutivos e devem ser considerados responsáveis por manter os animais persistentemente infectados. O presente estudo incluiu 479 amostras de soro de bezerro para exportação no estado do Rio Grande do Sul (RS). Todas as amostras foram rastreadas para BVDV por um antígeno ELISA. As amostras de ELISA positivas para o antigénio BVDV foram isoladas a partir de BVDV em cultura de células. Uma técnica de imunofluorescência indireta (IFT) foi utilizada para detectar anticorpos anti-*N caninum*. Das 479 amostras de soro tratadas para exportação, 361 foram positivas para antígenos de BVDV por ELISA e/ou teste de isolamento viral (361/479-75,36%) e 109 amostras positivas para IFT para *N. caninum* (109/479-22,75%). Apesar da detecção de anticorpos anti-*N. caninum* não diferiu estatisticamente entre animais infectados naturalmente BVDV e não BVDV sugerindo que não há interferência da infecção pelo BVDV na infecção ou taxa de detecção de animais com *N. caninum*, animais positivos em isolamento viral e alta DO em BVDV-Ag ELISA, pode apresentar doença ativa e consequente imunossupressão, contribuindo para uma potencial reativação de *N. caninum.*

TERMOS DE INDEXAÇÃO: Coinfecção, *Neospora caninum*, vírus da diarréia viral bovina, bovinos, Rio Grande do Sul, Brasil, exportação, BVDV.

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INTRODUCTION

Cattle reproductive diseases have great economic importance due to productive losses as return to estrus, abortion, long interval between births, birth of weak and unviable animals, birth reduction and increase in females discard rate (Dubey et al. 2007, Neta et al. 2010, Lanyon et al. 2014, Lilenbaum & Martins 2014). Diverse etiological agents are related to bovine reproductive diseases and the most frequent are *Neospora caninum* (NC), Bovine Viral Diarrhea virus (BVDV), *Leptospira* spp., Bovine Herpesvirus type 1 (BoHV-1), *Brucella* spp., *Campylobacter* spp., *Tritrichomonas* spp., *Chlamydia abortus*, *Coxiella burnetii* (Kirkbride 1992, Morris et al. 2018, Olmo et al. 2018, Softic et al. 2018). Among these agents NC and BVDV have significant importance because they to promote reproductive losses and have the ability to establish persistent infections (Chernick et al. 2018).

Bovine pestivirus are worldwide important pathogens of cattle promoting reproductive, enteric, hemorrhagic and/or respiratory disorders (Dubovi 1994, Pellerin et al. 1994, Flores et al. 2000, Fulton et al. 2002). Three species of pestivirus are recognized affecting bovine: Bovine viral diarrhea virus types 1 (BVDV-1, or Pestivurus A), Bovine viral diarrhea virus types 2 (BVDV-2, or Pestivirus B) and HoBi-like (Pestivirus H). The maintenance of BVDV (and HoBi-like) in herds occurred mainly by persistent infected (PI) animals, that are BVDV immunotolerant. These animals are produced when seronegative cows are infected by BVDV at 40 to 120 days of pregnancy, and after birth, calves allow the virus replication in a variety of tissues and shed virus in secretions and excretions for their lifetime (McClurkin et al. 1984). Immunosuppression is a common consequence of BVDV infection in adult animals that facilitates secondary infections and disease severity (Brownlie 1990).

NC is a protozoan belonging to the phylum Apicomplexa and family Sarcocystidae (Dubey et al. 1999), it is widely distributed in the world and considered one of the main agent causing abortion in bovines (Dubey & Schares 2011). The definitive hosts are domestic and wild canids (Gondim 2006) and NC is capable of infecting a wide range of intermediate hosts including bovine. In bovine, infection occurs via horizontal or vertical, and the vertical transmission is the most important in epidemiological aspect (Williams et al. 2009). Infection of pregnant cows promotes embryonic mortality, return to estrus, abortion, birth of weak animals, birth of animals with nervous signs and/or healthy but persistently infected animals (PI). PI calves are the main responsible for the maintenance of the agent in the herd and therefore they are extremely important in the epidemiology of the disease (Dubey et al. 2007). Besides that, transplacental infection by *N. caninum* may occur at any gestational stage and also may occur in subsequent pregnancies of persistently infected females due to the protozoa reactivation (Davison et al. 1999, Dubey et al. 2007).

BVDV frequently determine immunosuppression that contributes to subsequent infection by other agents or reactivation of latent agents and this viral infection also contributes to the severity of infection caused by other agents (Baker 1995). NC may be a secondary cause of abortion and also may potentialize abortion caused by other agents (Mineo et al. 2006, Asmare et al. 2012). BVDV and *N. caninum* co-infection is reported in other studies and suggested that should be related to higher rates of reproductive losses and consequently economic losses (Thurmond et al. 1997).

Thus, due the economic impact related to productive and reproductive losses caused by the infection with these two agents in herds, the present study aimed to determine the frequency of antibodies anti-*N. caninum*, BVDV antigen and the co-infection by *N. caninum* and BVDV in animals naturally infected destined to exportation.

MATERIALS AND METHODS

The current study included 479 sera samples of beef calves destined to exportation from Rio Grande do Sul state (RS). All samples were screened for BVDV by an antigen ELISA. Positive samples for BVDV antigen in ELISA were submitted to BVDV isolation in cell culture. Indirect immunofluorescence technique (IFT) was used for antibodies anti-*N. caninum* detection.

Samples. A total of 479 samples of beef sera were taken from calves destined for export was maintained under refrigeration (4°C) until analyses.

Antigen ELISA. All 479 sera samples (50µl) were submitted to BVDV antigen ELISA (ELISA BVDV-Ag) using IDEXX BVDV Ag/Serum Plus (code 9943830) and/or IDvet ID Screen BVDP80 Antigen Capture (code BVDAGP80-10P) kits. All procedures were performed according manufacture instructions. Samples were considered positive to BVDV antigen when corrected OD ration (OD sample - OD negative) was >0.3 and/or the S/P ration was ≥0.2, when IDEXX kit and IDVet kit were used, respectively.

Virus isolation. Virus isolation was performed into monolayers of MDBK cells by inoculating sera samples obtained from positive BVDV antigen ELISA (50µl/well of 24-wells plate). Samples were submitted to 3 passages of 5 days each. The presence of viral antigens was assessed by submitting inoculated cells (at passage 3) to an indirect fluorescente antibody assay (IFA), using a pool of BVDV-specific monoclonal antibodies (MAbs) as primary antibodies (Corapi et al. 1990, Kreutz et al. 2000) and anti-mouse conjugated with fluorescein as secondary antibody. Samples were evaluated at 400x magnification under epifluorescence microscope (Carl Zeiss - Germany, HBO 50/AC, Axiolab) using a 465-495nm excitation and 515-555nm emission filter.

Indirect immunofluorescence technique (IFT) for NC antibodies. The IFT was used to detect immunoglobulins-G against NC in the serum of animals. The IFT evaluation was performed using microscopy slides containing fixed tachyzoites of the NC-1 strain of NC. Sera samples were diluted 1:50 in PBS (pH 7.2) and incubated for 30 min at 37°C in a humid dark chamber. The secondary antibody used was bovine anti-IgG-fluorescein isothyocianate (FITC) conjugate for 30 min at 37°C in a humid dark chamber. Serum sample known as positive or negative was used as a control. Samples were evaluated at 400x magnification under epifluorescence microscope (Carl Zeiss - Germany, HBO 50/AC, Axiolab) using a 465 495nm excitation and 515-555nm emission filter. We considered positive samples reactions that showed a peripheral or diffuse fluorescence in the tachyzoites surface, in contrast to apical or polar fluorescence that were considered negative samples (Paré et al. 1995).

Statistical analysis. Comparisons between the direct ELISA results for BVDV, BVDV isolation and the detection of anti-NC antibodies were analyzed using the Chi-square test.

RESULTS AND DISCUSSION

From the 479 sera samples of calves destined to exportation, 361 were positive for BVDV antigens in ELISA and/or viral isolation test (361/479-75.36%), and 109 positive samples in IFT for NC. (109/479-22.75%). The detection of anti-*Neospora caninum* antibodies in animals naturally infected by BVDV was 23.27% (84/361) and 21.18% (25/118) in animals negative for BVDV antigen (Table 1).

The occurrence of anti-NC antibodies detected in the present study when the total serum samples were analyzed (22.75%) is in accordance with serological studies realized in some Brazilian regions/states, as Goiás (27%) (SANTIN et al., 2017) and Paraná (13.2%) (Snak et al. 2018). There was not statistical difference in the frequency detection of antibody anti-NC in animals naturally infected with BVDV (23.27%) and not infected (21.18%), suggesting that in the bovines tested, the frequency of animals persistently infected with *N. caninum* should not be influenced by previously BVDV infection.

Studies conducted by He et al. (2004) in Australia and Lassen et al. (2012) in Estonia also did not found correlation between BVDV infection and *Neospora* spp. However, Chi Duong et al. (2008) found association between the presence of anti-*N. caninum* and anti-BVDV antibodies in cows from small farms in Vietnam and this association between the occurrence of *Neospora* spp. and BVDV antibodies was been previously described by Björkman et al. (1996) in Sweden. In Brazil, Melo et al. (2004) detected anti *Neospora* spp. and anti-BVDV antibodies in milk from cows, demonstrating co-existence between these two agents in the analyzed herd. Therefore, the levels of BVDV or NC infection and co-infection are associated with herd characteristic as size, beef or milk production system and sanitary conditions (Thurmond et al. 1997).

The presence of anti-*Neospora* spp. in animals that did not ingest colostrum and non-vaccinated indicates the occurrence of persistent infection (Dubey et al. 2007). Therefore, all animals that have antibodies to *Neospora* spp. diagnosed are persistently infected. The detection of BVDV virion or viral antigens indicates both acute infection (transiently infected animals - TI) or persistent infection (persistently infected animals - PI) (Bachofen et al. 2010). Therefore, serologically positive animals for *Neospora* spp. and positive for BVDV antigen in viral isolation or ELISA-Ag tests are considered to be co infected by these two agents. The viral and parasitological coexistence should be related to a series of epidemiological factors mainly the immunosuppression determined by BVDV (Melo et al. 2004).

Detection frequency of anti-NC. in positive- BVDV animals from viral isolation was 18.75%, whereas positive - BVDV in ELISA-Ag and negative in viral isolation were 23.7% (Table 2). Concomitant infection by two agentes should be related to birth reduction rate, PI and TI animals usually suffer of immunosuppression caused by BVDV infection, which probably facilitate secondary infection by other agents, as *N. caninum* (Asmare et al., 2012). Pregnant cows co-infected with *N. caninum* and BVDV (female TI, female harboring PI calf, or female PI) would have higher rates of abortion and return to estrus than monoinfected cows and this occurs because co-infections potentialize the negative reproductive effects in cattle caused by both agents (Bjorkman et al. 2000, Wouda et al. 1998, Quinn et al. 2004).

Analyzing OD results obtained in ELISA, the detection frequency of anti-Neospora spp. antibodies is higher when OD is above 1.01 (Table 3). Higher OD in direct anti-BVDV ELISA is related to higher antigen detection and consequently higher probability of BVDV PI animals (Cornish et al. 2005). When BVDV isolation in cell culture was possible, it was observed that from the six positive animals for both agents BVDV and *N. caninum*, five had high OD in ELISA (Table 3). Analyzing the negative samples, a similar tendency is observed.

Monoinfection or co-infection with *N. caninum* and BVDV is associated with reproductive losses at any stage of gestation (Wouda et al. 1998) and despite of the detection of antibodies anti-*N.caninum* did not differ statistically between animals naturally infected by BVDV and not BVDV infected suggesting that there is not interference of BVDV infection in the infection or detection rate of animals with *N. caninum*, animals positive in viral isolation and with high OD in BVDV-Ag ELISA may

Table 1. Detection of anti-*Neospora caninum* in animals naturally infected by BVDV

ELISA BVDV-Ag	Anti-*Neospora caninum* IgG			
	Positive		Negative	
	Number	Percentual (%)	Number	Percentual (%)
Positive (361)	84	23.27	277	76.73
Negative (118)	25	21.18	93	78.82
Total (479)	109	22.75	370	77.25

Table 2. Detection from viral isolation

BVDV viral isolation	Anti-*Neospora caninum* IgG			
	Positive		Negative	
	Number	Percentual (%)	Number	Percentual (%)
Positive	6*	18,75	26	81,25
Negative	78	23,70	251	76,3
Total	84	100	277	100

Table 3. Results obtained in ELISA

ELISA BVDV-Ag	Positive BVDV isolation		Negative BVDV isolation		TOTAL	anti-*Neospora caninum* IgG	
	anti-*Neospora caninum* IgG		anti-*Neospora caninum* IgG				
OD	+	-	+	-		+	-
Lower 0.3*	-	-	25	93	118	25	93
0.3-1.0	1	8	57	194	260	58	202
1.01 – 2.0	0	4	14	36	54	14	40
Above 2,01	5	14	7	21	47	12	35
TOTAL	6	26	78	251	361	109	370

* Negative in ELISA BVDV antigen.

present active disease and consequently imunnosupression as disease consequence contributing to a potential *N. caninum* reactivation. Although the samples in this study were obtained from animals destined to exportation, and the reproductive history of the original properties is unknown, the results suggested the occurrence of infection with *N. caninum* and/or BVDV and that should be related to reproductive problems and consequently economic losses.

CONCLUSIONS

The present study reinforces the importance and occurrence of *Neospora caninum* and BVDV as pathogens infecting bovines and although the demonstrated low occurrence of co-infection, these agents are circulating in bovine herds and consequently causing damage to health, reproduction and animal production.

Further research should be conducted in animals infected by the two agents to more clearly determine the importance of BVDV and NC co-infection in the reproductive rates of cattle.

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

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Phenotypic and molecular characterization of erythromycin resistance in *Campylobacter jejuni* and *Campylobacter coli* strains isolated from swine and broiler chickens[1]

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ABSTRACT.- Dias T.S., Machado L.S., Vignoli J.A., Cunha N.C., Nascimento E.R., Pereira V.L.P. & Aquino M.H.C. 2020. **Phenotypic and molecular characterization of erythromycin resistance in *Campylobacter jejuni* and *Campylobacter coli* strains isolated from swine and broilers.** *Pesquisa Veterinária Brasileira 40(8):598-603*. Faculdade de Veterinária, Universidade Federal Fluminense, Rua Vital Brazil Filho 64, Niterói, RJ 24230-340, Brazil. E-mail: thomassalles@id.uff.br

Campylobacter spp. is a bacterial agent that causes gastroenteritis in humans and may trigger Guillain-Barré Syndrome (GBS) and is also considered one of the main foodborne diseases in developed countries. Poultry and pigs are considered reservoirs of these microorganisms, as well as raw or undercooked by-products are often incriminated as a source of human infection. Treatment in human cases is with macrolide, such erythromycin, that inhibits the protein synthesis of the microorganism. This study aimed to isolate *Campylobacter jejuni* and *Campylobacter coli* from intestinal content samples of broiler chickens (n=20) and swine (n=30) to characterize the erythromycin resistance profile of the strains and to detect molecular mechanisms involved in this resistance. The minimum inhibitory concentration was determined by agar dilution. The Mismatch Amplification Mutation Assay-Polymerase Chain Reaction (MAMA-PCR) was performed to detect mutations at positions 2074 and 2075 of 23S rRNA region, in addition to PCR test to detect the *erm*(B) gene. From the intestinal content of broiler chickens, 18 strains of *C. jejuni* and two strains of *C. coli* were isolated, whereas, from swine samples, no *C. jejuni* strain and 14 strains of *C. coli* were isolated. All *C. coli* strains were resistant, and three *C. jejuni* strains from broilers chickens were characterized with intermediate resistance to erythromycin. The MIC of the strains ranged from ≤0.5mg/μL to ≥128mg/μL. All resistant strains had the A2075G mutation, and one strain with intermediate resistance had the A2075G mutation. However, the A2074C mutation and the *erm*(B) gene were not detected. High resistance levels were detected in *C. coli* strains isolated from swine. The MAMA-PCR is a practical tool for detecting the erythromycin resistance in *Campylobacter* strains.

INDEX TERMS: Phenotype, molecular characterization, erthromycin resistance, *Campylobacter jejuni*, *Campylobacter coli*, strains, swine, broiler chickens, foodborne pathogens, MAMA-PCR, macrolides, A2075G.

RESUMO.- [**Caracterização fenotípica e molecular da resistência à eritromicina em cepas de *Campylobacter jejuni* e *Campylobacter coli* isoladas de suínos e frangos de corte.**]

Campylobacter spp. é um agente bacteriano causador de gastroenterite em humanos e associado à síndrome de Guillain–Barré, sendo a campilobacteriose considerada uma das principais enfermidades de origem alimentar. Aves e suínos são importantes reservatórios desses microrganismos e seus produtos derivados crus ou mal cozidos são muitas vezes incriminados como fonte de infecção humana. A primeira escolha para o tratamento em casos humanos são os antimicrobianos da classe dos macrolídeos como à eritromicina. Dentro desse contexto, o objetivo deste estudo foi isolar *Campylobacter jejuni* e *C. coli* a partir de 20 amostras

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de conteúdo intestinal de frangos de corte e de 30 de suínos ao abate e investigar a resistência à eritromicina das estirpes obtidas e os possíveis mecanismos moleculares envolvidos nesta resistência. A concentração inibitória mínima foi determinada pela diluição em ágar e a técnica MAMA-PCR foi utilizada para detecção de mutações nas posições 2074 e 2075 da região 23s rRNA, foi pesquisado também a presença do gene *erm*(B) pela PCR. A partir do conteúdo intestinal de frangos de corte foram isoladas 18 estirpes de *C. jejuni* e duas de *C. coli*, enquanto de suínos foram obtidas 14 estirpes de *C. coli* e nenhuma estirpe de *C. jejuni*. Todas as estirpes de *C. coli* de suínos foram identificadas como resistentes e três estirpes de *C. jejuni* de frangos foram caracterizadas com resistência intermediária. A CIM das estirpes variou de ≤0,5mg/μL a ≥128mg/μL. Todas as estirpes resistentes tinham a mutação A2075G e uma cepa com resistência intermediária também apresentou a mutação A2075G. Não foi detectada a mutação A2074C ou a presença do gene *erm*(B) em nenhuma das estirpes obtidas. Os resultados revelam um alto nível de resistência em estirpes de *C. coli* isoladas de suínos frente a eritromicina. A técnica MAMA PCR utilizada se constitui em uma ferramenta prática para detecção da resistência à eritromicina em estirpes de *C. jejuni e C. coli.*

TERMOS DE INDEXAÇÃO: Caracterização fenotípica, caracterização molecular, resistência à eritromicina, cepas, *Campylobacter jejuni*, *Campylobacter coli*, suínos, frangos de corte, patógenos de origem alimentar, macrolídeos, MAMA-PCR, A2075G.

INTRODUCTION

Campylobacter spp. is a bacterial agent that causes gastroenteritis in humans and despite its importance in unique health, studies on human campylobacteriosis in Brazil are scarce. However, in the European Union (EU), campylobacteriosis is the leading disease that has its agent transmitted by food, since 2004 (Gibbons et al. 2014, EFSA & ECDC 2018a, 2018b). Among the main reservoirs of these microorganisms, birds and pigs stand out, with the same genotypes of *Campylobacter* spp. being reported in some countries, circulating between humans and domestic animals, thus reinforcing their zoonotic potential. The transmission of microorganisms to people can occur through the ingestion of contaminated animal products and direct contact with animal feces (Wilson et al. 2008, Rosner et al. 2017, Asakura et al. 2019). Human campylobacteriosis is usually self-limiting, but in some cases treatment is performed with macrolide antibiotics (Bolinger & Kathariou 2017).

The use of (WHO 2013), especially in farm animals, allow the selection of resistant strains in livestock. Thus, there is the possibility of contamination of animal origin products with resistant strains, which can lead to the subsequent infection of people who consume these products (Pyörälä et al. 2014). Antibiotics of the macrolide class can act by inhibiting protein synthesis in the 50s subunit of ribosomal RNA by interrupting peptide translocation that prevents protein synthesis. Erythromycin is considered to be the representative of this class (Vázquez-Laslop et al. 2018).

The primary mechanism that confers high levels of resistance to macrolides in *Campylobacter* spp. involves a modification of the antimicrobial binding site to the ribosome by a point mutation at the target site in the peptidyl transferase region regarding the 23S region of the ribosomal RNA gene. In this mechanism, adenine is replaced by guanine at position 2075 and/or by cytosine at position 2074 (Alonso et al. 2005, Ladely et al. 2009). At the same time, low levels of resistance to macrolides occur due to mutations in genes that encode ribosomal proteins, such as L4 and L22 (Luangtongkum et al. 2009). For many years, it has been accepted that high levels of resistance to macrolides in *Campylobacter* spp. occurred exclusively due to mutations in the 23S rRNA region associated with efflux pumps, such as *Cme*ABC. However, Qin et al. (2014) identified the presence of the *erm*(B) gene in a strain of *C. coli*. This gene encodes a methylase that mediates high resistance levels to macrolides and can be transferred through bacterial transformation, a frequent mechanism in *Campylobacter* spp. (Wiesner et al. 2003). Through horizontal transmission, there is a higher possibility of spread resistance among strains (Qin et al. 2014, Wang et al. 2014). To date, strains of *Campylobacter* spp. possessing the *erm*(B) gene has only been described in isolates in China, Spain, and the United States of America (USA) (Qin et al. 2014, Wang et al. 2014, Florez-Cuadrado et al. 2016, Zhang et al. 2016, Chen et al. 2018).

The detection of these point mutations can be performed through sequencing, a technique with high accuracy, high cost, and limited availability in laboratories. Therefore, Alonso et al. (2005) described a specific Polymerase Chain Reaction (PCR) to detect mutations in the 23rRNA region, called Mismatch Amplification Mutation Assay-PCR (MAMA-PCR). This technique can detect these point mutations without the need for DNA sequencing, once these mutations are already known. The MAMA-PCR uses a conserved forward primer from the 23S rRNA region in conjunction with the ERY2075-R/ERY2074-R reverse primers to detect A2075G/A2074C mutations. A 485 bp PCR product is generated when the isolates have the corresponding mutation (Han et al. 2016). Several studies have already reported the efficiency of MAMA-PCR for detecting mutations in the 23S rRNA region when compared to DNA sequencing techniques (Qin et al. 2011, Giacomelli et al. 2012, Maćkiw et al. 2012, Han et al. 2016, Zhang et al. 2016).

This study aimed to isolate *Campylobacter* spp. from the intestinal content of swine and broiler chickens, characterize the profile of resistance to erythromycin of these strains, and to detect molecular mechanisms involved in this resistance.

MATERIALS AND METHODS

Material collection. The project was submitted to and approved by the Animal Ethics Council of "Universidade Federal Fluminense" (UFF) under number 5223141018. From September to November 2018, intestines of 20 broiler chickens (10 animals/batch), slaughtered under state sanitary inspection, and intestinal content of 30 swines (10 animals/batch), slaughtered under federal inspection were collected, the animals came from the states of Rio de Janeiro and Minas Gerais, respectively. The intestines and intestinal contents were sent to the "Laboratório de Doenças Infecciosas", at the "Faculdade de Veterinária" (UFF,) in isothermal boxes and processed on the same day of collection.

Isolation and identification. An aliquot of the content was diluted in 3mL of sterile distilled water with subsequent filtration through a 0.65μM filter membrane (Sartorius). The filtrate was streaked on Columbia Agar (Kasvi, Brazil) supplemented with 0.4% activated carbon and selective supplement CAMPYLOFAR® (CEFAR, Brazil). The plates were incubated at 37°C under microaerophilia for

48 hours, and colonies were selected for presumptive identification, due to their morphotintorial characteristics, and later identification by PCR. According to Sambrook et al. (2006), strains of DNA were extracted by the phenol-chloroform method. Multiplex PCR was performed to identify the species (Harmon et al. 1997 modified by Aquino et al. 2002). The amplification reaction was performed with a final volume of 50µL, containing 5µL of the sample DNA, 1X PCR Buffer (500mM KCl, 100mM Tris-HCl [pH 8.5]), 5.5mM/L $MgCl_2$, 0.4µM dNTP, 0.4µM of each primer pg3 and pg50, 0.2µM of each primer C1 and C4 and 2.5U of Taq DNA polymerase (Invitrogen, Brazil). Initial denaturation was carried out at 94°C for four minutes, followed by 25 amplification cycles consisting of one minute at 94°C, one minute at 55°C, one minute at 72°C and final extension at 72°C for seven minutes. Strains of *Campylobacter jejuni* ATCC 33560 and *Campylobacter coli* NCTC 11366 were used as positive controls of the reaction.

Minimum inhibitory concentration. The sensitivity of *Campylobacter* spp. to erythromycin was determined by the antibiotic dilution method on agar to determine the Minimum Inhibitory Concentration (MIC) according to the criteria determined by the Clinical and Laboratory Standards Institute (CLSI 2010). The concentrations of erythromycin used were 128µg/mL, 64µg/mL, 32µg/mL, 16µg/mL, 8µg/mL, 4µg/mL, 2µg/mL, 1µg/mL and 0.5µg/mL. The breakpoint for erythromycin was defined according to the established by CLSI (2013), where strains with a MIC of up to 8µg/mL were considered sensitive, 16µg/mL intermediate and ≥32µg/mL as resistant (Table 1).

MAMA-PCR. Strains characterized as resistant or intermediate, by MIC, were analyzed by MAMA-PCR (Table 2), described by Alonso et al. (2005). A 23SRNA-F forward primer was used in conjunction with the ERY2075 primer to detect the A2075G mutation. In parallel, the ERY2074 primer was used to detect the A2074C mutation. A 485 bp amplicon was obtained for each reaction in the strains that presented the mutation. The PCR reaction had a final volume of 25µl containing: 1X PCR buffer (10mM Tris HCl, 1.5mM $MgCl_2$, 50mM KCl (pH 8.3), 1.5mM $MgCl_2$, 5µL of DNA, 0.2µM of the 23S rRNA-F primer and 0.2µM of ERY 2074 or ERY 2075 primer, 0.2mM of dNTP and 1U of Taq polymerase (Invitrogen, Brazil). The initial denaturation was carried out at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30s, annealing at 59°C for 30s, extension at 72°C for 45s and final extension at 72°C for 5 min.

***erm(B)* gene detection.** The *erm*(B) gene detection was performed in strains characterized as resistant or intermediate, following the PCR proposed by Zhang et al. (2016). The reaction contained 1X PCR buffer (10mM Tris HCl, 1.5mM $MgCl_2$, 50mM KCl (pH 8.3), 1.5mM $MgCl_2$, 0.2mM dNTP, 0.5mM of each primer and 1U of Taq polymerase (Invitrogen, Brazil). An initial cycle was used for denaturation at 94°C for 5 min; followed by 30 cycles of amplification at 94°C for 30 s, 60°C for 30s for annealing and 72°C for 45s for extension. The final extension was performed at 72°C for 5 min.

RESULTS AND DISCUSSION

From the intestinal content of 30 swines, 14 (46.6%) strains of *Campylobacter coli*, and none of *Campylobacter jejuni* were isolated. The presence of *C. coli* in swine is more frequent than in birds, and this microorganism can be considered a natural inhabitant of the swine intestine (Varela et al. 2007). Kempf et al. (2017) and Gebreyes et al. (2005), as well as in this study, isolated only *C. coli* from swine feces. From the 20 samples of intestinal content from broiler chickens, 18 (90%) strains of *C. jejuni* and two (20%) of *C. coli* were obtained. Frasão et al. (2015), Chen et al. (2010), and Hald et al. (2000) also reported more significant colonization of *C. jejuni* in broiler chickens when compared to *C. coli*, demonstrating that this is the predominant species in broilers.

Table 1. Strains, origin, minimum inhibitory concentration (MIC) and molecular mechanisms involved in resistance in *Campylobacter jejuni* and *Campylobacter coli* strains

Species	Origin	MIC µl/mL*	A2075G	A2074C	*erm*(B)
C. coli	Swine/MG	>128	+	-	-
C. coli	Swine/MG	>128	+	-	-
C. coli	Swine/MG	>128	+	-	-
C. coli	Swine/MG	128	+	-	-
C. coli	Swine/MG	>128	+	-	-
C. coli	Swine/MG	>128	+	-	-
C. coli	Swine/MG	>128	+	-	-
C. coli	Swine/MG	128	+	-	-
C. coli	Swine/MG	>128	+	-	-
C. coli	Swine/MG	>128	+	-	-
C. coli	Swine/MG	>128	+	-	-
C. coli	Swine/MG	>128	+	-	-
C. coli	Swine/MG	>128	+	-	-
C. jejuni	Broiler chicken/RJ	16	+	-	-
C. jejuni	Broiler chicken/RJ	16	-	-	-
C. jejuni	Broiler chicken/RJ	16	-	-	-
C. jejuni	Broiler chicken/RJ	1	-	-	-
C. jejuni	Broiler chicken/RJ	0.5	-	-	-
C. jejuni	Broiler chicken/RJ	<0.5	-	-	-
C. jejuni	Broiler chicken/RJ	<0.5	-	-	-
C. jejuni	Broiler chicken/RJ	<0.5	-	-	-
C. coli	Broiler chicken/RJ	0.5	-	-	-
C. jejuni	Broiler chicken/RJ	<0.5	-	-	-
C. jejuni	Broiler chicken/RJ	<0.5	-	-	-
C. jejuni	Broiler chicken/RJ	<0.5	-	-	-
C. coli	Broiler chicken/RJ	0.5	-	-	-
C. jejuni	Broiler chicken/RJ	<0.5	-	-	-
C. jejuni	Broiler chicken/RJ	<0.5	-	-	-
C. jejuni	Broiler chicken/RJ	<0.5	-	-	-
C. jejuni	Broiler chicken/RJ	<0.5	-	-	-
C. jejuni	Broiler chicken/RJ	<0.5	-	-	-
C. jejuni	Broiler chicken/RJ	<0.5	-	-	-
C. jejuni	Broiler chicken/RJ	<0.5	-	-	-

* Interpretation parameters according to CLSI (2013): ≤8µl/mL = sensitive, 16µl/mL = intermediate, ≥32µl/mL = resistant.

Table 2. Primers used in the investigation of molecular mechanisms in *Campylobacter jejuni* and *Campylobacter coli* resistant to erythromycin

Gene	Primer	Sequence (5' → 3')	Fragment	Reference
23S rRNA	23S RNA-F	TTAGCTAATGTTGCCCGTACCG	485 pb	Alonso et al. 2005
	ERY2075-R	TAGTAAAGGTCCACGGGGTCGC		
	ERY2074-R	AGTAAAGGTCCACGGGGTCTGG		
erm(B)	*erm*(B)-F	GAAGGAGTGATTACATGAACAA	760 pb	Zhang et al. 2016
	erm(B)-R	TCATAGAATTATTTCCTCCCGT		

The MIC of *C. jejuni* strains ranged from ≤0.5μg/mL to 16μg/mL. In Brazil, Hungaro et al. (2015) did not observe the presence of strains resistant to erythromycin in isolates from chicken carcasses, however high frequency of strains resistant to this antimicrobial were reported in *C. jejuni* (75%) and *C. coli* (60%) isolated of children with diarrhea in the state of Minas Gerais (Rodrigues et al. 2015).

The *C. coli* strains from broiler chickens showed a MIC of 0.5μg/ml, being considered sensitive, while *C. coli* strains from swines were characterized as resistant, with two strains (15.3%) showing a MIC of 128μg/ml and twelve (85.7%) with MIC ≥128μg/ml. Asakura et al., 2019 also detected resistance to erythromycin in 92% of *C. coli* strains obtained from swines in Japan. It is accepted that *C. coli* from swines has a high level of resistance to erythromycin (Egger et al. 2012), possibly due to the greater survival capacity of strains with mutations associated with resistance to macrolides when compared to *C. jejuni* (Bolinger et al. 2017). Regarding *C. jejuni*, it could be noted that the resistance to erythromycin is accompanied by a reduced ability to colonize birds, potentially contributing to the low incidence of resistance to macrolides (Bolinger et al. 2018). In the laboratory, strains of *C. jejuni* that had these mutations induced, in the 23S rRNA region, grew more slowly than their non-mutant clones and showed higher mortality, which may partially contribute to the low levels of resistance to macrolides observed in *C. jejuni* (Han et al. 2009). Several studies (Chen et al. 2010, Wang et al. 2014, Lim et al. 2016, Zhou et al. 2016, Zhang et al. 2016) have shown higher levels of resistance to erythromycin in *C. coli* compared to *C jejuni*.

In this study, the A2075G mutation was found in all strains resistant to erythromycin, possibly one of the first reports. Studies on the molecular mechanisms involved in erythromycin resistance report this mutation as the leading cause of resistance in *C. jejuni* and *C. coli*. The A2074C mutation was not identified in any resistant strain in this study, which corroborates other studies that report being rare mutations and described in a few strains in the world (Payot et al. 2004, Lim et al. 2016, Wei & Kang 2018). It was observed that a strain of *C. jejuni* with intermediate resistance had the A2075G mutation, suggesting that intermediate levels of resistance may also be related to this mutation. In the other two strains of *C. jejuni* with intermediate resistance, this mutation was not detected. Other mechanisms, such as the presence of point mutations in genes encoding ribosomal proteins and/or efflux pump mechanisms may be involved (Lehtopolku et al. 2011).

None of the strains tested had the *erm*(B) gene. Except for a description of this gene in Spain, made by Florez-Cuadrado et al. (2016) and a case of a woman who had traveled to Asia, and on returning to the USA presented a picture of campylobacteriosis from which a strain of *C. jejuni* with the *erm*(B) gene was isolated (Chen et al. 2018). Except in this case, all reports of positive strains for this gene have been detected in China (Qin et al. 2014, Wang et al. 2014, Zhang et al. 2016, Liu et al. 2017, 2019). Bolinger et al. (2018), when studying strains of *Campylobacter* spp. isolated from commercial turkey farms in the USA, as well as Kempf et al. (2017), studying strains isolated from swines in France, did not detect this gene in any of the strains that are resistant to erythromycin. The mechanism reported by these studies was also the presence of the A2075G mutation, corroborating that this is the most common resistance to erythromycin.

CONCLUSIONS

A high level of resistance (≥128μg/ml) to erythromycin was detected in *Campylobacter coli* strains isolated from swine by MIC, and the A2075G mutation was observed in all strains resistant to this antimicrobial. The A2074C mutation and the *erm*(B) gene were absent in all strains studied.

The MAMA-PCR technique is a practical tool for detecting the molecular mechanisms involved in resistance to erythromycin in strains of *Campylobacter jejuni* and *C. coli*.

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Meta-analysis of brucellosis vaccinology in natural hosts[1]

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ABSTRACT.- Carvalho T.F., Haddad J.P.A., Paixão, T.A. & Santos R.L. 2020. **Meta-analysis of brucellosis vaccinology in natural hosts**. *Pesquisa Veterinária Brasileira 40(8):604-613.* Escola de Veterinária, Universidade Federal de Minas Gerais, Av. Pres. Antônio Carlos 6627, Pampulha, Belo Horizonte, MG 31270-901, Brazil. E-mail: rsantos@vet.ufmg.br

Brucellosis is a relevant zoonotic disease for which the most important tool for control is vaccination of susceptible animals. Assessment of vaccine efficacy in natural hosts is based on prevention of abortion and *Brucella* infection in organs of immunized animals. A meta-analysis of experimental vaccination of *Brucella* spp. natural hosts was performed, including 45 PubMed and/or Scopus-indexed publications, representing 116 individual experiments. Difference of risk was calculated as an indicator of protection, and a temporal analysis (1980-2016) demonstrated that experimental vaccines tested on natural hosts provided levels of protection that were stable over the past decades. The meta-regression model developed in this study included different vaccine categories (attenuated, inactivated, mutant, subunit, and vectored) considering the difference of risk as the dependent variable. The subcutaneous route of vaccination provided better protection when compared to the intramuscular and oral routes of vaccination. Surprisingly, inactivated vaccines provided better protection than live naturally attenuated vaccine strains (spontaneous mutations) that were considered the reference, whereas subunit vaccines provided lower levels of protection. This is the first meta-analysis of *Brucella* vaccinology in the natural hosts. These results are useful for the development of new vaccination protocols for controlling animal brucellosis.

INDEX TERMS: Meta-analysis, brucellosis, vaccinology, natural hosts, *Brucella*, vaccine, immunity.

RESUMO.- [**Meta-análise da vacinologia de brucelose em hospedeiros naturais.**] Brucelose é uma doença zoonótica relevante, para a qual a vacinação de animais susceptíveis é a ferramenta mais importante de controle. Avaliação da eficácia vacinal em hospedeiros naturais é baseada na prevenção de aborto e da colonização de órgãos pela *Brucella* spp. em animais imunizados. Foi realizada meta-análise de estudos de vacinação experimental de *Brucella* spp. em hospedeiros naturais, incluindo 45 publicações indexadas pela PubMed e/ou Scopus, representando 116 experimentos individuais. Diferença de risco foi calculada como indicador de proteção e uma análise temporal (1980-2016) demonstrou que vacinas experimentais testadas em hospedeiros naturais promoveram níveis de proteção que foram estáveis ao longo das últimas décadas. O modelo de meta-regressão desenvolvido neste estudo incluiu diferentes categorias de vacinas (atenuada, inativada, mutante, subunidade e vetorial) considerando a diferença de risco como variável dependente. A via de vacinação subcutânea promoveu melhor proteção quando comparada às vias intramuscular e oral. Surpreendentemente, vacinas inativadas promoveram melhor proteção que vacinas vivas atenuadas (com mutações espontâneas) que foram consideradas como referência, enquanto vacinas de subunidades promoveram menor proteção. Este é o primeiro estudo de meta-análise da vacinologia de *Brucella* em hospedeiros naturais. Estes resultados são úteis para o desenvolvimento de novos protocolos vacinais para controle de brucelose animal.

TERMOS DE INDEXAÇÃO: Meta-análise, vacinologia, brucelose, hospedeiros naturais, *Brucella*, vacina, imunidade.

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INTRODUCTION

Brucellosis is a highly relevant zoonotic disease worldwide caused by Gram-negative coccobacilli belonging to the genus *Brucella* (Pappas et al. 2005), which has very little genetic diversity and currently includes 12 species with particular host preferences, pathogenicity potential, and phenotypic features (Gándara et al. 2001, Al Dahouk et al. 2017). Classical *Brucella* species includes: *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. ovis*, and *B. neotomae*. Human brucellosis is often associated with *B. melitensis*, *B. abortus*, or *B. suis* (Atluri et al. 2011), although there is an increasing number of reported cases of human brucellosis due to *B. canis* infection (Nomura et al. 2010, Marzetti et al. 2013).

Human infection occurs through ingestion, inhalation or contact mucosae or ulcerated skin with contaminated animal products. Occupational exposure is also a relevant risk while manipulating virulent strains in the laboratory or exposure to live attenuated vaccine strains (Young 1995, Godfroid et al. 2005, Corbel 2006, Atluri et al. 2011). The most common clinical manifestation of brucellosis in human patients is recurrent and persistent fever, but several complications may occur, including: osteomyelitis, arthritis, spondylitis, neurobrucellosis, and endocarditis (Gotuzzo et al. 1982, Rajapakse 1995, Pendela et al. 2017). *Brucella* spp. may also cause epididymitis and orchitis in man, and infect the placenta during pregnancy, although *Brucella*-induced abortion in pregnant woman is rare (Queipo-Ortuño et al. 2006).

In domestic animals, brucellosis is associated with infertility due to abortion in pregnant females or epididymitis and/or orchitis in males (Anderson et al. 1986, Carvalho Neta et al. 2010, Poester et al. 2013), resulting in highly relevant economic losses for the animal industry (Santos et al. 2013). Official control programs in several counties are based on vaccination with live attenuated vaccine strains (Olsen & Stoffregen 2005). Currently, the most common vaccine strains commercially available are *B. abortus* S19, *B. abortus* RB51, and *B. melitensis* Rev.1 (Cheville et al. 1993, Corbel 2006). Although very useful and protective, these vaccines have some disadvantages including residual pathogenicity since they may cause abortion in pregnant animals and may result in human infections (Schurig et al. 1991, Tobias et al. 1992, Elzer et al. 2002, Davis & Elzer 2002). Furthermore, in the case of S19 e Rev.1, which has smooth LPS, there is interference with routinely used serologic tests (Cheville et al. 1992, Marín et al. 1999). Therefore, a large number of studies aiming to develop new vaccine protocols for brucellosis have been published in the past years. New vaccine technologies applied to brucellosis include subunit vaccines (Wyckoff et al. 2005, Estein et al. 2009), attenuated mutant strains (Kahl-McDonagh et al. 2006, Jacques et al. 2007, Fiorentino et al. 2008, Silva et al. 2015a, 2015b), and vectored vaccine protocols (Tabynov et al. 2014, Tabynov et al. 2016).

Complete eradication of brucellosis may not be achievable particularly due to wildlife reservoirs (Grégoire et al. 2012). Antibiotic treatment for human *Brucella* spp. infection is prolonged, expensive, and it is often followed by recurrence (Solera et al. 1998). Therefore, animal vaccination is the most important tool for controlling brucellosis in endemic areas (Corbel 2006), whereas there are no vaccines available for human use so controlling animal brucellosis is the most efficient approach for mitigating risks of human infections (Godfroid et al. 2005, Ko & Splitter 2003, Ficht et al. 2009).

The mouse has been extensively used as an animal model for brucellosis, particularly for vaccinology (Silva et al. 2011a). In a previous study, we analyzed all qualified experiments for *Brucella* vaccine development using the mouse model published over the past 30 years, which did not demonstrate clear progress towards the development of new vaccine protocols (Carvalho et al. 2016). This situation may reflect lack of potential of some experimental vaccine protocols, but also limitations of the mouse model. For instance, a *B. ovis* vaccine strain (Silva et al. 2011b) that provides moderate protection in the mouse model (Silva et al. 2015b) induces sterile immunity in its preferential host under experimental conditions (Silva et al. 2015a). Therefore, in this study we performed a comprehensive search of the literature to select all qualified experiments on *Brucella* spp. vaccine development using their natural preferential hosts, followed by the development of a meta-analysis model for identifying factors that may potentially improve vaccine efficacy in natural *Brucella* spp. hosts.

MATERIALS AND METHODS

Data source. Data was extracted from scientific articles indexed by Pubmed and Scopus until May 25, 2017. The following terms were used for literature search: "*Brucella*" and "vaccine". The list of publications was manually disambiguated, remaining only publications dealing with evaluation of vaccine protection of natural hosts after experimental challenge with pathogenic strains of *Brucella* spp. Criteria for inclusion of a given article was a clear statement of the number of experimental animals (immunized and non immunized) and the number of protected animals in each group, which allowed calculation of risk difference. All articles included in this study were written in English.

Meta-analysis was performed according to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses Criteria)[5]. Data from each experimental group were grouped according to the category of the experimental vaccine: (i) live naturally attenuated strains (from now on simply named attenuated); (ii) mutant attenuated strains (mutant); (iii) recombinant subunit vaccines (subunit); (iv) vectored vaccines (vector), and (v) inactivated vaccines (inactivated). Other parameters from each individual experiment that were considered in this analysis included: publication year, animal species, number of immunized and protected animals, immunization protocol (vaccination route, number of doses, and use of adjuvant), and parameters related to the experimental challenge (inoculation route, and *Brucella* species).

Protection of experimentally challenged males and females was based on prevention of abortion in pregnant females or prevention of infection of target organs.

Data transformation and meta-regression analysis. Data obtained from each article, including the following parameters: vaccine category, host species, use of adjuvant, *Brucella* species used for challenge, and challenge route, were evaluated to determine which parameters had influence on the risk difference. Original data were transformed into qualitative data, varying from 0 to 5. Thus, values were attributed to these parameters as follows: vaccine category ("0" attenuated, "1" mutant, "2" subunit, "3" vector, or "4"

[5] Available at <http://www.prisma-statement.org/>

inactivated); host species ("0" cattle, "1" bison, "2" sheep, "3" goat, "4" deer, or "5" water buffalo); use of adjuvant ("1" yes, or "2" no); challenge *Brucella* species ("0"*B. abortus*, "1" *B. melitensis*, or "2" *B. ovis*); and rout of challenge ("0" conjunctival, "1" conjunctival and prepucial, "2" subcutaneous, or "3" intravenous).

The index of protected animals was obtained from the immunized and non immunized experimental groups from each article, which were named "protected vaccinated" and "protected control", respectively. Risk values were estimated based on the number of protected animals divided by the total number of animals in each experimental group. Risk difference was calculated subtracting the risk value of the vaccinated group from the risk value of the control group from each individual experiment. Risk difference varies from -1 to 1. Therefore, risk difference was calculated according to the following formula:

Risk difference (RD) = (number of protected vaccinated animals/ total number of vaccinated animals) – (number of protected non vaccinated animals/total number of non vaccinated animals)

A linear regression analysis was performed using the year of publication and risk difference as variables. Furthermore, the influence of each parameter (vaccine category, host species, and parameters related to immunization and challenge) on the value of risk.

Statistical analysis. A bivariate meta-regression analysis was performed using vaccine category as the dependent variable. Independent variables included: host species, vaccination route, use of adjuvant, number of doses, challenge route, and *Brucella* species used for challenge. Selection of variables for the multivariate meta-regression analysis was based on a p value lower than 0.05 on the bivariate meta-regression. The Stata software (Statacorp, Texas, USA) was used for these analysis.

RESULTS

Literature search and study characteristics

This study included 45 articles with 116 individual experiments. Criteria for literature search and inclusion of articles are detailed on Figure 1.

Vaccine protection of natural hosts over the past decades

A correlation analysis between the year of publication of the experimental study and risk differences demonstrated that there was no statistically significant improvement of protection (p>0.05) according to the risk differences over the past 36 years (Fig.2). Interestingly, there was a tendency for decreasing protection provided by experimental vaccines from 1980 to 2000, whereas protection induced by experimental vaccines as indicated by risk difference tended to improve from 2000 to 2016 (Fig.2).

Figure 3 demonstrates trend lines for each individual experimental vaccine type, namely attenuated, mutant, subunit, vectored, and inactivated vaccines.

Meta-analysis estimations

Preliminarily, a bivariate meta-regression analysis was performed considering each of the variables controlled by vaccine category. Dependent variables included host species, route of vaccination, use of adjuvant, *Brucella* species, and route of challenge. From 1990 to 2000, considering attenuated vaccines as reference with a difference of risk of 0.4349, subunit vaccines were significantly less protective with a difference of risk of 0.0258 (p<0.05), whereas difference of risk values for mutant and inactivated vaccines (0.3673 and 0.2401, respectively) were statistically similar (p>0.05) to the difference of risk provided by naturally attenuated vaccine strains (Table 1). For meta-regression analysis of challenge route the conjunctival route was considered as reference with a difference of risk of 0.5770, which was significantly higher than the subcutaneous route (p<0.05), which had a difference of risk equal to zero (Table 1).

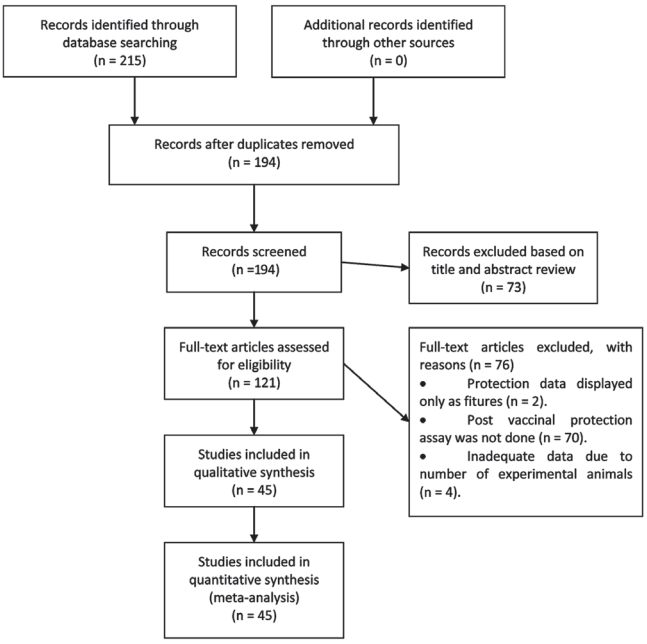


Fig.1. Flow chart describing the selection of articles from databases (Pubmed and Scopus) for inclusion in the meta-analysis, according to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses Criteria).

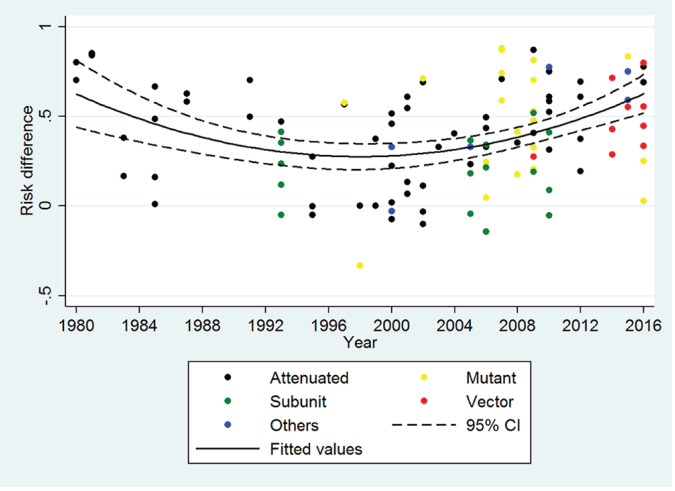


Fig.2. Linear regression analysis of risk of protection over time for different vaccines (attenuated, mutant, subunit, vectored, and inactivated) against *Brucella* spp. in their natural hosts. This analysis included 116 individual experiments with attenuated vaccines (n=62), mutant vaccine strains (n=23), subunit vaccines (n=17), vectored vaccines (n=9), and inactivated vaccines (n=5) (r=34.31, r2=11.77, p=0.159).

Protection provided by two immunizations (difference of risk = 0.5613) was statistically similar (p>0.05) to a single immunization (difference of risk = 0.4533) (Table 1). There were no statistically significant differences between host species, which included bison, sheep, goats, deer, and cattle (Table 1). Challenge with *B. melitensis* or *B. ovis* resulted in differences of risk of 0.2246 and 0.6269, respectively, which were statistically similar to the difference of risk after challenge with *B. abortus*.

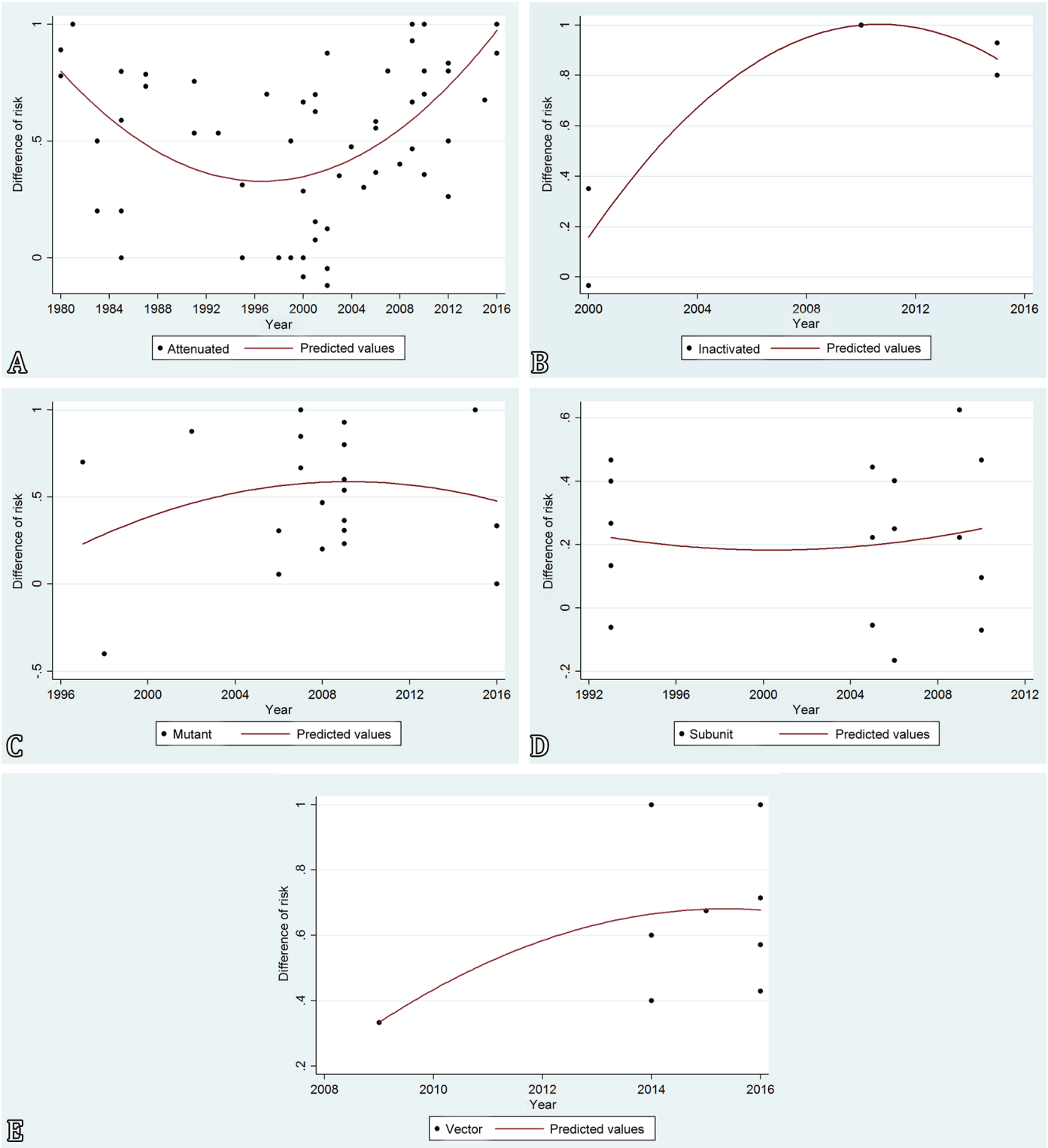


Fig.3. Linear regression analysis of risk of protection over time for each vaccine category against *Brucella* spp. in their natural hosts. (**A**) Naturally attenuated strains (n=62), (**B**) inactivated vaccine (n=5), (**C**) mutant vaccine strains (n=23), (**D**) subunit vaccines (n=17), and (**E**) vectored vaccines (n=9). Each data point represents one individual experiment with a solid trend line.

In the case of vaccination route, considering the occurrence of more than one route per experimental group, this variable was dichotomized prior to the analysis (Table 2).

Considering the period of 2000 to 2016 (Table 3), naturally attenuated vaccine strains with a difference of risk of 0.6320 performed significantly better that subunit vaccines (difference of risk = 0.169; p<0.001). Interestingly, the difference of risk provided by inactivated vaccines (1.005) was significantly higher than that provided by naturally attenuated vaccine strains (p<0.05) (Table 3).

Regarding the host species, considering cattle as the reference (difference of risk = 0.6320), bison, sheep, goat and water buffalos had differences of risk that were statistically similar. In contrast, deer had a statistically significant lower difference of risk (0.0701) when compared to cattle (p<0.0001) (Table 3).

As pointed out above, since there were more than one vaccination route in a given experimental group, this variable was dichotomized prior to analysis. Both the intramuscular and oral vaccination routes were significantly protective (Table 4).

Two or three vaccine doses resulted in difference of risk values (0.4787 and 0.3965, respectively) that were statistically similar to the difference of risk provided by a single vaccine dose (Table 3). The use of adjuvant did not have any statistically significant effect (Table 3).

Considering *B. abortus* as the reference challenge species (difference of risk = 0.4969), *B. melitensis* and *B. ovis* resulted in higher differences of risk (0.6777 and 0.7041, respectively) when compared to *B. abortus* (p<0.05).

The subcutaneous route of challenge resulted in a significantly higher difference of risk (0.89) when compared to the conjunctival route (difference of risk = 0.5298), which was considered the reference (p=0.01).

Multivariate meta-regression

The results of the bivariate meta-regression analysis were employed to select variables to be included in the multivariate meta-regression model. Therefore, the multivariate meta-

Table 1. Bivariate meta-regression analysis of variable that influenced difference of risk provided by brucellosis experimental vaccines from 1990 to 2000

Variable	Coefficient*	P value	Confidence intervals	
			Lower limit	Upper limit
Vaccine category - Reference: naturally attenuated - p<0.001				
Mutant	-0.3673	0.912	-0.7119	0.6384
Subunit	-0.4091	0.044**	-0.8056	-0.0126
Inactivated	-0.2401	0.411	-0.8273	0.3471
Constant	0.4349	<0.001	0.2619	0.6079
Number of doses - Reference: single vaccination - p<0.001				
Two doses	0.1080	0.640	-0.3571	0.5732
Constant	0.4533	<0.001	0.3055	0.6011
Host species - Reference cattle - p<0.001				
Bison	0.2309	0.405	-0.3278	0.7897
Sheep	0.1292	0.427	-0.1983	0.4569
Goats	-0.0513	0.860	-0.6413	0.5386
Deer	-0.1287	0.750	-0.9457	0.6884
Constant	0.4143	<0.001	0.2133	0.6154
Challenge species - Reference: *Brucella abortus* - p<0.001				
B. melitensis	-0.2103	0.346	-0.6585	0.2379
B. ovis	0.1920	0.221	-0.1217	0.5058
Constant	0.4349	<0.001	0.2619	0.6079
Challenge route - Reference: conjunctival - p<0.001				
Conjunctival and prepucial	-0.3174	0.101	-0.7007	0.0659
Subcutaneous	-0.6162	0.007**	-1.053	-0.1794
Intravenous	-0.5769	0.058	-1.1738	0.0199
Constant	0.5770	<0.001	0.4380	0.7158

* Positive regression coefficients indicate that the variable have better protection than the reference when statistically significant. Negative coefficients indicate the opposite; ** statistically significant differences (p<0.05).

Table 2. Dichotomized meta-regression analysis of the influenced route of vaccination on the difference of risk provided by brucellosis experimental vaccines from 1990 to 2000

Vaccination route	Coefficient*	P value	Confidence intervals	
			Lower limit	Upper limit
Subcutaneous	0.1478	0.525	-0.3204	0.6160
Constant	0.3311	0.139	-0.1133	0.7755
Intramuscular	-0.1846	0.636	-0.9701	0.6008
Constant	0.4703	<0.001	0.3278	0.6129

regression model included experiments published from 2000 to 2016, when there was a larger number of significant variables according to the bivariate meta-regression. The multivariate meta-regression model included the following vaccine categories: naturally attenuated vaccine strains, mutant vaccine strains, subunit vaccines, vectored vaccines, and inactivated vaccines, considering the difference of risk as the dependent variable and the other variables (vaccination route and challenge route) as independent variables (Table 5).

Subunit vaccines provided lower protection when compared to naturally attenuated vaccines (p=0.002), which was considered the reference. Conversely, inactivated vaccines

Table 3. Bivariate meta-regression analysis of variable that influenced difference of risk provided by brucellosis experimental vaccines from 1990 to 2000

Variable	Coefficient*	P value	Confidence interval	
			Lower limit	Upper limit
Vaccine category - Reference: naturally attenuated - p<0.001				
Mutant	0.0076	0.922	-0.1481	0.1634
Subunit	-0.4630	<0.001**	-0.6678	-0.2583
Vectored	0.0616	0.588	-0.1645	0.2878
Inactivated	0.3733	0.029**	0.0390	0.7076
Constant	0.6320	<0.001	0.4714	0.7927
Host species Reference: cattle - p<0.001				
Bison	-0.1324	0.314	-0.3927	0.1278
Sheep	0.6390	0.474	-0.1131	0.2409
Goat	0.0017	0.989	-0.2452	0.2487
Deer	-0.5619	0.001**	-0.8286	-0.2951
Water buffalo	0.2909	0.148	-0.1058	0.6876
Constant	0.6320	<0.001	0.4714	0.7927
Number of doses - Reference: single vaccination - p<0.001				
Two doses	-0.1310	0.293	-0.3775	0.1155
Three doses	0.2132	0.410	-0.2995	0.7259
Constant	0.6097	<0.001	0.4980	0.7215
Adjuvant - Reference: without adjuvant - p<0.001				
With adjuvant	0.0818	0.562	-0.1983	0.3618
Constant	0.5978	<0.001	0.4871	0.7086
Challenge species - Reference: *Brucella abortus* - p<0.001				
B. melitensis	0.1808	0.039**	0.0091	0.3527
B. ovis	0.2072	0.055	-0.0043	0.4188
Constant	0.4969	<0.001	0.3592	0.6346
Challenge route - Reference: conjunctival - p<0.001				
Conjunctival and prepucial	0.1274	0.199	-0.0688	0.3235
Subcutaneous	0.3602	0.010**	0.0893	0.6312
Constant	0.5298	<0.001	0.4094	0.6501

* Positive regression coefficients indicate that the variable has better protection than the reference when statistically significant. Negative coefficients indicate the opposite; ** statistically significant differences (p<0.05).

Table 4. Dichotomized meta-regression analysis of the influenced route of vaccination on the difference of risk provided by brucellosis experimental vaccines from 1990 to 2000

Vaccination route	Coefficient*	P value	Confidence intervals	
			Lower limit	Upper limit
Subcutaneous	0.0995	0.224	-0.0623	0.2614
Constant	0.5281	<0.001	0.3699	0.6862
Conjunctival	0.2011	0.054	-0.0039	0.4060
Constant	0.5732	<0.001	0.4618	0.6847
Intramuscular	-0.2916	0.009*	-0.5095	-0.0737
Constant	0.6488	<0.001	0.5373	0.7603
Oral	-0.5136	0.025**	-0.9618	-0.0654
Constant	0.5994	-	0.4929	0.7059
Intradermic	0.0022	0.995	-0.7494	0.7538
Constant	0.5978	<0.001	0.4870	0.7086

provided significantly better protection as indicated by the difference of risk when compared to the reference (p=0.007).

Regarding the vaccination routes, the subcutaneous route provided better protection than intramuscular and oral routes (p<0.05).

Experimental vaccines resulted in better protection when the challenge was performed by the subcutaneous route when compared to the conjunctival route (p=0.016), which was considered the reference.

DISCUSSION

Brucellosis is one of the most important zoonotic diseases worldwide. Importantly, the incidence of human brucellosis is strongly related to the prevalence in domestic animals (Gomez et al. 2013). Currently, vaccination is the most important tool for controlling infection and disease in animals, although vaccination alone is not sufficient for eradication of brucellosis (Corbel 2006, Grégoire et al. 2012). Therefore, there is still an enormous research effort for developing better and safer vaccines against *Brucella* (Saez et al. 2012, Curina et al. 2018, Paul et al. 2018). In this study we performed a meta-analysis that included a large series of experiments assessing protection against *Brucella* spp. in their natural host species. Meta-analyses of studies on the natural host is quite challenging since it requires comparisons among different host species, diagnostic methods, and parameters of protection, such as prevention of abortion, decrease of bacterial loads in tissues, prevention of lesions, among other parameters. In contrast, in the mouse model, in spite of variable experimental designs, the outcome is the same, which is protection index calculated based on the differences of bacterial loads in the spleen of vaccinated and non vaccinated mice (Carvalho et al. 2016). Therefore, our study was based on risk difference, which allowed comparison of original studies that employed different diagnostic methods to measure different outcomes of infection. This approach allowed us to compare traditional vaccines, including commercially available vaccine strains, with several different experimental vaccine protocols. A correlation analysis of year of publication and vaccine protection as predicted by the difference of risk indicated that protection provided by different types of experimental vaccines has remained stable over the past almost four decades. This finding is similar to our previous study in which a temporal analysis of protection induced by experimental *Brucella* vaccines in mice indicating stable protection indexes over the past three decades (Carvalho et al. 2016). Therefore, additional research efforts are needed for the development of the ideal *Brucella* vaccine, which may prevent infection and disease without adverse effects due to residual pathogenic potential for animal and humans (Dorneles et al. 2015, Xie et al. 2018).

To the best of our knowledge this is the first meta-analysis study of *Brucella* vaccinology in natural hosts. Our previous meta-analysis study was based on the mouse model (Carvalho et al. 2016), which is highly relevant since the mouse has been extensively used as a model for studies on pathogenesis, immune response and vaccine protection (Baldwin & Parent 2002, Silva et al. 2011a). Furthermore, the mouse is a convenient and inexpensive experimental model (Perkins et al. 2010). However, in spite of intensive *Brucella* vaccine research in this model, it is not clear how efficient is the mouse to predict protection in the different natural host species (Dorneles et al. 2015). For instance, in our experience an experimental vaccine with only moderate protection in the mouse (Silva et al. 2015b) induced sterile immunity in the natural host (Silva et al. 2015a). Experimental assessment of vaccine efficacy in natural hosts is therefore preferable, but experiments with pregnant cows for example are extremely expensive and require large animal biosafety level 3 facilities (Dorneles et al. 2015). In spite of these limitations, experimental assessment of vaccine protection is well established and highly relevant (Olsen et al. 1999, Poester et al. 2006). However, experimental assessment of vaccines in natural hosts may be influenced by several factors that do not affect the mouse model, including nutritional and immunological status, age, and environmental stress (Olsen et al. 2015).

Surprisingly, in spite of the dogma in the field of *Brucella* vaccinology that live attenuated vaccines are required for appropriate levels of protection, this study demonstrated that experimental vaccine formulations based on inactivated

Table 5. Multivariate meta-regression analysis of variables influencing the difference of risk provided by experimental brucellosis vaccines in natural hosts from 2000 to 2016

Variable	Coefficient*	Standard error	P value	Confidence interval	
				Upper limit	Lower limit
Vaccine category - Reference: naturally attenuated - p<0.001					
Mutant	0.0839	0.0905	0.357	-0.0968	0.2646
Subunit	-0.3979	0.1206	0.002**	-0.6387	-0.1572
Vectored	-0.1885	0.1446	0.197	-0.4772	0.1001
Inactivated	0.4742	0.1714	0.007**	0.1319	0.8164
Vaccination route - Reference: subcutaneous - p<0.001					
Intramuscular	-0.2232	0.1064	0.040**	-0.4356	-0.0106
Oral	-0.4547	0.2138	0.037**	-0.8817	-0.0277
Challenge route - Reference: conjunctival - p<0.001					
Conjunctival and prepucial	0.0666	0.093	0.477	-0.1191	0.2524
Subcutaneous	0.3139	0.1274	0.016**	0.0595	0.5682
Constant	0.5861	0.0607	<0.001	0.4650	0.7073

* Positive regression coefficients indicate that the variable has better protection than the reference when statistically significant. Negative coefficients indicate the opposite; ** statistically significant differences (p<0.05).

Brucella provided significantly higher protection as evidenced by their difference of risk when compared to naturally attenuated vaccine strains. A possible drawback of these inactivated vaccine formulations is long term immunity, which may justify the fact that the broadly used *Brucella* vaccines are live attenuated strains including *B. abortus* S19, *B. abortus* RB51, and *B. melitensis* Rev.1 (Cheville et al. 1993, Olsen & Stoffregen 2005, Corbel 2006). These strains promote good levels of long lasting protection and they are associated with a cellular immune response (Seder & Hill 2000, Titball 2008), which is required for controlling *Brucella* infection (Baldwin & Goenka 2006). Therefore, our meta-analysis data must be interpreted with caution since it does not assess long term immunity as most studies included in this analysis evaluated short term protection.

Together, the studies on inactivated vaccines included in this meta-analysis indicate that inactivated vaccines are capable of reducing incidence of infection in maternal and fetal tissues as well as preventing abortion in pregnant females. Olsen et al. (2015) demonstrated that an inactivated *Brucella* vaccine provided superior protection when two doses were administered with one year interval, resulting in reduction of the number of abortions when compared to non vaccinated bison or those vaccinated with a single dose. Development of inactivated *Brucella* vaccines are promising since in spite of good levels of protection, live attenuated vaccines retain virulence and are capable of causing abortion in vaccinated pregnant females. Live attenuated vaccines also often interfere with routinely used serologic tests. Furthermore, the Rev.1 vaccine strain is not allowed in areas free of *B. melitensis* (Cheville et al. 1992, Schurig et al. 2002, Vemulapalli et al. 2002, Dorneles et al. 2015). Furthermore, live attenuated strains are not applicable to humans since they have residual virulence, and can potentially cause human infection and disease (Waag et al. 2002, Rockx-Brouwer et al. 2012). Conversely, inactivated vaccines have desirable features for an ideal vaccine since it does not cause disease or infection in vaccinated animals, preventing any possibility of vaccine-induced abortion (Schurig et al. 1995, Ko & Splitter 2003). A vaccine is considered effective when it is capable of preventing development of the disease or minimizes risk of infection in vaccinated animals (Grilló et al. 2009). Therefore, the difference of risk is related to protection evidenced by reduction in abortion rates, generation of weak newborns, and decrease in bacterial loads in vaccinated animals (Elzer et al. 1998, Nol et al. 2016).

This study also demonstrated that subunit vaccines are less protective than naturally attenuated vaccine strains. Development of protective *Brucella* subunit vaccines is very challenging due to the difficulty to identify immunogenic and protective antigens, and the low probability of induction of protective immunity dependent on a single antigen (Titball 2008, Plotkin 2010, Yang et al. 2013). Subunit vaccines have also proven less protective in the mouse model (Carvalho et al. 2016), although some individual studies resulted in promising results, which may not correlate with protection in natural hosts (Dorneles et al. 2015).

Routes of vaccination and challenge influenced protection in natural host species under experimental conditions. This study demonstrated that the subcutaneous route of vaccination provides better results, which is in good agreement with previous reports (Marín et al. 1990, Todd et al. 2013, Carvalho et al. 2016). Importantly, the subcutaneous route is recommended for commercially available *Brucella* vaccines including S19, RB51, and Rev.1 (Beckett & Macdiarmid 1985, Xie et al. 2018).

CONCLUSION

Spite of new technologies for vaccine development, additional studies are needed to improve *Brucella* vaccine development since protection remained stable over the past decades. Interestingly, inactivated vaccine formulations demonstrated better protection, suggesting that this approach should be considered for future studies in the field of *Brucella* vaccinology.

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Original Article
Small Animal Diseases
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Canine cutaneous neoplasms in the metropolitan region of Goiânia, Goiás state, Brazil[1]

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ABSTRACT.- Santos I.R., Lima A.C.M.P., Ferreira H.H., Rezende B.R., Silva A.R. & Santos A.S. 2020. **Canine cutaneous neoplasms in the metropolitan region of Goiânia, Goiás state, Brazil.** *Pesquisa Veterinária Brasileira 40(8):614-620*. Laboratório de Histologia e Patologia Animal, Instituto Federal Goiano, Campus Urutaí, Rodovia Geraldo Silva Nascimento Km 2,5, Zona Rural, Urutaí, GO 75790-000, Brazil. E-mail: adriana.santos@ifgoiano.edu.br

The present study aimed to describe the occurrence and epidemiological features of skin neoplasms diagnosed in dogs in the metropolitan region of Goiânia, Goiás state, Brazil. Diagnoses from dog biopsies from 2011 to 2016 provided by a private veterinary pathology laboratory were analyzed. The main diagnoses were mast cell tumor, hemangiosarcoma, squamous cell carcinoma, malignant melanoma, and hemangioma. Highest frequency of neoplasms was found in female dogs, dogs aged >8 years, and purebred dogs, particularly the American Pit Bull Terriers and the Poodles. Most common sites affected by the neoplasms were the limb and the head. Using multiple correspondence analysis, groups of neoplasms were found to be associated with different epidemiological features and the size of the neoplasms was associated with the biological behavior. The results of this study described predispositions and verified the importance of different types of skin neoplasms in dogs in the region being studied.

INDEX TERMS: Canine, cutaneous neoplasms, Goiás state, Brazil, dermatopathology, dogs, skin, skin tumors.

RESUMO.- [**Neoplasias cutâneas caninas na região metropolitana de Goiânia, Goiás, Brasil.**] O objetivo deste estudo foi determinar a prevalência e as características epidemiológicas das neoplasias cutâneas em cães na região metropolitana de Goiânia, Goiás. Foram analisados os diagnósticos de um laboratório do setor privado de 2011 a 2016. Mastocitoma, hemangiossarcoma, carcinoma de células escamosas, melanoma maligno e hemangioma representaram os principais diagnósticos. A maioria dos casos ocorreram em cães de raças definidas, fêmeas e com idade >8 anos. American Pit Bull Terrier e Poodle foram as raças mais encontradas. As neoplasias acometeram principalmente regiões de membros e cabeça. Pela análise de correspondência múltipla, associou-se os grupos de neoplasias com diferentes características epidemiológicas e o tamanho da neoplasia com o comportamento biológico. A comparação dos resultados com pesquisas prévias possibilitou confirmar predisposições previamente descritas e verificar a importância dos diferentes tipos de neoplasias cutâneas em cães na região estudada.

TERMOS DE INDEXAÇÃO: Neoplasia cutânea, caninos, Goiás, Brasil, cão, dermatopatologia, pele, tumores cutâneos.

INTRODUCTION

Understanding the most common lesions affecting the animals in a particular region is an important tool in diagnostic investigation. Prevalence of canine cutaneous neoplasms has been reported in several countries. However, these studies showed differences in the frequency of different types of neoplasms (Bostock 1986, Rothwell et al. 1987, Dobson et al. 2002, Kaldrymidou et al. 2002, Pakhrin et al. 2007, Graf et al. 2018). These differences may be justified by the interference of environmental factors (Souza 2005) and the dog breeds in the respective regions.

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In Brazil, regional studies were performed in several states such as Bahia (Machado et al. 2018), Mato Grosso (Lima et al. 2018), Ceará (Bastos et al. 2017), Minas Gerais (Fernandes et al. 2015), São Paulo (Scattone & Fava 2014), Paraíba (Andrade et al. 2012), Rio de Janeiro (Silva et al. 2011), Rio Grande do Sul (Souza et al. 2006, Meirelles et al. 2010), and Paraná (Nardi et al. 2002). However, epidemiological data about canine skin neoplasms in the Goiás state are scarce.

The aim of present study was to describe the occurrence and epidemiological features of skin neoplasms diagnosed in dogs in the metropolitan region of Goiânia, Goiás state, Brazil.

MATERIALS AND METHODS

The present study was conducted at "Laboratório de Histologia e Patologia Animal" of "Instituto Federal Goiano", Campus Urutaí. The authors analyzed the reports from dog biopsy samples between 2011 and 2016 provided by a private veterinary pathology laboratory located in Goiânia, Goiás state. Information related to the histological diagnosis, breed, age, sex, size, and anatomical location of the neoplasms was obtained. For this study, primary cases of mammary neoplasms were excluded.

Original histological classification of the reports was considered, with standardization, when necessary, according to Goldschmidt & Goldschmidt (2017), Hendrick (2017), and Kiupel (2017) classifications. Neoplasms were grouped into epithelial neoplasms (subdivided into epidermal neoplasms, sebaceous gland neoplasms, hair follicle neoplasms, and apocrine glands neoplasms), mesenchymal neoplasms (subdivided into vascular neoplasms, adipose tissue neoplasms, connective fibrous tissue neoplasms, and other mesenchymal neoplasms), round cell tumors, and melanocytic neoplasms.

According to the anatomical locations, neoplasms were categorized into those of head, neck, thorax, abdomen, limbs, perineum, scrotum, tail, and multiple locations (Fernandes et al. 2015). When more than one histological classification of cutaneous neoplasms were identified, each classification was considered separately. Age groups of the animals were <1 year, 1-8 years, and >8 years (Souza et al. 2006).

Descriptive statistical analyses were used to evaluate the data. Additionally, multiple correspondence analysis was performed using R software (R Core Team, 2019) . The following associations were tested: i) groups of neoplasms with anatomical locations, ii) groups of neoplasms with age and sex (males <1 year, males 1-8 years, males >8 years, females <1 year, females 1-8 years, and females >8 years), and iii) size (<1cm, 1-2cm, 3-4cm, and >5cm) with biological behavior (benign and malignant). Results were presented in two-dimensional perception maps and Fisher's exact test was applied to the contingency tables to verify significant associations (p<0.05).

RESULTS

Dataset

From 2011 to 2016, 4336 canine tissue samples were received. Out of these, 2138 (49.3%) were from the skin and 1266 (59.2%) among the skin samples were diagnosed with cutaneous neoplasms. Fifty-nine (4.9%) out of the 1200 reports analyzed in this study reported that the animal had at least two histologically different cutaneous neoplasms.

Types of neoplasms

Neoplasms of epithelial origin (32.9%, n=417) were the most prevalent, followed by mesenchymal neoplasms (30.0%, n=380), round cell tumors (28.3%, n=359), and melanocytic neoplasms (8.6%, n=110). Among the epithelial neoplasms, 35.9% (n=150) were from the epidermis, 32.8% (n=137) from the sebaceous glands, 20.1% (n=84) from the hair follicles, and 10.0% (n=42) from the apocrine glands. Vascular neoplasms (63.6%, n=242), adipose tissue neoplasms (13.4%, n=51), and connective tissue neoplasms (7.8%, n=30) were the prevalent mesenchymal neoplasms.

Frequencies and epidemiological aspects of the 48 types of neoplasms diagnosed in this study are presented in Table 1. The most frequent types of individual neoplasms were mast cell tumor (14.9%, n=189), hemangiosarcoma (13.6%, n=173), squamous cell carcinoma (8.4%, n=107), malignant melanoma (7.0%, n=89), hemangioma (5.1%, n=65), histiocytoma (4.3%, n=55), lipoma (3.7%, n=47), hepatoid gland adenoma (3.6%, n=46), trichoblastoma (3.5%, n=45), and papilloma (2.8%, n=36). These 10 types of neoplasms represented 67.2% (n=852) of all neoplasms.

Distribution of neoplasms according to breed

Information about the animal breed was found in 1177 (92.9%) reports out of 1266 cases of cutaneous neoplasms. Purebred dogs (73.4%, n=930) had more neoplasms than mixed-breed dogs (19.5%, n=247). The most affected breeds were American Pit Bull Terrier (15.8%, n=201), Poodle (6.7%, n=85), Dachshund (6.2%, n=79), Boxer (6.0%, n=76), Shih Tzu (5.0%, n=64), Labrador Retriever (3.6%, n=46), Pincher (3.3%, n=43), English Cocker Spaniel (3.1%, n=40), Yorkshire Terrier (1.9%, n=25), and Rottweiler (1.8%, n=23). Description of these breeds according to the histological diagnoses is presented in Table 2.

Breeds not included in Table 2 due to low number of cases were Schnauzer (1.7%, n=22), Basset Hound (1.5%, n=20), Golden Retriever (1.4%, n=18), Lhasa Apso (1.4%, n=18), Brazilian Mastiff (1.2%, n=16), Chinese Shar Pei (1.1%, n=15), Pug (1.1%, n=14), Maltese (0.8%, n=11), German Shepherd (0.7%, n=10), Brazilian Terrier (0.7%, n=9), Beagle (0.6%, n=8), French Bulldog (0.5%, n=7), Bull Terrier (0.5%, n=7), West Highland White Terrier (0.4%, n=6), Dogo Argentino (0.4%, n=6), and 21 other breeds with an occurrence of less than 0.4% (n<6).

Distribution of neoplasms according to anatomical location

Only 889 (70.2%) reports contained data about the anatomical location of the skin neoplasms. Among these, 22.2% (n=198) were localized to the limbs, 22.2% (n=198) to the head, 19.6% (n=175) to the perineum, 13.3% (n=119) to the abdomen, 5.3% (n=48) to the thorax, 5.0% (n=45) to the neck, 3.9% (n=35) to the scrotum, and 1.3% (n=12) to the tail. Neoplastic lesions in multiple locations were found in 6.6% (n=59) of the cases. Three most frequent neoplasms in each location are shown in the Table 3.

Distribution of neoplasms according to age

The data included neoplasms from dogs aged 3 months to 19.5 years. Among the samples in which age was reported (80.2%, n=1016), 50.7% (n=516) were from dogs aged >8 years, 38.3% (n=486) from dogs aged 1-8 years, and only 1.3% (n=14) from dogs aged <1 year. The most prevalent neoplasms in dogs aged >8 years were mast cell tumor (15.6%, n=81), hemangiosarcoma (13.3%, n=69), and malignant melanoma (9.4%, n=49). The most prevalent neoplasms in dogs aged 1-8

Table 1. Canine cutaneous neoplasms in the metropolitan region of Goiânia, Goiás state, Brazil and the distribution of the neoplasms according to mean age (years), breed, anatomical location, and sex

Neoplasm type	Number (%)	Mean age	Breed (%)	Anatomical location (%)	Sex (%)
Epithelial	417				
Epidermal neoplasms	150				
Squamous cell carcinoma	107 (71.3)	8.3	Pit Bull (35.5)	Abdomen (17.7)	F (49.5)
Papilloma	36 (24)	5.5	Mixed (13.8)	Limbs (27.7)	F (63.8)
Basosquamous carcinoma	4 (2.6)	3.3	*	Limbs (25)	F/M (50)
Basal cell carcinoma	3 (2)	11	*	Limbs/Head (33.3)	M (66.6)
Sebaceous gland neoplasms	137				
Hepatoid gland adenoma	46 (33.5)	11.2	Mixed (34.7)	Perineum (80.4)	M (73.9)
Meibomian adenoma	26 (18.9)	9.3	Shih Tzu (19.2)	Head (92.3)	M (53.8)
Hepatoid gland carcinoma	20 (14.5)	10.5	Mixed (25)	Perineum (70)	M (75)
Sebaceous epithelioma	14 (10.2)	11.5	Poodle (21.4)	Head (42.8)	M (57.1)
Sebaceous adenoma	9 (6.5)	12.6	Cocker/Poodle (22.2)	Head (22.2)	F (55.5)
Meibomian epithelioma	8 (5.8)	10.9	Shih Tzu/Labrador (25)	Head (87.5)	F (62.5)
Sebaceous carcinoma	7 (5.1)	9.5	Poodle (42.8)	Head (28.5)	F (85.7)
Hepatoid gland epithelioma	6 (4.3)	11.5	Poodle (50)	Perineum (83.3)	M (66.6)
Meibomian carcinoma	1 (0.7)	5	Dachshund (100)	Head (100)	M (100)
Hair follicle neoplasms	84				
Trichoblastoma	45 (53.5)	7.7	Poodle (17.7)	Head (31.1)	M (51.1)
Trichoepithelioma	25 (29.7)	7.6	Shih Tzu/Mixed (16)	Limbs (16)	F (60)
Trichofolliculoma	5 (5.9)	6	Shih Tzu (40)	Limbs/Multiple (20)	F (60)
Infundibular ke. acanthoma	4 (4.7)	6.73	*	*	M (75)
Malignan. trichoepithelioma	4 (4.7)	9.3	*	Limbs (50)	F (75)
Pilomatricoma	1 (1.1)	Ni	Shih Tzu (100)	Ni	F (100)
Apocrine gland neoplasms	42				
Apocrine carcinoma	14 (33.3)	8.7	Dachshund (14.2)	Perineum (21.4)	F (64.2)
Apocrine ductal carcinoma	9 (21.4)	9.1	Mixed (22.2)	Limbs/Perineum (33.3)	M (55.5)
Ceruminous carcinoma	7 (16.6)	8.5	*	Head (85.7)	M (57.1)
Anal sac gland carcinoma	5 (11.9)	12.3	Mixed (40)	Perineum (80)	F (60)
Apocrine ductal adenoma	2 (4.7)	10	Golden (50)	Head/Tail (50)	F (100)
Apocrine adenoma	2 (4.7)	12	Shih Tzu (100)	Head (50)	M (100)
Complex cerumi. carcinoma	2 (4.7)	11	Cocker/Dachsh. (50)	Head (100)	F/M (50)
Anal sac gland adenoma	1 (2.3)	10	Cocker (100)	Ni	F (100)
Epithelial neoplasms NS	4				
Mesenchymal	380				
Vascular neoplasms	242				
Hemangiosarcoma	173 (71.4)	8.3	Pit Bull (45)	Perineum (21.9)	F (49.1)
Hemangioma	65 (26.8)	8.3	Pit Bull (32.3)	Limbs (13.8)	F (55.3)
Lymphagioma	2 (0.8)	8.5	Dalmat./Labrador (50)	Abdomen/Perineum (50)	F/M (50)
Lymphangiosarcoma	2 (0.8)	19	Labrador (50)	Abdomen (50)	F (50)
Adipose tissue neoplasms	51				
Lipoma	47 (92.1)	8.7	Mixed (17)	Limbs/Thorax (14.8)	F (78.7)
Liposarcoma	4 (7.8)	9.5	*	Abdomen (50)	F/M (50)
Fibrous con. tissue neoplasms	30				
Fibrosarcoma	17 (56.6)	10.3	Mixed (29.4)	Head (23.5)	F (76.4)
Fibroma	9 (30)	9	Labrador (22.2)	Limbs (22.2)	F (66.6)
Mixosarcoma	4 (13.3)	7.6	*	Limbs (50)	F (75)
Other mesenchy. neoplasms	21				

Neoplasm type	Number (%)	Mean age	Breed (%)	Anatomical location (%)	Sex (%)
Peripheral nerve she. tumor	14 (66.6)	8.7	Mixed (42.8)	Limbs (57.1)	F (57.1)
Osteosarcoma	3 (14.2)	6.3	⁎	Abdomen/Thorax (33.3)	F (100)
Hemangiopericytoma	2 (9.5)	16.5	Mixed/Poodle (50)	Head (50)	F (100)
Condrosarcoma	1 (4.7)	6	Pit Bull (100)	Limbs (100)	M (100)
Leiomyoma	1 (4.7)	9	Shih Tzu (100)	Perineum (100)	F (100)
Mesenchy. neoplasms NS	36				
Round cell tumors	359				
Mast cell tumor	189 (52.6)	8.5	Mixed (26.9)	Limbs (23.2)	F (61.3)
Histiocytoma	55 (15.3)	4.2	Mixed (14.5)	Head (36.3)	M (67.2)
Lymphoma	25 (6.9)	7	Mixed (16)	Multiple (60)	M (56)
Plasmacytoma	23 (6.4)	8.4	Mixed/Pit Bull (17.3)	Limbs (43.4)	M (52.1)
Transmissible vener. tumor	6 (1.6)	6.7	Mixed (83.3)	Perineum (83.3)	M (66.6)
Round cell neoplasms NS	61				
Melanocytic	110				
Maligant melanoma	89 (80.9)	9.5	Mixed (24.7)	Head (21.3)	F (53.9)
Melanocytoma	21 (19)	9.7	Dachsh./Pit Bull (23.8)	Head (23.8)	F (57.1)

cerumi. = Ceruminous, Cocker = English Cocker Spaniel, con. = connective, Dachsh. = Dachshund, Dalmat. = Dalmatian, F = female, Golden = Golden Retriever, ke. = keratinizing, Labrador = Labrador Retriever, Ni = not informed, NS = not specified, M = male, Malignan. = Malignant, mesenchy. = mesenchymal, Pit Bull = American Pit Bull Terrier, she. = sheath, vener. = veneral; ⁎ there are at least three specifications with the same occurrence.

Table 2. Ten most common breeds with canine cutaneous neoplasms in the metropolitan region of Goiânia, Goiás state, Brazil and the distribution of the main histological diagnoses

Breed	Main morphological diagnoses (%)
Pit Bull	Hemangiosarcoma (38.8) Squamous cell carcinoma (18.4) Hemangioma/Mast cell tumor (10.4)
Poodle	Trichoblastoma/Hepatoid gland adenoma (9.4) Squamous cell carcinoma (8.2) Lipoma/Mast cell tumor (5.8)
Dachshund	Maligant melanoma (16.4) Mast cell tumor (8.8) ⁎
Boxer	Mast cell tumor (25) Hemangiosarcoma (15.7) Squamous cell carcinoma (13.1)
Shih Tzu	Histiocytoma (10.9) Trichoblastoma (9.3) Hepatoid gland adenoma/Meibomian adenoma (7.8)
Labrador	Mast cell tumor (23.9) Squamous cell carcinoma (8.6) Meibomian adenoma/Trichoepithelioma (6.5)
Pinscher	Mast cell tumor (37.2) Maligant melanoma/Hemangiosarcoma (13.9) Hemangioma (6.9)
Cocker	Lipoma (15) Trichoblastoma (10) Mast cell tumor (7.5)
Yorkshire	Papilloma (16) Mast cell tumor/Histiocytoma (12) Malingant melanoma/Trichoblastoma (8)
Rottweiler	Lipoma/Maligant melanoma (16.6) Lymphoma/Hemangiosarcoma (8.3) ⁎

Cocker = English Cocker Spaniel, Labrador = Labrador Retriever, Pit Bull = American Pit Bull Terrier, Yorkshire = Yorkshire Terrier; ⁎ there are at least three specifications with the same occurrence.

years were mast cell tumor (15.4%, n=75), hemangiosarcoma (14.6%, n=71), and squamous cell carcinoma (8.8%, n=43). Histiocytoma alone represented more than half (57.1%, n=8) of the neoplasms in dogs aged <1 year.

Distribution of neoplasms according to sex

Among the samples, 51.8% (n=656) were female and 45.4% (n=576) were male. Sex was not reported in 34 samples (2.6%). The most common diagnoses in females were mast cell tumor (17.6%, n=116), hemangiosarcoma (12.6%, n=83), squamous cell carcinoma (8.0%, n=53), malignant melanoma (7.3%, n=48), and lipoma (5.6%, n=37). The most common diagnoses in males were hemangiosarcoma (14.2%, n=82), mast cell tumor (12.1%, n=70), squamous cell carcinoma (8.5%, n=49), malignant melanoma (6.4%, n=37), and hepatoid gland adenoma (6.4%, n=37).

Multiple correspondence analysis

Groups of neoplasms showed a significant association ($p<0.01$) with anatomical locations, age, and sex. Head, perineum, and tail were associated with epithelial neoplasms; abdomen with mesenchymal and melanocytic neoplasms; and limbs, thorax, scrotum, and multiple locations with round cell tumors. Irrespective of sex, dogs aged <1 year were more likely to develop round cell tumors and dogs aged >8 years were more likely to develop mesenchymal and melanocytic neoplasms. Dogs aged 1-8 years and the neck location showed a random relationship with the groups of neoplasms.

Among the neoplasms, 56.1% (n=711) were malignant and 33.5% (n=425) were benign. Non-specified neoplasms and epitheliomas were excluded. Size of the neoplasms was mentioned in 19.8% (n=251) reports. A significant association was observed between the size and the biological behavior ($p<0.01$), where neoplasms with sizes <1cm presented a higher association with benign behavior and those with sizes

Table 3. Main histological diagnoses according to the anatomical locations of canine cutaneous neoplasms in the metropolitan region of Goiânia, Goiás state, Brazil

Anatomical location	Main morphological diagnoses (%)
Limbs	Mast cell tumor (22.2) Hemangiosarcoma (7) Squamous cell carcinoma (6)
Head	Meibomian adenoma (12.1) Histiocytoma (10.1) Maligant melanoma (9.5)
Perineum	Hemangiosarcoma (21.7) Hepatoid gland adenoma (21.1) Squamous cell carcinoma (10.2)
Abdomen	Hemangiosarcoma (28.5) Squamous cell carcinoma (16.8) Maligant melanoma (12.6)
Multiple	Lymphoma (25.4) Hemangiosarcoma (23.7) Mast cell tumor (13.5)
Thorax	Mast cell tumor (25) Lipoma (14.5) Histiocytoma (12.5)
Neck	Hemangioma (15.5) Mast cell tumor/ Trichobastoma (11.1) Histiocytoma (6.6)
Scrotum	Mast cell tumor (54.2) Hemangiosarcoma (11.4) Squamous cell carcinoma (8.5)
Tail	⁎

⁎ There are at least three specifications with the same occurrence.

>5cm showed a higher association with malignant behavior. Neoplasms with sizes of 1-2cm and 3-4cm were associated with both types of biological behaviors.

DISCUSSION

In the present study, we analyzed 1266 skin neoplasms in dogs in the metropolitan region of Goiânia, Goiás state, Brazil. The data was derived from a single pathology laboratory in the region. Although the prevalence of lesions diagnosed in other laboratories may have similarities with this study, we assume that the exposed results are underestimated. It is also known that not every cutaneous tumor in dogs is sent for histological analysis.

About 9.8% of the dogs in this study had more than one skin neoplasms, irrespective of whether they were of the same histological type. Similar results were found in other studies, adding the chance of occurrence of non-neoplastic tumors (Souza et al. 2006, Machado et al. 2018). These data highlight the importance of the clinical veterinarians and the veterinary surgeons sending samples of all skin tumors for histological analysis, despite the possibility that they are of the same histological type.

Skin tissue samples comprised of almost 50% of all canine samples during the period under evaluation. Previous studies have shown that skin lesions constitute the highest number of pathological diagnoses in dogs (Meirelles et al. 2010, Silva et al. 2011, Graf et al. 2018, Machado et al. 2018). Neoplastic lesions can range from 49.9% to 75.8% of all skin lesions (Souza et al. 2006, Machado et al. 2018), a finding similar to the results found in the present study.

The high prevalence of skin neoplasms in dogs may be related to factors specific to the species such as genetic predisposition (Lima et al. 2018), and factors related to tutors such as ease of observation of the lesions (Goldschmidt & Goldschmidt 2017). Moreover, skin has a high rate of cell regeneration (Murphy 2006), is formed from numerous components in its structure (Bastos et al. 2017), and is directly exposed to oncogenic conditions (Martinez et al. 2006).

Epithelial neoplasms were the most common neoplasms in dogs in the studied region. Although round cell tumors are known to be of mesenchymal origin (Hendrick 2017), our criteria for classifying the neoplasm groups according to cell origin were defined to assist in the clinical diagnostic routine. In studies using a similar division of groups, round cell tumors (Graf et al. 2018) and mesenchymal neoplasms (Lima et al. 2018) presented high frequencies of 33.0% and 41.0%, respectively.

Regarding analysis of the relative frequency of the types of neoplasms, our results are partially similar to previous studies. Mast cell tumor is described as the main cutaneous neoplasm in dogs (Bostock 1986, Mukaratirwa et al. 2005, Graf et al. 2018, Lima et al. 2018) and has a multifactorial etiology (Welle et al. 2008). The high occurrence of mast cell tumor in this study may be associated with the number of samples evaluated from dogs with racial predisposition such as Boxers and Labrador Retrievers (Dobson et al. 2002).

Squamous cell carcinoma, hemangioma, and hemangiosarcoma are among the most common canine cutaneous neoplasms in other Brazilian states (Andrade et al. 2012, Fernandes et al. 2015, Lima et al. 2018). However, they have low prevalence in other countries (Bostock 1986, Pakhrin et al. 2007, Graf et al. 2018). The frequency of these neoplasms in dogs has a direct association with geographical location, as they are associated with prolonged sun exposure and breeds with little skin pigmentation and short hair (Hargis et al. 1992, Goldschmidt & Goldschmidt 2017).

Due to the low degree of differentiation, 7.9% of all evaluated neoplasms were diagnosed with non-specific neoplasia. In these cases, immunohistochemistry is indicated for the identification of the cell origin, a test little used due to the high cost and low availability in the laboratories. However, in some situations, the histological aspects and the biological behavior of the neoplasm may be sufficient for the clinical veterinarian to determine the treatment for the animal (Meirelles et al. 2010).

The evaluation of the distribution of neoplasms according to breeds, age groups, anatomical locations, and sex confirmed the previously reported predispositions such as sebaceous adenoma in Cocker Spaniels and in the head, hepatoid neoplasms in males (Goldschmidt & Goldschmidt 2017), mast cell tumor in Boxers and Labrador Retrievers (Kiupel 2017), histiocytoma in young animals, and lipoma in females (Hendrick 2017). However, due to the limited knowledge about the dog population in the studied region, there is a possibility of environmental bias in the samples.

In the present study, groups of neoplasms showed an association with different epidemiological features. It is suggested that these results were observed due to the main histological diagnoses found in the analyzed variables such as mast cell tumor in the limbs, thorax and scrotum, lymphoma in multiple locations, and histiocytoma in dogs aged <1 year.

It is noteworthy that some results may vary due to the large number of neoplasms with different behaviors in each group.

Frequent association between the size of the cutaneous neoplasms in dogs and their biological behavior has been previously reported (Lima et al. 2018). However, the growth rate of neoplasms may depend on a number of factors such as blood supply, unknown influence, and hormonal stimulation (Stricker & Kumar 2010). Therefore, the size of the neoplasm should not be used as a criterion while deciding to send the skin tumors for histological analysis.

Receiving examination requests with incomplete information is part of the routine for numerous veterinary pathology laboratories (Meirelles et al. 2010, Lima et al. 2018). This fact made it impossible to include other data such as the relationship between castration and the occurrence of neoplasms in the present study. It is worth remembering that epidemiological data, clinical history, and macroscopic characteristics of the lesion may help pathological diagnosis in numerous situations.

CONCLUSIONS

Cutaneous neoplasms constituted a large part of diagnoses in dogs in the metropolitan region of Goiânia, Goiás state. Mast cell tumor, hemangiosarcoma, and squamous cell carcinoma were the most common neoplasms.

In addition to confirming the previously reported predispositions, our results revealed that the groups of neoplasms showed association with different epidemiological features and the size of the neoplasms showed association with the biological behavior.

The present study may encourage new studies in future to improve animal welfare.

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Sinonasal neoplasms in 49 dogs: clinical, macroscopic, and histopathological aspects[1]

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ABSTRACT.- Ricaldi G.F.S., Hennig M.M., Tondo L.A.S., Irigoyen L.F., Fighera R.A., Flores M.M. & Kommers G.D. 2020. **Sinonasal neoplasms in 49 dogs: clinical, macroscopic and histopathological aspects.** *Pesquisa Veterinária Brasileira 40(8):621-629.* Laboratório de Patologia Veterinária, Departamento de Patologia, Universidade Federal de Santa Maria, Av. Roraima 1000, Cidade Universitária, Camobi, Santa Maria, RS, 97105-900, Brazil. E-mail: glaukommers@yahoo.com

This study aimed to quantify nasosinusal neoplasms diagnosed in dogs in 20 years (2000-2019) and characterize the main clinical, macroscopic, and histological aspects of these neoplasms. The sex, breed, age, skull conformation, the main clinical signs, and the anatomopathological characteristics (distribution, macroscopy, and histology) were computed. During this period, 49 dogs were affected by neoplasms in these regions, totaling 50 neoplasms (one dog had two neoplasms of different locations and histogenetic origins). Similar amounts of mixed-breed dogs (25/49) and purebred dogs (24/49) were affected, these distributed in 16 breeds. Among purebreds, it was noted that dogs with mesocephalic cranial conformation (12/24) were the most affected, followed by dolichocephalic (10/24) and brachycephalic (2/24). There were 22 cases in males and 27 in females, making a proportion of 1:1.23. There was an age variation from 11 months to 16 years old. The epithelial neoplasms have occurred in older dogs compared to those of other histogenic origins (mesenchymal and other origins/round cells). The main clinical signs were similar between the histogenetic categories, related to the involvement of the upper respiratory tract, sometimes accompanied by nervous signs (when there was brain invasion of nasal neoplasms or vice versa). The possible origin site was mostly in the nasal cavity concerning the paranasal sinuses (and other locations). Invasions occurred in different tissues adjacent to the nasal cavity and paranasal sinuses, resulting in cranial and facial deformities (21/49). The frequency was 48% of epithelial neoplasms, 32% of mesenchymal neoplasms, and 10% of neoplasms with other origins and round cells. The neoplasms most frequently observed, in decreasing order of frequency, were: adenocarcinoma (9/50), squamous cell carcinoma (9/50), transmissible venereal tumor (5/50), osteosarcoma (5/50), chondrosarcoma (4/50), and undifferentiated sarcoma (4/50). Through this study, it was possible to establish the frequency of these neoplasms in 20 years and their clinical, macroscopic, and histological characteristics.

INDEX TERMS: Sinonasal neoplasm, dogs, clinic, macroscopy, histopathology, nasal cavity, paranasal sinuses, upper respiratory tract, canine.

RESUMO.- [**Neoplasmas nasossinusais em 49 cães: aspectos clínicos, macroscópicos e histopatológicos.**] Este estudo teve como objetivo quantificar os neoplasmas nasossinusais diagnosticados em cães em 20 anos (2000-2019) e caracterizar os principais aspectos clínicos, macroscópicos e histológicos desses neoplasmas. Foram computados sexo, raça, idade, conformação do crânio, principais sinais clínicos e características anatomopatológicas (distribuição, macroscopia

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e histologia). Nesse período, 49 cães foram acometidos por neoplasmas nessas regiões, totalizando 50 neoplasmas (um cão tinha dois neoplasmas de localização e origens histogenéticas distintas). Foram acometidas quantidades semelhantes de cães sem raça definida (25/49) e de cães com raça definida (24/49), estes distribuídos em 16 raças. Entre os cães com raça definida, notou-se que os cães com conformação craniana mesocefálica (12/24) foram os mais acometidos, seguidos pelos dolicocefálicos (10/24) e braquicefálicos (2/24). Foram observados 22 casos em machos e 27 em fêmeas, perfazendo a relação de 1:1,23. Ocorreu uma variação de idade de 11 meses a 16 anos; tendo os neoplasmas epiteliais ocorrido em cães mais velhos quando comparado aos de outras origens histogênicas (mesenquimais e outras origens/células redondas). Os principais sinais clínicos foram semelhantes entre as categorias histogenéticas, sendo relacionados ao comprometimento do trato respiratório superior, por vezes acompanhados de sinais nervosos (quando houve invasão encefálica de neoplasmas nasais ou vice-versa). O possível local de origem em sua maioria foi na cavidade nasal em relação aos seios nasais (e de outras localizações). Ocorreram invasões para diferentes tecidos adjacentes à cavidade nasal e seios paranasais, tendo como consequência deformidades cranianas e faciais (21/49). A frequência foi de 48% de neoplasmas epiteliais, 32% de neoplasmas mesenquimais e 10% de neoplasmas com outras origens e de células redondas. Os neoplasmas mais frequentemente observados, em ordem decrescente de frequência, foram: adenocarcinoma (9/50), carcinoma de células escamosas (9/50), tumor venéreo transmissível (5/50), osteossarcoma (5/50), condrossarcoma (4/50) e sarcoma indiferenciado (4/50). Com isso, pode-se estabelecer a frequência desses neoplasmas em 20 anos, bem como suas características clínicas, macroscópicas e histológicas.

TERMOS DE INDEXAÇÃO: Neoplasma nasossinusal, cão, clínica, macroscopia, histopatologia, cavidade nasal, seios paranasais, trato respiratório superior, caninos.

INTRODUCTION

Nasosinusal neoplasms in dogs occur with rare frequency. Estimate that for every 100,000 dogs, only 2.5 have some neoplasm in the nasal cavity or paranasal sinuses (Wilson 2017). Lobetti (2009) showed that in 75 cases of dogs with chronic nasal diseases, 46.7% had some neoplasm that affected the nasal cavity; however, compared to all tumors that can affect dogs, its prevalence was between 0.3% and 1.4% (Ogilvie & Larue 1992). These data demonstrate how unusual it is to have nasosinusal neoplasms, both when related to the total population of dogs, and when compared among all tumors that affect this animal species.

The nasal cavity and sinuses are composed and directly related to different tissues, including the epithelia that line the nasal cavity and sinuses, as well as underlying bone and cartilage tissue. The epithelia in the nasal cavity vary between stratified nasal epithelium in the most rostral portion, the pseudostratified ciliated columnar epithelium, and, in the more caudal region, the olfactory epithelium (Bacha & Bacha 2012, Evans & Lahunta 2013). The sinus epithelium is composed of pseudo-stratified ciliated epithelium, with few goblet cells (König & Liebich 2011, Junqueira & Carneiro 2013). Because of this, neoplasms of different embryonic origins may arise, resulting in a large histogenetic variety of tumors in this region, which may vary according to the anatomical region affected (Wilson 2017).

Information regarding the frequency of occurrence of these neoplasms in the Brazilian literature has been limited mainly to case reports (Canal et al. 2006, Formighieri et al. 2012, Gonçalves et al. 2012). However, studies with a more expressive series are scarce (Auler 2010, Dadalto et al. 2019).

This study aimed to quantify nasosinusal neoplasms diagnosed in dogs at the "Laboratório de Patologia Veterinária" of the "Universidade Federal de Santa Maria" (UFSM) over 20 years and characterize the main clinical, macroscopic, and histological aspects of these neoplasms.

MATERIALS AND METHODS

All cases of neoplasms affecting the nasal cavity and/or nasal sinuses (nasosinusal neoplasms) in dogs were compiled, both from necropsies and biopsies analyzed at the "Laboratório de Patologia Veterinária" (LPV) of the "Universidade Federal de Santa Maria" (UFSM) from January 2000 to December 2019 (20 years). The number of necropsies and biopsies of the canine species received in the same period were also computed.

The data regarding the age, sex, breed, size, the cranial conformation of the dogs, and the clinical signs were obtained from the biopsy and necropsy protocols of the LPV-UFSM files. For purebred dogs, the classification was made according to the skull conformation in brachycephalic, mesocephalic, and dolichocephalic dogs, according to the characteristics described for each breed by the "Confederação Brasileira de Cinofilia" (CBKC 2019) and by the American Kennel Club (AKC 2019).

The macroscopic aspects, including the distribution of the lesions (considering the possible site of origin, invasion, and metastases), macroscopic descriptions, and the morphological diagnoses were based on what was contained in each protocol. Based on histopathological diagnoses, they were grouped according to histogenesis into epithelial, mesenchymal, or other neoplasms and round cells, according to the system proposed by Wilson (2017) and adapted according to Caswell & Williams (2016). The macroscopic characteristics were complemented by the photographs of the cases from the photographic collection of the LPV-UFSM.

In cases where paraffin blocks were available in the LPV-UFSM files, new histological slides were made to reassess the microscopic characteristics in selected cases, mainly when the neoplasm subtype had not been classified.

In some of these cases where a subclassification had not been defined, hematoxylin and eosin (HE) stain and the immunohistochemistry (IHC) techniques were also used to define the diagnosis better. The IHC technique was performed in three cases diagnosed as undifferentiated sarcoma. All sections were subjected to dewaxing and rehydration, blocking endogenous peroxidase with 3% hydrogen peroxide (H_2O_2) (2x times for 10 min.). Subsequently, antigen retrieval was performed with Tris-EDTA (pH 9.0) in a microwave oven (for 10 min. at full power) and blocking of non-specific reactions using casein (skimmed-milk powder). Primary antibodies were used in dilutions of 1:2,000 (pan cytokeratin; epithelial cell marker), 1:100 (vimentin; mesenchymal cell marker), 1:200 (KIT; mast cell marker), 1:200 (CD3; T lymphocyte marker), 1:100 (CD79αcy; B lymphocyte marker) and 1:500 (MAC-387; macrophage marker), all of which were incubated in a humid chamber at 37°C for 1 hour. Later, the EasyLink-One polymer (EasyPath) was used at 25°C for 20 min. As a chromogen, 3,3'diaminobenzidine (DAB) was used.

Harris hematoxylin was used as a counter-stain. As positive controls, previously tested cases for the respective antibodies were used, and as negative controls, only PBST was used in the tested sections.

RESULTS

From January 2000 to December 2019, 5,365 dogs were necropsied, and 11,489 biopsies of the same species were received, totaling 16,854 samples from canines. Forty-nine cases of nasosinusal neoplasms were accounted for, 29 from necropsies and 20 from biopsies, making up 0.29% of all dogs analyzed in the 20 years of the study.

Of the 49 dogs that had a nasosinusal neoplasm, twenty-seven (55%) were female, and twenty-two (45%) were male. About a half (51%) of the dogs were classified as mixed-breed. Of the 24 (49%) dogs that were purebred (distributed in 16 breeds), most dogs were classified as large breeds (63%), followed by small dogs (21%), giant dogs (13%), and medium-sized dogs (4%). Dogs were classified according to their skull shape: 8% were brachycephalic (2/24), 50% were mesocephalic (12/24), and 42% were dolichocephalic (10/24).

The clinical signs observed in dogs with neoplasms from the three histogeneses (epithelial, mesenchymal, and other origins/round cells) were similar. They mainly had an increase in volume in the nasal region (unilateral or bilateral) (21/49), causing partial or total obstruction of the nasal cavity; the presence of nasal discharge (9/49), sometimes bloody (14/49); difficulty breathing (7/49); sneezing (4/49) and coughing (1/49). Due to the invasion of some neoplasms to the adjacent tissues (mainly to the brain), the dogs showed less specific clinical signs of respiratory impairment, such as seizures (4/49), blindness (3/49), reduction in the level of consciousness (2/49), behavior change (1/49), howls (1/49), nystagmus (1/49), and proprioceptive deficiency (1/49). Additionally, there was an increase in volume in the skull region (2/49) and atrophy of the skull muscles (1/49).

In the 49 dogs, 50 tumors affecting the nasal cavity and/or the sinuses were counted. Of the 50 neoplasms, twenty-four were of epithelial origin (48%), sixteen were of mesenchymal origin (32%), and ten were of other origins/round cells (20%). One dog simultaneously presented two different types of neoplasms affecting the nasal cavity (undifferentiated sarcoma) and the frontal sinuses (adenocarcinoma). The macroscopic, histopathological, and immunohistochemical aspects of this case are illustrated in Figures 1 and 2. Of the 50 neoplasms, three were submitted to immunohistochemistry because they had a diagnosis of undifferentiated sarcoma. When submitted to IHC, all cases were positive for vimentin, and one case was also positive for CD3 (T lymphocyte marker). All tested cases were negative for the other antibodies. Four cases of carcinomas that had no subclassification were histologically reassessed. Of these cases, three were classified as adenocarcinomas and one as undifferentiated carcinoma, based on their morphological characteristics. Data on age, breed, distribution of neoplasms, and macroscopy were grouped according to the three histogenetic groups and will be detailed below.

Epithelial neoplasms

The 24 neoplasms of epithelial origin were diagnosed in twelve mixed-breed and twelve purebred dogs. The distribution between the breeds was heterogeneous and consisted of: three Rottweilers and one specimen each of the breeds: Cocker Spaniel, Weimaraner, Fila Brasileiro, Great Dane, Poodle, Saint Bernard, Boxer, German Shepherd, and Dachshund. Of the 21 cases in which the age was informed in the protocols, it ranged from 3 to 16 years, with an average of 10.33 years and a median of 10 years.

Histological subtypes and the distribution of neoplasms (considering the possible site of origin, invasion, and metastasis) are shown in Table 1. Among the epithelial neoplasms, nineteen had as probable site of origin, the nasal cavity, two in the sinuses, and three in other regions (nostril, skin, and palate). The main macroscopic and histological characteristics are detailed in Table 2.

Eleven cases were observed in which the neoplasms caused deformities in the head and/or face of the dogs. Of these cases, five were of squamous cell carcinoma, two of adenocarcinoma, two of transitional carcinoma, one case of neuroendocrine carcinoma, and one of adenosquamous carcinoma.

Table 1. Histological type, number of cases, and distribution of nasosinusal neoplasms with epithelial origin

Neoplasm	No. of cases (%)	Distribution
Adenocarcinoma	9 (18%)	(3) Nasal cavity PL
		(1) Nasal cavity PL, frontal sinus I, jaw I and nasal bone I
		(1) Nasal cavity PL, frontal sinus I, nasal bone I, palate I and gum I
		(1) Nasal cavity PL, soft palate I, brain I and frontal sinus I
		(1) Nasal cavity PL, hard palate I, lung M and submandibular lymph node M
		(1) Nasal cavity PL, nasal bone I, palate I, nasopharynx I and lung M
		(1) Nasal sinus PL, frontal bone I, temporal bone I and temporal muscles I *
Squamous cell carcinoma	9 (18%)	(2) Nasal cavity PL
		(1) Nasal cavity PL, brain I, soft palate I, and lung M
		(1) Nasal cavity PL, hard palate I, soft palate I, and jaw I
		(1) Nasal cavity PL and jaw I
		(1) Nasal cavity PL, frontal bone I and nasal bone I
		(1) Palate PL, frontal sinus I and jaw I
		(1) Nostril PL, nasal cavity I, nasal plane I, nasopharynx I and frontal sinus I
		(1) Skin PL and nasal cavity I
Transitional carcinoma	2 (4%)	(1) Nasal cavity PL and adrenal M
		(1) Frontal sinus PL, frontal bone I, parietal bone I, brain I and retropharyngeal lymph nodes M
Undifferentiated carcinoma	2 (4%)	(1) Nasal cavity PL
		(1) Nasal cavity PL and frontal sinus I
Adenosquamous carcinoma	1 (2%)	(1) Nasal cavity PL, frontal sinus I, frontal bone I, parietal bone I and brain I
Neuroendocrine carcinoma	1 (2%)	(1) Nasal cavity PL, nasal bone I, jaw I, frontal bone I, brain I and frontal sinus I
TOTAL	24/50 (48%)	

(1), (2), (3) Number of cases; PL = Possible place of origin, I = invasion, M = metastasis; * dog had undifferentiated sarcoma in the nasal cavity.

Mesenchymal neoplasms

The 16 neoplasms of mesenchymal origin were diagnosed in seven mixed-breed dogs and in nine purebred dogs (Dalmatian, Akita, Shetland Shepherd, Boxer, Collie, Fila Brasileiro, Pinscher, and two Rottweilers). Of the sixteen dogs, only in one case, the age was not informed. The age of the 15 dogs ranged from 11 months to 14 years, with an average of 7.79 years and a median of 8 years. The youngest dog in this study (11 months) had undifferentiated sarcoma, interpreted as multicentric.

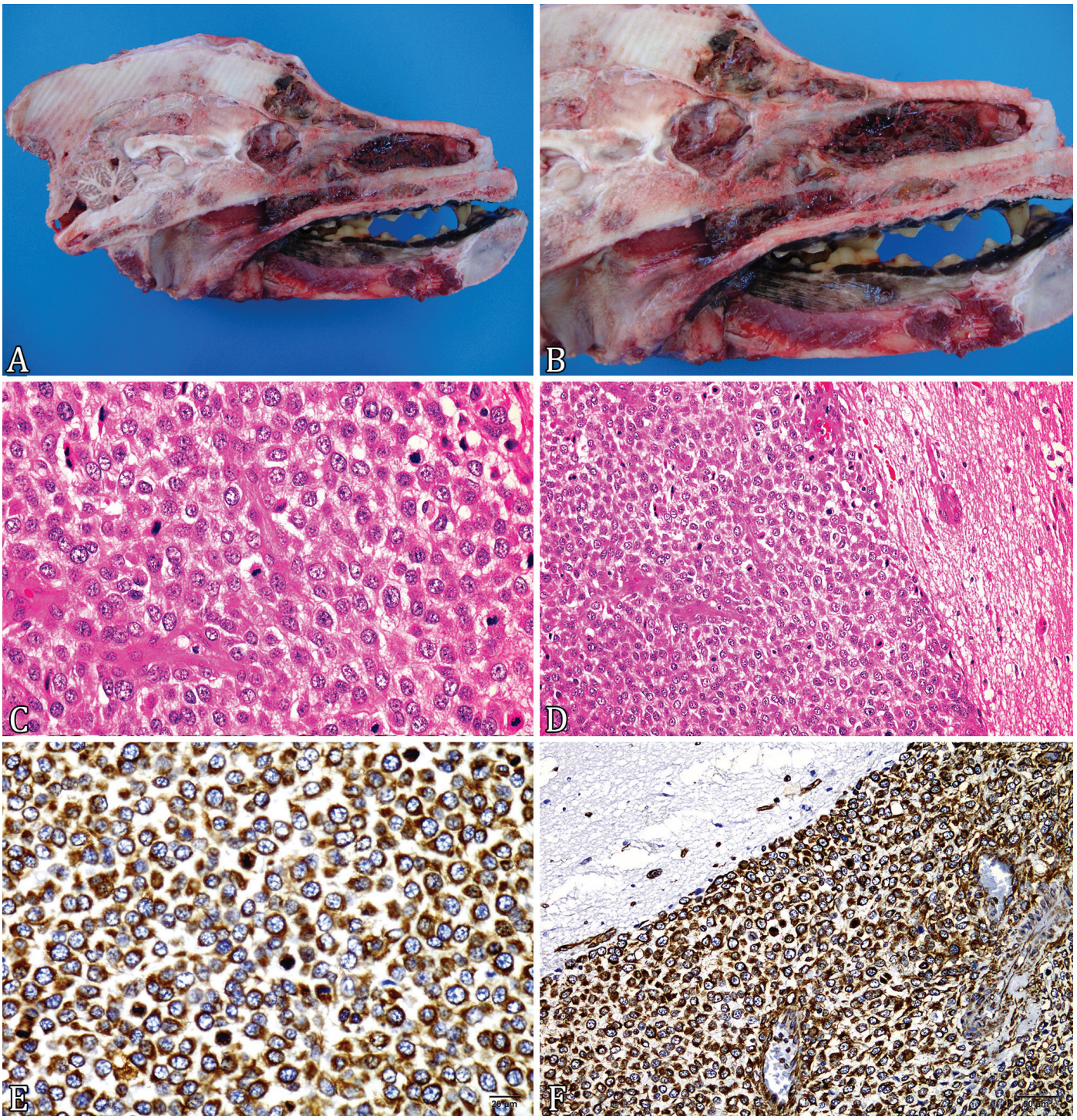


Fig.1. Undifferentiated sarcoma. (**A**) The nasal cavity is filled with a friable, reddish mass that invades the pharynx and crosses the ethmoidal region invading the cranial cavity. (**B**) Approximation of Figure 1A, showing the invasion of brain tissue in the frontal cortex region. (**C**) Neoplasm consisting of a solid mantle of large cells, round to polygonal, with moderate to severe pleomorphism. HE, bar = 20µm. (**D**) Brain invasion of the neoplastic cells described in Figure 1C. HE, bar = 50µm. (**E-F**) Immunohistochemistry for vimentin (streptavidin-biotin-peroxidase method). Neoplastic cells strongly immunostained in the nasal mass (**E**) and brain invasion (**F**) are observed. (**E**) IHC, bar = 20µm. (**F**) IHC, bar = 50µm.

Histological subtypes and neoplasm distribution (considering the possible site of origin, invasion, and metastasis) are shown in Table 3. Among the mesenchymal neoplasms, 12 had a probable place of origin in the nasal cavity, three in other regions (two in the maxilla and one in the palate), and one was multicentric. The main macroscopic and histological characteristics are described in Table 4.

Seven cases were observed in which the neoplasms caused deformities in the head and/or face. Of the seven dogs, four were diagnosed with osteosarcoma, two with chondrosarcoma, and one with fibrosarcoma.

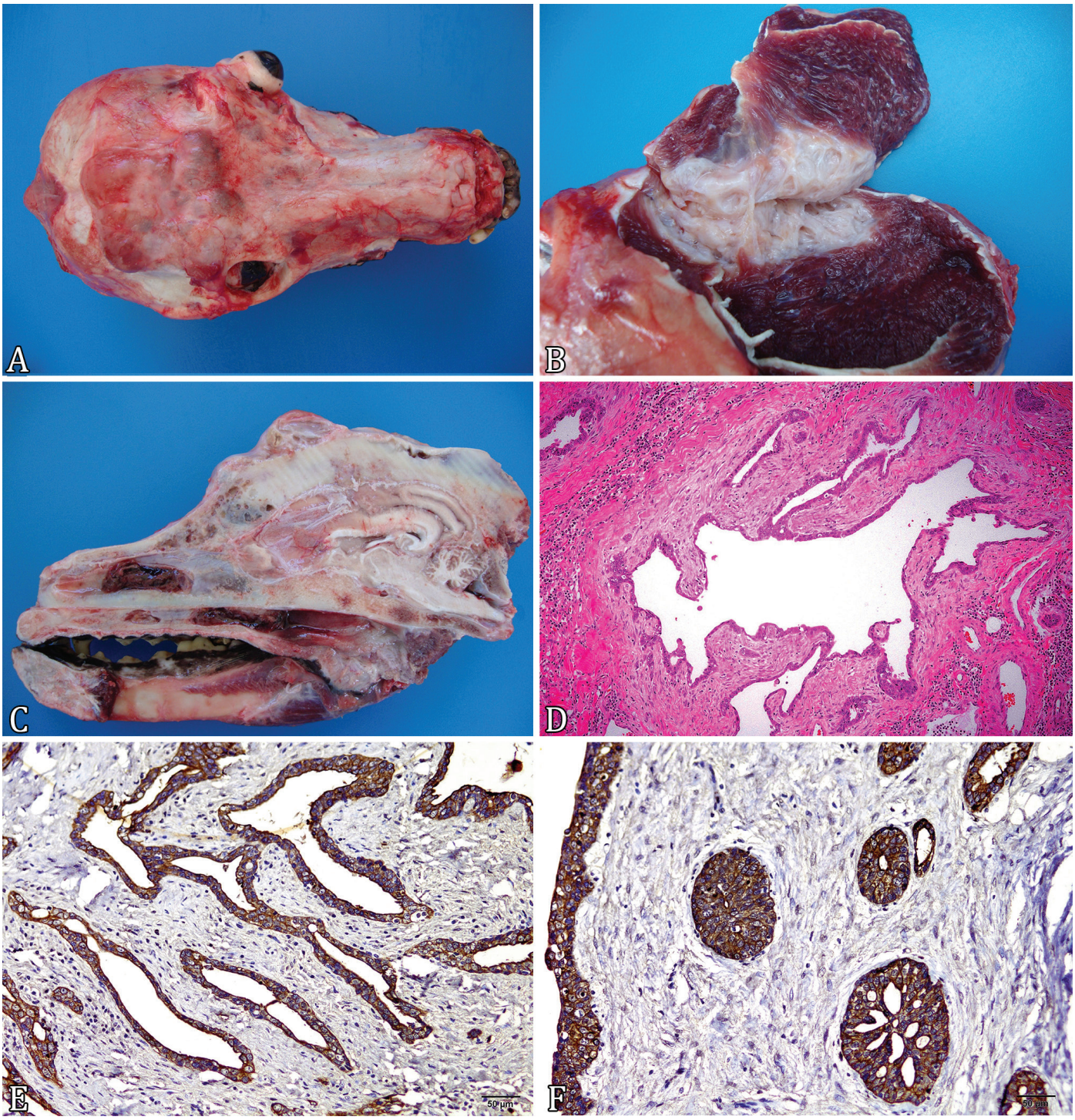


Fig.2. Adenocarcinoma of frontal sinuses. (**A**) Temporal region of the head with firm, cystic and coalescent nodules up to 3 cm in diameter. (**B**) The temporal muscles are invaded by the proliferation of cystic neoplasia. (**C**) Half-head (right side). Adenocarcinomatous proliferation is observed in the frontal sinus region. There are cystic formations that protrude, invade, and thicken the skull bones. (**D**) Neoplasm inside the frontal sinuses consisting of cystic epithelial formations, covered by one or more layers of cells. There are also some islands of neoplastic epithelial cells. HE, bar = 100µm. (**E-F**) Immunohistochemistry for pan cytokeratin (streptavidin-biotin-peroxidase method). Solid islands with luminous openings (**E**) or cystic epithelial formations (**F**) are strongly immunostained. IHC, bar = 50µm.

Neoplasms of other origins and round cells

The breeds affected by neoplasms from other origins/round cells were: Siberian Husky, German Shepherd, and Dachshund (two cases). Six were mixed-breed dogs. The age of these dogs ranged from 1 to 14 years; the mean age was 8.2 years, and the median was 8 years.

Histological subtypes and the distribution of neoplasms (considering the possible site of origin, invasion, and metastasis) are shown in Table 5. Among neoplasms from other origins/round cells, seven had the nasal cavity as the probable place of origin and three in the brain. The main macroscopic and histological characteristics are described in Table 6. In three cases, deformation of the face was observed, and all of which were diagnosed as transmissible venereal tumor (TVT).

DISCUSSION

Primary neoplasms of the nasal cavity or sinuses are rarely found and rarely reported. However, among the species of domestic animals, the most affected is the canine (Stünzi & Hauser 1976). The low frequency of occurrence of these neoplasms, according to the literature, meets the small number of cases found in 20 years in the diagnostic routine of the LPV-UFSM, where it accounted for only 0.29% of all cases of necropsies and biopsies from dogs received in the period. However, this percentage was higher than that reported by Wilson (2017), where for every 100,000 dogs, only 2.5 (0.0025%) would have some type of nasosinusal neoplasm.

Table 2. Histological type and macroscopic and histological characteristics of nasosinusal neoplasms with epithelial origin

Neoplasm		Main macroscopic and histological characteristics
Adenocarcinoma	M	Whitish, yellowish, and brownish masses and nodules, mostly friable, soft, firm, and mostly multilobular. Cystic areas when cutting.
	H	Polyhedral, cuboid or pleomorphic cells, forming acini with single or multiple layers and, sometimes, solid areas. Scarce to abundant cytoplasm. Round and oval nuclei, with loose chromatin and evident nucleoli.
Squamous cell carcinoma	M	Whitish to yellowish mass, sometimes pink or brownish, ranging from soft to firm.
	H	Polyhedral cells are grouped in cords, nests, and islands. Abundant cytoplasm. Round to oval nuclei, with loose chromatin and evident nucleoli. There are areas with individual keratinization and others with the formation of keratin pearls.
Transitional carcinoma	M	Whitish to light pink and firm mass, sometimes with cystic areas with dark brown content.
	H	Polyhedral cells forming acini. The cytoplasm is abundant. Polyhedral nuclei, round or elongated, central, with densely aggregated, loose, or vesicular chromatin.
Undifferentiated carcinoma	M	Whitish with reddish areas, ranging from soft to firm.
	H	Round polyhedral cells are grouped in the mantle. Abundant cytoplasm. Round nuclei with loose to vesicular chromatin and evident nucleoli.
Adenosquamous carcinoma	M	Whitish mass with yellowish and soft areas.
	H	Polyhedral cells forming single-walled acini. Moderate cytoplasm. Large, round, and oval core with loose chromatin. Presence of lamellar keratin ("keratin pearls").
Neuroendocrine carcinoma	M	Whitish, soft, friable, and multilobulated mass.
	H	Polyhedral cells are grouped in the mantle. Vacuolated cytoplasm. Round, oval or reniform nuclei with markedly aggregated chromatin.

M = Macroscopy, H = histology.

Table 3. Histological type, number of cases, and distribution of nasosinusal neoplasms with mesenchymal origin

Neoplasm	No. of cases (%)	Distribution
Chondrosarcoma	4 (8%)	(2) Nasal cavity PL
		(1) Nasal cavity PL and hard palate I
		(1) Nasal cavity PL and palate I
Undifferentiated sarcoma	4 (8%)	(2) Nasal cavity PL
		(1) Nasal cavity PL and pharynx I
		(1) Nasal cavity MC, skin MC, mammary gland MC, myocardium MC, lymph nodes MC, lungs MC, mediastinum MC, hard palate MC, abdominal lump MC, kidneys MC, ovaries MC, skeletal muscles MC, pharynx MC, brain MC and submandibular lymph nodes MC
Osteosarcoma	1 (2%)	(1) Nasal cavity PL
Chondroblastic osteosarcoma	1 (2%)	(1) Jaw PL, nasal cavity I and lung M
Osteoblastic osteosarcoma	1 (2%)	(1) Jaw PL, nasal cavity I and hard palate I
Telangiectatic osteosarcoma	1 (2%)	(1) Hard palate PL and nasal cavity I
Giant cell-type osteosarcoma	1 (2%)	(1) Nasal cavity PL and facial muscles I
Giant cell bone tumor	1 (2%)	(1) Nasal cavity PL and oral cavity I
Fibrosarcoma	1 (2%)	(1) Nasal cavity PL and facial muscles I
Hemangiosarcoma	1 (2%)	(1) Nasal cavity PL
TOTAL	16/50 (32%)	

(1), (2) Number of cases; PL = possible place of origin, I = invasion, M = metastasis, MC = multicentre; * dog had adenocarcinoma in the frontal sinuses.

In general (including the three histogenetic categories of neoplasms), it was observed that females were slightly more affected than males (male: female ratio of 1:1.23). The frequency of involvement of mesocephalic dogs was slightly higher than in dolichocephalic dogs; brachycephalics were much less affected. Twenty-four purebred dogs, distributed in 16 different breeds, were affected. It was also observed that approximately 80% of the purebred dogs were of large and giant breeds. These results demonstrate the profile of this group of 49 dogs analyzed, not allowing to affirm any predisposition of the canine population in general. According to the literature, there is variation in the male:female relationship between studies, and it is not possible to determine whether there is a predisposition by sex (Wilson 2017). For some authors, nasosinusal neoplasms usually affect dogs with dolichocephalic cranial conformation (with a longer elongated snout) compared to mesocephalic and brachycephalic (Lobetti 2009, Rassnick et al. 2006). However, this conclusion is considered controversial (Wilson 2017). It is believed that dogs when compared to other domestic species, are more prone to the development of neoplasms in the nasal cavity. This predisposition is possibly due to epidemiological conditions, such as higher exposure to pollutants in the environment, especially tobacco smoke, burning charcoal, and kerosene heat sources(Caswell & Williams 2016). Certain breeds have been identified in the literature as most at risk for this type of neoplasm, including

Table 4. Histological type and macroscopic and histological characteristics of nasosinusal neoplasms with mesenchymal origin

Neoplasm		Main macroscopic and histological characteristics
Chondrosarcoma	M	Masses ranging from whitish to yellowish and sometimes reddish, mostly firm and less often soft, usually with an uneven surface.
	H	Cells are spindle-shaped, polyhedral, round, or oval. Cytoplasm from scarce to abundant. Oval or round nuclei, elongated or reniform, with loose chromatin and evident nucleoli. Chondroid matrix formation areas.
Undifferentiated sarcoma	M	Whitish and reddish, friable, and irregular mass.
	H	Round cells grouped in the mantle. Scarce to abundant cytoplasm. Round, central nuclei with dense chromatin.
Osteosarcoma	M	Blackened, friable, and firm or hard fragments.
	H	Cells are round, polygonal, or starry, forming mantles. Abundant cytoplasm. Core with loose chromatin and evident nucleolus. Osteoid matrix, sometimes mineralized.
Chondroblastic osteosarcoma	M	Whitish, firm, and finely cross-linked mass.
	H	Pleomorphic cells are grouped in the mantle. Moderate cytoplasm. Round, oval or polyhedral nuclei with loose chromatin and evident nucleoli. Streaked by myxoid stroma. Presence of osteoid and chondroid matrix.
Osteoblastic osteosarcoma	M	Whitish, multilobulated, and soft mass
	H	Rounded polyhedral cells grouped in cords, groups, and mantles. Moderate cytoplasm. Round to oval nuclei with loose chromatin and evident nucleoli. With a small amount of bone matrix, sometimes mineralized.
Telangiectatic osteosarcoma	M	Whitish nodule with reddish areas, smooth and regular.
	H	Pleomorphic, elongated, oval, and round cells grouped in mantles. Scarce cytoplasm. Round, large nuclei with loose chromatin and nucleoli evident. A large number of newly formed vessels in the middle of the neoplastic cells.
Giant cell-type osteosarcoma	M	Reddish mass.
	H	Rounded polyhedral cells are arranged in mantles (anaplastic osteoblasts). Moderate cytoplasm. Round nuclei with finely dotted chromatin and evident nucleoli. Large numbers of giant cells are observed. Interwoven with a small amount of non-mineralized osteoid matrix.
Giant cell bone tumor	M	Invasive and ulcerated mass.
	H	Polygonal and spindle cells forming mantles and spindles, respectively. Moderate cytoplasm. Oval to elongated nuclei with loose chromatin and evident nucleoli. With multinucleated giant cells interspersed. Mineralized and non-mineralized osteoid matrix.
Hemangiosarcoma	M	Brownish, regular, friable, and soft plate.
	H	Fusiform and polyhedral cells are grouped in mantles, cords, and bundles. Scarce cytoplasm. Elongated and rounded nuclei, central with loose chromatin and evident nucleoli.

M = Macroscopy, H = histology.

Table 5. Histological type, number of cases, and distribution of nasosinusal neoplasms with other origins and round cells

Neoplasm	No. of cases (%)	Distribution
Transmissible venereal tumor	5 (10%)	(1) Nasal cavity PL
		(1) Nasal cavity PL and oral cavity I
		(2) Nasal cavity PL and hard palate I
		(1) Nasal cavity PL, soft palate I and skin I
Psammomatous meningioma	2 (4%)	(2) Brain PL and nasal cavity I
Anaplastic meningioma	1 (2%)	(1) Brain PL, nasal cavity I, nasal septum I, jaw bone I, the lacrimal bone I, and left orbit I
Olfactory neuroblastoma	1 (2%)	(1) Nasal cavity PL and brain I
T-cell lymphoma	1 (2%)	(1) Nasal cavity PL, brain I and submandibular lymph nodes M
TOTAL	10/50 (20%)	

(1), (2) Number of cases; PL = Possible place of origin, I = invasion, M = metastasis.

Table 6. Histological type and macroscopic and histological characteristics of nasosinusal neoplasms from other origins and round cells

Neoplasm		Macroscopic and histological characteristics
Transmissible venereal tumor	M	Mostly whitish nodules and masses, sometimes reddish, soft, and multilobulated (with the appearance of cauliflower).
	H	Round cells are arranged in a mantle. Abundant and vacuolated cytoplasm (ranging from finely to markedly). Round, large, central nuclei with loose chromatin and evident nucleoli.
Psammomatous meningioma	M	Masses ranging from whitish to reddish and soft in the olfactory bulb.
	H	Spindle cells grouping into islands, nests, and bundles forming swirls. Moderate to abundant cytoplasm. Round to oval nuclei with loose chromatin and few evident nucleoli. Among the neoplastic cells, there are concentric aggregates of basophilic material (psammomatous bodies).
Anaplastic meningioma	M	Irregular, soft, and whitish mass.
	H	Round cells in mantle. Scarce to moderate cytoplasm with very distinct cytoplasmic limits. Round nuclei, with condensed chromatin and evident nucleoli.
Olfactory neuroblastoma	M	Whitish, soft mass.
	H	Polyhedral cells forming mantles, rosettes, and microcysts. Scarce cytoplasm with indistinct boundaries. Round to oval, hyperchromatic cores with finely granular chromatin.
Lymphoma (T-cell)	M	Left side: reddish to blackish soft mass with gelatinous areas. Right side: white and soft mass.
	H	Round cells are arranged in mantles. Scarce cytoplasm. The nuclei are round and sometimes reniform with evident nucleoli.

M = Macroscopy, H = histology.

descending order, Airedale terrier, Basset hound, Old English Sheepdog, Scottish terrier, Collie, Shetland Shepherd, and German Shorthaired Pointer (Wilson 2017).

In general, there was a wide variation in the age of the affected dogs (11 months to 16 years old). However, when separated by histogenetic groups, it was observed that the dogs affected by epithelial neoplasms had a mean and median age higher than those affected by mesenchymal neoplasms and other origins. These results are in line with those described in the literature (Stünzi & Hauser 1976, Wilson 2017), but a specific cause for this observation was not identified.

The clinical signs described for the dogs in this study were mainly related to involvement in the upper respiratory tract, including partial or total obstruction of the nasal cavity, breathing difficulty, sneezing, presence of nasal discharge, and cough, being similar to that previously observed (Segal et al. 1986, Rocha et al. 2008, Cruz et al. 2009, Gonçalves et al. 2012, Galiazzo et al. 2017). However, attention was drawn to the frequency of dogs with neurological signs related to when the neoplasm invaded the brain and adjacent tissues or when it was primary in the brain, as in the case of the three meningiomas observed. Although nervous symptoms are described in dogs with nasosinusal neoplasms (Rassnick et al. 2006, Silva et al. 2009, Wilson 2017), brain invasion should not be ruled out due to the absence of neurological signs, because in some cases dogs may not display these signs (Turek & Lana 2013).

As to the origin (histogenesis) of the neoplasms, the epithelial ones accounted for 16% and 28% more than the mesenchymal neoplasms and other origins/round cells, respectively, in the 49 dogs studied. In the literature, most nasosinusal neoplasms have been described as having an epithelial origin, but the percentage relationship with neoplasms from other sources has not been reported (Patnaik 1989, Lobetti 2009, Wilson 2017).

Among the epithelial neoplasms, the most frequently described, in decreasing order of prevalence, are adenocarcinomas, transitional carcinomas, and squamous cell carcinomas (SCC) (Lobetti 2009, Wilson 2017). In this study, the most prevalent neoplasms of epithelial origin were adenocarcinoma and SCC, with the same number of cases each.

Among the 16 mesenchymal neoplasms diagnosed, the highest occurrence was osteosarcomas, chondrosarcomas, and undifferentiated sarcomas; the first had five cases, and the others had four cases each. Chondrosarcomas occur mainly in flat bones, whereas osteosarcomas usually occur in the appendicular skeleton (Thompson & Dittmer 2017). Because of this, it was believed that a higher number of chondrosarcoma cases occur when compared to those of osteosarcoma, in the nasal cavity, due to the presence of the nasal bone, which is a flat bone (Patnaik et al. 1984). However, in this study, similar numbers were observed for each of these neoplasms.

Of the ten neoplasms with other origins/round cells, half were classified as transmissible venereal tumor (TVT). This neoplasm is a tumor mainly related to the genitals, occurring mainly in the vulva, penis, and foreskin, for having its transmission during coitus (Hendrick 2017). However, dogs can acquire this neoplasm by licking, biting, and smelling another dog affected by the TVT. Because of this behavior of dogs from one sniff to another, neoplastic cells are transmitted to the nasal cavity (Woods 2013). The other cases were encephalic meningiomas that invaded the nasal cavity, olfactory neuroblastoma, and lymphoma. The latter two being neoplasms considered rare in this location (Wilson 2017).

As for cranial/facial deformation, it was observed that, among epithelial neoplasms, almost half of the cases were diagnosed as SCCs. This diagnosis indicates that this neoplasm has more destructive/proliferative tissue characteristics (with more external changes) than the other nasosinusal epithelial cell neoplasms. Among mesenchymal neoplasms, the one that caused the greatest deformities visible externally (facial and cranial asymmetries) was osteosarcoma, compared to chondrosarcoma and fibrosarcoma. As for neoplasms of other origins and round cells, the only one that caused external deformities was TVT. The other neoplasms (brain meningioma, olfactory neuroblastoma, and T-cell lymphoma) had their internal expansion in the nasal cavity, including invading the brain and vice-versa (in the case of brain meningioma).

CONCLUSIONS

Nasosinusal neoplasms had a low frequency in the diagnostic routine. However, their occurrence is relevant because they were all malignant, regardless of histogenesis, with a higher frequency of epithelial neoplasms, followed by mesenchymal and other origins/round cells. It was possible to trace the profile of the affected dogs.

Among purebred dogs, the mesocephalics were more affected concerning dolichocephalics and brachycephalics.

There was a wide variation in the age of the dogs, but older dogs were affected by epithelial neoplasms, compared to those of other origins.

There was a predominance of nasal over sinus neoplasms, with frequent invasions to adjacent tissues, often culminating in cranial and facial deformities accompanied mainly by respiratory and nervous signs, depending on the location and extent of the neoplasm. Despite being infrequent in the diagnostic routine, nasosinusal neoplasms can be challenging to diagnose, especially in small biopsies.

Considering that these neoplasms are usually malignant, the importance of an accurate and rapid diagnosis is emphasized to establish the most appropriate treatment for the affected dog.

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Electrocardiographic markers of myocardial conduction and repolarization in Boxer dogs[1]

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ABSTRACT.- Carvalho E.R, Zacché E., Fenerich M., Camacho A.A., Santos J.P. & Sousa M.G. 2020. **Electrocardiographic markers of myocardial conduction and repolarization in Boxer dogs**. *Pesquisa Veterinária Brasileira 40(8):630-636*. Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista "Júlio do Mesquita Filho", Via de Acesso Prof. Paulo Donato Castellane s/n, Jaboticabal, SP 14884-900, Brazil. E-mail: beth_rcarvalho@hotmail.com

Electrocardiographic markers have been used in people to classify arrhythmogenic risk. The aims of this study were to investigate electrocardiographic markers of conduction and repolarization in Boxers and non-Boxer dogs, and compare such findings between groups. Ten-lead standard electrocardiograms of Boxer dogs and non-Boxers recorded from 2015 to 2018 were retrospectively reviewed. Dogs ≥4 years of age and weighing >20kg were included. Animals with valvular insufficiencies, congenital cardiopathies, cardiac dilation, suspected systolic dysfunction, biphasic T-wave, bundle branch blocks, and those receiving antiarrhythmics were excluded. Electrocardiographic markers of conduction, QRS duration (QRS_d) and dispersion (QRS_D), and repolarization (corrected QT interval, T_{peak}-T_{end}, JT and JT_{peak}), as well as derived indices, were measured. Two hundred dogs met the inclusion/exclusion requirements, including 97 Boxers (8.1±2.5 years old; 30±7kg) and 103 non-Boxer (8.8±2.5 years old, 30±8kg). QRS_d and QRS_D, and repolarization markers in lead II and left precordial lead V4 were considered similar between groups. Dispersion of late repolarization on lead rV2, Tpeak-Tend interval, was considered longer in Boxers (45±8ms vs 38±10ms, P=0.01). The T_{peak}-T_{end}/JT_{peak} and the JT_{peak}/JT also differed between groups. Our results indicate that the dispersion of myocardial late repolarization in lead rV2 is slower in Boxers than other dog breeds.

INDEX TERMS: Electrocardiography, myocardium, conduction, repolarization, Boxer dogs, arrhythmias, canine, electrocardiography, premature cardiac complexes.

RESUMO.- [**Marcadores eletrocardiográficos de condução e repolarização miocárdica em cães da raça Boxer.**] Marcadores eletrocardiográficos têm sido estudados em seres humanos para estratificação do risco arritmogênico. Os objetivos deste estudo foram investigar os marcadores eletrocardiográficos de condução e repolarização miocárdica em Boxers e em cães de outras raças, e comparar tais resultados entre os grupos. Para tal, a eletrocardiografia convencional de 10 derivações registradas de 2015 a 2018 foram avaliadas de maneira retrospectiva. Cães com idade igual ou superior a 4 anos e pesando >20kg foram incluídos. Animais com insuficiência valvar, cardiopatias congênitas, dilatação cardíaca, suspeita de disfunção sistólica, onda T bifásica, bloqueio(s) de ramo(s), ou aqueles que recebiam antiarrítmicos foram excluídos. Variáveis eletrocardiográficas de condução, como a duração e dispersão do complexo QRS (QRS_d e QRS_D, respectivamente), e repolarização (intervalo QT corrigido, T_{pico}-T_{final}, JT e JT_{pico}), bem como índices derivados, foram mensurados. Duzentos cães que se adequaram aos critérios de inclusão/exclusão foram incluídos, 97 Boxers (8,1±2,5 anos; 30±7kg) e 103 não Boxers (8,8±2,5 anos; 30±8kg). O QRSd e o QRSD, e os marcadores de repolarização nas derivações II e V4 foram similares entre os grupos. O marcador de dispersão da repolarização tardia na derivação rV2, T_{pico}-T_{final}, foi considerado mais longo no

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Boxers (45±8ms *vs* 38±10ms, P=0.01). O T_{pico}-T_{final}/JT_{pico} e o JT_{pico}/JT também diferiram entre os grupos. Nossos resultados indicam que a dispersão da repolarização miocárdica tardia na derivação precordial direita, rV2, é mais lenta no Boxer do que nas outras raças.

TERMOS DE INDEXAÇÃO: Eletrocardiografia, condução, repolarização, miocárdio, cães, raça Boxer, arritmias, caninos, eletrocardiografia, complexos cardíacos prematuros.

INTRODUCTION

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an inherited myocardial disease commonly seen in adult Boxer dogs. The disease is characterized by the replacement of right ventricle (RV) cardiomyocytes by a fibrofatty tissue, a condition that eventually generates areas prone to the formation of ventricular arrhythmias, which may culminate in sudden death (Harpster 1983, 1991). There is no single and specific diagnostic test for ARVC, thus, in veterinary practice, the diagnosis is best based on a combination of findings which may include: the presence of ventricular tachyarrhythmia with no other documented causes for the arrhythmia, syncope, and family history of ARVC (Meurs 2004, 2017). Although many Boxers with ARVC live for years even without treatment, they are always at an increased risk of sudden cardiac death (Meurs 2017).

In people many electrocardiographic markers have been studied to identify individuals at risk for malignant arrhythmias and sudden death (Kurl et al. 2012, Kurosaki et al. 2013, Tse & Yan 2016, Zumhagen et al. 2016). These can be based on myocardial conduction, such as QRS duration (QRS_d), which is a surrogate marker of conduction velocity (CV), and QRS dispersion (QRS_D) reflecting CV dispersion. Conduction abnormalities associated with reduced CV, along with a functional or structural obstacle, are conditions for re-entry (Tse & Yan 2016).

Abnormal repolarization properties can increase the risk of triggered activity and re-entrant arrhythmias, the latter known to be involved in the pathogenesis of ARVC (Basso et al. 2015). Electrocardiographic markers based on repolarization include: corrected QT (QT_c), QT dispersion (QT_D), interval from the peak to the end of the T-wave (T_{peak}-T_{end}), and interval from J point to the peak and/or end of the T-wave (JT_{peak} and JT, respectively).

Although studied in many conditions known to be associated with increased arrhythmogenicity in man, such as ARVC (Zorzi et al. 2013), diabetes (Stettler et al. 2007), sepsis (Ozdemir et al. 2016), and myocardial infarction (Zhao et al. 2012), studies investigating electrocardiographic markers of conduction and repolarization in dogs are lacking. The aims of this study were to investigate electrocardiographic markers of conduction and repolarization in Boxers and non-Boxer dogs over 20kg, and compare such findings between the general population and a breed widely known to be predisposed to developing ventricular arrhythmias.

MATERIALS AND METHODS

Study Design and Ethics Statement. This retrospective study was approved by the institutional Animal Care and Use Committee (protocol 012277/17).

Animals. Boxer dogs (BG) evaluated from 2015 to 2018 at the Cardiology sections of two referral teaching practices were identified by searching the medical record databases. Only dogs that met the following inclusion criteria were admitted into the study: ≥4 years old, body weight >20kg, with a standard high-quality 10-lead electrocardiogram and an echocardiogram. The electrocardiograms (ECG) (ECGPCVet, Tecnologia Eletrônica Brasileira) were recorded for a minimum of 3 minutes with the animal positioned in right lateral recumbency. The electrodes for frontal and precordial leads were placed as described elsewhere (Tilley & Burtinick 1999). The echocardiogram (My Lab 30, Esaote) was obtained within a maximum of seven days from the evaluated ECG. Dogs found to have valvular insufficiencies, suspected systolic dysfunction (defined as shortening fraction ≤25%), left atrial dilation (defined as left atrial-to-aortic root ratio ≥1.6 (Hansson et al. 2002), ventricular dilation (defined as body weight normalized left ventricular internal diameter in diastole ≥1.7 (Cornell et al. 2004), congenital cardiopathies, biphasic T-wave, QRS complexes with bundle branch block morphology, and those receiving antiarrhythmics were excluded. In addition, matched non-Boxer dogs (NBG) were selected based on the same inclusion/exclusion requirements.

In BG 37 dogs had an available 24-Hour Holter monitoring recorded within seven days of the evaluated ECG and echocardiogram. Boxers were further classified according to the degree of ventricular ectopy, based on the following criteria: 0 to 20 ventricular premature complexes (VPCs) per 24 hours were interpreted as normal; 21 to 300 VPCs per 24 hours were interpreted as indeterminate; and animals with more than 300 VPCs/24h were considered likely to be affected by ARVC (Meurs 2017). Only the subgroup of Boxers considered normal (n=14) and the subgroup considered likely to be affected by ARVC (n=15) utilizing the Holter criteria were compared for all electrocardiographic parameters listed in Table 1. The subgroup considered indeterminate (n=8) was excluded from such comparisons.

The clinical history of syncope at the time of ECG and echocardiogram was searched in medical records of all dogs in BG and NBG. In NBG no animal had a recorded syncope, 88 Boxers in BG were unaffected but nine had suffered at least one syncopal episode. All electrocardiographic parameters listed in Table 1 were also compared for syncopal and non-syncopal Boxers.

Electrocardiographic evaluation. One investigator, who was blinded to the breed and clinical status of dogs, measured all the parameters listed in Table 1 and illustrated in Figure 1. The measurements were made in a run of five sequential sinus beats. The highest and lowest values obtained for each parameter were excluded and the final result was considered as the average of three measurements. Parameters obtained from precordial leads were measured using leads rV2 as right and V4 as left.

Statistical analysis. The Shapiro-Wilk test was used to investigate the normal distribution of data. Groups were compared using either Mann-Whitney test or Student's t-test according to distribution. Parametric data are represented as mean ± standard deviation, while non-parametric variables are shown as median [interquartile range]. The contingency was evaluated using the Fisher's exact test. Statistical processing was performed using commercially available statistical software (Prism Windows 6.0, GraphPad Software), and significance was defined as $P<0.05$.

RESULTS

A total of 200 dogs met the inclusion/exclusion requirements and were recruited for this study. The sample comprised 97

Boxers and 103 non-Boxer dogs. No differences in gender were documented within or between groups. The mean body weight did not differ between groups (Boxers 30±7kg, non-Boxers 30±8kg, P=0.82). The mean age was also considered similar between groups (Boxers 8.1±2.5 years old, non-Boxers 8.8±2.5 years old, P=0.16). Among NBG, mixed breed dogs were most represented (n=67, 65%), followed by Labrador Retrievers (n=11, 11%), Pitbulls (n=9, 9%), and Golden Retrievers (n=8, 8%). No animal from NBG had a history of syncope. In BG, nine dogs (9%) had a clinical history of syncope, and 88 (91%) had no previous history.

The electrocardiographic markers of conduction, QRS_d and QRS_D, were considered similar between BG and NBG. Also, no differences existed between BG and NBG regarding the myocardial repolarization markers obtained from leads II and V4. When looking at lead rV2, the dispersion of late repolarization evaluated as T_{peak}-T_{end} was longer in Boxers (45±8ms) than it was in non-Boxer dogs (38±10ms) (P=0.01). Similarly, the dispersion of repolarization normalized to JT_{peak} interval obtained from lead rV2, i.e. T_{peak}-T_{end} / JT_{peak}, was found to be higher in BG (0.41 [0.35-0.51]) than in NBG (0.37 [0.27-0.40]) (P=0.03). Curiously, the dispersion of repolarization normalized to JT interval, the JT_{peak}/JT, was found to be slightly lower in Boxers (0.72 [0.67-0.75] *vs* 0.73 [0.71-0.79], P=0.04). Our results are summarized in Table 2, and box-plots of markers found to be different between groups are represented in Figure 2.

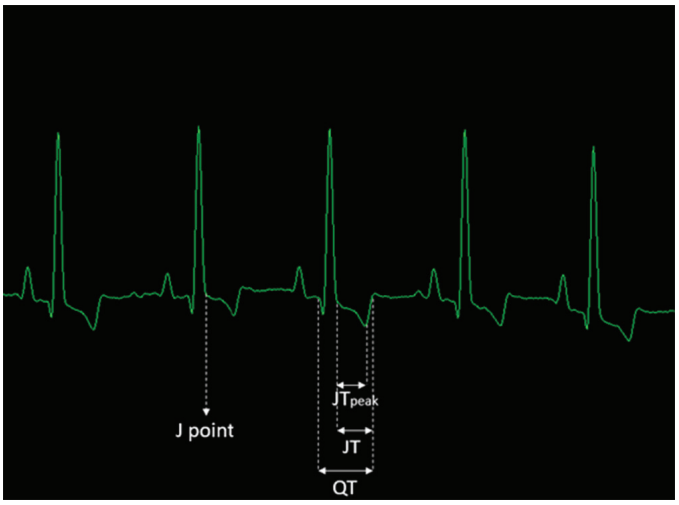


Fig.1. Schematic representation of J point, QT interval, JT interval and JT_{peak} interval measurements used to obtain electrocardiographic markers in lead II.

Interestingly, no differences were documented in the electrocardiographic markers between syncopal and non-syncopal Boxers (Table 3), or between Boxers considered normal (n=12) and those likely affected by ARVC (n=15) according to the 24-Hour Holter criteria for the degree of ventricular ectopy (Table 4).

DISCUSSION

In this study, traditional and novel electrocardiographic conduction and repolarization markers of augmented arrhythmogenesis were evaluated and compared between Boxers and dogs of other breeds. In these retrospective analyses, both Boxers who had history of syncope (n=9) and those without (n=88) were included, as well as Boxers considered normal (n=12) or likely affected by ARVC (n=15) according to Holter criteria. None of the dogs had received antiarrhythmic therapy at the time the electrocardiogram was recorded. The body weight and age were considered similar between BG and NBG, although only animals ≥ 4 years old and >20kg were included in the study to minimize the impact of the dog's size and age on electrocardiographic parameters.

Table 1. Summary of electrocardiographic markers evaluated in this study, which are based on either myocardial conduction or repolarization

Classification of risk marker	Electrocardiographic marker	Definition	Pre-clinical marker correlate	Reference
Conduction	QRS_d	QRS duration, interval between the beginning and the end of QRS complex measured in leads II, rV2 and V4	Conduction velocity (CV)	Kurl et al. 2012
	QRS_D	QRS dispersion, maximum difference between QRS durations measured in the right (rV2) and left (V4) precordial leads	CV difference between two regions	Tse et al. 2015
Repolarization	QT_c	QT interval at DII corrected for heart rate using the Van de Water' formulae	Action potencial duration (APD)	Surawicz 1987
	QT_D	Maximum difference between QT intervals in two leads of the 10-lead ECG (apllying the correction proposed by Malik & Batchvarov 2000)	Difference in APD values between two regions	Linker et al. 1992
	T_{peak}-T_{end}	Interval from the peack to the end of the T-wave, measured in leads II, rV2 and V4	Dispersion of late repolarization	Xia et al. 2005a
	T_{peak}-T_{end}/QT_c	Interval from the peak to the end of the T-wave divided by QT_c	Dispersion of repolarization divided by APD	Castro Hevia et al. 2006
	JT_{peak}/JT	JT_{peak}: interval from J-point to peak of the T-wave JT: interval from J-point to the end of T-wave. Measured in leads II, rV2 and V4	Dispersion of repolarization normalized to JT interval	Alvarado-Serrano et al. 2003, Brisinda et al. 2004
	T_{peak}-T_{end}/JT_{peak}	Measured in leads II, rV2 and V4	Dispersion of repolarization normalized to JT_{peak} interval	Alvarado-Serrano et al. 2003, Brisinda et al. 2004

The pathological features of ARVC in both people and Boxer dogs, which are considered as a spontaneous animal model for the study of the human ARVC, include cardiomyocyte atrophy and fibrofatty replacement (Marcus et al. 1982, Basso et al. 2015). Depolarization delay in right precordial leads is a common feature in people with ARVC (Nasir et al. 2004, Cox et al. 2008). Thus, the QRS prolongation (>110ms) in right precordial leads is considered a major criteria for diagnosis of ARVC in people (Marcus et al. 2010), and an independent predictor of risk of sudden cardiac death in the general population (Kurl et al. 2012). However, our findings suggest that the electrocardiographic surrogates of conduction velocity of myocardial depolarization impulse behave similarly in Boxers and in medium to large non-Boxer dogs, and Boxers with a high degree of ventricular ectopy (>300 VPCs/24h) compared to those with a low number of VPCs (0-20 VPCs/24h).

The QT interval reflects the duration of the action potential at the cellular level, being associated with depolarization and triggered activity. The QT_c interval in people with type 1 diabetes is considered an independent predictor of cardiovascular mortality (Stettler et al. 2007). However, although it is a poor indicator of heterogeneity in repolarization across the heart, it is known that increased heterogeneities in repolarization increase the risk of arrhythmias (Tse & Yan 2016). No

Table 2. Comparison of electrocardiographic surrogates of myocardial conduction and repolarization in Boxer dogs (n=97) and non-Boxer dogs (n=103) obtained from the 10-lead standard electrocardiography

Electrocardiographic marker	Boxer	non-Boxer	P	Pre-clinical marker correlate
QRS_d lead II	67 (61-76) ms	60 (57-67) ms	0.06	Conduction velocity (CV)
QRS_d lead rV2	70 (67-77) ms	67 (60-73) ms	0.11	
QRS_d lead V4	64 ± 9 ms	64 ± 10 ms	0.89	
QRS_D	10 (3-16) ms	7 (3-13) ms	0.50	CV difference between two regions
QT_c	257 (246-272) ms	254 (242-258) ms	0.22	Action potencial duration (APD)
QT_D	7 (5-11) ms	7 (5-8) ms	0.54	Difference in APD values between two regions
T_{peak}-T_{end} lead II	34 ± 9 ms	31 ± 7 ms	0.22	Dispersion of late repolarization
T_{peak}-T_{end} lead rV2	45 ± 8 ms	38 ± 10 ms	0.01*	
T_{peak}-T_{end} lead V4	35 ± 9 ms	34 ± 12 ms	0.51	
T_{peak}-T_{end} /QT_c lead II	0.14 ± 0.04	0.12 ± 0.03	0.34	Dispersion of repolarization divided by APD
T_{peak}-T_{end} /QT_c lead rV2	0.17 (0.15-0.19)	0.15 (0.13-0.18)	0.07	
T_{peak}-T_{end} /QT_c lead V4	0.13 (0.11-0.16)	0.12 (0.09-0.17)	0.67	
JT DII	146 ± 22 ms	150 ± 10 ms	0.47	Global dispersion of repolarization
JT rV2	150 ± 19 ms	148 ± 8 ms	0.77	
JT V4	153 ± 20 ms	151 ± 12 ms	0.74	
JT_{peak} DII	112 ± 25 ms	121 ± 11 ms	0.21	Dispersion of early repolarization
JT_{peak} rV2	105 ± 18 ms	110 ± 7 ms	0.29	
JT_{peak} V4	119 ± 20 ms	117 ± 14 ms	0.78	
JT_{peak}/JT lead II	0.76 (0.71-0.83)	0.82 (0.76-0.86)	0.08	Dispersion of repolarization normalized to JT interval
JT_{peak}/JT lead rV2	0.72 (0.67-0.75)	0.73 (0.71-0.79)	0.04*	
JT_{peak}/JT lead V4	0.78 (0.73-0.81)	0.77 (0.73-0.83)	0.64	
T_{peak}-T_{end} / JT_{peak} lead II	0.33 (0.23-0.42)	0.26 (0.19-0.29)	0.08	Dispersion of repolarization normalized to JT_{peak} interval
T_{peak}-T_{end} / JT_{peak} lead rV2	0.41 (0.35-0.51)	0.37 (0.27-0.40)	0.03*	
T_{peak}-T_{end} / JT_{peak} lead V4	0.29 (0.25-0.37)	0.27 (0.18-0.40)	0.50	

* Statistical significance (P<0.05).

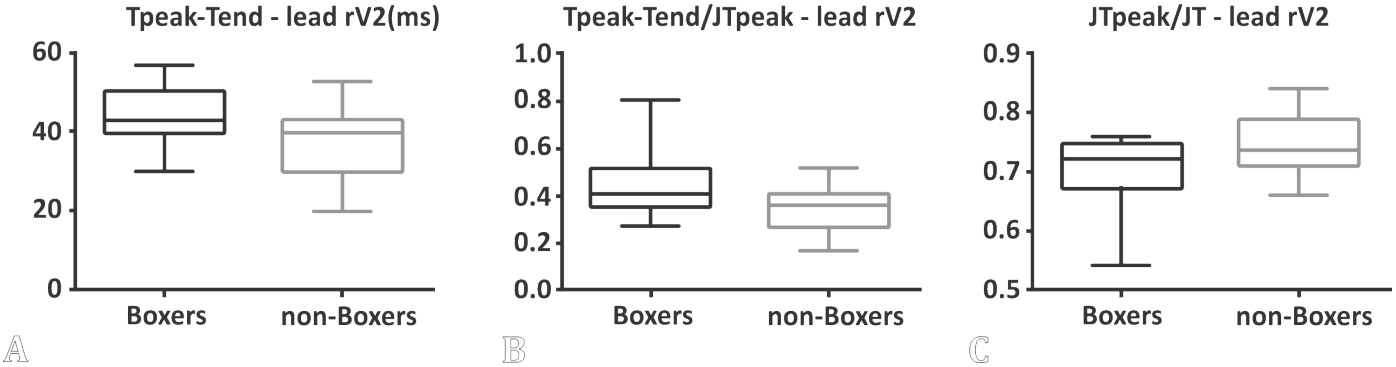


Fig.2. Box-plots of data distribution for electrocardiographic markers of repolarization found to differ between Boxer (n=97) and non-Boxer (n=103) dogs.

differences were noticed either in QT_c or QT_D between groups in this investigation.

The fibrofatty tissue that replaces healthy myocardium in ARVC is thought to contribute to the development of ventricular arrhythmias by slowing intra-ventricular conduction, and acting as a substrate for arrhythmias through a scar-related macro-reentry mechanism (Fontaine et al. 1984). The T_{peak}-T_{end} interval is important as a lead-dependent marker of global dispersion of late repolarization (Xia et al. 2005a, 2005b), since repolarization dispersion varies in different cardiac regions (Bieganowska et al. 2013). Therefore, it was proposed that T_{peak}-T_{end} interval should be determined from right precordial leads for right ventricular disorders (Tse & Yan 2016). When the right precordial lead rV2 is considered, our results for the electrocardiographic markers T_{peak}-T_{end} and T_{peak}-T_{end}/JT_{peak} suggest that the late dispersion of RV repolarization occurs more slowly in Boxer dogs than in other breeds. In people T_{peak}-T_{end} is increased in short QT syndrome (Watanabe et al. 2010), Brugada syndrome (Zumhagen et al. 2016), and patients with "torsade des pointes" (Yamaguchi et al. 2003).

While T_{peak}-T_{end} represents late repolarization, JT_{peak} represents early repolarization (Johannesen et al. 2014a, 2014b), and both are potential markers of abnormal repolarization (Clemente et al. 2012). In people, the JT_{peak}/JT and the T_{peak}-T_{end}/JT_{peak} showed a higher sensitivity and specificity than T_{peak}-T_{end} for identifying patients who suffered myocardial infarction (Alvarado-Serrano et al. 2006). The dispersion of repolarization normalized to JT or JT_{peak} interval at rV2 differs in Boxers form non-Boxers in our study. Unexpectedly, the marker JT_{peak}/JT was slightly lower in BG, mainly due to the interquartile range difference between groups, since median values were similar in both groups.

The small and unequal sample size between syncopal and non-syncopal Boxers may have contributed significantly to our findings in such a comparison. According to our results, myocardial conduction and repolarization is similar in supposedly healthy and syncopal Boxers. As previously mentioned, a diagnosis of ARVC in veterinary patients is based on a combination of findings, including ventricular arrhythmias, syncope, and familial history of ARVC. Many disorders, such as neurocardiogenic bradycardia (Thomason et al. 2008), can ultimately lead to syncopal events. Antiarrhythmic drugs such as sotalol and mexiletine act by blocking ion channels, potassium and sodium respectively, in the cellular membrane, thus, interfering in electrocardiographic measurements, especially those related to conduction and repolarization. Since most syncopal Boxers admitted to a referring cardiology service are already receiving antiarrhythmic therapy, it is challenging to study electrocardiographic markers in such individuals.

The results of this study should be interpreted in the context of its limitations. This was a retrospective investigation and accepts the inherent criticisms of the methodology. Moreover, it is difficult to determine the end of the T-wave on conventional electrocardiography, as previously reported by many researchers and this may introduce inaccuracies (Bieganowska et al. 2013, Kaplan et al. 2015, Tse & Yan 2016, Zumhagen et al. 2016). Also, the low number of syncopal Boxers, and those with a 24-hour Holter recording may have compromised the accuracy of our analysis.

CONCLUSIONS

Myocardial electrical conduction occurs similarly in Boxer dogs and non-Boxer dogs.

Global dispersion of myocardial late repolarization in the right precordial lead rV2 occurs more slowly in Boxers than dogs from other breeds.

Table 3. Comparison of electrocardiographic surrogates of myocardial conduction and repolarization in syncopal (n=9) and non-syncopal Boxer dogs (n=88) obtained from 10-lead standard electrocardiography

Electrocardiographic marker	Non-syncopal (n=88)	Syncopal (n=9)	*p*
QRS_d lead II	63 (60-70) ms	73 (63-80) ms	0.11
QRS_d lead rV2	67 (63-73) ms	73 (67-80) ms	0.14
QRS_d lead V4	64 ± 8 ms	63 ± 8 ms	0.60
QRS_D	6 (3-13) ms	10 (6-17) ms	0.21
QT_c	257 ± 16 ms	267 ± 28 ms	0.19
QT_D	5 (4-10) ms	7 (5-11) ms	0.26
T_{peak}-T_{end} lead II	31 ± 10 ms	35 ± 9 ms	0.26
T_{peak}-T_{end} lead rV2	43 (38-53) ms	50 (37-50) ms	0.77
T_{peak}-T_{end} lead V4	37 ± 11 ms	33 ± 9 ms	0.34
T_{peak}-T_{end}/QT_c lead II	0.12 ± 0.04	0.13 ± 0.04	0.38
T_{peak}-T_{end}/QT_c lead rV2	0.16 (0.15-0.20)	0.18 (0.16-0.19)	0.83
T_{peak}-T_{end}/QT_c lead V4	0.14 ± 0.05	0.12 ± 0.05	0.13
JT_{peak}/JT lead II	0.79 (0.73-0.85)	0.75 (0.73-0.82)	0.24
JT_{peak}/JT lead rV2	0.72 (0.70-0.75)	0.71 (0.65-0.73)	0.14
JT_{peak}/JT lead V4	0.77 ± 0.06	0.78 ± 0.07	0.52
T_{peak}-T_{end}/JT_{peak} lead II	0.26 (0.20-0.36)	0.34 (0.24-0.40)	0.21
T_{peak}-T_{end}/JT_{peak} lead rV2	0.38 (0.34-0.47)	0.44 (0.38-0.52)	0.30
T_{peak}-T_{end}/JT_{peak} lead V4	0.29 (0.25-0.37)	0.28 (0.20-0.34)	0.57

Table 4. Comparison of electrocardiographic surrogates of myocardial conduction and repolarization obtained from 10-lead standard electrocardiography in Boxer dogs considered normal (n=12) and affected by ARVC (n=15) according to the degree of ventricular ectopy on 24-hour Holter monitoring

Electrocardiographic marker	Normal Boxers (n=12)	ARVC Boxers (n=15)	P
QRS_d lead II	63 (60-67) ms	73 (63-80) ms	0.05
QRS_d lead rV2	69 ± 7 ms	72 ± 7 ms	0.15
QRS_d lead V4	67 (60-70) ms	63 (57-70) ms	0.48
QRS_D	8 ± 9 ms	9 ± 6 ms	0.37
QT_c	260 ± 15 ms	267 ± 28 ms	0.40
QT_D	5 (4-11) ms	7 (5-11) ms	0.25
T_{peak}-T_{end} lead II	32 ± 10 ms	35 ± 9 ms	0.33
T_{peak}-T_{end} lead rV2	43 (37-54) ms	50 (37-50) ms	0.99
T_{peak}-T_{end} lead V4	39 ± 12 ms	33 ± 9 ms	0.14
T_{peak}-T_{end}/QT_c lead II	0.13 ± 0.04	0.13 ± 0.04	0.45
T_{peak}-T_{end}/QT_c lead rV2	0.16 (0.15-0.19)	0.18 (0.16-0.19)	0.79
T_{peak}-T_{end} /QT_c lead V4	0.15 ± 0.05	0.12 ± 0.05	0.07
JT_{peak}/JT lead II	0.78 (0.72-0.87)	0.75 (0.73-0.82)	0.38
JT_{peak}/JT lead rV2	0.73 (0.69-0.75)	0.71 (0.65-0.73)	0.21
JT_{peak}/JT lead V4	0.76 ± 0.07	0.79 ± 0.06	0.36
T_{peak}-T_{end}/JT_{peak} lead II	0.24 (0.19-0.37)	0.34 (0.24-0.40)	0.23
T_{peak}-T_{end}/JT_{peak} lead rV2	0.43 ± 0.15	0.45 ± 0.10	0.30
T_{peak}-T_{end}/JT_{peak} lead V4	0.34 ± 0.15	0.29 ± 0.11	0.27

Traditional and novel electrocardiographic markers of myocardial conduction and repolarization were obtained in Boxers and non-Boxers dogs >20kg.

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Computed tomographic and radiologic anatomy of the lower respiratory tract in the red-foot tortoise (*Chelonoidis carbonaria*)[1]

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ABSTRACT.- Polanco J.B.A., Mamprim M.J., Silva J.P., Inamassu L.R. & Schimming B.C. 2020. **Computed tomographic and radiologic anatomy of the lower respiratory tract in the red-food tortoise (*Chelonoids carbonaria*).** *Pesquisa Veterinária Brasileira 40(8):637-646*. Departamento de Anatomia, Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rua Prof. Dr. Antônio Celso Wagner Zanin 250, Distrito de Rubião Junior, Botucatu, SP 18618-689, Brazil. E-mail: bruno.schimming@unesp.br

The diagnosis of several diseases in chelonians is a challenge in the veterinary clinic, because a detailed physical examination with auscultation and palpation is difficult due the presence of carapace and plastron. Imaging analysis such as radiography and computed tomography (CT) have been shown to be beneficial for diagnosis, prognosis and treatment in numerous animal species. Thus, this study aimed to identify and describe the structures of the lower respiratory tract in red-foot tortoises, by computed tomography, radiography and gross anatomy in twelve red-foot tortoises (*Chelonoidis carbonaria*), adults and of both sexes. The lower respiratory tract in these animals comprised the larynx, trachea, bronchi and the lungs. The presence of epiglottic cartilage was not observed in the animals studied. CT allowed the observation of the intrapulmonary part of the bronchi, which was accompanied by large intrapulmonary blood vessels. The lungs presented a reticulated parenchyma, without lobulations. Each lung had a small chamber located near the cranial and caudal poles. These structures were identified in CT and 3D CT reconstructions and these could suggest that these chambers could be non-respiratory structures, and could be comparable to the air sacs of birds. This study establishes normal CT anatomy of the lower respiratory tract of the red-foot tortoise; and may be used as a reference in the assessment of respiratory disorders in this tortoise.

INDEX TERMS: Computed tomographic, lower respiratory tract, red-food tortoise, *Chelonoids carbonaria*, chelonians, respiratory system, radiography, anatomy, wildlife animals.

RESUMO.- [**Anatomia radiológica e por tomografia computadorizada do trato respiratório inferior no jabuti-piranga (*Chelonoidis carbonaria*).**] O diagnóstico de diversas afecções em quelônios é um desafio para a clínica veterinária, já que um exame físico detalhado com auscultação e palpação é difícil devido à presença da carapaça e do plastrão. A radiografia e a tomografia computadorizada (TC) tem se mostrado benéficas para o diagnóstico, prognóstico e tratamento em muitas espécies animais. Assim, este estudo teve por objetivo identificar e descrever as estruturas do trato respiratório inferior no jabuti-piranga por meio da tomografia computadorizada, radiografia e anatomia em 12 jabutis-piranga (*Chelonoidis carbonara*), adultos e de ambos os sexos. Nos animais estudados, o trato respiratório

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inferior consistiu da laringe, traqueia, brônquios e os pulmões. A cartilagem epiglote não foi observada. A TC permitiu a observação da parte intrapulmonar dos brônquios, a qual estava acompanhada dos vasos sanguíneos intrapulmonares. Os pulmões possuíam um parênquima reticulado, sem lobações. Cada pulmão tinha uma pequena câmara localizada junto aos pólos cranial e caudal. Estas estruturas foram identificadas na TC e na reconstrução 3D a partir da TC e poderiam ser estruturas não-respiratórias, podendo ser comparadas aos sacos aéreos das aves. Este estudo identificou a anatomia normal por meio da TC do trato respiratório inferior do jabuti-piranga, o que pode ser usado como referência para diagnóstico de desordens respiratórias nesta espécie.

TERMOS DE INDEXAÇÃO: Anatomia, radiologia, tomografia computadorizada, trato respiratório inferior, jabuti-piranga, *Chelonoidis carbonaria*, quelônios, sistema respiratório, radiografia e anatomia, animais selvagens.

INTRODUCTION

The red-foot tortoise (*Chelonoidis carbonaria*) belongs to the order Testudines, and to the family Testudinidae (Schimidt-Nielsen 1999, Vought et al. 2015). Although this chelonium is not listed as endangered species (Rhodin et al. 2017), there is anthropogenic pressure, caused by habitat degradation, introduction of invasive species, pollution, diseases, unsustainable use, traffic and climate change. Therefore, the red-foot tortoise has been created as a pet, although it is a target for illegal trade and smuggling (Nunes et al. 2010, Vought et al. 2015). However, the lack of knowledge about its creation has led to management errors, compromising health and animal welfare (Nunes et al. 2010). This corroborates the fact that red-foot tortoise is the chelonium most bred in captivity in Brazil and represents an expressive casuistry of attendance in the wildlife clinic (Matias et al. 2006).

The diagnosis of the various respiratory diseases such as pneumonia in chelonians is a challenge in the veterinary clinic. A detailed physical examination with auscultation and palpation is hard since the lungs and others organs are contained by the carapace and the plastron (Murray 1996, McArthur et al. 2004). Noninvasive diagnostic imaging techniques provide detailed information on all organic systems of animals, therefore, anatomical knowledge for disease recognition in radiographic examinations and interpretation of images in CT is essential (Pinto 2007). The diagnosis of respiratory alterations in chelonians is generally based on radiographic studies, but in radiographic examination of the respiratory system there is overlapping of structures such as plastron and carapace, which make difficult the accurate diagnosis of these alterations (Pinto 2007). According to Mackey et al. (2008), the CT showed lesions that the radiographic examination had not indicated, with rich details like size and shape of the different structures that constitute this pulmonary system, besides demonstrating the internal anatomy without overlapping of adjacent structures.

There are few studies on red-foot tortoise and other Brazilian chelonians, especially in relation to imaging studies and anatomical descriptions. To date, there is a description of the appendicular skeleton (Bortolini et al. 2012) and about the kidneys, recently performed by Dadalto (2017), and despite the prevalence of red-foot tortoise in Brazil, there are no data on the respiratory system of this species. Thus, the objective of this study was to describe the lower respiratory tract of red-foot tortoise, clinically healthy, raised in captivity, by imaging studies and gross anatomy, in order to provide subsidies to clinicians, surgeons and researchers, allowing them to obtain a better diagnosis of respiratory disorders in this group of tortoises.

MATERIALS AND METHODS

Animals. Twelve red-foot tortoises (*Chelonoidis carbonaria*) were used in this study (ten males and two females). Ten live animals were used for radiography and computed tomography, and two dead animals were used to anatomical studies. The cadavers of these animals were kept frozen until to perform dissections and anatomical sections. All the animals came from the "Centro de Medicina e Pesquisa em Animais Selvagens" (CEMPAS) of the "Faculdade de Medicina Veterinária e Zootecnia", Unesp, Botucatu, Brazil. The present study was carried out in accordance with ethical principles and with the approval of the Committee on Ethics in the Use of Animals (CEUA 998/2017).

Anesthetic management. For radiographic and tomographic examination, the animals were submitted to 24 hours of fasting prior to the anesthetic procedure. The tortoises were anaesthetized with a combination of ketamine hydrochloride (0.5mg/kg) and propofol (7mg/kg) injected intravenously in the dorsal cervical sinus. Anesthetic maintenance was used in smaller doses: ketamine hydrochloride (0.2mg/kg) associated with propofol (4mg/kg). Anesthesia monitoring was performed with the aid of cardiac Doppler. Cardiac and respiratory frequences were monitored throughout the procedure and anesthetic recovery.

Radiographic examination. All the imaging examinations of each animal were performed on the same day, aiming at reducing the possibility of deleterious effects related to general anesthesia. All analyzes were performed with spontaneous breathing. The animals were positioned in the ventral recumbency to avoid compression of the respiratory structures, on a rectangular foam cushion, with the neck and limbs extended. This avoided the overlapping of images and allowed the positioning similar to the normal anatomical position of this animal. Direct digital radiography equipment (DR-F; GE Health Care Unit, Brazil) was used to obtain the radiographic images of the respiratory system. For this, the incidence of dorsoventral, craniocaudal and lateral (with the animal placed on a plastron stand), with 100cm film focus distance, were performed. The protocol of the radiographic technique was 60-68kVp and 4.0mAs. After obtaining the radiographic images, they were transferred and later accessed in a PACS medical imaging system (Synapse, Fuji Medical System, Tokyo, Japan).

Computed tomography (CT). The equipment used to obtain the tomographic images was a single-slice helical tomograph of the third generation of the Shimadzu Mark, model SCT-7800 TC. Areas of interest for the study with continuous cuts of 1mm and 2mm in axial plane (perpendicular to the greater body of the body) were selected in the craniocaudal direction, using a protocol of 120kVp and 130 (pitch 2, 1-2mm increment and 1 second tube rotation time), RF2 reconstruction filter (soft tissue) and W: 320 L: 35 window for reconstruction, MPR and 3D. The filter RF5 PF9 (hard lung filter) and window W: 1800 L:-600 were also used for pulmonary evaluation. Due to the size of red-foot tortoise and limitations of the tomographic equipment, it was necessary to perform two tomographic cuts with different thicknesses to obtain good resolution images and the evaluation of the different structures of the respiratory system

of these animals. The first cut was performed from the glottis to the beginning of the tracheal bifurcation with a thickness of 1mm. Subsequently, the second cut was performed, with a thickness of 2mm, which began at the tracheal bifurcation until the end of the carapace. The tomographic images were transferred to the software Voxar-3D® version 6.3 (Toshiba Medical Visualization; Edinburg, Scotland) for reconstruction of multiplanar images (MRP) in sagittal and dorsal planes and reconstruction in third dimension (3D) of the different structures. The two-dimensional tomographic images obtained and their three-dimensional reconstructions were evaluated in a PACS medical imaging system (Synapse, Fuji Medical System, Tokyo, Japan) for measurement of pulmonary volume, tracheal and extrapulmonary bronchi length, and tracheal caliber at the level of the first tracheal ring. The length of the trachea and right and left bronchi were measured.

Anatomical study. The dissection and anatomical description of the structures of the respiratory system were performed. The dissection was initiated by removal of the plastron, afterwards all anatomical structures were dissected and carefully removed from the body cavity. In order to establish a comparison with the different tomographic sections, cross-sectional anatomical sections were performed in slices of 4 cm. All findings were documented and named according to the Nomina Anatomica Veterinaria (2017).

RESULTS

The larynx is the most rostral structure and is located caudal to the base of the tongue, in the caudomedial portion of the oral cavity leading directly to the trachea. This structure and its muscles are supported by the hyoid apparatus (Fig.1A-C). Laryngeal cartilages observed were arytenoids, cricoids, and thyroid cartilages (Fig.1C). No epiglottis cartilage was identified in this animal. On the CT scan and on the radiographs, the glottis was easily identified, since it was closed at the moment of the tomographic scan and the radiographic image was obtained (Fig.1A,D,E and 2F).

The trachea was observed caudally to the glottis, following the midline of the neck of the red-foot tortoise (Fig.1C, 3C, 4C, 5D). Subsequently, the trachea deflects towards the left side of the neck, where it runs until its ends (Fig.1D and 3A). This was formed by complete cartilaginous rings (Fig.1C and 3A,C) and a left ventral position in relation to the esophagus (Fig.3A,C). The tracheal bifurcation occurs at the level of the fifth cervical vertebra, giving rise to the extrapulmonary parts of the left and right bronchi (Fig.3C, 4E, 5B, 2D). The trachea was easily identified in the radiographic projections (Fig.2D,F). The presence of air limited by the cartilaginous rings that constitute this tubular structure facilitated its visualization (Fig.1D,E, 3B,D, 2D,F). When the trachea was evaluated using

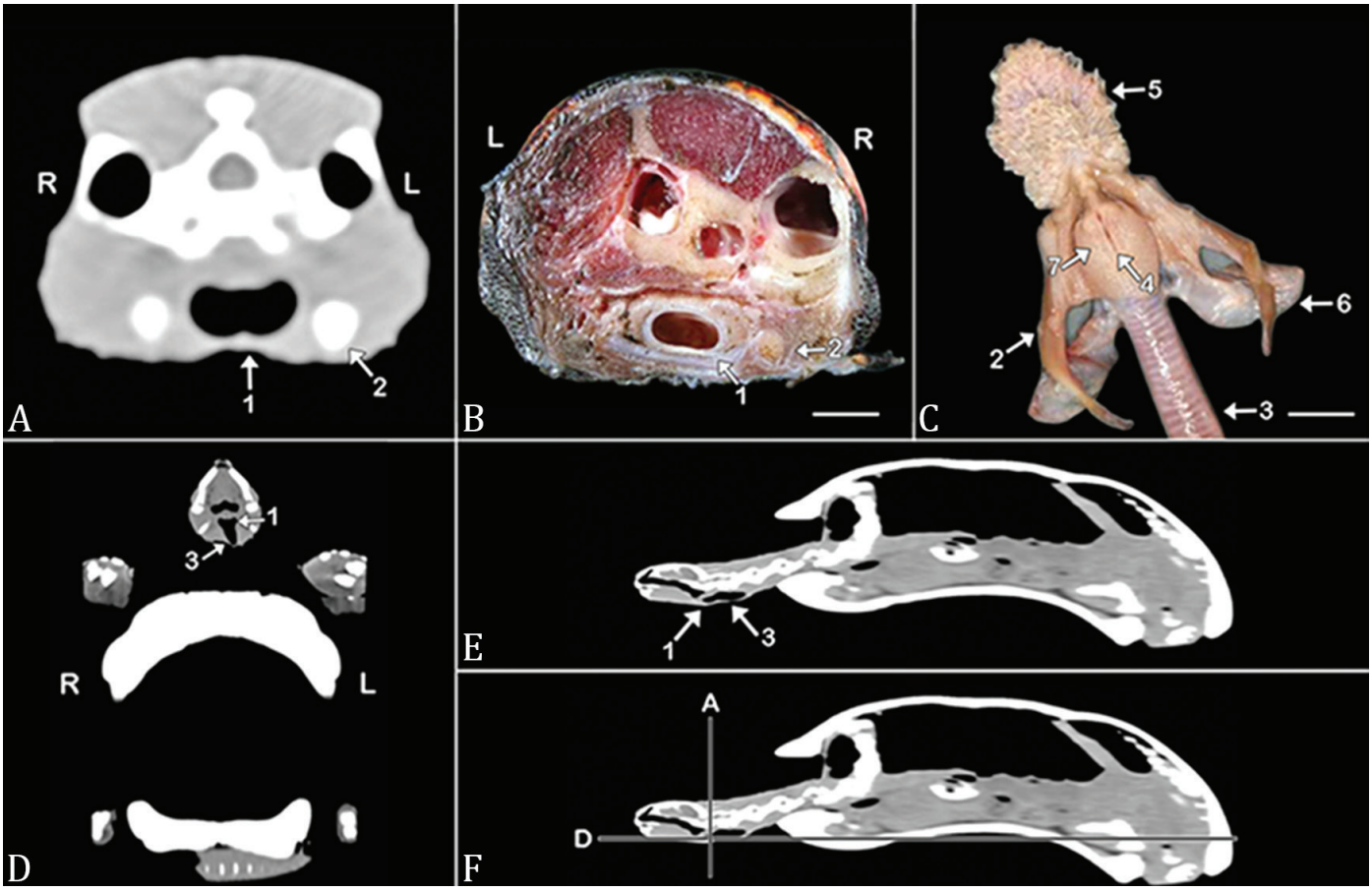


Fig.1. Tomographic and anatomical images of the glottis in the red-foot tortoise. (**A**) Computed tomography (CT) image, transverse plane (soft-tissue window). (**B**) Corresponding gross anatomical section in the level of glottis. Bar = 1cm. (**C**) Dorsal view of larynx. Bar = 1cm. (**D**) CT dorsal plane and (**E**) sagittal plane (soft-tissue window). (**F**) Reference to locate Figure A and D. Glottis (1), hyoid apparatus (2), trachea (3), opening of the glottis (4), tongue (5), thyroid cartilage wings (6), arytenoid cartilage (7), right side (R), and left side (L).

3D virtual bronchoscopy, the tracheal rings were confirmed to be complete and the tracheal mucosa had a smooth surface (Fig.4C). Positioning of the trachea in relation to the esophagus was difficult to evaluate on CT, since the esophagus had no gaseous content in its lumen, which prevented its visualization in the tomographic sections (Fig.3B). However, the anatomical sections allowed to confirm topographically the position of the trachea in relation to the esophagus described previously. The average length of the trachea was 6.67±0.84cm, and its caliber, at the level of the first tracheal ring, had a mean of 0.60±0.11cm. The length of the extrapulmonary parts of the right bronchus was 4.55±0.91cm and the left bronchus was 3.72±0.67cm, respectively.

The bronchi penetrated medially on the ventral surface of the lungs, originating the intrapulmonary part of the bronchi. The intrapulmonary bronchi were visualized in CT and anatomical sections, but were not identified in the anatomic dissection (Fig.6A,C). In tomographic evaluation, these intrapulmonary bronchi were accompanied by two blood vessels during their longitudinal course in the pulmonary parenchyma (Fig.6B,C,E). There were no branches of intrapulmonary bronchi, only chambers of different shapes and sizes, forming part of the pulmonary parenchyma (Fig.6A,C,E). The extra and intrapulmonary parts of the bronchi as well as the large pulmonary blood vessels were clearly identified in the 3D pulmonary reconstruction (Fig.7A-C).

The lungs were large structures with a reticulated aspect that extended dorsally, occupying a large part of the body cavity, from the region of the shoulder girdle to the cranial portion of the pelvic girdle (Fig.3A and 5D). The ventral surface of the lungs was separated from the other structures of the body cavity by a thin non-muscular membrane that completely recovered the lungs, there was difficulty in identifying this membrane in the anatomical dissection because of its fragility (Fig.5A,D). There is a close relationship of the dorsal surface of the lung with a large part of the spine, rib heads (*caput costae*) and dermal bone plates that form the dorsal part of the carapace (Fig.5C).

No pulmonary lobes were identified in the red-foot tortoise (Fig.5C,D). The parenchyma was described as strongly reticulated (Fig.5A and 6A). Pulmonary volume was calculated by means of 3D, being 69.04±15,55cm^3 on average. Hyperdensal areas were observed on the ventral surface of the lung, related to the intestinal loops (Fig.7C,E). Each lung had a small chamber located dorsomedially at the cranial and caudal poles, without the presence of calibrous blood vessels close to them. These structures were clearly identified in the 3D reconstruction (Fig.7A,C,F), as well as in all tomographic sections (Fig.8A-F) and in the Ld radiographic projection, however, they were not identified in the anatomical dissection. Radiographically, the lung was observed to be a radiolucent structure. In the craniocaudal and right lateral projections, it was possible to identify the close pulmonary relation with the carapace and with most of the organs of the body cavity (Fig.2E,F).

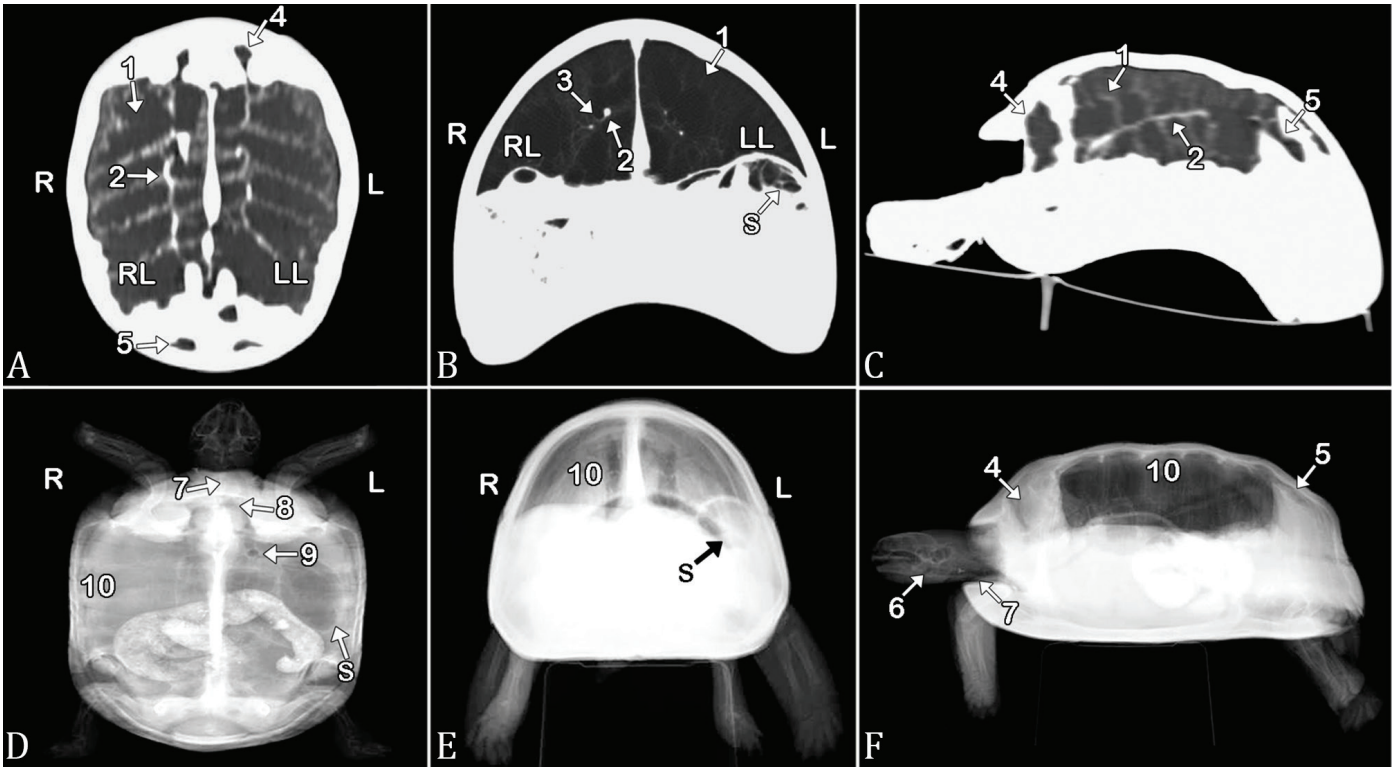


Fig.2. Tomographic and radiographic images of the lungs in the red-foot tortoise. (**A**, **B**, **C**) Tomographic sections: dorsal, transverse and sagittal planes, respectively (pulmonary window). Radiographic imagens in (**D**) dorsoventral, (**E**) craniocaudal and (**F**) right lateral projections. Pulmonary chambers (1), pulmonary blood vessel (2), intrapulmonary part of the right bronchus (3), cranial lung chamber (4), caudal pulmonary chamber (5), glottis (6), trachea (7), carina (8), entry of the left bronchus into the lung (9), lungs with overlapping structures (10), stomach (S), right side (R), and left side (L).

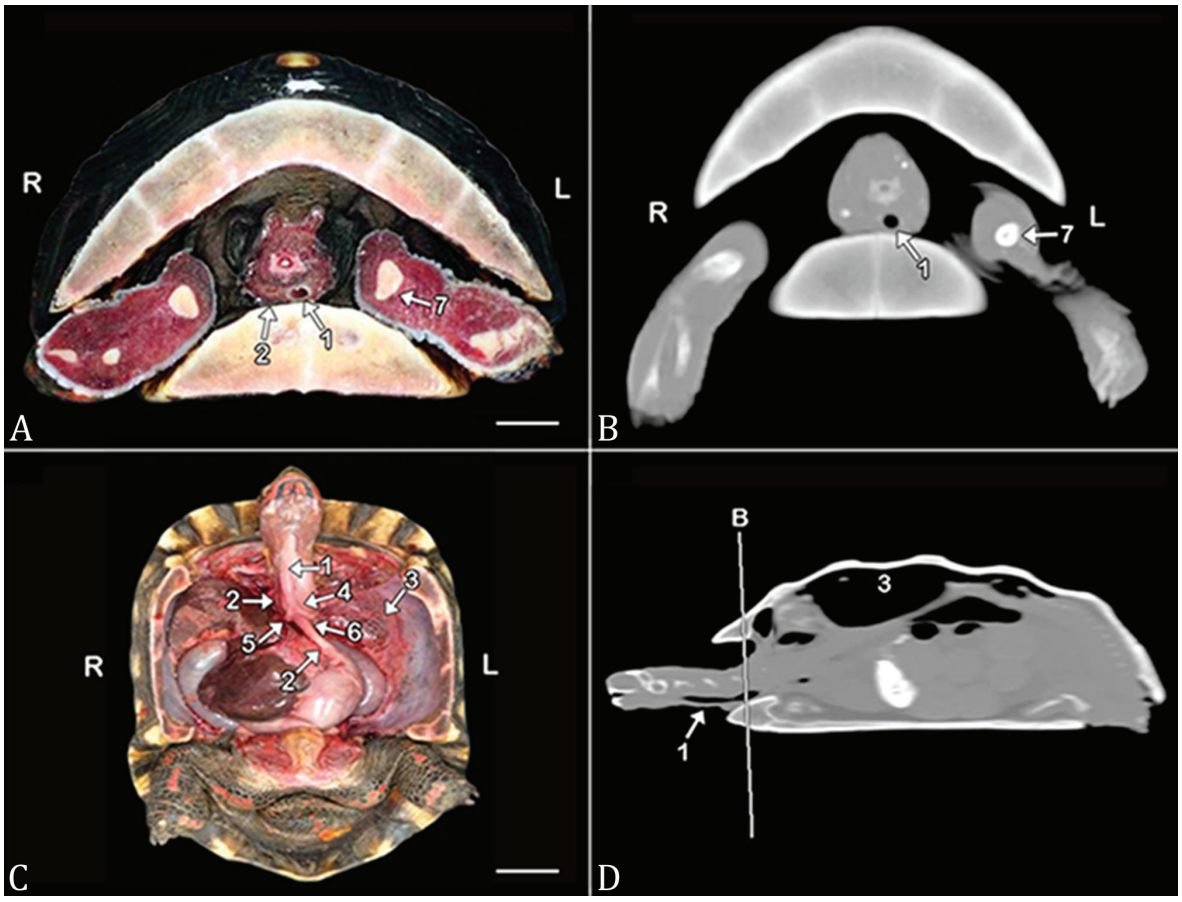


Fig.3. Anatomical and tomographic images of the trachea in the red-foot tortoise. (**A**) Corresponding gross anatomical section. Bar = 9cm. (**B**) Computed tomography (CT) image transverse plane (bone window). (**C**) Ventral view of lower respiratory tract. Bar = 9cm. (**D**) CT sagittal plane (bone window). Reference to locate Figure B. Trachea (1), esophagus (2), lung (3), tracheal bifurcation (4), extrapulmonary part of the right bronchus (5), extrapulmonary part of the left bronchus (6), humerus (7), right side (R), and left side (L).

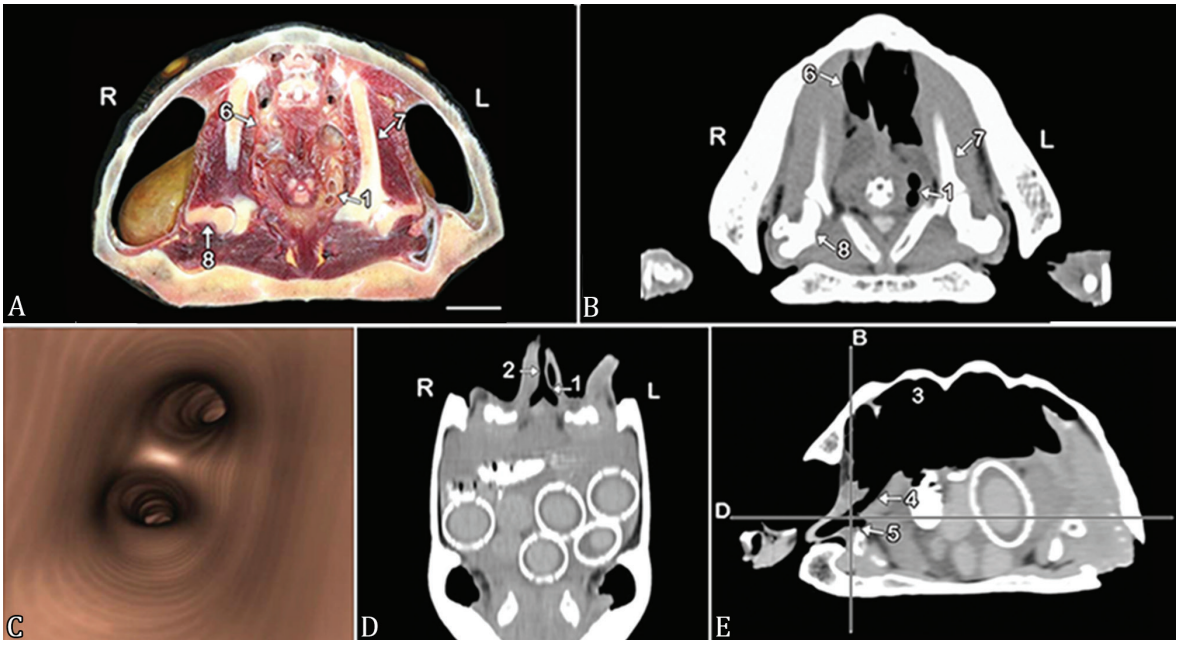


Fig.4. Anatomical and tomographic images of the tracheal bifurcation in the red-foot tortoise. (**A**) Corresponding gross anatomical section. Bar = 7cm. (**B**) Computed tomography (CT) transverse plane (soft-tissue window). (**C**) Virtual bronchoscopy image. (**D**) CT dorsal plane (soft-tissue window). (**E**) Reference to locate the Figure B and D. Tracheal bifurcation (1), trachea (2) left lung (3), extrapulmonary part of the left bronchus (4), extrapulmonary part of the right bronchus (5), cranial pulmonary chamber (6), scapula (7), humerus (8), right side (R), and left side (L).

DISCUSSION AND CONCLUSION

In the chelonians, the knowledge of anatomy of the lower respiratory tract is of great clinical importance, since inflammatory exudates particularly those associated with infectious diseases tend to accumulate in the lungs, due to the fact that the respiratory tract has a primitive mucociliary apparatus, and thus, chelonians could not effectively expel exudates from their lungs (Murray 1996). According to Tracchia (2018), is pivotal to know the anatomy and physiology of the chelonians respiratory tract because the absence of a real diaphragm, the absence of a bronchial ciliated epithelium, the opening of the bronchi in the dorsal aspect of the lungs, and their trabecular appearance cause the secretions produced by pneumonia and it tends to accumulate in the lungs and air sacs since they are scarcely eliminated by the airways. So, this study used CT, radiography and gross anatomy to describe the constituents of the lower respiratory system of red-foot tortoise (*Chelonoidis carbonaria*).

The lower respiratory tract in the red-foot tortoise is composed of larynx, trachea, right and left bronchi and lungs. These results were in agreement with those reported for the sea turtle (*Caretta caretta*) and Egyptian tortoise (*Testudo kleinmanni*) (Valente et al. 2007, Saber & Kamal 2010).

Mayor (2017) described the presence of the epiglottic cartilage in *Chelonoidis denticulata*. A different result was found in this study, in which the presence of this cartilage was not observed in the larynx of *C. carbonaria*, although the two species of tortoises belong to the same Genus. The absence of the epiglottis has been reported in chelonians (O'Malley 2005). In the absence of the epiglottis, the closed glottis is opened by the dilator of glottis muscle (O'Malley 2005, Devoe 2010).

Several previous studies (Christopher & Hernandez-Divers 2003, O'Malley 2005, Valente et al. 2007, Zehtabvar et al. 2014) have described that trachea is composed of complete cartilaginous rings, following the pattern described herein

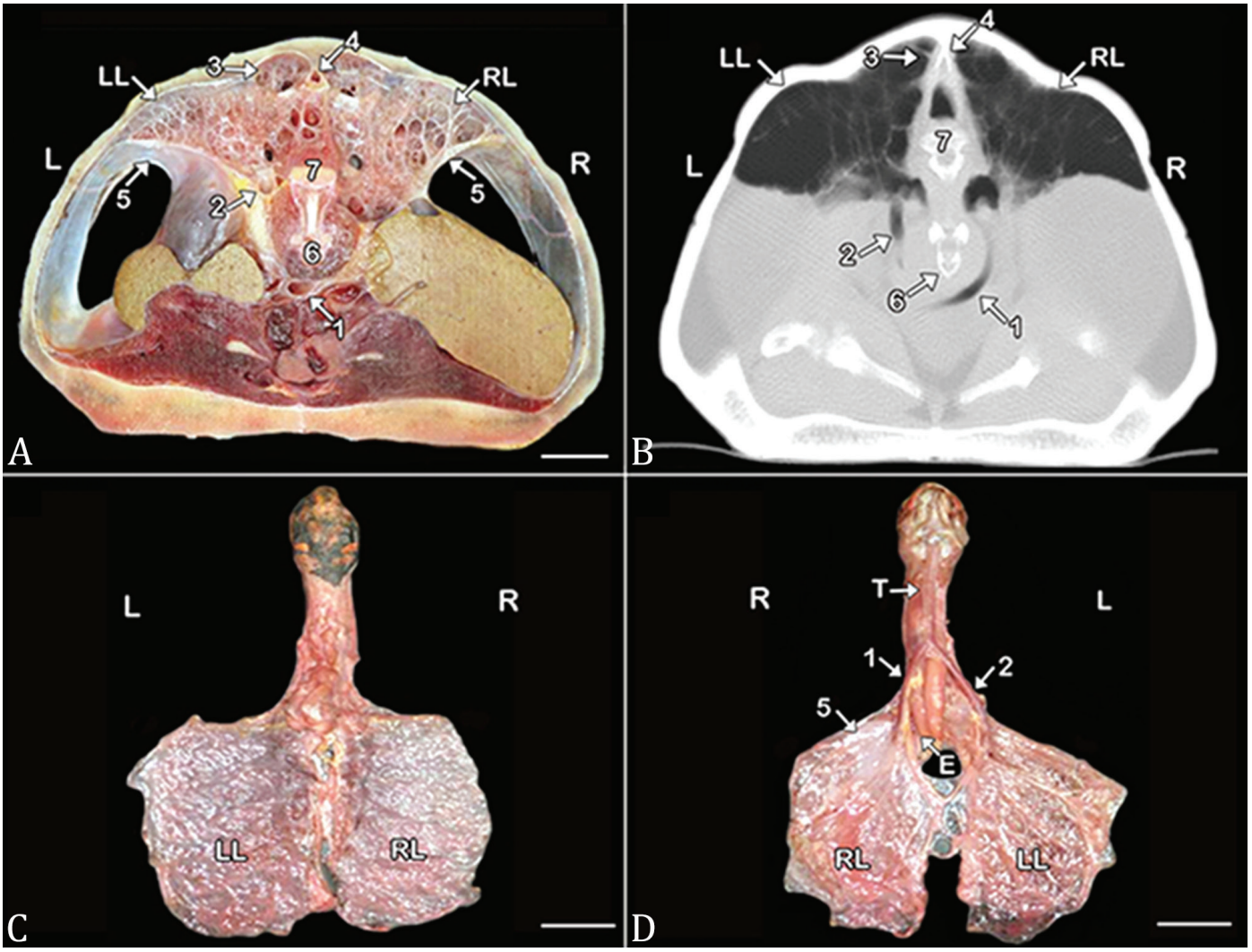


Fig.5. Anatomical and tomographic images of pulmonary bronchi in the red-foot tortoise. (**A**) Corresponding gross anatomical section. Bar = 7cm. (**B**) Computed tomography (CT) transverse plane (pulmonary window). (**C**) Dorsal view of the lungs. Bar = 4cm. (**D**) Ventral view of the respiratory system. Bar = 4cm. Extrapulmonary part of the right bronchus (1), extrapulmonary part of the left bronchus (2), pulmonary chambers (3), eighth cervical vertebra (4), pulmonary septum (5), sixth cervical vertebra (6), seventh cervical vertebra (7), esophagus (E), trachea (T), right lung (RL), left lung (LL), right side (R), and left side (L).

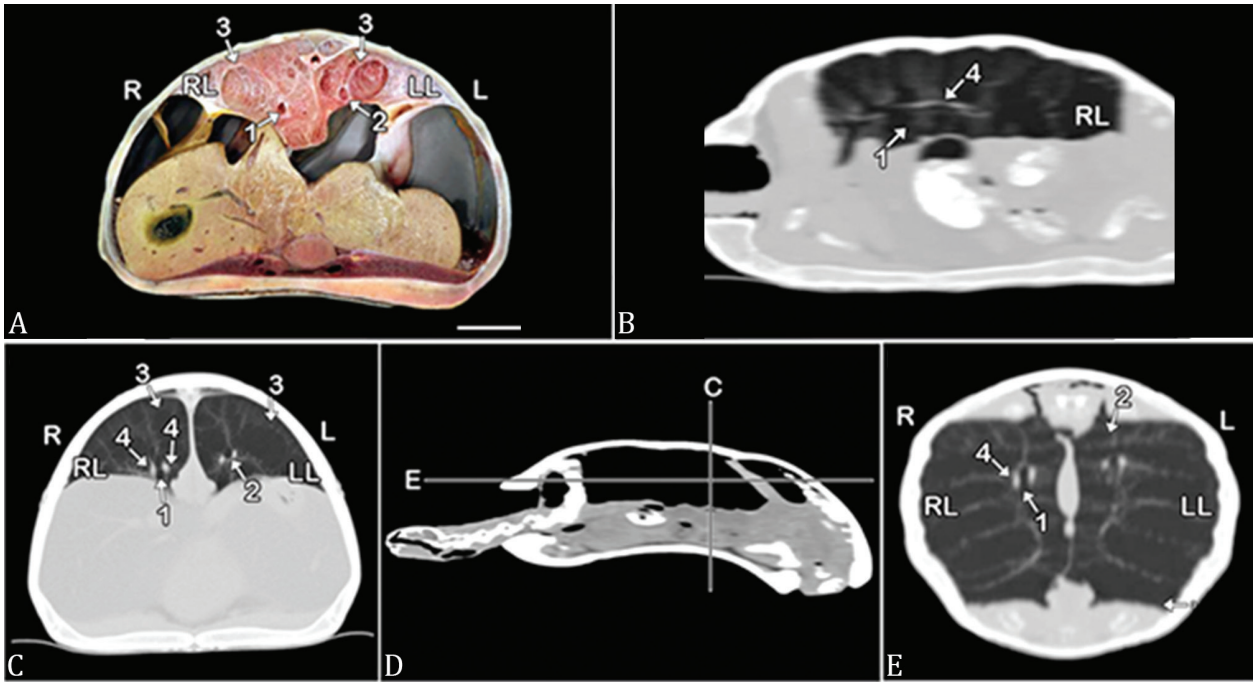


Fig.6. Anatomical and tomographic images of the lungs in the *Chelonoidis carbonaria*. (**A**) Corresponding gross anatomical section. Bar = 7cm. (**B**) Computed tomography (CT) sagittal plane and (**C**) transverse plane (pulmonary window). (**D**) Reference to locate Figure C and E. (**E**) CT dorsal plane. Intrapulmonary part of the right bronchus (1), left bronchus intrapulmonary part (2), pulmonary chambers (3), pulmonary blood vessels (4), right lung (RL), left lung (LL), right side (R), and left side (L).

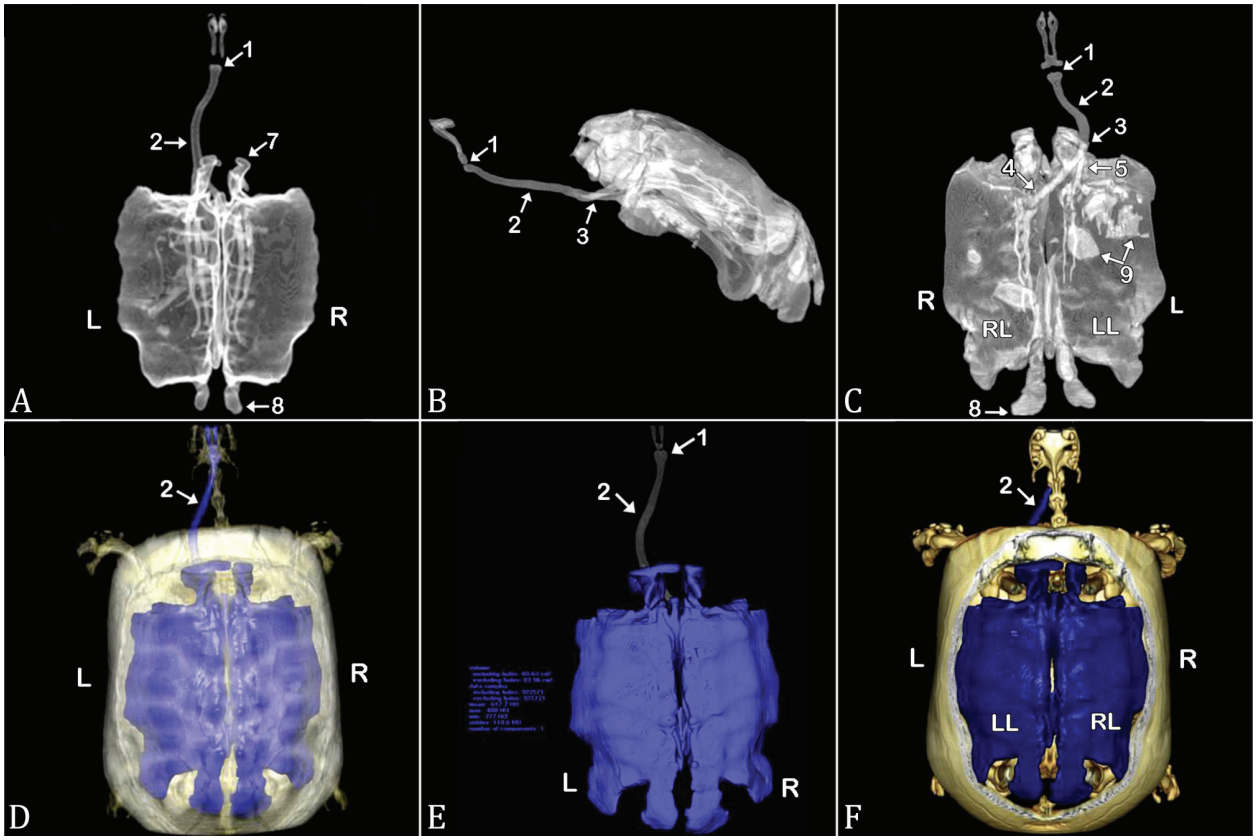


Fig.7. (**A**) Dorsal, (**B**) lateral, and (**C**) ventral views of 3D CT reconstructions of the lower respiratory tract in the red-foot tortoise. (**D**, **F**) Position of the respiratory tract in relation to the skeleton. (**E**) Representation of calculation of the pulmonary volume. Glottis (1), trachea (2), Carina (3), extrapulmonary part of right bronchus (4), extrapulmonary part of the left bronchus (5), pulmonary blood vessels (6), cranial lung chamber (7), caudal pulmonary chamber (8), remnants of intestinal loops with gaseous contents (9), right lung (RL), left lung (LL), right side (R), and left side (L).

for the red-foote tortoise. The trachea presents variable positions in the different species of chelonians. In sea turtle, it is positioned ventrally and to the right of the esophagus (Valente et al. 2007), whereas the trachea of the red-foot tortoise is ventrally and the left esophagus, similar to that observed in European pond turtle (*Emys orbicularis*) (Zehtabvar et al. 2014).

Regarding the length of the trachea, this was in agreement that described for European pond turtle *(*Zehtabvar et al. 2014), although they were species of different habits, morphological characteristics and families. In contrast, the length of the extrapulmonary part of the bronchi and the difference in length between the right and left bronchi observed in this study were considerably higher than that reported for that tortoise. Valente et al. (2007) with the use of virtual 3D bronchoscopy have described that the tracheal mucosa has a smooth surface, as observed in the red-foot tortoise. The position of the carina found in the present study is considerably more cranial than in sea turtles, where the trachea branches after the first dorsal vertebra (Valente et al. 2007) and in the European pond turtle, where it forks between the eighth and ninth cervical vertebra. However, in Egyptian tortoise, the carina was identified more cranially, at the level of the second to fourth cervical vertebra (Saber & Kamal 2010), although it is also a terrestrial chelonius as the species of this study. The difference in the position of the carina seems to be related to the morphofunctional characteristics of the neck observed in the different groups of the Testudines.

Perry (1983) stated that the intrapulmonary bronchus of the chelonians does not have branching. In contrast, Kiefer & Pees (2011) reported that it has its trajectory in the pulmonary tissue as a central airway and branches into smaller terminal bronchioles that reach the pulmonary chambers, in agreement with that observed in red-foot tortoise. The blood vessels that accompany the intrapulmonary part of the bronchus were identified histologically, as pulmonary artery and vein, located, respectively, dorsally and ventrally to the bronchus (Valente et al. 2007). In the present study it was not possible to observe this differentiation between these blood vessels.

The pulmonary anatomy of the red-foot tortoise is comparable to that found in other species of Testudines, but with differences in the relative size of the lungs. Terrestrial chelonians appear to have much more pulmonary volume than sea turtles (Valente et al. 2007). In CT at the base of the heart, in a sea turtle, the lungs occupied only the dorsal third of the body cavity (Valente et al. 2007), whereas in terrestrial chelonians, the lungs occupy about two-thirds of body cavity (Wilkinson et al. 2004), as seen in the specie of this study.

The red-foot tortoise have lungs morphologically characterized as large multi-chamber structure, with many septa and rich in reticulated parenchyma, similar to that reported for other terrestrial Testudines (Christopher & Hernandez-Divers 2003, Duncker 2004). Schachner et al. (2017) claimed that traditionally the morphology of the turtle lungs is multichambered. One of the most important clinical contributions of tomography examination is to describe the relationship of the respiratory system with other organs. Studies using CT in sea turtles (Valente et al. 2007) and terrestrial chelonians (Saber & Kamal 2010) reported that the lungs were not lobed and the pulmonary parenchyma was heavily reticulated. These findings were in agreement with that observed in *C. carbonaria*.

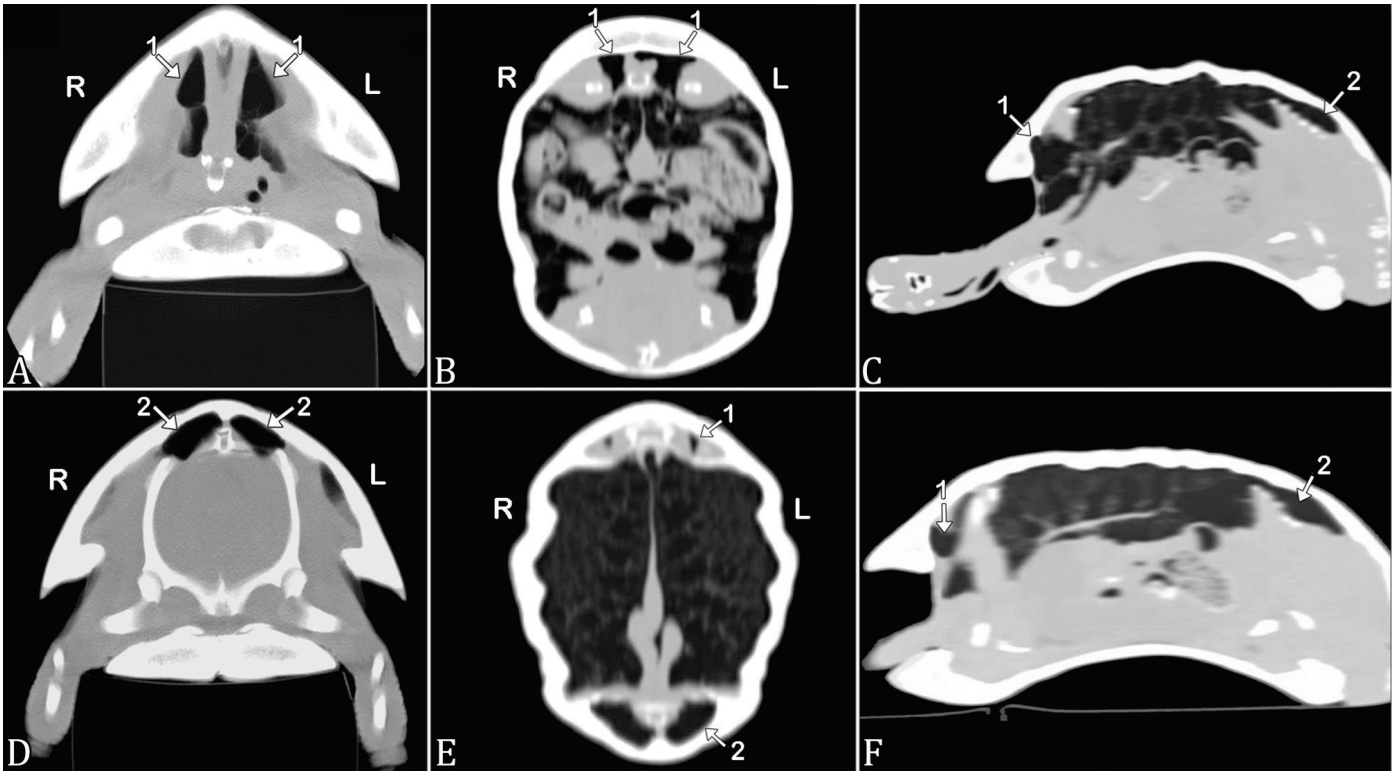


Fig.8. Tomograhic images of the cranial and caudal chambers of the lungs in the red-foot tortoise. (**A**,**D**) CT transverse, (**B**,**E**) dorsal, and (**C**,**F**) sagittal planes (soft-tissue window). Cranial lung chamber (1), caudal lung chamber (2), right side (R), and left side (L).

In the present study, it was observed that the lungs were separated from the other structures of the body cavity, by a thin non-muscular membrane. Some authors such as Perry (1983) and Schumacher (2011) reported the existence of this structure, called the "post-pulmonary septum" or "pleural membrane", which may cover all or part of the lungs depending on the species of chelonians. This structure can influence ventilatory mechanics (Schumacher 2011). This membrane was not described in some studies performed on chelonians (Valente et al. 2007, Saber & Kamal 2010, Zehtabvar et al. 2014). It was not possible to visualize this post-pulmonary septum in the radiographic and tomographic images performed in the red-foot tortoise. It is suggested that this was due to the low thickness of this membrane or due to the limitation of the CT employed here.

Small pulmonary chambers were identified in the tomographic sections and 3D reconstructions described in this study. Such chambers were not identified in the anatomical study, probably because the lungs were not inflated. We suggest that these pulmonary dilatations could be non-respiratory structures, with little vascularity, comparable to the air sacs of birds, based on the reports of O'Malley (2005) and Devoe (2010), who affirmed the presence of this type of structures in the lungs in others species of reptiles such as snakes and lizards. This finding suggesting performing other methods of investigation to address this question.

Craniocaudal and lateral radiographic projections are commonly used to detect pulmonary diseases in routine care in Testudines (Hernandez-Divers 2006). However, when associated with carapace fracture, the local inflammatory response and the overlap of the bone fragment usually prevent an accurate evaluation of the pulmonary parenchyma. Based on the results observed in the present study, the radiography did not allow a more detailed and reliable evaluation of all respiratory structures in red-foot tortoise. CT and 3D reconstructions plays an important role in the diagnosis of a wide several diseases in a variety of animals (Garland et al. 2002). Valente et al. (2007) postulated to the use of CT for the evaluation of the respiratory system of sea turtles and affirm that it provides accurate information about the lungs and airways, in agreement with that observed in this study in red-foot tortoise.

The comparison with the anatomical sections was indispensable in our study for the recognition of anatomical structures in CT images. Some soft tissue celomatous structures, such as the esophagus, could not be identified in tomographic images because of their small size, the lack of contrast between them and surrounding tissues, and the resolution of the conventional CT employed here. The CT avoids overlapping structures, both skeletal and soft tissue, observed on radiographs. The slow respiratory rate of these animals associated with air-lung contrast provided a detailed and high-quality visualization of the lungs and airways. The lower respiratory tract in the red-foot tortoise was similar to that reported to other Testudines and based on the findings of this study, we recommend that CT could be the better technique for a good diagnostic in a large variety of respiratory problems in Testudines.

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Granulomatous pneumonia due to pythiosis in captive South American coatis (*Nasua nasua*)[1]

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ABSTRACT.- Bezerra K.S., Santos T.A., Rosa J.M.A., Pescador C.A., Dutra V., Nakazato L., Driemeier D. & Colodel E.M. 2020. **Granulomatous pneumonia due to pythiosis in captive South American coatis (*Nasua nasua*)**. *Pesquisa Veterinária Brasileira 40(8):647-650*. Laboratório de Patologia Veterinária, Hospital Veterinário, Faculdade de Medicina Veterinária, Universidade Federal de Mato Grosso, Av. Fernando Corrêa da Costa 2367, Boa Esperança, Cuiabá, MT 78068-495, Brazil. E-mail: moleta@gmail.com

Pythiosis is an emerging infectious disease affecting captive and free-ranging wild animals. We report granulomatous pneumonia due to *Pythium insidiosum* in two South American coatis (*Nasua nasua)*, who were found dead without any clinical records. Severe granulomatous pneumonia associated with pleural effusion was revealed in the necropsy. Microscopically, variably sized granulomas and pyogranulomas presented negative hyphae profiles at the periphery of their necrotic cores. Grocott methenamine silver stain highlighted these structures, and immunostain (anti- *P. insidiosum*) was strongly positive. Molecular analysis by polymerase chain reaction amplified *P. insidiosum* specific DNA. These findings characterized *P. insidiosum* as a cause of granulomatous pneumonia in coatis and proved that pythiosis needs to be considered in the differential diagnosis of respiratory diseases affecting this species in endemic areas.

INDEX TERMS: Granulomatous pneumonia, pythiosis, captive animals, South America, coatis, *Nasua nasua*, pneumonia, Procyonidae, *Pythium insidiosum*, Southern coati, Stramenopila.

RESUMO.- [**Pneumonia granulomatosa decorrente de pitiose em quatis sul-americanos (*Nasua nasua*) de cativeiro.**] A pitiose é uma doença infecciosa emergente que afeta animais silvestres de cativeiro e em vida livre. Reportamos dois casos de pneumonia granulomatosa decorrentes da infecção por *Pythium Insidiosum* em quatis sul-americanos (*Nasua nasua*), que foram encontrados mortos sem apresentar nenhum quadro clínico prévio. Pneumonia granulomatosa severa associada a efusão pleural foi observada durante a necropsia. Na microscopia, foram observados múltiplos granulomas e piogranulomas de tamanhos variados que continham imagens negativas de hifas na periferia de seus centros necróticos. A coloração de metenamina de prata (Grocott) evidenciou estas estruturas, e a imunomarcação (anti-*P. insidiosum*) foi fortemente positiva. A análise molecular pela reação de polimerase em cadeia amplificou o DNA específico do *P. insidiousum*. Estes achados caracterizaram o *P. insidiosum* como a causa da pneumonia granulomatosa nos quatis e provou que a pitiose deve ser considerada um diagnostico diferencial para outras doenças respiratórias que afetam esta espécie.

TERMOS DE INDEXAÇÃO: Pneumonia granulomatosa, pitiose, quatis, América do Sul, *Nasua nasua*, cativeiro, Procynidae, *Pythium insidiosum*, quati, Stramenopila.

INTRODUCTION

Pythium insidiosum (kingdom: Stramenopila, phylum: Pseudofungi, class: Oomycetes, order: Pythiales, family: Pythiaceae) is the causative agent of pythiosis and an emerging pathogen in captive and free-ranging wild animals (Wellehan et al. 2004, Buergelt et al. 2006, Pesavento et al. 2008, Gaastra et al. 2010, Videla et al. 2012). It is commonly known for causing chronic and granulomatous disease in humans and domestic animals, particularly horses. Recently, it has become

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significant in zoos and feral herds, such as the wild ponies in Chincoteague Island (Gaastra et al. 2010, Hedgpeth 2018).

Usually associated with aquatic environments, it can manifest as mild to severe tumor-like masses or ulcerated lesions that can affect any part of the body depending on the species (Gaastra et al. 2010). They can be cutaneous, subcutaneous or gastrointestinal (Tabosa et al. 2004, Buergelt et al. 2006, Pesavento et al. 2008, Gaastra et al. 2010). As the disease progresses, the infection can spread to the lungs or other sites (Grooters 2003, Reis et al. 2003, Tabosa et al. 2004, Gaastra et al. 2010, Gaddis & Brinkman 2017). Pneumonia rarely occurs as a primary infection, and seems to have been limited to a single case report of a Central American jaguar among non-domestic species (Camus et al. 2004). As it is a life-threatening condition, an accurate and early diagnosis is critical to improve prognosis (Gaastra et al. 2010). However, unusual presentations like pulmonary pythiosis could be difficult to detect and easily overlooked.

South American coatis, Southern coatis, brown-nosed coatis, or ring-tailed coatis (*Nasua nasua*) are members of the Procyonidae family and broadly distributed in South America (Desbiez & Borges 2010). Here, we document the first report of pulmonary pythiosis infection in two captive coatis in midwest Brazil.

MATERIALS AND METHODS

Two coatis, a 1-year-old female (Coati 1) and a 4-month-old male cub (Coati 2), were found dead during the rainy season in an outdoor zoo enclosure at the "Universidade Federal do Mato Grosso" Zoo, Cuiabá, Brazil. The facility had 15 coatis in total, with a history of recurrent territorial and sexual fighting and consequent suppurative cutaneous wounds due to bites. During the wet season, respiratory signs like nasal discharge and respiratory distress occurred sporadically. The Coati 1, presenting a non-healing purulent bite wound on its dorsal part, was hospitalized at the time for suspected pneumonia.

The bodies of Coatis 1 and 2 were submitted for necropsy and samples of all organs were collected and fixed in 10% buffered formalin for routine histopathological processing and hematoxylin and eosin (HE) staining. Special Grocott methenamine silver stains (GMS) stain was performed in the lung to detect negative hyphae profiles, besides immunohistochemistry (IHC) using rabbit polyclonal anti-*Pythium insidiosum* antibody (non-commercial) (Pesavento et al. 2008), and polymerase chain reaction with primers PI-1: 5'-TTCGTCGAAGCGGACTGCT-3' and PI-2: 5'-GCCGTACAACCCGAGAGTCATA-3' encoding the sequence ITS1 and rDNA of *P. insidiosum* (Pesavento et al. 2008).

RESULTS

Grossly, Coati 1 and 2 presented unilateral, severe, and chronic granulomatous pneumonia (Fig.1A). The affected lung

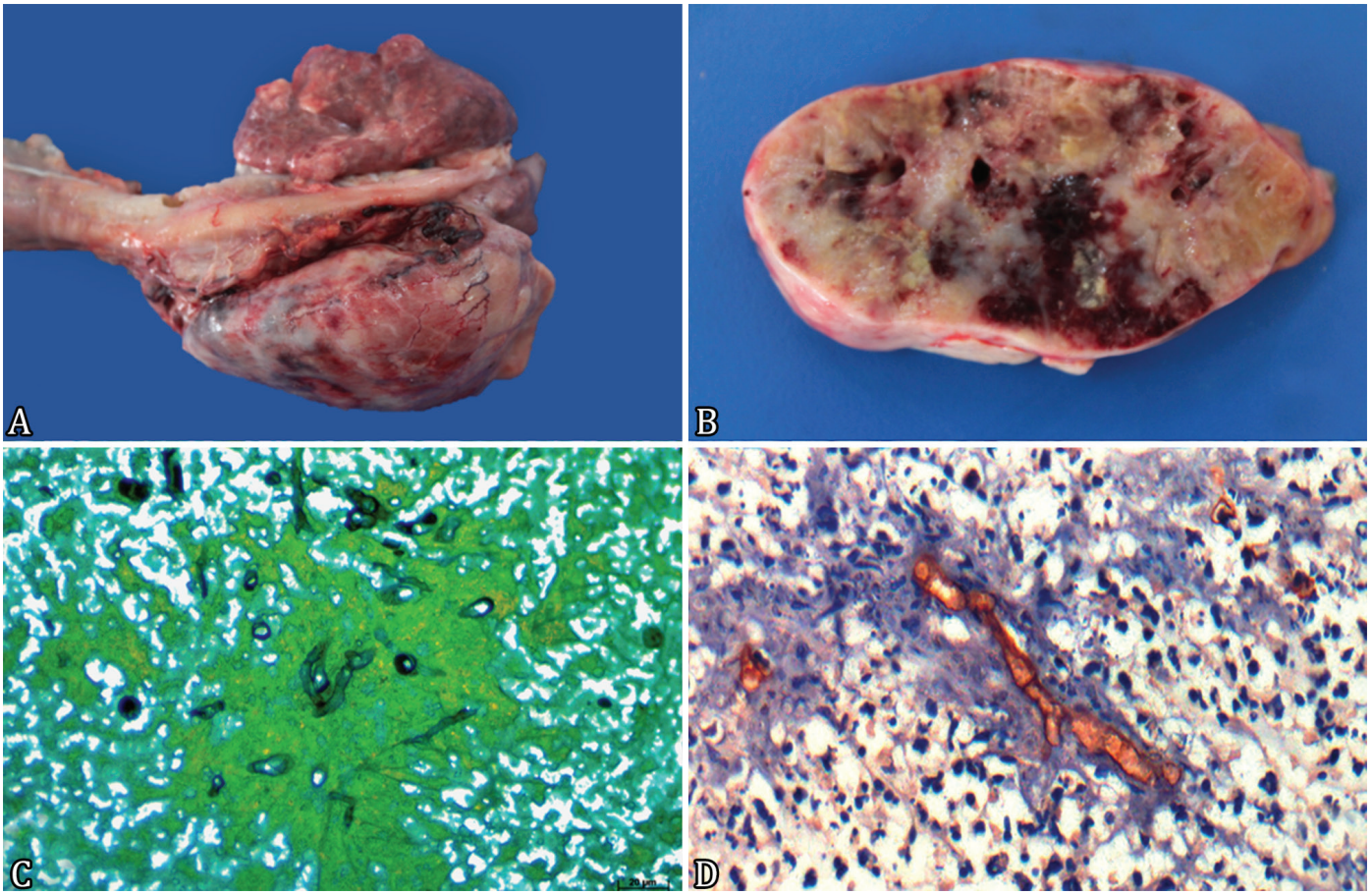


Fig.1. *Pythium insidiosum* infection, coati, lung. (**A**) The affected left lung was greatly enlarged. (**B**) Multiple granulomas scattered through the pulmonary parenchyma. (**C**) A hypha is situated in the necrotic center. GMS, obj.40x. (**D**) IHC for *Pythium insidiosum* shows strong immunostaining on the hyphae-like wall. IHC, obj.40x.

was greatly enlarged and consolidated, showing numerous coalescent nodular granulomas with yellowish caseous cores (Fig.1B). Coati 1 was emaciated and showed marked subcutaneous thoracic edema, as well as mild mucopurulent nasal flow. In Coati 2, the lung lesions seemed more severe, sometimes presenting a cavernous and lytic pattern. In both cases, a large amount of serofibrinous pleural exudate and multiple points of chronic pleural adherences surrounded the affected lung. Mediastinal lymph nodes were enlarged and embedded among the granulomatous masses.

Microscopically, the lesions were similar with variable intensity in both the coatis. Lung sections showed multiple coalescing granulomas and pyogranulomas characterized by extensive caseous necrosis associated with a dense collection of epithelioid macrophages, eosinophils, degenerate neutrophils, and a mild number of Langhans cells. There was an outer layer of lymphocytes, plasmocytes, and less reactive macrophages and marked proliferation of fibrous connective tissue. Hemorrhage, fibrin thrombi, and necrotizing vasculitis were frequent, but there was no evidence of angioinvasion. Intensely eosinophilic and radiant amorphous material (Splendore-Hoeppli reaction) was frequently observed in these granulomas. Negative hyphae-like profiles were often seen.

Grocott methenamine silver staining of the lung sections revealed several broad, sparsely septate, and irregularly branched hyphae-like organisms that were 3-7µm in diameter and located predominantly at the Splendore-Hoeppli reaction cores with only a few ones scattered throughout the adjacent areas (Fig.1C). Mediastinal lymph nodes presented lymphoid hyperplasia with marked granulomatous inflammation. The hyphae-like organisms were consistently found in the pulmonary granulomas. There was no angioinvasion or hyphae in the lymphnodes. Hyphae-like organisms were regularly marked using the rabbit polyclonal anti- *Pythium insidiosum* antibody (non-commercial) (Fig.1D)

Environmental enclosure water, nasal secretions, and a cutaneous punch biopsy of the coati 1 were collected for pathological and epidemiologic investigation. The skin biopsy showed pyogranulomatous dermatitis and granulation tissue. However, hyphae-like organisms were not observed. All samples tested positive by polymerase chain reaction with primers PI-1: 5'-TTCGTCGAAGCGGACTGCT-3' and PI-2: 5'-GCCGTACAACCCGAGAGTCATA-3' encoding the sequence ITS1 and rDNA of *P.insidiosum* as indicated by the amplification fragment 105pb. All attempts of culture and isolation of *P. insidiosum* from fresh tissues were unsuccessful.

DISCUSSION

Pythiosis is a well-described condition that manifests as a chronic and progressive granulomatous disease. Its etiologic agent, *Pythium insidiosum*, is a oomycete pathogen frequently associated with tropical or subtropical aquatic environments (Gaastra et al. 2010). Brazil is endemic for this disease and its warm temperatures with long rainy seasons through the year are highly favorable for the growth and proliferation of this pathogen (Tabosa et al. 2004, Machado et al. 2016). These cases were unique because the primary site of infection appeared to be the lungs and the presentation occurred in more than one animal from the same group, in contrast to the individual reports described till date (Goad 1984, Heath et al. 2002, Camus et al. 2004, Kepler et al. 2017). To the best of our knowledge, this is the first reported case of pythiosis in coatis. This report broadens the spectrum of the disease in non-domestic species. Pythiosis should be included as a differential diagnosis of coatis and zoo animals presenting unspecified respiratory signs or non-healing wounds, especially in endemic regions.

There are very few reports of pulmonary pythiosis. In most cases, it occurs as a consequence of metastatic spread within the progression of the disease in its systemic form, or through aspiration secondary to nasal infection as already described in sheep, horses, and dogs (Reis et al. 2003, Tabosa et al. 2004, Ubiali et al. 2013).

Pulmonary pythiosis as a primary infection is rare and includes only singular case reports in a horse, human, dog, and captive jaguar until now (Goad 1984, Heath et al. 2002, Camus et al. 2004, Kepler et al. 2017). Similar to these coatis, there was no evidence or previous history of wounds or infection at any other site, except in the equine case. This horse previously presented cutaneous pythiosis, but the pulmonary infection was not related to it.

The disease typically involves lesions on parts of the body that have come in contact with warm, stagnant water containing motile zoospores, and infection occurs due to a high tropism that the pathogen has for damaged tissue (Gaastra et al. 2010). In the coati from the group suspected of pneumonia and presenting the skin wound, amplification of the homologous sequence of *P. insidiosum* occurred. However, no hyphae-like organisms were observed in histological sections of skin biopsy of the affected site, and clinical correlation was not possible.

In these cases, the pulmonary infection most likely occurred through inhalation or ingestion with subsequent aspirations of contaminated water containing the pathogen. The pathogen was detected in a nasal swab from a coati of the same group, which suggests that the possible route was through inhaled aerosolized zoospores as seen in a similar case of a Central American jaguar (Camus et al. 2004)

Clinical signs of localized pulmonary pythiosis are usually non-specific, including chronic coughing, dyspnea, and exercise intolerance, as well as nasal discharge, anorexia, weight loss, decreased appetite, anemia, and hypoproteinemia (Goad 1984, Heath et al. 2002, Camus et al. 2004, Kepler et al. 2017). No clinical signs were observed in these two coatis, but their group had a history of respiratory distress and the pathological findings were consistent with these signs. The subcutaneous edema in the female most likely occurred due to hypoproteinemia, as implied by the poor body condition.

The gross and histologic appearance of the lesions of these cases were very similar to those previously described (Martins et al. 2012, Ubiali et al. 2013). When infection occurs through metastatic spread along lymphatics, the granulomas are randomly distributed in both lungs in a miliary pattern (Reis et al. 2003, Tabosa et al. 2004, Ubiali et al. 2013). When pythiosis is restricted to the lungs, the lesions tend to be more localized and form a large and compact mass containing multiple granulomas, as observed in the coatis of our report. The unilateral pulmonary consolidation seen here was also observed in the jaguar case (Camus et al. 2004). However, "kunkers", characteristic of the equine pythiosis, were not observed (Gaastra et al. 2010).

Microscopically, the eosinophilic pattern of necrosis was predominant and similar to that described in horses

and sheep (Grooters 2003, Martins et al. 2012, Ubiali et al. 2013). Non-eosinophilic granulomatous infiltrate is more common in cattle and was also present in these cases, but only sporadically (Martins et al. 2012). Despite the presence of severe necrotizing vasculitis within fibrin thrombus in many areas of the pulmonary parenchyma, there was no evidence of hyphae vascular invasion or hematogenous spread to other organs.

While the diagnosis is quite non-specific based only on the histopathological examination, these morphological findings narrow the range of possible etiologic agents to *P. insidiosum*, zygomycetes, and *Lagenidium* species. *Lagenidium* spp. primarily affects dogs, and it has been reported in cats and humans. The differential diagnosis between these infections is essential, because they are morphologically similar but differ in the epidemiology, choice of therapy, and prognosis (Grooters 2003, Ubiali et al. 2013).

CONCLUSIONS

The anatomopathological changes, histologic and molecular findings show that *Pythium insidiosum* is the cause of granulomatous pneumonia in these coatis.

Due the non-specific clinical signs, pythiosis needs to be considered in the differential diagnosis of respiratory diseases affecting this species in endemic areas.

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Conflict of interest statement.- The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

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➢ Artigos publicados em periódicos:

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

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

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

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- 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.

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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.

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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.

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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)

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Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

GUIDE FOR AUTHORS

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

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

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

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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.

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

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Exemples for References:

➢ Articles published in scientific journals:

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

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

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

➢ Books:

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

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

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

➢ Chapters of books:

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

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

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

➢ Dissertations and Theses:

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

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

➢ Abstracts published in Events:

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

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

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

(Note: Consult entire papers instead of only Abstracts)

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